Evaluation and Validation of Measures of Chronic Stress in Ring-tailed Lemurs (*Lemur catta*)

Thesis

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Abstract

Chronic stress can have detrimental long-term health effects but is challenging to measure. In humans, and recently gorillas (*Gorilla gorilla*), allostatic load index (ALI) has been utilized to measure the impact that exposure to chronic stressors has on somatic systems. The goal of this project was to validate ALI in ring-tailed lemurs (*Lemur catta*) as a potential means of evaluating health in captive and wild lemur populations. The specific objectives of the project were 1) validate the use of commercially available assays to measure six biochemical markers to calculate ALI in ring-tailed lemurs, and 2) determine the effects of age, sex, and stressors on ALI in ring-tailed lemurs.

Commercial ELISA assays were utilized to measure and validate the following ALI biomarkers: cortisol, DHEA-S, DNA oxidative damage and PGE2. Serial dilutions of pooled serum were run and compared to the standard curve to ensure that the antigen of interest was accurately being measured. Albumin and glucose were obtained through standard chemistry analysis at a commercial laboratory. Allostatic loads indexes were calculated for each individual by dividing the raw values for each biomarker into quartiles. Among the biomarkers chosen, albumin and DHEA-S have a higher likelihood

of causing impairment of the physiologic regulatory mechanisms when in the lowest quartile, while all other biomarkers show increased risk of physiologic dysregulation in the highest quartile. Since cortisol can be either elevated or depressed in association with chronic stress, a two-tailed cut-off was applied with the highest and lowest 12.5% of values being classified as high risk. Each biomarker within the high-risk quartile for an individual lemur was scored 1; biomarkers not within the high-risk quartile were scored zero. Scores were summed for each lemur as a composite allostatic load index creating an ALI from 0 to 6. The effects of sex and age were tested using two-sample t-test and linear regression, respectively. Associations between ALI and stressors (numbers of: anesthetic events, manual restraint, institutional transfers, enclosure changes, trauma, illnesses, pregnancy, group composition changes, % of time spent indoors/outdoors in semi-free ranging enclosures, participation in research trials (both frequency and time in minutes), and average group size) were tested using linear regression. When a significant association was found between ALI and a stressor males and females were evaluated separately to determine if there were differences by sex.

ALI was associated with percent time spent indoors/outdoors in semi-free ranging enclosures. Lemurs that spent a larger percentage of their time outside in a semi-free ranging habitat had a lower ALI. Average group size had an effect also, with individuals maintained in smaller social groupings having higher ALI. Allostatic load index in

females, but not males, was associated with group composition changes; animals with fewer group changes had higher ALI.

Continuing to validate allostatic load as an indicator of chronic stress in non-human primates may not only improve their care in zoological collections, but also provide a means of evaluating the impact of human disturbances on wild populations, providing quantitative data to inform management decisions and improve species conservation.

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Publications

Seeley KE, Baitchman E, Bartlett S, Debroy C, Garner MM. Investigation and Control of an Attaching and Effacing Escherichia Coli Outbreak in a Colony of Captive Budgerigars (Melopsittacus undulatus). Journal of Zoo and Wildlife Medicine.

2014;45(4):875–882.

Seeley KE, Clayton LA, Hadfield CA, Muth D, Mankowski JL, Kelly KM. Retrospective Review of Mortality in Giant Pacific Octopus (Enteroctopus dofleini). Journal of Zoo and Wildlife Medicine. 2016;47(1):271–274

Seeley KE, D'angelo M, Gowins C, Greathouse J. Prevalence of Batrachochytrium dendrobatidis in Eastern Hellbender (Cryptobranchus alleganiensis) Populations in West Virginia, USA. Journal of Wildlife Diseases.

Seeley KE, Garner MM, Waddell WT, Wolf KN. A Survey of Diseases in Captive Red Wolves (Canis rufus), 1997–2012. Journal of Zoo and Wildlife Medicine. 2016;47(1):83–90.

Seeley KE, Junge RE, Jennings RN, Cunha CW, Li H. Moose (Alces alces) Mortality Associated with Caprine Herpesvirus 2 (CpHV-2) in a Zoological Collection. Manuscript in preparation

Seeley KE, Wolf KN, Bishop MA, Turnquist M, Kukanich B. Pharmacokinetics of Long-acting Cefovecin in Copper Rockfish (Sebastes caurinus). American Journal of Veterinary Research. 2016;77(3):260–264.

Fields of Study

Major Field: Comparative and Veterinary Medicine

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Chapter 1: Literature Review

Over the last few decades, there has been growing interest in improving the health and quality of life for wildlife and animals housed under human care. While standard measures of health, such as hematology, are frequently utilized there need to be ongoing efforts to develop and refine additional health measures. A promising indicator of long-term animal health is the amount of chronic stress that the animal experiences. Chronic stress has been associated with negative health outcomes in humans and decreased production in farm animals, but less research has been done using wild animals and those housed in zoos. (McEwen, 2004; Sterling, 2012; Von Borell, 2007). In the human literature, biochemical markers in blood have been validated as measures of chronic stress or 'allostatic load index' (ALI) (Beckie, 2012; Edes & Crews, 2017; McEwen, 2010; McEwen & Stellar, 1993; Seeman et al., 2001). These same biomarkers have recently been validated in gorillas housed in zoological institutions (Edes et al., 2016a; Edes et al., 2016b). Results from this line of research offer a promising approach for monitoring animal health and mitigating stressors that may lead to poor health in both wild and zoo populations.

1.1 Definition of Stress

Stress can be defined in a variety of different ways and is often based on the field of study in which it is being evaluated. In the simplest terms, Hans Selye defined stress as "the nonspecific response of the body to any demand" (Fink, 2010; Selye, 1950). Since this initial definition we have learned a great deal and now realize that the stress response can vary widely depending on a variety of factors and is not as 'non-specific' as initially thought. When evaluating the physiologic and behavioral changes in an organism experiencing stress a more inclusive definition is "a real or interpreted threat to the physiological or psychological integrity of an individual that results in physiological and/or behavioral responses" (McEwen, 2010).

Any living organism encounters numerous stressors as part of day to day existence and has developed various coping mechanisms. Acute stress can be adaptive and promote survival (Dowd et al., 2009; Edes & Crews, 2017; McEwen, 2010). However, if stressors are prolonged or frequent, or the organisms lacks the ability to initiate adaptive mechanisms, stress can have long term negative consequences on health and behavior (Abbott et al., 2003; Dowd et al., 2009; Edes & Crews, 2017; Sapolsky, 2002).

Stress involves both a stressor and a stress response. The definition of a stressor varies based on the individual as well as the species. For instance, what a predator perceives to be a stressor is different than what a prey species perceives as a stressor

(Stankowich & Blumstein, 2005). Some examples of stressors include: physical insult (i.e. trauma), overexertion, noise, overcrowding, and illness (McEwan, 2010). The stress response can be either physiological (i.e. increased blood pressure, elevated heart rate) or behavioral (i.e. aggression, escape behavior, vocalization, freezing) (McEwen, 2010; Moberg, 2000). The degree of these responses varies based on the type and duration of the stressor the animal is encountering (Moberg, 2000).

The physiological stress response is mediated by the activation of the autonomic nervous system and the hypothalamic-pituitary-adrenal (HPA) axis resulting in the release of various hormones, catecholamines and cytokines. This response evolved to allow the organism to adapt and maximize survival (e.g., allow animals to escape from immediate threats to their survival such as predators), however they are energetically costly and physiologically demanding (Edes & Crews, 2017; Edes et al., 2016a). Additionally, when these responses are activated for prolonged periods of time they may result in cellular and organ damage as well as potentially altered CNS function (Edes & Crews, 2017; McEwan, 2010). Chronic stressors can be mediators of poor health outcomes and while they may not directly cause adverse health effects they may place an animal at an increased risk of succumbing to disease (Moberg, 2000)

1.2 Methods used to of Measure Stress

Due to the important role of stress in human and animal health, numerous ways of

measuring stress have been developed (Davis et al., 2008; Mohr et al., 2002; Tennant & Andrews, 1976). Given that stressors cause a complex physiological and behavioral response in animals, there is no one measure that can fully quantify the stress response or its long-term effects in an organism. Additionally, there is an increased challenge in measuring the stress response in animals, given that they are unable to communicate their perceptions in the same way that humans can.

Glucocorticoids, particularly cortisol, are the most commonly measured stress hormones (Bush & Hayward, 2009; Sheriff et al., 2011). When an animal encounters a stressor the HPA axis is triggered. The hypothalamus releases corticotropin releasing hormone (CRH) that stimulates the pituitary to release adrenocorticotropic hormone (ACTH) which then prompts the adrenal glands to secrete glucocorticoids and catecholamines (epinephrine, norepinephrine, and aldosterone) (Edes & Crews, 2017; Squires, 2006). In the short-term increased cortisol levels can be reflective of a "good", acute stress response which can be adaptive and maximize survival (Cabezas et al., 2007; Cavigelli et al., 2009). Chronic activation of the HPA axis can result in prolonged elevation of cortisol levels or abnormally low levels due to blunting of the adrenocorticotropin response (Edes et al., 2016a; Van Den Eede et al., 2007).

The challenge with using cortisol as the sole measure of a stress response is that glucocorticoids fail to change in a consistent, predictable manner and do not always show

a linear correlation with survival or reproduction (Bush & Hayward, 2009). The process of invasive sampling (i.e. capture and blood collection) may itself cause elevations in cortisol levels (Morton et al., 1995). Additionally, since cortisol is an essential component of normal physiological mechanisms there is significant variation based on the sleep/wake cycle, exercise, diet, season, and sex that need to be taken into account when interpreting cortisol levels (Brandenberger & Follenius, 1975; Huber et al., 2003; Monfort et al., 1993; Van Cauter et al., 1996; Weitzman et al., 1971).There are also limitations to using one physiological indicator of stress, as it does not provide a holistic picture across multiple physiologic systems within an organism (Cockrem, 2005; Shepherdson et al., 2004; Sheriff et al., 2011).

Behavioral observations are another means of evaluating stress in animal populations. This method requires an extensive knowledge of the species of interest since behavior varies widely from one species to another. For instance, dogs that experience an acute stressor show changes in body postures, restlessness and yawning (Beerda et al, 1998). In captive zoo settings, animals that are exposed to chronic stressors, barren enclosures, or are experiencing boredom often develop stereotypies, which are defined as "repetitive, invariant behavior patterns with no obvious goal" (Mason, 1991a). Stereotypies vary widely but common examples of behaviors seen in zoo housed animals include pacing, head-swaying, and bar-chewing (Mason, 1991a; Mason, 1991b;

Shepherdson et al., 2004; Vickery & Mason, 2004). A major limitation of this method of evaluation is that it varies widely by species. Additionally, individuals within a species vary in their response to the same stressor, as each individual perceives the stressor differently (Vickery & Mason, 2004; Stankowich & Blumstein, 2005).

Researchers have also used long-term impacts of a chronic stress response such as the animal's ability to grow normally and reproduce. Reproduction is very sensitive to the effects of stress and as such provides a potential means of measuring the stress levels of an individual or population. (Moberg, 2000). The role that stress plays in reproduction and the negative impacts it has have been documented widely in many species (Dobson & Smith, 2000; Von Borell et al., 2007)

Due to the complex nature of the stress response, multiple methods of evaluating stress are often used in conjunction (Shepherdson et al., 2004; Von Borrell et al., 2007). For example, Shepherdson et al. used both fecal cortisol analysis and behavioral observations try and quantify stress in polar bears (*Ursus maritimus)*, Hawaiian honeycreepers (*Drepanididae*) and clouded leopards (*Neofelis nebulosa*).

1.3 Allostasis, Allostatic Load (AL) and Allostatic Load Index (ALI)

Conceptualization of the stress response has expanded since Selye's initial definition. Homeostasis, which refers to the mechanisms that maintain vital physiological

parameters (pH, blood pressure, heart rate) within narrow margins, is complemented by allostasis. Allostasis is the process by which an organism makes physiological adjustments to predictable and unpredictable stressors (Edes & Crews, 2017; McEwen & Wingfield, 2003; Schulkin, 2003; Seeman et al., 2004). Allostasis relies on the integrated responses of the HPA axis and sympathetic-adrenal-medullary (SAM) axis and allows the organism to adapt to stressors in their environment (Edes & Crews, 2017; Edes et al., 2016a). This "adaptive plasticity" allows for the organism to have more complex responses to real and perceived threats in a more fluid manner and maintain "stability through change" (Edes & Crews, 2017).

Allostatic load (AL) refers to the cumulative cost to the body to maintain allostasis (Edes & Crews, 2017; McEwen & Wingfield, 2003; Schulkin, 2003; Seeman et al., 1997). There are daily and seasonal stresses experienced by any species that are part of normal life and the body appropriately adjusts when encountering these situations (Edes & Crews, 2017; McEwen & Wingfield, 2003; Seeman et al., 2004; Seeman et al., 1997). However, when the system is overloaded by excessive or constant stressors there can be 'wear and tear' of the physiologic state which may predispose the individual to poor health (Edes & Crews, 2017; Leahy & Crews, 2012; McEwen & Seeman, 1999; McEwen & Wingfield, 2004; Seeman et al., 2004; Seeman et al., 1997). This constant exposure to chronic stress results in dysregulation, which is defined as the "impairment of

a physiological regulatory mechanism, such as those that governing metabolism, immune response or organ function ("dysregulation", 2017).

Allostatic load provides a more integrated approach than evaluation of cortisol by accounting for changes in primary stress mediators and associated secondary outcomes resulting from ongoing or repeated exposure to stressors (Beckie, 2012; Edes & Crews, 2017; McEwen & Seeman, 1999). Since allostatic load increases as there is physiologic disruption in multiple allostatic systems it needs to be measured using a composite of physiological biomarkers that reflect this dysregulation (Beckie, 2012; Juster et al., 2010; Leahy & Crews, 2012; McEwen & Stellar, 1993). This collection of biomarkers is referred to as an allostatic load index and can be used to approximate the allostatic load of an organism. An advantage of using ALI instead of more traditional measures of stress responses is that the calculation of ALI uses various biomarkers that evaluate multiple physiologic systems and how they respond to stressors, both actual and perceived. In humans, many different biomarkers, representing neuroendocrine, metabolic, immune and cardiovascular function, have been used to calculate allostatic load index (Beckie, 2002; Edes & Crews, 2017). The information provided by these biomarkers about the function of multiple somatic systems is what provides a more holistic picture of physiological dysregulation than looking at any single biomarker by itself.

There are three physiologic responses that potentially contribute to allostatic load (McEwen, 1998; McEwen, 2010). Type 1 allostatic load is frequent stress, where either the magnitude or the frequency of the stressor is greater than the organism can appropriately adapt to, for example repeated blood pressure surges may trigger myocardial infarction (McEwen, 1998; McEwen, 2010). Type 2 allostatic load is a failure of the stress response to shut down. In this case, appropriate feedback mechanisms do not occur resulting in the overproduction or suppression of a biomarker (McEwen, 1998; McEwen, 2010). Type 3 allostatic load is an inadequate response in which the organism does not react to the stressor, as is the case of immunosuppression secondary to inadequate cortisol production (McEwen, 1998; McEwen, 2010).

Allostatic load has been used extensively in human populations, often in the fields of epidemiology, neurobiology, immunology, psychology, and public health (Beckie, 2012; Crews, 2007; Edes & Crews, 2017; Juster et al., 2010; Stewart, 2006). It was first operationalized into a working theory over two decades ago and has since been used to evaluate differences in human populations of varying economic, social and educational backgrounds as well as the evaluation of aging and associated morbidity (Dowd et al., 2009; Juster et al., 2010; Edes & Crews, 2017; Leahy & Crews, 2012; Sterling & Eyer, 1988).

