Spring 4-2020

Comparison of comprehensive health score in North American housed giraffe and free-ranging giraffe from South Africa

Haley Beer
University of Nebraska - Lincoln, haley.kizer@gmail.com

Comparison of comprehensive health score in North American housed giraffe and free-ranging giraffe from South Africa

by

Haley Beer

A THESIS

Presented to the Faculty of
The Graduate College at the University of Nebraska
In Partial Fulfillment of Requirements
For the Degree of Master of Science

Major: Animal Science
Under the supervision of Professor Lisa Karr
Lincoln, NE
April, 2020

COMPARISON OF COMPREHENSIVE HEALTH SCORE IN NORTH AMERICAN HOUSED GIRAFFE AND FREE-RANGING GIRAFFE FROM SOUTH AFRICA

Haley Nicole Beer, M.S.

University of Nebraska, 2020

Advisor: Lisa K. Karr

As in humans, stress is evident in many animal species and has been correlated to disease prevalence; yet a value for reliable quantification of chronic stress is unestablished. During stressful events, allostasis, an adaptive process, is initiated by physiologic systems to maintain or reestablish homeostasis to protect an organism's viability. Over time, the acclimation to frequent stress causes systematic dysregulation, leading to the phenomena of increased allostatic load. In recent studies, allostatic load has been assessed in animal species via serum through selection of representative, multi-system biomarker indices. Perception and number of stress events an individual experiences may impact dysregulation severity, yielding allostatic load as a valuable tool in predicting future outcomes. However, the allostatic load methodology poses application limitations to individuals without historical data and those lacking a conscious recognition of stress. Comprehensive health score may be more encompassing of populations, as it targets biomarkers dysregulated by life events associated with pathology, despite an unknown level of cognition or history. Serum samples were obtained from zoo-housed (n=18) and free-ranging (n=11) giraffe to predict subclinical risk of morbidity and mortality caused by chronic stress. Serum concentrations of selected biomarkers were investigated using colorimetric ELISAs (cortisol, DHEA-S), the CHOD-PAP method (cholesterol), colorimetric enzymatic method (NEFA), and

nitroblue tetrazolium assay (fructosamine). Selection of these biomarkers were based on the ability to estimate dysregulation of physiologic processes impacted by stress accumulation, such as the neuroendocrine, metabolic, and inflammatory systems. Free-ranging giraffe were younger (m = 7.9 years) on average and had lower cholesterol (p = 0.016) and fructosamine (p = 0.039) levels when compared to captive giraffe (m = 12.8 years). Additionally, free-ranging giraffe had higher cortisol (p = 0.007) levels and NEFA (p = 0.004) status, while DHEA-S (p = 0.548) was found at relatively similar concentrations between the populations. Although suitable composites rely heavily on specific species and environmental factors, comprehensive health score provides a foundation for a more applicable tool in conservation research through comparison of biomarkers across populations.

Key words: allostatic load, chronic stress, clinical pathology, *Giraffa camelopardalis,* morbidity, mortality, wildlife

DEDICATION

This thesis is dedicated to Dr. Trent Shrader for always demonstrating unrelenting encouragement and support, in addition to providing the most extraordinary and unforgettable opportunities.

“Sometimes when you follow your passion, it works out for you.”

ACKNOWLEDMENTS

From fiction to fruition, the completion of this thesis would not be an attainable endeavor without the steadfast support and assistance of many individuals, only some of whom are specifically mentioned here. Any omission does not signify lack of thanks.

First and foremost, I am grateful for the Lord instilling inspiration, courage, and enthusiasm in me to embark on this journey and for providing strength and perseverance to this accomplishment. A mere expression of thanks does not suffice.

Immeasurable appreciation and deepest gratitude go to my principle advisor, Dr. Lisa Karr for your unequivocal patience and organization. Thank you for providing learning opportunities and demonstrating lenience while I traveled for my research. Additionally, I would like to extend a sincere and heartfelt obligation toward my Committee members, Dr. Brett White and Dr. Trent Shrader. Your active guidance, availability, and unsurpassed knowledge was instrumental to my success.

Ineffably, I am indebted to all those that contributed funding and resources that made my research possible. A profound thank you goes to the Lincoln Children's Zoo and Dr. Coleen Stice for your financial support, Omaha Henry Doorly Zoo and Aquarium and the Save the Giraffes foundation for providing blood samples, Ampath Laboratories, Cornell University's Veterinary Diagnostic Center, and the St. Louis Endocrinology Lab for running tests, and UNL's SC3L Statistics Lab for additional data analysis.

At last but not least, gratitude goes to all of my family and friends who directly or indirectly supported me. Thank you to my husband, Evan, for your unwavering love and support. Lastly, I could not have started this journey without you Bella, Remi, and Spud—your many years of comfort were thoroughly appreciated. May you rest in peace.

TABLE OF CONTENTS

Chapter **Page**

1. Introduction……...…...1

2. Literature Review……...…...4

Risk of Extinction……..…….....4

Stress Mechanism and Accumulation……..…...…..5

Index Composition…….....…...9

Body Condition Score……...……10

Cortisol...……...……12

DHEA-S……...…….……..14

Cholesterol……...……...…….……...15

Non-esterified Fatty Acids…….…….…17

Fructosamine…….…18

Comprehensive Health Score Utilization…….…..…..19

Literature Cited…….…….....22

3. Comparison of comprehensive health score in North American housed giraffe and free-ranging giraffe from South Africa……...….29

Abstract…….……..29

Introduction…….……....30

Methods and Materials…….…….…..…32

Results…….…….…….…..37

Discussion…….…….…43

Implications…….……...…..49

Conclusions…….…….…..…..52

Literature Cited……..……..53

APPENDIX

Figure **Page**

1. Current distribution of free-ranging *Giraffa camelopardalis*…….…..…....4

2. HPA Axis in response to a stress event……...……...…….6

3. Allostatic load accumulation model……...…….8

4. Stress events from the captive giraffe population……..35

5. Example of photograph used to determine BCS in FR giraffe…….....……..…36

6. Demographics of captive and free-ranging giraffe populations……...….……...38

Table **Page**

1. Comparison of Biometric and Biomarker Value Means in Captive and FR Giraffe…...……..……...38

2. Correlations of Stress Variables in Captive Giraffe...…….…..41

3. Correlations of Stress Accumulation and Subjective Values in FR Giraffe....….40

4. Correlations of Biometric and Biomarker Values in FR Giraffe.…...….……..42

LIST OF ABBREVIATIONS

Item	Meaning
ACS	acyl-CoA synthetase
ACOD	acyl-CoA oxidase
ACTH	adrenocorticotropic hormone
AL	allostatic load
AVP	arginine vasopressin
AVT	arginine vasotocin
ANS	autonomic nervous system
BCS	body condition score
BP	blood pressure
C	Celsius
CNS	central nervous system
CoA	coenzyme A
CHS	comprehensive health score
CRH	corticotropin-releasing hormone
DHEA	dehydroepiandrosterone
DHEA-S	dehydroepiandrosterone sulfate
E	epinephrine
FFA	free fatty acid
GAS	general adaptation syndrome
GC	glucocorticoid
GOSG	Giraffe and Okapi Specialist Group

HbA1c	glycated hemoglobin
HDL	high-density lipoproteins
HR	heart rate
HPA	hypothalamic-pituitary-adrenal
IUCN	International Union for Conservation of Nature
LDL	low-density lipoproteins
MEHA	3-methyl-N-ethyl-N-(β-hydroxyethyl)-aniline
NEB	negative energy balance
NEFA	non-esterified fatty acid
NE	norepinephrine
POD	peroxidase
SC3L	statistical cross-disciplinary collaboration and consulting lab
SNS	sympathetic nervous system
UV	ultraviolet
VLDL	very low-density lipoproteins

Chapter 1

INTRODUCTION

The concept of "stress" has been depicted quite consistently throughout history. From being used synonymously with "hardship" in the seventeenth century, describing "passions," like nerves, vapors, and hysteria in the eighteenth century, to deemed the culprit of "nervous exhaustion" in the nineteenth century, the perception of stress has historically carried negative connotations (Cooper & Dewe, 2004). Regardless of the century, explanations of this phenomenon mention some aspect of environmental influence on individuals (Doublet, 2000). However, the term "stress," used as an analogue in biological sciences to describe a possible cause of ill health and mental disease, was not coined until the twentieth century (Bartlett, 1998). Before the early 1900s, the idea and discussion of stress was limited to the engineering profession, but as the century of science and technology evolved, the term's meaning followed suit (Cooper & Dewe, 2004).

Researchers, Walter Cannon and Hans Selye, are often recognized as the founding fathers that paved the way into understanding stress (Cooper & Dewe, 2004; Romero & Gormally, 2019). Cannon first described what is now referred to as "homeostasis" and explored instincts that gave rise to the "fight or flight" response. Following the work of Cannon, Selye linked the hypothalamic-pituitary-adrenal (HPA) axis to the way the body copes with stress (Cooper & Dewe, 2004). Identifying stress as nonspecific signs or symptoms of illness, Selye termed the general adaption syndrome (GAS) which distinguishes physiological changes during stress (Selye, 1982). The theories coupled

together gave rise to the mechanism known as the stress response (Romero & Gormally, 2019).

With the foundation of stress established, understanding the physiological, hormonal, and behavioral response became a source of immense interest in human studies, given that the pace of life in the twentieth century was often viewed as the root cause of much illness and disease (Cooper & Dewe, 2004). As work on stress progressed over the ensuing years, it is now viewed as a familiar condition, originating in the mind, that is expressed differently among individuals depending on major life events and daily life pressures (McEwen, 2006). The stress response in acute situations is typically beneficial, though if mounted consistently or consecutively without proper termination following a stressor, pathological states may occur. The level of physiological system elevation, or chronic stress, influenced by genetic variations and life-style habits (diet, exercise, and substance abuse, etc.), leads to pathophysiology which increases the instances of morbidity and mortality (Juster et al., 2010; McEwen, 1998; Rodrigues et al., 2009).

