Seasonal physiological responses in the greater thick-tailed galago (*Otolemur crassicaudatus*)

by

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Declaration

I, Channen Long, student number 14390800, hereby declare that this thesis, "*Seasonal physiological responses in the greater thick-tailed galago (Otolemur crassicaudatus),*" is submitted in accordance with the requirements for the Doctor of Philosophy degree at University of Pretoria, is my own original work and has not previously been submitted to any other institution of higher learning. All sources cited or quoted in this research paper are indicated and acknowledged with a comprehensive list of references.

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Channen Long

31 May 2023

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As always, to my parents. As with all my other endeavours and accomplishments, this thesis is dedicated to you. Although Mum was only around for the first few months of my PhD, you have both and will always be my Number One supporters. I am forever grateful. To my other family members – especially Shel and Bronwyn – I am so grateful for your help, support, and advice.

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Thesis Summary

Primate populations over the globe are facing declines as a result of several factors including climate change. It has become imperative to gain further insight into how primate species respond to these changes in weather to ensure appropriate conservation approaches. For this study, I chose to monitor the physiological changes of a population of greater thick-tailed galagos (*O. crassicaudatus*) residing in a highly seasonal, temperate environment. Research of this strepsirrhine species has been lacking for over two decades and the scientific community is unaware how they respond to seasonal weather changes. In this study, we assessed their glucocorticoid and thyroid hormone levels to monitor their hormonal responses, gut microflora, and metabolite profiles associated with changes in temperature and food availability. We successfully validated the immunoassays necessary to measure hormone metabolites in this species. The results revealed an increase in hormone levels during the summer season which may be caused by an increase in energy expenditure as food availability and temperatures increase. Furthermore, lactating females during this time require additional energy and nutrition to sustain themselves and their offspring. The results of the metabolite analyses indicate these concentrations were affected by changes in diet. However, it appears the dominant microflora and metabolic pathways adapt to seasonal fluctuations of nutrient intake to ensure the body receives the essential amino acids needed for ATP generation. Overall, this project has given further information into the mechanisms undertaken by this species during times of low food availability and will assist in future primate conservation.

Keywords: *Otolemur crassicaudatus*, strepsirrhine, diet ecology, glucocorticoids, thyroid hormones, microbiome, metabolites, metabolomics, faeces, urine, serum, polysaccharides, ketones

Ethical Permits

Ethical clearance to conduct the hormone, microbiome, and metabolomic analyses, including the capture and housing of animals, and seasonal analysis, requiring the capture and sampling of free-ranging animals, was received from the Research and Ethical Sciences Committee at the South African National Biodiversity Institute's (SANBI) National Zoological Gardens (NZG; Project 18/26) and the Animal Ethics Committee of the University of Pretoria (Project V037-17).

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Chapter 1

Literature Review

Background

A seasonal environment and resource availability

Seasonal changes will alter many different factors in the environment such as rainfall temperature, and soil, which, in turn will affect the nutrient availability (Coelho, 1986). Temperate climates experience four seasons per year with warmer temperatures in summer (>10°C) and colder temperatures in winter (>-3°C; Trewartha & Horn, 1980). In temperate environments, an increase in rainfall in summer is expected with very little precipitation in winter (McColl & Young, 2005; Simmons, 2015). These weather changes will influence the availability of resources (Foerster *et al.*, 2012; Tecot, 2007). Across differing seasons, food resources will become available or disappear depending on an individual's preferred environment. For instance, insect density may decrease during the less favourable period where rainfall is reduced in a high-altitude area (Williams, 1961). Subsequently their numbers will be replenished when the environmental conditions improve in the wet season. Species will most likely have evolved adaptations to these changes in their natural habitat through behavioural and physiological plasticity (Nowack *et al.*, 2013). Many primate species are generalist feeders and capable of dietary flexibility which allows them to survive seasonal change (Hemingway & Bynum, 2005). Animals can adapt to climatic changes using evolutionary and adaptive processes; however, the current rate of change in temperature and weather patterns are greater and more extreme than experienced during evolutionary history, and largely driven by anthropogenic activities. This rate of change puts pressure on species, and it has become apparent some species are not able to cope with the current rate of change (for instance, the review by Estrada *et al*., 2017). Estrada *et al*. (2017) discussed the current population densities of primates situated all over the

world. The results of the review concluded that many species of primates are severely affected by the current changes in their environments, and how some species populations are decreasing dramatically. Scientists have made several projections for climate changes, for instance, the sea levels, carbon dioxide levels, air temperature (Cubasch *et al*., 2001) and vegetation (Svenning & Sandel, 2013) will be widely affected in the next 50 years.

Considering the progressing climatic changes expected (Cubasch *et al*., 2001), it has become imperative for scientists over the last two decades to develop models and strategies to monitor and predict the survivability of wild animal populations. This information could then be used to implement adequate conservation schemes to improve the maintenance of the species' habitat and for the species itself. Primates are ideal for studying the effects of environmental changes as they inhabit almost all terrestrial habitats; for instance, the Japanese macaques surviving in the high-altitude mountains of Japan (Hanya, 2004), and the lorises in the forests of Borneo (Nekaris *et al*., 2007). Primates also rely on a variety of different diets, some more generalist feeders, such as the omnivorous yellow baboons in Amboseli (Gesquiere *et al*., 2012), while other primate are more specialist such as the folivorous black howler monkeys of Mexico (Amato *et al*., 2013). Primates will implement foraging strategies in order to reach their daily energy requirements, which may involve relying on fallback foods in less favourable conditions for instance, the Taihangshan macaque (*Macaca mulatta tcheliensis*; Cui *et al*., 2018) mostly residing in highly seasonal environments, preferably feeding on seeds when available while eating tree and herbaceous leaves as fallback foods (Shao *et al*., 2023).

Changes in climate are likely to affect the survival of primates because of changes to the environment and food availability. In recent years, scientists have more frequently investigated the effects of climate changes on primate behaviour and physiology. With the fluctuations in food resources, this would require a degree of physiological and

behavioural plasticity from individuals to attain the necessary daily nutritional requirements (Tecot, 2007). For instance, some species will increase time searching for food during low food abundance, while other species will reduce their activity levels to reserve energy or shift their ranges accordingly. There is a trade-off between energy gain and energy expenditure (Tecot, 2007). Exploring the seasonal fluctuations in the physiological changes will inform on how species respond to challenges, such as changes in weather and availability of resources. Subsequently, investigating a species' physiological plasticity in response to these seasonal changes will give an indication how they may react to more challenging scenarios.

Glucocorticoid hormone monitoring

The body secretes hormones to help maintain homeostasis within the body. When certain bodily systems are confronted with a sudden or unpredictable stimulus, hormones may be used to counteract any effects that may occur or re-establish the hormonal status (Sapolsky, 1994; Chrousos *et al*., 1998). These responses may be initiated by natural changes (for instance, predation and competitive behaviour), or even external environmental challenges (Sapolsky *et al*., 2000). These factors that may instigate a hormonal response from the individual has been termed a "stressor", namely, a stimulus that is unpredictable (Levine & Ursin, 1991).

Once the stressor has occurred, this will initiate a cascade of hormonal effects occur that will lead to behavioural and physiological changes to improve the well-being of the individual. When an unpredictable stimulus occurs, the hypothalamic-pituitary-adrenal axis will be triggered. The stimulus will activate the hypothalamus to secrete corticotropin-releasing hormone (CRH), which then travels to the anterior pituitary. The anterior pituitary is then induced to release adrenocorticotropin releasing hormone (ACTH). The ACTH will travel throughout the body until it reaches in adrenal glands whereby the glands will secrete

glucocorticoid hormones, such as cortisol and corticosterone, which will travel through the body via the bloodstream to the target areas (Fig 1-1).

The concentration of glucocorticoids is controlled by negative feedback, whereby once there is an excess of glucocorticoids within the body, receptors will trigger the hypothalamus and anterior pituitary to cease the cascade of hormones to produce glucocorticoids (Fig 1-1; Beehner & Bergman, 2017). Once secreted, the glucocorticoid will induce physiological and behavioural adjustments in response to the stressor (Charmandari *et al.*, 2005). These include, increasing analgesia, inhibition of non-essential metabolic functions, improving mental cognition and vigilance (Nelson *et al.*, 2011). Interestingly, glucocorticoids are involved in many other functions within the body, for instance gluconeogenesis, controlling cell metabolism and growth (Bereschenko *et al*., 2018), plays important roles during reproduction including foetal development during pregnancy, mammary development, and lactation (Marciniak *et al*., 2011).

Hormone monitoring has become a popular technique for investigating the stress response in wild and captive individuals (Möstl *et al*., 2003; Palme, 2005). Initially, scientists used blood samples to measure hormones, however, less invasive techniques and matrices have been shown to be a reliable measure of hormone concentrations such as, faeces and urine (Sheriff *et al*., 2010). There have been many previous studies evaluating the glucocorticoid concentrations in primate species. Most studies have focused on the glucocorticoid responses in more social species such as macaques (for instance *Macaca mulatta*; Maestripieri *et al.*, 2008), baboons (for instance, *Papio cynocephalus*; Beehner *et al.*, 2006), and chimpanzees (*Pan troglodytes*; for instance, Muehlenbein, 2006). However, in the last two decades, researchers have also started to address environmental challenges primates may face such as, the intrinsic effects of external changes such as habitat destruction and seasonal changes, by monitoring glucocorticoid responses in longitudinal studies.

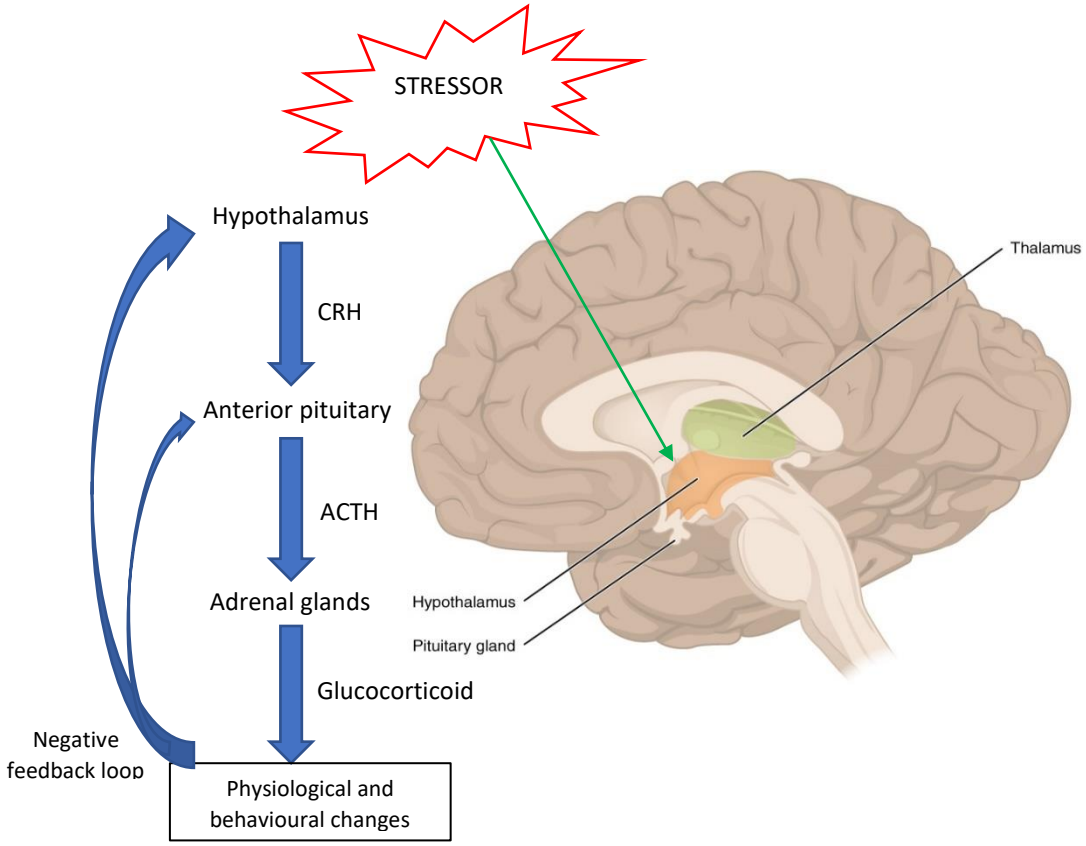


Figure 3-1. Demonstration of the glucocorticoid hormone cascade effect after a stressor event via the hypothalamic-pituitary adrenal axis. Corticotropin releasing hormone (CRH) is released by the hypothalamus at the initiation of a stressor. The CRH activates the anterior pituitary axis to secrete adrenocorticotropic hormone (ACTH), which targets the adrenal glands to release glucocorticoids (for instance corticosterone). A negative feedback loop is implemented when sufficient levels of the hormone have been released and will stimulate the hypothalamus to reduce secretion of CRH. The brain image is taken from https://www.lecturio.com/concepts/hypothalamus/

Exploring energetic condition

The main goal of wild animals is expending the least amount of energy while acquiring sufficient nutrition for survival and achieving reproductive success (Stearns 1992, Roff, & Fairburn, 2007; Burton *et al.*, 2011). Monitoring the energy balance of a species is necessary to understand how they allocate their energy and subsequently, how environmental and food density changes may affect their energetic status. This information will help scientists understand their capabilities to cope in the future to other changes that may occur within their environment. When food resources are scarce, individuals will need

to be flexible with their energy allocation and use this energy for processes necessary for the survival of the individual (Stearns & Hoekstra, 2005; Schaebs *et al.,* 2016).

Thyroid hormones have been shown to regulate seasonal adaptations (Yousef, 1975; Dierderich, 2022). Thyroid hormones play a major role in the regulation of the metabolic rate (Schaebs *et al*., 2016; Behringer *et al*., 2018; Chen *et al*., 2021). Studies have shown that when an animal has limited food available, their thyroid hormone levels are significantly reduced and this is a strategy to slow metabolic rate and preserve energy (Behringer *et al*., 2018). Consequently, the thyroid hormone levels in wild impala (*Aepyceros melampus*) in the Serengeti were mostly influenced by ambient temperature, rather than food availability (Hunninck *et al*. 2020). These authors found that ambient temperature inversely influenced thyroid levels, so that when ambient temperature dropped, thyroid levels increased. The authors suggest that the ungulates were increasing their metabolic rate, and thus increasing energy expenditure, in cooler temperatures to help maintain body temperature.

In mammals, thyroid hormone secretion is regulated by the hypothalamic pituitary thyroid (HPT) axis (Fig. 1-2). Once there is a change in stimulus, such as temperature or nutritional intake, thyrotropin releasing hormone (TRH) is secreted by the hypothalamus. This stimulates the anterior pituitary to secrete thyroid stimulating hormone (TSH) into the circulatory system. Eventually, this will activate the secretion of thyroxine (T4) and triiodothyronine (T3) hormones from the thyroid gland. Only a small concentration of T3 is secreted, the rest is produced when T4 is broken down during monodeiodination (Power *et al*., 2001). This means there are higher concentrations of T4 in the bloodstream (Burke & Eastman, 1974). The secretion of thyroid hormones is controlled by a negative feedback loop, which will induce or inhibit TRH and TSH release.

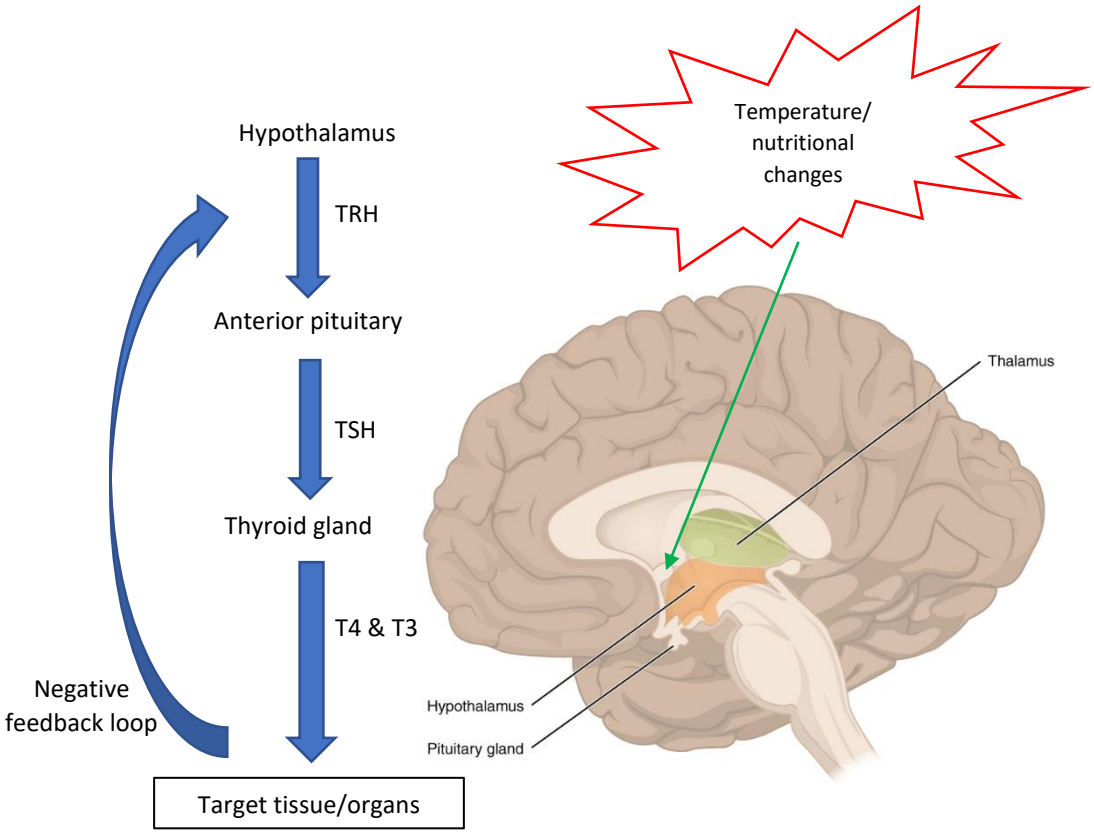


Figure 1-4. Demonstration of the thyroid hormone cascade via the hypothalamic-pituitary axis. Thyroid releasing hormone (TRH) is released by the hypothalamus at the initiation of a change in temperature or diet. The TRH activates the anterior pituitary axis to secrete thyroid secreting hormone (TSH), which targets the thyroid gland to release thyroxine (T4). T4 travels to the liver where it is broken down to produce triiodothyronine (T3), which then travels to the target organs or tissues. A negative feedback loop is implemented when sufficient levels of the hormone have been released and will stimulate the hypothalamus to reduce secretion of TRH. The brain image is taken from https://www.lecturio.com/concepts/hypothalamus/

In recent years, scientists have progressively moved away from sampling thyroid concentrations from blood samples and have focused on non-invasive monitoring techniques such as hormone metabolite concentrations in excreta, such as urine (Deschner *et al.*, 2008; Girard-Buttoz *et al.*, 2011) and faeces (Dias *et al.*, 2017). Faeces has been used successfully to monitor levels of glucocorticoid metabolites (for instance, Long *et al.*, 2021) and is gradually becoming a more popular sample matrix for measuring thyroid hormone levels, for instance, Barbary macaques (*Macaca sylvanus*; Cristobal-Azkarate *et al.*, 2016), African elephants (*Loxodonta africana*; Szott *et al.*, 2020), and North Pacific grey whales (*Eschrictius robustus*; Lemos *et al.*, 2020).

The faecal microbiome

The microflora inhabiting the gut of a host individual includes a tremendous variety of microorganisms from the bacterial, viral, archaeal, and fungal phyla (Xu *et al*., 2013). Overall, it is estimated the human gut contains approximately 4 million genes (Ventura *et al*., 2009).

In the past, the most common method for assessing microbes included culture-dependent techniques (Bauchop, 1971). In the late 1970s, analysis using 16S rRNA (culture-independent) was developed (Woese 1987; Woese *et al.*, 1990). This method allowed for more in-depth assessments of the bacteria present and was even used to discover archaebacteria. Since this time, culture-independent studies have dramatically increased in host-microbe interactions and environmental bacterial communities (Clayton *et al*., 2018). The host-microbe relationship has been well-studied in recent years with many studies relating the microbiota to mental health, diseases such as obesity (Bäckhed *et al*., 2004), diabetes and irritable bowel syndrome (Delzenne & Cani, 2011; Carroll *et al*., 2012),), and general host immunity (for instance, Kinross *et al*., 2011) in humans. Wildlife studies concerning the gut microbiome have also been growing. Many studies have focused on the anthropogenic effects on the gut microbiome (for instance, Hernandez-Gomez, 2020; Fackelmann *et al.*, 2021; Heni *et al*., 2023), exposure to disease (for example, Williams *et al*., 2018), and conservation (for instance, Guo *et al*., 2020; Zhu *et al.*, 2021).

The gut microflora has a symbiotic relationship with the host, in which the microorganisms are housed in the gut intestines where they can feed on food matter that cannot be broken down or fermented by digestive enzymes (Hume, 1997), while the host uses the microbiota to digest the food material (Clayton *et al*., 2018) and also functions in nutrient absorption and energy consumption (Hume, 1997). The microbial organisms are

essential for breaking down and fermenting polysaccharides in short-chain fatty acids found in plants that are necessary as an energy source in the host (Hume, 1997). Primate species rely of a variety of food sources; for instance, some are more insectivorous (insect-eating), some are more folivorous (leaf-eating), and others are more frugivorous (fruit-eating). However, there are few species that rely solely on one type of food type (Harding, 1981). Many primate species rely on plants as their main source of nutrients (Milton, 1987). Subsequently, the gut anatomy of primates differs between species. Some species have an enlarged hindgut, while others will contain a foregut that contain large populations of bacteria necessary for the fermentation of these complex carbohydrates (Edwards & Ullrey, 1999). The microbial flora also play an important role in the immunity of the host. Specific bacteria are found in various locations within the host gut, some are more likely to be found in the lumen in amongst the food matter, while other species are located towards the mucosal lining and form a barrier preventing harmful bacteria from entering into the bloodstream (McFall-Ngai, 2007; Lee & Mazmanian, 2010). An unbalanced diet could lead to disruptions (dysbiosis) in the gut flora and may cause gastrointestinal problems for the host including inhibited immune response (Bäckhed *et al*., 2004), obesity (Turnbaugh *et al*., 2006), and other gastrointestinal discomforts.

Many studies have been conducted on the microbe-host interactions in primates in regard to immunity and human-related health problems as primates serve as a good model. Despite this, research into understanding the functionality of these specific bacteria is only now starting to gain momentum in the scientific field. More specifically, little research has been conducted addressing the gut microbiome of primate species in mainland Africa. Gomez *et al*. (2015) determined the changes in the gut microbiome of western lowland gorillas (*Gorilla gorilla gorilla*) are influenced by changes in feeding behaviour, and Long *et al*. (2018) determined seasonal differences in the gut microbiome of the lesser bushbaby

(*Galago moholi*) also related to changes in diet. Tung *et al*. (2015) determined the important role of sociality and how it influences the composition of the gut microbiome in the Amboseli baboons (*Papio cynocephalus*) and how these effects health.

This information of primate species residing in Africa would show more insight into host ecology and better our knowledge of their diet preferences.

Metabolomics in animal research

Metabolomics is a scientific technique to identify and quantify all metabolites in a biological system (Dettmer *et al*., 2007) that has been used for over 40 years (Wishart, 2019) in a number of scientific fields, including research into diseases, metabolism and nutrition, and the pharmaceutical industry (Lindon *et al*., 2000; Wishart, 2019; Wang *et al*., 2022). Metabolites are the final product of various biochemical processes and the interaction of genetics and environmental changes occurring (Fiehn, 2001). Metabolomics research is valuable as it not only identifies the metabolites, but also gives insight and understanding into the metabolic mechanisms required for that process. Most importantly, metabolites provide direct information about the influence the extrinsic environment has on an organism.

The analysis of metabolomics can be performed using various platforms. Mass-Spectronomy (MS) and nuclear magnetic resonance (NMR) are the most common analytical instruments used. MS is useful for diagnosing in-born errors of metabolism (Dettmer *et al*., 2012). However, MS is useful for providing more in-depth information such as accurate mass determination, structural identification, and is highly sensitive. MS is good for detecting compounds, mostly volatile compounds, and provides high resolution data (Stettin *et al*., 2020). Currently, MS is most commonly used in targeted studies. NMR is low cost and provides an overall outlook on the metabolite profile. It is reproducible, non-destructive, non-biased analytical instrument and gives quantitative accuracy. NMR is ideal for qualitative and

quantitative analysis of metabolites. Most NMR research is focused on 1H atoms (1H-NMR; Bharti & Roy, 2012), as these atoms demonstrate the most intensity with high isotopic natural abundance. (1H-NMR). The limitations with NMR spectroscopy include the likelihood of overlapping of compounds on the spectra, which causes uncertainty when identifying and quantifying compounds (Liu *et al.*, 2021).

In animal research, metabolomics has been limited with most studies being performed on livestock health (for instance, Tran & Conville, 2020) and production (for instance, Goldansaz *et al.*, 2017). Metabolomic studies can help identify the metabolic strategies to understand how species will react to changes within their environment and are essential in understanding their evolution and ecology (Cristobal-Azkarate *et al*., 2016). In primates, metabolomics research is substantially lacking with most studies based upon faecal metabolomics to help understand the functionality of the microbial flora within the gut (for instance black howler monkeys, Mallott *et al*., 2022) or has been focused on the effects of radiation exposure (Pannkuk *et al.*, 2015; 2016). More research using biofluids would be better to understand the metabolic processes and mechanisms involved during environmental changes.

Study Species

The thick-tailed greater galago (*Otolemur crassicaudatus*, hereafter "*O. crassicaudatus*") is an arboreal, nocturnal non-human primate (NHP; Bearder, 1974). In South Africa, this species is widely distributed in the northern and eastern areas in which tree density is high and generally inhabit montane woodlands and evergreen forests (Charles-Dominique & Bearder, 1979). As such, this species is considered "least concern" according to the International Union for Conservation of Nature (IUCN, 2016). *O. crassicaudatus* are income breeders, meaning they will undergo parturition and lactation during periods of high food abundance to help counteract

the taxing energetic costs of these reproductive states (Souza-Alves, 2019). Throughout the year *O. crassicaudatus* relies on gum exudates (complex polysaccharides), insects, and fruit for nourishment. It is generally accepted that, in correlation with seasonal changes, food availability fluctuates through the year, whereby food abundance is low in winter and higher in the summer months (Bearder, 1974; Masters, 1988).

Research pertaining to this species has been lacking in the last twenty years, thus making it an ideal species of interest. With the increasing primate population declines, it is worth investigating this species prior to any extreme changes in their environment or climate to understand how their physiology reacts to these deviations.

Research Site

The Lajuma Research Centre (23°2'16.8" S, 29°26'34.08" E) is situated in the Soutpansberg Mountains, on the Western end of the Venda region in Limpopo, South Africa (Fig. 1-3).

Figure 1-3. Map illustrating the research site for this study, Lajuma Research Centre. Situated in the Soutpansberg mountains of the Limpopo province, South Africa. The image on the right shows the location zoomed-in and was captured by Google Earth. Th image is also able to highlight the vegetation density at the research site as opposed to the surround areas below the mountain.

Lajuma Research Centre is recognised as a Natural Heritage Site and is part of the Luvhondo Nature within the UNESCO Vhembe Biosphere Reserve (Mphidi, 2019). The site is at a high-altitude (between 1500m and 1750m above sea level), with a range of varying biomes including thicket, wetland, montane woodland, and mist-belt forest (Lane, 2009).

Essentially, there is a broad variety of vegetation with over 600 species found on the research site alone. Five species of primates occur on-site (chacma baboons, lesser galagos, thick-tailed greater galagos, samango monkeys, and vervet monkeys). There are a number of predators including several raptor species (black eagles, owls), caracals, genets, mongooses, otters, and leopards. The area is part of the temperate region, thus experiences highly seasonal changes of temperature and rainfall. The warm summers can reach over 35°C and receive approximately 250 mm of rain, and in winter will drop to an average of 21°C and can reach a minimum of 0°C at night and rainfall occurs rarely (Hahn, 2002). After analysing the weather data captured from the on-site weather monitor (Davis® Instruments) from 2012 to 2018, a resounding drop in rainfall is noted, with a gradual increase in temperature.

Aim of the study

The overall aim of the research described in this document was to investigate the physiological responses of *O. crassicaudatus* affected by seasonality. This study should provide some insight and adding on from the current limited knowledge of physiological mechanisms implemented by this species to survive successfully in a highly seasonal temperate environment. To achieve this, we have combined several contemporary research techniques, such as identifying and quantifying hormonal responses, faecal microbial communities, and metabolic variations, to evaluate the impact of seasonal changes in *O. crassicaudatus*.

In Chapter 2, I validate the most appropriate hormone assay for monitoring faecal glucocorticoid metabolite concentrations in *O. crassicaudatus*. Then, I describe the potential drivers of faecal glucocorticoid metabolite levels via least invasive methods. The ambient temperature and rainfall for the sampling period is also reported, and any significant changes between seasons are calculated. I, also, estimate the seasonal food resource availability and report the volumes of each food that were previously identified from faecal samples.

In Chapter 3, I report the results of the validation test in order to accurately measure faecal triiodothyronine metabolite concentrations. I determine the seasonal profiles of faecal triiodothyronine metabolite levels. Then, I investigate the impact of temperature, rainfall, and diet fluctuations on the metabolic activity of *O. crassicaudatus* across seasons.

Chapter 4 describes the faecal microbiome across seasons and how this is affected by changes in diet. I identify the most prominent bacterial phyla, families, and genera across seasons.

Chapter 5 explores the urinary and serum metabolite profiles for *O. crassicaudatus* and how the metabolite concentrations are affected by seasonal changes in resources and weather. I report the significant seasonal changes in metabolites from both matrices. Also, I report in which metabolic pathways these metabolites play a role. Then, I discuss the importance of the metabolites and how their presence is essential in the metabolic pathways to ensure homeostasis within the body, especially during harsh weather conditions.

Finally, in Chapter 6, I incorporate all findings and discuss the implications for this wild population of *O. crassicaudatus* and add to the growing array of information signifying the physiological capabilities of primates during environmental change. Lastly, I highlight the importance of these research findings and propose future research pathways.

Chapter 2

Seasonal drivers of faecal glucocorticoid metabolite concentrations in an African strepsirrhine primate, the thick-tailed greater galago (*Otolemur crassicaudatus*)

Published paper based on this chapter:

Long, C., Tordiffe, A., Sauther, M., Cuozzo, F., Millette, J., Ganswindt, A., Scheun, J. (2021). Seasonal drivers of faecal glucocorticoid metabolite concentrations in an African strepsirrhine primate, the thick-tailed greater galago (*Otolemur crassicaudatus*). *Cons Physiol*, 9: coab081.

Abstract

As global non-human primate populations show dramatic declines due to climate change, land transformation, and other anthropogenic stressors, it has become imperative to study physiological responses to environmental change in order to understand primate adaptability and enhance species conservation strategies. We examined the effects of seasonality on faecal glucocorticoid metabolite (fGCM) concentrations of free-ranging male and female thick-tailed greater galagos (*Otolemur crassicaudatus*) in an Afromontane habitat. To do so, we established an enzyme immunoassay (EIA) for monitoring fGCM concentrations in the species using a biological validation. Following this, faecal samples were collected each month over the course of a year from free-ranging males and females situated in the Soutpansberg Mountains, Limpopo, South Africa. Multivariate analyses revealed lactation period was a driver of fGCM levels in females. Whereas sex and food availability mostly influenced seasonal fGCM concentrations in the total population. Thus far, the results of this study show that drivers of fGCM levels, an indication of increased adrenocortical activity, in *O. crassicaudatus* are numerous and complex within the natural environment. The species may be adapted to such conditions and an extreme change to any one component may result in elevated fGCM levels. This increases our understanding of strepsirrhine primate physiology and offers initial insights into species adaptability to a challenging environment.

Introduction

More than 60% of all non-human primate populations have been identified as highly susceptible to changes in the environment (Schloss, 2012; Schwitzer *et al*., 2019) and are consequently threatened with extinction (Estrada *et al*., 2017). Seasonal change can play an important role in the life history of different organisms, across varied habitats and time, and at least some primates will also experience naturally occurring environmental change at varying life history stages (Carré & Cheddadi, 2017). Environmental change influences reproductive success, availability of food resources, and available habitat types (van Schaik & Brockman, 2005; Frederiksen *et al*., 2014; Campos *et al*., 2017). Similarly, reproductive state will affect an individual's physiology (Rangel-Negrín *et al.*, 2009; Charpentier *et al*., 2018). A number of fields (such as behavioural and physiological) have been developed to monitor the effect of life history stages and environmental change in a given species (Homyack *et al.*, 2010; Takeshita *et al*., 2018).

Behavioural (Pruetz *et al*., 2009) and hormone-based studies have been used to assess adaptability of animals to extrinsic stressors (environmental change/reproduction) to ensure their survival (Dettmer *et al*., 2012; Goymann 2012). Naturally occurring seasonal change, such as temperature and rainfall variation, as well as fluctuations in resource availability (Feng *et al*., 2013), can induce physiological changes via the hypothalamic-pituitary-adrenal (HPA) axis leading to an increase in the production and secretion of glucocorticoids (GCs; Romero, 2002; Sapolsky, 2002). Environmental temperatures may have an inverse relationship with GC concentrations; for example, to maintain optimal body temperature, endotherms must increase their energy expenditure through the hyperactivation of the HPA axis (McKechnie and Wolf. 2019). Furthermore, behavioural attributes (such as decreased activity) will also be implemented to withstand the seasonal high and low temperatures (Wingfield 2013). In seasonal environments, there is a strong association between the dry season and increased GC concentration owing to food and water shortages (Davies *et al.*, 2013; Hernandez *et al*., 2018).

For example, an increase in GC levels during seasonal dry periods ensures a constant source of energy through the metabolism of stored fat as shown in Magellanic penguins (*Spheniscus magellanicus*; Walker *et al.* 2005). However, once energy reserves are depleted, a decrease in GC production is found to maintain homeostasis. In addition to meeting energy requirements, elevated GC levels have also been found during periods of reproductive activity (Romero, 2002). For example, in male primates, the mating season seems to significantly influence GC concentrations as increased aggression and activity prevail as documented in red-fronted lemurs (*Eulemur fulvus rufus*) by Ostner *et al*. (2008). Whereas, in female primates, elevated GC levels are associated with reproduction: GC secretions are necessary both during the late gestation, owing to placental and foetal lung development (Bolt *et al*., 2001; Grier *et al*., 2004), and lactation stages as these are considered energy costly (Cavigelli 1999; Williams *et al*., 2007; Rimbach *et al*., 2013). There can also be sex and age-related differences in the physiological stress response in a species. For example, wild ring-tailed lemurs (*Lemur catta*), responded differently to the ecological consequences of cyclones and droughts, with adult females' responses being more variable during droughts, adult males' responses more variable during cyclones and subadults having higher cortisol values during cyclones (Fardi *et al*., 2017). An acute increase in GC concentrations can be adaptive in nature, leading to an increase in available energy and alterations in behaviour (Romero, 2002; Sapolsky, 2002). However, a chronic increase in GC concentrations can lead to the suppression of the immune response and reproductive abilities (McEwen 1998; Crossin *et al.*, 2016; Fardi *et al*., 2017). Hence, assessing GC concentrations of free-ranging populations, as a model for stress-associated environmental alterations, has become an important technique to better understand the effect of stressors on individuals and populations and the likelihood of survival during challenging periods (Romero 2002; Sapolsky 2002; Beehner and McCann, 2008).

Non-invasive hormone monitoring, through the utilization of faeces, has become a preferred technique when studying wildlife. The collection of faecal samples requires little to no direct human-animal interaction, while allowing for longitudinal and repeated sampling (Heistermann 2010; Touma and Palme, 2005). Furthermore, related hormone metabolite concentrations within a matrix like faeces are less affected by the episodic fluctuations of hormone secretions observed within blood (Vining *et al*., 1983; Russell *et al*., 2012). However, prior to the use of matrices like faeces for monitoring stress-related GC metabolite concentrations in a species for the first time, it is important to reliably investigate the applied test system to ensure suitable quantification of GCs for the species in question (Touma and Palme 2005; Heistermann *et al*., 2006). It is widely accepted that the stimulation of the adrenal cortex via administration of adrenocorticotropic hormone (ACTH) adequately mimics a physiological stress response within an individual (Palme 2005). However, conducting an ACTH challenge is often not possible due to the lack of accessibility to the species, the size of the individual, the research locality, or their IUCN status. Therefore, the use of a biological validation has become increasingly popular as an alternative approach (Touma and Palme, 2005; Sheriff *et al*., 2011), in which individuals are exposed to a biological stressor (such as captivity or predation cues) known to activate the HPA axis, resulting in a rise in GC secretion (Kersey and Dehnhard, 2014). The use of non-invasive measures to monitor fGCM levels in response to external stressors has been successfully demonstrated in several strepsirrhine primate species including red-bellied lemurs (*Eulemur rubriventer*; Tecot, 2008) and ring-tailed lemurs (*Lemur catta*; Gabriel *et al.*, 2018). Studies focusing on the stress response associated with reproductive state and environmental challenges has been conducted in numerous primate species including yellow baboons (*Papio cynocephalus*; Gesquiere *et al*., 2008), pileated gibbons (*Hylobates pileatus*; Pirovino *et al*., 2011), and white-faced capuchins (*Cebus capucinus*; Carnegie *et al*., 2011). On mainland Africa, endocrine monitoring to

determine adrenocortical activity has only been applied to one species of strepsirrhine primate, the African southern lesser bushbaby (*Galago moholi*; Scheun *et al*., 2015; 2016). Therefore, there is a need to increase our understanding of the influence of seasonality on the stress response in strepsirrhine primates.

The greater thick-tailed galago, *Otolemur crassicaudatus* (currently rated as Least Concern; IUCN Redlist; Masters and Génin, 2016), is an arboreal, nocturnal primate found in montane, riverine, and evergreen forests of Botswana, Swaziland (Eswatini), Uganda, Kenya, Tanzania, Zambia, Angola, Zimbabwe, Mozambique and northern/north-Eastern South Africa (Masters and Génin 2016). Within its distribution range, this species feeds predominantly on a variety of fruits, insects, and tree exudates as available (Hladik 1979; Clark 1985; Happold and Happold, 1992; Nekaris and Bearder 2011; Masters and Génin 2016). *O. crassicaudatus* is known to be polygamous (Bearder 1974), with mating occurring during the Austral winter months (Eaton *et al*. 1973; Bearder 1974). Female gestation length is estimated to be between 130 to 135 days (Manley 1966; Bearder 1974), resulting in the birth of one or two individuals during October and November (Nekaris and Bearder 2011; Masters and Génin 2016). Thus far, there is little information regarding their physiological responses to seasonality and reproductive activity. Therefore, in this study, longitudinal hormonal monitoring in this species was conducted.

This study was conducted to monitor seasonal variation in glucocorticoid hormone output in a free-ranging *O. crassicaudatus* population across differing seasonal and environmental conditions. More specifically, this study a) evaluated the suitability of four enzyme-immunoassays (EIAs) to measure faecal glucocorticoid metabolite (fGCM) concentrations in *O. crassicaudatus* by monitoring a handling event as a form of a biological

validation, and b) assessed correlated effects, such as reproductive period, climatic variables, and food availability with fGCM concentrations over a longitudinal period.

This study hypothesised that: 1) differences in fGCM concentrations between sexes will be notable; 2) fGCM concentrations in males will be elevated during the mating season caused by increased competition-related aggression and activities; 3) females will experience raised fGCM concentrations during the gestation and lactation periods owing to increased energy expenditure; and 4) fGCM concentrations will be affected by the seasonal fluctuations in ambient temperatures, rainfall, and food availability.

Materials and Methods

Study site

This study was conducted at the Lajuma Research Centre (23.0381° S, 29.4429° E), Soutpansberg mountains, Limpopo province, South Africa. Here, *O. crassicaudatus* individuals frequent the woodland and mist belt forest habitats within this highly seasonal, temperate, relatively high altitude (study area ranges from approximately 1200m to 1400m) environment (Hughes *et al*., 2005; Hahn 2017). In addition to the diverse vegetation and food resources, the site also has few human settlements. The area sampled is approximately 3 km^2, with individuals captured along one of the designated transects running in a general south-east to north-west direction (Phukuntsi *et al*., 2020). Importantly, *O. crassicaudatus* at this site appear to have large home ranges, and certain individuals travelled for up to 2 km within the sampling range (personal observations: ML Sauther, FP Cuozzo). Some individuals observed within the study site appear to travel into the site but do not necessarily reside on the property (52 of the 94 distinct *Otolemur* individuals, 55%, captured 2013 to 2018 were only captured once; unpublished data: ML Sauther, FP Cuozzo).

Weather data

For this study the seasons are referred to as winter (June–August), spring (September–November), summer (December–February), and autumn (March–May), with apparent dry and wet periods similarly defined by Nowack *et al*., (2013). Mean daily ambient temperature (°C), relative humidity (%), dew point (°C), and heat stress index (°C) were recorded from August 2017 to June 2018 using the Kestrel Drop D2 climate monitor and data loggers positioned throughout the study area (Kestrel© Instruments, Nielsen-Kellerman Co, Pennsylvania, USA). Additional climate data were logged using a Davis Instruments sensor suite linked via wireless to a Davis Pro 2™ console (Davis© Instruments, California, USA) and results were provided by the Ndlovu Node of the North-Eastern Mountain Observatories project of the South African Environmental Observation Network (SAEON). The mean daily high and low temperatures (°C) and wind chill factor (°C) were calculated for each season, while a cumulative rainfall (mm) total for each day and month were calculated. All weather monitors were placed within the study site, near trapping locations to ensure on-site weather was recorded.

Determining food availability

To assess insect availability two light traps were set up near the field campsite (trap 1: 23°02'19.6"S 29°26'34.8"E; trap 2: 23°02'20.4"S 29°26'34.6"E), approximately 20m from any other source of light, from September 2017 to June 2018. A nylon lamp was secured less than one metre from the ground, with a catching container placed below the light. The second light and catching container were secured on a tree branch approximately three metres above the ground. Both lights remained in the same location for the entirety of the sampling periods. Each day, the lights were turned on prior to sunset (~17:00–19:15) and turned off prior to sunrise (~04:30–06:00) each day. Once captured, the number of invertebrates and order and/or common name classification, if possible, were recorded. Insects were then stored in vials containing 70% ethanol or 90% isopropanol. In addition to these insect captures, *O.*

crassicaudatus faeces (n = 65), collected during the monthly animal captures from August 2017 to June 2018 (see below), were assessed for insect and seed abundance. Individually identified freeze-dried faecal samples were dissected and seed and insect parts separated. All seeds were counted with the presumption that each seed represented one fruit, except for fig (*Ficus* sp.) fruit which constitute numerous, tiny seeds. The insect remains were measured in a 1.7 ml vial to the nearest 0.1 ml. The remains were identified if possible, and the volumes of the content were recorded for each sample. Additionally, monthly collections of gum from all trapping sites were conducted to assess gum availability from July 2017 to June 2018. Gum samples were removed from trees using a sharp knife, avoiding removal of tree bark, and once transferred to the laboratory, were weighed in grams. Trees were selected based on visible presence of gum, however as observations of *O. crassicaudatus* feeding activities increased, more trees were added (personal observations: C Long, JB Millette). The gum samples were stored in a frozen state for future nutritional content analysis.