1.4 Allostatic Load in Non-Human Species

Until recently, allostatic load had only been applied conceptually to animal species and not evaluated in the same rigorous manner as in humans. Allostatic load has been discussed in multiple species including: rhesus macaques, rats, baboons, and European white storks (Blas et al., 2007; Cavigelli & Caruso, 2015; Cavigelli et al., 2009; Goyman & Wingfield, 2004; Hoffman et al., 2011; Roth et al., 2004; Sapolsky, 2005; Soderholm et al., 2002). Most published investigations on non-human primates report one or two biomarkers. A study evaluating the impact of allostatic load on health in rhesus macaques (*Macaca mulatta*) measured cortisol, interleukin-6, and epinephrine (Maestripieri & Hoffman, 2011). Although they did not follow published methodology to determine an allostatic load index, the validation and measure of hormones and other biomarkers provides a vital foundation from which allostatic load indexes for non-human primates may be built.

There are multiple potential applications to animal populations if allostatic load index is found to be a valid measure of chronic stress. It could provide insight into health on both the individual and population levels. It could also potentially allow for better evaluation of the welfare of individuals under human care and inform husbandry decisions (Korte et al., 2007). Additionally, ALI may provide a tool by which to evaluate conservation measures and how they are impacting the population (Edes et al., 2016a).

Although applied theoretically to several different animal species, the only non-human species that allostatic load has been validated in is captive gorillas (*Gorilla gorilla*) (Edes et al., 2016a; Edes et al., 2016b). This work showed significant associations between ALI and age with older individuals having higher ALI (Edes et al., 2016a). They also found that female gorillas had higher ALI than males and that overall ALI was associated with the number of stress events an animal experienced over a lifetime (Edes et al., 2016a). Lastly, they found that gorillas taken from the wild had higher ALI than those born in captivity (Edes et al., 2016b).

Given its potential utility as another way of evaluating health it is important to continue investigating ALI in other species. When expanding this foundational work, it is important to consider the species' popularity in captive institutions as well as their endangered status. For example, a good potential model for further research assessing the impact of allostatic load on health outcomes in captive and wild settings are lemurs (Primates: Prosimii: Lemuriformes). Lemurs are considered one of the most threatened groups of mammals (LaFleur et al., 2016). They are indigenous only to the island of Madagascar, and over half of the 101 lemur species are listed as threatened or endangered, primarily due to human encroachment, land use (e.g., mining and logging) and poaching (IUCN, 2016; LaFleur et al., 2016). These human activities have a direct impact on lemurs, and introduce novel stressors (e.g. capture, translocation, noise) which

may contribute to chronic stress responses.

Ring-tailed lemurs (*Lemur catta*) are a particularly good model for research in allostatic load, as they are very popular in zoos and other settings, including a large research center at Duke University (Duke Lemur Center). Additionally, their populations are declining in the wild and conservation efforts are imperative (LaFleur et al., 2016). Lemurs housed at the Duke Lemur center had the banked serum samples and detailed records that were essential to complete validation research on allostatic load.

Ring-tailed lemurs are a diurnal species inhabiting the Southeast portion of the island of Madagascar (Figure 1; Hilton-Taylor, 2000; Lang, 2005b). They can be found in various habitats ranging from dry brush and scrub forests to closed canopy rainforest (Hilton-Taylor, 2000; Lang, 2005b; Wilson & Hanlon, 2010). Due to the seasonal variability in their habitat they rely on several different food resources throughout the year and are considered "opportunistic omnivores" (Lang, 2005b).

Social structure and dominance hierarchy has been shown to impact stress in many primate species, including ring-tailed lemurs (Cavigelli & Caruso, 2015; Jolly et al., 2002; Sclafani et al., 2012). In the wild, ring-tailed lemurs have a complex social structure and live in social groups ranging in size from 3-21 with an average of about 12 individuals (Gould et al., 2003; Sussman, 1991). The optimum group size depends on several factors including resource availability (Pride, 2005b). Females typically remain

with their natal group for life whereas males migrate to other troops (Nakamichi & Koyama, 1997). Unlike other old-world monkey species, the dominance hierarchy in lemurs is not linear; however adult females are almost always dominant over adult males (Nakamichi & Koyama, 1997; Sclafani et al., 2012).

In the wild, potential stressors for lemurs include: obtaining resources, finding mates, disruptions in social structure (e.g., births, deaths, etc.), and social status (Cavigelli et al., 2003; Pride, 2005b; Sclafani et al., 2012). Research has shown that changes in social status in primates may cause a stress response and lead to health risks, but the impact of social status depends on the individual, the species, and other factors (Abbott et al., 2003; Sopolsky, 2000). Ring-tailed lemurs are seasonal breeders and females are only receptive for 10-24 hours during the weeks that comprise the breeding window (Jolly, 1966; Evans & Goy, 1968; Koyama, 1988; Cavigelli & Pereira, 2000). During the breeding season mate selection can become intense and dominance hierarchies can change, this has been associated with increased cortisol secretion and behaviors suggestive of anxiety such as scratching (Cavigelli et al., 2003; Sclafani et al., 2012)

In captive situations, animals are provided with ample resources (shelter, food), and often their breeding opportunities are controlled. However, animals in captive environments experience different stressors compared to those in the wild, including

human interactions, limited space allowance, and imposed social groupings (Morgan & Tromborg, 2007). Given the highly social nature of ring-tailed lemurs and the matriarchal social structure we anticipate that group composition changes would significantly contribute to stress, as has been documented for other ring-tailed lemur populations (Cavigelli et al., 2003; Starling et al., 2010). Additionally, we would anticipate that females experience greater stress than males, although for different reasons than reported for gorillas. In captive gorillas females express higher stress with greater proximity to the male (Edes et al., 2016a). Ring-tailed lemurs have a female-dominated despotic social system wherein dominant females are likely to experience greater stress in their efforts to maintain their rank (Sapolsky, 2005).

Literature discusses stress in ring-tailed lemurs; however, this work almost exclusively involves quantification stress by cortisol (Cavigelli et al., 2003; Pride, 2005 a & b; Starling et al., 2010). This is logical given that cortisol can be extracted from feces and therefore be obtained noninvasively. However, it is insufficient to characterize the overall physiologic effects of prolonged stress with cortisol alone. In situations where additional biomarkers may be sampled, application of more robust measures of stress will improve understanding of such physiological outcomes.

There is a plethora of observational information on wild lemur populations ranging from behavioral studies to evaluations of effects of environmental stressors

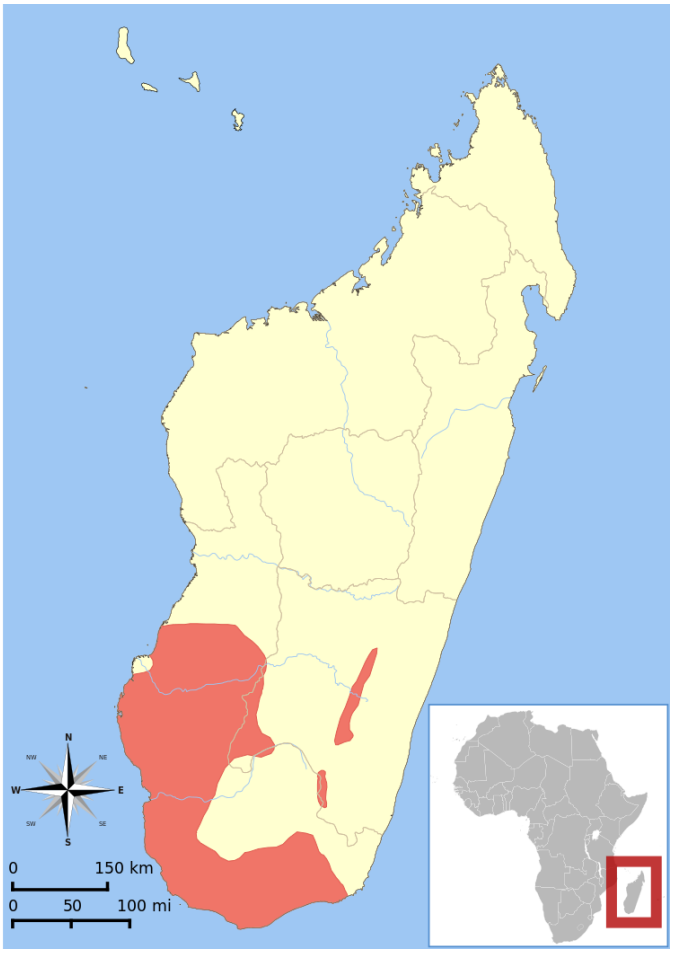
(Gould et al., 1999; Jolley et al., 2002; Sclafani et al., 2012; Starling et al., 2010). Jolly et al., conducted a long-term demographic study of ring-tailed lemurs in Madagascar and provided crucial information regarding optimal group size and the female dominance hierarchy (Jolly et al., 2002). Work by Gould et al. (1999) illustrated population decline and subsequent rebound after severe drought conditions in Madagascar. Starling et al. (2010) investigated the effect of seasonality, sociality and reproduction on fecal cortisol and found elevations in association with breeding season and social circumstances with group composition playing a major role in cortisol levels regardless of season (Starling et al., 2010). Additionally, extensive work has documented health parameters in wild lemurs and how these are affected by factors such as climate change (Barrett et al., 2013; Junge, 2006). To date no work that has been reported quantifying effects of chronic stress on lemur populations in captivity or the wild. Nor have reports determining factors likely contributing to chronic stress been published. In an effort to address gaps in this literature the primary objectives of this project were:

1) Validate the use of commercially available assays to measure six biochemical markers and determine their effectively for calculating ALI in ring-tailed lemurs, and

2) Determine the associations effects of age, sex, and stressor exposure on ALI in a

sample of ring-tailed lemurs.

Figure 1. Map of the island of Madagascar with the home range of ring-tailed lemurs (*Lemur catta*) shown in red.



Chapter 2: Validation of Assays to Measure Biomarkers Associated with Allostatic Load in *Lemur catta*

2.1. Introduction

Allostasis is the process by which an organism makes physiological adjustments to predictable and unpredictable stressors (Edes & Crews, 2017; McEwen & Wingfield, 2003; Schulkin, 2003; Seeman et al., 2004). Allostasis relies on integrated responses by the HPA and SAM axes that allows organisms to adapt to stressors in their environment in a flexible fashion (Edes & Crews, 2017; Edes et al., 2016a). This "adaptive plasticity" allows the organism to make complex responses to real and perceived threats in a fluid manner and thereby maintain "stability through change" (Edes & Crews, 2017).

Allostatic load (AL) refers to the cumulative cost to the body to maintain allostasis (Edes & Crews, 2017; McEwen & Wingfield, 2003; Schulkin, 2003; Seeman et al., 1997). There are daily and seasonal stresses experienced by any species that are part of normal life and the body appropriately adjusts when encountering these situations (Edes & Crews, 2017; McEwen & Wingfield, 2003; Seeman et al., 2004; Seeman et al., 1997). However, when the system is overloaded by excessive or constant stressors there can be 'wear and tear' of the physiologic state which may predispose the individual to

poor health (Edes & Crews, 2017; Leahy & Crews, 2012; McEwen & Seeman, 1999; McEwen & Wingfield, 2004; Seeman et al., 2004; Seeman et al., 1997). This constant exposure to chronic stress results in dysregulation, which is defined as the "impairment of a physiological regulatory mechanism, such as those that governing metabolism, immune response or organ function ("dysregulation", 2017).

Since allostatic load increases as there is physiologic disruption in multiple allostatic systems it needs to be measured using a composite of physiological biomarkers that reflect this dysregulation (Beckie, 2012; Juster et al., 2010; Leahy & Crews, 2012; McEwen & Stellar, 1993). This collection of biomarkers is referred to as an allostatic load index and can be used to approximate the allostatic load of an organism. An advantage of using ALI instead of more traditional measures of stress responses is that the calculation of ALI uses various biomarkers that evaluate multiple physiologic systems and how they respond to stressors, both actual and perceived. In humans, many different biomarkers, representing neuroendocrine, metabolic, immune and cardiovascular function, have been used to calculate allostatic load index (Beckie, 2002; Edes & Crews, 2017). The information provided by these biomarkers about the function of multiple somatic systems is what provides a more holistic picture of physiological dysregulation than looking at any single biomarker by itself.

In order to appropriately calculate an allostatic load index it is essential that

species-specific assays are utilized to ensure that the antigen of interest is being reliably measured and results are reflective of the physiology of the focal species. As such, the first objective was to validate six commercial assays to accurately measure allostatic-load biomarkers in lemurs.

2.2. Material and Methods

2.2.1. Animals, Sample Collection and Storage

The study population consisted of 38 ring-tailed lemurs (16 males, and 22 females) ranging in age from 2-30 years that were housed at the Duke Lemur Center (DLC) between 1983 and 2016. Historical banked serum samples (n=27) from the DLC were utilized for this work. If a banked sample was not available for an individual, but they were still part of the collection (n=11), a fresh blood sample was collected. The procedure was conducted with oversight from Duke University's Institutional Animal Care and Use Committee (#A027-15-01, "Staff support for research at the DLC"; PI: Ehmke). The blood was centrifuged (10 minutes, 1000 X g) within 15 minutes and the serum was pipetted off and stored in 1.8mL cryovials. Samples were stored at -80C while at Duke and were transported on dry ice and then stored at -18C until assays were run.

2.2.2. Validation Technique

A biological validation approach was taken to ensure that assays being used to quantify the biomarkers utilized in allostatic load index accurately measured these compounds in this species (Wildlife Endocrinology Manual, 2008). Since the species of interest are non-domestic species for this initial exploratory phase manipulations were not done to the animals to elicit the release of the hormone of interest. Instead individuals that were presumed to have some degree of acute and chronic stress were utilized with the assumption that the biomarkers of interest were being produced.

To evaluate that the analyte of interest was being measured, samples were serially diluted to evaluate parallelism with the standard curve. Percent recovery was performed when possible to test for potential interference of other compounds within the sample. To do this a pooled sample, with a presumed low concentration of the hormone of interest, was spiked with a known quantity of standard. The un-spiked pooled standard was also assayed to provide quantification of the background hormone present. The following formula was utilized to determine the percent recovery:

$$\% \text{ Recovery} = (\text{Amount Observed}/\text{Amount Expected}) * 100$$

Intra-assay precision was calculated by running three individual's samples multiple times and determining the % covariance (CV) using the following formula.

$$\%CV = \frac{\text{standard deviation of data-set} \times 100}{\text{Mean of data-set}}$$

Inter-assay coefficients of variation were not performed since all assays were run within a 24-48-hour period by the same individual.

2.2.3. Assay Selection, Specific Assays and Protocols

Enzyme linked immunoassays (ELISAs), either sandwich assays or competitive assays, were utilized to evaluate each of the biomarkers. Sandwich assays utilize two different antibodies that bind to different portions of the antigen **(**Figure 1**)**. The plate is coated with the first antibody (capture antibody) and the sample is added. The antigen of interest binds to the capture antibody. The second antibody (detection antibody) is then added and binds to a second site on the antigen. Linked to the second antibody is an enzyme that changes color after the addition of appropriate chemicals. Since the enzyme linked antibody is bound to the antigen the strength of the color change is positively associated with the amount of antigen present. Because binding to both antibodies is required for detection of the antigen of interest the binding affinity of the antibodies is the greatest determinant of sensitivity (Cox, 2012; Wildlife Endocrinology Manual, 2008).