As evolution proves through instinctual defense mechanisms, animals also activate the stress response to help cope with environmental threats. Because the anatomical and physiological backbone of the stress response is considerably well-conserved across vertebrates, researchers have recently transposed this human methodology to various taxa, with concerns that other organisms are also at risk of succumbing to the impact of chronic stress. Similar in theory, prolonged activation of the stress system in animals is proposed to result in adverse effects on overall health status (Edes et al., 2018; Gundlach et al., 2018; Romero, 2004). In addition to the myriad of other mammals threatened with extinction, free-ranging giraffe (*Giraffa camelopardalis*) may be at high risk for

developing secondary psychosomatic conditions, given the increasingly threatening environment (Muller et al., 2018). The objective of the work presented in this thesis was to evaluate chronic stress in captive and free-ranging giraffe as a means of validating the concept that individuals who experience more stress will reflect greater subclinical risk for future development of chronic degenerative conditions and shortened lifespan.

Chapter 2

LITERATURE REVIEW

Extinction Risk

After reassessing the tallest living terrestrial animal and largest ruminant, the International Union for Conservation of Nature (IUCN) Giraffe and Okapi Specialist Group (GOSG) moved giraffe (*Giraffa camelopardalis)*, currently recognized as a single species with nine subspecies, from "least concern" to "vulnerable" in 2016 (Muller et al., 2018). Within the last thirty years, past and ongoing populations have declined 36 to 40% due to the combined impacts of habitat loss, habitat fragmentation, human population growth, poaching, and war and civil unrest (Dagg, 2014; Muller et al., 2018). Since the 1980's when well over 160,000 giraffes were documented across the African continent, research and conservation for the species was grossly overlooked. Though location dependent, of the nine subspecies, five are declining, one has maintained population numbers, whereas the remaining three have slightly increased (Dagg, 2014). Today, with approximately 68,000 free-ranging, mature individuals remaining (Figure 1) and with the

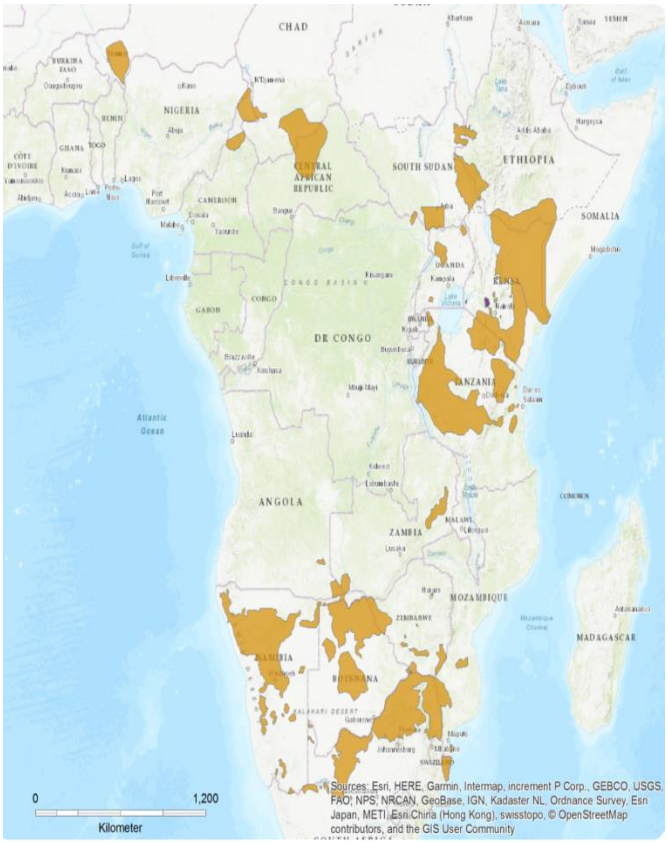

FIGURE 1. Current distribution of (*G. camelopardalis)*. © International Union for Conservation of Nature and Natural Resources (Muller et al., 2018).

ongoing pressure from humans, this iconic species will soon be a victim of 'silent extinction' if conservation resources are not prioritized immediately.

Stress Mechanism and Accumulation

Free-ranging animals living in unregulated environments are more likely to experience fear than those conditioned to cohabitate with humans. Perceived high risk is evoked by events that are threatening, intense, novel, or sudden. Evolved to help animals survive noxious environmental stimuli, adaptive physiological and behavioral responses are initiated in natural circumstances. As in humans, animal brains perceive risk and illicit defensive behaviors such as freeze or startle (Asli & Flaten, 2012). Reactions like these are intrinsically interconnected with stress and are intended to aid self-preservation.

The anatomy of a stress response is a well-conserved function attributed to regulating fitness in many vertebrate species (Romero & Gormally, 2019). To increase survivability, an acute stress event is generally beneficial to animals due to rapid and protective physiological responses. Allostasis is a restorative process responsible for responding to both internal stress and the external environment resulting in the ability to achieve stability through change (McEwen, 1998). Unlike homeostatic systems that have narrower ranges to maintain such as body temperature or blood oxygen, allostatic or adaptive systems, have much broader ranges. These are multisystemic and include the hypothalamic-pituitary-adrenal (HPA) axis, the immune system, and metabolic systems like the thyroid axis, insulin, glucagon, and gut permeability. Allostasis promotes adaptation to activities such as locomotion and aversive stimuli in situations that an individual may be experiencing isolation, hunger, extreme temperature changes, microbial or parasitic infections and threats to safety.

Upon encountering an environmental threat, fear arousal leads to the initiation of the stress response which is composed of two pathways that promote autonomic and endocrine changes. The first response activated is commonly known as fight-or-flight, or the fast arm of the reaction. Mediated by the sympathetic nervous system (SNS), a component of the autonomic nervous system (ANS), an "action potential" propagates along axons to the adrenal medulla and sympathetic nerves which then release the catecholamines, epinephrine (E) and norepinephrine (NE) (Dickens et al., 2010; Rodrigues et al., 2009). In turn, heart rate (HR) elevates, blood pressure (BP) increases, and energy sources mobilize to the central nervous system (CNS) and somatic muscle.

As shown in Figure 2, the second pathway of the stress response is a slower hormonal cascade along the HPA axis following fight-or-flight and resulting in glucocorticoid (GC) secretion. The HPA axis reaction begins with stimulation of the paraventricular nucleus in the hypothalamus which secretes corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP) or arginine vasotocin (AVT), depending on the species (Romero & Gormally, 2019).

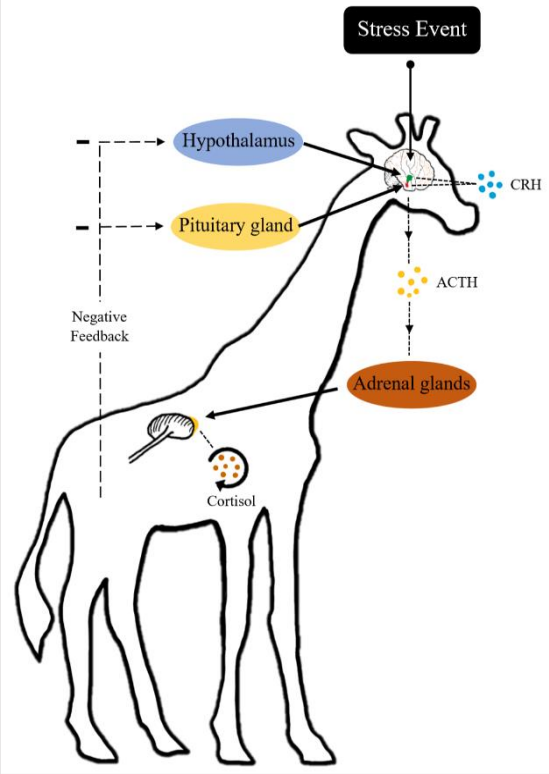

FIGURE 2. Activation of a hormonal cascade along the HPA axis when coping with stress.

The pituitary is then signaled to release adrenocorticotropin (ACTH) hormone which, in turn, mediates the secretion of glucocorticoids from the adrenal cortex (McEwen, 1998). For most fish and mammalian species, cortisol is the predominate GC secreted; whereas

in rodent, bird, amphibian, and reptile taxa, corticosterone is the most prevalent GC (Romero, 2004). Until they reach their target tissues and interact with intracellular receptors such as mineralocorticoid receptors (MR) and glucocorticoid receptors (GR), GCs circulate through plasma bound to corticosteroid binding globulins (CBG). Once homo- or heterodimers are formed for function, GCs affect many tissues throughout the body, depending on receptor ratio (Romero & Gormally, 2019). Glucocorticoids in the brain exert negative feedback effects which quickly suppress GC release once a stressor ceases (Dickens et al., 2010). Inactivation of the HPA axis returns systems to base-line levels of catecholamine and cortisol secretion.