Animal captures

Captures were conducted once a month from May 2017–June 2018 utilizing a total of 48 traps, which were set-up throughout the study site. In addition to the monthly captures, a more comprehensive capturing period was conducted over seven days approximately every three months. These sampling seasons were used to identify different reproductive periods based on Bearder (1974) and Masters *et al*. (1988) and Millette, Sauther, and Cuozzo (person. obvs): June 2017 (mating 1), September 2017, January 2018 (lactating), March/April 2018 (post-lactating), May/June 2018 (mating 2). These interval events were accompanied by a certified veterinarian to perform health checks and confirm reproductive status on the captured individuals.

Throughout the study an attempt was made to trap and sample a minimum of 20 adult animals (10 males; 10 females) every month. Havahart® traps (Woodstream Corporation,

43cm x 17.8cm x 17.8cm, Lititz, PA, USA) were placed into designated trees and baited with a mixture of peanut butter and bananas, Traps were set at sunset (~17h00-18h30) and checked at sunrise (~05h00-07h00). Previous sampling periods determined the individuals were more visibly stressed (for instance, pacing) when traps were checked throughout the night prior to being transported to the field lab area for sampling; thus, individuals were kept in the traps throughout the night surrounded by familiar sounds and conditions. Furthermore, previous camera trap evidence indicated multiple periods of *O. crassicaudatus* activity throughout the night (pers. comm. ML Sauther, FP Cuozzo). Only fresh faecal samples were collected, namely, evident of moist, soft texture, further eliminating the possibility that old samples were collected. Samples that fell from the trap or were contaminated with urine were not analysed in this study. Traps were cleaned with a mixture of isopropanol (90%) and water after each successful capture. Additionally, all fresh faeces collected from an individual in one night (constituting one sample) were pooled together during the extraction procedure to avoid any effects of glucocorticoid diurnal cyclicity.

When captured, an individual was removed from the trap using a carrier bag, where it was weighed, sexed, scanned for a passive identification transponder (see below), to determine the animal's identity, and then released at the site of capture. All faecal samples within the trap were collected and stored at -20°C until analysis.

During the comprehensive captures, the project veterinarian assessed the health, and confirmed the age-class and reproductive status of all captured individuals. Pregnancy was determined by abdominal palpation in September 2017, and lactation confirmed in January 2018. However, some females were not recaptured (7 out of 20 different individuals), and therefore their reproductive status could not be confirmed. The status of the vulva (open/closed – if open this indicates receptive to mating) was noted, and the length of the vulva and nipple in females were measured to determine reproductive maturity and lactation (for instance, long

nipples are associated with adult lactating females). In males, testes length and width were measured to support confirmation of reproductive status (for instance, large testes are associated with the mating season). Males captured in other sampling periods (when females are categorised as lactating/post-lactating) of the year were identified under the "non-mating" period. All individuals captured for the first time were injected subcutaneously on the dorsal side (mid-back region) with a passive identification transponder (ID100 Trovan®, EURO I.D., Weilerswist, Germany) to determine individual identity during future captures.

Biological validation experiment

A biological validation (handling event) was conducted in May 2018 using one known male and female from the free-ranging population to establish a method for measuring fGCM concentrations, as an indication of adrenocortical activity, in *O. crassicaudatus.* As no facility within South Africa could provide captive *O. crassicaudatus* for the validation process at the time of the study, a male and female from the free-ranging population at the Lajuma Research Station were used.

The veterinarian confirmed the female was not pregnant or lactating at the time of capture. Once all morphometric samples were collected, both individuals were housed in separate outside cages (350 cm x 120 cm x 230 cm) for a total of seven days. To remove the possibility of environmental contamination of faecal samples, a sheet of tarpaulin was placed at the bottom of each cage to catch the faecal samples and allow the run-off of urine. A black tarpaulin sheet and shade cloth were used to cover the top of the cage to limit sunlight during the day. Branches were placed throughout each cage and a makeshift nest was hung in a dark corner for the comfort of both individuals. The cages were placed in an area away from human activity to decrease anthropogenic stressors on the animals. Individuals were fed a diet of available fruit, dry cat food pellets (Catmor®, South Africa), and peanut butter. Water was

available *ad libitum*. A veterinarian ensured the health of the study animals throughout the validation period.

Both individuals were left to acclimatise to the new environment for two days. During this period, no samples were collected, and human interaction was limited to reduce possible psychological distress. The nature of the validation process and the need for a registered veterinarian on-site during the process limited the time available for animal acclimation. On day three and four, faecal samples were collected hourly during the night (18h00–05h00) and bi-hourly during the day (06h00–18h00) to determine baseline fGCM concentrations of both individuals. At 18h00 on the fourth day, individuals were captured in baited Havahart® traps within the enclosure, then were appropriately held by the back of the neck and pelvic region for two minutes to prevent any risk of injury to the animals by the veterinarian in order to facilitate a stress response. The entire process was completed in less than five minutes. Sample collection continued for a further 72 hours until 18h00 on the seventh day when both individuals were released at their respective site of initial capture. Samples were collected by reaching into the enclosures through a mesh opening, while preventing additional distress on the individuals. Faecal samples were collected by using sterilised forceps and placed into a 1.5 ml microcentrifuge tube, labelled, and stored at –4°C within 20 minutes of collection. Samples were kept frozen for approximately one week until reaching the Endocrinology Laboratory of the SANBI National Zoological Garden Pretoria.

Although fGCM patterns may have been elevated during the acclimation period, the aim of this validation experiment was to determine whether one of the available EIAs could measure an increase in fGCM levels (and adrenocortical activity) following a stressful event. The results of the validation process confirmed that the chosen EIA could do so.

Faecal steroid extraction and analysis

Sample extraction was conducted following the methods used by Fieß *et al*., (1999). Faecal samples were lyophilised, pulverised, and sieved through a thin mesh to remove non-faecal matter. Subsequently, 1.5 ml of 80% ethanol was added to 0.050–0.055 g of faecal powder and vortexed for fifteen minutes, before being centrifuged at 1500 x *g* for ten minutes. The supernatant was then transferred to a clean 2.5 ml microcentrifuge tube and stored at -20°C until analysis.

A total of 30 faecal samples from the validation experiment were extracted for analysis: pre-handling: male = 5 samples, female = 5 samples; post-handling: male = 10 samples, female = 10 samples. Faecal extracts resulting from the biological validation process were measured for immunoreactive fGCM concentrations using four EIAs: (i) oxoaetiocholanolone I (detecting 11,17 dioxoandrostanes), (ii) oxoaetiocholanolone II (detecting fGCMs with a 5β-3α-ol-11-one structure), (iii) cortisol, and (iv) 11β-hydroxyaetiocholanolone. Details of the assays, including cross-reactivities, are described by Palme and Möstl (1997) for oxoaetiocholanolone I, Möstl *et al.,* (2002) for oxoaetiocholanolone II and cortisol, and Frigerio *et al*., (2004) for 11β-hydroxyaetiocholanolone. The test of parallelism confirmed serial dilutions of faecal extracts gave displacement curves that were parallel to the respective standard curve (slope < 4%). Inter-assay coefficients of variation (CV) of high- and low-value quality controls were a) 11.72% and 11.75% for oxoaetiocholanolone I, b) 10.70% and 10.97% for oxoaetiocholanolone II, c) 12.20% and 14.38% for cortisol (combined both validation and seasonal CVs), and d) 6.13% and 14.47% for 11β-hydroxyaetiocholanolone. Two plates were used for each EIA for the inter-assay CVs. The intra-assay CV of high- and low-value quality controls were a) 5.65% and 6.11% for oxoaetiocholanolone I, b) 2.15% and 2.21% for oxoaetiocholanolone II, c) 5.67% and 6.90% for cortisol, and d) 7.43% and 6.96% for 11β-hydroxyaetiocholanolone. The sensitivities of the EIAs used were 0.6 ng/g DW for all assays

except 11β hydroxyaetiocholanolone, which was 1.2 ng/g DW. Assays were conducted on microtiter plates as described by Scheun *et al*. (2016).

For the seasonal fGCM evaluation, 185 samples from 24 male (total samples = 76, 3.44 ± 2.58 SD samples/individual) and 21 female (total samples = 109, 5.30 ± 5.36 SD samples/individual) free-ranging individuals were analysed using the cortisol EIA (see Results: Biological validation).

Data analysis

EIA validation

To calculate baseline fGCM concentrations during the biological validation experiment, an iterative process was implemented that excluded baseline samples greater than the mean plus 1.5 SD for both *O. crassicaudatus* individuals (Ganswindt *et al*., 2014; Scheun *et al*. 2015). Here, all fGCM concentrations collected before and after the handling stressor greater than the mean plus 1.5 SD were excluded, the average recalculated, and the process repeated until no values exceed the new mean (the baseline) plus 1.5 SD. To determine the effect of handling, the absolute change in fGCM concentrations was determined by calculating the quotient of baseline and post-handling peak fGCM samples. Subsequently, a 100% (1-fold) increase in the calculated response indicated baseline value and not a change in HPA response (as demonstrated in Scheun *et al*. 2018).

Seasonal analysis

All statistical analyses were conducted in R (R Core Team, 2020). All fGCM concentrations were presented as micrograms of immunoreactive hormone metabolites per gram of dry faecal powder weight (µg/g DW). P-values < 0.05 were deemed significant.

Seasonal fluctuations (winter, spring, summer, autumn) in food availability were assessed by conducting ANOVA tests on insect counts from the light traps, faecal seed counts

and faecal insect volumes. ANOVA tests were conducted on the seasonal values for rainfall and ambient temperature to determine any significant fluctuations. Mean fGCM concentrations (± standard deviation) were determined separately for males and females.

Covariates of free-ranging male and female fGCMs were assessed by analysing the variation of fGCM concentrations from 25 male (n = 76) and 20 female (n = 109) individuals. Initially, t-tests were used to identify any significant differences in fGCMs between sexes and seasons (Winter, Autumn, Summer, Spring). Linear mixed models (LMMs) were used to explore the variation in fGCM hormone response within the population in response to annual seasonal effects using the *lmer* function from the 'lme4' package (Bates *et al*., 2014). Global model sets were used. After using Q-Q plots to visually assess that normality of the data met the assumptions of the model, log-transformed fGCM concentrations were used as the response variable. All quantitative fixed effects were z-transformed (Schielzeth, 2010) for more accurate model fittings and to facilitate model estimate comparisons. Two models were used for analysis as reproductive status was only confirmed in individuals sampled during the comprehensive sampling including health checks. In the first model the effects of environmental and dietary factors were assessed: sex, ambient temperature (°C), rainfall (mm), insect count, seed count, and gum availability were used as fixed effects. In the second model, the influence of sex, each reproductive status (lactating, mating 1, mating 2, post-lactating) and the interaction of sex and reproductive status on fGCM concentrations was described. Both models combined both sexes in the analyses. For both models, Individual ID was used as the random effect to avoid pseudo-replication (Millar and Anderson, 2004). Collinearity was checked by determining Variance Inflation Factors (VIF; variables were excluded if VIF > 3; Field 2009; Neter *et al*., 1996; Dias *et al.*, 2017; Chaves *et al.*, 2019) using the *vif* function from the "car" package applied to standard linear models for both sexes excluding the random effects (Fox and Weisberg, 2016). Models were selected using the *dredge* function in MuMIn" package (Barton 2018). The

candidate models were ranked using AIC small sample correction (ΔAICc; Anderson, 2007). Marginal (mR^2) and conditional *R*-squared (cR^2) values were calculated for each model, explaining variance by the random (mR^2) and the random and fixed variables (cR^2), to justify the model selection (Nakagawa and Schielzeth 2013; Nakagawa *et al*. 2017). R^2 values were determined using the *rsquared* function in the "piecewiseSEM" package (Lefcheck *et al.*, 2016). Following the method suggested by Grueber *et al.* (2011), model averaging was performed, considering models with $\Delta AIC_C < 2$ to have a strong support. The *confint* function was used to determine confidence intervals. Likelihood ratio tests were conducted (using the *anova* function in the "car" package) to assess whether each selected model performed better when compared to the null model.

Results

EIA Validation

All four EIAs showed a considerable increase in fGCM concentrations following the handling event (Tab. 2-1). However, in the male it was the cortisol EIA which showed the highest peak fGCM increase (585%) at 10.5 h post-handling (Tab. 2-1; Fig 2-1a.). Similarly, the cortisol EIA also showed the highest fGCM increase (168%) 9.5 h post-handling in the female (Tab. 2-1; Fig 2-1b). As such, the cortisol EIA was chosen as the most suitable assay for monitoring fGCM concentrations in both sexes of *O. crassicaudatus*.

Table 2-1. The baseline + 1.5 SD, peak values (µg/g DW), and the change from the baseline peak percentage (%) determined for the male (n = 1) and the female (n = 1) *O. crassicaudatus* individuals during the biological validation. Bolded percentages indicate the highest percentage peak change from baseline.

	Oxoaetiocholanolone I	Oxoaetiocholanolone II	Cortisol	11β-hydroxyaetiocholanolone
Male				
Baseline + 1.5 SD	0.07	0.27	2.15	0.79
Peak (ug/g DW)	0.11	0.67	14.72	3.28
Peak response (%)	57.14	149.22	**584.82**	312.83
Female				
Baseline + 1.5 SD	0.10	0.30	4.43	1.69
Peak (ug/g DW)	0.10	0.65	11.89	2.50
Peak response (%)	71.83	117.95	**168.41**	48.06

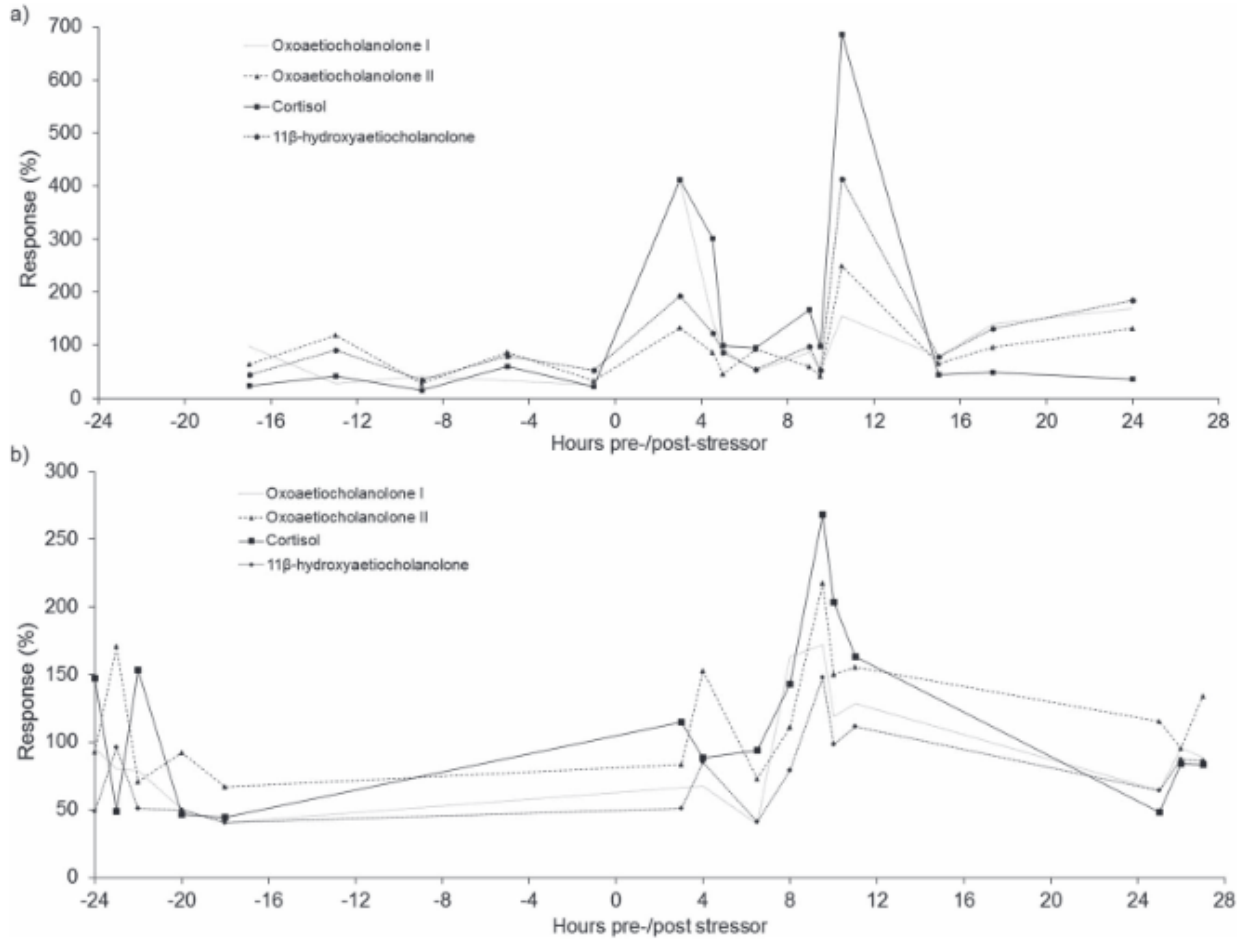


Figure 2-1. The a) male and b) female *O. crassicaudatus* fGCM response (%) for each EIA before and after the stressor (handling event). Time zero indicates handling event.

Seasonal variation in food abundance

A total of 3820 insects were collected from the light traps from September 2017 to June 2018 (185 nights). Overall, there were significant seasonal differences in insect abundance (ANOVA: $F_{(287)} = 6.89$, $p = 0.002$), specifically between autumn and spring (Tukey honest significant [HSD]: $p = 0.019$; Tab. 2-2), and autumn and summer (Tukey HSD: $p = 0.001$). The lowest number of insects were captured during the winter period and was significantly different to the summer period (Tukey HSD: $p = 0.038$).

Table 2-2. Measurements of food availability for *O. crassicaudatus* collected. Insects were trapped in light traps, and their number counted, from September 2017 to June 2018. Subsequently, the number of seeds and insect content in faeces (ml) for each month were counted and weighed, respectively, from a subset of the total faecal samples. Gum density (grams) was determined by weighing the samples collected from July 2017 to June 2018. Gum and faecal samples were not collected in December 2017. Sample size is denoted by the number in brackets following the total value. Mean values are followed by standard deviation values.

Season	Insect Count	Faecal Seed Count	Mean (±SD) Faecal Insect Volume (ml)	Mean (±SD) Gum Density (g)
Winter	27 (8)	12 (3)	0.50 ± 0.21 (21)	0.45 ± 0.04 (62)
Spring	1212 (29)	281 (12)	1.23 ± 0.42 (8)	0.45 ± 0.04 (62)
Summer	1793 (85)	361 (13)	3.48 ± 1.98 (21)	1.34 ± 0.18 (69)
Autumn	747 (63)	1378 (37)	2.21 ± 1.43 (19)	0.78 ± 0.06 (64)

A total of 2032 seeds were recovered from 65 faecal samples. The majority of seeds found in faeces originated from fig (*Ficus* sp.) and jojoba (*Simondsdia chinensis*) fruits. The highest number of seeds collected were during the autumn season (Tab. 2-2); however, the analysis of faecal seed count revealed no significant differences between seasons (ANOVA: $F_{(61)} = 0.52$; $p = 0.67$).

A total volume of 18.27 ml of insect content was recovered within the faecal samples. Documentation of insects in the faeces was limited as identification was difficult but social insects such as termites and ants were present. The analysis of faecal insect content was significantly lower in autumn (0.18 ± 0.29 SD) compared to the summer months (0.54 ± 0.60 SD; $F_{(61)}=3.22$, $p = 0.029$; Tukey HSD: $p = 0.016$).

Vachellia karroo is the only tree species from which *O. crassicaudatus* individuals were observed consuming gum. Total gum volume of 182.7 g from 257 samples were retrieved (one week per month) and a significant difference in seasonal volume was determined ($F_{(253)}$ = 12.48, $p < 0.001$). Gum density measured in summer was significantly higher than in autumn (Tukey HSD: $p = 0.010$), spring (Tukey HSD: $p<0.001$) and winter (Tukey HSD: $p<0.001$; Tab. 2-2).

Seasonal weather variations

Seasonal differences were determined for rainfall and ambient temperature. Rainfall peaked in February 2018 (232mm) with a significant rise in the summer months, compared to winter ($F_{(9)}$ = 3.45, $p = 0.065$; Tukey HSD: $p = 0.05$) in which the study site received less than 1 mm of precipitation (Fig. 2-2). Ambient temperatures were significantly warmer in the spring (range = 9.60–30.30 °C; mean = 18.74 ± 0.96 °C; $F_{(9)}$ = 21.49, $p = 0.001$) and summer months (range = 11.3–34.0 °C; mean = 21.04 ± 1.13 °C; Tukey HSD: $p < 0.001$) than in winter (range = 4.4–29.6 °C; mean = 14.85 ± 0.59 °C; Fig. 2-2).

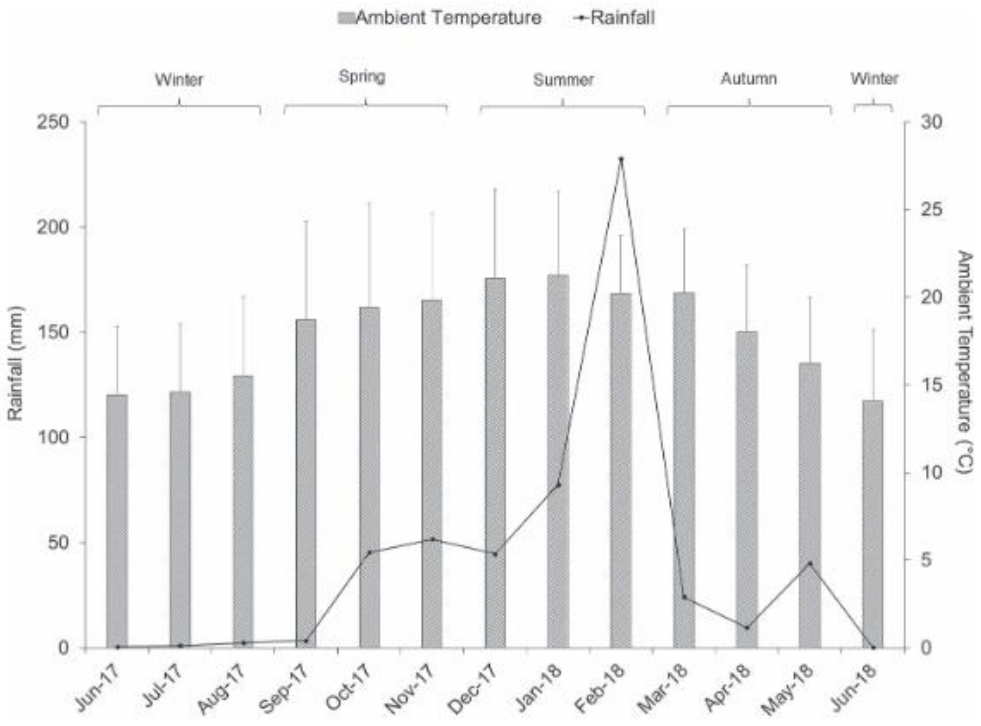


Figure 2-2. Cumulative monthly rainfall (mm) and mean monthly ambient temperature (°C) throughout the sample period (June 2017 to June 2018) for Lajuma Research Station in the Soutpansberg Mountains, Limpopo. Error bars indicate the standard deviation.

Seasonal reproductive differences

No significant differences were seen in adult male fGCM levels between the reproductive states (ANOVA: $F_{(27)} = 1.92$, $p = 0.153$) with only slight fGCM elevations during the non-breeding period (Tab. 2-3; Fig. 2-3). Additionally, testes length also exhibited no significant difference across the reproductive periods; however, a slight increase in testicle length was measured in both mating periods compared to the non-breeding period (Tab. 2-3). Adult female fGCM levels during the lactating period were significantly higher than during the mating 1 (ANOVA: $F_{(37)} = 4.65$, $p = 0.008$; Tukey HSD: $p = 0.014$) and post-lactating period (Tukey HSD: $p = 0.043$; Tab 2-3; Fig. 2-3); however, they did not significantly differ from the mating 2 period (Tukey HSD: $p = 0.580$). Furthermore, nipple length increased significantly in lactating period (ANOVA: $F_{(19)} = 5.46$. $p = 0.015$) between mating 1 (Tukey HSD: $p = 0.018$) and mating 2 (Tukey HSD: $p = 0.031$; Tab. 2-3); however, vulva length expressed no significant changes across the reproductive periods (ANOVA: $F_{(21)} = 0.25$, $p = 0.785$; Tab. 2-3).

Table 2-3. Mean ± standard deviation of female and male fGCM concentrations, vulva length, nipple length, testes width, and testes length of the O. crassicaudatus population at the Lajuma Research Centre over the reproductive periods between June 2017 and June 2018. Sample size is given in brackets.

	Reproductive period				
	Mating1	Lactating	Post-lactating	Mating2	Non-breeding
Female [fGCM]	0.31 ± 0.15 (10)	0.88 ± 0.64 (11)	0.27 ± 0.15 (5)	0.66 ± 0.33 (12)	0.46 ± 0.42
Male [fGCM]	0.35 ± 0.25 (7)	0.43 ± 0.29 (7)	0.12 ± 0.06 (5)	0.40 ± 0.20 (9)	0.43 ± 0.29
Vulva length	2.90 ± 0.94 (9)	2.99 ± 0.79 (7)		2.92 ± 0.69 (11)	
Nipple length	0.37 ± 0.08 (6)	0.56 ± 0.21 (7)		0.40 ± 0.16 (8)	
Testes width	1.56 ± 0.23 (5)	1.06 ± 0.41 (8)		1.54 ± 0.32 (7)	1.60 ± 0.30 (3)
Testes length	2.46 ± 0.53 (5)	1.89 ± 0.51 (7)		2.40 ± 0.19 (7)	1.89 ± 0.51 (3)

Seasonal variation in fGCM concentrations

Overall, fGCM concentrations differed significantly between females (n = 109, mean = 0.55 ± 0.45 µg/g DW) and males (n = 76, mean = 0.34 ± 0.29 µg/g DW; $t_{(181)} = 3.55$ $p < 0.001$). There was no seasonal difference in fGCM concentrations in male individuals ($F_{(72)} = 0.40$, $p = 0.751$). Female fGCM concentrations were significantly different between seasons: $F_{(104)} = 2.83$, $p = 0.04$; specifically, summer (n = 14, mean = 0.86 ± 0.56 µg/g DW) was significantly higher than winter (n = 27, mean = 0.46 ± 0.41 µg/g DW; Tukey HSD: $p = 0.03$) and autumn (n = 45, 0.51 ± 0.43 µg/g DW; Tukey HSD: $p = 0.049$).

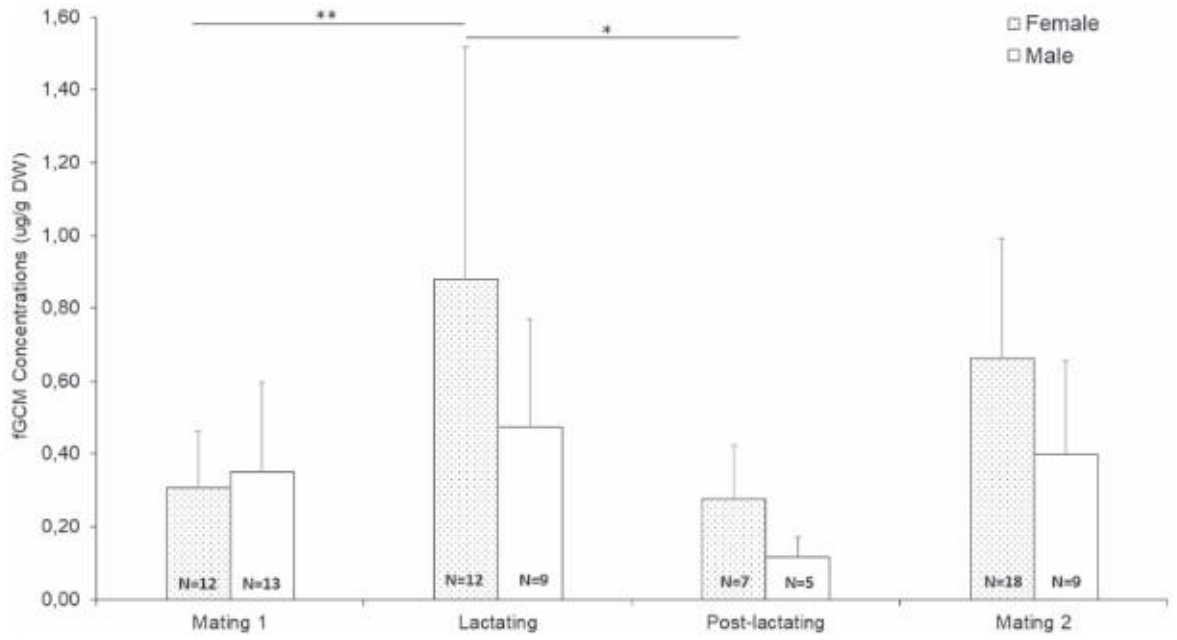


Figure 2-3. Mean fGCM concentrations for male and female *O. crassicaudatus* populations depicted by the allocated reproductive periods (Mating 1 = June–July 2017, Lactating = January 2018; Post-lactating = March 2018, Mating 2 = May–June 2018). Bars indicate the standard deviation for values for each season. Significant differences in fGCM concentrations between periods are illustrated by bar and asterisk (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). Sample size is indicated by N.

From the two global models, significant effects of sex ($\chi^2(1) = 9.56$, $p = 0.002$, $R_{m/c} = 0.08/0.19$), insects ($\chi^2(1) = 4.74$, $p = 0.03$, $R_{m/c} = 0.02/0.20$), gum availability ($\chi^2(1) = 9.27$, $p = 0.002$, $R_{m/c} = 0.04/0.23$; Tab. 2-4; Fig. 2-4) contributed to the environmental model, and sex ($\chi^2(1) = 2.825$, $p = 0.03$, $R_{m/c} = 0.01/0.20$), lactating ($\chi^2(3) = 22.08$, $p = 0.001$, $R_{m/c} = 0.22/0.40$; Tab. 2-4; Fig. 2-4), and post-lactating reproductive status ($\chi^2(3) = 2.641$, $p = 0.020$, $R_{m/c} = 0.10/0.18$) had an effect on fGCM concentrations. When the sex and reproductive status interaction was added to the model, sex ($\chi^2(1) = 13.852$, $p = 0.003$, $R_{m/c} = 0.12/0.26$), lactating ($\chi^2(3) = 3.000$, $p = 0.014$, $R_{m/c} = 0.08/0.20$), and post-lactating ($\chi^2(3) = 2.600$, $p = 0.022$, $R_{m/c} = 0.06/0.20$) reproductive status showed significant effects to the reproductive status model. The best-fit model included the variables sex, gum availability, ambient temperature, and insect availability ($\chi^2(7) = 26.58$, AICc = 417.58, $R_{m/c} = 0.16/0.26$; Tab. 2-5); however, no significant effect of ambient temperature was determined ($\chi^2(1) = 3.204$, $p = 0.073$, $R_{m/c} = 0.02/0.18$; Tab. 2-5).

Table 2-4. Model-averaged LMMs of faecal glucocorticoid hormone variation in free-ranging *Otolemur crassicaudatus.* Two global models were used to firstly identify the main environmental parameters (rainfall, ambient temperature, food availability, and sex), and second, the reproductive stages influencing fGCM concentrations. Parameters shown are model-averaged parameter estimates (β), standard error (SE), chi-squared values (ChiSq) and corresponding p-values, and 95% confidence intervals.

Predictors	***(β)***	***SE***	***ChiSq***	***P-value***	***95% CI***
Environmental					
Intercept	-0.540	0.274			-1.081, 0.000
Rainfall	0.033	0.028	0.852	0.356	-0.023, 0.089
Ambient temperature	-0.039	0.016	3.204	0.073	-0.069, -0.008
SexMale	-0.434	0.142	**9.563**	0.002	-0.718, -0.150
Gum	0.030	0.012	**9.270**	0.002	0.006, 0.055
Insects	0.009	0.005	**4.738**	0.029	-0.002, 0.019
Seeds	0.001	0.001	0.985	0.321	-0.001, 0.002
Reproduction					
Intercept	0.853	0.101			0.653, 1.054
ReproductiveState			**2.573**	**0.019**	
Lactating	-0.570	0.161	**22.080**	**0.031**	-0.892, -0.251
Mating1	-0.583	0.141	0.830	0.270	-0.864, -0.301
Mating 2	-0.224	0.126	0.960	0.204	-0.477, 0.026
Post-lactating	-0.234	0.089	**2.641**	**0.020**	
SexMale	-0.410	0.150	**13.852**	**0.003**	-0.709, -0.111
Sex:ReproductiveState			**25.731**	**<0.001**	
Sex:Lactating	0.212	0.071	**3.000**	**0.014**	0.481, 0.816
Sex:Mating1	0.612	0.204	0.882	0.380	0.209, 1.016
Sex:Mating2	0.174	0.205	1.119	0.355	-0.230, 0.766
Sex:Post-lactating	0.269	0.251	**2.600**	**0.022**	-0.233, 0.583

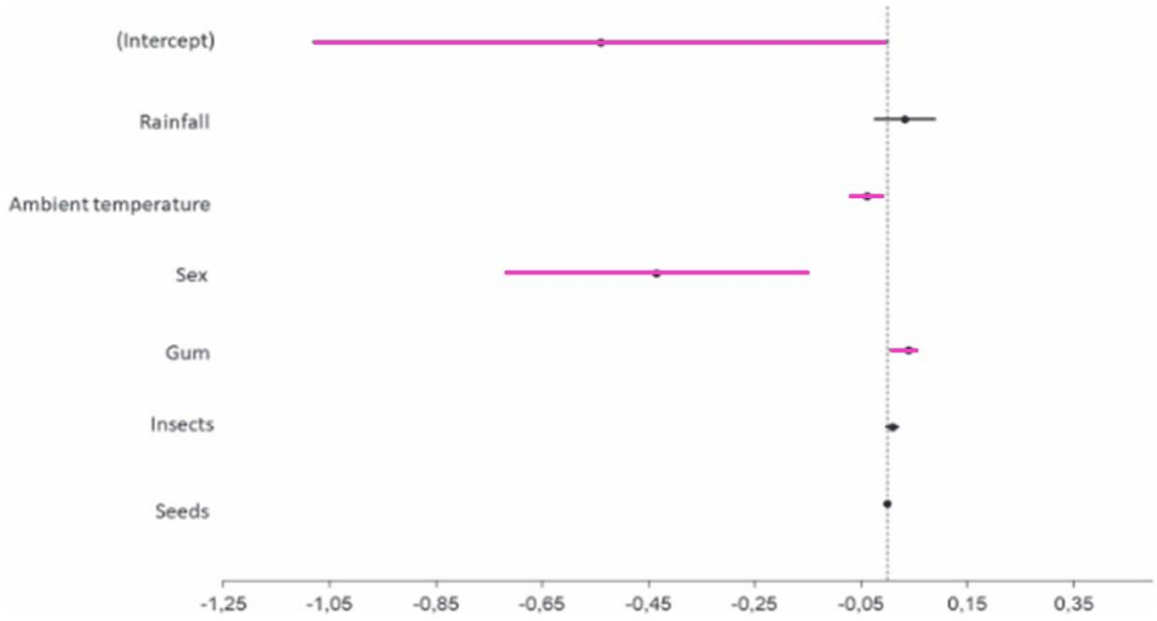


Figure 2-4. Coefficient plot depicting the beta coefficients (β) for each parameter in the model selected *O. crassicaudatus* fGCM concentrations. These parameters indicate a negative or positive influence on the fGCM concentrations. The parameters significantly influencing fGCMs are highlighted in pink. The error bars extending from the marker indicate 95% confidence intervals.

Table 2-4. Best fit model selection results for averaged mixed effects models with modelling fGCM levels in free-ranging *O. crassicaudatus* individuals (n = 185). Individual ID was used as the random effect in all the models (1|ID). For each model, we report marginal (mR^2) and conditional R-squared (cR^2) values, number of parameters (K), Akaike's Information Criterion with small sample correction (AICc), AICc distance from the best model (Δ), Akaike weight (w_i), and likelihood ratio tests (ChiSq) comparing the null models to the selected models. *** ($p<0.001$) indicating significant difference between selected model and the null model.i.

Model: log~	mR^2	cR^2	**df**	**AICc**	**ΔAICc**	w_i	**ChiSq**
Gum + Ta[1] + Sex + Insects + (1|ID)	0.16	0.26	7	417.58	0.00	0.37	26.58***
Gum + Ta + Sex + (1|ID)	0.15	0.25	6	418.36	0.78	0.25	23.63***
Gum + Ta + Sex + Insects + Rainfall + (1|ID)	0.16	0.27	8	418.50	0.92	0.23	27.84***
Gum + Ta + Sex + Insects + Seeds + (1|ID)	0.16	0.26	8	419.52	1.94	0.14	26.82***

[1]Ta refers to ambient temperature.

Discussion

EIA Validation

This is the first study to successfully monitor seasonal fGCM concentrations as an indication of adrenocortical activity, in *O. crassicaudatus* using a validated EIA. Our study, therefore, attests to the use of this method for validating EIAs in wild individuals and provides new insight into the seasonal physiological responses of this nocturnal African primate species. The use of a biological stressor (capture, captivity, and physical restraint) sufficiently induced an acute stress response in the *O. crassicaudatus* individuals, resulting in elevated fGCM levels. Similar approaches for establishing EIAs for measuring fGCM concentrations have been successfully used in several mammal species, for instance the grey mouse lemur (*Microcebus murinus*; Hämäläinen *et al*., 2015) and southern elephant seals (*Mirounga leonina*, Engelhard *et al*., 2002). The results indicate a second peak prior to the highest peak in the cortisol EIA in both the male and female data three hours after the handling stress occurred. This could be due to an extrinsic event or stressor such as another animal nearing the enclosures or urine contamination causing an earlier peak. There is a possibility the results shown in this study were as a result of trapping rather than as a representative of the physiological state of an

individual. As the enclosures are situated outside, the exact cause of fGCM elevation cannot be accounted for.

Sex differences in individual baseline fGCM values

During the biological validation, we report a considerable difference between the male and female individual baseline fGCM values, with the female expressing higher baseline fGCM levels. Such differences have been reported in other primate species such as the pied tamarin (*Saguinus bicolor*; Armstrong and Santymire 2013) and the common marmoset (*Callithrix jacchus*; Johnson *et al*., 1996). This may be a result of sex-related differences in metabolic processes and dietary differences altering gut passage time (Goymann 2012; Hämäiläinen *et al*. 2016), or that female adrenocortical activity may be elevated in this species. Differences in metabolic processes and reproductive activities could be factors contributing to these changes (Millspaugh and Washburn 2004; Goymann 2012).

Reproduction

More energy-demanding reproductive conditions, such as lactation, could also cause significant changes to the concentration of hormone metabolites secreted (Boonstra *et al*., 2001). Sex differences in fGCM concentrations can be caused by a number of factors associated with reproduction (such as, gonadal steroid hormone synthesis; Handa *et al*., 1994; Cavigelli *et al*., 2003). In the results, females exhibited greater fGCM concentrations than males in all reproductive states except Mating 1. Male primates can increase their GC secretions during the mating period where they exhibit greater aggression and may expend more energy such as seen in ring-tailed lemurs (*Lemur catta*; Starling *et al*., 2010), Verreaux's sifakas (*Propithecus verreauxi*; Fichtel *et al*., 2007), and collared brown lemurs (*Eulemur collaris*; Balestri *et al*., 2014). In contrast, primates who exhibit a low degree of mate competition, such as the tufted

capuchin monkey (*Cebus apella*; Lynch *et al*., 2002), red-bellied lemurs (*Eulemur rubriventer*; Tecot 2013), and rhesus macaques (*Macaca mulatta*; Higham *et al.*, 2013), show limited no HPA hyperactivation during the breeding season.

The models revealed lactating reproductive status as a driver of adrenocortical activity in female *O. crassicaudatus*. In this study, females in the lactating period had the highest fGCM concentrations and the model revealed lactation state to have an influence on fGCMs. Energy-demanding reproductive conditions, such as lactation, could cause significant changes to the concentration of hormone metabolites secreted (Boonstra *et al*., 2001) for mammals (McLean and Smith, 1999) and specifically primates (Dufour and Sauther, 2002), and is often associated with elevated GC concentrations when compared to other non-reproductive stages (Rimbach *et al.*, 2013; Charpentier *et al.*, 2018). An increased secretion of glucocorticoid may assist in mobilising energy reserves during the lactation process (Romero and Wingfield, 2015) as reported in rhesus macaques (*Macaca mulatta*; Maestripieri *et al.*, 2008). Therefore, elevated female fGCM levels in the summer period could reflect lactation and increased energy expenditure owing to offspring protection (Ostner *et al*., 2008), as reported in chacma baboons (Beehner *et al*., 2005). It must be noted that elevated glucocorticoid hormones may also be affected by other physiological activities. For instance, glucocorticoid hormone secretion may increase in consequence of the development of foetal organs and adrenal gland during the late gestation phase (Ishimoto and Jaffe, 2011; Huang *et al.*, 2012). Furthermore, inter-female competition and aggression for resources has been shown to elevate glucocorticoid secretions within animals (Creel *et al*., 2013), previously demonstrated in *G. moholi*, (Scheun *et al*., 2015), and could contribute to the elevated female fGCM concentrations during summer.

No significant increase was found in male fGCM levels and testes size during the mating period. Contrasting patterns have been observed in several group-living strepsirrhine species in which males will express significantly elevated fGCMs during the mating season, as

seen in male ring-tailed lemurs (*Lemur catta*; Pride, 2005), Verreaux's sifakas (*Propithecus verreauxi*; Fichtel *et al*., 2007), and brown-collared lemurs (*Eulemur collaris*; Balestri *et al*., 2014). This could indicate *O. crassicaudatus* males were not psychologically or energetically affected by the type of inter-male competition seen in strepsirrhine primates that live in complex social groups, such as *Lemur catta* (Sauther, 1991). Factors affecting testes size could be related to spermatogenesis is occurring in this species in the absence of testis size increases, male age or quality which could affect the variation in the dataset.