Competitive assays rely on a plate coated with antibody to which the antigen of interest (in serum) binds. A second chromogenic enzyme-linked antigen is added to compete for antibody binding sites, so the color (absorbance) is inversely proportional to the concentration of the biomarker of interest **(**Figure 2) (Cox, 2012; Wildlife

Endocrinology Manual, 2008).

All samples were run in duplicate on 96-well plates. Plates were read using a Tecan microplate reader (Tecan Group Ltd., Seestrasse 103, 8708 Männedorf, Switzerland) at a wavelength of 450 nm. All raw data were analyzed using the MyAssay Analysis Software Solutions (https://www.myassays.com/welcome.aspx).

Cortisol EIA: Competitive ELISA was conducted using monoclonal antibodies for the detection of cortisol (NCal^{TM} International Standard Kit, DetectX, Cortisol Enzyme Immunoassay Kit, Species independent, Arbor Assays Interactive Assay Solutions, 1514 Eisenhower Place, Ann Arbor, MI 48108-3284). The kit was run following manufacturer instructions and samples were diluted at the recommended 1:100 concentration. An exploratory plate was run and, based on the initial results, serum from individuals with high cortisol was pooled and serial dilutions were done to evaluate parallelism.

Insulin EIA: Competitive ELISA was conducted using monoclonal antibodies for the detection of insulin (DetectX, Insulin Enzyme Immunoassay Kit, multi-species, catalog #K046-H1, Arbor Assays Interactive Assay Solutions, 1514 Eisenhower Place, Ann Arbor, MI 48108-3284). The kit was run following manufacturer instructions and samples were diluted at the recommended 1:4 dilution.

DHEA-S EIA: Competitive ELISA was conducted using monoclonal antibodies

for the detection of DHEA-S in serum (DetectX® Dehydroepiandrosterone sulfate (DHEA-S) Immunoassay Catalog # K054-H1, Arbor Assays Interactive Assay Solutions, 1514 Eisenhower Place, Ann Arbor, MI 48108-3284). The kit was run following manufacturer instructions and samples were diluted at 1:2 per standard instructions. Two samples were outside the standard curve and were rerun at a dilution of 1:10. Select samples were serially diluted to compare to the standard curve.

DNA Damage EIA: The DNA damage ELISA is a competitive monoclonal assay designed to measure RNA and DNA oxidized guanine species (DetectX® DNA Damage Immunoassay Catalog # K059-H1, Arbor Assays Interactive Assay Solutions, 1514 Eisenhower Place, Ann Arbor, MI 48108-3284). The kit was run following manufacturer instructions. Samples were diluted 1:8. Select samples were serially diluted for comparison to the standard curve.

PGE-2 EIA: Competitive ELISA was conducted using monoclonal antibodies for the detection of PGE-2 in serum (Product: DetectX ® PROSTAGLANDIN E2 Enzyme Immunoassay Kit catalog #K051-H1, Arbor Assays Interactive Assay Solutions, 1514 Eisenhower Place, Ann Arbor, MI 48108-3284). The kit was run following manufacturer instructions and samples were diluted at 1:10 per standard instructions. With this dilution several samples were outside of the standard curve so these specific samples were run at a dilution of 1:20. Serial dilutions were performed to compare to the standard curve.

IL-6 EIA: A monoclonal, quantitative sandwich enzyme assay was utilized for the detection of IL-6 (Human IL-6 Quantikine ELISA Kit R&D Systems Inc., 614 McKinley Place NE, Minneapolis, MN 55413 USA). The kit was run following manufacturer instructions with standard dilutions.

C-reactive protein (CRP) EIA: A monoclonal, quantitative sandwich enzyme assay was utilized for the detection of CRP (Human C-Reactive Protein/CRP Quantikine ELISA Kit R&D Systems Inc., 614 McKinley Place NE Minneapolis, MN 55413 USA). The kit was run following manufacturer instructions with standard dilutions.

TNF-α: A monoclonal, quantitative sandwich enzyme assay was utilized for the detection of TNF-α (Human TNF-α Quantikine ELISA Kit R&D Systems Inc., 614 McKinley Place NE Minneapolis, MN 55413 USA). The kit was run following manufacturer instructions with standard dilutions.

2.3. Results

2.3.1. Cortisol

The cortisol EIA showed parallelism to the standard curve (Figure 3). The intra-assay precision ranged from 11.8-25.8% (Table 1) which is was slightly higher than the reported precision for the assay.

2.3.2. Insulin

The commercial insulin assay that was used consistently produced results above the provided standard curve so the values were invalid and not utilized in the study.

2.3.3. DHEA-S

The serial dilutions showed parallelism to the standard curve (Figure 4).

2.3.4. DNA Damage

The serial dilutions showed parallelism to the standard curve (Figure 5).

2.3.5. PGE-2

Serial dilutions of sample serum showed parallelism to the standard curve (Figure 6). The intra-assay coefficient of variation was 18.25%.

2.3.6. IL-6

IL-6 was not detectable in any samples (equivalent to blank well), indicating that this assay is not appropriate for this species.

2.3.7. CRP

Initial plate run at 1:100 dilutions that was recommended led to values that were outside the standard curve. A second plate was then run with serum run at a 1:200 dilution, however there was no detection of CRP indicating that this is assay is not appropriate for this species.

2.3.8. TNF-α

TNF- α was not detectable in any samples (equivalent to blank well), indicating that this assay is not appropriate for this species.

2.4. Discussion

The results of this validation provide an important tool in allowing for the calculation of allostatic load index in lemurs. There were inherent limitations to the study and the types of validation that could be performed. Since the validations were part of a larger study on allostatic load (see chapter 3) the priority was placed on running a sufficient variety of assays to compose the allostatic load index. Serum samples were obtained from the Duke Lemur Center and were typically banked and of limited volume. As such the ability to perform percent recovery or run samples multiple times to determine intra assay variation was limited.

The choice of biomarkers validated in this study was based on the human literature (Beckie, 2012; Juster et al., 2010; Seeman et al., 2001). Similar biomarkers have been measured in gorillas using human assays (Edes et al., 2016a; Edes et al., 2016b). Of the assays that were used cortisol, DHEA-S, PGE2 and DNA damage were all found to be valid in ring-tailed lemurs based on parallelism to the standard curve. The assay kits used to measure insulin, IL-6, C-reactive protein, and TNF-α, however, did not

provide valid results for those biomarkers.

Reasons these specific assay kits did not work for lemurs most likely is related to the targeted antigens. In the insulin assay a few samples yielded results but the majority did not. The kit antibody was designed for binding sites on human insulin antigens and these amino acid sequences may not be highly conserved across primate species.

The other three non-validating assays were sandwich ELISAs, requiring two antibodies to appropriately bind to the antigen. Even small variation in the amino acid sequences of these antigens would preclude binding. A future possibility may be to sequence the proteins of interest and compare them with the kits utilized to ensure antibodies are appropriately matched. However, such work was beyond the scope of this project.

Given the exploratory nature of this research there were limitations and additional work needs to be done. Due to plate size and sample availability intra-assay precision and % recovery were not completed for all assays. While the parallelism seen between the serial dilutions and the standard curve are suggestive that the tests used are valid, these additional assessments should be performed in the future. Additionally, only a limited number of commercial assays were investigated, others may be available with antigens that were not detected by those used here.

Overall, these validations revealed that commercially available assays for cortisol,

DHEA-S, DNA damage and PGE2 likely are valid for measuring these analytes in ring-tailed lemur serum. Insulin, IL-6, CRP and TNF-α were not identified in ring-tailed lemur serum, consequently they are not currently useful for assessing allostatic load in this species.

Figure 2. Schematic of sandwich ELISA. Two antibodies (capture and detection) bind to sites on the antigen. The capture antibody is linked to an enzyme that causes color development. The amount of color (absorbance) is directly proportional to the concentration of

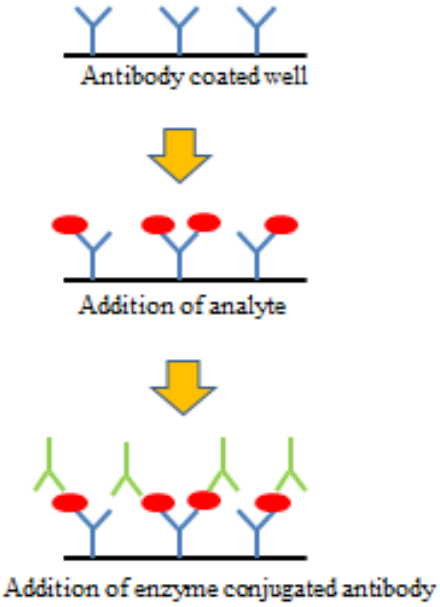


Figure 3. Schematic of competitive binding assay. The analyte of interest and the enzyme-conjugated analyte are added and compete for binding sites on the antibodies. The amount of color is indirectly proportional to the concentration of the analyte of interest.

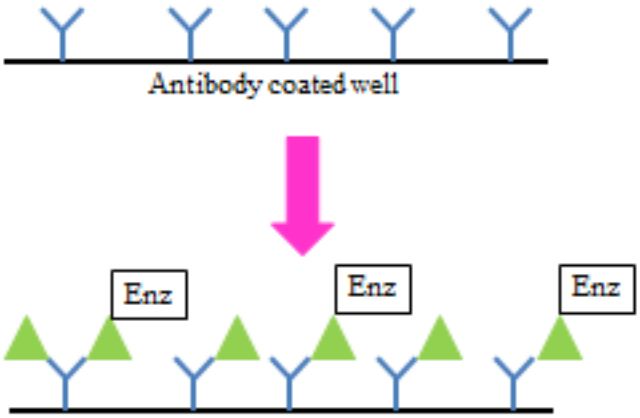


Figure 4. Parallelism of serial dilutions of pooled lemur serum in comparison to the standard curve for cortisol.

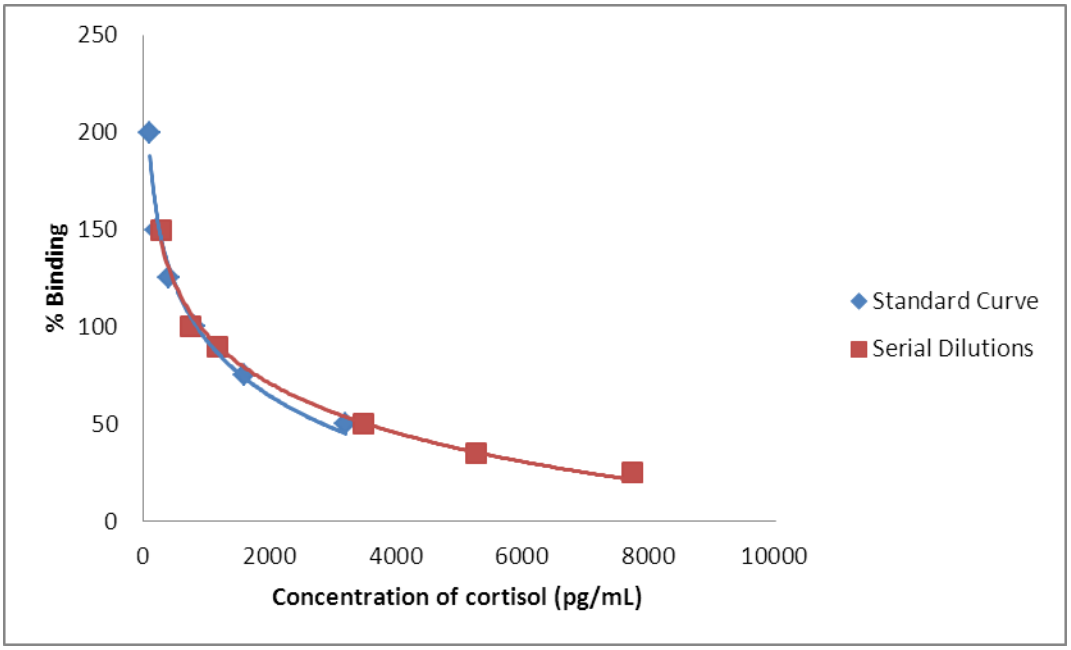


Figure 5. Parallelism of serial dilutions of pooled lemur serum in comparison to the standard curve for DHEA-S.

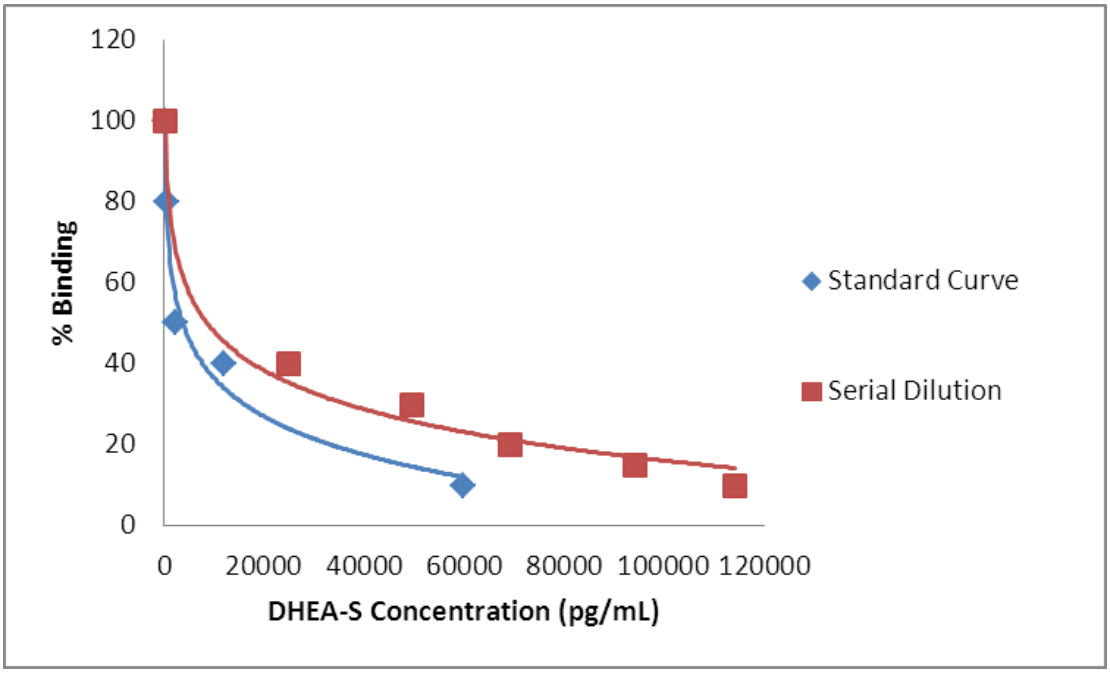


Figure 6. Parallelism of serial dilutions of pooled lemur serum in comparison to the standard curve for DNA Damage.