Enhancing the possibility of survival, the acute stress response is the culmination of allostasis, with the activation of the fight-or-flight and glucocorticoid pathways. Because the level of activation of the stress response has been correlated with the overall health of an animal, those that experience acute stress events intermittently over a lifetime, allowing ample time to initiate and terminate the complex adaptive pathway between stressors, are projected to live longer (Romero, 2004). While adaptive acutely, chronic activation, with little to no recovery time between stressful events, can have a negative impact on an individual's physical and mental health. In other words, when the activation of the stress pathway is overactivated, or constantly adapting to internal and external stressors, the body is exposed to an excessive amount of stress hormones. Persistent secretion of epinephrine, norepinephrine, and cortisol begins to damage the brain and body, rather than protect it like it was evolved to do (McEwen, 2006). Responsible for this degradation are primary mediators, or stress hormones and their antagonists, in conjunction with inflammatory cytokines (McEwen, 2003). Affecting cellular activities like enzymes, receptors, and ion channels, physiological integrity of

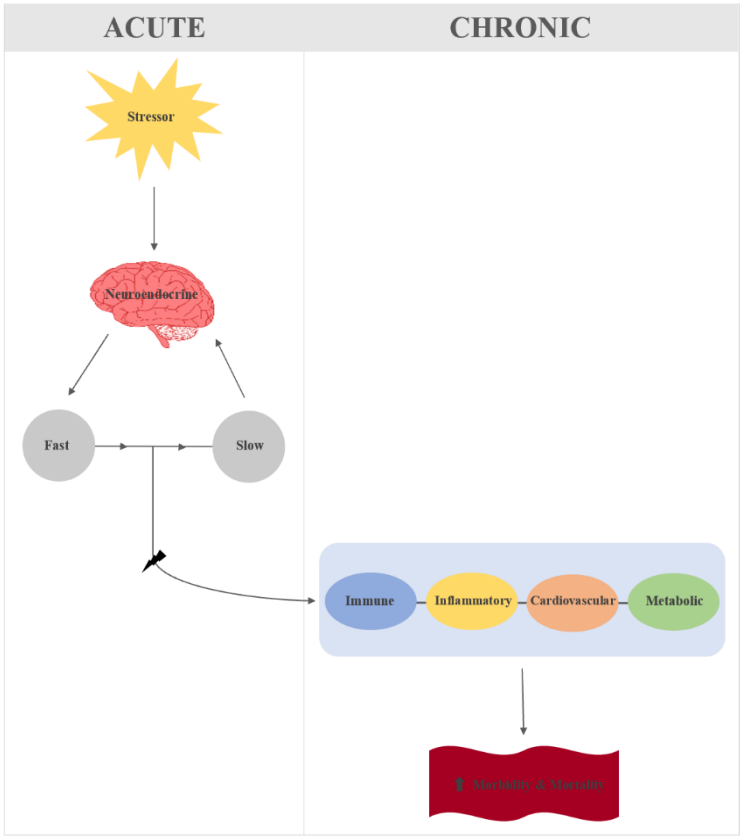

FIGURE 3. Frequent stress causes dysregulation of allostatic systems and has been associated with morbidity and mortality.

allostatic systems become compromised to the point that operating ranges to maintain chemical, tissue, and organ functions shift. Over weeks, months, or years, this accommodation to stress progresses to secondary outcomes where neuroendocrine, inflammatory, cardiovascular, metabolic, and immune parameters become abnormal at sub-clinical levels (as illustrated in Figure 3). The collapse of these interconnected biological processes lead to tertiary outcomes that encompass disease predisposition (Juster et al., 2010). When allostatic systems become exhausted or overworked they leave the organism susceptible to stress-related diseases, promoting pathophysiology called "allostatic load" (McEwen, 1998).

The three types of physiological responses that make up allostatic load are frequent stress of different consistency and magnitude, failed shut-down of the stress process, and inadequate response to a challenge, often times leading to overcompensation of other systems (McEwen, 1998). When an individual experiences this temporal cascade of multi-systemic physiological dysregulation, chronic degenerative conditions like compromised immune function, hypertension, insulin resistance and many other

proposed ills are substantially more likely to develop (Rodrigues et al., 2009). However, not all allostatic load accrual is created equal. Depending on the number of stressful events an individual will experience in a lifetime and to stress perception, the rate at which allostatic load accumulates is not uniform across a population. Predisposing factors such as demographic, genetic, environment, and developmental conditions dictate sensitivity to stress and the degree or rate at which allostatic load will accumulate (Edes et al., 2016).

Index Composition

Regardless of how stress is perceived, the extent of physiologic dysregulation, or allostatic load, can be estimated using a composite of biomarkers indexing a wide range of biological systems (McEwen, 1998). Constructing allostatic load indices from members of a sample not only identifies which systems are at higher risk of 'wear and tear' but serves as a warning of future outcomes that suggest morbidity and mortality. Multiple tissue types such as saliva, urine, feces, hair, and serum can be used when creating an allostatic load index but should be assessed at the same time (Edes et. al, 2018). Using quartile methodology where data is divided into lower quartiles, medians, and upper quartiles with each group measuring the spread of values above and below the mean, allostatic load is a comprehensive value that sums high risk biomarkers into a single number (McEwen, 1998). Extreme values are those in the top or bottom quartiles from a tested population.

The highest allostatic load score an individual can obtain is the number of biomarkers included in the composite, but generally most indices range between eight to fourteen (Beckie, 2012). If a greater number of biomarkers can be targeted that selectively evaluate for morbidity and mortality, the index will be more precise than a

composite with fewer biomarkers. For example, the initial attempt to determine allostatic load in humans used an index composed of ten biomarkers that represented the neuroendocrine, cardiovascular, and metabolic systems. The results from the study indicated individuals that had low allostatic load were high functioning, whereas those with high scores were more likely to develop cardiovascular disease and had the greatest decline in cognitive measures (McEwen, 1998)

Traditionally, physiological changes that individuals undergo following a stressful event are measured by the misleading, short-hand phrase “stress hormones” like glucocorticoid differences. However, not only have studies demonstrated that glucocorticoid levels were independent of induced chronic stress in selective individuals and populations, but oftentimes, common metrics used in field studies fail to adequately represent the breadth of risk perception (Boudreau et al., 2019; MacDougall-Shackleton et al., 2019). Given the affordability, convenience to existing data, and predictive power, the allostatic load framework has started to appear in many animal population studies. While many biomarkers have been used for assessing allostatic load in humans, not every traditional stress-activated biomarker will be transferrable to other species and requires validation for each population from an accredited institution (Edes et al., 2018).

Body Condition Score

When evaluating free-ranging animals within a population, simple, rapid, and noninvasive methods are vital in effectively assessing health and fitness. Body condition offers a quantitative measurement that indicates nutritional status, health, and wellbeing of individuals (Stringer et al., 2010). Although more accurate methods for measuring markers of body composition exist, they often require destruction of an individual. Instead of utilizing a complex extraction process or relying on a panel of clinical

observations, an indirect technique called body condition score (BCS) may be a useful guide in understanding population dynamics (Hill et al., 2003). A subjective assessment of subcutaneous body fat stores, BCS evaluates muscle tone and key skeletal elements either visually or tactilely (Stringer et al., 2010). Depending on the species of interest, a 5- or 9- point scale is utilized where low scores indicate less body fat and higher scores represent more body fat. Respectively, a score of 3 or 5 are considered ideal body conditions. In giraffe (*Giraffa camelopardalis*), however, a 1-8 point scale is used to for body scoring, with 4-6 being the optimal range (Kearney & Ball, 2001).

Although the typical scoring technique in most mammals involves observing and palpating (if accessible) the flesh over the bony protuberances of the hips, body condition standards vary between animal species (Hickman & Swan, 2010; Stringer et al., 2010). Due to the subjective nature of body condition scoring, individuals should be trained to accurately assess the species of interest which will in turn, reduce interobserver variation. Though assessing body fat ratios using BCS is subjective, many strategies such as photographs, MRIs, CT scans, X-ray absorptiometry, and ultrasonography have since validated the method in a multitude of species (Bjornvad et al., 2017; David et al., 2007; Gant et al., 2016; Gifford et al., 2014; Hill et al., 2003; Morfeld et al., 2014; Kobayashi et al., 2014; Mellish et al., 2004; Payan-Carreira et al., 2016; Stephenson et al., 1998). Other studies indicate that kidney fat content accurately reflects BCS with actual fat content and demonstrates a significant influence on allometric measurements, respiration rate, and various reproductive parameters like performance, conception rate, parturition, and birth weight (Menchetti et al., 2015; Sejian et al., 2010). Commonly used in veterinary medicine for its simplicity, effectiveness, and affordability, BCS poses as a sensitive

indicator of progressive illness that can be directly linked to survival probability (Hickman & Swan, 2010; Hill et al., 2003).

Cortisol

Fluctuations of cortisol, a prevalent glucocorticoid or steroid hormone in mammals, has been used as a bio-indicator to assess physiological and psychological states of animals influenced by habitat quality and anthropogenic disturbances (Pride, 2005; Hajduk et al., 1992; Wilkening et al., 2016; Yamanashi et al., 2016). Although these hormones are mediated by multiple receptor types and have innumerous other functions unrelated to stress, increased glucocorticoid levels have been associated with health risks and suggest lower fitness (Pride, 2005). More accurately referred to as metabolic hormones, glucocorticoids' primary role consists of what the name suggests, carbohydrate metabolism regulation (MacDougall-Shackleton et al., 2019). Synthesized by the adrenal cortex following the release of adrenocorticotropic hormone (ACTH), cortisol, catabolic in nature, stimulates resource allocation (Lennartsson et al., 2012). Rapid energy mobilization from long-term storage aids in coping with immediate crises and are essential to life. Without hormone replacement and behavioral change during exercise, exertion, and stress in vertebrates, death ensues (Hajduk et al., 1992; Wilkening et al., 2016).

However, when reallocation of resources from normal behaviors like reproduction and survival are continuous, individuals are likely to experience serious health risks (Bonier et al., 2009; Boonstra et al., 2018). Exposure to prolonged glucocorticoid secretion at elevated concentrations eventually produces hippocampal dysfunction. Over time, this impairment affects stress perception and suppresses inflammatory cytokine

production, which reduces the rate of wound healing in animals (Ebrecht et al., 2004; Lupien et al., 1998).