Insects and Gum Availability

Gum availability was correlated with fGCM values. Gum density decreased in the colder temperatures and increased during the summer months. Exudates are an essential food source for *O. crassicaudatus* (Bearder 1974) that are consumed throughout the year at Lajuma (pers. obs. C. Long, J. Millette) as such, it is to be expected that a lower-quality diet may indicate individuals have fewer energetic resources and thus higher levels of fGCMs to help combat this metabolic challenge (Beehner and McCann, 2008). Insects are an ideal energy source comprised of fats, protein, and micronutrients (Rumpold and Schlüter 2013; O'Malley and McGrew 2014). For *O. crassicaudatus*, insects most likely play an important role in meeting energy demands that gum and fruits cannot provide, especially in the warmer months during high insect density. A dramatic decline in insect populations was observed during the winter season (low ambient temperature and rainfall). However, from the results, low fGCM levels during the winter (low food availability) may indicate that this species is capable of finding an alternative method of coping, either by adjusting their metabolic rate or by opportunistically feeding on available food sources such as fruit. Nevertheless, insects can provide an important source of both protein and energy if they can be eaten in sufficient quantities (Rothman *et al*., 2014).

Conclusion

This study not only successfully validated the most appropriate EIA for monitoring fGCMs in *O. crassicaudatus* but implemented the technique in a free-ranging population to facilitate determining the drivers of adrenocortical activity within the natural environment. This study shows that seasonal changes in sex, insect and gum availability, as well as reproductive state were found to influence GC output. From the results it is apparent that energetic consequences are a major factor contributing to GC secretion, namely, reproductive state and food availability. This indicates that GC assessments constitute a reliable tool to monitor the responses of this species to changes in their environment and opens the way to further research to enhance our understanding of species-specific responses to environmental and biological changes.

Chapter 3

Seasonal drivers of faecal triiodothyronine metabolite patterns in the thick-tailed greater galago (*Otolemur crassicaudatus*)

Abstract

Natural environmental change will force species to adjust their physiology and behaviour to survive. An important component responsible for individual survival, through metabolic and thermoregulatory regulation, are the thyroid hormones. Despite their importance in this regard, few studies have determined the effect of a changing environment on thyroid hormone production. With this in mind, the aim of this study was to assess the change in faecal thyroid hormone metabolite concentrations (fTMs), as a proxy of thyroid hormone patterns, within a free-ranging population of the thick-tailed greater galago (*O. crassicaudatus*) in response to environmental and dietary changes in a seasonal environment. First, we conducted a TSH challenge to validate the most appropriate enzyme immunoassay for monitoring faecal triiodothyronine metabolite (fTM) concentrations. Following this, the seasonal impact of biotic factors (rainfall and ambient temperature), food availability, sex, and reproductive status on fTM concentrations were assessed in a free-ranging population of *O. crassicaudatus*. Overall, we found a positive relationship between food availability and ambient temperature. We were successfully able to measure fTMs for both sexes of this species. In the seasonal analysis, no significant sex differences were determined; however, fTM levels were significantly affected by changes in insect availability. We found significantly lower fTM levels during the dry months, suggesting the *O. crassicaudatus* population restricts energy expenditure during periods of low food availability. This study has provided insight into the effects of seasonality on the metabolic patterns present within the species.

Introduction

Primates have evolved a number of behavioural and physiological adaptations to survive often extreme environmental change (such as climate and resource availability; Hemingway & Bynum, 2005; Grow *et al*., 2014, Thompson *et al*., 2017). In terms of the former, species such as the Japanese macaques (*Macaca fuscata*) survive the cold northern hemisphere owing to bathing in the hot springs (Hanya, 2004; Takeshita *et al*., 2018). In the latter adaptation, the geladas (*Theropithecus gelada*) residing in the high altitudes of Ethiopia have adapted to consuming abundant, although low-quality food sources (Dunbar, 1997). Along with such physiological adaptation is the hypothalamic pituitary-thyroid (HPT) axis, which assists in restoring and maintaining homeostasis within an organism (Schmid & Kappeler, 2005).

Potential drivers activating the HPT axis include fluctuations in temperature (Low, 2011), limited resource availability or requirements (Costa-e-Sousa & Hollenburg, 2012), or reproductive activities (Rasmussen *et al*., 2021). Once activated, a cascade of events results in the production of biologically active triiodothyronine (T3) and inactive thyroxine (T4; Flier *et al*., 2000; Silva, 2003; Hunt *et al*., 2004). It is the primary role of T3 and T4 to regulate metabolism, thermoregulation, and the development of an organism under various environmental conditions (Kaack *et al*., 1979; Silva, 2006; Basset & Williams, 2016). Periods of low environmental temperature or increased resource availability will result in the hyperactivation of the HPT axis and T3 production to increase body temperature and convert excess resources into energy (Pijl *et al*., 2001; Silva, 2003; Rasooli *et al*., 2004). In contrast to this, higher ambient temperature and low resource availability will result in lower T3 concentrations but elevate inactive T4 levels to conserve energy (Blake *et al*., 1991; Flier *et al*., 2000; Douyon & Schteingart, 2002).

The change in T3 concentrations during periods of decreased resource availability has been observed in a number of wildlife species. For instance, the Stellar sea lion (*Eumetopias*

jubatus; du Dot *et al*., 2009), black-legged kittiwake (*Rissa tridactyla*; Welcker *et al*, 2013) and the African striped mouse (*Rhabdomys pumilio*; Rimbach *et al*, 2018). Additionally, differing stages of reproductive state (lactating and pregnancy) have been shown to coincide with elevated T3 levels, most likely resulting from increased energy expenditure (Rangel-Negrín *et al*, 2018).

Although monitoring endocrine patterns in serum and plasma of an individual provides real-time hormone concentrations, the need for capture and restraint is often less than ideal. Non-invasive endocrine sampling through the collection of faeces offers a means to monitoring hormone metabolite patterns as a proxy for the hormone, without the need for increasing the risk of stress and injury on an animal (Chen *et al*., 2021). Non-invasive endocrine monitoring of thyroid metabolites has been successfully implemented in several primate species such as yellow baboons (*Papio cynocephalus*; Gesquiere *et al*, 2018), yellow-breasted capuchins (*Sapajus xanthosternos*; Schaebs *et al*, 2016), Barbary macaques (*Macaca Sylvanus*; Cristóbal-Azkarate *et al*, 2016) and golden snub-nosed monkeys (*Rhinopithecus roxellana*; Chen *et al*., 2021). As such, implementing faecal thyroid hormone metabolite monitoring can provide feedback on the metabolic requirements of a primate species throughout periods of seasonal change and various life history stages. To date, no study has attempted to implement non-invasive faecal triiodothyronine metabolite (fTM) monitoring in any African strepsirrhine primate species.

O. crassicaudatus (Nash *et al*., 1989) is a nocturnal primate found throughout the northern and eastern regions of South Africa. During the wet months (October–March), *O. crassicaudatus* will feed predominantly on insects, gum exudates, and fruit which are available in high densities (Bearder, 1974; Hladik, 1979; Clark, 1985). However, as insect and fruit availability decrease during the dry months (April–September), galagos increase their gum intake (Clark, 1985; Harcourt, 1986). Mating and conception occur during the cold months of

June and July annually (Bearder, 1974; Masters, 1988); following a 130-day gestation period, parturition occurs between September and November, with lactation observed until February (Eaton et al., 1973; Ehrlich, 1974).

No information currently exists on the possible drivers of thyroid function in this species. The aim of this study was, first, to validate the most appropriate enzyme immunoassay (EIA) for monitoring fTM concentrations in the galagos. Second, to implement this validated EIA to monitor thyroid function in the species across naturally occurring seasonal changes. Here, we hypothesise an elevation in fTM concentrations as a response to 1) high resource availability increasing energy expenditure, 2) increased ambient temperature causing an increase in metabolic rate, and 3) specific reproductively active states such as pregnancy for females and the mating season in males.

Materials and Methods

Weather data

Climate data were collected from the on-site weather station (Davis Instruments sensor suite linked via wireless to a Davis Pro 2™ console, Measurement and Console Systems CC, Cape Town, South Africa). As the weather station was located on-site, the climate data retrieved was comparable to that experienced by the trapped individuals. The data is made available by the Ndlovu Node of the North-eastern Mountain Observatories project of the South African Environmental Observation Network (SAEON). Data were collected on an hourly basis. The mean (± standard deviation; SD) daily ambient temperature (°C) was calculated, while a daily cumulative rainfall (mm) total for the sampling was calculated. All weather monitors were located near trapping sites to ensure accurate on-site weather data was collected.

Determining food availability

Gum volume, and seed and insect counts were collected and recorded as described by Long *et al.* (2021). Briefly, From June 2017 to June 2018, gum exudates were collected directly from trees by scraping the gum and placing in a sealable bag. These bags were stored at -4°C until weighing. Seeds were collected, counted, and identified (where possible), taken from additional faecal samples collected during the seasonal trapping. Light traps were set up in two locations 15m apart. The lights were positioned 1m and 2m, respectively, above a bucket. Each bucket had a lid used to prevent the insects from escaping. The lights were switched on at sunset and switched off at sunrise, after which, the insects were counted and, if possible, identified. In July 2018 and December 2018 months, pitfall traps were used at two different sites to evaluate the crawling insect density. Five pitfall traps were placed two metres apart at each site. The traps were checked for insects every two weeks for the remaining duration of the study. Insects captured were counted and stored in 70% ethanol.

Seasonal fTM analysis

We identified four seasons over the two-year sampling period: dry1 (May – August 2017), wet1 (November 2017 – February 2018), dry2 (May – July 2018), and wet2 (November – December 2018) in which we collected samples. We selected specific months within the wet and dry periods to monitor fTMs as these provided the most extreme periods of temperature and rainfall. In total, 115 faecal samples were collected from 15 adult females (n = 63; average = 4.20 ± 3.91 samples/individual) and 22 adult males (n = 59; average = 2.32 ± 1.32 samples/individual) during the dry (total samples collected: n = 26), dry2 (n = 33), wet (n = 30), and wet2 (n = 25) periods.

Animal Captures

The sampling was completed over a period from June 2017 to December 2018, in which sampling occurred two weeks each month. The study follows a standardised trapping method

used in Long *et al*. (2021). Briefly, 20 Havahart® traps were secured to tree branches and baited with banana and peanut butter and set up just before sunset (17:00–18:30) and checked early in the morning (05:00–07:00). If present in the trap, individuals were trapped in capturing bags, weighed, sexed, scanned for identification using a passive identification transponder (ID100 Trojan, EURO I.D., Weilerswist, Germany), and released at the source of capture. We collected faecal samples that had collected in the bottom of the traps and placed these into plastic sample bags. Only samples which appeared fresh (dark, moist, and soft in texture) were collected. Old samples (white, hard and crumbly texture), or samples that looked to be contaminated by urine were not used for analysis. Traps were cleaned at the end of each sampling day. In addition, all samples collected from one individual in one night were pooled together as one sample to reduce the effects of hormonal diurnal cyclicity.

A comprehensive sampling event was conducted over seven days approximately every three months. These sampling periods were used to perform health checks, implant identification microchips in new individuals and confirm reproductive status of the individuals. Pregnancy was determined by abdominal palpation by the veterinarian. Testis length was measured in males to support mating status (larger testes is usually associated with the mating season). In females, the status and length of the vulva was checked (open/closed) and nipple length (more predominant nipples are associated with lactating females) was measured. Reproductive seasons were identified by Bearder (1974), Masters et al. (1988), and Cuozzo, Sauther, and Millette et al. (pers. obs): mating 1 (June 2017), lactation (January 2018), mating 2 (June 2018).

Sample storage and extraction

Throughout the validation and seasonal study, all faecal samples were placed into a microcentrifuge tube and stored in -4°C freezer at the field site. Frozen samples were transferred to the National Zoological Gardens, South African National Biodiversity Institute

in Pretoria, South Africa, for the extraction process. Here, samples were lyophilised, pulverised, and sieved through a thin mesh to remove non-faecal matter (Fieß *et al*., 1999). Subsequently, 1.5 ml of 80% ethanol was added to 50-55 mg of faecal sample and vortexed for fifteen minutes, before being centrifuged at 1500 x *g* for another ten minutes (Wasser *et al.*, 1993). The supernatants were then stored at -20°C until hormone analysis.

Physiological validation

To validate whether the enzyme immunoassay used during this study could robustly measure fTM concentrations in *O. crassicaudatus*, a thyroid stimulating hormone (TSH) challenge was conducted. As no facility in South Africa contained any captive individuals, one male and female individual from the free-ranging population found at the study site were used. This type of EIA validation has proven to be sufficient in several species (see Keech *et al*., 2009; Wasser *et al.*, 2010; Mondol *et al*., 2020). Both individuals were captured using baited Havahart® traps following the standardised trapping method, morphometric measurements recorded, and the female confirmed not pregnant or lactating by a veterinarian, then transferred to separate on-site enclosures (350 cm x 120 cm x 230 cm). A black tarpaulin sheet covered the top of the enclosure to reduce sun exposure during the daytime. A plastic sheet was laid along the bottom of the enclosure to catch faecal samples and help prevent dirt contamination. Branches were placed throughout, and a makeshift nest was constructed in a dark corner for the comfort of each individual. Individuals were fed a variety of available fruit, cat pellets (Catmor®, South Africa), and peanut butter, while water was available *ad libitum* throughout the housing period. Both individuals were left to acclimatise to the new environment for two days following introduction into the cages. During this period, human presence and interaction was limited to reduce possible distress. From the onset of the third day to the fifth day, faecal samples were collected hourly during the night (18h00–06h00) and bi-hourly during the day (06h00–18h00) in order to determine baseline fTM concentrations of both individuals. At 18h00 on the fifth

day, individuals were captured and injected with 2 IU/kg TSH (Sigma Aldrich©, Sigma Aldrich Inc, USA). Sample collection continued until 18h00 on the eighth day when both individuals were released back to their site of initial capture.

Enzyme immunoassay analysis

A total of 54 faecal samples were collected from adult galagos, pre-injection: n = 20 (female: n = 10; male: n = 10) and post-injection: n = 34 (female: n = 17; male: n = 17) were selected for analysis. Total fTM levels were determined using the DetectX® Triiodothryronine (T3) Enzyme Immunoassay Kit (Arbor Assays™, USA), following the manufacturer's instructions. T3 antibody cross reacts 100% with T3 (L-triiodothyronine), 0.88% with thyroxine (T4), and 0.1% with reverse T3 (3,3',5'-triiodo-L-thyronine).

The intra- and inter-assay coefficients of variation (CV), determined by repeated measurements of high- and low-value quality controls, ranged from 5.5% to 14.7%. The assay sensitivity of the triiodothyronine EIA was 37.6 pg/ml dry weight and limit of detection was determined as 46.6pg/ml.

Statistical Analysis

All statistical analyses were conducted in R version 3.6.3 (R Development Core Team; 2019). All values are expressed as mean ± standard deviation, and differences considered significant at $p < 0.05$. FTM concentrations are expressed at microgram per millilitre for dry weight of faecal matter (ug/ml DW).

fTM assay validation

Individual baseline values during the TSH challenge were calculated using the median of all pre-injection samples for both the male and female individuals. Peak percentage response for both individuals was calculated by dividing the peak fTM value post-injection by the calculated

baseline and multiplied by 100 (Scheun *et al.*, 2018). As such, the baseline is indicative of 100% (1-fold) response. As a result of our small sample size, no formal statistical analysis was possible and only a comparison of the pre-injection and post-injection values was conducted.

Weather fluctuations

We compared the seasonal fluctuations (dry1, wet1, dry2, wet2 periods) in ambient temperature and rainfall using the analysis of variance (ANOVA) test. If found to be significant, *post-hoc* Tukey tests were performed to determine which seasons differed significantly.

Food availability

Comparisons between seasonal insect population counts, seed counts and gum volumes were assessed using ANOVA tests. If found to be significant, *post-hoc* Tukey tests were performed to determine which seasons differed significantly.

Seasonal analysis

Mean seasonal concentrations (± standard deviation) were determined for both sexes. We explored any significant differences in fTM levels between sexes using the Mann Whitney U-test. Then, significant differences in fTM concentrations were assessed separately between reproductive periods for males and females using Kruskal Wallis testing. Seasonal changes in testes, nipple, and vulva length were assessed using ANOVA and Tukey's HSD. Seasonal differences (dry and wet periods in 2017 and 2018) in fTM concentrations were evaluated using the Kruskal Wallis test, followed by Dunn's Test if found to be significant.

To explore the impact of environmental changes and reproductive states, we evaluated potential factors driving changes in fTM levels over the sampling period. Linear mixed models (LMMs) were performed to identify the most significant variables affecting fTM concentrations in free-ranging male and female individuals over a longitudinal period using the *lmer* function from the "lme4" package (Bates *et al.*, 2010). The dependent variable was not

normally distributed (Shapiro-Wilk test: W=0.699, p<0.001), hence the fTM concentration was log-transformed prior to analysis. A global model was conducted using the fixed effects: sex, ambient temperature:insect availability, insect availability, ambient temperature, rainfall, gum, seed. Individual ID was included as a random effect controlling for non-independence. As we did not have the reproductive status, seed count, and gum availability for the entire sampling period, this was not included in the global model. For the reproductive model (Model 2), the fixed variables included reproductive period (mating 1, mating 2, lactating, non-mating) and sex, while animal ID was used as the random effect. We performed a model including reproductive status (lactating, post-lactating, mating1, mating2), seed count, gum volume, insect availability, ambient temperature, rainfall, and sex as fixed factors, while animal ID was used as a random effect. Variance inflation factor (VIF) was determined using the *vif* function in the "car" package (Barton, 2018), any factor above the threshold (VIF>3; Zuur *et al*., 2011) was discarded from the model. Model selection and averaging were conducted using the *dredge* function of the "MuMIn" package. Akaike information criterion small size sample correction (AICc) and Akaike's weight (w_i) were used to rank the best models. Models with a difference from the AICc by less than two (ΔAICc < 2) were reported. Pseudo-R squared values were also determined to support the best model selection (Grueber *et al*., 2011; Nakagawa *et al*., 2017) using the "piecewiseSEM" package. This approach was used to account for model uncertainty, especially if the best model support is not strong (w_i < 0.9; Burnham & Anderson, 2003). Candidate models were averaged using the *model.avg* function of the "MuMIn" package to obtain estimates. The *confint* function was used to determine confidence intervals. Lastly, we tested the individual effect of each predictor using the *drop1* function in "lme4" to perform likelihood ratio tests to compare the full model with the reduced models lacking a variable one at a time (Bates *et al*., 2015).

Results

Physiological validation

The male *O. crassicaudatus* fTM concentration levels increased by 58% from the pre-injection baseline value (0.18 ± 0.08 ug/ml; Fig. 3-1) to the highest peak, post-injection value (0.31 ug/ml) with a lag time of 10.5 hours. Similarly, the fTM concentrations in the female showed a 70% increase from mean baseline (0.21 ± 0.05 ug/ml) to peak post-injection values (0.29 ug/ml; Fig. 3-1) 6.5 hours following the TSH injection. No significant difference was determined between the male and female fTM levels when comparing all the sample values for pre- and post-injection (T-test: t(45) = -0.73, p = 0.23).

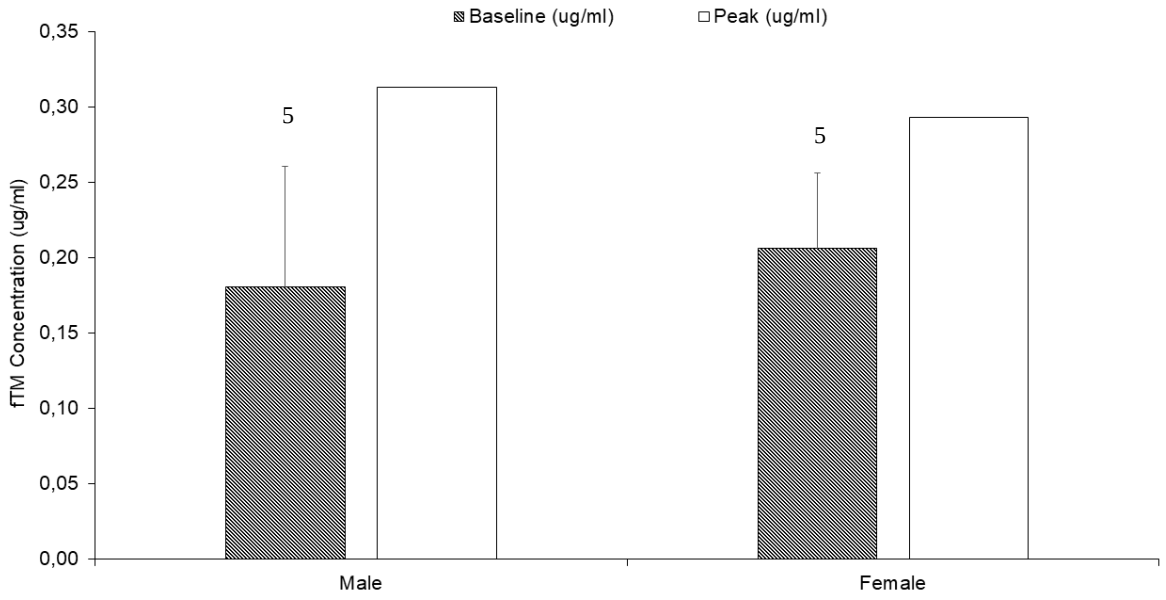


Figure 3-1. The median and peak fTM values of the male and female *O. crassicaudatus* in response to the 2.0 IU TSH stimulation. The peak value indicates the highest peak response measured post-injection. The numbers above the error bars represent the sample size used to calculate the baseline value. Error bars represent the standard deviation. Peak response in males was seen at 10.5 hours, while females showed a peak response at 6.5 hours post-injection.

Seasonal weather fluctuations

Overall, there was a significant difference in daily rainfall between both seasons (summer and winter) for both years (2017 and 2018; $F_{(136)}$= 4.71, p < 0.001; Tukey HSD: p < 0.001; Fig 3-

2a). In winter 2017, an average of 0.06 ± 0.03 mm per day was experienced, while in the summer months, 2.70 ± 2.80 mm per day were observed. Subsequently, in 2018 the winter season had an average of 0.42 ± 0.60 mm of rainfall per day and mean summer rainfall of 4.03 ± 3.11 mm/day (Fig. 3-2a). In December 2018, there was an average of 1.09 ± 1.51 mm rainfall per day.

Significant differences in ambient temperature were experienced between dry1 and both wet periods ($F(136)= 38.03$, $p < 0.001$). Specifically, the temperatures in 2017 increased from 16.02 ± 1.69°C in dry1 season to 19.98 ± 0.61°C in wet1 (Tukey HSD: $p < 0.001$); subsequently, in 2018 temperatures increased from 15.23 ± 2.41°C in dry1 to 21.36 ± 3.20°C in the wet period ($p < 0.001$; Fig. 3-2a).

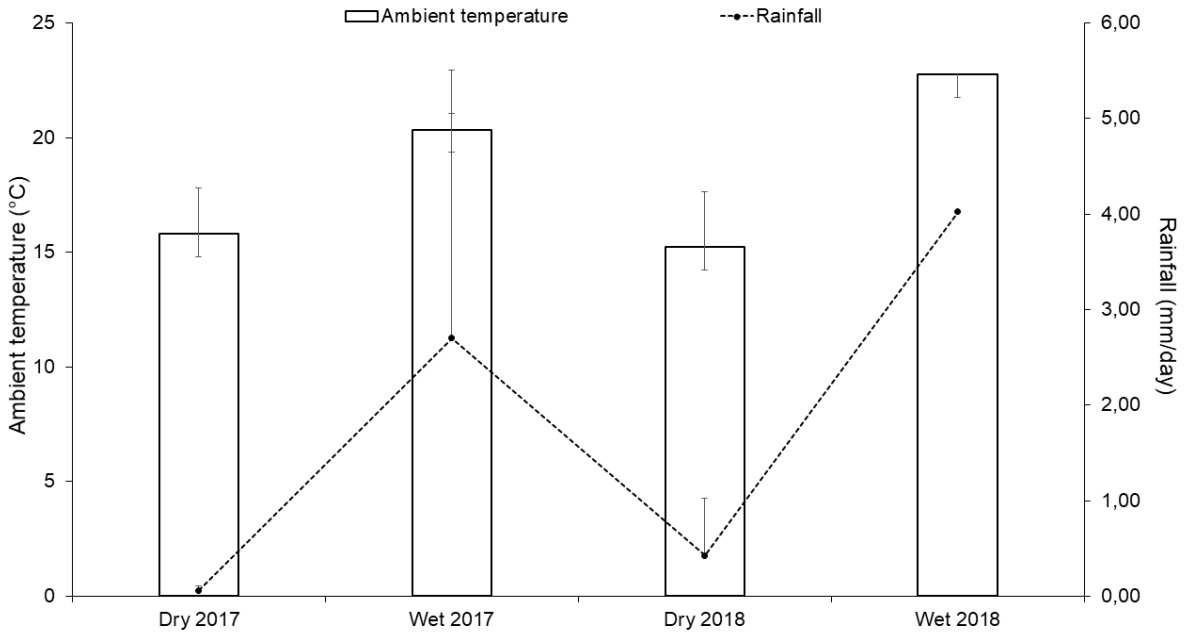


Figure 3-2. Seasonal fluctuations in average daily rainfall and ambient temperature experienced over the study period. Error bars indicate standard deviation.

Seasonal food availability

A total of 5017 insects were captured throughout the sampling period. Insects were significantly more abundant during the summer months (2017: n = 108 sampling days; mean = 18.27 ± 9.12 insects; 2018: n = 132 sampling days; mean = 21.55 ± 14.78 insects) when compared to the winter months (2017: n = 7 sampling days, mean = 3.71 ± 4.42 insects; 2018: n = 118 sampling days, mean = 6.03 ± 41.07 insects; ANOVA: $F(318)= 16.01$, $p < 0.001$; Tukey HSD($_{\text{Summer 2017-Winter}}$): $p < 0.001$; Tukey HSD($_{\text{Summer 2018-Winter}}$): $p < 0.001$) with the highest number of insects found in December 2018 (mean = 38.86 ± 44.97 insects/day), and the lowest number of insects captured in June 2018 (mean = 1.93 ± 1.30 insects/day).

The *Vachellia karroo* tree was most frequented by *O. crassicaudatus* (pers. obs.). Significant seasonal variation in gum availability was determined between the summer (n = 109; mean = 0.87 ± 0.18 g) and winter seasons (n = 148; mean = 0.56 ± 0.20 g; t-test: $t(136)= -2.49$, $p = 0.014$). The highest gum weight (mean = 1.34 ± 0.19 g) was observed in the summer months from December 2017 to February 2018 and the lowest (mean = 0.42 ± 0.01 g) from July to September 2017.

A total of 2032 seeds were recovered from 65 faecal samples. The majority of seeds found in faeces originated from fig (*Ficus* sp) and jojoba (*Simondsdia chinensis*) fruits. The seed count in the 2017 seasons (dry1: n = 6; mean = 11.83 ± 19.84 seeds per day; wet1: n = 29; mean = 20.28 ± 41.89 seeds per day) was lower than that recovered in the 2018 dry2 months (n = 30; mean = 45.77 ± 63.02 seeds/day), however no significant differences were determined between seasonal periods (ANOVA: $F(62) = 2.61$; $p = 0.08$).

Seasonal body mass variations

Body weight in females was highest in June 2018 (dry2 season) and lowest in September 2017 (dry1; Tab. 3-1) with the average body mass fluctuating approximately 250g throughout the

sampling period. Body weight in males peaked in May 2018 (wet2) and was lowest in November 2017 with body mass fluctuating 395 grams throughout the sampling period.

Table 3-1. Average monthly body mass (and standard deviation) from male and female *O. crassicaudatus* individuals captured across four sampling seasons. In December 2017, no females and only one male were captured for sampling.

Year	Season	Month	Female body weight (± SD)	Male body weight (± SD)
2017	Dry1	June	964.1 (± 153.1)	963.6 (± 342.0)
		July	917. 5 (± 39.3)	1003.3 (± 293.9)
		August	866.7 (± 37,5)	871.3 (± 265.7)
		September	838.3 (± 184,7)	851.8 (± 286.7)
	Wet1	November	900.7 (± 143.9)	842.5 (± 141.9)
		December	N/A	885,0
2018		January	1007.4 (± 106.8)	1103.0 (± 146.4)
		February	970.0 (± 78.6)	1080.0 (± 145.1)
	Dry2	May	997.8 (± 226.2)	1291.9 (± 112.9)
		June	1088.4 (± 101.7)	1180.0 (± 86.7)
		July	880.0 (± 195.0)	1028.3 (± 85.4)
	Wet2	December	930.3 (± 129.5)	1237.7 (± 87.1)

Seasonal reproductive variations

Seasonal reproductive data analysis revealed no significant sex differences in fTM concentrations. As published in Long et al. (2021), no significant differences were observed in testes length across reproductive periods; however, we observed a slight increase in testes length in the first mating and second mating periods when compared to the non-breeding period. In the lactating period, females exhibited significantly higher fTM levels than the mating 2 period. Nipple length significantly increased in the lactating period (ANOVA: $F_{(19)}$ = 5.46, p = 0.02) between mating 1 (Tukey HSD: p = 0.02) and mating 2 (Tukey HSD: p = 0.03). We saw no significant seasonal differences in vulva length (ANOVA: $F_{(21)}$ = 0.25, p = 0.79).

Seasonal analysis of fTM concentrations

Seasonal differences were noted (Kruskal Wallis: Chisq = 34.04, df = 3, p < 0.001); specifically, the fTM levels in the Wet1 sampling period were significantly higher compared to Dry1 (p = 0.018) and Dry2 (p = 0.003); and the fTM concentrations were significantly higher

in Wet2 period when compared to Dry1 ($p < 0.001$), Dry2 ($p < 0.001$), and Wet1 ($p = 0.003$; Fig. 3-3).

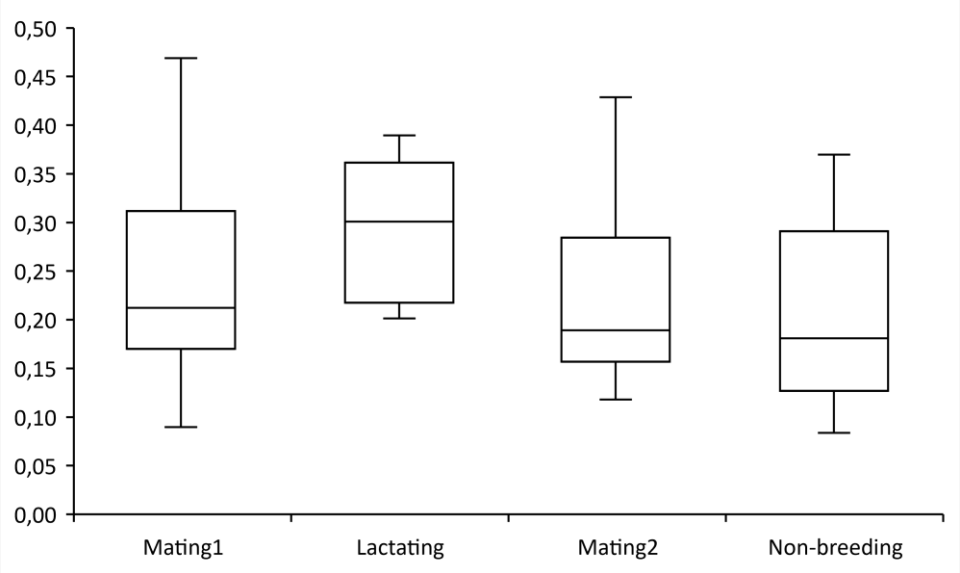


Figure 3-3. Boxplots demonstrating the variation in mean fTM concentrations during the distinguished reproductive periods. Boxplots indicate the median, and 1st (25% percentile) and 3rd quartiles (75% percentile).

Overall, both models fitted the data better than the null models (Model 1: Chisq = 53.50, df = 6, $p < 0.001$; Model 2: Chisq = 32.25, df = 5, $p < 0.001$; Tab. 3-2). In the environmental model, insect availability (Chisq = 22.62, df = 1, $p < 0.001$, $R^2 = 0.32$) had a significant effect on fTM concentrations (Tab. 3-2). Ambient temperature and rainfall were also included in the best-fit model for model 1. Lactating state significantly affected fTM (Chisq = 20.880, $p < 0.043$, $R^2 = 0.35$; Tab. 3-2 in model 2.

Table 3-2. The parameter estimates (β), standard errors (SE), likelihood ratio tests (LRT), 95% confidence intervals (CI) of predictors for both models assessing the influence of environmental and reproductive period factors on the log fTM concentrations.

Predictors	***(β)***	***SE***	***LRT***	***95% CI***	***Df***	***P***
Environmental				***lower; upper***		
Intercept	-1.872	0.171		-2.210; -1.533		
Rainfall	0.047	0.027	3.010	-0.006; 0.099	1	0.080
Ta[1]	0.015	0.011	1.942	-0.006; 0.038	1	0.160
Sex	0.011	0.078	0.022	-0.143; 0.164	1	0.890
Insects	**0.015**	**0.003**	**22.624**	**0.009; 0.021**	**1**	**<0.001**
Gum	0.001	0.007	1.687	-0.002; 0.002	1	0.890
Seeds	0.001	0.001	1.401	-0.013; 0.015	1	0.770
Reproductive Period						
Intercept	-1.251	0.135		-1,518; -0.982		
Reproduction			**31.840**		**4**	**<0.001**
Mating1	-0.049	0.171	2.465	-0.569; 0.111		0.230
Mating 2	-0.298	0.171	3.810	-0.638; 0.042		0.290
Lactating	**0.361**	**0.169**	**20.880**	**0.026; 0.697**		**0.043**
Non-breeding	0.387	0.222	1.561	-0.053; 0.827		0.347
SexMale	-0.043	0.120	0.153	-0.281; 0.196	1	0.720

[1]Ambient temperature

Table 3-3. Selected models best describing the variation in seasonal fTM concentrations of *O. crassicaudatus*. Models were selected based on the Akaike information criterion small sample correction distance from the best model (ΔAICc < 2), pseudo-R squared (R^2) values indicating percentage of variance explained by fixed and random factors, number of parameters (df), Akaike information criterion small sample correction (AICc), and model probability Akaike weight (w_i) are also shown.

Environmental Model: log~	R^2	**df**	**AICc**	**ΔAICc**	w_i
Insects + Ta[1] + Rainfall + (1|ID)	0.35	6	146.3	0.00	0.15
Insects + (1|ID)	0.32	4	146.9	0.57	0.12
Insects + Rainfall + (1|ID)	0.33	5	147.1	0.80	0.10
Insects + Ta + (1|ID)	0.33	5	147.3	0.99	0.09
Reproductive Model: log~					
Reproductive_state + (1|ID)	0.34	7	95.2	0.00	0.77
Reproductive_state + Sex + (1|ID)	0.34	8	97.6	2.37	0.23

[1]Ambient temperature

Discussion

This study has provided the first look at fTM within the *O. crassicaudatus* population and provides insight into the physiological responses of this African primate living in a highly seasonal temperate environment. We were able to validate the appropriate EIA for monitoring fTM concentrations in this species. Subsequently, we determined important drivers of thyroid function using fTM concentrations as a proxy.

EIA Validation experiment

The TSH challenge confirmed the ability of the chosen EIA to robustly monitor changes in fTM concentrations, with peak fTM levels as an indication of gut passage time at 10.5- and 6.5-hours post-injection for the male and female, respectively. This gut passage time is similar to what has been found in other Strepsirrhini primates using comparable feeding sources, such as the African lesser bushbaby, *G. moholi* (14 hours, Scheun *et al.*, 2015) and previous research on *O. crassicaudatus* (9.5 to 10.5 hours; Long *et al*., 2021), common marmosets, *Callithrix jacchus* (8-24 hours, Bahr *et al*., 2000), and yellow-breasted capuchins, *Sapajus xanthosternos* (8 hours, Schaebs *et al*., 2017). Generally, diet and season play a role in gut passage time and hormone metabolite concentrations in faeces (Goymann, 2012). Body temperature fluctuations will affect metabolic rate, either increasing or decreasing the gut passage time; while a shift to a low- or high-fibre diet will impact the excretion rate (Goymann, 2012). These factors should be considered when conducting validations as well as monitoring fTM levels in free-ranging animals.

The effects of food availability

The results from this study demonstrate a positive relationship between fTM concentrations and food availability. Food resource fluctuations are an important factor during differing seasons and thermoregulatory demands. The decrease in fTM levels during periods of resource scarcity could be indicative of down-regulation of the HPT axis and metabolic function within

O. crassicaudatus (Eales, 1988; Amin *et al*., 2011). It is as a result of such a mechanism that animals can survive during periods of nutritional deprivation. In black bears (*Ursus americanus*), Tomasi *et al*. (1998) noted a significant decrease in plasma T3 levels during the reduced-food and hibernation periods. The results showed insect availability had a significant effect on fTM concentrations. In this study, we noted *O. crassicaudatus* increased gum feeding in the winter months to sustain their daily nutritional requirements and has been a method used in previous studies (Harcourt, 1986). Patterns of increased T3 production during periods of nutrient abundance, as an indication of elevated metabolic function, have been observed in several mammalian species, including Barbary macaques (*Macaca sylvanus*; Cristóbal-Azkarate *et al*., 2016), mantled howler monkeys (*Alouatta palliata*; Dias *et al*., 2017), and killer whales (*Orcinus orca*; Ayres *et al*., 2012). Therefore, we can suggest the insect availability may have influenced fTM levels by either increasing activity levels (for catching flying insects) and also increasing nutritional intake. Long *et al.* (2021) revealed an increase in seed presence in the faeces of *O. crassicaudatus* at the onset of the 2018 winter season, suggesting these individuals may have increased fruit consumption (along with gum consumption) opportunistically while insect densities drop at the onset of more unfavourable conditions. The fruit of the fig tree (*Ficus* sp.) is high in sugar and soluble fibre (Milton *et al.*, 1982) with some trees flowering throughout the year while others only from summer to midwinter (Milton *et al*., 1982; Damstra *et al.*, 1996), and could provide additional energy sustenance required during the less favourable dry season (Harcourt, 1986). Yellow baboons (*P. cynocephalus)* expressed similar behaviour by decreasing activity levels and converting to a lower-quality diet when preferred food resources diminished (Gesquiere *et al*., 2018). In sum, the results from this current study suggest the role of thyroid hormones as a metabolic strategy to preserve energy during long periods of nutritional stress (Eales, 1988; Moon *et al.*, 1999; Dias *et al*., 2017).

Weather fluctuations

Seasonal fluctuations in weather conditions, and the resulting limitation of resource availability, may result in a number of negative responses from an organism such as nutritional imbalance (Wingfield, 2013; du Dot *et al*., 2009; Dausmann, 2014). Animals will adjust both behavioural and physiological mechanisms in order to survive changes in weather. For instance, Japanese macaques (Hanya, 2004), Barbary macaques (*M. sylvanus*; Majolo *et al*, 2013), and collared brown lemurs (*Eulemur collaris*; Donati *et al*, 2011) have been shown to decrease their activity budgets during colder periods in order to reduce thermoregulatory costs. From the results, we saw a non-significant positive relationship between environmental factors, ambient temperature and rainfall, and T3 production. Which suggests the galagos reduced their activity levels or nutritional intake (low fTM levels) during the winter periods. Additionally, we see a positive relationship between ambient temperatures and food availability. The decrease in ambient temperatures in winter dramatically affect the production of gum and fruit, and the occurrence of insects. Conversely, we see a rise in all three main food sources in the warmer months. In *G. moholi*, Nowack *et al*. (2013) reported behavioural changes whereby individuals reduced activity levels and foraging behaviour, and increased sleeping behaviour all while maintaining normothermy during the winter season. Harcourt (1986) observed similar behavioural changes in *O. crassicaudatus* which could suggest from the results of this study, that *O. crassicaudatus* reduced activity during the colder months while downregulating circulating T3 levels. Conversely, several studies have shown no influence of ambient temperature on metabolic activity, including Dauncey (1990) studying the effects of nutrition and temperature on pigs, and Dias *et al.* (2017) who found no significant effect of ambient temperature on mantled howler monkeys. Cold exposure generally leads to an increase in metabolic rate and thyroid hormone concentrations (McBride *et al*., 1985; Burger & Denver, 2002) as supported by Tomasi (1991) and Rimbach *et al*. (2018) who observed a negative

relationship between T3 levels of the study species and temperature. These previous studies indicate thyroid hormones play a role in thermoregulation, however, most studies evaluating the effects of temperature on thyroid hormones and basal metabolic rate were conducted on free-ranging species residing in areas of extreme environments, such as the desert (Pritchard *et al*., 2020), or high latitudes (Cristóbal-Azkarate *et al*., 2016; Thompson *et al*., 2017). For this study, it was difficult to distinguish between the effects of temperature and diet on fTM concentrations, as both temperature and food availability are correlated.

As predicted, fTM levels were elevated during the wet season when climatic conditions improved. Although rainfall did not have a significant effect on fTM concentrations, there may be an indirect influence of rainfall. The more favourable wet weather conditions may contribute to food production, encouraging exudate and fruit production, as well as insect availability rather than being directly related to fTM concentrations, as supported by Emmons (1980) and Levings & Windsor (1983). Interestingly, Cristóbal-Azkarate *et al*. (2016) also noted a positive relationship between thyroid hormones and rainfall in Barbary macaques, suggesting a thermoregulatory function by increasing basal metabolic rate as a response to the cooling effects of wet fur. It likely the results indicate ambient temperature together with rainfall may improve resource abundance causing the increase in individual activity and the elevation in fTM levels.

The effects of reproductive period

In females, lactating is an energetically expensive stage as mothers are required to produce sufficient milk (Altmann, 1980; Sauther, 1998; Dufour and Sauther, 2002), and several studies have observed increased feeding habits during the lactation periods (for instance, *T. gelada*; Dunbar *et al*, 2002). During the lactating, females exhibited higher levels of fTM than females in the mating, supporting previous studies suggesting heightened metabolic demands are

required to provision the developing foetus (Reynolds *et al*., 2010) and lactating females by regulating prolactin and oxytocin (Akasha *et al*., 1987). Although the results of this current study indicate higher female fTM concentrations during the lactating periods, these reproductive states are also synchronous with the wet season, and the elevated fTM levels are possibly influenced by both food availability and the more favourable environmental conditions (increased energy expenditure) and lactation which requires substantial energy usage to develop the lactose.

Conclusion

The study successfully validated an appropriate EIA for monitoring fTM concentrations in the species. This was the first study to provide insight into the seasonal changes in thyroid hormones in free-ranging *O. crassicaudatus* and how they are affected by food abundance rather than changes in temperature. The results suggest HPT functionality changes in response to alterations in insect availability. Such results will help provide more knowledge as to how these wild African nocturnal strepsirrhine are affected by changes in food abundance and will add to the growing information on primate physiology.