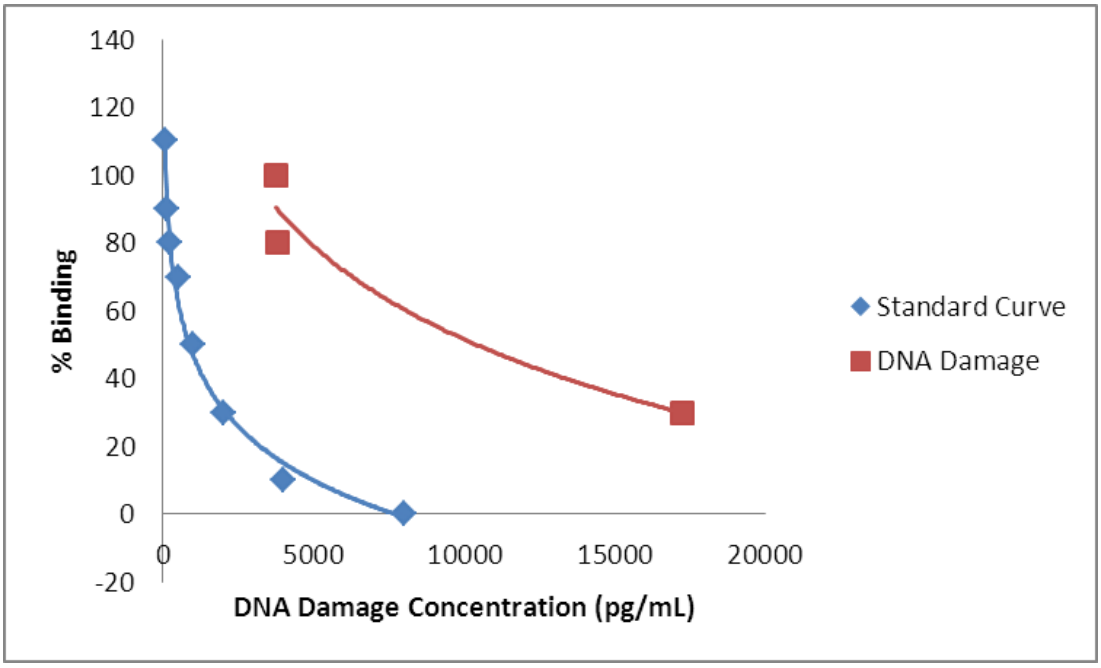


Figure 7. Parallelism of serial dilutions of pooled lemur serum in comparison to the standard curve for PGE-2.

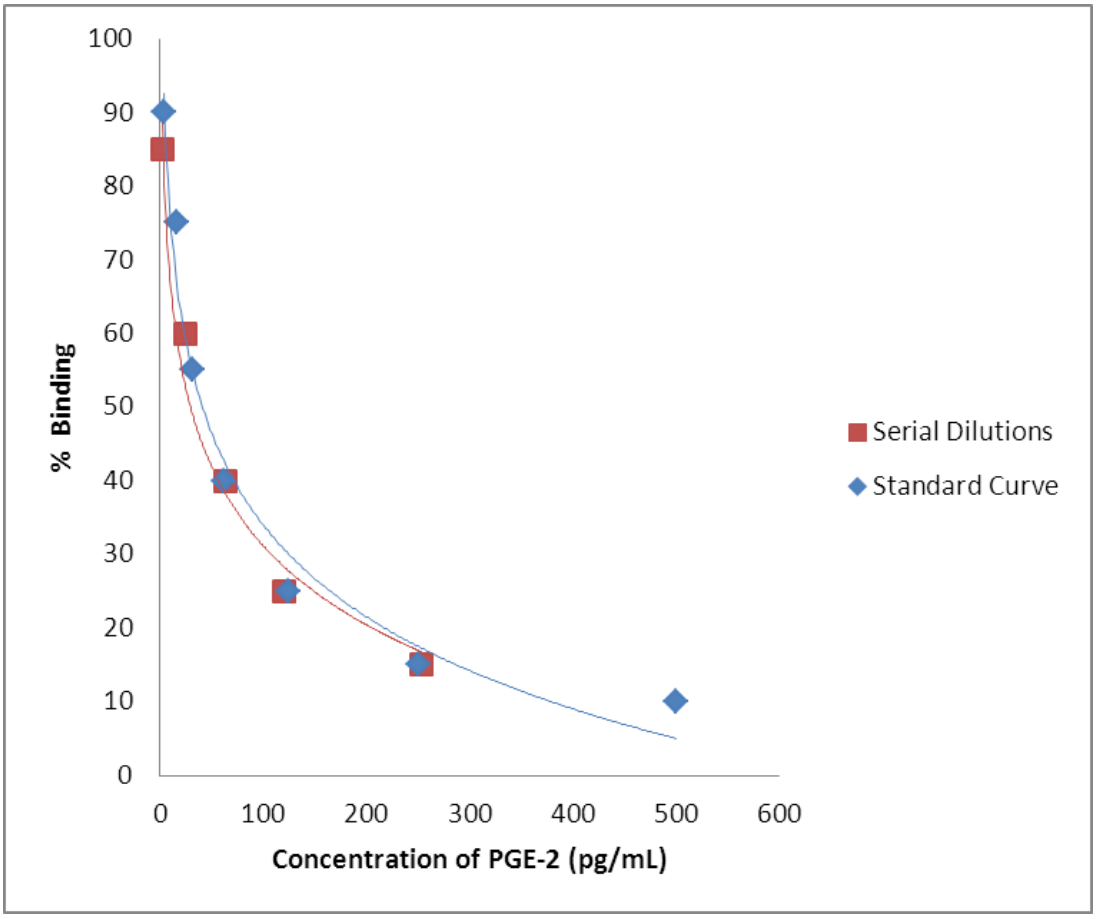


Table 1. Intra-assay precision for cortisol and PGE2

Assay	Sample	%Covariance
Cortisol	1	16.88
	2	25.9
	3	11.8
PGE2	1	18.25%

Chapter 3: The Relationship between Allostatic Load Index and Stressors in *Lemur catta*

3.1. Introduction

In Chapter 2, possible biomarkers for evaluating allostatic load in lemurs were validated. The second objective of this project was to evaluate allostatic load index in relation to various stressors experienced by lemurs in a captive environment to determine if allostatic load index provides a valid way of measuring chronic stress in this species.

Allostatic load index has been used as a measure of chronic stress in humans and Gorillas (Edes et al., 2016a; Edes et al., 2016b; Edes and Crews, 2017), but there is no research to date assessing if this tool can also be used in lemurs. Allostatic load index has been used extensively in human populations, often in the fields of epidemiology, neurobiology, immunology, psychology, and public health (Beckie, 2012; Crews, 2007; Edes & Crews, 2017; Juster et al., 2010; Stewart, 2006). It used to evaluate differences in human populations of varying economic, social and educational backgrounds as well as the evaluation of aging and associated morbidity (Dowd et al., 2009; Juster et al., 2010;

Edes & Crews, 2017; Leahy & Crews, 2012).

Although applied theoretically to several different animal species, the only non-human species that allostatic load has been validated in is captive gorillas (*Gorilla gorilla*) (Edes et al., 2016a; Edes et al., 2016b). This work showed significant associations between ALI and age with older individuals having higher ALI (Edes et al., 2016a). They also found that female gorillas had higher ALI than males and that overall ALI was associated with the number of stress events an animal experienced over a lifetime (Edes et al., 2016a).

The objective of this study was to determine the effect of age, sex, and stressors on ALI in ring-tailed lemurs. Age has been shown to be associated with increases in allostatic load index since as the body ages there is increased 'wear and tear' on the physiologic systems (Crews, 2007; Edes & Crews, 2017; Seeman et al., 1997; Seeman et al., 2001). Sex differences have also been shown, most notably in work done on gorillas (Edes et al., 2016a, Edes et al., 2016b). In humans, long-term chronic stressors like socio-economic status have been associated with increased allostatic load and greater mortality risk (Dowd et al., 2009; Seeman et al., 2004).

Stress research in evaluating cortisol in primates and other species has shown that social status and group dynamics play a large role in mediating stress (Bartolomucci, 2007; Cavigelli & Caruso, 2015; Sapolsky, 2005). For that reason, social factors

including group composition changes and average group size were included as stressors as well.

3.2. Materials and Methods

3.2.1. Animals, Housing, and Inclusion Criteria

A total of 198 Ring-tailed lemurs have been housed at the Duke Lemur Center since its opening. From this group, individuals were selected for inclusion in the study based on the following criteria: 1) the animal is/was sexually mature (> 2 years) at the time of at least one banked blood sample collection for serum; 2) medical records with details of health events existed for the individual; 3) Duke Lemur Center is/was the final institution where the animal was held; and 4) if an individual did not have adequate banked serum, but remained part of the collection and a blood sample could still be collected. Thirty-eight lemurs met all 4 of these criteria (16 male and 22 female) and were included in the sample.

At the Duke Lemur Center, lemurs are housed in a variety of settings (Table 2) ranging from completely indoor enclosures to the semi-free ranging areas where they are solely housed outdoors. Some of the indoor enclosures contained small areas where the animals could go outdoors, but remained confined. The larger semi-free ranging enclosures are on 1.5-14.3 ha tracts of land with indoor access. During periods of extreme

weather (cold, snow, hurricanes) the lemurs were kept in heated indoor areas but otherwise were kept entirely outdoors.

Individuals were fed daily rations of primate diet (Purina®MonkeyDiet5038, PMI Nutrition International, Inc., Brentwood, MO) which was supplemented with fresh fruits and vegetables. When lemurs had access to the outdoor semi-free ranging enclosures they were able to forage from the forest. Clean water always was readily available.

3.2.2. Serum Sample Collection

Banked serum was utilized for 27 individuals. If banked serum was not available (n = 11) fresh blood was collected. Blood sampling procedures were overseen by Duke University's Institutional Animal Care and Use Committee (#A027-15-01, "Staff support for research at the DLC"; PI: Ehmke). All samples were kept frozen at -18C until analysis. Samples were aliquotted into several smaller vials to minimize the need to freeze and thaw a sample multiple times.

3.2.3. Biomarkers to Estimate Allostatic Load

Biomarkers used to estimate allostatic load in ring-tailed lemurs were selected based on the following criteria: 1) successfully used in work focusing on gorillas (Edes et al., 2016a; Edes et al., 2016b); 2) used in human models of allostatic load (Edes &

Crews, 2017; Juster et al., 2010; Seeman et al., 2001); 3) measurable in serum and 4) validated using commercially available assays (Chapter 2). Table 3 shows the biomarkers used to calculate allostatic load in the lemurs. Albumin and glucose are routinely run for health evaluations and these values were obtained from medical records. All other biomarkers were measured using competitive binding enzyme linked immunoassays (ELISAs) run in-house as outlined in Chapter 2.

Several biomarkers were considered including: albumin, cortisol, C-reactive protein (CRP), dehydroepiandrosterone-sulfate (DHEA-S), DNA damage, glucose, insulin, IL-6, PGE2 and TNF-α. The biomarkers ultimately utilized in this allostatic load index were selected based on successful validation of a commercially available assay (Chapter 2) and the ability to represent neuroendocrine, immune, cardiac and metabolic functioning.

Albumin is a negative acute phase protein and a marker of the immune system that decreases in the face of acute inflammation. It is a protein produced by the liver and in the face of trauma, inflammation, neoplasia, or other insult its transcription is down-regulated (CUCVM, 2013). Albumin has also been shown to be an indicator of cardiovascular health in humans and is associated with increased risk of heart failure and cardiovascular disease (Gopal et al., 2010; Shah & Dumler, 2008).

Cortisol is a glucocorticoid produced by the adrenal gland in response to the

stimulation by the HPA axis. It plays an essential role in the maintenance of most homeostatic functions, and is commonly used as a biomarker for stress in lemurs (Cavigelli, 1999; Pride, 2005a; Pride, 2005b). Dysregulation of the HPA axis can result in either inadequate production of cortisol or prolonged elevation, both of which can potentially have deleterious effects and increase allostatic load.

Dehydroepiandrosterone-sulfate (DHEA-S) is a hormone produced by the adrenal gland that impacts the production of androgens and estrogens. DHEA-S has been shown to have significant impacts on health as it stimulates the immune system and has anti-glucocorticoid effects (Hazeldine et al., 2010).

The DNA damage test identifies oxidized guanine species as a measure of cumulative oxidative stress in the body. Oxidative stress is defined as "a disturbance in the balance between the production of reactive oxygen species (free radicals) and antioxidant defenses" (Betteridge, 2000). Oxidative stress can cause damage to DNA leading to modifications that can have pathological consequences such as cancer or Alzheimer's in humans (Nunomura et al., 2006; Valko et al., 2006).

Glucose is derived from dietary carbohydrates and is the primary energy source for mammalian cells. The regulation of glucose is mediated by multiple hormones including insulin, catecholamines and glucocorticoids (CUCVM, 2013). In the face of stressors glucose can be elevated transiently or for prolonged periods which can have

adverse health effects.

Prostaglandin E_2 (PGE_2) is a principal mediator of inflammation that is synthesized as part of the arachidonic acid cascade (Park et al., 2006). Production is stimulated by specific trauma or through signaling molecules. Under normal circumstances PGE2 has a homeostatic effect in helping the body mediate inflammation (Park et al., 2006).

3.2.4. Allostatic Load Index (ALI) Estimation

An allostatic load index was calculated for each lemur using the methodology described by Seeman et al. (2001). Raw values for each biomarker (Table 3) were divided into quartiles. Among the biomarkers chosen, albumin and DHEA-S have a greater risk of impairing physiological regulatory mechanisms when they fall in the lowest quartile, while all other biomarkers show increased risk in the highest quartile (Edes et al., 2016a; Edes et al., 2016b). Since cortisol can be either elevated or depressed when dysregulated, a two-tailed cut-off was applied with the highest and lowest 12.5% of values being classified as high risk. Although there are clinical cut-points for some of these biomarkers, these cut-points were not used in order to include individuals that may appear clinically healthy but have increased allostatic load and potentially increased risk for adverse health outcomes in the future. Each biomarker within the high-risk quartile

for an individual lemur has was be scored 1; biomarkers not within the high-risk quartile were be scored zero. Scores were summed for each lemur as a composite allostatic load score (min = 0, max = 6).

3.2.5. *Age, Sex and Stressors*

The sex and the age of the individual lemurs at the time of sample collection were determined from the medical records.

Stressors were defined as events which were likely to cause disruption of allostasis due to their chronic or severe nature. Information about stressors were collected from individual medical and husbandry records. Stressors (Table 4) included: 1) number of anesthetic events 2) number of times an animal was manually restrained, 3) number of times an animal was transferred between institutions, 4) number of times an animal experienced an enclosure change, 5) number of times an individual sustained trauma, 6) number of illnesses an individual had, 7) number of pregnancies for female lemurs, 8) number of group composition changes (minor, major and total) 9) percentage of time an animal spent outdoors in semi-free ranging enclosures, 10) percentage of time an animal spent in indoor enclosures, 11) number of times each lemur participated in research projects at the Duke Lemur Center and 12) average group size. Research projects were

further categorized as either minor or major. Major research events were defined as those requiring direct manipulation of the lemur (i.e. handling or moving to another location); minor events involved changes to the environment with no direct handling. Some research projects included anesthesia; these cases were coded as an anesthetic event and not a research project. Some research projects included blood samplings that were opportunistically collected for medical reasons; these cases were coded as a manual restraint event and not a research project. Research projects were documented by both number of events and time in minutes.

The number of group composition changes and the average group size were recorded as stressors. Group structure changes were further classified as major or minor: major group structure changes included first-time separation from generational sibling or dam, the addition of a group of another species into the enclosure, the introduction of two individuals for the first time for breeding purposes, isolation of the focal animal from conspecifics, and birth in the group. All other group structure changes, including removal or addition of unrelated females or males, were considered minor. In addition to being evaluated separately, a composite total number of group changes was also calculated by adding the number of major and minor group changes.