Circulating hormones secreted into the bloodstream by the adrenal glands are typically metabolized by the liver and excreted as metabolites into the gut (Wilkening et al., 2016). Whether detected in blood, saliva, hair, urine, or feces, cortisol readings have been associated with the activity of the HPA axis in certain species (Ebrecht et al., 2004; Hajduk et al., 1992). In general, cortisol measures relatively short-term stress. Due to the convenience and noninvasive nature, field studies often utilize temporal waste products from free-ranging animals (Bashaw et al., 2016). Though species dependent, peak excretion in urine is typically at 4.8 hours and recovers in feces 22.2 hours after induced stress (Yamanashi et al., 2016). For animals without habituation to human handling, significant physiological effects on hematological characteristics are observed, with a peak cortisol reading at 6 hours after capture (Hajduk et al., 1992). In a study comparing glucocorticoid levels in northern elephant seals, capture and blood collection considerably impacted cortisol in wild populations versus captive seals that were trained for the procedure (Gundlach et al., 2018). Thus, the time of blood sampling in relation to capture should be considered when assessing hematology of free-ranging animals and suggests other parameters be included to measure chronic stress more robustly.

Of the physiological measurements, hair follicles represent the longest duration of cortisol in vertebrates. Absorbing substances from blood, cortisol accumulates in the shaft over several months as hair grows (Yamanashi et al., 2016). Although blood and hair methods require animal disturbance, sample integrity and accuracy are maintained. Additionally, type of diurnal rhythm, storage method, and duration from initial collection can also alter cortisol concentrations (Yamanashi et al., 2016). Given that cortisol levels

are easily influenced by sampling situations and present many limitations when quantifying a component in a complex response, it is recommended to measure more than glucocorticoid levels when estimating ecological, social, and environmental stressors of animals (MacDougall-Shackleton et al., 2019).

DHEA-S

Because the stress response interacts with more than just the nervous system, modulators of immune functions are often evaluated when assessing status and may conjunctionally represent HPA axis functionality more accurately. Precursors to androgens, dehydroepiandrosterone (DHEA) and its sulphated metabolite, dehydroepiandrosterone sulfate (DHEA-S), are described as the most abundant anabolic steroid hormones in humans (Gundlach et al., 2018). Suggested to have protective properties against negative consequences of stress, the ratio of catabolic (cortisol) to anabolic (DHEA) activity may provide insight on neuro-immune-endocrine system interactions (Gundlach et al., 2018; Lennartsson et al., 2012). Although cortisol originates from the zona fasciculata layer and DHEA/DHEA-S from the zona reticularis layer of the adrenal cortex, both share the same regulatory mechanism and are secreted in response to ACTH. Acting as a glucocorticoid antagonist, DHEA is proposed to provide protection against cortisol effects. However, cortisol production causes DHEA concentrations to deplete in humans, directly influencing ability to respond to acute psychosocial stress over time (Lennartsson et al., 2012).

Serving as a reservoir to the biologically active DHEA, DHEA-S has a longer half-life and is found in higher concentrations which may represent an ideal quantitative measurement when compared to cortisol levels (Lennartsson et al., 2012; Takeshita et al., 2016). Originally established in humans, animals that present with high cortisol/DHEA

ratios via blood, urine, or fecal sampling reflect a higher level of HPA dysregulation (Takeshita et al., 2016). Although inter-individual variation influences the magnitude for concentrations, a majority of animals with depleted DHEA-S levels are prone to experience chronic stress, resulting in cognitive and reproductive disorders (Takeshita et al., 2016). As described in many species, DHEA levels inherently decline over time as 17,20-demolase, the enzyme responsible for DHEA and DHEA-S biosynthesis, decreases (Boonstra et al., 2018; Lennartsson et al., 2012). Peak concentrations in humans are reached between 20 and 30 years of age and gradually decline throughout a lifetime as the zona reticularis changes (Lennartsson et al., 2012). However, validation is required in many species, as a study confirmed that this typical age-related change was not observed in phocids, also known as true seals (Gundlach et al., 2018). When comparing DHEA-S differences between males and females, the protective effects of DHEA appear to be stronger in men than women, yet age and pubertal status for either sex can influence DHEA levels, as well as HPA axis activation (Farooqui et al., 2019; Goldman & Glei, 2007; Roberts et al., 2016). Furthermore, measuring individual differences of DHEA to cortisol levels may reliably represent systemic dysregulation and predict health outcomes for both sexes, given the neuroprotective, antioxidative, anti-inflammatory, and anti-glucocorticoid properties (Lennartsson et al., 2012; Vieira-Marques et al., 2016).

Cholesterol

In situations of sudden metabolic need, cholesterol produced from various amino acids, carbohydrates, and fatty acids are supplied to organisms (Coblentz, 1975). Obtained through biosynthesis and diet, cholesterol is responsible for major biological functions. The essential metabolite is distributed throughout cells in the body and provide structural scaffolding for steroid synthesis of sex hormones, bile acids,

corticosteroids, and vitamin D (Schmidt et al., 2006; Valenzuela et al., 2003). Biosynthesis of cholesterol is highly-regulated and occurs in almost all animal tissues, but the adrenal gland, the ovaries, the testis, and liver represent the most significant activity in mammals, with 75% of the total cholesterol production originating in the liver (Coblentz, 1975; Valenzuela et al., 2003). Required for transport within tissues and blood, cholesterol must be paired with amphipathic lipids and protein, or lipoproteins (Schmidt et al., 2006). Total cholesterol is composed of chylomicrons that transport dietary fat from the intestinal tract to the circulatory system, very low-density lipoproteins (VLDL) that transport synthesized fat from the liver to adipose cells during excessive energy intake, low-density lipoproteins (LDL) which transport cholesterol to damaged cell membranes, and high-density lipoproteins (HDL) which help remove cholesterol remnants from blood circulation (Schmidt et al., 2006). Although total serum cholesterol ratios of VLDLs, LDLs, and HDLs vary between species, humans have a composition of 10-15%, 60-70%, and 20-30%, respectively. Also common in most mammals, humans typically convert cholesterol to bile to be excreted from the body at a rate eight times greater than the volume converted to hormones (50 mg) per day (Valenzuela et al., 2003).

Utilization of blood chemistry may offer relevant information about the health, pathology, and nutritional status of an individual (Coblentz, 1975). Several studies have identified energy metabolism as a contributing culprit in disease development (Quiroz-Rocha et al., 2009). Increased LDL concentrations have been linked to cardiovascular dysregulation, given the associated atherogenic properties which are known to increase the risk of cardiovascular disorders and predispose individuals to developing specific diseases like gallstones (Ginnett et al., 2003). Low HDL levels, however, have been

strongly correlated with metabolic dysregulation. Although factors claimed to lower serum cholesterol, such as malnutrition and high inflammation levels, are not fully understood, studies have indicated that older humans with low HDL levels are more likely to develop coronary heart disease, enhanced inflammation (known to suppress new blood vessel growth), endothelial activation, and oxidative stress (Hu et al., 2003; Schmidt et al., 2006; Wan Ahmad et al., 2015). An energy imbalance, such as low or high total cholesterol, may be attributed to an individual's genetics, energy expenditure, and diet (Quiroz-Rocha et al., 2009; Schmidt et al., 2006). Excitability and seasonal food quality have a definitive influence on cholesterol (Card et al., 1985). However, lipids are less affected by severe, short-term physiological stresses in ruminants and therefore, serum cholesterol may have significant value as a reliable index of nutritional condition, particularly adding relevance for animals with substantial BCS variations between seasons (Coblentz, 1975).

Non-esterified fatty acids

Essential to energy and carbohydrate homeostasis in ruminants, fat metabolism is often measured using a form of lipid called free fatty acids (FFAs), also referred to as non-esterified fatty acids (NEFAs). Gastrointestinal and energy-stored triglycerides from dietary intake undergo lipolysis, or the process of breaking down adipose tissue, and are released into the blood as albumin-bound fatty acids (Adewuyi et al., 2005; Leroy et al., 2005). Additionally, the energy substrate can be oxidized into acetyl CoA and converted into ketone bodies (acetone, acetoacetate, and beta-hydroxybutyrate) or energy is exported into peripheral circulation through VLDLs as a result of lipogenesis in the liver (Dijkstra et al., 2005). Consisting of only 5% of the total plasma lipid, the oxidizable energy source from NEFA plays an important role during periods where energy

requirements exceed energy intake or negative energy balance (NEB) (Adewuyi et al., 2005; Dijkstra et al., 2005). Representative of inadequate metabolic resources, NEB increases the potential for secondary and tertiary negative effects, resulting from acute situations such as exercise or physiological imbalances like decreased body condition scores, plasma glucose, and insulin or increased plasma (beta-hydroxybutyrate) and TAG concentrations (Adewuyi et al., 2005).

Ideally, NEFA concentrations should be maintained at relatively low levels (less than 0.2 nM in cows) throughout an animal's life, unless faced with situations of sparse food abundance during cold and dry seasons or throughout pregnancy when mobilization of nutrients from body reserves are required for survival (Adewuyi et al., 2005; Valckx et al., 2012). However, excessive NEFA diffusion into the blood may become toxic to an individual, increasing the risk of developing fatty liver syndrome (from hepatic TAG accrual) or ketosis (an accumulation of unused ketones from peripheral tissues) (Adewuyi et al., 2005). Furthermore, results from previous studies in ruminants indicate that NEFAs are particularly vulnerable to adrenergic stimulation, suggesting that fat mobilization may be a useful marker in predicting morbidity in wildlife (Dijkstra et al., 2005). Unlike other biomarkers that normally undergo significant changes in body condition score, body composition ratio, and other management strategies due to seasonality of resources, NEFA can still be evaluated as an indicator of ongoing metabolic activity, whether anabolic or catabolically, so long as the fat stores have not been entirely depleted.

Fructosamine

When measuring long-term glucose control in clinical practice, glycated hemoglobin (HbA1c) has traditionally been the standard measurement and is a marker of future cardiovascular risk (Selvin et al., 2015). Although glucose concentrations are

reflected over a 2- to 3-month period with this biomarker, HbA1c could present bias results, given that its validity is influenced by red blood cell condition and survival. Therefore, individuals with existing disorders like anemia, kidney and liver disease, or altered red cell lifespan may demonstrate difficulty in interpreting HbA1c levels. The glycemic marker is also limited to only subjects that have been fasted, holding an unrealistic requirement for field application (Malmström et al., 2014). However, utilizing nontraditional, short-term markers like fructosamine are increasing in interest. Data from current literature is investigating the sensitivity and specificity of various fructosamine cut-offs (Malmström et al., 2014).