Chapter 4

Seasonal effects on the faecal microbial composition in a wild population of nocturnal African strepsirrhine primates, the greater thick-tailed galago (*Otolemur crassicaudatus*)

Published article based on this chapter:

Long C., Scheun, J., Sauther, M.L., Cuozzo, F.P., Millette, J., Tordiffe, A.S.W. (2023). Seasonal effects on the fecal microbial composition of wild greater thick-tailed galagos (*Otolemur crassicaudatus*). *Int J Primatol*, 1–21. DOI: 10.1007/s10764-023-00407.

Abstract

Changes in diet and the environment are major factors affecting the composition and diversity of the faecal microbiome. In addition to changes in ambient temperature and rainfall, primates living in seasonal temperate environments also need to adapt to the changes in food resource quantity and quality occurring across seasons. In this study, we compared the possible effects of seasonal dietary and environmental changes to the faecal microbial diversity and composition in the strepsirrhine primate, the greater thick-tailed galago (*Otolemur crassicaudatus*) using 16S rRNA next-generation sequencing. We assessed the food availability and weather differences between seasons at our field site. The results showed significant increases in the rainfall and ambient temperature in summer and a significant decrease in food availability during winter. No significant changes were determined in the overall diversity of bacterial species present between seasons. However, significant changes in the abundance of certain bacterial families during the winter period suggest the bacterial presence are influenced by a change in diet. As we do not find significant differences in body mass across seasons and reproductive success, this could suggest the overall population is not faced with severe affects to their health. This investigation provides the first insight into the faecal microbiome of *O. crassicaudatus*. This information will also add to the growing

knowledge and understanding of the faecal microbiomes in primates and their effectiveness to sustain populations during current and future changes to their environments.

Introduction

Primates have adapted to living in a variety of different habitats such as forests, mountains, and savannahs and show a vast array of dietary preferences such as insectivory, folivory, gummivory, and frugivory. Non-human primates (NHPs) will adjust their activity patterns and feeding behaviour in response to changes in their environment (Strier *et al*., 2009; Reyna-Hurtado et al., 2018). These developments may include changes to their social structure or habitat use (Chevalier *et al.*, 2015). Changes may also occur in the gut microbiome, which consists of a symbiotic community of microbial organisms residing in the gut of the host individual (Amato et al., 2014). Bacterial communities present in the host digestive tract are linked to changes in the host diet, health, and the extrinsic environment (David *et al*., 2014; Bjork *et al.*, 2019; Orkin *et al.*, 2019). These communities are important for the breakdown and absorption of nutrients required by the host (Cabana *et al*., 2018). Microbes will breakdown protein and carbohydrate food matter into vitamins (Said & Mohammed, 2006) and branched-chain fatty acids and short-chain fatty acids (SCFAs; Scheppach, 1994; Nicholson *et al*., 2012), which can be used as an energy source for the host (Macfarlane & Macfarlane, 2012) that cannot be absorbed by the gut itself. Carbohydrate fermentation is a critical function of the gut in the catabolism of these important nutrient sources in the metabolic homeostasis of the host (Scheppach, 1994; Nicholson *et al*., 2012; Yuan *et al.*, 2020).

In addition, gut microbes are able to produce energy that the host can then absorb (Hildebrandt *et al.*, 2009; De Fillipo *et al*., 2010; Wu *et al*., 2011; Claesson *et al.*, 2012). Previous studies have demonstrated the influence of seasonal shifts in climate and resource use, as well as the captive and natural environment, on the gut microbial composition of individuals (Amato *et al.*, 2014; Sun *et al*., 2016). However, studies regarding the effects of

changes in the natural environment on the gut microbiome of wild NHP populations have only been addressed in a limited number of species (Amato *et al*., 2014, 2015; Gomez *et al*., 2015; Mallott *et al*., 2018).

In South Africa, only two nocturnal strepsirrhine primate species are found; the southern lesser galago (*Galago moholi*) and the thick-tailed greater galago (*Otolemur crassicaudatus*). Few studies have determined how a highly seasonal habitat impacts the gut microbial profiles of mainland African strepsirrhine species (for instance, Long, 2018). In South Africa, several populations of *O. crassicaudatus* reside in highly seasonal regions (Hill, 1953). The thick-tailed greater galago (*O. crassicaudatus*) feeds mostly on gum, insects, and fruits to sustain itself across seasons (Bearder, 1974). In South Africa, several populations of *O. crassicaudatus* reside in highly seasonal regions (Hill, 1953). Seasonality will affect the rainfall and temperature across the four designated seasons (winter, spring, summer, autumn) and, inevitably, affects the availability of their natural food resources.

The *O. crassicaudatus* diet is mostly dependent on gum exudates throughout the year; however, this species will sustain their nutritional requirements with insects in summer and fruit in winter (Clark, 1976; Nowak & Paradiso, 1983; Bearder, 1987; Happold & Happold, 1992). Gum is an unusual food source as it is low in nutrients (but high in calories) (Nash, 1986), but is also found in limited quantities (O'Malley & Power, 2012). Therefore, above a certain body size gum would not be able to sustain the energy requirements of the individual (Kay, 1979). Bearder & Martin (1980) suggested gummivory may be beneficial for an insectivorous primate as it may provide calcium to complement the phosphorous taken from insects (Génin *et al.*, 2010). Gum exudates and insect exoskeletons may also provide some minerals and carbohydrates in the form of soluble β-linked complex polysaccharides (O'Malley & Power, 2012). The polysaccharides from gum are comprised of cellulose and the mammalian gut is incapable of digesting these molecules without the assistance of gut

microbes. As a result, fermentation and the digestive abilities of gut microbes are required in the gut for the primate to absorb and utilise these carbohydrates (Caton *et al.*, 2000; O'Malley & Power, 2012). In addition, insects provide phosphorous and are high in fat content (O'Malley & Power, 2012) and would help increase weight gain within the species. Gut microbes are not specifically required to digest insects in the same way as cellulose as they have the specific gut anatomy and enzymes to help do so. Gut microbes would help to ferment the fruit that is stored in the gut. Essentially, the gut microflora would change based on the dietary changes but would also benefit the host by assisting in the breakdown of these complex carbohydrates.

To contribute to our understanding of the effect of seasonality on the gut microbiome in strepsirrhines, we aimed to identify the main microbial taxa found in the fecal microbiome of thick-tailed greater galagos inhabiting an isolated temperate environment in two contrasting seasons. Under the hypothesis that the fecal microbiome is affected by changes in diet, we predict a decrease in microbial taxonomy and relative abundance during winter when a considerable shift in diet occurs in comparison to summer. We also explore potential influences on the fecal microbiome, including seasonal changes in food availability, rainfall, and ambient temperature.

Materials and Methods

Animal captures and faecal sampling

Sampling occurred in June 2017, December 2017/January 2018, March 2018, and May/June 2018 in which we aimed to collect samples from at least 20 adult individuals (10 males and 10 females; Table A1) from each trapping season. Each night (total trapping nights = 47) we focused one area of the trapping range. These areas were approximately 50 – 100m^2. In each sampling area, between four and nine traps were located (depending on the density of *O. crassicaudatus* and the proximity of the traps to one another). A licenced veterinarian was present during all animal capture and sampling events. Havahart® traps (Woodstream

Corporation Inc, Lancaster, PA, USA) baited with peanut butter and banana were set before sunset (between 17h00 and 18h30) and were checked at sunrise (between 04h30 and 06h00) with time depending on the season. We believe the interval time between setting traps to sample collection was apt as a previous study found the time taken for cortisol to be egested was approximately 10 hours depending on sex (Long et al, 2021). Previous research has shown that a long gut retention time is seen in animals feeding on gum as fermentation is required to help breakdown the substance. Empty traps were cleaned with a solution of 99% rubbing alcohol and water and closed till the next trapping session. If an animal was present in the trap, a capture bag was used to collect the animal, identified using a microchip transponder reader (ID100 Trojan, EURO I.D., Weilerswist, Germany), then placed into a pet travel box and translocated to the field laboratory. Faecal samples necessary for seed availability assessment were collected from the trap once the animal was safely placed in the travel box. Once at the field lab, the animal was anaesthetised with isoflurane and the veterinarian completed a health check on the individual. If no microchip was detected on the animal, they were given a transponder microchip which was inserted subcutaneously, and the transponder identity number recorded as the animal's ID. The weight of each individual was recorded. A FLOQ® faecal swab (FLOQSwabs®, Copan Group, Brescia, Italy) was inserted into the rectum to retrieve a sufficient volume of faecal matter to discolour the swab. Once removed, the head of the swab was cut off and immediately placed into a 2 ml sterile tube and stored immediately in a - 4°C freezer. Once transported back to the University of Pretoria, approximately one week after the start of sampling, the samples were stored in a - 80°C freezer until DNA isolation. Captured animals were released the evening following trapping, at the source of capture, once completely conscious, and the veterinarian was content with their health condition.

Seasonal food availability

Insect, gum, and seed samples were collected to reflect the annual food availability for O. crassicaudatus. Insect and gum data were collected from the environment, whereas seed data were ascertained from ingested fruit. Insects were collected from September 2017 to June 2018. We set up two light traps at the field site before sunset approximately 15 metres apart. Both traps were found in an area where O. crassicaudatus had been observed frequently. These lights were set up on consecutive days before and after trapping sessions. One light trap was set on the ground to estimate the ground dwelling insect density, while the second light trap was set up two metres above the ground to assess the abundance of flying insects present. The light was set above a collecting chamber with a hole in the lid preventing most insects from escaping from the trap. We checked traps every morning after sunrise and all insects collected in the trap were sprayed with 70% ethanol and counted. If possible, we identified insects and then all specimens were stored in 90% ethanol until further analysis.

We collected gum exudate samples monthly from a total of 50 trees (10 trees per sampling section). Trees were selected after personal observations (J.B. Millette and C. Long) identified trees frequented by *O. crassicaudatus* individuals or trees that were observed to have gum exudates. Once identified, gum exudates were scraped off the tree bark using a multi-tool and placed in a sample bag. These samples were stored in a -20°C freezer until analysis.

Seeds were collected from a subset of the faecal samples gathered from July 2017 to June 2018. These samples were excess faecal samples collected with the microbiome analysis samples. Faeces were dried and seeds were extracted using tweezers. Each seed was designated as representative of one fruit except for multiple *Ficus* seeds which were identified as one fruit per faecal sample (Long *et al*., 2021). The seeds retrieved were counted and identified if possible.

Seasonal weather differences

We captured rainfall and ambient temperature data from the on-site weather station (Davis Instruments sensor suite linked via wireless to a Davis Pro 2™ console, Measurement and Console Systems CC, Cape Town, South Africa) and thus was analogous to the conditions experienced by the sampled individuals. The data are made available by the Ndlovu Node of the North-eastern Mountain Observatories project of the South African Environmental Observation Network (SAEON). Data were collected every hour throughout the study period. Subsequently, the mean (± standard deviation; SD) daily ambient temperature (°C) and daily cumulative rainfall (mm) total was calculated.

DNA Isolation and Next-Generation Sequencing

We extracted the total faecal bacterial DNA from 47 individuals (males: N=28; females: N=21) using the DNeasy® PowerSoil® Kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. The quality of DNA was assessed using a QuBit 2.0 Fluorometer (Invitrogen, Carlsbad, CA, USA). DNA samples were stored in a -80 °C freezer prior to polymerase chain reaction (PCR) amplification. PCR amplification, library preparation, next-generation sequencing, and data process and analysis procedures were completed at the Inqaba Biotec Industries (Pty) Ltd (Muckleneuk, Pretoria). Bacterial gene libraries were created using the PacBio Full-Length 16S Amplification, SMRTbell® Library Preparation and Sequencing protocol. Briefly, the workflow implements two PCR rounds, the first with the universal primer-tailed 16S primers (27F; 1492R) and the second with PacBio Barcoded Universal Primers. Amplification was performed with an initial denaturation at 95 °C for 30 s, 23 cycles of denaturing (95 °C for 30 s), annealing (55 °C for 30 s) and elongation (72 °C for 60 s). At each PCR step amplicons were generated using a KAPA HiFi PCR kit (Kapa Biosystems, Wilmington, MA, USA), and quantified on a Qubit 2.0 Fluorometer (Invitrogen, Carlsbad, CA, USA). The second round of amplification was performed with the same thermocycler

instructions. Libraries were prepared using SMRTbell Template Prep kit (Pacific Biosciences, Menlo Park, CA, USA) and purified using AMPure® PB beads (Pacific Biosciences, Menlo Park, CA, USA).

The prepared libraries were sequenced on the PacBio Sequel system (PacBio, www.pacbio.com). Raw subreads were processed through the SMRTlink (v7) Circular Consensus Sequences (CCS) algorithm to produce highly accurate reads (>QV40). These reads were then processed through vsearch (https://github.com/torognes/vsearch) and taxonomic information was determined based on QIIME2.

Statistical analyses of bacterial taxa

Statistical analyses were performed in RStudio (R 3.2.1, R Development Core Team, 2019). Significance was set at $p < 0.05$. The *p*-values were corrected for using Bonferroni correction.

Seasonal analysis

The data for food availability was divided into the three sampling seasons: June 2017 – August 2017 (winter 1), November 2017 to February 2018 (summer), and May to July 2018 (winter 2). We assessed seasonal differences in insect count, gum mass, and seed count data using Kruskal-Wallis tests with a chi-square distribution as the data were not normally distributed. We used Dunn's *post-hoc* tests to test for differences between seasons. For insects, we analysed the cumulative number of insects from both light traps per month. For gum mass, we analysed the sum of the gum mass from all trees per month. We also tested for seasonal differences (between winter 1, summer, and winter 2) in body mass for both sexes using Kruskal-Wallis tests with chi-squared distribution.

If the Kruskal-Wallis analysis found seasonal differences, we used post-hoc Dunn's tests to test for differences between the three seasons. Prior to analysis, we used the Levene's test to determine that variances were significantly different for both rainfall and ambient temperature. If seasonal differences were found after the Kruskal-Wallis analysis, we

performed *post-hoc* Dunn's tests to determine which seasons experienced the highest ambient temperature and rainfall.

We compared relative abundances of the phyla present across the three seasons (winter 1, summer, winter 2) using a Kruskal-Wallis test and post hoc Dunn's tests. We assessed the seasonal presence of the most predominant microbial families by performing Kruskal-Wallis tests with post hoc Dunn's tests. Venn diagrams were created using Venny 2.1 (Olivieros, 2015) to demonstrate the unique and shared bacterial families present between sexes and seasons. As there was little variation between the bacterial families present between winter 1 and winter 2, we combined the data. We used rarefaction curves to evaluate the depth of sequencing. Analysis of Similarities (ANOSIM) comparing gut microbial abundance differences between seasons was determined using the *anosim* function in the "vegan" package. Effects of seasonal (winter 1, summer, winter 2) and sex variation on the microbial composition was determined using a permutational multivariate analysis of variance (PERMANOVA) test using the *adonis2* function. The number of permutations was set at 9999. The Mann-Whitney U-test was employed to evaluate any significant seasonal differences in the bacterial diversity We used the Kruskal-Wallis test to assess differences in the relative abundance (log10 transformed) of genera between winter 1, summer, and winter 2, and Dunn's test to identify which seasons significantly differed.

We used linear mixed models (LMMs) to determine the factors potentially affecting gut microbial composition. Fixed parameters in the global model include daily insect count, gum volume, seed count, rainfall, ambient temperature, sex, and reproductive status. Animal ID was set as the random variable to control for non-independence. The linear model was run using the *lmer* function in the 'lme4' package (Bates *et al*., 2010). Model selection and averaging were performed using the *dredge* function (Barton *et al*., 2018). Prior to modelling, the dataset was normalised using the *preProcess* function. The best models were identified by ranking the

models with the lowest Akaike Information Criterion small correction (AICc). Models with a difference of AICc > 2 were reported. Pseudo R-squared values were calculated to detect the degree of influence of the fixed and random variables (Grueber *et al*., 2011; Nakagawa *et al*., 2017). The effect of each individual predictor was assessed using the *drop1* function in "lme4" to perform likelihood ratio tests to compare the global model with the reduced models lacking a variable one at a time (Bates *et al*., 2014). After assessing the variance inflation factor (VIF > 3; Zuur & Ieno, 2016), gum was removed as a fixed variable owing to collinearity with the insect availability variable. We then investigated the correlations between the most abundant phyla and specific environmental factors to assess their effects on the faecal microbial community composition within *O. crassicaudatus* by assessing the correlation coefficients.

Results

Seasonal food availability

A total of 2414 insects were captured from June 2017 to June 2018. The number of insects captured varied significantly with season (Kruskal Wallis: $X^2_{(2)} = 23.12$, $p < 0.001$). Insects were significantly more abundant during summer (102 capture events) than winter 1 (56 capture events) and winter 2 (48 capture events) with the highest number of insects found in December 2017 and the lowest in June 2018 (Fig 4-1a). Winter 2 also had significantly higher abundance of insects than winter 1 (Fig 4-1a).

We found significant variation in gum availability between seasons ($X^2_{(2)} = 14.69$, $p < 0.001$) with the highest gum mass in summer (n = 69, median = 0.5 ml, interquartile range = 0.4-0.7 ml) and the lowest in winter 1 (n = 48; median = 0.2 ml, interquartile range = 0.1-0.6 ml; Fig. 4-1b). Gum volume in summer was significantly higher than winter 2. We also found significantly lower gum volumes in winter 1 compared to winter 2 (Fig 4-1b).

A total of 919 seeds were recovered from 42 faecal samples. Most of the seeds originated from fig (*Ficus* sp) and jojoba (*Simondsdia chinensis*) fruits. We found no

significant variability between seasons ($X^2_{(2)}$ = 1.43, p = 0.489), however winter 2 had the highest count of seeds present in the faeces (Fig. 4-1c).

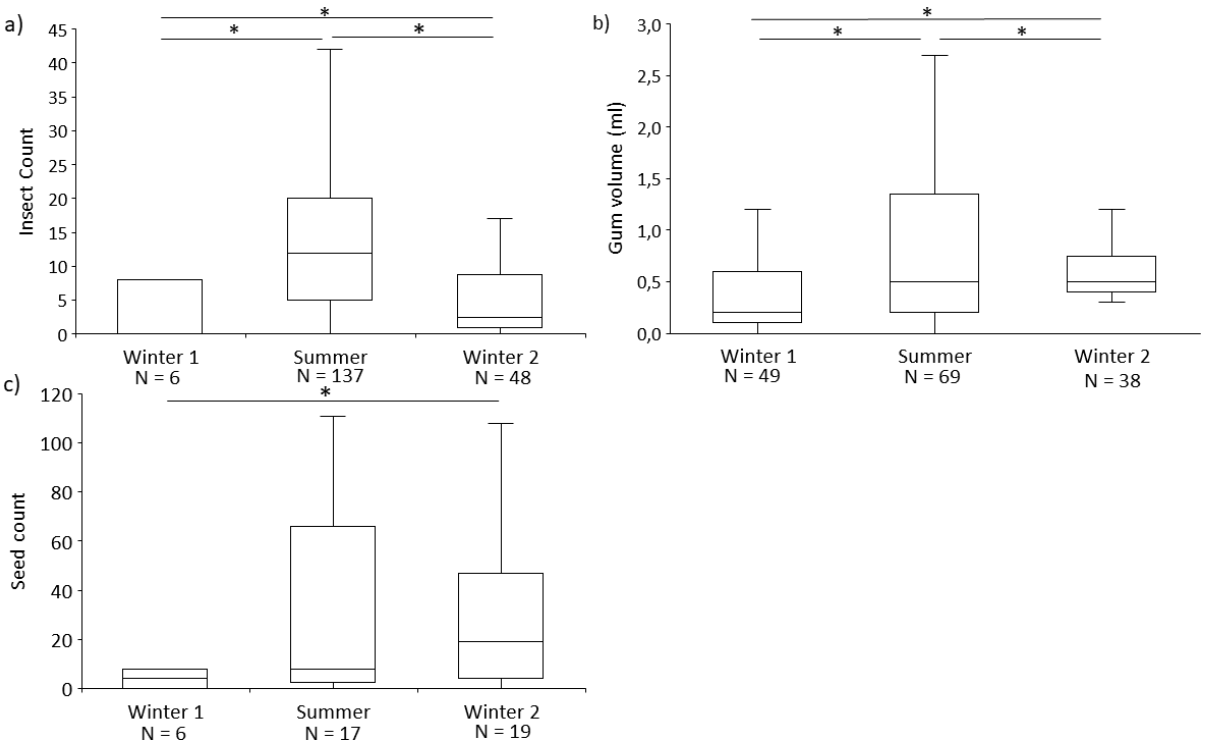


Figure.4-1 Boxplots indicating the seasonal differences between a) insect count, b) gum volume, and c) seed count over the sampling periods. Asterisks represent significant difference between seasons (p<0.05). Each boxplot represents the median and the interquartile range.

There was no significant difference in body mass between seasons for both males (Kruskal-Wallis: $X^2_{(2)}$ = 4.34, p = 0.11) and females ($X^2_{(2)}$ = 0.31, p = 0.86; Table 4-1).

Table 4-2. Body mass of male and female galagos (*Otolemur crassicaudatus*) across seasons at Lajuma Research Centre in South Africa. Numbers in brackets indicate sample size.

Sex	**Mean body mass ± SD (g)**		
	Winter 1 (June to August 2017)	**Summer** (November 2017 to February 2018)	**Winter 2** (May to July 2018)
Male	1029.8 ± 327 (19)	1131.1 ± 165.9 (15)	1298.5 ± 109.7 (7)
Female	922.8 ± 177.7 (12)	907.9 ± 211.6 (15)	919.3 ± 219.3 (13)

Seasonal weather analysis

Rainfall was significantly higher in the summer season ($X^2_{(2)}$ = 7.03, p = 0.029; *post hoc* Tukey HSD: p = 0.044), with peak levels observed in February 2018 (mean: 8.2 mm per day; Fig. 2) compared to winter 2, which received <1 mm of precipitation per day. Summer was not

significantly higher than winter 1 (mean: 1.2 mm per day; Tukey HSD: $p = 0.281$). Ambient temperatures were significantly higher in summer (range = 9.9 – 35.6 °C; mean = 20.58 ± 4.59 °C; $X^2_{(2)} = 24.72$, $p < 0.001$) than in winter 1 (range = 4.4 – 29.6 °C; mean = 14.85 ± 0.59 °C; Tukey HSD: $p < 0.001$) and winter 2 (Tukey HSD: $p < 0.001$ Fig. 4-2).

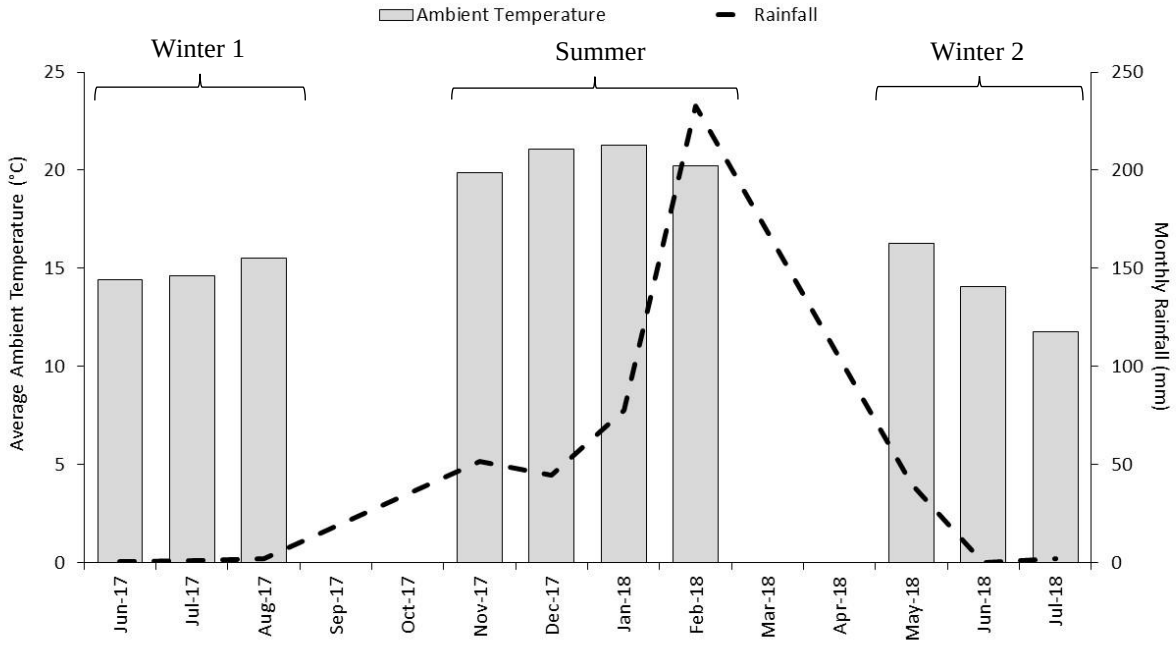


Figure.4-2 Weather patterns from June 2017 to July 2018 at Lajuma Research Centre in South Africa. The line indicates the cumulative monthly rainfall (mm). Data for months September and October 2017, March and April 2018 have been omitted as they were not used in the analyses.

Diversity of bacterial taxa

Overall, 75 samples were sequenced and, after excluding certain samples owing to low read numbers, only 49 faecal samples were used in the analyses. In total we collected samples from 15 adult males and 16 adult females (mean: females = 1.38 ± 0.62; males = 1.40 ± 0.83 samples from each individual). Rarefaction curves used to evaluate the depth of sequencing are illustrated in Fig. A1. In total, 17269 sequences were identified and a total of 295 unique OTUs were detected. A total of 12 distinct phyla and 53 families were detected in the faeces of the *O. crassicaudatus* population. All taxa were identified to at least family level; 136 taxa were further identified to genus level, and 72 were identified to species level. Neither the effects of

season (PERMANOVA: F = 0.984, R^2 = 0.021, p = 0.444) or sex (PERMANOVA: F = 0.593, R^2 = 0.013, p = 0.695) had a significant effect on the *O. crassicaudatus* faecal microbiome.

At phylum level, the faecal microbiome of the *O. crassicaudatus* population was dominated by Actinobacteria (65%), Bacteroidetes (15%), Firmicutes (14%), Proteobacteria (3%), with unclassified (>1%; Fig A2). Several other phyla, including Cyanobacteria, Fusobacteria, Planctomycetes, and Tenericutes were detected but represented less than 1% of the total taxa present. We detected 10 phyla in samples from winter 1 and winter 2 and eight in samples from summer (Fig. 4-3).

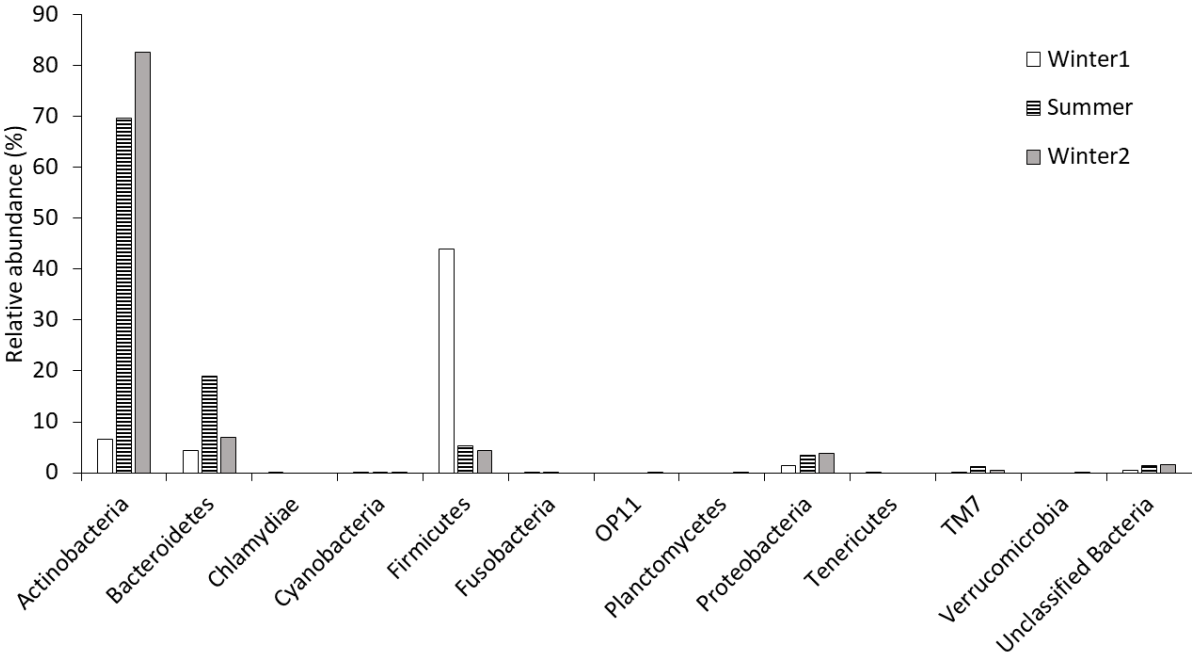


Figure 4-3. Relative abundance of phyla in the feces of galagos (*Otolemur crassicaudatus*) in summer (n = 25) and winter (n = 24) collected at the Lajuma Research Centre in South Africa. Data are mean ± standard deviation.

The relative abundance of Actinobacteria significantly differed between all seasons (winter 1 = 6%, summer = 69%, winter 2 = 82%; Kruskal-Wallis: $X^2_{(2)}$ = 47.08, p < 0.001). Winter 2 had a significantly higher relative abundance of Actinobacteria than summer and winter 1 (*post hoc* Dunn's test: p < 0.001) and made up 82% of the total bacterial population. Summer had a significantly higher relative abundance of Actinobacteria than winter 1 (Dunn's test: p < 0.001). An inverse relationship was present between Bacteroidetes and Firmicutes;

with Bacteroidetes increasing in summer (winter 1= 4%, summer = 19%, winter 2 = 7%) and Firmicutes relative abundance highest in winter 1 (winter 1 = 44%, summer = 5%; winter = 4%); however, season did not have a significant impact on the abundance of Bacteroidetes ($X^2_{(2)}$ = 1.90, p = 0.387). Firmicutes showed significant differences between all seasons ($X^2_{(2)}$ = 52.96, p < 0.001; Fig. 4-3).

The most prominent families recognised include Bifidobacteriaceae (62%), Prevotellaceae (13%), Clostridiaceae (6%), and Micrococcaceae (3%; Fig. A3). Overall, *O. crassicaudatus* shares 51 bacterial families between seasons. A heatmap (Fig. A4) indicates the most prevalent families for each host individual over the sampling period. It is apparent Bifidobacteriaceae was dominant throughout each sampling period. In summer, 23 unique families are observed including Neisseriaceae, Gemellaceae, and Rhodospirillaceae; in winter, 21 taxa were uniquely identified including Peptostreptococcaceae, Turicibacteraceae, and Lactobacillaceae (Fig. ~~4~~-4; Table A1).

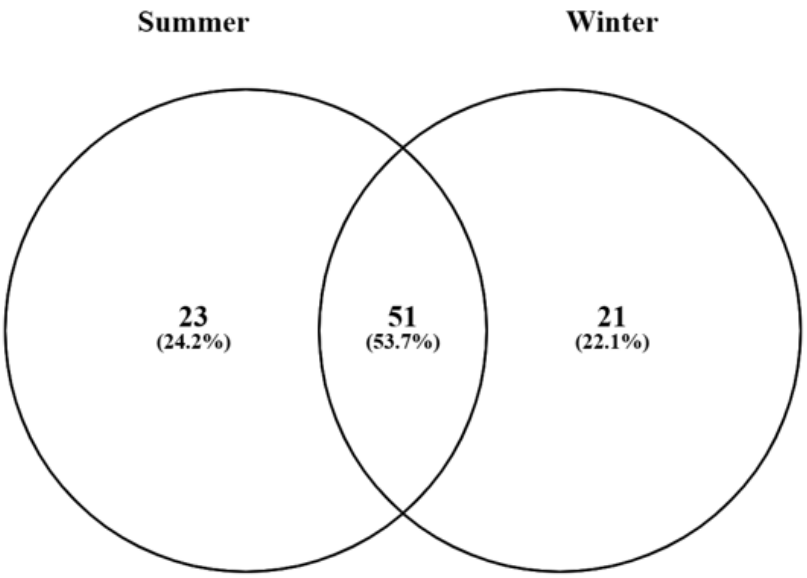


Figure 4-4. Venn diagram demonstrating shared and unique bacterial families found in galagos (O. crassicaudatus) during summer and winter in the Lajuma Research Centre in South Africa. The top number is the number of unique families, the number in brackets is the percentage of the total families unique to the season.

The most abundant genera comprised >85% of the total. *Bifidobacterium* (55%), *Prevotella* (14%), *Clostridium* (6%) were the most dominant genera detected with lesser contributions from *Staphylococcus, Arthrobacter, Gardnerella*, and unclassified genera of Proteobacteria, Micrococcaceae and Bacteria (Fig. 4-5). *Bifidobacterium* bacteria increased significantly during summer (Kruskal-Wallis: *Bifidobacterium*: $X^2_{(2)} = 23.66$, $p < 0.001$) compared to both winter 1 (Dunn's test: $Z = 4.79$, $p < 0.001$) and winter 2 ($Z = 0.78$, $p < 0.001$). *Prevotella* also significantly increased in summer ($X^2_{(2)} = 22.02$, $p < 0.001$) when compared to the relative abundance in winter 1 ($Z = 4.70$, $p < 0.001$) and winter 2 ($Z = 1.78$, $p < 0.001$), while unclassified Micrococcaceae decreased in summer ($t_{(2)} = 2.81$, $p = 0.011$). *Clostridium* bacteria were only present in the winter 2 samples while *Gardnerella*, unclassified *Micrococcaceae* and *Staphylococcus* were absent from the winter 2 samples altogether.

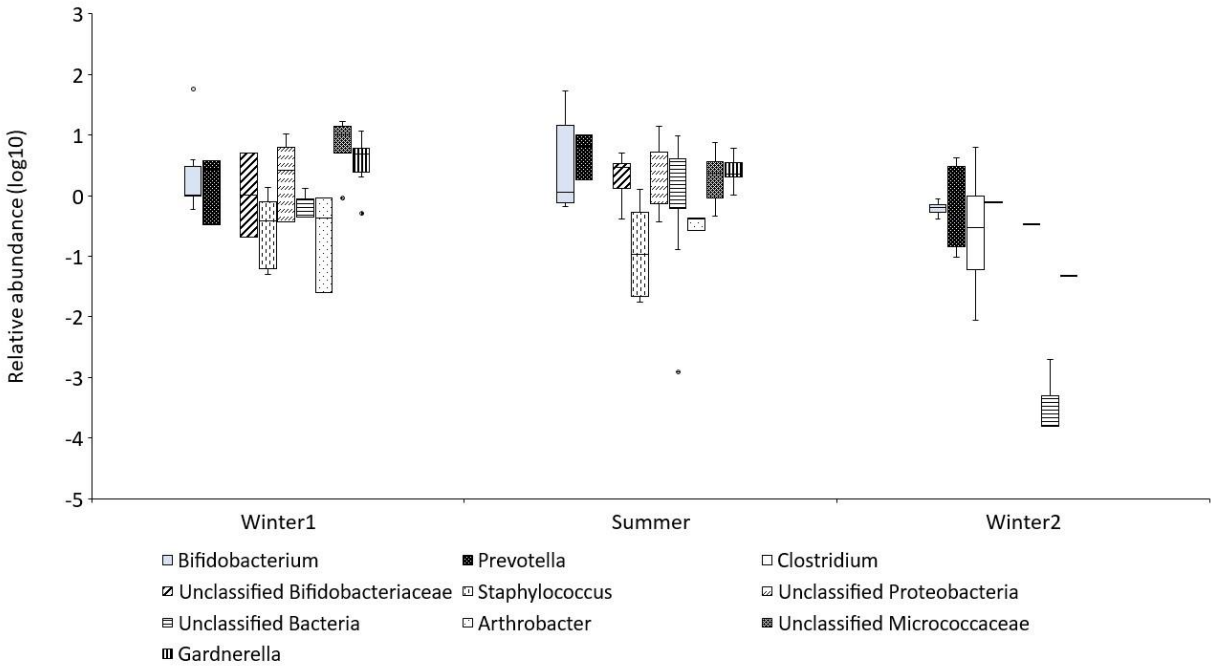


Figure 4-5. Seasonal differences in the relative abundance (log10 transformed) of the 10 most abundant genera in the fecal microbiome of galagos (*Otolemur crassicaudatus*) at Lajuma Research Centre, South Africa, between June 2017 and July 2018. Lines show the median, boxes the interquartile range, whiskers the standard deviation, and points outliers.

In winter, the most prominent species found in the female population are *Bifidobacterium* sp. (50%), *Clostridium perfringens* (10%), and *Prevotella* unclassified (3%);

in the males, *Bifidobacterium* sp. (19%), *Clostridium perfringens* (17%), and *P. ruminicola* (5%) were most evident. In summer, the most abundant species in both males and females include *Bifidobacterium* sp., *Prevotella* sp., and *P. ruminicola.*

The LMM we fitted to explore which predictors influenced bacterial composition had moderate variation (marginal R^2 = 0.26). It showed that bacterial composition was most influenced by insects (β = 2.62, Standard Error (SE) = 6.40, t = 0.41), gum (β = -3.33, SE = 10.23, t = -0.33), rainfall (β = -21.56, SE = 42.56, t = -0.51), and seeds (β = 0.68, SE = 0.82, t = 0.82). We found significant effects of rainfall, insects, gum, and seeds (Table 4-2). Reproductive status showed no significant effect ($X^2_{(4)}$ = 1.12, p = 0.89; Table 4-2). Ambient temperature, gum mass, and insect availability were positively correlated with Proteobacteria (Table 4-2).

Table 4-2. A table describing the correlations between the most abundant phyla and specific environmental factors to assess their effects on the faecal microbial community composition within *Otolemur crassicaudatus*. Bolded text indicates significant correlation.

Phylum	Parameter				
	Ambient temperature	**Rainfall**	**Gum**	**Insects**	**Seeds**
Actinobacteria	**R = 0.37** ***p* = 0.01**	R = 0.13 *p* = 0.37	**R = 0.34** ***p* = 0.02**	**R = 0.35** ***p* = 0.02**	R = 0.16 *p* = 0.28
Proteobacteria	**R = 0.49** ***p* < 0.01**	R = 0.04 *p* = 0.79	**R = 0.54** ***p* < 0.01**	**R = 0.51** ***p* < 0.01**	R = -0.12 *p* = 0.42
Firmicutes	R = 0.17 *p* = 0.23	R = 0.09 *p* = 0.51	R = 0.28 *p* = 0.05	R = 0.26 *p* = 0.06	R = 0.13 *p* = 0.39
Bacteroidetes	R = 0.09 *p* = 0.52	R = -0.05 *p* = 0.76	R = 0.05 *p* = 0.72	R = 0.18 *p* = 0.22	R = -0.12 *p* = 0.42

Discussion

Wild animals will generally adapt to changes in their environment by either physiological or behavioural adjustments (Sun *et al*., 2016). Scientists have demonstrated the adaptive abilities of the faecal microbial community as a mechanism for the host to adapt to extrinsic changes (for instance, Amato et al., 2013; Hanya *et al*., 2020). The aim of this study was to determine

the faecal microbial community profile of a population of *O. crassicaudatus* residing in a temperate environment in response to the seasonal change in biotic and abiotic parameters within their environment.

The seasonal environment and food resources

During the summer season a significant increase in relative abundance of *Prevotella* is apparent, which is associated with a carbohydrate and plant-based diet. In common marmosets, (*Callithrix jacchus;* Sheh *et al*., 2022), who use tree exudates as an important food source, *Prevotella* was also present when characterising the gut microbiome and is important for the metabolism of carbohydrates (Wu *et al.*, 2011; Amato *et al*., 2014; Kovatcheva-Datchary *et al*., 2015; Gorvitovskaia *et al*., 2016). Similarly, the gut microbiome of the golden snub-nosed monkey (*Rhinopithecus roxellanae*) also relied on *Prevotella* for carbohydrate metabolism. (Zeng *et al*., 2022). As *Prevotella* presence is linked to carbohydrate consumption and glucose metabolism, the high numbers of this genus in the *O. crassicaudatus* population are possibly linked to the increased consumption of insects and gum in the summer months, by which, the non-digestible food matter can be broken down by *Prevotella*.

The results support our prediction that there is a relationship between seasonal changes of weather and the faecal microbiome of *O. crassicaudatus*. The weather parameters, ambient temperature and rainfall, have a direct effect on the availability food depending on the season. Elevated temperatures and rainfall, as well as resource availability, are important drivers of insect population dynamics and availability (Williams, 1961). The decrease in insect availability during colder, drier periods will result in a change in the diversity and composition of the faecal microbiome as is apparent in the results. Additionally, the presence of rainfall and high ambient temperatures induces the migration and mating period for many species of insects (Williams, 1961) and a drop in temperature and low precipitation reduces the food available

and also causes a restriction of host activity patterns in order to preserve energy (Esteve, et al., 2017).

Body weight and health condition of individuals did not appear to show much variation between seasons. Nash (1986) suggested that gum exudates were high in calories (averaging approximately 12.1 kcal/g). Subsequently, the preliminary findings by Jayne (2020) addressing the nutritional components within the gum exudates found at the Lajuma Research Station determined that the gum exudate samples contained an average of 380.8 ± 12.5 kcal/100g. The study also concluded no significant changes in gum nutritional status between seasons and is a stable food resource for *O. crassicaudatus*. Therefore, feeding from these gum sources could potentially provide enough sustenance to maintain a general health status across the year.

The results showed an inverse relationship between Firmicutes and Bacteroidetes. Fruit consumption is commonly associated with increased Firmicutes relative abundance (Mallott et al., 2018) and is also associated with producing high-energy SCFAs (Schleifer, 2009). Whereas, a high prevalence of Bacteroidetes and decreased relative abundance of Firmicutes are associated with increased fibre consumption and lower consumption of high-glycaemic index sugars in several mammal species (for instance, De Fillipo *et al.*, 2010; Ley, 2010; Wu *et al.*, 2011; Nagpal *et al.*, 2018). It is possible the Firmicutes population density increased during winter, possibly, in relation to the increase in fruit consumption which could inevitably lead to the rise in SCFAs. Bacteroidetes species are capable of breaking down the hard exoskeleton of insects (Wu *et al.*, 2011; Rothman *et al.*, 2014), therefore their increased presence in summer may be directly related to the increase in insect consumption.