3.2.6. Sample Size Calculations and Statistical Analysis

Given the matriarchal dominance hierarchy and previous literature showing higher cortisol levels in dominant individuals, females were expected to have average higher ALI compared to males (Cavigelli, 1999; Cavigelli et al., 2003). Considering this large effect size (Cohen's d = 0.9), we anticipated needing at least 32 lemurs (approximately 16 males and 16 females) to achieve sufficient power (80% with an α-level of 0.05). Thus, our sample size of 38 was considered more than sufficient to reach our objective. To determine the impact of age, sex, and stressors on allostatic load, an ALI (ordinal; 1 to 6) for each lemur (n = 38) was calculated.

All statistical analyses were completed using SPSS Statistics Base (IBM Corporation, Armonk, NY, United States). Data were screened for extreme outliers before analysis and none were found. Descriptive statistics were calculated to determine the variation and distribution of biomarkers used in allostatic load indexes in this sample of animals. To ensure that males and females had the same general distribution for each biomarker, two-sample t-tests were conducted. No significant differences between males and females for any biomarker were observed, thus the same quartile cut points were used for males and females. Although ALI is an ordinal variable it commonly is normally distributed in human samples, therefore linear models were sufficiently robust to allow slight deviations from normality so linear regressions were applied also.

Differences in ALI between male and female lemurs were compared using a two-

sample t-test. Linear regression was used to determine the relationships between ALI, age, and stressors (continuous predictor variables; Table 3).

Independent stressors include: anesthetic events, manual restraint, institutional transfers, enclosure changes, trauma, illness, pregnancy, number of group composition changes, percentage of time spent in semi-free ranging enclosures, number of research events/time participating in research, and average group size. If an association between ALI and a stressor had a tendency or was marginally significant males and females were compared separately to determine if there was a stronger association within one sex.

In addition to measuring relationships between each stressor and ALI, a total 'stressor score' was calculated by summing the total number of anesthetic events, number of manual restraints, number of institutional transfers, number of enclosure changes, number of traumas and number of illnesses. Calculation of this 'stress score' was based on previous work in gorillas (Edes et al., 2016a). Group composition changes and research participation were not added to this stress score because they were not evaluated as part of the score in previous literature. The relationship between ALI and total stress score was determined using linear regression.

To determine if a high ALI was associated significantly with age at death, linear regression was used. In these models, ALI was considered the predictor variable, and age at death (for n=11 individuals) was considered the outcome variables.

To evaluate the relationship of each individual biomarker to each stressor, linear regression was performed. Given the exploratory nature of this work, a principal components analysis (PCA) was performed to evaluate whether some biomarkers had more influence on the associations with stressors than others. It was hypothesized that allostatic load index is strongly affected by principal components (PC) with eigenvalues ≥1. Linear regressions were conducted with each such PC, sex, age, and stressors. Differences at $P \leq 0.05$ were considered significant and a trend at $0.05 > P \leq 0.10$.

3.3. Results

3.3.1. Descriptive Statistics for Allostatic load index (ALI)

In this sample of animals, allostatic load indexes ranged from 0 to 4 out of a possible score of 6. The mean ALI was 1.7 with a standard deviation of 1.0 (Figure 7).

3.3.2. Effect sex, age and stressors on ALI

There was no effect of sex or age on ALI (sex: P=0.94, t=0.07, DF=36; age: P=0.14, R^2=0.04).

There was no effect of total 'stress score' on ALI (P=0.72, R^2= -0.02). Most stressors examined were not associated significantly with ALI, including number of

anesthetic events (P=0.25, R^2=0.01), manual restraint events (P=0.64, R^2= -0.02), number of institutional transfers (P=0.27, R^2=0.01), number of enclosure changes (P=0.39, R^2= -0.01), number of trauma events (P=0.21, R^2=0.017), the number of illnesses (P=0.48, R^2= -0.01), and number of pregnancies (P=0.54, R^2=0.01). In addition, there was no relationship between ALI and either frequency or time spent involved as part of a minor research project (frequency: P=0.44, R^2=-0.11; minutes in research project: P=0.28, R^2=0.01), or a major research project (frequency: P=0.97, R^2=-0.03; minutes in research project: P=0.36, R^2= -0.00).

A positive association was found between ALI and percentage of time spent indoors (P=0.05, R^2= 0.08). Similarly, there was a tendency for a negative association between ALI and percentage of time spent outdoors in semi-free ranging enclosures (P=0.07, R^2=0.066) (Figure 8).

There was a tendency for the number of group composition changes to affect ALI (P=0.09, R^2=0.05). This tendency for an association was driven by the number of changes classified as minor (P=0.07, R^2=0.06), not the number of major group changes (P=0.23, R^2=0.01). When minor and major group changes were summed and data were analyzed separately by sex, there was a negative association between ALI and total number of group changes in females (P=0.012, R^2= 0.25) (Figure 9), but not males (P=0.59, R^2=-0.05) **(**Figure 10**).**

There was a negative association between average group size and ALI (P=0.02, R^2=0.13); the smaller the group size, the higher the ALI (Figure 11).

Associations and tendencies were seen when each individual biomarker was run as a predictor for each stressor (Table 5). There were significant associations between anesthetic events (P= 0.01, R^2= 0.14) and manual restraint events and glucose (P= 0.00, R^2= 0.22); manual restraint and cortisol (P= 0.04, R^2= 0.08) and DNA damage (P= 0.04, R^2= 0.08); enclosure changes and DNA damage (P= 0.01, R^2= 0.14); illness and cortisol (P=0.02, R^2= 0.13), glucose (P= 0.00, R^2= 0.22), and PGE-2 (P= 0.03, R^2= 0.1); group changes and DNA damage (P= 0.04, R^2= 0.09); % time indoors and DHEA-S (P= 0.04, R^2= 0.10), glucose and research event frequency (P= 0.01, R^2= 0.16), albumin and average group size (P= 0.01, R^2= 0.16), and PGE-2 and average group size (P= 0.02, R^2= 0.14).

There was a tendency for associations between research event frequency and cortisol (P= 0.06, R^2= 0.08), research time in minutes and glucose (P= 0.08, R^2= 0.06), and average group size and DHEA-S (P= 0.06, R^2= 0.07).

When the six biomarkers were analyzed using PCA, the top three components had eigenvalues greater than or equal to 1. Cumulatively, these three components explained 78.9% of the total variation in the variance-covariance matrix. The first PC (33.9%) loaded on the biomarkers glucose, PGE-2, and cortisol with a minor influence of

albumin. The second PC (28.8%) loaded on albumin and DHEA-S with minor influence from glucose and cortisol. The third PC (16.29%) loaded on DNA damage. All six biomarkers that were used in the allostatic load matrix were included in the top three PCs.

PC1 was associated significantly with the number of manual restraints (P= 0.01, R^2=0.17), average group size (P=0.03, R^2 =0.12), number of illnesses (P=<0.01, R^2=0.2) and had a tendency to associate significantly with age (P=0.06, R^2 =0.05) and number of research events (P=0.09, R^2 =0.06).

PC2 did not significantly associate significantly with any of the stressors evaluated. PC3 had a tendency with % of time spent outdoors in semi-free ranging enclosure (P=0.07, R^2 =0.07), enclosure changes (P=0.06, R^2 =0.09), total group composition changes (P=0.07, R^2 =0.07) and average group size (P=0.09, R^2 =0.06).

3.4. Discussion

The objective of this study was to determine if allostatic load score was associated with age, sex or stressors. The results provide several insights into the relationship between ALI and stressors in ring-tailed lemurs. Based on these results there are significant associations between allostatic load index and social stressors in this species.

Choosing appropriate biomarkers reflecting neuroendocrine, metabolic, inflammatory and cardiovascular dysregulation due to stressors on animals is vital to

developing an accurate allostatic load index. In human research, biomarkers used to calculate AL are variable. To extrapolate to other species, it is necessary to first evaluate whether each biomarker is appropriate. As exploratory research, biomarkers were chosen based on our ability to assess them from a single serum sample and availability of commercial assays. In principal components analysis all 6 biomarkers explained significant variation in stressors, indicating all contributed to interactions among stressors and ALI. Additionally, although several biomarkers were associated with specific stressors, no one biomarker replicated associations observed between stressors with ALI.

We observed no significant association of sex and age with ALI in lemurs. In contrast, research on gorillas reported females and older gorillas had higher allostatic load compared to males and younger animals (Edes et al., 2016a; Edes et al., 2016b). This also contrasts with humans among whom allostatic load generally increases with age and varies by sex (Crimmins et al., 2003, Crews, 2007; Crews et al., 2012; Seeman et al., 2002).

Lack of a significant association between ALI and sex could be due to both methodological and species differences. Gorillas have a male dominant social structure with one silverback and multiple females. While males are always dominant there is also a hierarchy among females (Lang, 2005a). A sex difference in captive gorillas likely relates to there being only one adult male in any group. Zoo gorillas do not need to

defend their territory as they do in the wild. Females are unable to avoid aggression from either the male or other females as in the wild, thereby increasing exposure to male aggression and chronic stress (Edes et al., 2016a).

Captive lemur social structure is markedly different, with animals housed in multi-male, multi-female groups. While a female dominance hierarchy exists, it is disrupted by intense mate competition during the breeding season (Starling et al., 2010). Hierarchical fluidity and large social groups may lead to males and females experiencing chronic stress equally.

The lack of significant association between allostatic load and age is more difficult to explain since there is often a significant association in humans (Crews, 2007; Crews et al., 2012; Edes & Crews, 2017; Seeman et al., 2002). It may indicate gaps in the allostatic load index. Perhaps additional biomarkers need to be validated to construct a more robust estimate of allostatic load in lemurs.

Most individual stressors were not associated significantly with allostatic load. This contrasts to research with captive gorillas where total stress score was associated significantly with allostatic load (Edes et al., 2016a). This may indicate that ring-tailed lemurs are more flexible or resilient to chronic stressors examined here.

Average group size negatively associated with allostatic load. Animals that were maintained in smaller groups had higher allostatic load. Typically wild ring-tailed lemurs

live in groups of 10-20. Benefits may accrue to those in larger cohorts and inversely there may be negative effects of being housed in small groups (Pride et al., 2005b) This finding may also be related to the way in which small groups are housed at the DLC. While the large groups reside in the semi-free ranging area the smaller groups are housed in smaller enclosures. When social conflict arises in smaller spaces the ability to retreat is limited and agonistic interactions may be more frequent. Additionally, there is increased activity and human presence in the buildings than in the semi-free ranging. A limitation to the evaluation of this parameter was that it was not possible to evaluate the amount of time that individuals spent in varying group sizes, so it may be duration of time in any particular group size instead of the average that is exerting the effect.

Time spent indoors was positively associated with allostatic load, and time spent outdoors in semi-free ranging enclosures tended to be negatively associated with allostatic load. There are several potential explanations for these associations. The first is that the outdoor semi-free ranging enclosures are more similar to the lemurs natural forested habitat. Work has shown that naturalistic habitats in captive settings are more suitable than non-naturalistic enclosures (Fabregas et al., 2012). The semi-free ranging enclosure also allows the lemurs to participate in more natural feeding behaviors, like foraging. It has been illustrated in other species that promoting natural feeding behaviors improves health both physically and psychologically (Bond & Lindberg, 1990).

Another potential reason for the lower allostatic load in animals housed outdoors more often may have to do with an increase in control over their environment. In other species, a perceived lack of control and unpredictable husbandry routines are often associated with increased levels of stress hormones and behavioral changes (Carlstead et al., 1993; Koolhaas et al., 1999). In the semi-free ranging enclosure areas, the lemurs could choose where to spend their time and who to spend their time with. This provides the ability to form more natural relationships and exhibit normal behaviors.

Lastly the association between decreased allostatic load and time spent outdoors in semi-free ranging enclosures may be associated with exposure to natural sunlight. In humans and animal species vitamin D deficiency has been linked to a number of poor health outcomes (Nair et al., 2012; Zhang & Naughton, 2010). More work would be needed to more definitively illustrate an association between time outdoors and vitamin D levels in this species.

There was a negative association between the number of group composition changes to allostatic load in females, while no association was seen in males. We had anticipated that group structure changes would be a source of stress in lemurs, especially females, however, we found that the opposite may be true. One potential explanation for this result is that more frequent changes in the group structure meant that the dominance hierarchy was more often altered so no one individual was always dominant or always

subordinate. Perhaps this allowed for increased fluidity in the group dynamic and decreased the chronic stress on any one individual. Although dominance has been shown to be linked to stress in many primate species whether the dominant or the subordinate experiences the bulk of stress is species-dependent (Abbott et al., 2003; Goyman, 2004; Sapolsky, 2002). In ring-tailed lemurs, dominance is not linear and often changes, making it more complicated to sort out the potential impacts it may play on chronic stress.

The lack of an association between what were classified by major group changes, specifically separation from a dam or generational sibling, was also surprising given the close familial bonds maintained in wild social groups (Budnitz & Dainis, 1975; Cavigelli, 1999; Cavigelli & Caruso, 2015; Starling et al., 2010). This may indicate that there are more subtle interactions occurring and that any group composition change could potentially have an impact on allostatic load index and the events that were classified as minor were not. There is also the potential that minor group changes may have a protective effect and changing the group composition more frequently plays a role in the alleviation of chronic stress. The mechanism by which this occurs is unclear.

There was no relationship detected between allostatic load and research projects in this study. Research on non-human primates is essential to understand their social structure, cognitive capabilities and natural history which in turn can be used to aid in

their conservation in the wild. However, it is important when conducting this work to ensure that undue stress is not being placed upon the animals and that their welfare is paramount. Additional work incorporating other biomarkers would be needed to draw further conclusions but these results suggest that the study population housed at Duke Lemur Center is not showing increased allostatic load in association with research activities.

The findings of this project illustrate that many factors that were anticipated to be stressful did not show an association with allostatic load index. The major drivers of chronic stress were found to be linked to social groupings and environment which has a potential impact on husbandry and management decisions and how these are made.

There were limitations to this study. The retrospective nature of the project means that information had to be obtained from medical and husbandry records which are not always complete. The statistical analysis showed several tendencies which can sometimes be a reflection of small sample size. Based on our sample size calculations 38 individuals should have been large enough to reveal associations between ALI and the predictor variable; however a larger study population may have caused some of the tendencies that were seen to become significant associations as well.

Overall the allostatic load index used in ring-tailed lemurs, while still in need of refining, appears to show significant associations with chronic stressors. Further research

into the use of ALI in lemur species is warranted.

Table 2. Descriptions of locations that ring-tailed lemurs were housed at the Duke Lemur Center from 1983-2016.

Enclosure Name	Description	Dimensions in feet (Length x Width x Height)	Total Area (Ha or Square feet)
NHE-2	Semi-free ranging enclosure surrounded by chain link fencing		8.3 ha
NHE-3	Semi-free ranging enclosure surrounded by chain link fencing		14.3 ha
NHE-6	Semi-free ranging enclosure surrounded by chain link fencing		11 ha
Triplex	Indoor enclosure with access to attached outdoor yards	Indoor: 6 x 6 x 8 Outdoor: 14 x 5.17 x 7.8	Indoor: 36 sq. ft. Outdoor: 72.4 sq. ft.
Core	Indoor enclosure	15 x 17.75 x 16.42	266.3 sq. ft.
OR	Indoor enclosure	23.5 x 12.6 x 11.6	296.1 sq. ft.
NHE Barn	Indoor enclosure, winter housing	12 x 10 x 10	120 sq. ft.