A ketoamine formed from the binding of fructose to total circulating serum protein through glycosylation, fructosamine includes all glycated proteins (Ji-Eun, 2015). Reflecting blood glucose concentrations over the previous 2 to 4 weeks, serum fructosamine values may offer earlier indication of poor glycemic control (Selvin et al., 2015). Alterations in protein metabolism, causing increased fructosamine levels, have been largely associated with the same pathophysiological processes responsible for raising blood glucose concentrations (Selvin et al., 2015). Used independently or in conjunction with glucose and HbA1c, fructosamine assays are not only simple and affordable, but may yield as an advantage in epidemiological studies as a potential predictor of myocardial infarction, ischemic stroke, and diabetic complications (Ji-Eun, 2015; Malmström et al., 2014; Selvin et al., 2015).

Comprehensive Health Score Utilization

Allostatic load has aided researchers in measuring chronic stress and predicting future disease in humans for the past two decades (McEwen, 1998). More recently, this concept has been transposed to populations of animals which have yielded studies that

suggest similar implications (Arlettaz et al., 2015). However, allostatic load application may be limited to only subjects with medical histories and those that elicit high-level cognition. Measuring allostatic load presents great benefits for animals in controlled populations such as captive wildlife, production animals or even certain pets, given that investigators have access to detailed health records. These reports help distinguish and quantify perceived acute stressors (immobilizations, illness, transportation, rearing history, etc.) that an individual may experience through life, directly corresponding to total stress (Edes et al., 2018). Clearly, in-depth medical documents for free-ranging wildlife are nonexistent, as well as impractical. Furthermore, the allostatic load model heavily relies on secondary psychosomatic stress or conscious recognition of a potential stressor. For example, a gorilla surrendered from the wild is more likely to mount the stress response in the presence of a human, in comparison to one that was raised in captivity who lacks that negative association. Anticipation of stress, learned from a previous event, has not yet been documented in relatively low-level thinking animals and therefore, allostatic load may be too narrow of a value.

The medical history evaluations and serum biomarker data obtained and compared through this study, may indicate that health outcomes of free-ranging individuals can be predicted without knowledge of specific stress events or individual mental capacity. Using allostatic load as a tool to establish baseline serum levels in captive animals that are systematically less-stressed, chronic stress may still be measured in free-ranging animals using a new scoring system called comprehensive health score (CHS). Although details as to what events specifically contribute to somatic malfunction remain unknown, the impact of historical stressors can still be evaluated within wild populations. Biomarkers that reflect allostatic dysregulation should be included in

comprehensive health score indices for the purpose of predicting future outcomes as they relate to chronic stress. With possibilities to protect vulnerable species and allocate resources, comprehensive health score can contribute to decisions regarding environmental decline.

LITERATURE CITED

Adewuyi, A. A., Gruys, E., & van Eerdenburg, F. J. C. M. (2005). Non esterified fatty acids (NEFA) in dairy cattle. A review. *Veterinary Quarterly, 27*(3), 117-126. doi:10.1080/01652176.2005.9695192

Arlettaz, R., Nusslé, S., Baltic, M., Vogel, P., Palme, R., Jenni - Eiermann, S., Genoud, M. (2015). Disturbance of wildlife by outdoor winter recreation: allostatic stress response and altered activity–energy budgets. *Ecological Applications, 25*(5), 1197-1212. doi:10.1890/14-1141.1

Asli, O., & Flaten, M. (2012). In the Blink of an Eye: Investigating the Role of Awareness in Fear Responding by Measuring the Latency of Startle Potentiation. *Brain Sciences, 2*(1), 61-84. doi:10.3390/brainsci2010061

Bartlett, D. (1998). Stress: perspectives and processes: Buckingham: Open University Press ; Philadelphia: Open University Press.

Bashaw, M. J., Sicks, F., Palme, R., Schwarzenberger, F., Tordiffe, A. S. W., & Ganswindt, A. (2016). Non-invasive assessment of adrenocortical activity as a measure of stress in giraffe (Giraffa camelopardalis). *BMC veterinary research, 12*(1), 235. doi:10.1186/s12917-016-0864-8

Beckie, T. M. (2012). A Systematic Review of Allostatic Load, Health, and Health Disparities. *Biological Research For Nursing, 14*(4), 311-346. doi:10.1177/1099800412455688

Bjornvad, C., Nielsen, M., Hansen, S., & Nielsen, D. (2017). The effect of position on the precision of dual-energy X-ray absorptiometry and correlation with body condition score in dogs and cats. *Journal of Nutritional Science, 6*. doi:10.1017/jns.2017.16

Bonier, F., Martin, P. R., Moore, I. T., & Wingfield, J. C. (2009). Do baseline glucocorticoids predict fitness? *Trends in Ecology & Evolution, 24*(11), 634-642. doi:10.1016/j.tree.2009.04.013

Boonstra, R., Dušek, A., & Flynn, R. W. (2018). DHEA and territoriality during the nonbreeding season in male American martens (*Martes americana*). *Journal of Mammalogy, 99*(4), 826-835. doi:10.1093/jmammal/gyy067

Boudreau, M. R., Seguin, J. L., Boonstra, R., Palme, R., Boutin, S., Krebs, C. J., & Murray, D. L. (2019). Experimental increase in predation risk causes a cascading stress response in free-ranging snowshoe hares. *Oecologia, 191*(2), 311. doi:10.1007/s00442-019-04500-2

Card, W. C., Kirkpatrick, R. L., Webb, K. E., & Scanlon, P. F. (1985). Nutritional Influences on Nefa, Cholesterol, and Ketones in White-Tailed Deer. *The Journal of Wildlife Management, 49*(2), 380-385. doi:10.2307/3801537

Coblentz, B. E. (1975). Serum Cholesterol Level Changes in George Reserve Deer. *The Journal of Wildlife Management, 39*(2), 342-345. doi:10.2307/3799912

Cooper, C. L., & Dewe, P. (2004). Stress: A Brief History. Oxford, UK: Blackwell. doi:10.1002/9780470774755

Dagg, A. I. (2014). Giraffe: Biology, Behaviour and Conservation. United States: Cambridge University Press.

David, D. G., Katherine, L. P., & Douglas, C. H. (2007). Using ultrasound measurements of rump fat to assess nutritional condition of woodland caribou in northern British Columbia, Canada. *Rangifer, 27*(4). doi:10.7557/2.27.4.351

Dickens, M. J., Delehanty, D. J., & Michael Romero, L. (2010). Stress: An inevitable component of animal translocation. *Biological Conservation, 143*(6), 1329-1341. doi:10.1016/j.biocon.2010.02.032

Dijkstra, J., Forbes, J. M., & France, J. (2005). Quantitative aspects of ruminant digestion and metabolism (2nd ed.). Wallingford, Oxfordshire, UK ; Cambridge, MA: Wallingford, Oxfordshire, UK ; Cambridge, MA: CABI Pub.

Doublet, S. (2000). The stress myth: Chesterfield, MO: Science & Humanities Press.

Ebrecht, M., Hextall, J., Kirtley, L.-G., Taylor, A., Dyson, M., & Weinman, J. (2004). Perceived stress and cortisol levels predict speed of wound healing in healthy male adults. *Psychoneuroendocrinology, 29*(6), 798-809. doi:10.1016/S0306-4530(03)00144-6

Edes, A. N., Wolfe, B. A., & Crews, D. E. (2016). Rearing history and allostatic load in adult western lowland gorillas (*Gorilla gorilla gorilla*) in human care. *Zoo Biology, 35*(2), 167-173. doi:10.1002/zoo.21270

Edes, A. N., Wolfe, B. A., & Crews, D. E. (2018). Evaluating allostatic load: a new approach to measuring long-term stress in wildlife. *49*(2), 272-282. doi:10.1638/2016-0070.1

Pride, E. (2005). High faecal glucocorticoid levels predict mortality in ring-tailed lemurs (*Lemur catta*). *Biology letters, 1*(1), 60. doi:10.1098/rsbl.2004.0245

Farooqi, N. A. I., Scotti, M., Yu, A., Lew, J., Monnier, P., Botteron, K. N., Nguyen, T. V. (2019). Sex-specific contribution of DHEA-cortisol ratio to prefrontal-hippocampal structural development, cognitive abilities and personality traits. *Journal of Neuroendocrinology, 31*(2), n/a-n/a. doi:10.1111/jne.12682

Gant, P., Holden, S. L., Biourge, V., & German, A. J. (2016). Can you estimate body composition in dogs from photographs?(Report). *BMC Veterinary Research, 12*(17). doi:10.1186/s12917-016-0642-7

Gifford, A., Kullberg, J., Berglund, J., Malmberg, F., Coate, K. C., Williams, P. E.,Welch, E. B. (2014). Canine body composition quantification using 3 tesla fat–water MRI. *Journal of Magnetic Resonance Imaging, 39*(2), 485-491. doi:10.1002/jmri.24156

Ginnett, D. A., Theis, J. H., & Kaneko, J. J. (2003). Spontaneous gallstone formation in deer mice: interaction of cholesterol, bile acids, and dietary fiber. *Journal of wildlife diseases, 39*(1), 105. doi:10.7589/0090-3558-39.1.105

Goldman, N., & Glei, D. A. (2007). Sex differences in the relationship between DHEAS and health. *Experimental Gerontology, 42*(10), 979-987. doi:10.1016/j.exger.2007.05.005

Gundlach, N. H., Schmicke, M., Ludes-Wehrmeister, E., Ulrich, S. A., Araujo, M. G., & Siebert, U. (2018). New approach to stress research in phocids--potential of dehydroepiandrosterone and cortisol/dehydroepiandrosterone ration as markers for stress in harbor seals (*Phoca vitulina*) and gray seals (*Halichoerus grypus*). *Journal of Zoo Wildlife Medicine.* 49: 556-563. doi:10.1638/2017-0191.1

Hajduk, P., Copland, M. D., & Schultz, D. A. (1992). Effects of capture on hematological values and plasma cortisol levels of free-range koalas (Phascolarctos cinereus). *Journal of wildlife diseases, 28*(3), 502. doi:10.7589/0090-3558-28.3.502

Hickman, D. L., & Swan, M. (2010). Use of a body condition score technique to assess health status in a rat model of polycystic kidney disease. *Journal of the American Association for Laboratory Animal Science : JAALAS, 49*(2), 155.