The gut is a fermentative environment

A major role of the anatomical and physiological aspects of *O. crassicaudatus* gut and the major taxa characterised within the faecal microbiome is to allow fermentation of food matter

(Nash, 1986; Langer & Clauss, 2018). Gut fermentation is a critical mechanism in several galago species that allows for the breakdown and absorption of nutrients from low-quality and difficult to digest foods that are necessary for survival in the less ideal conditions of winter (Nash, 1986). The microbes present in the gut are imperative for sufficient absorption of nutrients and energy required, especially in the cold dry winter conditions in which low-quality resources are available and in which the mating season occurs. Previous studies have described the fermentation abilities necessary for the digestion of gum exudates in *G. moholi* and *O. crassicaudatus* (Nash, 1986; Caton *et al*., 2000) in which fermentation spaces are present in the foregut to elongate the gut passage time and increase the volume of food matter broken down. The phylum Firmicutes, the family Bifidobacteriaceae, and the genus *Clostridium* are noted as being primary fermenters and could contribute to the fermentation abilities within the gut of *O. crassicaudatus* in this study. Interestingly, *Clostridium* is only present in the Dry season. Although there is no clear reason for this, some species of *Clostridium* can also cause harm when an individual's health is compromised (McClane *et al.*, 2012). *C. perfringens* (the most common species found in the faecal gut microbiota of *O. crassicaudatus* in this study), is an opportunistic pathogen that can form part of the mucosa lining in the gastrointestinal tract (Pluvinage *et al*., 2019). It is possible the individuals sampled during this season were not well, hence their preference to enter the set traps to rather preserve energy and gain food that required no additional effort. No visible signs of bad health were observed at the time of sampling there could have been an internal biological reasoning for *Clostridium* present within the body.

Interestingly, the presence of the genus *Clostridium* in winter, while absent in summer, could suggest a role in fermentation and the breakdown of cellulose and dietary fibre as has been observed in Tibetan macaques (*Macaca thibetana*; Sun *et al*., 2016). It is a genus commonly found in decaying vegetation, soil, and is known to play a role in the fermentation of polysaccharides. Similarly, a study by Licht *et al*. (2010) observed an increase in

Clostridiales and a decrease in *Bacteroides* in rat specimens when fed fruit pectin. The results showed a slight increase in unclassified Proteobacteria genera in winter. As Proteobacteria plays a role in energy acquisition, this increase may be a coping mechanism during cold weather when energy loss is inevitable (Koren et al., 2012; Amato et al., 2014).

Unlike other primate host species studied, such as black howler monkeys (*Alouatta pigra*; Amato *et al*., 2014) and ring-tailed lemurs (*Lemur catta*, Bennett *et al*., 2016), *Bifidobacterium* was the most abundant genus in *O. crassicaudatus* over the entire sampling period. It is a commensal genus that plays a key role in carbohydrate metabolism and is also beneficial to the host health by protecting the gut mucosal lining from harmful bacteria (Pinzone *et al*., 2012; Ghouri *et al*., 2014; Turroni *et al*., 2014). *Bifidobacterium* has also been associated with the degradation of arabinogalactan (Crociani *et al*., 1994; Grieshop, 2002; Bornbusch *et al*., 2019; Long, 2018) and was abundant in the faecal microbiome in the lesser galago (*G. moholi*; Long, 2018) during the winter period when gum exudates were the main food source. Zhu *et al*. (2021) determined that certain genes within the genome of *B. aesculapii* are necessary for the breakdown of *β-arabinans* in gum exudates from the Acacia senegal trees that are a main food source by common marmosets (*C. jacchus*). Similarly, *Bifidobacterium* was prevalent in exudativorous emperor tamarins (*Sanguinus imperator*; Duranti *et al*., 2017). A more definitive assessment of functional data of the faecal microbiome may provide more insight as to whether *Bifidobacterium* also plays a functional role in the degradation of gum in *O. crassicaudatus*.

Together, our results provide a first look into the faecal microbiota present within *O. crassicaudatus*. Ultimately, we find evidence of seasonal changes within the faecal microbiota linked to changes in their diet across seasons. This study has given us the first opportunity to characterise distinct bacterial species and genera. Overall, the faecal microbiota does not appear to substantially shift between seasons, possibly linked to the consistent consumption of

gum; however, several unique bacterial taxa are present in each season. The results from this study encourage further research into the functionality of these present bacteria.

Conclusion

In this study, we demonstrate the first look at the gut microbial community profile for *O. crassicaudatus*, a nocturnal NHP situated in a temperate environment that depends on food sources generally found in low quantities. These results indicate the variation and versatility in the microbial community within the gut of *O. crassicaudatus* and their symbiotic relationship with their host. This study highlights the importance of the gut microbiota in wild primate nutrition and feeding ecology and provide more information for future research into strepsirrhine primates. Alternate approaches, such as shotgun metagenomics to help identify bacterial functioning coupled with more refined characterization of bacterial diversity and metabolic function would further enhance our understanding of the relationship between diet and microbial communities. Overall, by identifying the gut microbial profile for *O. crassicaudatus*, this study provides further insight into the ecology of the relationship between host and microbiome and will add further information for primate conservation.

Chapter 5

Seasonal variation in the urinary and serum metabolite profiles of the thick-tailed greater galago (*Otolemur crassicaudatus*) using ^{1}H-NMR spectroscopy

Abstract

Temperate environments have highly seasonal changes in temperature and rainfall. These changes also alter the quality and quantity of foods available and will force animals to adapt to these fluctuations in diet. In this study, the physiological capabilities of *Otolemur crassicaudatus*, at the metabolite level were assessed to compare the serum and urinary metabolite profile of a wild population between two seasonal extremes, winter and summer. The results were able to generate the first look into the urinary and serum profile of *O. crassicaudatus*. The results indicated significant changes in the concentrations of certain metabolites between seasons, however overall concentration of metabolites did not significantly change between seasons. Importantly, ketones and branched-chain amino acids significantly increased in the winter period, indicating the necessity of alternative mechanisms to achieve energy homeostasis within the body. There is an increase in galactose in winter and an increase in specific amino acids and purines potentially influenced by the presence of available food resources. These findings help us to understand how this species is capable of surviving during less ideal conditions and provide useful guidance for future research into the survival threshold of this species.

Introduction

Primates will forage for a variety of foods in order to meet their daily nutritional requirements (Parker, 2003; Amato & Garber, 2014; Righini, 2014; Coiner-Collier *et al*., 2016). This is mostly seen as a response to challenges with changes in seasons, including fluctuations in food resources (Hemingway & Bynum, 2005). Some primate species are generalist feeders, for

instance, baboons (Gesquiere *et al*., 2008), geladas (Baniel *et al*., 2021), and macaques (van Schaik & van Noordwijk, 1985; Cui *et al*., 2019), which allows them to rely on alternative food sources when preferred foods are unavailable (Foerster *et al*., 2012). These have been termed fallback foods and usually have lower energetic value, however, are essential for the survival of a species (Marshall *et al*., 2009). Alternatively, some species of primates are specialist feeders, for instance, western tarsiers (*Tarsius bancanus*) which are carnivorous (Jablonski & Crompton, 1994) and spectral tarsiers which are insectivorous (Gursky, 2000). Sufficient energy intake is critical for triggering ovulation and reproductive success in females (Trivers, 1972; Bronson, 1985; Schneider, 2004) and, essentially, the survival of a species. Thus, understanding the nutritional ecology is important to gain insight into the relationship between an individual and its selection and use of available food resources. Animals will opt for a diet that allows them to gain the correct nutrients in ideal conditions (Simpson *et al*., 2004; Jensen *et al*., 2012; Cui *et al*., 2018). The correct use of nutrients, proteins, and lipids are necessary by individuals at all life history stages (Altmann, 1991; Thompson, 2017). In generalist feeders, it is expected that metabolic systems are more flexible to changes and can withstand nutrient deficits or excesses (Cui *et al*., 2018). For example, if an animal's diet is dominated by one nutrient type (for instance, a high protein diet), the individual amino acids could be converted into energy to compensate for the carbohydrate or fat deficiency (Raubenheimer *et al*., 2012; Simpson *et al*., 2004). Rhesus macaques (*Macaca mulatta*), which are generalist primates, were shown to regulate their macronutrients dependent on the energy requirements of an individual (for instance, lactating or non-lactating; Cui *et al*., 2018; 2019). Individuals living in temperate environments experience seasonal variations in extrinsic conditions throughout the year. This includes changes in day length, temperature and rainfall which will, inevitably, affect the availability of food (Grueter *et al*., 2009).

The greater thick-tailed galago (*Otolemur crassicaudatus*, referred to as such for the remainder of the study) is an arboreal, nocturnal Strepsirrhine primate species residing in the temperate Northern to south-Eastern regions of South Africa. Contrary to the lesser galago (Scheun *et al.*, 2014, 2015; Engelbrecht, 2016), few studies have addressed the sociality (Clark, 1975; Bearder *et al.*, 2003), reproduction (Dixson, 1989; Pullen *et al*., 2000), behaviour (Bearder *et al*., 2003), and diet (Bearder & Doyle, 1974; Bearder & Martin, 1980) of *O. crassicaudatus* within the last two decades. *O. crassicaudatus* feeds mainly on insects and gum exudates in the summer season and when food densities diminish in winter, the species depend more on gum exudates and available fruits (Bearder & Doyle, 1974). It is widely understood that behavioural and physiological mechanisms are critical for the survival of an individual. Little is known about the physiological strategies that are implemented for *O. crassicaudatus* to withstand seasonal changes in weather and resulting food availability. This information will then be able to give insight about how these mechanisms could be affected by the progression of climate change.

Metabolomics is the comprehensive analysis of small molecules in a cell, tissue, organ, or their environment (Oliver *et al.*, 1998). This form of analysis has become a powerful tool in several research fields such as nutrition, pharmacology, and toxicology. Metabolic responses to challenges are reflected in the metabolome as changes in the concentration and presence of certain metabolites are associated with specific physiological processes (Suàrez *et al.*, 2017). Various sample matrices such as biofluids and tissues can be used in metabolite profiling to identify biomarkers. Most commonly in primate studies, metabolomics has been used to assess the response of microbial metabolism to seasonal changes using faeces, for instance, in black howler monkeys (Mallott *et al*., 2022) and gorillas (Gomez, 2015). Few metabolomics studies on free-ranging primates have specifically targeted serum or urinary metabolites, although these matrices have been used to assess the impact of radiation exposure (for instance, Pannkuk

et al., 2015; 2016). There are various analytical platforms that can be implemented to assess the metabolites. Most commonly, mass spectrometry is used with the addition of either light- or gas-chromatography to analyse specific molecules. Nuclear magnetic resonance (NMR) spectroscopy is another frequently used technology to analyse the metabolites in biological samples.

NMR has become a popular analytical tool used to acquire complex data from metabolites. Its popularity over mass spectrometry includes it being highly reproducible; the samples are not destroyed; a short acquisition time to obtain results; a large range of metabolites are detected and quantified (Zheng *et al*., 2011; Takis *et al*., 2017; Röhnisch *et al*., 2018; Emwas *et al*., 2019; Wang *et al*., 2022). NMR is used to analyse the metabolites in biological samples such as urine and serum and has been used in the detection of various diseases, contaminants, and metabolic mechanisms within various tissues found within living organisms (Emwas *et al*., 2019; Wishart, 2019). The platform allows for the identification and quantification of a variety of metabolites (Dunn *et al*., 2011).

Understanding food choice and nutritional ecology of wild primates provides more insight into the factors that have developed the nonhuman primate diet, physiological mechanisms, and behaviour (Righini, 2014). Metabolomics can be used to assess the physiological processes and metabolic pathways implemented by an individual to ascertain adequate energy. In Chapter 2, it was deduced there were no significant seasonal changes in glucocorticoid concentrations; however, it was apparent lactating females may be affected by the increased energy requirements. In Chapter 3, the results indicated a significant increase in T3 hormone levels during the summer season suggesting an increase in metabolism within individuals. In Chapter 4, a dramatic seasonal shift in certain phyla was determined, suggesting a different combination of food resources across seasons.

Therefore, in this study, we aimed to assess the profiles for the urinary and serum metabolites of *O. crassicaudatus*. From this we will be able to investigate the effects of seasonal dietary changes and how certain physiological mechanisms are used to maintain homeostasis within the body. Overall, we expect to see changes in the metabolite concentrations owing to the variation in food types and availability across seasons.

Materials and Methods

Study site

Sampling was conducted at the Lajuma Research Centre (23°2'16.8" S, 29°26'34.08" E) located in the Soutpansberg mountains in the Limpopo province of South Africa. This is a highly seasonal temperate environment comprised of several habitats including montane forest and savanna.

Weather monitoring

The on-site weather station (Davis Instruments sensor suite linked via wireless to a Davis Pro 2™ console, Measurement and Console Systems CC, Cape Town, South Africa) provided the hourly ambient temperature and rainfall data used in this study. The mean (± standard deviation; SD) daily ambient temperature (°C) was calculated, and daily cumulative rainfall (mm) total for the sampling period was calculated. The proximity of the weather station data was used to ensure the data was similar to conditions experienced by the *O. crassicaudatus* population on-site.

Animal captures

Sampling was completed over a period from June 2017 to June 2019, in which sample collection occurred approximately during June-July 2017, January and March 2018, May-June 2018, and June 2019 to gather samples from the winter and summer seasons. The study follows

a standardised trapping method used in Chapter 2. Briefly, throughout the sampling period, Havahart® traps were secured to tree branches and baited with banana and peanut butter and set up just before sunset (17:00–18:30) and checked early in the morning (05:00–07:00). If captured, individuals were collected from the trap in capturing bags and scanned for identification using a passive identification transponder (ID100 Trojan, EURO I.D., Weilerswist, Germany). Individuals were taken back to the field laboratory for sample collection.

Sample collection

Animals were captured and transported to the field laboratory as described in Chapter 4. Each animal was anaesthetised with an intramuscular dose of tiletamine and zolazepam (Zoletil 100mg/ml, Virbac RSA, Centurion, South Africa). After weighing each animal, they were placed on a covered hot water bottle for the duration of the anaesthesia to maintain body temperature. A rectal thermometer (Hanna Instruments, USA) and pulse-oximeter (Edan H100B Vet, Edan Instruments, Pingshan, China) with a reflectance probe were lubricated and placed into the rectum to monitor body temperature, heart rate and SpO2. Heart rate, respiratory rate, rectal temperature, SpO2 and anaesthetic depth were monitored continuously, but recorded at 5-minute intervals to ensure each individual was stable during sample collection. Time of handling was kept to less than 10 minutes to reduce distress for the individuals. Once all the sampling procedures were completed, 10 ml of warm Ringer's lactate was given subcutaneously, and the animals were placed in a pet carrier to recover. If recovered and the veterinarian was satisfied they were ready, at sunset, individuals were returned to the initial site of capture for release.

Urine samples were collected by expressing the bladder while the individual was anaesthetised. Each sample was collected in a 2 ml Eppendorf tube and stored in the -4°C freezer. Whole blood samples were collected by venipuncture of the jugular vein from each captured individual using a 2.5 ml syringe and 21-gauge needle. Blood samples were then placed in plain serum tubes and allowed to stand at room temperature for 40 minutes before being centrifuged for 10 minutes at 1500 x g. The serum was then pipetted into Eppendorf tubes and stored at -20°C until transported back to the Paraclinical Laboratory at the Faculty of Veterinary Science, University of Pretoria, Pretoria, where samples were stored at -80°C until analysis.

Sample preparation

Samples were transported to the Centre for Human Metabolomics at North-West University in Potchefstroom, South Africa on dry ice for processing and NMR spectroscopy.

Buffer solution preparation

The urine buffer solution (1.5M KH_2PO_4) was developed following the methods in Mason *et al.* (2018). Briefly, the solution was prepared by dissolving 20.4 g of the buffer reagent in 80 mL of deuterated water (D_2O). Thereafter, 100 mg of TSP (trimethylsilyl-2,2,3,3-tetradeuteropropionic acid, 0.5805mM) and 13 mg of NaN_3 was dissolved in 6-10 mL of D_2O. Sonication was used to mix the solutions together. The pH was adjusted to 7.4 (by adding KOH pellets) and then transferred to a 100 mL volumetric flask and the volume adjusted with D_2O.

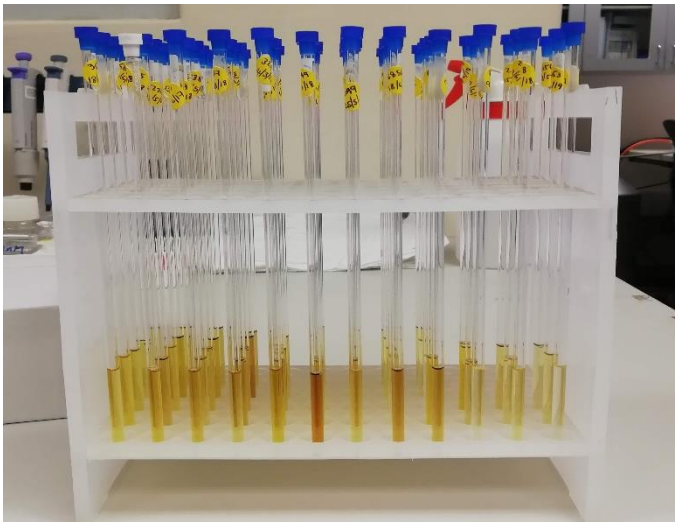
Urine sample preparation

For the urine sample preparation, a minimum of 1 mL urine was centrifuged at 12,000 g for five minutes. Then, 540 µL of ultrafiltrate was collected and added to 60 µL of buffer solution in a microcentrifuge tube. This solution was vortexed and centrifuged at 12,000 g for five minutes, the ultrafiltrate collected and transferred to a 5 mm NMR tube (Fig. 5-1a) and sealed.

Miniaturised ^{1}H-NMR protocol for serum

Majority of the serum samples collected were below an acceptable volume to apply the same method of sample processing as the urine samples. Alternatively, we implemented the miniaturised protocol devised by Mason *et al*. (2018) which only requires 10% of the original volume of serum buffer solution. Briefly, samples were filtered using Amicron Ultra – 2 ml centrifugal units with 10 kDa membrane filters (Merck, Ref: UFC201024). Each filter unit was pre-rinsed twice using ~2 ml dH_2O and centrifuged at 4500 g for 10 minutes to remove trace amounts of glycerol and glycerine from the filters, which are capable of interfering with the NMR signals. A volume of 100 µL of serum was filtered using the pre-washed filters. The samples were centrifuged for 20 minutes at 4500 g. The filtered samples were prepared in a 2 mm NMR tube (Fig. 5-1b) using the eVol® NMR digital syringe and a 180 mm-long bevel-tipped needle. A pipetting sequence was programmed: 1) aspirate 6 µL NMR buffer solution; 2) aspirate 54 µL of the filtered sample – maintaining the 10:90% ratio of D_2O:H_2O; 3) purge 60 µl to dispense the prepared sample into the 2 mm NMR tube; 4) aspirate 60 µL; 5) purge 60 µL (mixing the sample once inside the NMR tube) and then followed by a wash sequence; 6) aspirate 100 µL distilled water; 7) purge 100 µL (considered waste); 8) aspirate 100 µL distilled water; 9) purge 100 µL (considered waste); 10) aspirate 100 µL distilled water; 11) and finally, purge 100 µL (considered waste). For the miniaturized method, the Bruker MATCH system was implemented in which an adapter with a gripper was used to hold the 2 mm NMR tube. This was then inserted into a 10 mm spinner. Each NMR MATCH assembly was loaded onto a SampleXpress autosampler for the NMR analysis.

a)



b)

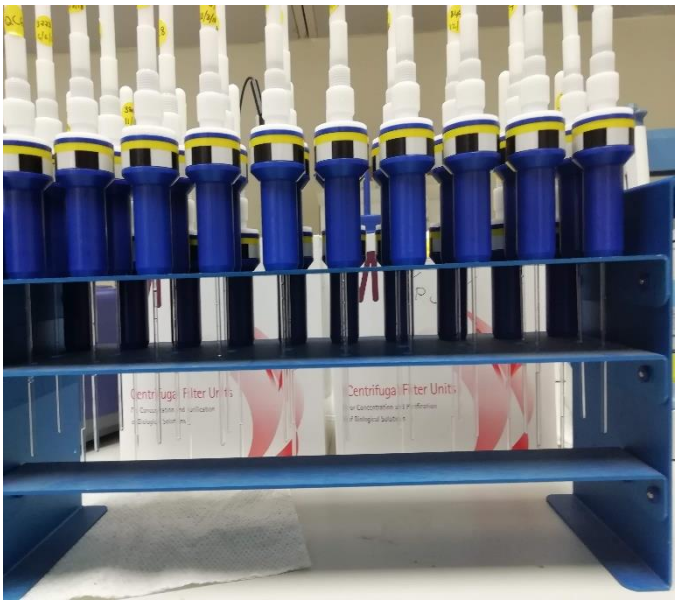


Figure 5-1. For the processing of samples, a) urine samples were aliquoted into 5 mm NMR tubes, and b) 2 mm NMR tubes for the serum samples prior to being placed in the NMR instrument.

Quality control

For both serum and urine sample processing, quality control samples (QCs) were implemented. For every 10 urine samples, 100 µL of each sample was added to a microcentrifuge tube to create the QC. This was repeated for the next ten samples, and so on. This solution was vortexed and added as an additional sample. This pooled QC was aliquoted into 9 smaller analytical QCs, which were then added to the sample analysis queue at constant interval between samples.

For the serum samples, only 10 ϑL of each sample was pooled and filtered. The pooled QC was aliquoted into 10 smaller analytical QC's. The QCs were added to the sample analysis line-up at constant intervals between samples. The QC samples were analysed following the same methods as that used for the urine samples. Serum QC samples were treated the same way and analysed with the serum samples.

^{1}H-NMR Spectroscopy

Samples were measured at 500 MHz on a Bruker Avance III HD NMR spectrometer equipped with a triple-resonance inverse (TXI) ^{1}H{15N, 13C} probe head and x, y, z gradient coils. For the urine samples, ^{1}H spectra were acquired as 128 transients in 32,000 data points with a spectral width of 10504 Hz and acquisition time of 3.12 seconds. Receiver gain was set to 90.5. For the serum samples, ^{1}H-NMR spectra were acquired as 128 transients in 32 K data points with a spectral width of 6002 Hz and an acquisition time of 2.72 seconds. The receiver gain was set to 64. For both sample matrices, the sample temperature was maintained at 300 K and the H_2O resonance was presaturated by single-frequency irradiation during a relaxation delay of 4 seconds (NOESY-presat), with a 90° excitation pulse of 8 µs. Shimming of the sample was performed automatically on the deuterium signal. Fourier transformation and phase and baseline correction were done automatically. Processing of ID ^{1}H-NMR data was conducted using Bruker Topspin (version 3.5; Bruker Corporation, Billerica, Massachusetts, USA) software. Spectral metabolite labelling was performed in Chenomx NMR Suite Chenomx Profiler (version 9.02; Chenomx Inc, Edmonton, Canada).

Statistical Analyses

To convert ^{1}H-NMR spectra into a metabolite relative concentration table, the R package 'ASICS' (R software package version 4.1.3 https://bioconductor.org/packages/ASICS; Lefort *et al.*, 2021). Briefly, it contains an automatic identification approach and quantifies the metabolites in the ^{1}H-NMR spectra based upon unique peak (Lefort *et al.*, 2021). The water

zone along the spectra was excluded from the analysis as it would cause noise in the spectra, making the analysis and identification of compounds difficult. A table including the metabolites detected and their concentrations was generated.

Analysis was conducted using MetaboAnalyst© (www.metaboanalyst.ca). This is an interactive platform in which all statistical analyses were conducted using R commands. Metabolite values were compared between winter and summer using t-tests. The urine and serum metabolite datasets were normalised using the mean, and then auto scaled. False discovery rates (FDRs) for each metabolite are estimated. These will indicate the degree of false positives within the results. The FDR determines the adjusted p-value which will signify which result are truly significant. Urinary and serum metabolite datasets were explored by principal component analysis (PCA; Hotelling, 1993) to identify the most significant principal components. Partial least squares-discriminant analysis (PLS-DA) was performed to highlight the metabolites distinguishing the two seasons. The quality of fit of the models was estimated by the proportion of cumulative explained variance (R^2) for the variables (x = metabolites and y = seasons).

Results

Weather variables

Seasonal differences were determined for rainfall and ambient temperature. Rainfall peaked in February 2018 (232 mm) with a significant rise in the summer months, compared to winter ($F_{(9)} = 3.45$, $p = 0.065$; Tukey HSD: $p = 0.049$) in which the study site received less than 1 mm of precipitation each day. Ambient temperatures were significantly warmer in summer, ranging from 9.4 – 38.0 °C, than in winter (range = 4.4 – 29.6 °C; mean = 14.85 ± 0.59◦C; Tukey HSD: $p < 0.001$).

Metabolite analyses

Overall, we processed a total of 99 samples: urine (total = 45 samples; females = 23 samples, males = 22 samples) and serum (total = 44 samples; females = 24 samples, males = 20 samples). From these samples, we were able to obtain the metabolite profiles for both urine and serum for a total of 18 individuals across the entire sampling period (Fig. 5-1).

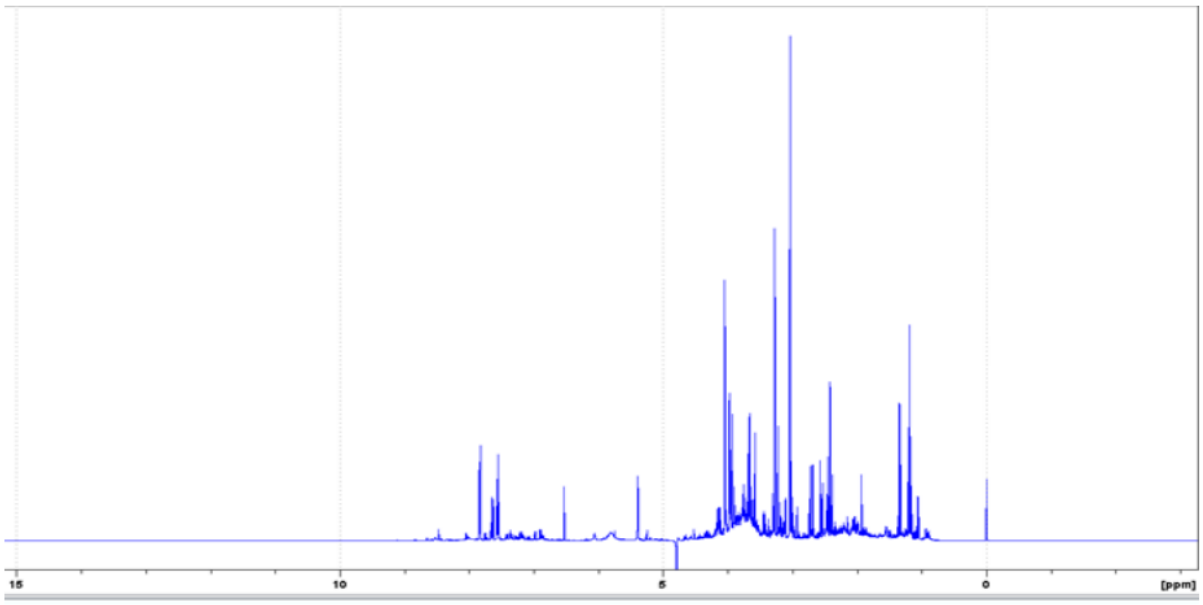
Urine Metabolites

After data cleaning, a total of 113 urine metabolites were detected for this set of 45 spectra (summer = 14 samples, winter = 31 samples). The most concentrated metabolites that significantly increased during winter include 3-hydroxyisovalerate, glutamic acid, 3-hydroxybutyrate, hippuric acid, and 2-aminobutyric acid (Tab. 5-2). Creatine, allantoin, uridine, succinate, and glyceric acid were some of the urinary metabolites that significantly increased in concentration during summer.

Table 5-1. Table depicting the urine metabolites that have indicated a significant seasonal difference. The metabolite class and the season in which there is a significant increase in the respective metabolite is given. False discovery rate (FDR) is also given.

Metabolite class	Metabolite	p-value	FDR	Change
Amino acid	Glutamic acid	<0.0001	0.006	↑ winter
	Citrulline	0.0006	0.023	↑ winter
	3-Methylhistidine	0.001	0.024	↑ winter
	Creatine	0.002	0.028	↑ summer
	Isoleucine	0.003	0.035	↑ winter
	Proline	0.013	0.070	↑ winter
	Sarcosine	0.014	0.075	↑ summer
	Arginine	0.001	0.024	↑ winter
Antioxidant	Glutathione-oxidised	0.011	0.066	↑ summer
Dicarboxylic acid	Quinolinic acid	0.011	0.066	↑ winter
Ketoacid	2-Oxoisovaleric acid	0.011	0.066	↑ winter
Glucuronic acid derivative	Glucaric acid	0.003	0.034	↑ winter
Purine	Uridine	0.010	0.066	↑ summer
	Allantoin	0.003	0.034	↑ summer
Ketone body	2-Hydroxybutyric acid	<0.0001	0.006	↑ winter
	3-Hydroxybutyric acid	0.002	0.028	↑ winter
	2-Aminobutyric acid	0.003	0.028	↑ winter
Metabolite intermediate	Dihydrothymine	0.009	0.066	↑ winter
	Indoxysulfate	0.019	0.093	↑ winter
	Succinic acid	0.013	0.057	↑ summer
Gut microbial derivative	4-Hydroxyphenylacetic acid	0.0005	0.023	↑ winter
	Hippuric acid	0.003	0.028	↑ winter
	3-Phenylpropionic acid	0.003	0.034	↑ winter
	Benzoic acid	0.010	0.066	↑ winter
Styrene product	Phenylglyoxylic acid	0.003	0.034	↑ winter
Plant metabolite	Vanillic acid	0.003	0.028	↑ winter
Saturated fatty acid	Isovaleric acid	0.008	0.066	↑ winter
Sugar derivative	Fucose	0.020	0.096	↑ summer
Sugar acid	Glyceric acid	0.010	0.066	↑ summer
	Threonic acid	0.015	0.077	↑ winter
Sugar alcohol	Galactitol	0.003	0.034	↑ winter
Triglyceride	Glycerol	0.003	0.063	↑ winter

a)



b)

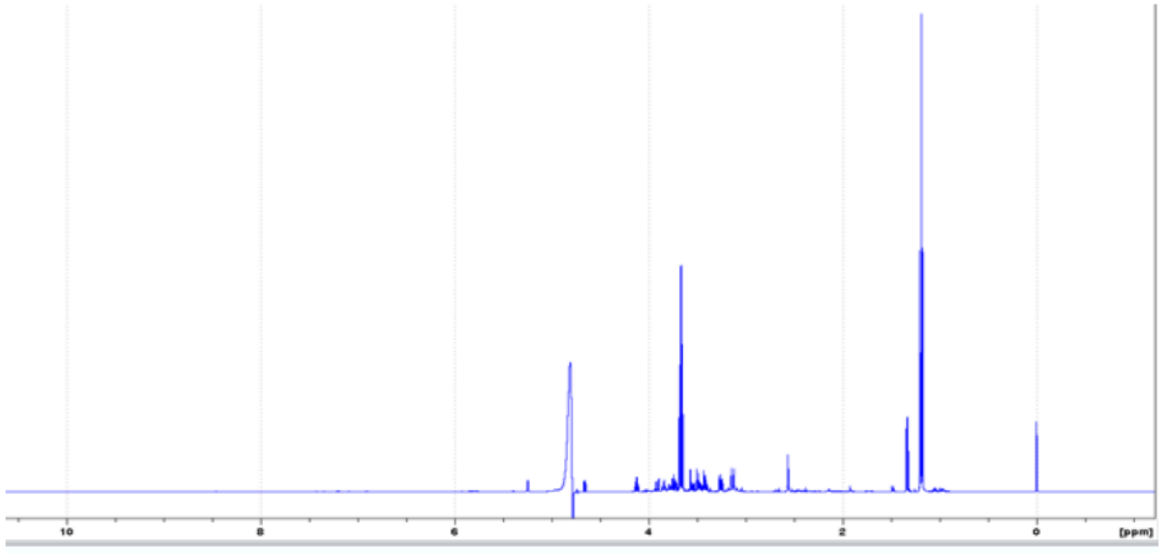


Figure 5-2. The a) urine and b) serum spectral profiles for *O. crassicaudatus.* The x-axis indicates the parts per millisecond (ppm) that the molecules are aligned. This a representative spectrum of all samples within this study. The blue peaks indicate the molecules present within the sample.

In the PCA, the first principal component (PC1) explained 14.7% of all the samples' variance with overlap between the winter and summer samples in the PCA plot. The PLS-DA showed separate insignificant clusters of summer and winter samples (p = 0.74; Fig. 5-3a).

Serum Metabolites

In total, 133 serum metabolites were detected for this set of 44 spectra (summer = 19 samples, winter = 25 samples), however, after removing metabolites absent from at least 50% of the samples, only 30 compounds were used in this analysis. Overall, 21 metabolites showed significant differences in concentration between seasons when assessing t-tests, these include arabitol, galactitol, glutamine, and propanol that increased during winter, while the metabolites lactate, ascorbic acid, and mannose increased in summer (Tab. 5-2).

Table 5-2. A table showing the serum metabolites of *O. crassicaudatus* that differ significantly between seasons. The metabolite class and the season in which there is a significant increase in the respective metabolite is given.

Metabolic class	Metabolite	p-value	FDR	Seasonal change
Adenosine receptor antagonist	7-Methylxanthine	<0.0001	0.005	↑ summer
Secondary alcohol	2-Propanol	<0.0001	0.001	↑ winter
Amine oxide	TMAO	0.002	0.006	↑ winter
Amino acid	Glutamine	<0.0001	0.0004	↑ winter
	Alanine	0.002	0.006	↑ winter
	Taurine	0.002	0.006	↑ winter
	Proline	0.022	0.038	↑ winter
	Glycine	0.034	0.049	↑ winter
Glycolysis-related metabolites	Glycerol	0.034	0.049	↑ winter
	Lactic acid	<0.0001	<0.0005	↑ summer
	Pyruvate	0.009	0.020	↑ winter
	Glucose	0.020	0.038	↑ winter
Ketone body	3-Hydroxybutyric acid	0.005	0.012	↑ winter
	2-Hydroxybutyric acid	0.023	0.038	↑ winter
Sugar	Glucuronic acid	0.002	0.006	↑ winter
Sugar alcohol	Arabitol	<0.0001	<0.0005	↑ winter
	Galactitol	<0.0001	<0.0005	↑ winter
	Xylitol	0.014	0.027	↑ winter
	Sorbitol	0.028	0.045	↑ winter
Sugar monomer	Mannose	0.005	0.012	↑ summer
Vitamin C	Ascorbic acid	0.001	0.006	↑ summer

In the serum metabolite analysis, PC1 component explained 33.9% of all the sample's variance with both the summer and winter samples overlapping extensively. From the PCA, the winter samples appear at lower PC1 scores, while the summer samples appear at high PC1 scores. PLS-DA for serum metabolites found a significant separation between seasons (p < 0.001; Fig. 5-3b).

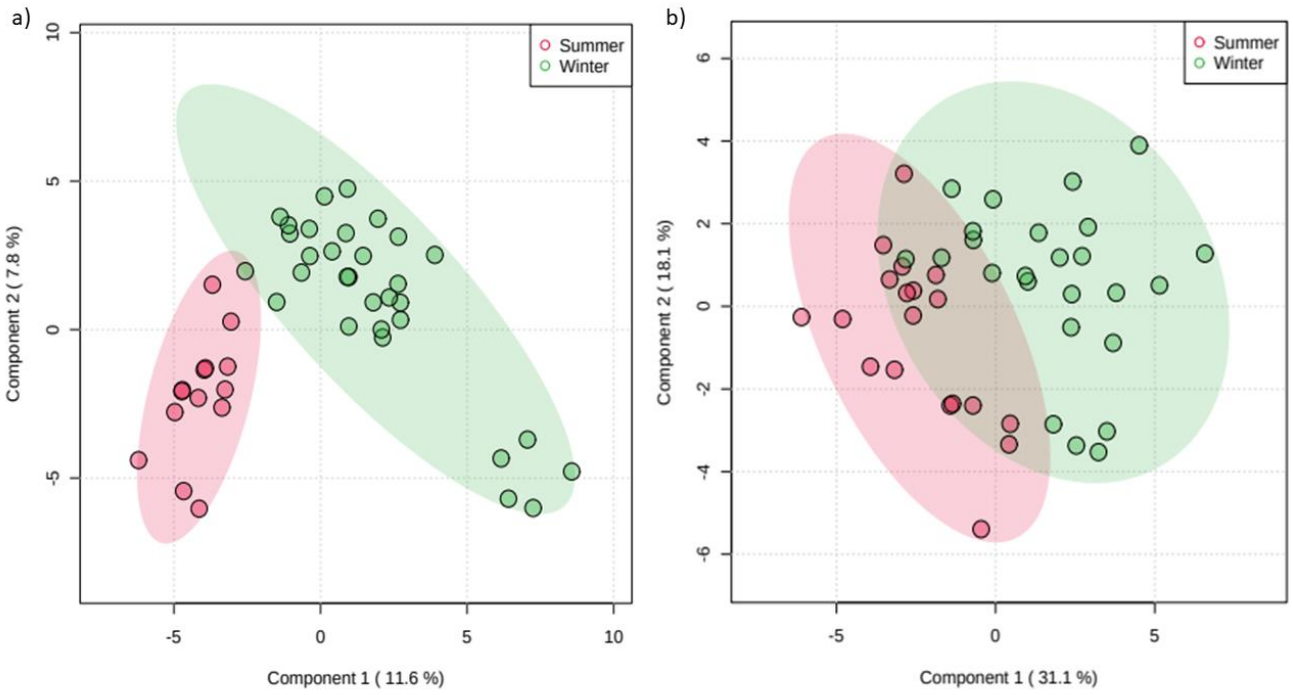


Figure 5-3. Plot of the first and second components for partial least square discriminant analysis (PLS-DA) plots for a) urine and b) serum samples demonstrating clusters between the summer (red dots) and winter (green dots) samples.

Pathway enrichment analysis

Metabolic pathways were identified for both urine and serum. In urine, 40 metabolic pathways were found to be associated with the present metabolites, however only nine pathways showed significant variation. These include valine, leucine and isoleucine biosynthesis (FDR = 0.0002; p < 0.001), aminoacyl- tRNA biosynthesis (FDR = 0.01; p < 0.001), alanine, aspartate and glutamate metabolism (FDR = 0.06; p = 0.002), and phenylalanine metabolism (FDR = 0.07; p = 0.003; Tab. 5.3).

Table 5-3. Over representation analysis (ORA) of significant urine metabolites. The pathways detected indicate the pathways significantly associated with the present metabolites. The total metabolites present in the pathway, the total number of impacted metabolites (Total hits) from the current dataset that are found within each pathway, the p-value, the FDR-adjusted p-value, and Holm p-value are shown.

Pathway	Total metabolites present	Total hits	*p*-value	Holm *p*-value	FDR
Valine, leucine and isoleucine biosynthesis	8	6	<0.01	<0.01	0.02
Aminoacyl-tRNA biosynthesis	48	11	<0.01	0.02	0.01
Alanine, aspartate and glutamate metabolism	28	7	<0.01	0.19	0.06
Phenylalanine metabolism	10	4	<0.01	0.27	0.07
Glycine, serine, and threonine metabolism	33	7	<0.01	0.49	0.10
Ascorbate and aldarate metabolism	8	3	0.01	1	0.20
Phenylalanine, tyrosine and tryptophan biosynthesis	4	2	0.03	1	0.32
Purine metabolism	65	9	0.03	1	0.33
Galactose metabolism	27	5	0.03	1	0.33

In the serum pathway enrichment analysis, 33 metabolic pathways were found to be associated with the present metabolites, however only seven pathways showed significant variation. These include aminoacyl- tRNA biosynthesis (FDR = 0.002; $p < 0.001$), galactose metabolism (FDR = 0.005; $p < 0.001$), valine, leucine and isoleucine biosynthesis (FDR = 0.001; $p < 0.001$), alanine, aspartate and glutamate metabolism (FDR = 0.038; $p = 0.002$), glyoxylate and dicarboxylate metabolism (FDR = 0.047; $p = 0.003$), glycine, serine and threonine metabolism (FDR = 0.047; $p = 0.003$), and pentose and glucuronate interconversions (FDR = 0.056; $p = 0.005$; Tab. 5-4).

Table 5-4. Over representation analysis (ORA) of significant urine metabolites. The pathways detected indicate the pathways significantly associated with the present metabolites. The total metabolites present in the pathway, the total number of impacted metabolites (Hits) from the current dataset that are found within each pathway, the p-value, the FDR-adjusted p-value, and Holm p-value are shown.

Pathway	Total metabolites present	Total hits	*p*-value	Holm *p*-value	FDR
Aminoacyl-tRNA biosynthesis	48	7	<0.001	0.002	0.002
Galactose metabolism	27	5	<0.001	0.010	0.005
Valine, leucine and isoleucine biosynthesis	8	3	<0.001	0.030	0.01
Alanine, aspartate and glutamate metabolism	28	4	0.002	0.150	0.038
Glyoxylate and dicarboxylate metabolism	32	4	0.003	0.240	0.047
Glycine, serine and threonine metabolism	33	4	0.003	0.270	0.047
Pentose and glucuronate interconversions	18	3	0.005	0.360	0.056

Discussion

This study aimed to examine whether there was any seasonal variation in the serum and urinary metabolite profiles of a wild population of greater thick-tailed galago (*O. crassicaudatus*) living in a seasonal temperate environment using ^{1}H-NMR spectroscopy. The results revealed significant changes of several metabolites related to either the physiological pathways and processes, or the diet preferred by *O. crassicaudatus*. The findings of this study partially support our prediction that seasonal (and, thus dietary) changes have a profound effect on the physiological pathways involved in the daily maintenance of an individual. These results may also suggest the physiological mechanisms employed to maintain energetic homeostasis despite the nutritional challenges faced in the winter period. Results from the previous chapters suggest that food availability and environmental factors (such as ambient temperature) influence the physiological mechanisms within *O. crassicaudatus*. From Chapter 2 we deduced there is an increase in food consumption and variety of food available for *O. crassicaudatus*

during summer including higher density of gum and insect populations. In winter, individuals were reliant on gum exudates and available fruit. Lactation is noted as a main factor affecting glucocorticoid secretion increase in summer, it demands high energy usage for the production of milk for offspring. Chapter 3 describes a substantial increase in triiodothyronine (T3) levels during the summer months, suggesting an increase in metabolism and activity. Chapter 4 also suggests the microbiota detected in the faeces across seasons were useful for the maximum breakdown of food matter for the absorption of nutrients. The results from this chapter indicate there is a substantial loss in food nutrition during winter indicated by the presence of several metabolites involved in the process of ketosis and energy usage. In this discussion, we discuss several of the metabolites from the urine and serum data that show significant differences between seasons and help to describe what may be occurring, physiologically, within *O. crassicaudatus*. We also discuss some significant pathways that support our metabolite results.