Table 3. Biomarkers used to estimate of allostatic load

Biomarker	Function	Response to chronic stress
Albumin	Plasma antioxidant	Decreases
Cortisol	Suppresses inflammation, and induces gluconeogenesis	Increases or Decreases
DHEA-S	Functional HPA- axis antagonist, suppresses inflammatory cytokines	Decreases
DNA Damage	Measure of oxidative stress and indicative of potential free radical damage.	Increases
Glucose	Main source of metabolic energy	Increases
PGE-2	Part of the inflammatory cascade, associated with neoplasia and Alzheimer's in humans	Increases

Table 4. Stressors and their descriptions extracted from medical and husbandry records for 38 ring-tailed lemurs (*Lemur catta*) housed at the Duke Lemur Center, 1983-2016.

Stressor	Description	Type of Variable
Anesthetic Events	The number of times an individual was placed under sedation or general anesthesia. Used for longer, more involved, or painful procedures such as surgery.	Continuous
Manual Restraint	The number of times an individually was physically restrained without the use of drugs. Used for short procedures such as blood collection or vaccinations.	Continuous
Institutional Transfers	The number of times an individual was moved from one zoological institution to another.	Continuous
Enclosure Changes	The number of times an individual was moved from one enclosure to another at the Duke Lemur Center	Continuous
Trauma	The number of events where an individual was wounded from agonistic encounters or other events (i.e. falling)	Continuous
Illness	The number of times an individual had an illness that was medically addressed	Continuous
Pregnancy	The number of times an individual was detected to be pregnant.	Continuous
Group Composition	The number of times where there were changes in the group (lemurs removed or added)	Continuous
% Time Indoors/ Outdoors	The percentage of total time that individuals spent in outdoor semi-free ranging enclosures and inversely in indoor enclosures.	Continuous
Research Time	Number and amount of time (in minutes) that individuals were used in research projects, both major and minor.	Continuous
Average Group Size	The average number of lemurs the focal animal was housed with.	Continuous

Table 5. Association of sex, age and stressors with 6 biomarkers comprising an allostatic load index for 38 ring-tailed lemurs housed at the Duke Lemur Center. Significant and trending P values bolded, based on linear regression.

Biomarker	Sex	Age			Anesthetic events		
	P	*P*	R^2	β	*P*	R^2	β
Albumin	0.64	0.29	0.00	-0.18	0.71	-0.02	-0.06
Cortisol	0.39	0.13	0.04	0.25	0.13	0.04	0.25
DHEA-S	0.55	0.47	-0.01	-0.13	0.46	-0.01	-0.13
DNA Damage	0.25	**0.05**	**0.08**	**-0.32**	0.81	-0.03	-0.04
Glucose	0.38	**0.07**	**0.06**	**0.30**	**0.01**	**0.14**	**0.40**
PGE-2	0.11	0.13	0.04	-0.26	0.61	-0.02	-0.09

Biomarker	Manual Restraint			Institutional Transfers		
	P	R^2	β	*P*	R^2	β
Albumin	0.14	0.03	-0.25	0.77	-0.03	0.05
Cortisol	**0.04**	**0.08**	**0.33**	0.41	-0.01	-0.14
DHEA-S	0.92	-0.03	0.02	0.60	-0.02	-0.09
DNA Damage	**0.04**	**0.08**	**-0.33**	0.64	-0.02	0.08
Glucose	**0.00**	**0.22**	**0.49**	0.39	-0.01	-0.14
PGE-2	0.27	0.01	-0.019	0.72	-0.03	-0.06

Continued

Table 5 continued

Biomarker	Enclosure Changes			Trauma		
	P	R^2	β	*P*	R^2	β
Albumin	0.69	-0.02	0.07	0.97	-0.03	0.01
Cortisol	0.54	-0.02	-0.10	0.25	0.01	0.19
DHEA-S	0.30	0.00	-0.19	0.76	-0.03	-0.06
DNA Damage	**0.01**	**0.14**	**0.41**	0.53	-0.02	-0.11
Glucose	0.81	-0.03	0.04	0.47	-0.02	0.11
PGE-2	0.51	-0.02	0.11	0.81	-0.03	0.04

Biomarker	Illness			Pregnancy		
	P	R^2	β	*P*	R^2	β
Albumin	0.42	-0.01	-0.14	0.33	-0.00	-0.22
Cortisol	**0.02**	**0.13**	**0.39**	0.20	0.03	0.28
DHEA-S	0.60	-0.02	-0.10	0.85	-0.06	-0.05
DNA Damage	0.90	-0.03	0.02	0.80	-0.05	-0.06
Glucose	**0.00**	**0.22**	**0.50**	0.11	0.08	0.36
PGE-2	**0.03**	**0.10**	**-0.35**	0.14	0.06	-0.33

Continued

Table 5 continued

Biomarker	Group Composition Changes			%Time Indoors		
	P	R^2	β	*P*	R^2	β
Albumin	0.66	-0.02	-0.07	0.19	0.02	0.22
Cortisol	0.12	0.04	0.26	0.72	-0.02	-0.06
DHEA-S	0.61	-0.02	-0.09	**0.04**	**0.11**	**-0.36**
DNA Damage	**0.04**	**0.09**	**-0.34**	0.21	0.02	0.21
Glucose	0.19	0.02	0.22	0.92	-0.03	-0.02
PGE-2	0.50	-0.02	-0.14	0.35	-0.00	0.16

Biomarker	Research Time (Minutes)			Research Events (#)		
	P	R^2	β	*P*	R^2	β
Albumin	0.53	-0.02	-0.11	0.72	-0.03	-0.06
Cortisol	0.50	-0.01	0.13	0.06	0.08	0.32
DHEA-S	0.40	-0.01	-0.15	0.43	-0.01	-0.15
DNA Damage	0.50	-0.02	-0.12	0.49	-0.02	0.12
Glucose	0.08	0.06	0.30	0.01	0.16	0.43
PGE-2	0.36	-0.00	-0.16	0.34	-0.00	-0.17

Continued

Table 5 continued

Biomarker	Average Group Size		
	P	R^2	β
Albumin	**0.01**	**0.16**	**-0.43**
Cortisol	0.2	0.02	0.21
DHEA-S	**0.06**	**0.07**	**-0.31**
DNA Damage	0.19	0.02	-0.22
Glucose	0.11	0.05	0.27
PGE-2	**0.02**	**0.14**	**0.41**

Note: *P*=0.00 indicates that *P*<0.005

Table 6. Principal components of allostatic load (AL PC) and relationships with stressors based on linear regression, for 38 ring-tailed lemurs at the Duke Lemur Center. Significant and trending P values in bold.

	AL PC1			AL PC 2			AL PC3		
	P	R^2	ß	*P*	R^2	ß	*P*	R^2	ß
Age	**0.06**	**0.09**	**0.34**	0.47	-0.02	0.13	0.12	0.05	-0.28
Age at Death	0.74	-0.11	0.12	0.48	-0.05	0.26	0.43	-0.04	-0.28
# Anesthetic Events	0.13	0.04	0.27	0.4	-0.01	0.15	0.72	-0.03	0.07
# Manual Restraint Events	**0.01**	**0.17**	**0.44**	0.64	-0.02	0.09	0.14	0.04	-0.26
# Institutional Transfers	0.42	-0.01	-0.15	0.83	-0.03	-0.04	0.87	-0.03	0.03
# Enclosure Changes	0.26	0.01	-0.2	0.95	-0.03	0.01	**0.06**	**0.09**	**0.34**
Trauma	0.96	-0.03	0.01	0.57	-0.02	0.1	0.98	-0.03	-0.01
Illness	**0.01**	**0.2**	**0.48**	0.55	-0.02	0.11	0.28	0.01	0.2
Pregnancy	0.2	0.04	0.32	0.73	-0.05	-0.06	0.8	-0.06	-0.06
# Group Composition Changes	0.29	0.01	0.19	0.37	-0.01	0.16	**0.07**	**0.08**	**-0.32**
% Time Outdoors	0.38	-0.01	0.16	0.2	0.02	-0.23	**0.07**	**0.07**	**-0.32**
Research Time (Minutes)	0.14	0.04	0.27	0.43	-0.01	0.15	0.69	-0.03	-0.07
# of Research Events	0.09	0.06	0.31	0.36	-0.0	0.17	0.19	0.03	0.24
Average Group Size	**0.03**	**0.12**	**0.39**	0.12	0.05	-0.28	**0.09**	**0.06**	**-0.3**
Total Stress Score	0.15	0.04	0.26	0.59	-0.02	0.1	0.98	-0.03	0.01

Figure 8. Histogram showing distribution of allostatic load indexes, mean and standard deviation in sample of 38 ring-tailed lemurs (*Lemur catta*).

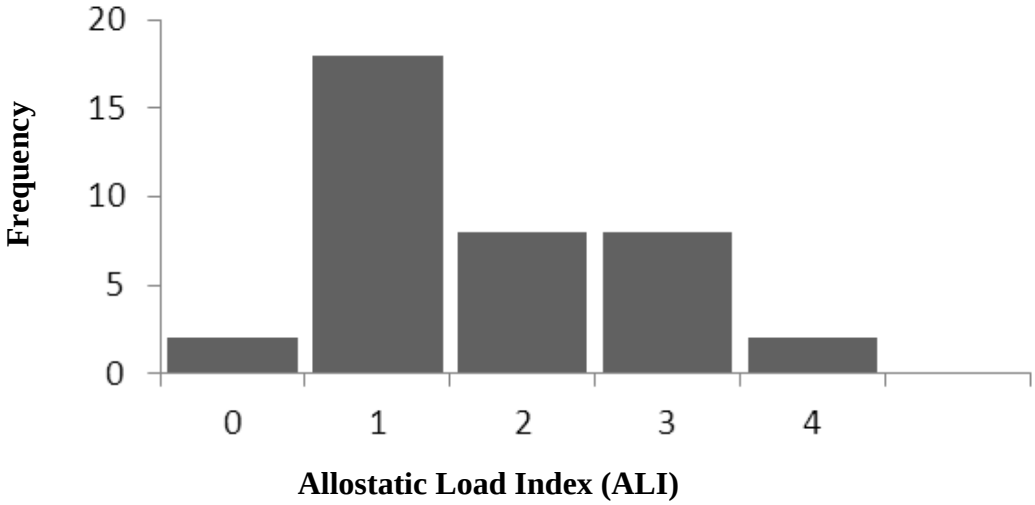


Figure 9. Scatter plots illustrating the tendency between allostatic load index the percentage of time an individual spent housed outdoors in semi-free ranging enclosures. P=0.07, R^2=0.07

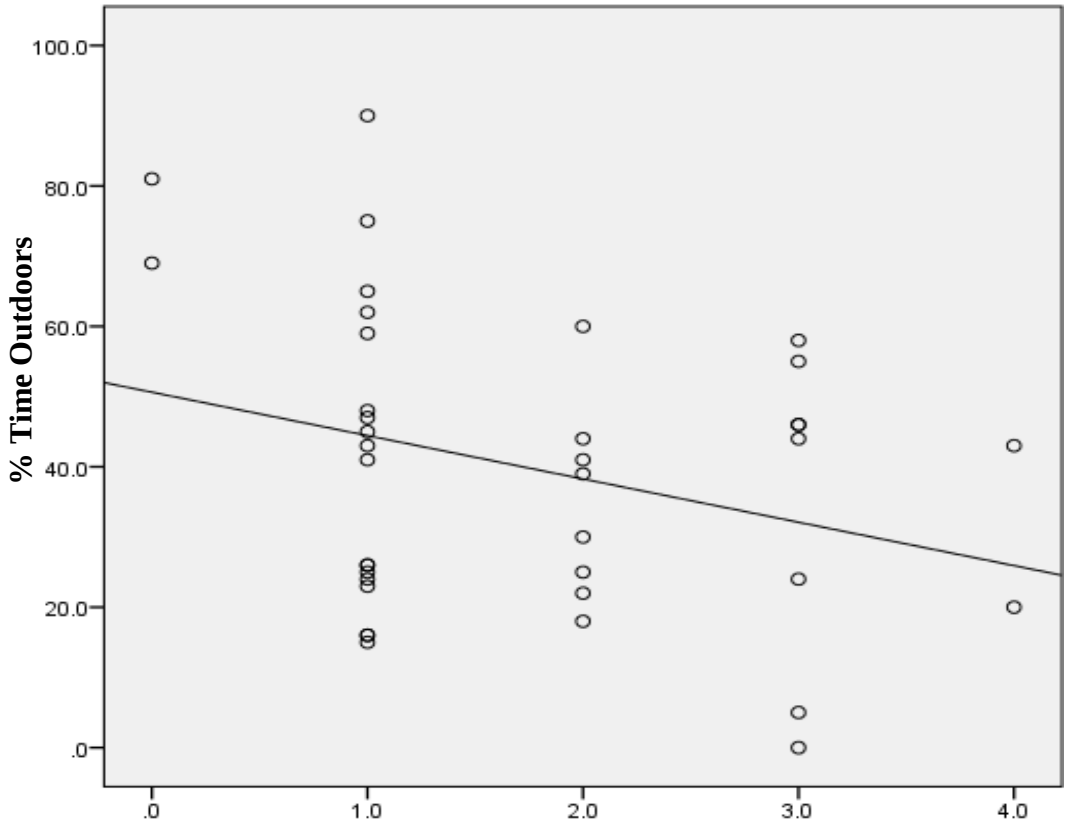


Figure 10. . Scatter plot of allostatic load by total number of group dynamic changes experienced, females (n = 22), P=0.01, R^2=0.25

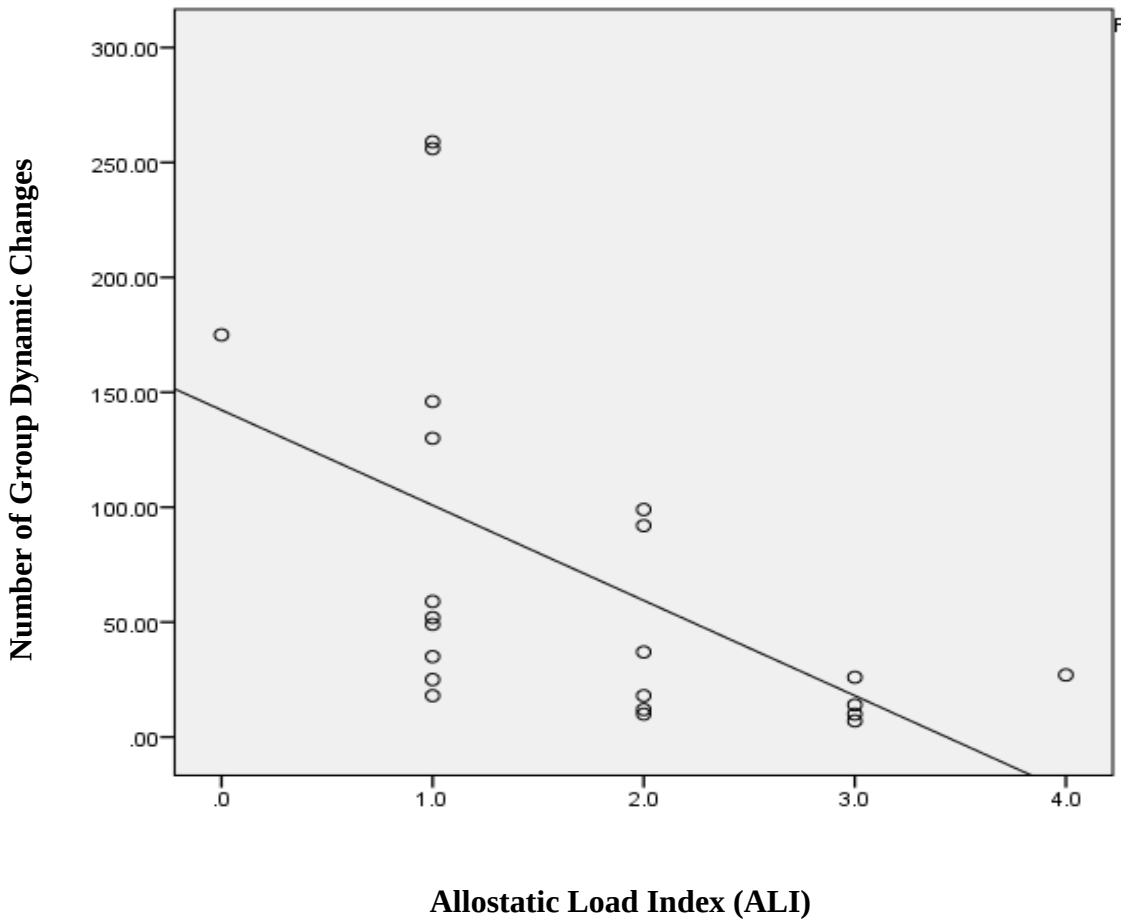


Figure 11. Scatter plots of allostatic load by total number of social group changes experienced, males (N=16), P=0.59, R^2= 0.05