Hill, M. R. J., Alisauskas, R. T., Ankney, C. D., & Leafloor, J. O. (2003). Influence of body size and condition on harvest and survival of juvenile Canada geese. *The Journal of Wildlife Management, 67*(3), 530-541. doi:10.2307/3802711

Hu, P., Seeman, T. E., Harris, T. B., & Reuben, D. B. (2003). Does Inflammation or Undernutrition Explain the Low Cholesterol–Mortality Association in High-Functioning Older Persons? MacArthur Studies of Successful Aging. *Journal of the American Geriatrics Society, 51*(1), 80-84. doi:10.1034/j.1601-5215.2002.51014.x

Ji-Eun, L. (2015). Alternative biomarkers for assessing glycemic control in diabetes: fructosamine, glycated albumin, and 1,5-anhydroglucitol. *Annals of Pediatric Endocrinology & Metabolism, 20*(2), 74-78. doi:10.6065/apem.2015.20.2.74

Juster, R.-P., McEwen, B. S., & Lupien, S. J. (2010). Allostatic load biomarkers of chronic stress and impact on health and cognition. *Neuroscience and Biobehavioral Reviews, 35*(1), 2-16. doi:10.1016/j.neubiorev.2009.10.002

Kari, A. M., John, L., Christina, A., Jeff, B., & Janine, L. B. (2014). Development of a body condition scoring index for female African elephants validated by ultrasound measurements of subcutaneous fat. *PLoS ONE, 9*(4), e93802. doi:10.1371/journal.pone.0093802

Kearney, C. C., & Ball, R. L. (2001). Body Scoring System for Captive Giraffe *(Giraffa camelopardalis). Giraffe Resource Centre.* Paper presented at the AAZV.

Kobayashi, T., Koie, H., Kusumi, A., Kitagawa, M., Kanayama, K., & Otsuji, K. (2014). Comparative Investigation of Body Composition in Male Dogs Using CT and Body Fat Analysis Software. *The Journal of Veterinary Medical Science, 76*(3), 439-446. doi:10.1292/jvms.13-0397

Lennartsson, A.-K., Kushnir, M. M., Bergquist, J., & Jonsdottir, I. H. (2012). DHEA and DHEA-S response to acute psychosocial stress in healthy men and women. *Biological Psychology, 90*(2), 143-149. doi:10.1016/j.biopsycho.2012.03.003

Leroy, J. L. M. R., Vanholder, T., Mateusen, B., Christophe, A., Opsomer, G., de Kruif, A., Van Soom, A. (2005). Non-esterified fatty acids in follicular fluid of dairy cows and their effect on developmental capacity of bovine oocytes in vitro. *Reproduction (Cambridge, England), 130*(4), 485. doi:10.1530/rep.1.00735

Lupien, S., Mony De, L., Susan De, S., Antonio, C., Chaim, T., Nair, N. P. V., Michael, J. M. (1998). Cortisol levels during human aging predict hippocampal atrophy and memory deficits. *Nature Neuroscience, 1*(1), 69. doi:10.1038/271

MacDougall-Shackleton, S. A., Bonier, F., Romero, L. M., & Moore, I. T. (2019). Glucocorticoids and "Stress" Are Not Synonymous. *Integrative Organismal Biology, 1*(1). doi:10.1093/iob/obz017

Malmström, H., Walldius, G., Grill, V., Jungner, I., Gudbjörnsdottir, S., & Hammar, N. (2014). Fructosamine Is a Useful Indicator of Hyperglycaemia and Glucose Control in Clinical and Epidemiological Studies – Cross-Sectional and Longitudinal Experience from the AMORIS Cohort (Fructosamine and Hyperglycaemia - AMORIS Cohort). *PLoS One 9*(10), e111463. doi:10.1371/journal.pone.0111463

McEwen, B. S. (1998). Protective and damaging effects of stress mediators. (Seminars in Medicine of the Beth Israel Deaconess Medical Center)(Review Article). *The New England Journal of Medicine, 338*(3), 171. doi:10.1056/NEJM199801153380307

McEwen, B. S. (1998). Stress, adaptation, and disease. Allostasis and allostatic load. *Annals of the New York Academy of Sciences, 840*, 33.

McEwen, B. S. (2003). Early life influences on life- long patterns of behavior and health. *Mental Retardation and Developmental Disabilities Research Reviews, 9*(3), 149-154. doi:10.1002/mrdd.10074

McEwen, B. S. (2006). Protective and damaging effects of stress mediators: central role of the brain. *Dialogues in Clinical Neuroscience, 8*(4), 367-381.

Mellish, J.-A. E., Tuomi, P. A., & Horning, M. (2004). Assessment of ultrasound imaging as a noninvasive measure of blubber thickness in pinnipeds. *Journal of zoo and wildlife medicine : official publication of the American Association of Zoo Veterinarians, 35*(1), 116. doi:10.1638/03-028

Menchetti, L., Brecchia, G., Cardinali, R., Polisca, A., & Boiti, C. (2015). Feed restriction during pregnancy: effects on body condition and productive performance of primiparous rabbit does. *World Rabbit Science,* 23 (1), 1-8. doi:10.4995/wrs.2015.1703

Muller, Z., Bercovitch, F., Brand, R., Brown, D., Brown, M., Bolger, D., Carter, K., Deacon, F., Doherty, J.B., Fennessy, J., Fennessy, S., Hussein, A.A., Lee, D., Marais, A., Strauss, M., Tutchings, A. & Wube, T. 2018. *Giraffa camelopardalis* (amended version of 2016 assessment). *The IUCN Red List of Threatened Species* 2018: e.T9194A136266699. https://dx.doi.org/10.2305/IUCN.UK.2016-3.RLTS.T9194A136266699.en.

Payan-Carreira, R., Martins, L., Miranda, S., Olivnrio, P., & Silva, S. R. (2016). In vivo assessment of subcutaneous fat in dogs by real-time ultrasonography and image analysis.(Report). *Acta Veterinaria Scandinavica, 58*. doi:10.1186/s13028-016-0239-y

Quiroz-Rocha, G. F., Leblanc, S., Duffield, T., Wood, D., Leslie, K. E., & Jacobs, R. M. (2009). Evaluation of prepartum serum cholesterol and fatty acids concentrations as predictors of postpartum retention of the placenta in dairy cows. *Journal of the American Veterinary Medical Association, 234*(6), 790. doi:10.2460/javma.234.6.790

Roberts, A., Geiss, E., Fawaz, L., & Lopez-Duran, N. (2016). Impact of DHEA to cortisol ratios on HPA-axis reactivity: Moderation by sex and age. *Psychoneuroendocrinology, 71*(sS), 28-28. doi:10.1016/j.psyneuen.2016.07.080

Rodrigues, S. M., LeDoux, J. E., & Sapolsky, R. M. (2009). The Influence of Stress Hormones on Fear Circuitry. *Annu. Rev. Neurosci., 32*(1), 289-313. doi:10.1146/annurev.neuro.051508.135620

Romero, L. M. (2004). Physiological stress in ecology: lessons from biomedical research. *Trends in Ecology & Evolution, 19*(5), 249-255. doi:10.1016/j.tree.2004.03.008

Romero, L. M., & Gormally, B. M. G. (2019). How Truly Conserved Is the "Well-Conserved" Vertebrate Stress Response? *Integrative and comparative biology, 59*(2), 273. doi:10.1093/icb/icz011

Schmidt, D. A., Ellersieck, M. R., Cranfield, M. R., & Karesh, W. B. (2006). Cholesterol Values in Free-Ranging Gorillas (*Gorilla gorilla gorilla* and *Gorilla beringei*) and Bornean Orangutans (*Pongo pygmaeus*). *Journal of Zoo and Wildlife Medicine, 37*(3), 292-300. doi:10.1638/05-040.1

Sejian, V., Maurya, V. P., Naqvi, S. M. K., Kumar, D., & Joshi, A. (2010). Effect of induced body condition score differences on physiological response, productive and reproductive performance of Malpura ewes kept in a hot, semi-arid environment. *Journal of Animal Physiology and Animal Nutrition, 94*(2), 154-161. doi:10.1111/j.1439-0396.2008.00896.x

Selvin, E., Rawlings, A. M., Lutsey, P. L., Maruthur, N., Pankow, J. S., Steffes, M., & Coresh, J. (2015). Fructosamine and Glycated Albumin and the Risk of Cardiovascular Outcomes and Death. *Circulation, 132*(4), 269. doi:10.1161/CIRCULATIONAHA.115.015415

Selye, H. (1982). History and present status of the stress concept. In L.Goldberger and S. Breznitz, (eds). *Handbook of Stress: Theoretical and Clinical Aspects* (New York: Free Press), 7-17.