Metabolite analysis

Energy metabolism

During the summer period an increase in energy metabolites is observed. This increase in activity, feeding strategies (catching insects), and potential maternal stressors will require additional energy reserves. Results from Chapter 3 showed that lower concentrations of the hormone triiodothyronine (T3) in the winter period indicate lower metabolism. We suggest less energy is being used for during this time by reducing their activity levels.

The metabolites glucose and pyruvate are necessary for energy production via the tricarboxylic (TCA) cycle (also known as the Krebs or citric cycle; Roznere *et al*., 2014). In serum, the increase in glucose during the period of food limitation could suggest glucose is produced via gluconeogenesis (Roznere *et al*., 2014) and is specifically targeting the major bodily systems (such as the nervous, renal, and immune system) for energy usage (Rabinowitz *et al*., 2018).

Some species, such as the Japanese quail (*Coturnix japonica*; Lamasova *et al*., 2004) and freshwater mussel (*Amblema plicata*; Roznere *et al*., 2014), exhibit a glucose spike soon after food availability is reduced, and then a slow decrease in glucose concentrations was recorded.

Succinate is a crucial metabolite part of the TCA cycle necessary in the production of ATPs and is also a metabolic end-product of gut bacteria (Sharon *et al*., 2014). *Prevotella sp* are renowned for their production of succinate and has been linked to improved glycaemic control via intestinal gluconeogenesis (De Vadder *et al*., 2016; Kovatcheva-Datchary *et al*., 2015). In black howler monkeys, succinate has been linked to a highly fibrous diet (Mallot *et al*., 2022). In *O. crassicaudatus*, succinic acid is elevated in urine during the winter period and could, perhaps, be related to the increase in a high fibre diet from relying mainly on gum exudates and figs. Succinate also plays an important role within the mitochondria in which it is involved in catabolic and anabolic processes (Grimolizzi & Arranz, 2018).

Lactate is a major source of energy for animals (Bergman *et al*., 1999; Chen *et al*., 2016; Hui *et al*., 2017). An increase in serum lactate in winter months and a decrease during summer is accepted as the normal response in human starvation studies (Kochan & Eseva, 2009) and is possibly related to the demands of glycolysis while the body maintains energy levels during decreased glucose concentrations (Brooks, 2018). Briefly, during excessive energy expenditure or anaerobic activity in which oxygen is limited, lactate is produced during carbohydrate metabolism (Landaas & Petersen, 1975; Brown & Poon, 2005; Yang, 2012). Conversely, studies have suggested that increased insulin sensitivity caused by decreased food intake, leading to progressive weight loss, will lead to decreased lactate levels. This response was first suggested in humans (Chondronikola *et al*., 2018). This has also been demonstrated in obese dogs whereby lactate concentrations rise and individuals lose weight progressively (Vendramini *et al*., 2020). Dairy cows have also exhibited similar responses of insulin

sensitivity during progressive weight gain (Garnsworthy & Tropps, 2010). As *O. crassicaudatus* had increased serum lactic acid during the summer when food availability was adequate (leading to increases in body mass), suggests this species may also developed greater insulin sensitivity in winter, and increased lactate levels when weight gain occurred.

Lipolysis

When animals are challenged with food limitations, they will start to use their fat stores as a source of energy (Reshef *et al*., 2003; Roznere, 2014). There are several alternative energy sources mammals can implement to produce ATPs during periods of low energy availability. For instance, fats are an ideal source of energy as they contain as much as seven times more calories per weight than glycogen (Kolb *et al*., 2021) and can supply energy for weeks in humans (Owen *et al*., 1979). Fatty acids are used as an alternative in the TCA cycle when glucose is insufficient to generate ATPs. When glucose reserves are low, fatty acids are released from fat stores and utilised for fuel in cells through beta oxidation forming Acetyl Coenzyme A (Acetyl CoA). Acetyl CoA can be further broken down through the TCA cycle by condensation with oxaloacetate to form citric acid. However, since glucose levels are low, oxaloacetate becomes unavailable as it is required for gluconeogenesis. Subsequently, Acetyl CoA cannot enter the TCA cycle, concentrations build up and is alternatively metabolised in the liver to form acetoacetate. Acetoacetate can then be converted to 3-hydroxybutyric acid using 3-hydroxybutyrate dehydrogenase or can break down into another ketone, namely, acetone. These end-products are then distributed to target organs and tissues for energy usage (McPherson & McEneny, 2012). The increased presence of 3-hydroxybutyric acid within the urine and serum samples in winter is indicative of this lipolysis, which then leads to ketosis.

Ketosis

The results reflect significant effects of scarce or poor-quality food sources during winter with an increase in ketone bodies, such as 3-hydroxybutyric acid, in the urine and serum profiles. The increase in ketone bodies could be an indication of negative energy balance, low carbohydrate intake, or excessive exercise (Mierziak *et al*., 2011; Newman *et al*., 2017) as they are used as alternative products of fatty acid oxidation (Moyes *et al*., 1990; McCue *et al*., 2010) and then secreted secondary to gluconeogenesis as an energy source. The metabolite 3-hydroxybutyrate is a main indicator of ketosis in animal cells (Dedkova & Blatter, 2014; Mierziak *et al*., 2021) and is also prevalent in the regulation of lipid metabolism and a signalling function for regulating energy expenditure and homeostasis during malnutrition (Puchalska *et al*., 2017; Kolb *et al*., 2021; Mierziak *et al*., 2021). The metabolite 2-hydroxybutyric acid is produced in tissues that catabolise threonine and synthesise glutathione and has been suggested as a biomarker for metabolic acidosis within an individual (Landass & Pettersen, 1975; Rubio-Aliaga *et al*., 2011). It has also been suggested as an indicator of insulin resistance (Gall *et al*., 2010). In this study, a significant increase in 2-Hydroxybutyric acid was determined in both serum and urine during the winter period, indicating an increase in ketones and poor nutritional intake.

Pathway analysis
Galactose metabolism

In this study, *O. crassicaudatus* had an increase in several sugar molecules, such as galactitol, and arabitol in the winter indicating that *Acacia* gum was a predominant source of food. The sugar alcohols present in the spectral profile were most likely metabolites of *Acacia* gum consumption and fruit consumption. *Acacia* gum is a complex polysaccharide predominantly made up of different sugars, such as galactose (~40%), arabinose (~25%), and rhamnose (~15%) and also contains approximately 2-10% protein material, approximately 0.3% nitrogen, and is comprised of a multitude of amino acids, such as serine, threonine, and hydroxyproline

(Idris *et al*., 1998; Azzaoui *et al*., 2015). Galactose metabolism is a necessary metabolic pathway in which we then see the end-product metabolites such as arabitol, glycerol, galactitol, glucuronic acid. Sorbitol and xylitol are usually found in fruit, they metabolise into fructose once consumed and then converted into glucose through glucose-6-phosphate. These sugars were both elevated in serum during winter months and most likely reflect increased fruit consumption.

Amino acid metabolism

Valine, leucine and isoleucine biosynthesis was a significant pathway determined in the analysis. Amino acids are essential for protein synthesis in the body tissues. Isoleucine, leucine, and valine are branched chain amino acids (BCAAs) necessary for lipid and glucose metabolism (Yoshizawa, 2015) and blood glucose regulation (Doi *et al*., 2005). If glucose or energy levels are low within the body, BCAAs derived mostly from muscle tissue (Brosnan & Brosnan, 2006) are one of several alternative sources in the process of gluconeogenesis to produce glucose from the muscles (Doi *et al*., 2005). If glucagon levels are low and cannot supplement the blood with suitable glucose levels, proteins in skeletal muscle tissue are broken down into amino acids. The results showed an elevation in BCAAs in the winter period, suggesting they were necessary for proteolysis (Kettlehut *et al.*, 1994; Finn & Dice, 1996). Subsequently, there was a significant increase in overall amino acids during winter. Similarly, in humans, elevated levels of gluconeogenic amino acids, such as alanine, have been observed several weeks after starvation (Felig *et al*., 1970) and lower blood sugar concentrations. Therefore, in *O. crassicaudatus*, it is possible the rise in amino acids is caused by low energy intake.

Creatine is a molecule necessary in supplying energy to the muscle cells via their function in ATP regeneration. Creatinine is produced from the dehydration of creatine and is

an indicator of body muscle mass in humans (Heymsfield *et al*., 1983) and can be used as a biomarker for individual health status (Heymsfield *et al*. 1983; Nemoto *et al*, 2001). An increase in strenuous exercise and metabolism is associated with rising levels of creatine and creatinine (Heymsfield *et al*., 1983). However, endogenous production of creatine requires methionine, arginine, and glycine. These amino acids are found in greater quantities in insects (Rumpold & Schlüter, 2013), so increases in urinary creatine seen in *O. crassicaudatus* will most likely be attributed to the increased consumption of insects during summer.

Purine metabolism

Nucleotides are the fundamentals for all genetic makeup within the body and are comprised of purines and pyrimidines. They are critical in the synthesis of DNA and RNA, cell signalling, and metabolism (Wu *et al*., 2022). An increase in nucleotides indicates the degradation of proteins for fuel (Gillis & Ballantyne, 1996). In this study, a significant increase in the nucleotides allantoin and uridine was found in the summer indicating there may be an increase in protein consumption derived from their diet as has previously been theorised by Kiriyami & Ashida (1964). Pak *et al.* (1973) confirmed allantoin is also linked to nitrogen metabolism and weight gain (indirectly related to protein consumption). Therefore, these nucleotide elevations in *O. crassicaudatus* are likely to be linked to the increase in total body nitrogen and weight gain caused by protein (insect) consumption rises.

Aminoacyl-tRNA synthesis is necessary for the translation of the messenger RNA (mRNA) in the generation of new amino acids and, inevitably, necessary for protein synthesis (Ibba *et al*., 1997; Ibba & Söll, 1999). As mentioned above, proteins are critical for energy homeostasis within the body. Therefore, the production of proteins is necessary. The process involves pairing specific translation RNAs (tRNAs) and amino acids for the transmission of genetic information (Ibba & Söll, 2001). Thus, the process includes up to 21 pathways for

aminoacyl-tRNA synthesis. It is clear the production of aminoacyl-tRNAs is a critical cellular process required for the generation of proteins.

Conclusion

The body is a professional at regulating itself. The results suggest significant effects of scarce or poor-quality food sources during winter with an increase in ketone bodies and fatty acids metabolites demonstrating the body's physiological response by using alternative sources to maintain energy and health within the body. Although the findings are preliminary, the urine and serum profiles give us a first look into the physiological mechanisms implemented by this species to cope while residing in a temperate environment in which food resources shift in quality and quantity across seasons.

Chapter 6

Conclusion

This thesis investigated the physiological responses of the thick-tailed greater galago (*O. crassicaudatus*) by exploring their glucocorticoid, triiodothyronine (T3), faecal microbiome, and serum and urine metabolite profiles across seasons. The purpose of this research was to understand how this species, internally, reacts to changes in the external environment. This information is important to broaden our informational baseline of *O. crassicaudatus* to provide a platform for future research pertaining to the capabilities of this species to cope during less ideal conditions expected for the future. Subsequently, changes in the environment have been shown to cause severe or detrimental results in other non-human primate species. This usually results in the primate populations less adaptive to change to respond poorly; or the more flexible species will adapt to change by shifting their location or adjusting their physiology or behaviour.

At the onset of this study, I had expectations regarding certain traits of *O. crassicaudatus*. For instance, I presumed that this species had distinctive differences in food choices across different seasons as has been documented in *Galago moholi* (Scheun *et al*., 2015; Long, 2018). I found that gum is their main source of food that sustains them throughout the year, but they add additional energy and nutrients through the consumption of insects and fruit. In addition, I expected to see a greater influence on temperature changes in their glucocorticoid concentrations as an indicator of general physiology.

Chapter 2 explored the seasonal glucocorticoid concentrations of *O. crassicaudatus*. First, the cortisol EIA was most appropriate to measure GC metabolites in the faeces of *O. crassicaudatus*. Subsequently, the seasonal analysis of faecal glucocorticoids indicated that no significant seasonal factors (these included food availability, ambient temperature, and rainfall) were evident. Despite this, the results concluded that the lactation state of females appeared to

have a significant influence on their cortisol levels. However, as the results also indicated slight increases during the mating and winter period, there is the potential that other extrinsic factors may have still had a biological effect on this population of *O. crassicaudatus*.

Subsequently, the investigation of thyroid hormone concentrations in Chapter 3 suggested that metabolic regulation was the main role of T3 rather than as a thermoregulatory function. The results displayed an oscillating pattern of T3 concentrations that suggests a positive relationship with the environmental factors ambient temperature and rainfall. Food availability was also correlated with the extrinsic factors suggesting an increase in resources causes a rise in metabolic rate. During the cold, dry period, *O. crassicaudatus* may reduce activity while reducing their metabolism and also maintain energy reserves. These findings corroborate well with the final two chapters of this thesis in which the faecal microbial and urine and serum metabolite profiles were explored.

The results of Chapter 4 explored the most prominent taxa present within the faecal microbiome of this population of *O. crassicaudatus*. Interestingly, the results from this study did not demonstrate the clear differentiation between seasonal diets as was expected (in line with the dietary preferences found in Jayne, 2020). The results showed several dominant genera that were present throughout all seasons (for instance, *Bifidobacterium* sp.) analysed. Despite this lack of seasonal distinction in their faecal microbiota, other bacterial phyla such as Firmicutes and Bacteroidetes displayed changes between the seasons. Although this dataset limited my abilities to analyse the functionality of these taxa, I proposed that certain taxa were present to help degrade certain food sources at certain times of the year. Namely, Actinobacteria to help process insects in the wet, hot period and Firmicutes to help breakdown fruit in winter.

Chapter 5 was able to demonstrate the extensive abilities the body is able to accomplish in order to maintain homeostasis. The results show significant differences of certain

metabolites, but these are important indicators as to the metabolic condition of the body between both seasons. For instance, the increase in ketones suggests some degree of food restrictions during the winter period even though there were no significant changes in body weight (Chapter 4). The reduction in faecal T3 hormones while there is an increase in ketones could further support the previous claim that *O. crassicaudatus* reduces activity levels during the cold, dry season to preserve energy (Bearder, 1974).

Intrinsic factors such as age, health condition, sex, and reproductive state have a significant influence physiological aspects of *O. crassicaudatus*. Each of these factors will require levels of energy to accommodate the changes within the body. The findings from this study indicate these parameters will induce changes in their glucocorticoid and T3 hormone levels to help adapt. Although I did not see much differentiation between sexes, it seems that the lactation period is an energy-demanding time for females. Further research into the effects of these factors will provide more insight into this species' ability to cope with change. This study was limited to a small samples size of known individuals of which we were confident of their status.

External environmental factors such as ambient temperature and rainfall are important in shaping habitats. These elements will affect the landscape and the food availability accessible for *O. crassicaudatus*. As is becoming a prominent problem with many primate species, climate change will most likely impact important factors mentioned above (Estrada *et al.*, 2017). If rainfall continues to decrease while temperatures rise, this will surely affect the yield of gum exudates and fruit, and the presence of insects. It is important to continue sharing information that will add to the growing knowledge of *O. crassicaudatus* and the rest of the non-human primates and how they may be affected in future years when climate change progresses. Limitations from this study will allow for gaps and thus future research to be conducted on this species.

This thesis has demonstrated the astonishing capabilities of this species to survive on glucose reserves, and what metabolic mechanisms are implemented to maintain body condition. Despite this, with the increasing rate of weather changes, food availability may be dramatically affected causing hardships for the galago population at the study site and may cause the population to either have to adapt and make dietary choices, or may result in the range shift for this population to areas with better resources.

Chapter 7

References

Akasha, M.A., Anderson, R.R., Ellersieck, M., Nixon, D.A. (1987) Concentration of thyroid hormones and prolactin in dairy cattle serum and milk at three stages of lactation. *J Dairy Sci*, 70: 271–276.

Allana, A.T.L., LaBargeb, L.R., Howletta,C., Bailey, A.L., Jones, B., Mason, Z., Pinfield, T., Schröder, F., Whitaker, A., White, A.F., Wilkinson. H., & Hill, R.A. (2023). Patterns of predation and meat-eating by chacma baboons in an Afromontane environment. *Folia Primatologica*, 94: 13–36.

Altmann, S.A. (1991) Diets of yearling female primates (*Papio cynocephalus*) predict lifetime fitness. *Proc Natl Acad Sci USA*, 88: 420–433.

Altmann, J. (1980) Baboon Mothers and Infants. Cambridge, Harvard University Press. Pp: 242.

Amato, K.R., Yeoman, C.J., Kent, A., Righini, N., Carbonero, F., Estrada, A., Rex Gaskins, H., Stumpf, R.M., Yildirim, S., Torralba, M. & Gillis, M. (2013) Habitat degradation impacts black howler monkey (*Alouatta pigra*) gastrointestinal microbiomes. *The ISME Journal*, *7*(7): 1344–1353.

Amato, K.R., Leigh, S.R., Kent, A., Mackie, R.I., Yeoman, C.J., Stumpf, R.M., Wilson, B.A., Nelson, K.E., White, B.A. & Garber, P.A. (2014) The role of gut microbes in satisfying the nutritional demands of adult and juvenile wild, black howler monkeys (*Alouatta pigra*). *Amer J Phys Anthropol*, 155: 652–664.

Amato, K.R., Leigh, S.R., Kent, A., Mackie, R.I., Yeoman, C.J., Stumpf, R.M., Wilson, B.A., Nelson, K.E., White, B.A. & Garber, P.A. (2015) The gut microbiota appears to compensate for seasonal diet variation in the wild black howler monkey (*Alouatta pigra*). *Microbial Ecol*, 69: 434–443.

Amin, A., Dhillo, W.S., Murphy, K.G. (2011) The central effects of thyroid hormones on appetite. *J Thyroid Res*, 306510. https://doi.org/10.4061/2011/306510.

Anderson, D.R. (2007) Model based inference in the life sciences: a primer on evidence. Springer Science & Business Media. Pp: 60–64.

Armstrong, D., Santymire, R. (2013) Hormonal and behavioral variation in pied tamarins housed in different management conditions. *Zoo Biol*, 32(3): 299–306.

Arnold, T.W. (2010), Uninformative parameters and model selection usin' Akaike's Information Criterion. *J Wildl Manage* 74(6): 1175–1178.

Azzaoui, K., Hammouti, B., Lamhamdi, A., Mejdoubi, E., Berrabah, M. (2015) The Gum Arabic in the southern region of Morocco. *Mor J Chem*, 3(1): 99–107.

Bäckhed, F., Ding, H., Wang, T., Hooper, L.V., Koh, G.Y., Nagy, A., Gordon, J.I. (2004). The gut microbiota as an environmental factor that regulates fat storage. *Proc Nat Acad Sci,* 101(44): 15718–15723.

Balestri, M., Barresi, M., Campera, M., Serra, V., Ramanamanjato, J.B., Heistermann, M., Donati, G. (2014) Habitat degradation and seasonality affect physiological stress levels of *Eulemur collaris* in littoral forest fragments. *PloS One* 9(9): e107698.

Baniel, A., Amato, K.R., Beehner, J.C., Bergman, T.J., Mercer, A., Perlman, R.F., Petrullo, L., Reitsema, L., Sams, S., Lu, A. (2021) Seasonal shifts in the gut microbiome indicate plastic responses to diet in wild geladas. *Microbiome*, 9(1): 1–20.

Barton, K. (2016). Package 'MuMIn': Multi-model Inference. R Package Version 1.15.6.

Barton K. (2018). MuMIn: multi-model inference. R package version 3.6 https://cran.r-project.org/web/packages/MuMIn/MuMIn.pdf (last accessed 23 December 2019).

Bassett, J.H.D. & Williams, G.R. (2016) Role of thyroid hormones in skeletal development and bone maintenance. *Endocr Rev*, 37: 135–187. https://doi.org/10.1210/er.2015-1106.

Bates, D.M., Mächler, M., Bolker, B., Walker, S. (2014) Fitting linear mixed-effects models using lme4. *J Stat Softw* 67(1): 1–48. doi:10.18637/jss.v067.i01.

Bates, D.M. (2010). lme4: Mixed-effects modeling with R. Springer, Madison, WI, USA.

Bearder, S.K. (1974). Aspects of ecology and behaviour of the thick-tailed bushbaby, *Galago crassicaudatus*. Doctoral dissertation, University of Witwatersrand.

Bearder, S.K. & Doyle, G.A. (1974) Ecology of bushbabies *Galago senegalensis* and *Galago crassicaudatus*, with some notes on their behaviour in the field. In: Martin, R.D.., Doyle, G.A., Walker, A.C. (eds.), Prosimian Biology. London, Duckworth. Pp: 109–130.

Bearder S.K. & Martin, R.D. (1980) Acacia gum and its use by bushbabies, Galago senegalensis (Primates: Lorisidae). *Int J Primatol*, 1: 103–128.

Bearder, S.K. (1987). Lorises, bushbabies and tarsiers: Diverse societies in solitary foragers. In D. Cheney, R. Seyfarth, B. Smuts, T. Struhsaker, R. Wrangham (Eds.), Primate Societies. Chicago: University of Chicago Press. Pp: 11–24.

Bearder, S. & Martin, R. (1980) Acacia gum and its uses by bushbabies, *Galago senegalensis* (Primates: Lorisidae). *International Journal of Primatology*, 1(2): 103–128.

Bearder, S.K., Ambrose, L., Harcourt, C., Honess, P., Perkin, A., Pimley, E., Pullen, S., Svoboda, N. (2003) Species-typical patterns of infant contact, sleeping-site use and social cohesion among nocturnal primates in Africa. *Folia Primatol*, 74(5-6): 337–354.

Bergman, B.C., Wolfel, E.E., Butterfield, G.E., Lopascuk, G.D., Casazza, G.A., Horning, M.A., Brooks, G.A. (1999) Active muscle and whole-body lactate kinetics after endurance training in men. *J Appl Physiol*, 87: 1684–1696.

Beehner, J.C., Bergman, T.J., Cheney, D.L., Seyfarth, R.M., Whitten, P.L. (2005) The effect of new alpha males on female stress in free-ranging baboons. *Anim Behav*, 69: 1211–1221.

Beehner, J.C. & McCann, C. (2008) Seasonal and altitudinal effects on glucocorticoid metabolites in a wild primate (*Theropithecus gelada*). *Physiol Behav* 95(3): 508–514.

Bennett, G., Malone, M., Sauther, M.L., Cuozzo, F.P., White, B., Nelson, K.E., Stumpf, R.M., Knight, R., Leigh, S.R., & Amato, K.R. (2016). Host age, social group, and habitat type influence the gut microbiota of wild ring-tailed lemurs (*Lemur catta*). *Amer J Primatol*, 78(8): 883–892.

Behringer, V., Deimel, C., Hohmann, G., Negrey, J., Schaebs, F.S., Deschner, T. (2018). Applications for non-invasive thyroid hormone measurements in mammalian ecology, growth, and maintenance. *Horm Behav*, 105: 66–85.

Bereschenko, O., Bruscoli, S., Riccardi, C. Glucocorticoids, sex hormones, and immunity. *Front Immunol*, 9: 1332.

Bharti S.K. & Roy R. (2012) Quantitative ^{1}H NMR spectroscopy. *TrAC Trend Anal Chem*, 35:5–26. doi: 10.1016/j.trac.2012.02.007.

Bianco, A.C., Maia, A.L., Da Silva, W.S., Christoffolete, M.A. (2005) Adaptive activation of thyroid hormone and energy expenditure. *Bioscience Reports*, 25(3-4): 191–208.

Björk, J.R., Dasari, M., Grieneisen, L., & Archie, E.A. (2019). Primate microbiomes over time: longitudinal answers to standing questions in microbiome research. *Amer J Primatol*, 81(10–11): e22970.

Blake, N.G., Eckland, D.J.A., Foster, O.J.F. Lightman, S.L. (1991) Inhibition of hypothalamic thyrotropin-releasing hormone messenger ribonucleic acid during food deprivation. *Endocrinol*, 129: 2714–2718.

Bolt RJ, van Weissenbruch MM, Lafeber HN, Delemarre-van de Waal HA (2001) Glucocorticoids and lung development in the fetus and preterm infant. *Pediatr Pulmonol*, 32(1): 76–91. doi: 10.1002/ppul.1092. PMID: 11416880.

Boonstra, R., Hubbs, A.H., Lacey, E.A., McColl, C.J. (2001) Seasonal changes in glucocorticoid and testosterone concentrations in free-living arctic ground squirrels from the boreal forest of the Yukon. *Can J Zool*, 79(1): 49–58.

Bornbusch, S.L., Greene, L.K., McKenney, E.A., Volkoff, S.J., Midani, F.S., Joseph, G., Gerhard, W.A., Iloghalu, U., Granek, J., & Gunsch, C.K. (2019). A comparative study of gut microbiomes in captive nocturnal strepsirrhines. *Amer J Primatol*, 81(10-11): e22986.

Bronson, F.H. (1985) Mammalian reproduction: An ecological perspective. *Biol Reprod*, 32: 1–26.

Brooks, G.A. (2018) The science and translation of lactate shuttle theory. *Cell Metab*, 27: 757–785.

Burger, M.F. & Denver, R.J. (2002) Plasma thyroid hormone concentrations in a wintering passerine bird: their relationship to geographic variation, environmental factors, metabolic rate, and body fat. *Physiol Biochem Zool*, 75: 187–199.

Burnham, K.P. & Anderson, D.R. (2013) Model selection and multimodel inference. Springer, New York.

Burnham, K.P., Anderson, D.R., Huyvaert, K.P. (2011) AIC model selection and multimodel inference in behavioral ecology: some background, observations, and comparisons. *Behav Ecol Sociobiol*, 65(1): 23–35.

Burke, C.W. & Eastman, C.J. (1974) Thyroid hormones. *Br Med Bull*, 30: 93–99. https://doi.org/10.1093/oxfordjournals.bmb.a071175.

Burton, T., Killen, S.S., Armstrong, J.D., Metcalfe, N.B. (2011) What causes intraspecific variation in resting metabolic rate and what are its ecological consequences? *Proc R Soc B*, 278: 3465–3473.

Cabana, F., Clayton, J.B., Nekaris, K.A.I., Wirdateti, W., Knights, D., Seedorf, H. (2019). Nutrient-based diet modifications impact the gut microbiome of the Javan slow loris (*Nycticebus javanicus*). *Scientific Reports* 9: 4078.

Campos, F.A., Morris, W.F., Alberts, S.C., Altmann, J., Brockman, D.K., Cords, M., Pusey, A., Stoinski, T.S., Strier, K.B., Fedigan, L.M. (2017) Does climate variability influence the demography of wild primates? Evidence from long-term life-history data in seven species. *Glob Chang Biol* 23(11): 4907–4921.

Carnegie, S.D., Fedigan, L.M., Ziegler, T.E. (2011) Social and environmental factors affecting fecal glucocorticoids in wild, female white-faced capuchins (*Cebus capucinus*). *Am J Primatol* 73: 861–869.

Carré, M. & Cheddadi, R. (2017) Seasonality in long-term climate change. Quaternaire. Revue de l'Association française pour l'étude du Quaternaire 28(2): 173–177.

Carroll, I.M., Ringel-Kulka, T., Siddle, J.P., Ringel, Y. (2012). Alterations in composition and diversity of the intestinal microbiota in patients with diarrhea-predominant irritable bowel syndrome. *Neurogatroentrol Motil*, 24: 521–530.

Castro, M.I., Alex, S., Young, R.A., Braverman, L.E., Emerson, C.H. (1986) Total and free serum thyroid hormone concentrations in fetal and adult pregnant and nonpregnant guinea pigs. *Endocrinol*, 118(2): 533–537.

Caton, J.M., Lawes, M., Cunningham, C. (2000). Digestive strategy of the south-east African lesser bushbaby, *Galago moholi*. *Comp Biochem Physiol A: Mol Integ Physiol*, 127(1): 39–48.

Cavigelli SA (1999) Behavioural patterns associated with faecal cortisol levels in free-ranging female ring-tailed lemurs, *Lemur catta*. *Anim Behav*, 57(4): 935–944.

Cavigelli, S.A., Dubovick, T., Levash, W., Jolly, A., Pitts, A. (2003) Female dominance status and fecal corticoids in a cooperative breeder with low reproductive skew: ring-tailed lemurs (*Lemur catta*). *Horm Behav*, 43: 166–179.

Celi, F.S., Brychta, R.J., Linderman, J.D., Butler, P.W., Teresa, A., Smith, S., Courville, A.B., Lai, E.W., Costello, R., Skarulis, M.C., Csako, G., Remaley, A., Pacak, K., Chen, K.Y. (2011) Minimal changes in environmental temperature result in a significant increase in energy expenditure and changes in the hormonal homeostasis in healthy adults. *Eur J Endocrinol*, 163(6): 863–872.

Charpentier, M.J.E., Givalois, L., Faurie, C., Soghessa, O., Simon, F., Kappeler, P.M. (2018) Seasonal glucocorticoid production correlates with a suite of small-magnitude environmental, demographic, and physiological effects in mandrills. *Am J Phys Anthropol:* 1–14. DOI: 10.1002/ajpa.23329.

Chen, Y.J., Mahieu, N.G., Huang, X., Singh C., Crawford, P.A., Johnson, S.L., Gross, R.W., Schaefer, J., Patti, G.J. (2016) Lactate metabolism is associated with mammalian mitochondria. *Nat Chem Biol*, 12: 937–943.

Chen, H., Yao, H., Yang, W., Xiang, Z., Ostner, J., Cristóbal-Azkarate, J. (2021). Validation of a fecal T3 metabolite assay for measuring energetics in wild golden snub-nosed monkeys (*Rhinopithecus roxellana*). *Int J Primatol*, 42(5): 759:763.

Chevalier, C., Stojanović, O., Colin, D.J., Suarez-Zamorano, N., Tarallo, V., Veyrat-Durebex, C., Rigo, D., Fabbiano, S., Stevanović, A., Hagemann, & S. Montet, X. (2015). Gut microbiota orchestrates energy homeostasis during cold. *Cell*, 163(6): 1360–1374.

Chondronikola, M., Magkos, F., Yoshino, J., Okunade, O.L., Patterson, B.W., Muelhbauer, M.J., Newgard, C.B., Klein, S. (2018) Effect of progressive weight loss on lactate metabolism: A randomized controlled trial. *J Obes*, 26: 683–688. doi:10.1002/oby.22129.

Cifani, C., Alboni, S., Mucci, A., Benatti, C., Botticelli, L., Brunello, N., Micioni Di Bonaventura, M.V., Righi, V. (2021) Serum metabolic signature of binge-like palatable food consumption in female rats by nuclear magnetic resonance spectroscopy. *NMR Biomed*, *34*(4): p.e4469.

Claesson, M.J., Jeffery, I.B., Conde, S., Power, S.E., O'connor, E.M., Cusack, S., Harris, H., Coakley, M., Lakshminarayanan, B., O'sullivan, O. & Fitzgerald, G.F. (2012) Gut microbiota composition correlates with diet and health in the elderly. *Nature*, 488(7410): 178–184.

Clark, A.B. (1975) Olfactory communication by scent marking in a prosimian primate, *Galago crassicaudatus*. The University of Chicago.

Clark, A.B. (1985) Sociality in a nocturnal "solitary" prosimian: *Galago crassicaudatus*. *Int J Primatol*, 6(6): 581–600.

Clark, A.B. (1986) Contrast in communication of *Galago crassicaudatus* and *G. garnettii*. *Primate Report*, 14: 216.

Coelho, A.I., Berry, G.T., Rubio-Gozalbo, M.E. (2015) Galactose metabolism and health. *Curr Opin Clin Nutr Metab Care*, 18: 422–427.

Coiner-Collier, S., Scott, R.S., Chalk-Wilayto, J., Cheyne, S.M., Constantino, P., Dominy, N.J., Elgart, A.A., Glowacka, H., Loyola, L.C., Ossi-Lupo, K., Raguet-Schofield, M. (2016) Primate dietary ecology in the context of food mechanical properties. *J Hum Evol*, 98: 103–118.

Cornick, N.A., Jensen, N., Stahl, D., Hartman, P. & Allison, M. (1994) *Lachnospira pectinoschiza* sp. nov., an anaerobic pectinophile from the pig intestine. *Int J Syst Evol Microbiol*, 44: 87–93.

Costa-e-Sousa, R.H., & Hollenburg, A.N. (2012). Minireveiw: THe neural regulation of the hypothalamic-pituitary-thyroid axis. *Endocrinol*, 153(9): 4128–4135.

Crawley, M.J. (2013) The R Book, Second Edition. John Wiley & Sons, UK.

Creel, S., Dantzer, B., Goymann, W., Rubenstein, D.R. (2013) The ecology of stress: effects of the social environment. *Funct Ecol* 27(1): 66–80.

Crociani, F., Alessandrini, A., Mucci, M.M., & Biavati, B. (1994). Degradation of complex carbohydrates by *Bifidobacterium* spp. *Int J Food Microbiol*, 24(1-2): 199–210.

Crossin, G.T., Love, O.P., Cooke, S.J., Williams, T.D. (2016) Glucocorticoid manipulations in free-living animals: considerations of dose delivery, life-history context and reproductive state. *Funct Ecol* 30(1): 116-125.

Cristóbal-Azkarate, J., Maréchal, L., Semple, S., Majolo, B., MacLarnon, A. (2016) Metabolic strategies in wild male Barbary macaques: Evidence from faecal measurement of thyroid hormone. *Biology Letters*, 12:1–4.

Cubasch, U., Meehl, G.A., Boer, G.J., Stouffer, R.J., Dix, M., Noda, A., Senior, C.A., Raper, S., Yap, K.S. (2001) Projections of future climate change. In: Houton, J.T>, Ding, Y., Griggs, D.J., Noguer, M., Van der Linden, P.J., Dai, X., Maskell, K, Johnson, C.A. (eds): Climate Change 2001: The Scientific Basis: Contribution of Working Group I to the Third Assessment Report of the Intergovernmental Panel. Pp: 526–582.

Cui, Z-W., Wang, Z-L., Shao, Q., Raubenheimer, D., Lu, J-Q. (2018). Macronutrient signature of dietary generalism in an ecologically diverse primate in the wild. *Behav Ecol*, 29(4): 804–813.

Cui, Z-W., Shao, Q., Grueter, C.C., Wang, Z-L., Lu, J-Q., Raubenheimer, D. (2019) Dietary diversity of an ecological and macronutritional generalist primate in a harsh high-latitude habitat, the

Taihangshan macaque (*Macaca mulatta tcheliensis*). *Am J Primatol*, e22965. https://doi.org/10.1002/ajp.22965.

Cuozzo, F.P., Halajian, A., Sauther, M.L., Rampedi, K.M., & Millette, J.B. (2021). First report of the thick-tailed bushbaby (*Otolemur crassicaudatus*) being preyed upon by an endemic carnivore (*Caracal caracal*) in South Africa. *African Zool*. 56(3): 231–235.

Damstra, K.S.J., Richardson, S., Reeler, B. (1996) Synchronized fruiting between trees of *Ficus thonningil* in seasonally dry habitats. *J Biogeo*, 23: 495–500.

Danforth, E. & Burger, A. (1984) The role of thyroid hormones in the control of energy expenditure. *Clin Endocrinol Metabol*, 13(3): 581–595.

Dauncey, M.J. (1990) Thyroid hormones and thermogenesis. *Proc Nutri Soc*, 49(2): 203–215.

Dausmann, K.H. (2014) Flexible patterns in energy savings: Heterothermy in primates. *J Zool*, 292(2): 101–111.

David, L.A., Maurice, C.F., Carmody, R.N., Gootenberg, D.B., Button, J.E., Wolfe, B.E., Ling, A.V., Devlin, A.S., Varma, Y., Fischbach, M.A., & Biddinger, S.B. (2014) Diet rapidly and reproducibly alters the human gut microbiome. *Nature*, 505(7484): 559–563.

Davies, N.A., Gramotnev, G., McAlpine, C., Seabrook, L., Baxter, G., Lunney, D., Rhodes, J.R., Bradley, A. (2013) Physiological stress in koala populations near the arid edge of their distribution. *PLoS One* 8: e79136. https://doi.org/10.1371/journal.pone.0079136.

Dedkova, E.N. & Blatter, L.A. (2014) Role of β-hydroxybutyrate and inorganic polyphosphate in mammalian health and disease. *Front Phys*, 5: 260.

De Filippo, C., Cavalieri, D., Di Paola, M., Ramazzotti, M., Poullet, J.B., Massart, S., Collini, S., Pieraccini, G. & Lionetti, P. (2010) Impact of diet in shaping gut microbiota revealed by a comparative study in children from Europe and rural Africa. *Proc Nat Acad Sci*, 107(33): 14691–14696.

Delzenne, N.M., & Cani, P.D. (2011). Gut microbiota and the pathogenesis of insulin resistance. *Current diabetes reports*, 11: 154–159.

Dettmer, K., Aronov, P.A., Hammock, B.D. (2007) Mass spectrometry-based metabolomics. *Mass Spectrom Rev*, 26(1): 51–78. doi: 10.1002/mas.20108. PMID: 16921475; PMCID: PMC1904337.

Dettmer, A.M., Novak, M.A., Suomi, S.J., Meyer, J.S. (2012) Physiological and behavioral adaptation to relocation stress in differentially reared rhesus monkeys: hair cortisol as a biomarker for anxiety-related responses. *Psychoneuroendocrinol* 37(2): 191–199.

Dias, P.A.D., Coyohua-Fuentes, A., Canales-Espinosa, D., Chavira-Ramírez, R., Rangel-Negrín, A. (2017) Hormonal correlates of energetic condition in mantled howler monkeys. *Horm Behav*, 94: 13–20.

Dierdrich, V., Haugg, E., Dreier, C., Herwig, A. (2020) What can seasonal models teach us about energy balance? *J Endocrinol*, 244(2): 17–32.

Dixson, A.F. (1989) Sexual selection, genital morphology, and copulatory behavior in male galagos. *Int J Primatol*, 10(1): 47–55.

Doi, M., Yamaoka, I., Fukunaga, T. & Nakayama, M. (2003) Isoleucine, a potent plasma glucose-lowering amino acid, stimulates glucose uptake in C2C12 myotubes. *Biochem Biophys Res Comm*, 312: 1111–1117.

Doi, M., Yamaoka, I., Nakayama, M., Moshizuki, S., Sugahara, K., Yoshizawa, F. (2005) Isoleucine, a blood glucose-lowering amino acid, increases glucose uptake in rat skeletal muscle in the absence of increases in amp-activated protein kinase activity. *Am Soc Nutri Sci*: 2103–2108.

Douyon, L. & Schteingart, D.E. (2002) Effect of obesity and starvation on thyroid hormone, growth hormone, and cortisol secretion. *Endocrinol Metabol Clin Nor Amer*, 31: 173–189.

Doyle, G.A. & Bearder, S.K. (1977) The galagines of South Africa. *Primate Conserv*: 1-35.

Dufour, D.L. & Sauther, M.L. (2002) Comparative and evolutionary dimensions of the energetics of human pregnancy and lactation. *Am J Hum Biol*, 14: 584–602.

Dunbar, R.I.M., Hannah-Steward, L., Dunbar, P. (2002) Forage quality and cost of lactation for female gelada baboons. *Anim Behav,*, 64(5): 801–805.

Duranti, S., Magifesta, M., Lugli, G.A., Turroni, F., Anzalone, R., Milani, C., Mancabelli, L., Ossiprandi, M.C., & Ventura, M. (2017). *Bifidobacterium vansinderenii* sp. nov., isolated from faeces of emperor tamarin (*Saguinus imperator*). *Int J Syst Evol Microbiol*, 67(10): 3987–3995.

du Dot, T.J., Rosen, D.A.S., Richmond, J.P., Kitaysky, A.S., Zinn, S.A., Trites, A.W. (2009) Changes in glucocorticoids, IGF-I and thyroid hormones as indicators of nutritional stress and subsequent refeeding in Steller sea lions (*Eumetopias jubatus*). *Comp Biochem Physiol - A Mol & Integr Physiol*, 152(4): 524–534.

Eales, J.G. (1988) The influence of nutritional state on thyroid function in various vertebrates. *American Zoologist*, 28(2): 351–362.

Eaton, G.G., Slob, A., Resko, J.A. (1973) Cycles of mating behaviour, oestrogen and progesterone in the thick-tailed bushbaby (*Galago crassicaudatus crassicaudatus*) under laboratory conditions. *Anim Behav*, 21(2): 309–315.

Eberle, M. & Kappeler, P.M. (2004) Sex in the dark: determinants and consequences of mixed male mating tactics in Microcebus murinus, a small solitary nocturnal primate. *Behav Ecol Sociobiol*, 57: 77–9.

Ebling, F.J.P. (2014) On the value of seasonal mammals for identifying mechanisms underlying the control of food intake and body weight. *Horm Behav*, 66(1):56–65.

Ebling, F.J.P. (2015) Hypothalamic control of seasonal changes in food intake and body weight. *Front Neuroendocrinol*, 37: 97–107.

Ehrlich, A. (1977) Social and individual behaviors in captive greater galagos. *Behaviour*, 5: 195–220.

Emmons, L.H. (1980) Ecology and resource partitioning among nine species of African rain forest squirrels. *Ecol Monog*, 50(1):31–54.

Emwas, A.-H., Roy, R., McKay, R.T., Tenori, L., Saccenti, E., Gowda, G., Raftery, D., Alahmari, F., Jaremko, L., Jaremko, M. (2019) NMR spectroscopy for metabolomics research. *Metabolites*, 9: 123.

Engelbrecht, D. (2016) Galagos as avian nest predators in South Africa. *Primates*, 57(4): 455–458.

Engelhard, G.H., Brasseur, S.M., Hall, A.J., Burton, H.R., Reijnders, P.J.H. (2002) Adrenocortical responsiveness in southern elephant seal mothers and pups during lactation and the effect of scientific handling. *J Comp Physiol B,* 172(4): 315–328.

Esteve, R., Lopez-Martinez, A.E., Peters, M.L., Serrano-Ibanez, E.R., Ruiz-Parraga, G.T., Gozalez-Gomez, H., Ramirez-Maestre, C. (2017). Activity pattern profiles: relationship with affect, daily functioning, impairment, and variables related to life goals. *J Pain*, 18(5): 546–555.

Estrada, A., Garber, P.A., Rylands, A.B., Roos, C., Fernandez-Duque, E., Di Fiore, A., Nekaris, K.A., Nijman, V., Heymann, E.W., Lambert, J.E. (2017) Impending extinction crisis of the world's primates: why primates matter. *Sci Adv* 3(1): e1600946. doi: 10.1126/sciadv.1600946.