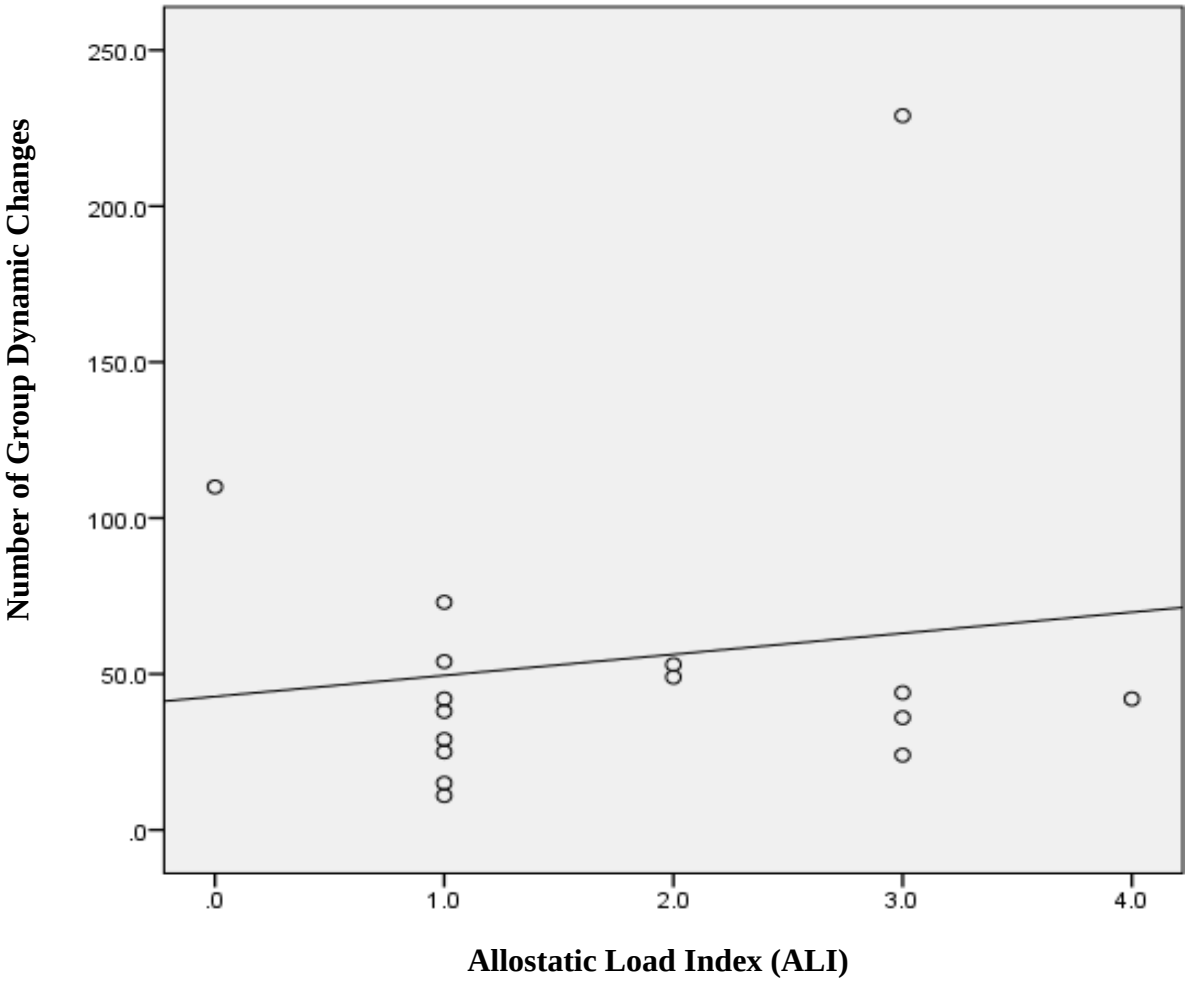
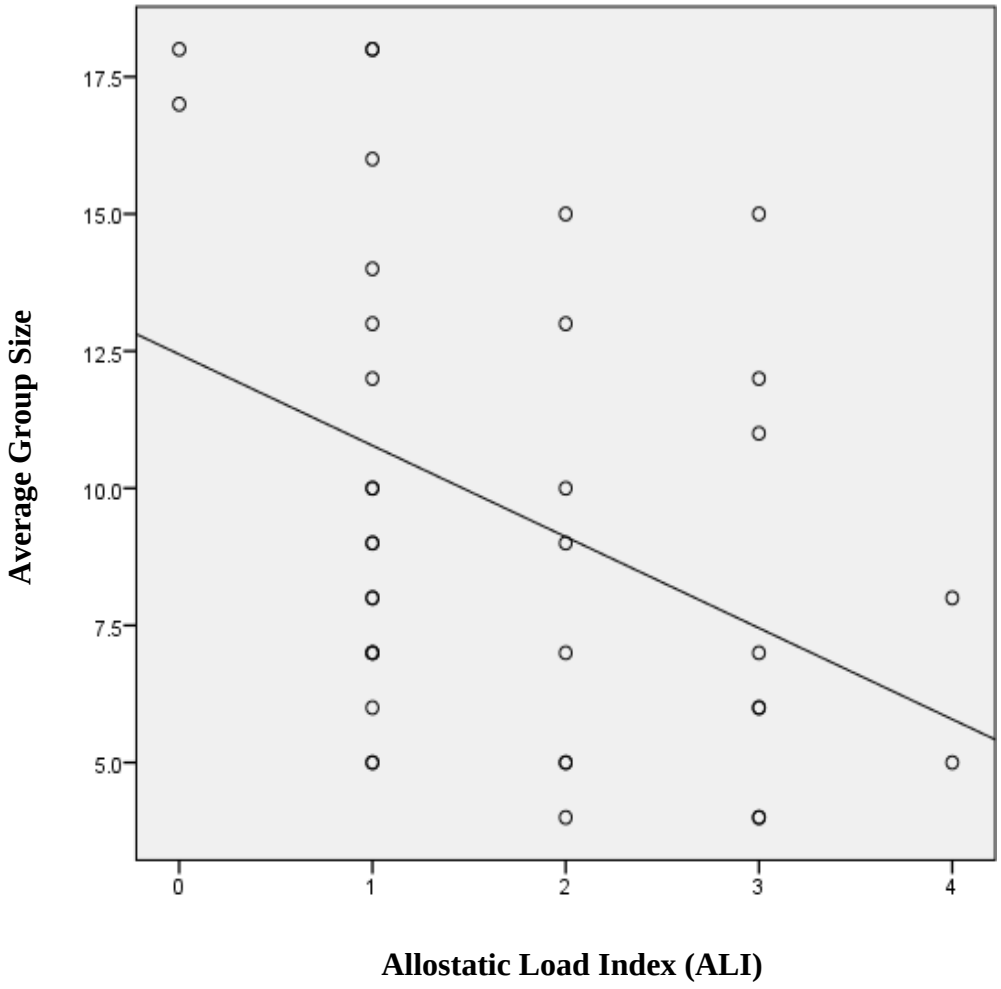


Figure 12. Scatter plot of allostatic load and average group size, P=0.02, R^2=0.13



Chapter 4: Discussions and Conclusions

This project has only begun to explore the various ways that allostatic load, once validated, may be used to monitor chronic stress in animal populations. The results from this preliminary work show that social factors appear to have the most significant effect on allostatic load in ring-tailed lemurs. This was not reflected in previous work in gorillas since gorillas have much less fluid social dynamics and are often housed in static groups in captive situations (Cawthon, 2005b). This is a stark contrast to lemurs who have large, dynamic groups that are often changing, particularly in a facility like the Duke Lemur Center where there are multiple animals that can be moved from one group to another. Given these findings and the complexity of the social dynamics, further work investigating what types of group changes affect allostatic load and the role that dominance hierarchy plays would likely yield additional insight.

In human populations, stressors often involve some degree of perception and different individuals perceive stress differently. Future work will be done with this data attempting to evaluate whether personality impacts allostatic load. This will be done by surveying the husbandry staff using previously validated matrices of personality.

Although it is impossible to quantify personality in the same way as is done in humans, there is growing research indicating that personality likely plays a large role in stress in animals (Anestis et al., 2006; Chadwick, 2014; Gartner, 2013; Tetley & O'Hara, 2012).

Investigations are also ongoing to continue to refine the allostatic load index to incorporate the biomarkers that are most appropriate and predictive for this species. This type of research will continue as long as new physiologic biomarkers of cardiovascular, immune, neuroendocrine and immune status are identified. While ALI was associated with the stressors that were evaluated there was no association seen between ALI and age or sex, as would be expected from the literatures (Crews et al., 2012; Edes et al., 2016a; Edes et al., 2016b; Seeman et al., 2002). Given that theses associations, particularly between ALI and age, have been illustrated in other research it may be that the biomarkers used to measure ALI in this case did not completely encompass all the physiologic systems and more biomarkers need to be investigated. Work is being done to evaluate A1C (a glucose regulation biomarker) and ST2 (a cardiac inflammatory marker) to assess whether these biomarkers could potentially be added to the allostatic load index in ring-tailed lemurs.

Additionally, no associations were seen between ALI and age at death. Some potential reasons these associations weren't seen could be that the proportion of individuals in the population who were deceased was small and many animals in the

population were relatively young.

Since the utilization of allostatic load in non-human primates is so new, a great deal of work also needs to be done to determine the best methodology to use. In humans, there are several ways to calculate the allostatic load index (i.e. quartiles vs. deciles vs. clinical cut points). Colleagues are in the process of investigating these different methods in the calculation of allostatic load in gorillas and, based on the findings of that work, similar comparisons should be made in ring-tailed lemurs.

The retrospective nature of this project creates inherent limitations in the data that is available and the conclusions that can be made. All information regarding stressors was taken from medical records. In some cases, these records are incomplete with several years unaccounted for. In other cases, that information of interest was only intermittently included.

The goal was to try to quantify the stressors encountered in the lives of the focus population; however, this requires leaps of logic and subjective interpretations of the information provided. This is supported by the fact that there was no association seen between what were assumed to be stressors contributing to chronic stress and allostatic load. What we think to be stressors may be when experienced acutely but may not contribute to long-term physiologic dysregulation.

A long-term goal is to utilize allostatic load in wild populations as a potential tool

to evaluate the success of conservation measures. The focal species of this work would be the highly endangered Diademed sifaka (*Propithecus diadema*) with a focus on a population directly impacted by the largest open pit nickel mine in the world. The hope is to utilize existing banked serum samples that were collected for ongoing surveillance work over the last five years to evaluate three distinct subpopulations of sifaka. The first would be individuals whose primary habitat was destroyed when the land was clear cut for mining operations and consequently had to relocate to adjacent habitat. The second group would consist of individuals that live on the margin of mining activity and as such are exposed to sequelae such as air, noise and light pollution. The final group would be lemurs residing in a protected area of forest with minimal human disturbance.

The aim of applying allostatic load to these groups is to compare them to each other to evaluate the effects that large anthropogenic disturbances have on animal populations. Additionally, the hope is look at how allostatic load changes over time by comparing multiple time points over a 5-year period to determine if allostatic load can be decreased by moving these individuals away from the mine site into suitable habitat.

The potential that allostatic load index has to provide additional measures of health and evaluation of chronic stress is promising and we are excited to continue to explore the possibilities.

References

Abbott DH, Keverne EB, Bercovitch FB, et al. 2003. Are subordinates always stressed? A comparative analysis of rank differences in cortisol levels among primates. Horm Behav 43:67-82.

Anestis SF, Bribiescas RG, Hasselschwert DL. 2006. Age, rank, and personality effects on cortisol sedation stress response in young chimpanzees. Physiol Behav 89: 287-294.

Barrett MA, Brown JL, Junge RE, Yoder AD. 2013. Climate change, predictive modeling and lemur health: assessing impacts of changing climate on health and conservation in Madagascar. Biolog Conserv 157:409-422.

Bartolomucci, A. (2007). Social stress, immune functions and disease in rodents. *Frontiers in neuroendocrinology*, *28*, 28-49.

Beckie TM. 2012. A systematic review of allostatic load, health and health disparities. Biol Res Nurs 14: 311-346.

Beerda B, Schilder MBH, van Hoof JARAM, de Vries HW, Mol JA. 1998. Behavioural, saliva cortisol and heart rate responses to different types of stimuli in dogs. App Anim Behav Sci 58:365–381.

Betteridge DJ. 2000. What is oxidative stress? Metabolis 49: 3-8.

Blas J, Bortolotti GR, Tella JL, Baos R, Marchant TA. 2007 Stress response during development predicts fitness in a wild, long lived vertebrate. P Natl Acad Sci USA 104: 8880–8884.

Bond JC, Lindburg DG. 1990. Carcass feeding of captive cheetahs (Acinonyx jubatus): the effects of a naturalistic feeding program on oral health and psychological well-being. Appl Anim Behav Sci 26:373-382.

Brandenberger G, Follenius M. 1975. Influence of timing and intensity of muscular exercise on temporal patterns of plasma cortisol levels. J Clin Endocrinol Metab 40:845-849.

Budnitz N, Dainis K. 1975. Lemur catta: Ecology and behavior. In: Tattersall I, Sussman RW, editors. Lemur biology. New York, New York: Springer. p. 219-235.

Busch DS, Hayward LS. 2009. Stress in a conservation context: A discussion of glucocorticoid actions and how levels change with conservation-relevant variables. Biol Conserv 142: 2844-2853.

Cabezas S, Blas J, Marchant TA, Moreno S. 2007. Physiological stress levels predict survival probabilities in wild rabbits. Horm Behav 51: 313–320.

Carlstead K, Brown JL, Strawn W. 1993. Behavioral and physiological correlates of stress in laboratory cats. Appl Anim Behav Sci 38:143-158.

Cavigelli SA, Caruso MJ. 2015. Sex, social status and physiological stress in primates: the importance of social and glucocorticoid dynamics. Philos T Roy Soc B 370: 20140103.

Cavigelli SA, 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.

Cavigelli SA, Ragan CM, Michael KC, Kovacsics CE, Brliscke AP. 2009. Stable behavioral inhibition and glucocorticoid production as predictors of longevity. Physiol Behav 98:205–14.

Cavigelli SA, Pereira ME. 2000. Mating season aggression and fecal testosterone levels in male ring-tailed lemurs (Lemur catta). Horm Behav 37:246-255.