Stephenson, T. R., Hundertmark, K. J., Schwartz, C. C., & Ballenberghe, V. V. (1998). Predicting body fat and body mass in moose with ultrasonography. *Canadian Journal of Zoology, 76*(4), 717-722. doi:10.1139/z97-248

Strandberg, T. E., Strandberg, A., Rantanen, K., Salomaa, V. V., Pitkälä, K., & Miettinen, T. A. (2004). Low cholesterol, mortality, and quality of life in old age during a 39-year follow-up. *Journal of the American College of Cardiology, 44*(5), 1002-1008. doi:10.1016/j.jacc.2004.04.057

Stringer, E. M., Stoskopf, M. K., Simons, T., O'Connell, A. F., & Waldstein, A. (2010). Ultrasonic Measurement of Body Fat as a Means of Assessing Body Condition in Free-Ranging Raccoons (*Procyon lotor*). *International Journal of Zoology, 2010*(2010). doi:10.1155/2010/972380

Takeshita, R. S. C., Huffman, M. A., Mouri, K., Shimizu, K., & Bercovitch, F. B. (2016). Dead or alive? Predicting fetal loss in Japanese macaques (*Macaca fuscata*) by fecal metabolites. *Animal Reproduction Science, 175*, 33-38. doi:10.1016/j.anireprosci.2016.10.006

Valckx, S. D. M., De Pauw, I., De Neubourg, D., Inion, I., Berth, M., Fransen, E., Leroy, J. L. M. R. (2012). BMI-related metabolic composition of the follicular fluid of women undergoing assisted reproductive treatment and the consequences for oocyte and embryo quality. *Human Reproduction, 27*(12), 3531-3539. doi:10.1093/humrep/des350

Valenzuela, A., Sanhueza, J., & Nieto, S. (2003). Cholesterol oxidation: health hazard and the role of antioxidants in prevention. *Biological research, 36*(3-4), 291. doi:10.4067/S0716-97602003000300002

Vieira-Marques, C., Arbo, B. D., Ruiz-Palmero, I., Ortiz-Rodriguez, A., Ghorbanpoor, S., Kucharski, L. C., Ribeiro, M. F. M. (2016). Dehydroepiandrosterone protects male and female hippocampal neurons and neuroblastoma cells from glucose deprivation. *Brain Research, 1644,* 176-182. doi:10.1016/j.brainres.2016.05.014

Wan Ahmad, W. N. H., Sakri, F., Mokhsin, A., Rahman, T., Mohd Nasir, N., Abdul-Razak, S., Nawawi, H. (2015). Low Serum High Density Lipoprotein Cholesterol Concentration is an Independent Predictor for Enhanced Inflammation and Endothelial Activation (Low HDL Is an Independent Predictor). *PLoS One,* 10(1), e0116867. doi:10.1371/journal.pone.0116867

Wilkening, J. L., Ray, C., & Varner, J. (2016). When can we measure stress noninvasively? Postdeposition effects on a fecal stress metric confound a multiregional assessment. *Ecology and Evolution,* 6 *(*2), 502-513. doi:10.1002/ece3.1857

Yamanashi, Y., Teramoto, M., Morimura, N., Hirata, S., Inoue-Murayama, M., Idani, G. i., & Latzman, R. D. (2016). Effects of Relocation and Individual and Environmental Factors on the Long-Term Stress Levels in Captive Chimpanzees (*Pan troglodytes*): Monitoring Hair Cortisol and Behaviors. *PLoS ONE, 11*(7). doi:10.1371/journal.pone.0160029

Chapter 3

COMPARISON OF COMPREHENSIVE HEALTH SCORE IN NORTH AMERICAN HOUSED GIRAFFE AND FREE-RANGING GIRAFFE FROM SOUTH AFRICA

ABSTRACT

As in humans, stress is evident in many animal species and has been correlated to disease prevalence; yet a value for reliable quantification of chronic stress is unestablished. During stressful events, allostasis, an adaptive process, is initiated by physiologic systems to maintain or reestablish homeostasis to protect an organism's viability. Over time, the acclimation to frequent stress causes systematic dysregulation, leading to the phenomena of increased allostatic load. In recent studies, allostatic load has been assessed in animal species via serum through selection of representative, multi-system biomarker indices. Perception and number of stress events an individual experiences may impact dysregulation severity, yielding allostatic load as a valuable tool in predicting future outcomes. However, the allostatic load methodology poses application limitations to individuals without historical data and those lacking a conscious recognition of stress. Comprehensive health score may be more encompassing of populations, as it targets biomarkers dysregulated by life events associated with pathology, despite an unknown level of cognition or history. Serum samples were obtained from zoo-housed (n=18) and free-ranging (n=11) giraffe to predict subclinical risk of morbidity and mortality caused by chronic stress. Serum concentrations of selected biomarkers were investigated using colorimetric ELISAs (cortisol, DHEA-S), the CHOD-PAP method (cholesterol), colorimetric enzymatic method (NEFA), and nitroblue tetrazolium assay (fructosamine). Selection of these biomarkers were based on

the ability to estimate dysregulation of physiologic processes impacted by stress accumulation, such as the neuroendocrine, metabolic, and inflammatory systems. Free-ranging giraffe were younger (m = 7.9 years) on average and had lower cholesterol ($p = 0.016$) and fructosamine ($p = 0.039$) levels when compared to captive giraffe (m = 12.8 years). Additionally, free-ranging giraffe had higher cortisol ($p = 0.007$) levels and NEFA ($p = 0.004$) status, while DHEA-S ($p = 0.548$) was found at relatively similar concentrations between the populations. Although suitable composites rely heavily on specific species and environmental factors, comprehensive health score provides a foundation for a more applicable tool in conservation research through comparison of biomarkers across populations.

INTRODUCTION

Given its complex relationship with disease development, "stress" has been a concept of interest for many centuries. Thought to be influenced by an individual's environment, the physiological, hormonal, and behavioral stress from major life events and daily pressures have been linked to ill health (Cooper & Dewe, 2004). Vulnerable free-ranging animal populations, such as giraffe (*Giraffa camelopardali*s), could be subjected to increased hypervigilance, a component associated with stress, due to environmental decline and human infringement (Edes et al., 2018).

Encountering a dangerous event in an unregulated environment initiates autonomic and endocrine compensation through allostasis, enhancing the possibility of survival (Dickens et al., 2010; Romero & Gormally, 2019). However, overactivation of the stress response due to the animal continuously coping with environmental threats may cause allostatic systems to fatigue, leading to chronic stress, or allostatic load, accumulation over time (McEwen, 1998). Stress assessment methodology, originally

established in humans, has the potential to be transposed to animal research which may effectively evaluate multi-system dysregulation and predict future health outcomes.

For validation purposes, a baseline model of clinically normal parameters is warranted for an accurate comparison. Captive animals living in more regulated environments are expected to experience less systematic stress than free-ranging individuals that are less conditioned to cohabitating with humans (Gundlach et al., 2018). Representation of system dysregulation may be offered through serum biomarker selection and scored using a summation of high-risk quartiles resulting in the animal's comprehensive health score or allostatic load. Additionally, body condition score, a biometric value, may also be useful in assessing health and fitness of populations (Stringer et al., 2010).

To be able to encompass animals of any consciousness and without historical data, an extension to the existing scoring system is necessary. Utilizing comprehensive health score in free-ranging populations may yield similar implications as allostatic load methodology. Although causes of somatic exhaustion are left unidentified with comprehensive health score, biomarkers that indicate dysregulation may aid in predicting future outcomes in relation to stress and can contribute to conservation decisions regarding free-ranging animals. The objective of this study was to evaluate comprehensive health score as a measurement of chronic stress in giraffe (*Giraffa camelopardalis*) and compare accumulation of North American housed individuals to those free-ranging in South Africa.

METHODS & MATERIALS

Animals

To evaluate chronic stress in captive giraffe (*Giraffa camelopardalis)*, eighteen frozen serum samples were obtained from individuals housed in a single North American zoo (Omaha Henry Doorly Zoo and Aquarium, Omaha, NE). The procedure was carried out in a low stress environment, as only individuals trained to stand for voluntary blood draws from the jugular were considered. Animals ranged in age from 2 to 26 years old and were clinically healthy (not receiving medication or treatment) at the time of the collection procedure. Of samples from 18 individuals, 16 were collected between 2010, 2011, or 2012 and two were from more recent blood collections in 2017 and 2018. Samples were immediately placed on ice and were moved into storage at -80 C within six hours of collection until requested in the summer of 2018.

For comparison, 22 fresh blood samples were opportunistically collected from a population of free-ranging giraffe (*Giraffa camelopardalis*) composed of 26 animals in the Rooipoort Nature Reserve of Kimberly, South Africa. As part of ongoing management of the reserve, animals between the ages of 3 to 12 years were immobilized for the application of tracking collars and health evaluations. Collections on distinct individuals took place over a period of two years.

Sample Collection and Analysis from Captive Giraffe

Blood was collected from the jugular vein of each animal, using an 18 gauge or larger hypodermic needle and drawn into a 20 ml syringe. Before the time of shipment, samples were distributed into eight tubes (six serum separator tubes and two Ca-EDTA whole blood tubes. BD, Franklin Lakes, NJ), each containing 2.5 ml per tube. Serum separator tubes were centrifuged at 2000 RPM, revolutions per minute, for ten minutes. The

supernatant was transferred off the clotted red cells using a clean Pasteur pipette into cryogenic storage vials (ThermoFisher Scientific, Waltham, MA). Samples were maintained in an ultralow, -80 C freezer (ULT1390-10-D, Revco CxF Series, ThermoFischer Scientific) from the time of collection until packaged in a Styrofoam container on ice and shipped overnight from Fedex to the Cornell Clinical Pathology Lab and the St. Louis Zoo Endocrinology Lab for analysis.