Fackelmann, G., Gillingham, M.A., Schmid, J., Heni, A.C., Wilhelm, K., Schensow, N., Sommer, S. (2021). *Communications Biology*, 4: 800.

Fardi, S., Sauther, M.L., Cuozzo, F.P., Jacky, I.A.Y., Bernstein, R.M. (2017) The effect of extreme weather events on hair cortisol and body weight in a wild ring-tailed lemur population (*Lemur catta*) in southwestern Madagascar. *Am J Primatol:* e22731. https://doi.org/10.1002/ajp.22731.

Feldman, E.C., Nelson, R.W., Reusch, C.E. (2014) Canine and Feline Endocrinology, 4th ed. St Louis, Mo: Elsevier.

Felig, P., Pozefsk, T., Marlis, E., Cahill, G.F. (1970) Alanine: key role in gluconeogenesis. *Science*, 167(3920): 1003–1004.

Feng, X., Porporato, A., Rodriguez-Iturbe, I. (2013) Changes in rainfall seasonality in the tropics. *Nat Clim Chang*, 3(9): 811–815.

Fichtel, C., Kraus, C., Ganswindt, A., Heistermann, M. (2007) Influence of reproductive season and rank on fecal glucocorticoid levels in free-ranging male Verreaux's sifakas (*Propithecus verreauxi*). *Horm Behav*, 51(5): 640–648.

Fiehn, O. (2001) Combining genomics, metabolome analysis, and biochemical modelling to understand metabolic networks. *Comp Funct Genom*, 2: 155–168. doi: 10.1002/cfg.82

Field, A. (2009) Discovering Statistics through SPSS:(and sex and drugs and rock'n'roll), Third Edition. Sage Publications, Thousand Oaks, CA. Pp: 197–220.

Finn, P.F. & Dice, J.F. (2006) Proteolytic and lipolytic responses to starvation. *J Nutri*, 22(7-8): 830–844. https://doi.org/10.1016/j.nut.2006.04.008.

Flier, J.S., Harris, M., Hollenberg, A.N. (2000) Leptin, nutrition, and the thyroid: the why, the wherefore, and the wiring. J *Clin Invest*, 105: 859–861.

Fieß, M., Heistermann, M., Hodges, J. (1999) Patterns of urinary and fecal steroid excretion during the ovarian cycle and pregnancy in the African elephant (*Loxodonta africana*). *Gen Comp Endocrinol*, 115(1): 76–89.

Foerster, S., Cords, M., Monfort, S.L. (2012) Seasonal energetic stress in a tropical forest primate: proximate causes and evolutionary implications. *PLoS ONE*, 7(11): e50108. doi:10.1371/journal.pone.0050108.

Fox J, Weisberg S (2011) An R Companion to Applied Regression, 2nd Edition. Sage Publications, Thousand Oaks, CA, pp 141-142.

Freake, H.C. & Oppenheimer, J.H. (1995) Thermogenesis and thyroid function. *Ann Rev Nutr*, 15: 263–291.

Frederiksen, M., Lebreton, J.D., Pradel, R., Choquet, R., Gimenez, O. (2014) Identifying links between vital rates and environment: a toolbox for the applied ecologist. *J Appl Ecol* 51(1): 71–81.

Frigerio, D., Dittami, J., Möstl, E., Kotrschal, K. (2004) Excreted corticosterone metabolites co-vary with ambient temperature and air pressure in male Greylag geese (*Anser anser*). *Gen Comp Endocrinol* 137(1): 29–36.

Gabriel, D., Gould, L., Cook, S. (2018) Crowding as a primary source of stress in an endangered fragment-dwelling strepsirrhine primate. *Anim Conserv*, 21(1): 76–85.

Garnsworthy, P.C. & Tropps, J. (1982) The effects of body condition at calving, food intake and performance in early lactation on blood composition in dairy cows given complete diets. *Anim Sci*, 35(1): 121–125. doi:10.1017/S000335610000088X.

Ganswindt, S.B., Myburgh, J.G., Cameron, E.Z., Ganswindt, A. (2014) Non-invasive assessment of adrenocortical function in captive Nile crocodiles (*Crocodylus niloticus*). *Comp Biochem Physiol A Mol Integr Physiol* 177: 11–17.

Génin, F.G., Masters, J.C., & Ganzhorn, J.U. (2010). Gummivory in cheirogaleids: primitive retention or adaptation to hypervariable environments? In: Burrows, A., Nash, L. (eds) The Evolution of Exudativory in Primates. Developments in Primatology: Progress and Prospects. Springer, New York, NY. Pp: 123–140. https://doi.org/10.1007/978-1-4419-6661-2_6

Gesquiere, L., Khan, M., Shek, L., Wango, T., Wango, E. (2008) Coping with a challenging environment: Effects of seasonal variability and reproductive status on glucocorticoid concentrations of female baboons (*Papio cynocephalus*). *Horm Behav*, 54: 410–416.

Gesquiere, L.R., Onyango, P.O., Alberts, S.C., Altmann, J. (2011) Endocrinology of year-round reproduction in a highly seasonal habitat: environmental variability in testosterone and glucocorticoids in baboon males. *Am J Phys Anthropol*, 144(2): 169–176.

Gesquiere, L.R., Pugh, M., Alberts, S.C., Markham, A.C. (2018) Estimation of energetic condition in wild baboons using fecal thyroid hormone determination. *Gen Comp Endocrinol*, 260: 9–17.

Ghouri, Y.A., Richards, D.M., Rahimi, E.F., Krill, J.T., Jelinek, K.A., & DuPont, A.W. (2014) Systematic review of randomised controlled trials of probiotics, prebiotics, and synbiotics in inflammatory bowel disease. *Clin Exp Gastroenterol*, 9: 473–487.

Gillis, T.E. & Ballantyne, J.S. (1996) The effects of starvation on plasma free amino acid and glucose concentrations in lake sturgeon. *J Fish Biol*, 49: 1306–1316.

Girard-Buttoz, C., Heistermann, M., Krummel, S., Engelhardt, A. (2009) Seasonal and social influences on fecal androgen and glucocorticoid excretion in wild male long-tailed macaques (*Macaca fascicularis*). *Physiol Behav*, 98(1-2):168–175.

Gomez, A., Petrzelkova, K., Yeoman, C.J., Vlckova, K., Mrázek, J., Koppova, I., Carbonero, F., Ulanov, A., Modry, D., Todd, A., & Torralba, M. (2015) Gut microbiome composition and metabolomic profiles of wild western lowland gorillas (*Gorilla gorilla gorilla*) reflect host ecology. *Molecular Ecology*, 24(10): 2551–2565.

Goldansaz, S.A., Guo, A.C., Sajed, T., Steele, M.A., Plastow, G.S. and Wishart, D.S., 2017. Livestock metabolomics and the livestock metabolome: A systematic review. *PloS one*, *12*(5): p.e0177675.

Gorvitovskaia, A., Holmes, S.P., & Huse, S.M. (2016) Interpreting *Prevotella* and *Bacteroides* as biomarkers of diet and lifestyle. *Microbiome*, 4: 15.

Goymann, W. (2012) On the use of non-invasive hormone research in uncontrolled, natural environments: The problem with sex, diet, metabolic rate and the individual. *Methods in Ecol Evol*, 3(4): 757–765.

Grier, D.G. & Halliday, H.L. (2004) Effects of glucocorticoids on fetal and neonatal lung development. *Treat Respir Med*, 3(5): 295–306. doi: 10.2165/00151829-200403050-00004. PMID: 15606220.

Grieshop, C.M., Flickinger, E.A., & Fahey, Jr G.C. (2002). Oral administration of arabinogalactan affects immune status and faecal microbial population in dogs. *J Nutr*, 132(3): 478–482.

Grimolizzi, F. & Arranz, L. (2018) Multipe faces of succinate beyond metabolism in blood. *Haematologica*, 103(10): 1586–1592.

Grueber, C., Nakagawa, S., Laws, R., Jamieson, I. (2011) Multimodel inference in ecology and evolution: challenges and solutions. *J Evol Biol*, 24(4): 699–711.

Grueter, C.C., Li, D., Ren, B., Wei, F., Xiang, Z., van Schaik, C. (2009) Fallback foods of temperate-living primates: a case study on snub-nosed monkeys. *Am J Phys Anthropol*, 140(4): 700–715.

Guo, W., Ren, K., Ning, R., Li, C., Zhang, H., Li, D., Xu, L., Sun, F., Dai, M. (2020). Fecal microbiota transplantation provides new insight into wildlife conservation. *Glob Ecol Cons*, 24: e01234.

Gursky, S. (2002). The behavioural ecology of the spectral tarsier, *Tarsius spectrum*. *Evol Anthropol*, 11(6): 226–234.

Hahn, N. (2017) Endemic flora of the Soutpansberg, Blouberg and Makgabeng. *South African J Botany*, 113: 324–336.

Hämäläinen, A., Heistermann, M., Kraus, C. (2015) The stress of growing old: sex-and season-specific effects of age on allostatic load in wild grey mouse lemurs. *Oecologia*, 178(4): 1063–1075

Handa, R.J., Burgess, L.H., Kerr, J.E., O'keefe, J.A. (1994) Gonadal steroid hormone receptors and sex differences in the hypothalamo-pituitary-adrenal axis. *Horm Behav*, 28:464–476.

Hanya, G. (2004) Seasonal variations in the activity budget of Japanese macaques in the coniferous forest of Yakushima: effects of food and temperature. *Amer J Primatol*, 63(3): 165–177.

Hanya G., (2010) Ecological adaptations of temperate primates: population density of Japanese macaques. In: N. Nakagawa, M. Nakamichi, H. Sugiura, (Eds.), The Japanese macaques. Tokyo: Springer. Pp: 79–97.

Hanya, G., Tackmann, J., Sawada, A., Lee, W., Pokharel, S.S., de Castro Maciel, V.G., Toge, A., Kuroki, K., Otsuka, R., Mabuchi, R. & Liu, J. (2020). Fermentation ability of gut microbiota of wild Japanese macaques in the highland and lowland Yakushima: In vitro fermentation assay and genetic analyses. *Micro Ecol*, 80: 459–474.

Happold, D. & Happold, M. (1992a) The ecology of three communities of small mammals at difference altitudes in Malawi, Central Africa. *J Zool*, 228(1): 81–101.

Happold, D. & Happold, M. (1992b) Termites as food for the thick-tailed bushbaby (*Otolemur crassicaudatus*) in Malawi. *Folia Primatol,* 58(2): 118–120.

Harcourt, C. (1986) Seasonal variation in the diet of South African galagos. *Int J Primatol*, 7:491–506.

Harding, R.S.O. (1981). An order of omnivores: nonhuman primate diets in the wild. In: Omnivorous primates. Gathering and hunting in human evolution. University of Columbia. Pp: 191–214.

Heistermann, M. (2010) Non-invasive monitoring of endocrine status in laboratory primates: methods, guidelines and applications. *Adv Sci Res* 5: 1–9.

Heistermann, M., Palme, R., Ganswindt, A. (2006) Comparison of different enzyme immunoassays for assessment of adrenocortical activity in primates based on fecal analysis. *Am J Primatol* 68(3): 257–273.

Hemingway, C.A. & Bynum, N. (2005) The influence of seasonality on primate diets and ranging. In: Brockman, D.K., Van Schaik, C.P. (eds). Seasonality in Primates. Cambridge: Cambridge University Press. Pp: 57–104.

Heni, A.C., Fackelmann, G., Eibner, G., Kreinert, S., Schmid, J., Schwensow, N.I., Wiegand, J., Wilhelm, K., Sommer, S. (2023). Wildlife gut microbiomes of sympatric generalist species respond differently to anthropogenic landscape disturbances. *Animal Microbiome*, 5(1): 1–8.

Hernandez, S.E., Strona, A.L.S., Leiner, N.O., Suzán, G., Romano, M.C. (2018) Seasonal changes of faecal cortisol metabolite levels in *Gracilinanus agilis* (Didelphimorphia: Didelphidae) and its association to life histories variables and parasite loads. *Conserv Physiol* 6: 1–11.

Hernandez-Gomez, O. (2020). Climate change disturbs wildlife microbiomes. *Nature Climate Change*, 10(11): 981–982.

Heymsfield, S.B., Arteaga, C., McManus, C., Smith, J., Moffitt, S. (1983) Measurement of muscle mass in humans: validity of the 24-hour urinary creatinine method. *Am J Clin Nutr*, 37: 478–494.

Higham, J.P., Heistermann, M., Maestripieri, D. (2011) The energetics of male-male endurance and rivalry in free-ranging rhesus macaques, *Macaca mulatta*. *Anim Behav*, 81(5): 1001–1007.

Higham, J.P., Heistermann, M., Maestripieri, D. (2013) The endocrinology of male rhesus macaque social and reproductive status: a test of the challenge and social stress hypotheses. *Behav Ecol Sociobiol* 67:19–30.

Hildebrandt, M.A., Hoffmann, C., Sherrill–Mix, S.A., Keilbaugh, S.A., Hamady, M., Chen, Y.Y., Knight, R., Ahima, R.S., Bushman, F., & Wu, G.D. (2009) High-fat diet determines the composition of the murine gut microbiome independently of obesity. *Gastroenterology*, 137(5), 1716–1724.

Hill, W.C.O. (1953) Primates. Comparative Anatomy and Taxonomy. I: Strepsirhini. Edinburgh: University Press.

Hladik, C.M. (1979) Diet and Ecology of Prosimians. In: Doyle GA, Martin RD, eds. The Study of Prosimian Behavior. Academic Press, Inc, New York. Pp 307–357.

Holsberger, D.R. & Cooke, P.S. (2005) Understanding the role of thyroid hormone in Sertoli cell development: a mechanistic hypothesis. *Cell Tissue Res*, 322(1): 133–140.

Homyack, J.A. (2010) Evaluating habitat quality of vertebrates using conservation physiology tools. *Wildl Res* 37: 332–342.

Hotelling, H. (1993). Analysis of a complex of statistical variables into principal components. *J Educ Psychol*, 24: 417–441.

Huang, C.C., Shih, M.C., Hsu, N.C., Chien, Y., Chung, B.C. (2012) Fetal glucocorticoid synthesis is required for development of fetal adrenal medulla and hypothalamus feedback suppression. *Endocrinol* 153(10): 4749–4756.

Hughes, M., Möller, M., Bellstedt, D.U., Edwards, T.J., De Villiers, M. (2005) Refugia, dispersal and divergence in a forest archipelago: a study of *Streptocarpus* in eastern South Africa. *Mol Ecol* 14: 4415–4426.

Hui, S., Ghergurovich, J.M., Morscher, R.J., Jang, C., Teng, X., Lu, W., Esparza, L.A., Reya, T., Le, Z., Yanxiang Guo, J., White, E., Rabinowitz, J.D. (2017) Glucose feeds the TCA cycle via circulating lactate. *Nature*, 551: 115–118.

Hunt, K.E., Trites, A.W., Wasser, S.K. (2004) Validation of a fecal glucocorticoid assay for Steller sea lions (*Eumetopias jubatus*). *Physiol Behav*, 80(5): 595–601.

Hume, I. D. (1997) Fermentation in the hindgut of mammals. In R. I. Mackie, B. A. White, (Eds.), Gastrointestinal microbiology. Volumne 1. Gastrointesinal Ecosystems and Fermentations. New York, NY. Pp: 84–115

Hunninck, L., Jackson, C.R., May, R., Roskaft, E., Palme, R., Sheriff, M.J. (2020). Triiodothyronine (T3) levels fluctuate in response to ambient temperature rather than nutritional status in a wild tropical ungulate. *Cons Physiol*, 8: coaa105.

Ibba, M., Curnow, A.W., Söll, D. (1997) Aminoacyl-tRNA synthesis; divergent routes to a common goal. *Trends Biochem Sci*, 22(2): 39–42.

Ibba, M. & Söll, D. (1999) Quality control mechanisms during translation. *Science*, 287(5446): 479–482.

Ibba, M. & Söll, D. (2001) The renaissance of aminoacyl-tRNA synthesis. *Embo Rep*, 2(5): 382–387.

Idris, O.H.M., Williams, P.A., Phillips, G.O. (1998). Characterisation of gum from *Acacia senegal* trees of different age and location using multi-detection gel permeation chromatography. *Food Hydrocoll*, 12: 379–388.

Ishimoto, H. & Jaffe, R.B. (2011) Development and function of the human fetal adrenal cortex: a key component in the feto-placental unit. *Endocr Rev* 32(3): 317–355.

Jablonski, N.G., & Crompton, R.H. (1994). Feeding behaviour, mastication, and tooth wear in the western tarsier (*Tarsius bancanus). Int J Primatol*, 15: 29–59.

Jannini, E.A., Crescenzi, A., Rucci, N., Screponi, E., Carosa, E., De Matteis, A., macchia, E., d'Amati, G., D'Armiento, M. (2000) Ontogenic pattern of thyroid hormone receptor expression in the human testis. *J Clin Endocrinol Metab*, 85(9): 3453–3457.

Jayne, R. (2020) Gum. It's What's for Dinner: The Dietary Significance and Nutritional Content of Acacia Gum for Five Sympatric Primate Species Living in a High-Altitude Temperate Environment. Masters Thesis. University of Colorado.

Jensen, K., Mayntz, D., Toft, S., Clissold, F.J., Hunt, J., Raubenheimer, D., Simpson, S.J. (2012) Optimal foraging for specific nutrients in predatory beetles. *Proc Roy Soc B*, 279(1736): 2212–2218.

Johnson, E.O., Kamilaris, T.C., Carter, C.S., Calogero, A.E., Gold, P.W., Chrousos, G.P. (1996) The biobehavioral consequences of psychogenic stress in a small, social primate (*Callithrix jacchus jacchus*). *Biol Psychiatry* 40(5): 317–337.

Kaack, B., Walker, L., Brizzee, K.R., Wolf, R.H. (1979) Comparative normal levels of serum triiodothyronine and thyroxine in nonhuman primates. *Lab Anim Sci*, 29: 191–194.

Kaack, B., Walker, M., Walker, L. (1980) Seasonal changes in the thyroid hormones of the male squirrel monkey. *Arch Androl*, 4: 133–136.

Keech, A.L., Rosen, D.A.S., Booth, R.K., Trites, A.W., Wasser, S.K. (2009) Fecal triiodothyronine and thyroxine concentrations change in response to thyroid stimulation in Steller sea lions (*Eumetopias jubatus*). *Gen Comp Endocrinol*, 166: 180–185.

Kersey DC, Dehnhard M (2014) The use of noninvasive and minimally invasive methods in endocrinology for threatened mammalian species conservation. *Gen Comp Endocrinol* 203: 296–306.

Kettelhut, I.C., Pepato, M.T., Migliorini, R.H., Medina, R., Goldberg, A.L. (1994) Regulation of different proteolytic pathways in skeletal muscle in fasting and diabetes mellitus. *Braz J Med Biol Res*, 27(4): 981–993.

Khonmee, J., Vorawattanatham, N., Pinyopummin, A., Thitaram, C., Somgird, C., Punyapornwithaya, V., Brown, J.L. (2016). Assessment of faecal glucocorticoid metabolite excretion in captive female fishing cats (*Prionailurus viverinus*) in Thailand. *Cons Physiol*, 4(1): cow021; doi:10.1093/ conphys/cow021.

Kinross, J.M., Darzi, A.W., Nicholson, J.K. (2011) Gut microbiome-host interactions in health and disease. *Genome Med*, 3: 1–12.

Kiriyami, S. & Ashida, K. (1964) Effect of the quality of dietary protein on nitrogen compounds in the urine of rats. *J Nutr*, 82: 127–134.

Klein, Á., Kulcsár, M., Krízsik, V., Mátics, R., Rudas, P., Török, J., Huszenicza, G. (2006) Effects of environmental temperature on thyroid hormones in the barn owl (*Tyto alba*). *Acta Veterinaria Hungarica*, 54(3): 321–331.

Kochan, T.I., Eseva, T.V. (2009) Seasonal dynamics of clinically significant metabolic parameters in northern residents of different age. *Bull Exp Biol Med*, 147(6): 757–759.

Kolb, H., Kempf, K., Röhling, M., Lenzen-Schulte, M., Schloot, N.C., Martin, S. (2021) Ketone bodies: from enemy to friend and guardian angel. *BMC Med*, 19(313). https://doi.org/10.1186/s12916-021-021185-0.

Koren, O., Goodrich, J.K., Cullender, T.C., Spor, A., Laitinen, K., Bäckhed, H.K., Gonzalez, A., Werner, J.J., Angenent, L.T., Knight, R., Bäckhed, F. (2012). Host remodeling of the gut microbiome and metabolic changes during pregnancy. *Cell*, 150(3): 470–480.

Kovatcheva-Datchary, P., Nilsson, A.C., Akrami, R., Lee, Y.S., De Vadder, F., Arora, T., Hallén, A., Martens, E.C., Björck, I.M., & Bäckhed, F. (2015). Dietary fiber-induced improvement in glucose metabolism is associated with increased abundance of *Prevotella*. *Cell Metab, 22(6):* 971–982.

Landaas, S. & Petersen, J.E. (1975) Clinical conditions associated with urinary excretion of 2-hydroxybutyric acid. *Scand J Clin Lab Inv*, 35(3): 259–266.

Langer, P. & Clauss, M. (2018). Morphological adaptation of the Eutherian gastrointestinal tract to diet. *Vert Zool*, 68: 237–252. https://doi.org/10.5167/uzh-157906

Lee, Y.K. & Mazmanian, S.K. (2010) Has the microbiota played a critical role in the evolution of the adaptive immune system? *Science*, 330(6012): 1768–1773.

Lefcheck, J.S. (2016) "piecewiseSEM: Piecewise structural equation modeling in R for ecology, evolution, and systematics. *Methods Ecol Evol* 7(5): 573–579.

Lefort, G., Liaubet, L., Marty-Gasset, N., Canlet, C., Vialaneix, N., Servien, R. (2021) Joint automatic metabolite identification and quantification of a set of 1H-NMR spectra. *Anal Chem*, 93: 2861–2870.

Lemos, L.S., Olsen, A., Smith, A., Chandler, T.E., Larson, S., Hunt, K. and Torres, L.G. (2020) Assessment of fecal steroid and thyroid hormone metabolites in eastern North Pacific gray whales. *Cons Physiol*, *8*(1): p.coaa110.

Levings, S.C. & Windsor, D.M. (1983) Seasonal annual variation in litter arthropod populations. In: E.G. Leigh, A.S. Rand, D.M. Windsor (Eds). The Ecology of a Tropical Forest. Oxford University Press, Oxford.

Ley, R.E. (2010). Obesity and the human microbiome. *Curr Opin Gastroenterol*, 26(1): 5–11.

Licht, T.R., Hansen, M., Bergstrom, A., Poulsen, M., Krath, B.N., Markowski, J., Wilcks, A. (2010). Effects of apples and specific apple components on the cecal environment of conventional rats: Role of apple pectin. *BMC Microbiol*, 10(1): 13. https://doi.org/10.1186/1471-2180-10-13.

Lindon, J.C., Nicholson, J.K., Holmes, E., Everett, J.R. (2000) Metabonomics: metabolic processes studied by NMR spectroscopy biofluids. *Concepts Magn Reson*, 12(5): 289–320.

Liu, R., Bao, Z.X., Zhao, P.J., Li, G.H. (2021) Advances in the study of metabolomics and metabolites in some species interactions. *Mol*. 26(11): 3311. doi: 10.3390/molecules26113311. PMID: 34072976; PMCID: PMC8197931.

Long, C. (2017). A seasonal comparison of the gut microbiome of the Southern Lesser Galago, *Galago moholi* (A.Smith 1836). Master's dissertation. University of Pretoria, South Africa.

Long, C., Tordiffe, A.S.W., Sauther, M.L., Cuozzo, F.P., Millette, J.L., Ganswindt, A., Scheun, J. (2021). Seasonal drivers of faecal glucocorticoid metabolite concentrations in an African strepsirrhine primate, the thick-tailed greater galago (*Otolemur crassicaudatus*). *Cons Physiol*, 9(1): coab081. doi:10.1093/conphys/coab081.

Low, M.J. (2012). Neuroendicrinology. In: Williams textbook of Endocrinology. Melmed, S., Olonsky, K.S., Larsen, P.R., Kronenberg, H.M. (eds). 12th Edition. Elsevier Inc. Pp: 1829–1897.

Lynch, J.W., Ziegler, T.E., Strier, K.B. (2002) Individual and seasonal variation in fecal testosterone and cortisol levels of wild male Tufted Capuchin monkeys, *Cebus apella nigritus*. *Horm Behav* 41: 275–287.

Macfarlane, G.T. & Macfarlane, S. (2012) Bacteria, Colonic Fermentation, and Gastrointestinal Health. *J AOAC Int*, 95(1): 50–60.

Maestripieri D, Hoffman CL, Fulks R, Gerald MS (2008) Plasma cortisol responses to stress in lactating and nonlactating female rhesus macaques. *Horm Behav* 53(1):170-176.

Mallott, E.K., Amato, K.R., Garber, P.A., & Malhi, R.S. (2018) Influence of fruit and invertebrate consumption on the gut microbiota of wild white-faced capuchins (*Cebus capucinus*). *Amer J Phys Anthropol*, 165(3): 576–588.

Mallott, E.K., Skovmand, L.H., Garber, P.A., Amato, K.R. (2022) The faecal microbiome of black howler monkeys (*Alouatta pigra*) varies in response to seasonal dietary changes. *Mol Ecol*, 31(15): 4146–4161.

Marciniak, B., Patro-Malysza, J., Poniedzialek-Czakowska, E., Kimber-Tojnar, Z., Leszczynska-Gorzelak, B., Oleszczuk, J. (2011). Glucocorticoids in pregnancy. *Curr Pharm Biotechnol*, 12(5): 750–757.

Marshall, A.J., Boyko, C.M., Feilen, K.L., Boyko, R.H., Leighton, M. (2009) Defining fallback foods and assessing their importance in primate ecology and evolution. *Am J Phys Anthropol*, 140: 603–614.

Mason, S., Terburgh, K., Louw, R. (2018) Miniaturized H-NMR method for analyzing limited-quantity samples applied to a mouse model of Leigh disease. *Metabolomics*, 14(74): 1–12.

Masters, J. & Génin, F. (2016) A conservation assessment of *Otolemur crassicaudatus*. In: M.F. Child, L. Roxburgh, E., Do Linh San, D., Raimondo, H.T., Davies-Mostert (Eds). The Red List of Mammals of South Africa, Swaziland and Lesotho. South African National Biodiversity Institute and Endangered Wildlife Trust, South Africa.

McBride, G., Christopherson, R., Sauer, W. (1985) Metabolic rate and plasma thyroid hormone concentrations of mature horses in response to changes in ambient temperature. *Canadian J Anim Sci*, 65: 375–382.

McClane, B.A., Robertson, S.L., Li, J. (2012) Clostridium perfringens. *Food Microbiology: fundamentals and frontiers*. Pp: 465–489.

McColl, J.C. & Young, M.D. (2005). Managing change: Australian structural adjustment lessons for water. CSIRO: 17–53.

McEwen, B.S. (1998) Protective and damaging effects of stress mediators. *N Engl J Med* 338(3): 171–179.

McFall-Ngai, M. (2007) Adaptive immunity—Care for the community. *Nature*, 445(7124): 153.

McKechnie, A.E. & Wolf, B.O. (2019) The physiology of heat tolerance in small endotherms. *Physiol*, 34(5): 302–313.

McLean, M. & Smith, R. (1999) Corticotropin-releasing hormone in human pregnancy and parturition. *Trends Endocrinol Metab* 10(5): 174–178.

McNab, BK (2002). The physiological ecology of vertebrates: a view from energetics. Cornell University Press: Ithaca.

McPherson, P.A. & McEneny, J. (2012) The biochemistry of ketogenesis and its role in weight management, neurological disease and oxidative stress. *J Physiol Biochem*, 68(1): 141–151.

Mierziak, J., Burgberger, M., Wojtasik, W. (2021) 3-Hydroxybutyrate as a metabolite and signal molecule regulating processes of living organisms. *Biomol*, 11(402): 1–21.

Millar, R.B. & Anderson, M.J. (2004) Remedies for pseudoreplication. *Fish Re,s* 70(2-3): 397–407.

Millspaugh, J.J. & Washburn, B.E. (2004) Use of fecal glucocorticoid metabolite measures in conservation biology research: considerations for application and interpretation. *Gen Comp Endocrinol*, 138: 189–199.

Milton, K. (1987). Primate diets and gut morphology: Implications for hominid evolution. Food and Evolution: Toward a Theory of Human Food Habits. Pp: 93–115.

Moon, D.Y., Owens, D.W., MacKenzie, D.S. (1999) The effects of fasting and increased feeding on plasma thyroid hormones, glucose, and total protein in sea turtles. *Zool Sci*, 16: 579–586.

Mondol, S., Booth, R.K., Wasser, S.K. (2020) Fecal stress, nutrition and reproductive hormones for monitoring environmental impacts on tigers (*Panthera tigris*). *Cons Physiol*, 8(1): 1–15.

Mostert, T., Bredenkamp, G., Klopper, H., Verwey, C. (2008) Major vegetation types of the Soutpansberg Conservancy and the Blouberg Nature Reserve, South Africa. *Koedoe*, 50(1): 32–48. Retrieved from http://www.koedoe.co.za

Möstl, E., Maggs, J., Schrötter, G., Besenfelder, U., Palme, R. (2002) Measurement of cortisol metabolites in faeces of ruminants. *Vet Res Commun* 26(2): 127–139.

Mphidi, M.F. (2019) The effectiveness of biosphere reserve as a tool for sustainable natural resource management in Vhembe District municipality, Limpopo Province, South Africa. University of Venda. PhD dissertation.

Muehlenbein, M.P. (2006). Intestinal parasite infections and fecal steroid levels in wild chimpanzees. *Amer J Phys Anthropol*, 130(4): 546–550.

Muller, M.N. & Wrangham, R.W. (2004) Dominance, aggression, and testosterone in wild chimpanzees: a test of the 'challenge hypothesis'. *Anim Behav*, 67(1):113–123.

Murray, C.M., Heintz, M.R., Lonsdorf, E.V., Parr, L.A., Santymire, R.M. (2013) Validation of a field technique and characterization of fecal glucocorticoid metabolite analysis in wild chimpanzees (*Pan troglodytes*). *Amer J Primatol*, 75(1): 57–64.

Nagpal, R., Shively, C.A., Appt, S.A., Register, T.C., Michalson, K.T., Vitolins, M.Z., & Yadav, H. (2018). Gut microbiome composition in non-human primates consuming a Western or Mediterranean diet. *Front Nutr*, 5: 28.

Nakagawa, S., Johnson, P.C., Schielzeth, H. (2017). The coefficient of determination R 2 and intra-class correlation coefficient from generalized linear mixed-effects models revisited and expanded. *Journal of the Royal Society Interface*, 14: 20170213. http://dx.doi.org/10.1098/rsif.2017.0213.

Nash, L.T. (1986). Dietary, behavioral, and morphological aspects of gummivory in primates. *American Journal of Physical Anthropology*, 29(S7): 113–137.

Nash, L.T. & Bearder, S.K., Olson, T.R. (1989) Synopsis of *Galago* species characteristics. *Int J Primatol*, 10(1): 57–80.

Nakagawa, S., Johnson, P.C., Schielzeth, H. (2017) The coefficient of determination R2 and intra-class correlation coefficient from generalized linear mixed-effects models revisited and expanded. *J Res Soc Inter*, 14(134): e20170213. http://dx.doi.org/10.1098/rsif2017.0213.

Nakagawa S, Schielzeth H (2013) A general and simple method for obtaining R2 from generalized linear mixed-effects models. *Methods Ecol Evol* 4(2):133-142.

Nekaris, K.A.I. & Jaffe, S. (2007). The strepsirrhine primates of Asia and Mainland Africa: diversity shrouded in darkness. In Campbell, C., Fuentes, A., MacKinnon, K., Panger, M., Bearder, S.K., (eds). Primates in Perspective. Oxford University Press, Oxford, pp 24–54.

Nekaris, KA.I. & Bearder, S. (2011) The lorisiform primates of Asia and mainland Africa: diversity shrouded in darkness. In: Campbell CJ, Fuentes A, MacKinnon KC, Bearder SK, Stumpf RM (eds), Primates in Perspective (2nd ed). Oxford: Oxford University Press. Pp: 34–54.

Nemoto, T., Burne, M.J., Daniels, F., O'Donnell, M.P., Crosson, J., Berens, K., Issekutz, A., Kasiske, B.L., Keane, W.F., Rabb, H. (2001) Small molecule selection ligand inhibition improves outcome in ischemic acute renal failure. *Kidney Int*, 60(6): 2205–2214.

Neter, J., Wasserman, W., Kutner, M.H. (1996) In: Neter J, Wasserman W, Kutner MH, eds. Applied Linear Statistical Models 4th Edition. WCB McGraw-Hill/Irwin, New York. Pp: 278–289.

Nicholson, J.K., Holmes, E., Kinross, J., Burcelin, R., Gibson, G., Jia, W., & Pettersson, S. (2012). Host-gut microbiota metabolic interactions. *Science*, 336: 1262–1267. https://doi.org/10.1126/science.1223813.

Nowack, J., Wippich, M., Mzilikazi, N., Dausmann, K.H. (2013) Surviving the cold, dry period in africa: behavioral adjustments as an alternative to heterothermy in the African lesser bushbaby (*Galago moholi*). *Int J Primatol*, 34: 49–64.

Nowak, R. & Paradiso, J. (1983). Galagos, Bush Babies. In *Walker's Mammals of the World*. Baltimore and London: The John Hopkins University Press. Pp: 364–367.

O'Malley, R.C. & Power, M.L. (2012). Nutritional composition of actual and potential insect prey for the Kasekela chimpanzees of Gombe National Park, Tanzania. *Amer J Phys Anthropol*, 149(4), 493–503.

Oliver, S.G.S., Winson, M.K.M.M.K., Kell, D.B.D.B.; Baganz, F., Böttcher, C., Neumann, S. (1998). Systematic functional analysis of the yeast Genome. *Trends Biotechnol*, 16(9): 373–378. DOI: 10.1016/S0167-7799(98)01214-1.

Olivieros, J.C. (2015). Venny. An interactive tool for comparing lists with Venn's diagrams. https://bioinfogp.cnb.csic.es/tools/venny/index.html.

Orkin, J.D., Campos, F.A., Myers, M.S., Hernandez, S.E.C., Guadamuz, A. & Melin, A.D. (2019). Seasonality of the gut microbiota of free-ranging white-faced capuchins in a tropical dry forest. *The ISME Journal* 13: 183–196.

Osman Hill, W.C. (1953) Primates (comparative anatomy and taxonomy). I. Strepsirrhini. University Press, Edinburgh. Pp: 25.

Ostner, J., Kappeler, P., Heistermann, M. (2008) Androgen and glucocorticoid levels reflect seasonally occurring social challenges in male redfronted lemurs (*Eulemur fulvus rufus*). *Behav Ecol Sociobiol* 62: 627–638.

Pak, N., Donoso, G., Tagle, M.A. (1973) Allantoin excretion in the rat. *Br J Nutr*, 30: 107–112.

Palme R (2005) Measuring fecal steroids: guidelines for practical application. *Ann NY Acad Sci* 1046(1):75-80.

Palme, R. & Möstl, E. (1997) Measurement of cortisol metabolites in faeces of sheep as a parameter of cortisol concentration in blood. *Int J Mammal Biol (Suppl II)* 62: 192–197.

Pannkuk, E.L., Laiakis, E.C., Authier, S., Wong, K., Fornace, A.J, Jr. (2015) Global metabolomic identification of longer-term dose dependent urinary biomarkers in non-human primates exposed to ionizing radiation. *Radiation Research*, 184(2): 121–133.

Pannkuk, E.L., Laiakis, E.C., Mak, T.D., Astarita, G., Authier, S., Wong, K., Fornace Jr, A.J. (2016) A lipidomic and metabolomic serum signature from nonhuman primates exposed to ionizing radiation. *Metabolomics*, 12(80): 1–11.

Petter-Rousseaux, A. (1984) Annual variations in the plasma thyroxine level in *Microcebus murinus*. *Gen Comp Endocrinol*, 55: 405–409.

Phukuntsi, M.A., Du Plessis, M., Dalton, D.L., Jansen, R., Cuozzo, F.P., Sauther, M.L., Kotze, A. (2020) Population genetic structure of the thick-tailed bushbaby (*Otolemur crassicaudatus*) from the Soutpansberg Mountain range, Northern South Africa, based on four mitochondrial DNA regions. *Mitochondrial DNA A DNA Mapp Seq Anal*, 31(1): 1–10.

Pijl, H., de Meijer, P.H.E.M., Langius, J., Coenegracht, C.I.G.M., van den Berk, A.H.M., Chandie, S., Boom, P.K., Schoemaker, R.C., Cohen, A.F., Burggraaf, J., Meinders, A.E. (2001) Food choice in hyperthyroidism: potential influence of the autonomic nervous system and brain serotonin precursor availability. *J Clin Endocrinol Metab*, 86: 5848–5853.

Pinzone, M.R., Celesia, B.M., Di Rosa, M., Cacopardo, B., & Nunnari, G. (2012). Microbial translocation in chronic liver diseases. *Int J Microbiol*.

Pluvinage B, Massel PM, Burak K, Boraston AB. (2019) Structural and functional analysis of four family 84 glycoside hydrolases from the opportunistic pathogen Clostridium perfringens. *Glycobiol*, 30(1): 49–57. doi: 10.1093/glycob/cwz069. PMID: 31701135; PMCID: PMC6925667.

Pontes, J.G.M., Brasil, A.J.M., Cruz, G.C., de Souza, R.N. and Tasic, L., 2017. NMR-based metabolomics strategies: plants, animals and humans. *Analytical Methods*, *9*(7): 1078–1096.

Power, D.M., Llewellyn, L., Faustino, M., Nowell, M.A., Björnsson, B.T., Einarsdottir, I.E., Canario, A.V.M., Sweeney, G.E. (2001) Thyroid hormones in growth and development of fish. Comp. Biochem. *Physiol C Toxicol Pharmacol*, 130: 447–459. https://doi. org/10.1016/S1532-0456(01)00271-X.

Pozzi, L., Disotell, T.R., Masters, J.C. (2014) A multilocus phylogeny reveals deep lineages within African galagids (Primates: Galagidae). *BMC Evolut Biol*.

Pride RE (2005) Foraging success, agonism, and predator alarms: behavioral predictors of cortisol in *Lemur catta*. *Int J Primatol* 26(2): 295–319.

Pritchard, C.E., Palme, R., Langkilde, T. (2020) Glucocorticoid and triiodothyronine concentrations do not correlate with behavior in vicuñas (*Vicugna vicugna*). *Gen Comp Endocrinol*, 286: e113299.

Pruetz, J.D. & Bertolani, P. (2009) Chimpanzee (*Pan troglodytes verus*) behavioral responses to stresses associated with living in a savanna-mosaic environment: implications for hominin adaptations to open habitats. *PaleoAnthropol*, 252–262.

Pucci, E., Chiovato, L., Pinchera, A. (2000) Thyroid and lipid metabolism. *Int J Obesity*, 24:109–112.

Pullen, S.L., Bearder, S.K., Dixson, A.F. (2000) Preliminary observations on sexual behaviour and the mating system in free-ranging lesser galagos (*Galago moholi*). *Am J Primatol*, 51(1): 79–88.

R Core Team. (2020) R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. https://www.R-project.org/.

Rangel-Negrín, A., Alfaro, J.L., Valdez, R.A., Romano, M.C., Serio-Silva, J.C. (2009) Stress in yucatan spider monkeys: Effects of environmental conditions on fecal cortisol levels in wild and captive populations. *Anim Conserv* 12(5): 496–502.

Rangel-Negrín, A., Coyohua-Fuentes, A., Chavira-Ramírez, D.R., Canales-Espinosa, D., Dias, P.A.D. (2018) Energetic constraints on the reproduction of female mantled howlers. *Amer J Primatol*, 80(11): 1–9.

Feldt-Rasmussen, U., Effraimidis, G., Klose, M. (2021). The hypothalamus-pituitary-thyroid (HPT)-axis and its role in physiology and pathophysiology of other hypothalamus-pituitary functions. *Mol Cel Endocrinol*, 525: 1–12.

Rasooli A., Nouri M., Khadjeh G.H., Rakesh, A. (2004) The influence of seasonal variations on thyroid activity and some biochemical parameters of cattle. *Irani J Vet Res,* 5(2):1383–1391.

Raubenheimer, D., Simpson, D.S., Tait, A.H. (2012) Match and mismatch: conservation physiology, nutritional ecology and the timescales of biological adaptation. *Philos Trans Royal Soc A*, 367(1596): 1628–1646.

Ray, I., Wren, B.T., Bowers, E.J. (2016). Documentation of plant consumption by *Galago moholi* in South Africa. *African Primates*, 11(1): 45–48.

Reyna-Hurtado, R., Teichroeb, J. A., Bonnell, T.R., Hernández-Sarabia, R.U., Vickers, S. M., Serio-Silva, J.C., Sicotte, P. & Chapman, C.A. (2018). Primates adjust movement strategies due to changing food availability. *Behavioral ecology*, *29*(2): 368–376.

Reynolds L.P., Borowicz P.P., Caton J.S., Vonnahme K.A., Luther J.S., Buchanan D.S., Hafez S.A., Grazul-Bilska A.T., Redmer D.A. (2010) Uteroplacental vascular development and placental function: an update. *Int J Devel Biol*, 54: 355–366.

Righini, N. (2014) Primate nutritional ecology: the role of food selection, energy intake, and nutrient balancing in Mexican black howler monkey (*Alouatta pigra*) foraging strategies. University of Illinois at Urbana-Champaign. PhD Thesis.

Rimbach, R., Heymann, E.W., Link, A., Heistermann, M. (2013) Validation of an enzyme immunoassay for assessing adrenocortical activity and evaluation of factors that affect levels of fecal glucocorticoid metabolites in two New World primates. *Gen Comp Endocrinol* 191: 13–23.

Rimbach, R., Pillay, N., Schradin, C. (2018) Correction: Both thyroid hormone levels and resting metabolic rate decrease in African striped mice when food availability decreases. *J Exp Biol*, 221(11): jeb185363. doi:10.1242/jeb.151449.

Röhnisch, H.E., Eriksson, J., Müllner, E., Agback, P., Sandström, C., Moazzami, A.A. (2018) AQuA: An automated quantification algorithm for high-throughput NMR-based metabolomics and its application in human plasma. *Anal Chem*,90: 2095–2102.

Roff, D.A. & Fairburn, D.J. (2007) The evolution of trade-offs: where are we? *J Evol Biol*, 20: 433-447.