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

Cawthon Lang KA. 2005a. Primate Factsheets: Gorilla (Gorilla) Behavior. Primate Info Net. http://pin.primate.wisc.edu/factsheets/entry/gorilla/behav.

Cawthon Lang KA. 2005b. Primate Factsheets: Ring-tailed lemur (*Lemur catta*). Primate Info Net. http://pin.primate.wisc.edu/factsheets/entry/ring-taileded_lemur.

Chadwick C. 2014. Social behaviour and personality assessment as a tool for improving the management of cheetahs (Acinonyx jubatus) in captivity. [dissertation]. Manchester: University of Salford.

Cockrem JF. 2005. Conservation and behavioral neuroendocrinology. Horm Behav 48:492-501.

[CUCVM] Cornell University College of Veterinary Medicine. 2013 EClinPath. http://www.eclinpath.com/

Cox KL, Devanarayan V, Kriauciunas A, et al. 2012. Immunoassay Methods. In: Sittampalam GS, Coussens NP, Brimacombe K, et al., editors. Assay Guidance Manual [Internet]. Bethesda (MD): Eli Lilly & Company and the National Center for Advancing Translational Sciences. Available from: https://www.ncbi.nlm.nih.gov/books/NBK92434/

Crews DE. 2007. Assessing composite estimates of physiological stress in American Samoans. Am J Phys Anthropol 133:1028-1034.

Crews DE, Harada H, Aoyagi K, Maeda T, Alfarano A, Sone Y, Kusano Y. 2012. Allostatic load among elderly Japanese living on Hizen-Oshima Island. Int J Phys Anthropol 31:18-29.

Crimmins EM, Johnston M, Hayward M, Seeman TE. 2003. Age differences in allostatic load: an index of physiological dysregulation. Exp Geront 38: 731-734.

Davis AK, Maney DL, Maerz JC, 2008. The use of leukocyte profiles to measure stress in vertebrates: a review for ecologists. Functional Ecology, 22(5), pp.760-772.

Dobson H, Smith RF. 2000. What is stress and how does it affect reproduction? Anim Repro Sci 60–61: 743–752.

Dowd JB, Simanek AM, Aiello AE. 2009. Socio-economic status, cortisol and allostatic load: a review of the literature. Int J Epidemiol 38: 1297-1309.

"dysregulation" Merriam-webster.com. 2017. https://www.merriamwebster.com/medical/dysregulation (23 March 2017)

Edes AN, Crews DE. 2017. Allostatic load and biological anthropology. Am J Phys Anthropol 162: 44-70.

Edes AN, Wolfe BA, Crews DE. 2016a. Assessing stress in zoo-housed Western lowland gorillas (*Gorilla gorilla*) using allostatic load. Int J Primatol 37:241-259.

Edes AN, Wolfe BA, Crews DE. 2016b. Rearing history and allostatic load in adult western lowland gorillas (Gorilla gorilla) in human care. Zoo Biol 35: 167-173.

Evans CS, Goy RW. 1968. Social behavior and reproductive cycles in captive ring-tailed lemurs (*Lemur catta*). J Zoo Lond 156:181-197.

Fàbregas MC, Guillén-Salazar F, Garcés-Narro C. 2012. Do naturalistic enclosures provide suitable environments for zoo animals?. Zoo Biol 31:362-373.

Fink G. 2010. Stress: Definition and history. In: Fink G, editor. Stress Science: Neuroendocrinology. San Diego, California: Elsevier. P 3-9.

Fraser D. 2010. Toward a synthesis of conservation and animal welfare science. Anim Welf 19:121-124.

Gartner MC, Weiss A. 2013. Scottish wildcat (Felis silvestris grampia) personality and subjective well-being: Implications for captive management. Appl Anim Behav Sci 147:261-267.

Gopal D, Kalogeropoulos AP, Georgiopoulou VV, Tang WWH, Methvin A, Smith A L et al. 2010. Serum Albumin Concentration and Heart Failure Risk: The Health, Aging, and Body Composition Study. Am Heart J 160: 279–285.

Gould L, Sussman RW, Sauther ML. 1999. Natural disasters and primate populations: the effects of a 2-year drought on a naturally occurring population of ring-tailed lemurs (*Lemur catta*) in southwestern Madagascar. Int J Primatol 20:69-84.

Gould L, Sussman RW, Sauther ML. 2003. Demographic and life-history patterns in a population of ring-tailed lemurs (*Lemur catta*) at Beza Mahafaly Reserve, Madagascar: A 15 year Perspective. Am J Phys Anthropol 120:182-194.

Goyman W, Wingfield JC. 2004. Allostatic load, social status and stress hormones: The costs of social status matter. Anim Behav 67: 591-602.

Hazeldine J, Wiebke A, Lord JM. 2010. Dehydroepiandrosterone as a regulator of immune cell function. J Steroid Biochem 120: 127-136.

Hilton-Taylor C. 2000. IUCN Red List of Threatened Species. International Union for the Conservation of Nature, Gland, Switzerland.

Hoffman CK, Higham JP, Heistermann M, Coe CL, Prendergast BJ, Maestripieri D. 2011. Immune function and HPA axis activity in free-ranging rhesus macaques. Physiol Behav 104:507-514.

Huber S, Palme R, Arnold W. 2003. Effects of season, sex, and sample collection on concentrations of fecal cortisol metabolites in red deer (Cervus elaphus). Gen Comp Endocrinol 130:48-54.

Jolly, A. 1966. Lemur behavior: a Madagascar field study. University of Chicago Press, Chicago.

Jolly A, Dobson A, Rasamimanana HM, Walker J, O'Connor S, Solberg M, Perel V. 2002. Demography of *Lemur catta* at Berenty Reserve, Madagascar: Effects of troop size, habitat and rainfall. Int J Primatol 23: 327-353.

Junge RE. 2006. Overview on the health and disease ecology of wild lemurs: conservation implications. In: Gould L, Sauther ML editors. Lemurs. New Mexico, Springer US:423-440.

Juster RP, McEwen BS, Lupien SJ. 2010. Allostatic load biomarkers of chronic stress and impact on health and cognition. Neurosci Biobehav R 35:2-16.

Koolhaas JM, Korte SM, De Boer SF, Van Der Vegt BJ, Van Reenen CG, Hopster H, De Jong IC, Ruis MAW, Blokhuis HJ. 1999. Coping styles in animals: current status in behavior and stress-physiology. Neurosci Biobehav Rev 23: 925-935.

Korte SM, Olivier B, Koolhaas JM. 2007. A new animal welfare concept based on allostasis. Physiol Behav 92:422-428.

Koyama N. 1988. Mating behavior of ring-tailed lemurs (*Lemur catta*) at Berenty, Madagascar. Primates 29:163-175.

LaFleur M, Clarke TA, Reuter K, Schaeffer T. 2016. Rapid decrease in populations of wild ring-tailed lemurs (*Lemur catta)* in Madagascar. Folia Primatol 87: 320-330.

Leahy R, Crews DE. 2012. Physiological dysregulation and somatic decline among elders: Modeling, applying and re-interpreting allostatic load. Collegium Antropol 36: 11-22.

Mason GJ. 1991a. Stereotypies and suffering. Behav Proc 25:103-115.

Mason GJ. 1991b. Stereotypies: a critical review. Anim Behav 41:1015-1037.

McEwen BS. 1998. Stress, adaptation, and disease: Allostasis and allostatic load. Ann NY Acad Sci 840:33-44.

McEwen BS. 2004. Protection and damage from acute and chronic stress: allostasis and allostatic overload and relevance to the pathophysiology of psychiatric disorders. Ann NY Acad Sci 1032:1-7.

McEwen BS. 2010. Stress: Homeostasis, rheostasis, allostasis and allostatic load. In: Fink G, editor. Stress Science: Neuroendocrinology. San Diego, California: Elsevier. P 10-14.

McEwen BS, Seeman T. 1999. Protective and damaging effects of mediators of stress: Elaborating and testing the concepts of allostasis and allostatic load. Ann NY Acad Sci 896: 30-47.

McEwen BS, Stellar E. 1993. Stress and the individual — mechanisms leading to disease. Arch Intern Med 153:2093–2101.

McEwen BS, Wingfield JC. 2003. The concept of allostasis in biology and biomedicine. Horm Behav 43: 2–15.

Moberg GP. 2000. Biological response to stress, implications for animal welfare. In: Moberg GP, Mench JA, editors. The Biology of Animal Stress: Basic Principles and Implications. New York, New York: CABI publishing. P 1-20.

Mohr E, Langbein J, Nürnberg G. 2002. Heart rate variability: a noninvasive approach to measure stress in calves and cows. Physiol Behav 75: 251-259.

Monfort SL, Brown JL, Wildt DE. 1993. Episodic and seasonal rhythms of cortisol secretion in male Eld's deer (*Cervus eldi thamin*). J Endocrinol 138:41-49.

Morgan KN, Tromborg CT. 2007. Sources of stress in captivity. Appl Anim Behav Sci 102:262-302.

Morton DJ, Anderson E, Foggin CM, Kock MD, Tiran EP. 1995. Plasma cortisol as an indicator of stress due to capture and translocation in wildlife species. Vet Rec 136:60-63.

Nair R, Maseeh A. 2012. Vitamin D: The "sunshine" vitamin. J Pharmacol Pharmacother 3: 118–126.

Nakamichi M, Koyama N. 1997. Social relationship among ring-tailed lemurs (*Lemur catta*) in two free-ranging troops at Berenty Reserve, Madagascar. Int J Primatol 18; 73–93.

[NZPCRC] National Zoological Park Conservation & Research Center. 2008. Wildlife Endocrinology Manual.

Nunomura A, Castellani RJ, Zhu X, Moreira PI, Perry G, Smith M A. 2006. Involvement of oxidative stress in Alzheimer disease. J Neuropath Exp Neur 65: 631 – 641.

Park JY, Pillinger MH, Abramson SB. 2006. Prostaglandin E2 synthesis and secretion: The role of PGE2 synthases. Cl Immunol 119: 229-240.

Pride RE. 2005a. High faecal glucocorticoid levels predict mortality in ring-tailed lemurs (Lemur catta). Biol Lett 1: 60-63

Pride RE. 2005b. Optimal group size and seasonal stress in ring-tailed lemurs (*Lemur catta*). Behav Ecol 16: 550-560.

Roth GS, Mattison JA, Ottinger MA, Chachich ME, Lane MA, Ingram DK. 2004. Aging in rhesus monkeys: relevance to human health interventions. Science 305:1423–1426.

Sapolsky, R.M., 2002. Endocrinology of the stress-response. In: Becker J, Breedlove S, Crews D, McCarthy M editors. Behavioral Endocrinology, 2nd edition. MIT Press, Cambridge, Massachusetts, pp. 409–450.

Sapolsky RM. 2005. The influence of social hierarchy on primate health. Science 308:648–652.

Sclafani V, Norscia I, Antonacci D, Palagi E. 2012. Scratching around mating: factors affecting anxiety in wild *Lemur catta.* Primates 53: 247-254.

Schulkin J. 2003. Allostasis: a neural behavioral perspective. Horm Behav 43:21–27.

Seeman TE, Crimmins E, Huang MH, Singer B, Bucur A, Gruenewald T, Berkman LF, Reuben DB. 2004. Cumulative biological risk and socio-economic differences in mortality: MacArthur Studies of Successful Aging. Soc Sci Med 58: 1985–1997.

Seeman TE, McEwen BS, Rowes JW, Singer BH. 2001. Allostatic load as a marker of cumulative biological risk: MacArthur studies of successful aging. PNAS [serial online]. Available at: www.pnas.orgycgiydoiy10.1073ypnas.081072698.

Seeman TE, Singer BH, Rowe JW, Horwtiz RI, McEwen BS. 1997. Price of adaptation-allostatic load and its health consequences. Intern Med 157:2259-2268.

Selye H. 1950. Stress and the general adaptation syndrome. Brit Med J 1383-1393.

Shah NR, Dumler F.2008. Hypoalbuminaemia- A Marker of Cardiovascular Disease in Patients with Chronic Kidney Disease Stages II – IV. Int J Med Sci 5: 366–370.

Shepherdson DJ, Carlstead KC, Wielebnowski N. 2004. Cross-institutional assessment of stress responses in zoo animals using longitudinal monitoring of faecal corticoids and behavior. Anim Welf 13: S105-113.

Sheriff MJ, Dantzer B, Delehanty B, Palme R, Boonstra R. 2011. Measuring stress in wildlife: techniques for quantifying glucocorticoids. Oecologia 166:869-887.

Soderholm JD, Yang PC, Ceponis P, Vohra A, Riddell R, Sherman PM, et al. 2002. Chronic stress induces mast cell-dependent bacterial adherence and initiates mucosal inflammation in rat intestine. Gastroenterology 123:1099–108.

Starling AP, Charpentier MJE, Fitzpatrick C, Scordato ES, Drea CM. 2010. Seasonality, sociality, and reproduction: Long-term stress of ring-tailed lemurs (*Lemur catta*). Horm Behav 57: 76-85.

Sterling P. 2012. Allostasis: a model of predictive regulation. Physiol Behav 106:5-15.

Sterling P, Eyer J. 1988. Allostasis:a new paradigm to explain arousal pathology. In: Fisher K, Reason J, editors. Handbook of Life Stress, Cognition and Health. Hoboken, New Jersey: Wiley. p. 629–649.

Stewart JA. 2006. The detrimental effects of allostasis: Allostatic load as a measure of cumulative stress. J Physiol Anthropol 25:133-145.

Squires, EJ. 2003. Applied Animal Endocrinology. Accessed online at:

https://www.researchgate.net/publication/228375855_Applied_Animal_Endocrinology CABI publishing, Cambridge, MA, 2003.

Sussman RW. 1991. Demography and social organization of free-ranging Lemur catta in the Beza Mahafaly Reserve, Madagascar. Amer J Phys Anthropol 84:43-58.

Tennant C, Andrews G. 1976. A scale to measure the stress of life events. Aust NZ J Psychiat 10:27-32.

Tetley CL, O'Hara SJ. 2012. Ratings of animal personality as a tool for improving the breeding, management and welfare of zoo mammals. Anim Welf 21:463.

Valko M, Rhodes CJ, Moncol J, Izakovic MM, Mazur M. 2006. Free radicals, metals and antioxidants in oxidative stress - induced cancer. Chem-Biol Interact 160:1-40.

Van Cauter E, Leproult R, Kupfer DJ. 1996. Effects of gender and age on the levels and circadian rhythmicity of plasma cortisol. J Clin Endocrinol Metab 81:2468-2473.

Van Den Eede F, Moorkens G, Van Houdenhove B, Cosyns P, Claes SJ. 2007. Hypothalamic-pituitary-adrenal axis function in chronic fatigue syndrome. Neuropsychobiology 55:112-120.

Vickery S, Mason G. 2004. Stereotypic behavior in asiatic black and malayan sun bears. Zoo Biol 23:409-430.

Von Borell E, Dobson H, Prunier A. 2007. Stress, behaviour and reproductive performance in female cattle and pigs. Horm Behav 52: 130-138.

Weitzman ED, Fukushima D, Nogeire C, Roffwarg H, Gallagher TF, Hellman L. 1971. Twenty-four hour pattern of the episodic secretion of cortisol in normal subjects. J Clin Endocrinol Metab 33:14-22.

Wilson DE, Hanlon E. 2010. Lemur catta (Primates: Lemuridae). Mamm Spec 42:58-74.

Zhang R, Naughton DP. 2010. Vitamin D in health and disease: current perspectives. Nutr J 9:65.