Romero LM (2002) Seasonal changes in plasma glucocorticoid concentrations in free-living vertebrates. *Gen Comp Endocrinol*, 128(1):1-24.

Romero LM, Wingfield JC (2015) Tempests, poxes, predators, and people: stress in wild animals and how they cope. Oxford University Press

Rothman, J.M., Raubenheimer, D., Bryer, M.A.H., Takahashi, M., & Gilbert, C.C. (2014). Nutritional contributions of insects to primate diets: Implications for primate evolution. *Journal of Human Evolution*, 71, 59–69.

Roznere, I., Watters, G.T., Wolfe, B.A., Marymegan, D. (2014) Nontargeted metabolomics reveals biochemical pathways altered in response to captivity and food limitation in the freshwater mussel *Amblema plicata*. *Comp Biochem Physiol*, 12: 53–60. doi: 10.1016/j.cbd.2014.09.004.

Rubio-Aliaga, I., de Roos, B., Duthie, S.J., Crosley, L.K., Mayer, C., Horgan, G., Colquhoun, I.J., Le Gall, G., Huber, F., Kremer, W., Rychlik, M. (2011) Metabolomics of prolonged fasting in humans reveals new catabolic markers. *Metabolomics*, *7*(3): 375–387.

Rumpold, B.A. & Schlüter, O.K. (2013) Nutritional composition and safety aspects of edible insects. *Mol Nutr Food Res*, 57: 802–823. DOI: 10.1002/mnfr.201200735.

Russell, E., Koren, G., Rieder, M., Van Uum S. (2012) Hair cortisol as a biological marker of chronic stress: current status, future directions and unanswered questions. *Psychoneuroendocrinol* 37(5): 589–601.

Said, H.M. & Mohammed, Z.M. (2006) Intestinal absorption of water-soluble vitamins: an update. *Curr Opin Gastroenterol*, 22: 140–146. https://doi.org/10.1097/01.mog.0000203870.22706.52.

Sapolsky, R.M. (2002) Endocrinology of the Stress-Response. In: Becker J.B., Breedlove, S.M., Crews, D., McCarthy, M.M. (eds). Behavioral Endocrinology, Second Edition. MIT Press, Cambridge. Pp 408–450.

Sauther, M.L. (1991) Reproductive behavior of free-ranging *Lemur catta* at Beza Mahafaly Special reserve, Madagascar. *Amer J Phys Anthropol*, 84:463-477.

Sauther, M.L. (1998) Interplay of phenology and reproduction in ring-tailed lemurs: implications for ring-tailed lemur conservation. In: C.S., Harcourt, R.H., Crompton, A.T.C., Feistner, (Eds.), *Biol Cons Prosimians*. *Folia primatological*, 69: 309–320. DOI:10.1159/000052719.

Schaebs, F.S., Wolf, T.E., Behringer, V., Deschner, T. (2016). Fecal thyroid hormones allow for the noninvasive monitoring of energy intake in capuchin monkeys. *J Endocrinol*, 231: 1–10.

Scheun, J., Bennett, N.C., Ganswindt, A., Nowack, J. (2014) Spicing up the menu: evidence of fruit feeding in *Galago moholi*. *Primates*, 55(3): 359–363.

Scheun, J., Bennett, N.C., Ganswindt, A., Nowack, J. (2015) The hustle and bustle of city life: monitoring the effects of urbanisation in the African lesser bushbaby. *Sci Nat* 102(9-10): 57–67.

Scheun J, Nowack J, Bennett NC, Ganswindt A (2016) Female reproductive activity and its endocrine correlates in the African lesser bushbaby, *Galago moholi*.

Scheun, J., Greeff, D., Ganswindt, A. (2018) Non-invasive monitoring of glucocorticoid metabolite concentrations in urine and faeces of the Sungazer (*Smaug giganteus*). *PeerJ,* 6(e6132): 115. Retrieved from www.nzg.ac.za

Schneider, J.E. (2004) Energy balance and reproduction. *Physiol Behav*, 81: 289– 317.

Scheppach, W. (1994). Effects of short chain fatty acids on gut morphology and function. *Gut* 35, 35–38. https://doi.org/10.1136/gut.35.1_suppl.s35.

Schielzeth, H. (2010) Simple means to improve the interpretability of regression coefficients. *Methods Ecol Evol* 1(2): 103–113.

Schleifer, K.-H. (2009). In Bergey's Manual® of Systematic Bacteriology. Springer: 19–1317.

Schloss, C.A., Nuñez, T.A., Lawler, J.J. (2012) Dispersal will limit ability of mammals to track climate change in the Western Hemisphere. *Proc Natl Acad Sci USA* 109(22): 8606–8611.

Schwitzer, C., Mittermeier, R.A., Rylands, A.B., Chiozza, F., Williamson, E.A., Byler, D., Wich, S., Humle, T., Johnson, C., Mynott, H., McCabe, G. (2019) Primates in Peril: The World's 25 Most Endangered Primates 2018–2020. IUCN SSC Primate Specialist Group, International Primatological Society, Global Wildlife Conservation, and Bristol Zoological Society, Washington, DC. Pp: 130.

Shao, Q., Cui, Z., Lui, C., Tian, J., Lu, J. (2023). Strategy in Taihangshan macaques (Macaca mulatta tcheliensis) in a temperate forest, North China. *Int J Primatol*. Pp: 1–17.

Sharma, M.K., Parchwani, D., Maheria, P., Upadhyah, A. (2012) Relationship between thyroid profile and semen quality. *Nat J Comm Med*, 3(1): 20–24.

Sharon, G., Garg, N., Debelius, J., Knight, R., Dorrestein, P.C., Mazmanian, S.K. (2014) Specialised metabolites from the microbiome in health and disease. *Cell Metab*, 20: 719–730.

Sheh, A., Artim, S.C., Burns, M.A., Molina-Mora, J.A., Lee, M.A., Dzink-Fox, J., Muthupalani, S. Fox, J.G. (2022). Analysis of gut microbiome profiles in common marmosets (*Callithrix jacchus*) in health and intestinal disease. *Sci Rep*, 12: 4430. https://doi.org/10.1038/s41598-022-08255-4

Sheriff, M.J., Dantzer, B., Delehanty, B., Palme, R., Boonstra, R. (2011) Measuring stress in wildlife: techniques for quantifying glucocorticoids. *Oecologia* 166(4):869–887.

Sidiropoulos, D.N., Al-Ghalith, G.A., Shields-Cutler, R.R., Ward, T.L., Johnson, A.J., Vangay, P., Knights, D., Kashyap, P.C., Xian, Y., Ramer-Tait, A.E., Clayton, J.B. (2020). Wild primate microbiomes prevent weight gain in germ-free mice. *Anim Microb* 2: 16. doi: 10.1186/s42523-020-00033-9.

Silva, J.E. (2003) The thermogenic effect of thyroid hormone and its clinical implications. *Amer Journ Int Med*, 139: 205–213.

Silva, J.E. (2006) Thermogenic mechanisms and their hormonal regulation. *Physiol Rev*, 86: 435–464.

Simmons, M.T. (2015). Climates and microclimates: challenges for extensive green roof design in hot climates. *Green roof ecosystems*: 63–80.

Simpson, S.J., Sibly, R.M., Lee, K.P., Behmer, S.T., Raubenheimer, D. (2004) Optimal foraging when regulating intake of multiple nutrients. *Anim Behav*, 68: 1299–1311.

Snodgrass, J.J., Leonard, W.R., Robertson, M.L. (2007) Primate bioenergetics: an evolutionary perspective. In: M.J. Ravosa & M. Dagosto (Eds.). Primate origins: adaptations and evolution. Boston, Massachusetts: Springer. Pp: 703–737.

Souza-Alves, J. P., Caselli, C. B., Gestich, C. C., & Nagy-Reis, M. B. (2019) Should I store, or should I sync? The breeding strategy of two small Neotropical primates under predictable resource availability. *Primates*, 60(2): 113–118.

Starling, A.P., Charpentier, M.J.E., Fitzpatrick, C., Scordato, E.S., Drea, C.M. (2010) Seasonality, sociality, and reproduction: long-term stressors of ring-tailed lemurs (*Lemur catta*). *Horm Behav* 57: 76–85.

Stearns, S.C. & Hoekstra, R.F. (2005). Evolution: An Introduction. Oxford, UK; New York: Oxford University Press.

Stettin, D., Poulin, R.X., Pohnert, G. (2020) Metabolomics benefits from orbitrap GC–MS-comparison of low- and high-resolution GC–MS. *Metabolites*, 10:143. doi: 10.3390/metabo10040143.

Strier, K.B. (2009). Seeing the forest through the seeds: mechanisms of primate behavioral diversity from individuals to populations and beyond. *Curr Anthropol*, *50*(2): 213–228.

Strier, K.B., Lynch, J.W., Ziegler, T.E. (2003) Hormonal changes during the mating and conception seasons of wild northern muriquis (*Brachyteles arachnoides hypoxanthus*). *Am J Primatol* 61(2): 85–99.

Sun, B., Wang, X., Bernstein, S., Huffman, M.A., Xia, D.P., Gu, Z., Chen, R., Sheeran, L.K., Wagner, R.S., & Li, J. (2016) Marked variation between winter and spring gut microbiota in free-ranging Tibetan Macaques. (*Macaca thibetana*) *Sci Rep*, 6(1): 6035.

Svenning, J-C. & Sandel, B. (2013) Disequilibrium vegetation dynamics under future climate change. *Amer J Bot*, 100: 1266-286.

Szott, I.D., Pretorius, Y., Ganswindt, A. and Koyama, N.F. (2020). Normalized difference vegetation index, temperature and age affect faecal thyroid hormone concentrations in free-ranging African elephants. *Cons Physiol*, *8*(1): p.coaa010.

Takeshita, R.S.C., Bercovitch, F.B., Kinoshita, K., Huffman, M.A. (2018) Beneficial effect of hot spring bathing on stress levels in Japanese macaques. *Primates*, 59(3): 215–225.

Takis, P.G., Schäfer, H., Spraul, M., Luchinat, C. (2017) Deconvoluting interrelationships between concentrations and chemical shifts in urine provides a powerful analysis tool. *Nat Comm*, 8: 1662.

Tardif, S.D. (1997) The bioenergetics of parental behaviour and evolution of alloparental care in marmosets and tamarins. *In*: N.G., Solomon & J.A. French (Eds). Cooperative breeding in mammals**.** Cambridge, Cambridge University Press. Pp:11–33.

Tecot, S. (2007) Seasonality and predicatibility: the hormonal and behavioural responses of the red-bellied lemur, *Eulemur rubriventer*, in southern Madagascar. The University of Texas at Austin. PhD Thesis.

Tecot, S., Gerber, B.D., King, S.J., Verdolin, J.L., Wright, P.C. (2013) Risky business: Sex differences in mortality and dispersal in a polygynous, monomorphic lemur. *Behav Ecol:* 987–996. doi:10.1093/beheco/art008.

Thompson, E.M. (2017) Energetics of feeding, social behaviour, and life history in non-human primates. *Horm Behav*, 91: 84–96.

Thompson, E.M. & Georgiev, A.V. (2014) The high price of success: costs of mating effort in male primates. *Int J Primatol*, 35: 609–627.

Thompson, C.L., Powell, B.L., Williams, S.H., Hanya, G., Glander, K.E., Vinyard, C.J. (2017) Thyroid hormone fluctuations indicate a thermoregulatory function in both a tropical (*Alouatta palliata*) and seasonally cold-habitat (*Macaca fuscata*) primate. *Amer J Primatol*, e22714. https://doi.org/10.1002/ajp.22714.

Tomasi, T.E. (1991) Utilization rates of thyroid hormones in mammals. *Comp Biochem & Physiol*, 100: 503–516.

Touma C, Palme R (2005) Measuring fecal glucocorticoid metabolites in mammals and birds: the importance of validation. *Ann NY Acad Sci* 1046(1): 54–74.

Tran, H., McConville, M., Loukopoulos, P. (2020) Metabolomics in the study of spontaneous animal diseases. *J Vet Diag Inv*, *32*(5): 635–647.

Trivers, R.L. (1972) Parental investment and sexual selection. In: Campbell B, editor. Sexual Selection and the Descent of Man 1871–1971. Chicago: Aldine Publishing Company. Pp: 136–179.

Tung, J., Barreiro, L.B., Burns, M.B., Grenier, J.-C., Lynch, J., Grieneisen, L.E., Archie, E.A. (2015). Social networks predict gut microbiome composition in wild baboons. eLife, 4: e05224

Turnbaugh, P.J., Ley, R.E., Mahowald, M.A., Magrini, V., Mardis, E.R., Gordon, J.I. (2006) An obesity-associated gut microbiome with increased capacity for energy harvest. *Nature*, 444(7122): 1027–1031.

Turroni, F., Duranit, S., Bottacini, F, Guglielmetti, S., Van Sinderen, D., & Ventura, M. (2014). *Bifidobacterium bifidum* as an example of a specialised human gut commensal. *Frontiers in Microbiology*, 21(5): 437.

Van Wyk, A.E., & Smith, G.F. (2001). Regions of floristic endemism in southern Africa: a review with emphasis on succulents. In: Regions of floristic endemism in Southern Africa: a review with emphasis on succulents. Van Wyk, A.E & Smith, G.F. (Eds). Umdaus Press, Pretoria, South Africa.

van Schaik CP, Brockman DK (2005) Seasonality and reproductive function. In: Brockman DK, Van Schaik CP (eds.). Seasonality in primates: Studies of living and extinct human and non-human primates. Cambridge University Press: 44:269.

van Schaik, C.P. & van Noordwijk, M.A. (1985) Interannual variability in fruit abundance and the reproductive seasonality in Sumatran long-tailed macaques (*Macaca fascicularis*). *J Zool*, 206: 533–549.

Vendramini, T.H.A., Macedo, H.T., Zafalon, R.V.A., Macegoza, M.V., Pedrinelli, V., Risolia, L.W, Ocampos, F.M.M., Jeremias, J.T., Pontieri, C.F.F., Ferriolli, E., Colnago, L.A., Brunetto, M.A. (2020) Serum metabolomics analysis reveals that weight loss in obese dogs results in a similar metabolic profile to dogs in ideal body condition. *Metabolomics*, 17: 27–40. https://doi.org/10.1007/s11306-020-01753-4.

Ventura, M., Turroni, F., Cancheva, C., Vaughan, E.E., O'Toole, P.W. (2009). Microbial diversity in the human intestine and novel insights from metagenomics. *Fronts Biosc*, 14(1): 3214–3221.

Vining, R.F., McGinley, R.A., Maksvytis, J.J., Ho, K.Y. (1983) Salivary cortisol: a better measure of adrenal cortical function than serum cortisol. *Ann Clin Biochem* 20(6):329-335.

Von Maltitz, G.P., Mucina, L., Geldenhuys, C., Lawes, M., Eeley, H., Adie, H., Vink, D., Flemming, G. & Voster, T. (2003) Classification system for South African Indigenous Forests: an objective classification for the Department of Water Affairs and Forestry. Environmentek Report ENV–P–C 2003–017. Pretoria: CSIR.

Wagner, M.S., Wajner, S.M., Maia, A.L. (2008) The role of thyroid hormone in testicular development and function. *J Endocrinol*, 199: 351–365.

Walker, B.G., Wingfield, J.C., Boersma, P.D. (2005) Age and food deprivation affects expression of the glucocorticosteroid stress response in Magellanic penguin (*Spheniscus magellanicus*) chicks. *Physiol Biochem Zool* 78(1):78–89.

Walker, H.K., Ottka, C., Lohi, H., Handel, I., Clements, D.N., Gow, A.G., Mellanby, R.J. (2020) Seasonal variation in serum metabolites of northern European dogs. *J Vet Intern Med*, 36: 190–195.

Wang, X., Mickiewicz, B., Thompson, G.C., Joffe, A.R., Blackwood, J., Vogel, H.J., Kopciuk, K.A. (2022) Comparison of two automated targeted metabolomics programs to manual profiling by an experienced spectroscopist for 1H-NMR spectra. *Metabolites*, 12(227): 1–14. https://doi.org/10.3390/metabo12030227.

Wasser, S.K., Azkarate, J.C., Booth, R.K., Hayward, L., Hunt, K., Ayres, K., Vynne, C., Gobush, K., Canales-Espinosa, D., Rodríguez-Luna, E. (2010) Non-invasive measurement of thyroid hormone in feces of a diverse array of avian and mammalian species. *Gen Comp Endocrin*, doi:10.1016/j.ygcen.2010.04.004.

Wasser, S.K., Lunidin, J.I., Ayres, K., Seely, E., Giles, D., Balcomb, K., Hempelmann, J., Parsons, K., Booth, R. (2017) Population growth is limited by nutritional impacts on pregnancy success in endangered Southern Resident killer whales (*Orcinus orca*). *PLoS One*, 12(6): e0179824. https://doi.org/10.1371/journal.pone.0179824.

Welcker, J., Chastel, O., Gabrielsen, G.W., Guillaumin, J., Kitaysky, A.S., Speakman, J. R., Tremblay, Y., Bech, C. (2013) Thyroid hormones correlate with basal metabolic rate but not field metabolic rate in a wild bird species. *PLoS ONE*, 8(2): e56229. doi:10.1371/journal.pone.0056229.

Whittow, G.C., Guernsey, D.L., Morishige, W.K. (1979) Thyroid activity in a hypometabolic primate, the owl monkey (*Aotus trivirgatus*). *Arch Physiol and Bioch*, 87:963–967.

Wingfield, J. (2013) Ecological processes and the ecology of stress: the impacts of abiotic environmental factors. *Funct Ecol*, 27: 37–44.

Williams, E.J. (1961). The distribution of larvae of randomly moving insects. *Austr J Biol Sci*, 14(4), 598–604.

Williams, N.I., Berga, S.L., Cameron, J.L. (2007) Synergism between psychosocial and metabolic stressors: impact on reproductive function in cynomolgus monkeys. *Am J Physio Endocrinol Metab* 293(1):270-276.

Williams, C.L., Willard, S., Kouba, A., Sparks, D., Holmes, W., Falcone, J., Williams, C.H., & Brown, A. (2012). Dietary shifts affect the gastrointestinal microflora of the giant panda (*Ailuropoda melanoleuca*). *Journal of Animal Physiology and Animal Nutrition*, 97, 577–585.

Williams, C.L., Caraballo-Rodrigez, A.M., Allaband, C., Zarrinpar, A., Knight, R., Gauglitz, J.M. (2018). Wildlife microbiome interactions and disease: exploring opportunities for disease mitigation across ecological scales. Drug Discovery Today: Disease Models, 28: 105–115.

Wingfield J (2013) Ecological processes and the ecology of stress: the impacts of abiotic environmental factors. *Func Ecol* 27:37-44.

Wishart, D.S. (2019) NMR metabolomics: a look ahead. *J Magn Reson*, 306: 155–161. https://doi.org/10.1016/j.jmr.2019.07.013.

Wu, G.D., Chen, J., Hoffmann, C., Bittinger, K., Chen, Y.Y., Keilbaugh, S.A., Bewtra, M., Knights, D., Walters, W.A., Knight, R., & Sinha, R. (2011). Linking long-term dietary patterns with gut microbial enterotypes. *Science*, 334, 105–108.

Xu, X., Xu, P., Ma, C., Tang, J., Zhang, X. (2013). Gut microbiota, host health, and polysaccharides. *Biotechnol Adv*, 31(2): 318–337.

Yoshizawa, F. (2015) Effects of Leucine and Isoleucine on Glucose Metabolism. In: Rajendram, R., Preedy, V., Patel, V. (eds) Branched Chain Amino Acids in Clinical Nutrition. Nutrition and Health. Humana Press, New York, NY. https://doi.org/10.1007/978-1-4939-1923-9_6.

Yousef, M.K. (1975) Thyroid and adrenal function in desert animals: a view on metabolic adaptations. In: Environmental Physiology of Desert Organisms. Hadley, N.F. (ed). Pp: 188–206. Dowdin. Hutchinson & Ross, Stroundsburg.

Yuan, D., Li, C., You, L., Dong, H., & Fu, X. (2020) Changes of digestive and fermentation properties of Sargassum pallidum polysaccharide after ultrasonic degradation and its impacts on gut microbiota. *Int J Biol Macromol*, 164: 1443–1450.

Zeng, Y., Pu, Y., Niu, L.L., Deng, J.B., Zeng, D., Amato, K.R., Li, Y., Zhou, Y., Lin, Y.C., Wang, J., & Wu, L.Q. (2022). Comparison of gastrointestinal microbiota in golden snub-nosed monkey (*Rhinopithecus roxellanae*), green monkey (*Chlorocebus aethiops sabaeus*), and ring-tailed lemur (*Lemur catta*) by high throughput sequencing. *Global Ecol Cons*, 33: e01946.

Zheng, C., Zhang, S., Ragg, S., Raftery, D., Vitek, O. (2011) Identification and quantification of metabolites in 1H NMR spectra by Bayesian model selection. *Bioinformatics*, 27: 1637–1644.

Zhu, L., Yang, Q., Suhr Van Haute, M.J., Kok, C.R., Gomes-Neto, J.C., Pavlovikj, N., Pillai, R., Sinha, R., Hassenstab, H., Mustoe, A., & Moriyama, E.N. (2021) Captive common marmosets (*Callithrix jacchus*) are colonised throughout their lives by a community of *Bifidobacterium* species with species-specific genomic content that can support adaptation to distinct metabolic niches. *Mbio*, 12(4), e01153-21.

Zhu, L., Wang, J., & Bahrndorff, S. (2021). The wildlife gut microbiome and its implication for conservation biology. *Front Microbiol*, 12: 1617.

Zuur, A., Ieno, E.N., Walker, N., Saveliev, A.A., Smith, G.M. (2009) Mixed effects models and extensions in ecology with R. Springer Science and Business Media, New York.

Supplemental Material

Table A1. A table showing the animals sampled over the course of the sampling periods. All rows highlighted red are samples that were removed due to poor sequencing quality and read depth.

Sample #	Date	Animal ID	Weight	Season	Sex
1	30-May-17	21A9	1055	Dry	Female
9	01-Jun-17	3339	984	Dry	Female
6	02-Jun-17	3138	743	Dry	Male
13	02-Jun-17	3433	780	Dry	Male
17	02-Jun-17	3605	1405	Dry	Male
2	02-Jun-17	O957	624	Dry	Male
19	06-Jun-17	3673	1272	Dry	Male
20	06-Jun-17	3773	1115	Dry	Female
21	06-Jun-17	3779	672	Dry	Female
5	07-Jun-17	3130	1257	Dry	Male
8	07-Jun-17	3234	625	Dry	Female
18	07-Jun-17	3609	1267	Dry	Male
25	07-Jun-17	4595	1305	Dry	Male
7	08-Jun-17	3230	1349	Dry	Male
15	09-Jun-17	3486	1443	Dry	Male
16	09-Jun-17	3497	682	Dry	Male
22	09-Jun-17	3805	1111	Dry	Female
23	09-Jun-17	3837	941	Dry	Female
26	09-Jun-17	6268	1551	Dry	Male
3	09-Jun-17	964A	1104	Dry	Female
11	13-Jun-17	3366	769	Dry	Male
14	13-Jun-17	3482	1273	Dry	Male
4	14-Jun-17	2156	956	Dry	Female
10	15-Jun-17	3380	705	Dry	Male
24	15-Jun-17	3843	667	Dry	Female
12	02-Jul-17	3323	981	Dry	Male
51	13-Sep-17	3138	628	Dry	Male
42	13-Sep-17	21A9	957	Dry	Female
49	14-Sep-17	2156	887	Dry	Female
53	14-Sep-17	3482	1040	Dry	Male
55	14-Sep-17	3497	716	Dry	Male
111	05-Jan-18	3351	1195	Wet	Male
110	05-Jan-18	3380	947	Wet	Male
112	05-Jan-18	21A9	991	Wet	Female
113	06-Jan-18	2156	904	Wet	Female
114	06-Jan-18	3579	882	Wet	Male
115	06-Jan-18	6268	1164	Wet	Male

116	07-Jan-18	2263	981	Wet	Female
117	07-Jan-18	3843	809	Wet	Female
118	07-Jan-18	4873	1218	Wet	Male
119	08-Jan-18	3527	1137	Wet	Male
120	08-Jan-18	3569	1272	Wet	Male
122	10-Jan-18	3138	917	Wet	Male
123	10-Jan-18	3573	946	Wet	Male
124	11-Jan-18	3805	1132	Wet	Female
125	12-Jan-18	8499	1091	Wet	Female
129	13-Mar-18	3377	995	Wet	Female
131	13-Mar-18	3551	1289	Wet	Male
136	13-Mar-18	21A9	1004	Wet	Female
127	14-Mar-18	2263	1079	Wet	Female
128	14-Mar-18	3231	1114	Wet	Male
133	14-Mar-18	3605	1351	Wet	Male
135	14-Mar-18	3843	937	Wet	Female
139	14-Mar-18	8454	1063	Wet	Male
126	15-Mar-18	2156	956	Wet	Female
130	15-Mar-18	3380	1040	Wet	Male
132	15-Mar-18	3569	1431	Wet	Male
134	15-Mar-18	3805	1127	Wet	Female
140	15-Mar-18	8455	456	Wet	Female
137	16-Mar-18	8452	547	Wet	Female
138	16-Mar-18	8453	609	Wet	Female
173	25-May-18	3380	1191	Dry2	Male
156	25-May-18	8451	850	Dry2	Female
157	25-May-18	8453	659	Dry2	Female
155	25-May-18	21A9	1089	Dry2	Female
158	26-May-18	8499	1152	Dry2	Female
159	27-May-18	2156	1021	Dry2	Female
160	27-May-18	8455	531	Dry2	Female
161	28-May-18	3230	1362	Dry2	Male
162	28-May-18	3843	1054	Dry2	Female
163	28-May-18	8457	1146	Dry2	Male
164	28-May-18	8458	632	Dry2	Female
165	28-May-18	8459	1391	Dry2	Male
166	28-May-18	8460	1017	Dry2	Female
167	29-May-18	1055	1089	Dry2	Female
168	29-May-18	3805	1137	Dry2	Female
169	29-May-18	8456	1290	Dry2	Male
170	30-May-18	3551	1411	Dry2	Male
171	01-Jun-18	2263	1043	Dry2	Female
172	01-Jun-18	8452	677	Dry2	Female

Table A2. A table depicting the shared and unique taxa at family level found in the gut of *O. crassicaudatus* during the summer and winter sampling periods.

Summer	**Winter**	**Shared**
mitochondria	Microthrixaceae	Tissierellaceae
Neisseriaceae	Peptostreptococcaceae	Weeksellaceae
Oxalobacteraceae	Pirellulaceae	Acetobacteraceae
Paenibacillaceae	Promicromonosporaceae	Aerococcaceae
Pasteurellaceae	Pseudanabaenaceae	Bacillaceae
Polyangiaceae	Rikenellaceae	Bacteroidaceae
Pseudomonadaceae	Turicibacteraceae	Beijerinckiaceae
Rhodospirillaceae	Solirubrobacteraceae	Bifidobacteriaceae
Solirubrobacterales unclassified	Ruminococcaceae	Bradyrhizobiaceae
Alphaproteobacteria unclassified	Nocardioidaceae	Campylobacteraceae
Nocardiaceae	Cyanobacteria unclassified	Comamonadaceae
Methylophilaceae	Marinilabiaceae	Coriobacteriaceae
Paraprevotellaceae	Barnesiellaceae	Enterobacteriaceae
Caulobacteraceae	Carnobacteriaceae	Enterococcaceae
Deinococcaceae	Clostridiaceae	Erysipelotrichaceae
Dietziaceae	Corynebacteriaceae	Fusobacteriaceae
Flavobacteriaceae	Desulfovibrionaceae	Helicobacteraceae
Gemellaceae	Erythrobacteraceae	Intrasporangiaceae
Hyphomicrobiaceae	Halanaerobiaceae	Lachnospiraceae
Leptotrichiaceae	Lactobacillaceae	Listeriaceae
Leuconostocaceae	LD19	Methylobacteriaceae
YS2 unclassified		Microbacteriaceae
Gemellales unclassified		Micrococcaceae
		Bacilliunclassified
		Moraxellaceae
		Actinomycetales unclassified
		Bacillales unclassified
		Planococcaceae
		Porphyromonadaceae
		Prevotellaceae
		Propionibacteriaceae
		Actinobacteria unclassified
		Rhizobiaceae
		Rhodobacteraceae
		Bacteriaunclassified
		Rs-045
		S24-7
		Gammaproteobacteria unclassified
		Staphylococcaceae
		Streptococcaceae
		Streptomycetaceae
		Veillonellaceae
		Xanthomonadaceae
		Bacteroidales unclassified
		Bacteroidetes unclassified
		Burkholderiales unclassified
		Clostridiales unclassified

		Firmicutes unclassified Proteobacteria unclassified Rhizobiales unclassified Lactobacillales unclassified

Figures

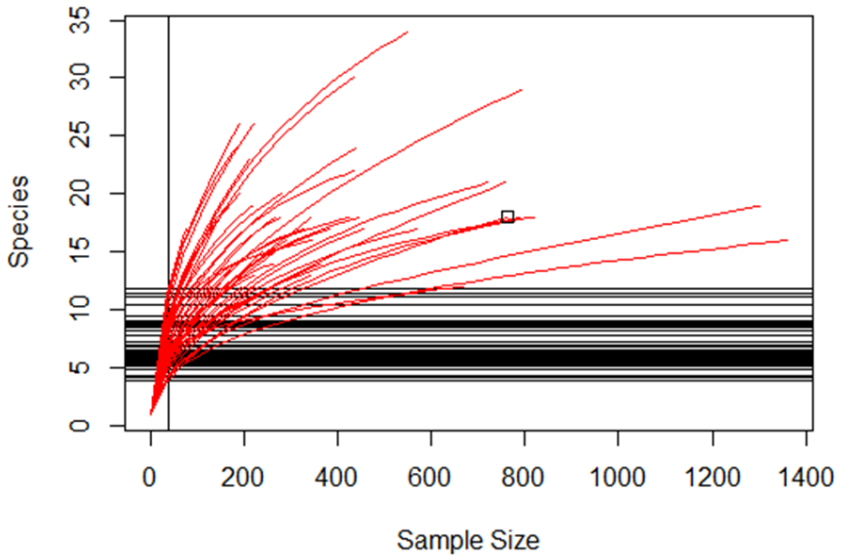


Fig. A1. Rarefaction curves demonstrating the gut bacteria sequencing of 49 individuals of *O. crassicaudatus*. OTUs were assigned based on 97% similarity cut-off.

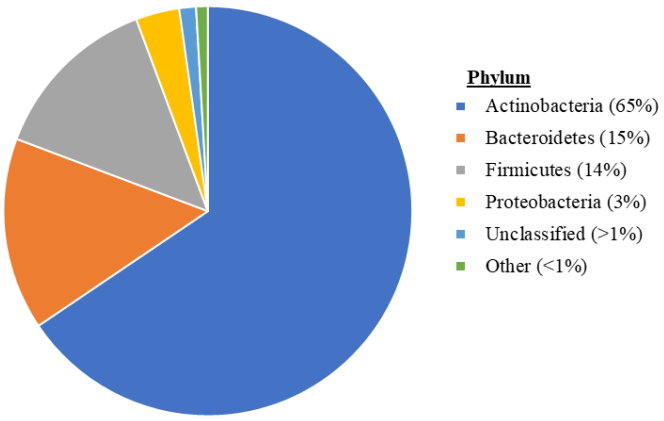


Fig. A2. A pie chart demonstrating the composition of the phyla comprising of the gut microbiome of *O. crassicaudatus*

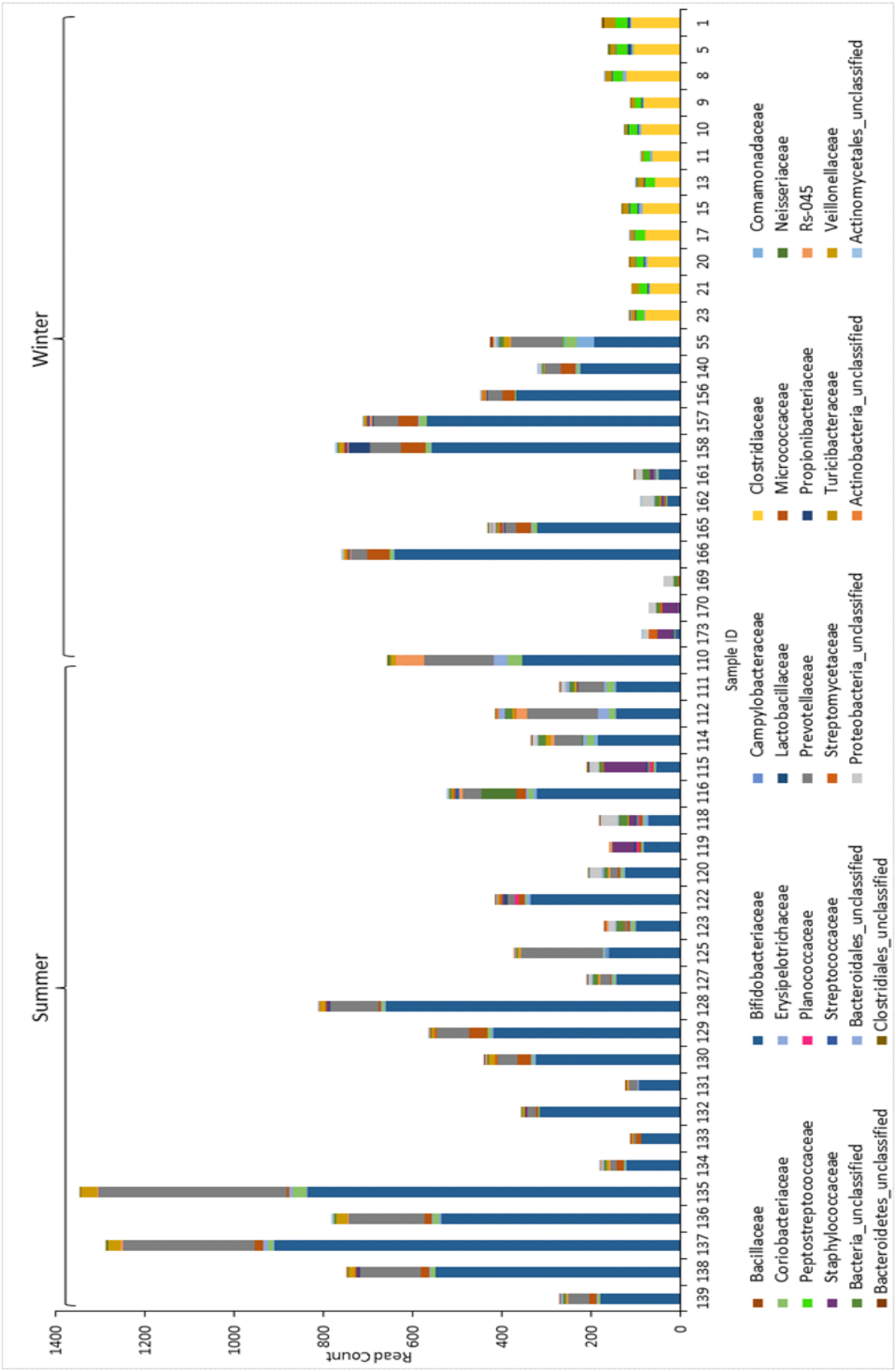
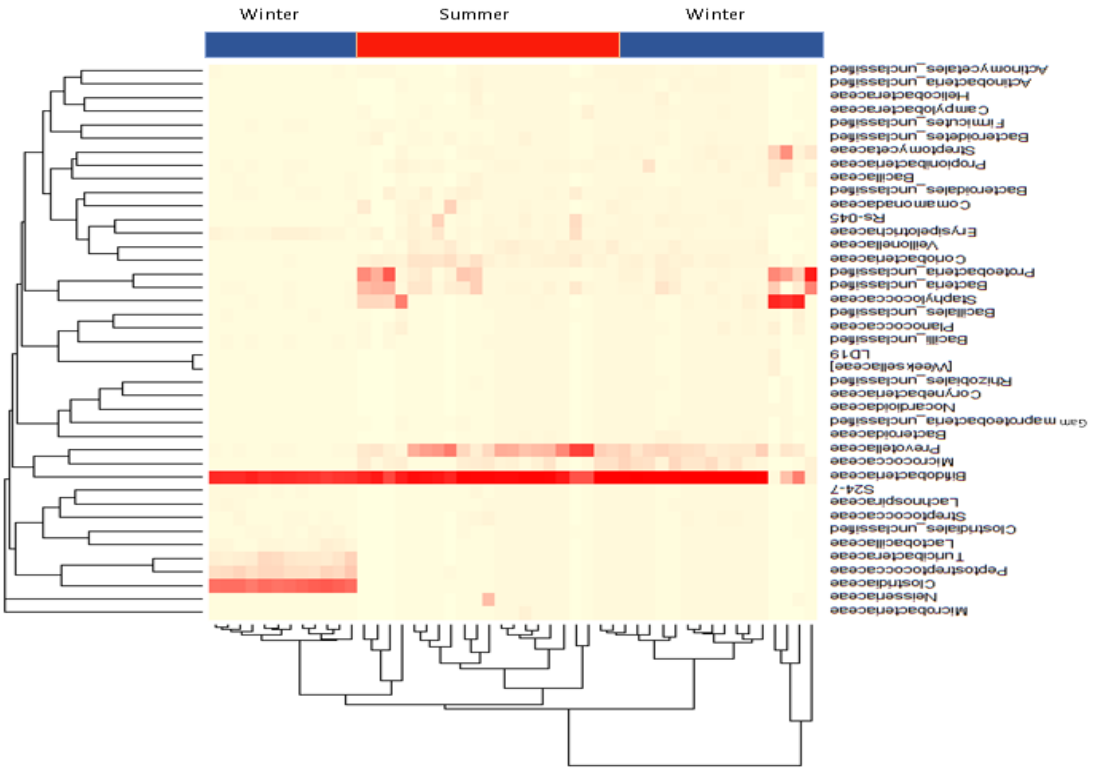


Fig. A3. A bar chart presenting the gut bacterial composition at family level for all samples from the summer and winter sampling periods. Only taxa with an overall relative abundance >0.1% are reflected in the graph.

Fig. A4. Heatmap illustrating the most abundant OTUs at family level detected in 49 faecal samples of O. crassicaudatus during summer and winter.



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Biodiversity for Life
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NATIONAL ZOOLOGICAL GARDEN

SANBI NZG/RES/P18/26

28 September 2018

Channen Long

University of Pretoria (UP)

OUTCOME OF SUBMITTED RESEARCH PROPOSAL

This letter serves to inform you that your submitted research proposal titled "Physiological responses to seasonal changes in a strepsirrhine primate, *Otolemur crassicaudatus*" was **approved** by the SANBI NZG Research Ethics and Scientific Committee (RESC).

The following provisos should be taken into consideration:

1. Inform the RESC of completion or termination (with reason) of your research at the SANBI NZG.
2. Submission of an annual progress report in November of each year. Failure to submit a progress report may result in approval to be withdrawn.
3. Submission of a written request for an extension or for any changes within the research project.
4. The SANBI NZG should be acknowledged in all reports, scientific publications and conference contributions as follows:
   - The South African National Biodiversity Institute, National Zoological Garden is acknowledged for providing samples/research platform.
5. Submission of a final report in December 2020.
6. This approval is only valid from September 2018 to December 2020.

IMPORTANT: It is your responsibility to ensure compliance to Section 20 of the Animal Diseases Act 1984 (Act 35 of 84) that applies to "investigation, experiment or research". A copy of your section 20 permit must be sent to this office before research can commence.

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Animal Ethics Committee

PROJECT TITLE	**Extreme primates: Physiological and behavioural responses to a temperate primate niche using a strepsirrhine model**
PROJECT NUMBER	**V037-17**
RESEARCHER/PRINCIPAL INVESTIGATOR	**Dr. ASW Tordiffe**

STUDENT NUMBER (where applicable)	
DISSERTATION/THESIS SUBMITTED FOR	**Academic**

Condition: Water has to be easily available to ensure no dehydration during recovery

ANIMAL SPECIES	**Galago moholi**	**Otolemur crassicaudatus**
NUMBER OF ANIMALS	**20**	**20**
Approval period to use animals for research/testing purposes		April 2017 – April 2018
SUPERVISOR	**Dr. ASW Tordiffe**	

KINDLY NOTE:

Should there be a change in the species or number of animal/s required, or the experimental procedure/s - please submit an amendment form to the UP Animal Ethics Committee for approval before commencing with the experiment

APPROVED	Date 9 May 2017
CHAIRMAN: UP Animal Ethics Committee	Signature

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Animal Ethics Committee

PROJECT TITLE	**Extreme primates: Physiological and behavioural responses to a temperate primate niche using a strepsirrhine model**
PROJECT NUMBER	**V037-17 (Amendment 1)**
RESEARCHER/PRINCIPAL INVESTIGATOR	**C Leng**

STUDENT NUMBER (where applicable)	----------
DISSERTATION/THESIS SUBMITTED FOR	**Academic**

ANIMAL SPECIES	**Otolemur crassicaudatus (Greater Galago)**
NUMBER OF ANIMALS	**20 with additional faecal samples obtained**
Approval period to use animals for research/testing purposes	**February 2018 – February 2019**
SUPERVISOR	**Dr. ASW Tordiffe**

KINDLY NOTE:

Should there be a change in the species or number of animal/s required, or the experimental procedure/s – please submit an amendment form to the UP Animal Ethics Committee for approval before commencing with the experiment

APPROVED(* with condition)	Date 27 February 2018
CHAIRMAN: UP Animal Ethics Committee	Signature

Condition: Water has to be easily available to ensure no dehydration during recovery

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UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
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Animal Ethics Committee

Extension No. 2

PROJECT TITLE	**Extreme primates: Physiological and behavioural responses to a temperate primate niche using a strepsirrhine model**
PROJECT NUMBER	**V037-17 (Amendment 1 and Amendment 2)**
RESEARCHER/PRINCIPAL INVESTIGATOR	**C Long**

STUDENT NUMBER (where applicable)	**U_14390800**
DISSERTATION/THESIS SUBMITTED FOR	**PhD**

Condition: Water has to be easily available to ensure no dehydration during recovery

ANIMAL SPECIES	**Galago moholi**	**Otolemur crassicaudatus**
NUMBER OF ANIMALS	**2/month**	**20/month**
Approval period to use animals for research/testing purposes		**March 2019 – March 2020**
SUPERVISOR	**Prof. ASW Tordiffe**	

KINDLY NOTE:

Should there be a change in the species or number of animal/s required, or the experimental procedure/s – please submit an amendment form to the UP Animal Ethics Committee for approval before commencing with the experiment

APPROVED	Date 5 March 2019
CHAIRMAN: UP Animal Ethics Committee	Signature

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