Samples were further divided for additional diagnostic testing. All samples processed for cortisol and DHEA-S were quantified with commercially available enzyme immunoassay (EIA) kits (DetectX © Cortisol EIA K003-H5W and DetectX © DHEA-S EIA K054-H5, Arbor Assays, Ann Arbor, MI) with lowest detection limits of 45.4 pg/ml and 75.6 pg/ml, respectively at the St. Louis Endocrinology Laboratory as validated for ruminants (Möstl et al., 2002). Samples were diluted either 1:100 or 1:1000 with assay buffer (catalog number: X053-55ML, Arbor Assays, Ann Arbor, MI), and assays were performed according to the manufacturer's protocol. Cholesterol concentration in serum was evaluated using the CHOD-PAP method (Lumb & Slavin, 1993), NEFA were measured via a colorimetric enzymatic method (Mizuno et al., 1980), and fructosamine levels were detected using a nitroblue tetrazolium assay (Chung et al., 1988), each modified to meet quality control standards of the Cornell University College of Veterinary Medicine Pathology Laboratory as provided below:

In the first step of the three stage reaction, the cholesterol oxidase/peroxidase aminophenazone (CHOD-PAP) method, enzyme cholesterol esterase hydrolyzes cholesterol esters to yield free fatty acids and cholesterol. In the next step, cholesterol oxidase catalyzes the oxidation of cholesterol to cholest-4-en-3-one. Under the catalytic action of peroxidase, hydrogen peroxide produced in the previous reaction oxidizes the

chromophore 4-aminophenazone, in the presence of phenol, to the red dye compound 4-(p-benzoquinone-monoimino)-phenazone. The color intensity change at 500-550 nm is measured photometrically and is directly proportional to the concentration of cholesterol in the sample (Burtis & Ashwood, 1999).

In the first step of the three stage reaction of NEFA colorimetric methodology, acyl-CoA synthetase (ACS) catalyzes the acylation of NEFAs to (coenzyme A) CoA resulting in the formation of acyl-CoA. Hydrogen peroxide is then generated from the oxidation of acyl-CoA through the catalytic action of acyl-CoA oxidase (ACOD). Lastly, hydrogen peroxide in the presence of peroxidase (POD) allows for the oxidative condensation of 4-aminoantipyrine with 3-methyl-N-ethyl-N-(β-hydroxyethyl)-aniline (MEHA) to form a blue-purple end product with an absorbance maximum at 550 nm. NEFA concentration is directly proportional to the measured optical density of the dye product (Miksa et al., 2004).

Fructosamine nitroblue tetrazolium assays consist of rearranging ketoamines in alkaline conditions, forming eneaminol, which reduces the chemical compound nitroblue tetrazolium, in a one-step reaction, to formazan. A ten-minute preincubation period is recommended to circumvent interference effects from fast-reacting, nonspecific reducing substances. The formation of formazan is quantified from an absorbance change over time (Cefalu et al., 1991).

Additionally, because body condition score (BCS) is seen as a useful metric for determining fitness and health status in a variety of species, it was visually evaluated and recorded for each animal at the time of collection. BCS scores were documented by the examining veterinarian in the medical record at the time of blood collection and were recorded on either a 1-5 or 1-9 point scale. Values collected on the 1-5 scale were

adapted to fit the 1-9 scale as such: 1=1, 1.5=2, 2=3, 2.5=4, 3=5, 3.5=6, 4=7, 4.5=8, and 5=9. Physical condition correlating with the BCS value are enumerated below:

1 - Emaciated condition with absent visible fat, prominent skeletal details, and muscle wasting
3 - Lean body condition with suboptimal fat stores, noticeable skeletal details, and lean muscling
5 – Ideal condition with adequate fat, only notable features of skeletal frame (pelvis & spine)
7 - Overweight condition with lost definition of notable features and fat deposition on the chest
9 - Obese condition with extensive fat, palpable loss of skeletal features, protruding abdomen

Documented "stress events," including immobilizations, transfers, illness, or pregnancy/birth, were also recorded using Zoo Information Management Software (ZIMS) digital medical records. Stressful events (Figure 4) were tabulated by the authors interpreting medical records and husbandry records provided by the cooperating institution. Immobilizations were defined as events during which an anesthetic drug was injected to facilitate or accompany restraint. Transfers were defined as events during which the animal was loaded into a trailer for transport. Newly documented diseases or injuries that required medical intervention were classified as illnesses, including

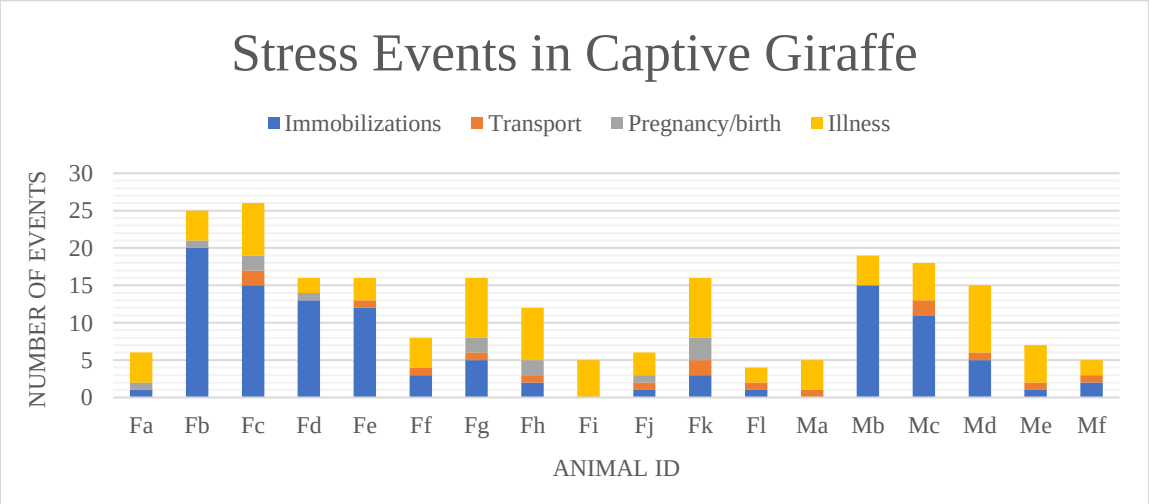

FIGURE 4. Documented stress events (immobilizations, transport, pregnancy/birth/illness) obtained from ZIMS for allostatic load calculation in captive giraffe (n=18). Uppercase F and M letters denote female and male individuals, respectively; lowercase letters represent individual identifiers of the captive population.

recurrence of a previously resolved illness. Pregnancy/birth was defined as a confirmed pregnancy, either by observed birth or rectal ultrasound, and included stillbirths and abortions. All medical records were evaluated by the same individual. The biomarker results were compiled and submitted for statistical analysis.

Sample Collection and Analysis from Free-Ranging Giraffe

Free-ranging, sexually mature giraffe were immobilized using etorphine (M99; Novartis Animal Health SA) alone administered via Pneu-Dart (Pneu-Dart; Williamsport, PA) from a helicopter after a short pursuit. Anesthesia of immobilized animals were reversed using Naltrexone (Wildlife Pharmaceuticals; White River, South Africa). immediately upon gaining physical restraint. Immobilized animals all belonged to the Rooipoort Nature Reserve (De Beers Group, South Africa). Blood was collected from the jugular vein using a 60 ml syringe and 18-gauge needle (BD; Johannesburg, South Africa). All samples processed for cortisol, DHEA-S, cholesterol, NEFA, and fructosamine concentrations were run following kit instructions on bovine validated ELISA kits (MyBioSource © Catalog Numbers MBS166285, MBS008229, MBS737068, MBS748204, and MBS737068, respectively, San Diego, CA, performed by Ampath Laboratories, Zwartkop, South Africa). Body condition scores for free-ranging giraffe were determined by a single zoo veterinarian, with experience in evaluating giraffe body

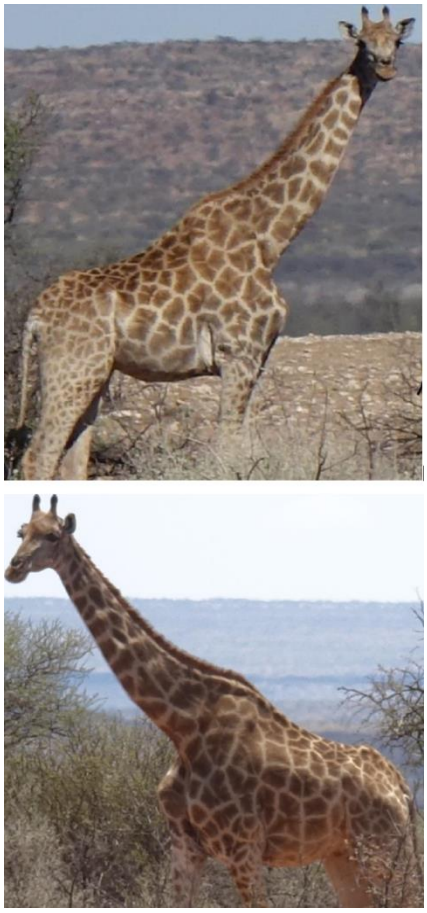

FIGURE 5. Examples of photos provided by field collection team for body condition scoring evaluation (Ciska Scheijen, University of the Free State, South Africa).

condition, using a series of photos provided by the field collection team (Figure 5). Habituation level was established as “well,” “semi,” or “not” as determined by an experienced ecologist onsite. The delineating distance between “well” and “semi” habituated animals was a flight distance of greater than or less than 150 meters. For statistical analysis numerical values were substituted for the subjective assessments: 0=not habituated, 1=semi habituated, and 2=well habituated.

Statistical Analysis

Statistical analyses were performed using JASP software (version 0.10) with each biomarker as independent experimental units. The CORR procedure of JASP was used to determine Pearson correlation coefficients between each individual biomarker and between biomarkers and stress accumulation. Concentrations of cortisol, DHEA-S, cholesterol, NEFA, and fructosamine were analyzed as continuous outcomes, while BCS, age, and life events were evaluated as ordinal values. Pearson correlations were also used to evaluate habituation level and its relationship with BCS in free-ranging giraffe. Additionally, the Statistical Cross-disciplinary Collaboration and Consulting Lab (SC3L) at the University of Nebraska-Lincoln utilized a binomial distribution model (PROC GLIMMIX in SAS) to represent response variables such as gender and age in relation to stress accumulation. P-values less than or equal to 0.05 were considered significant and values between 0.05 and 0.10 indicated a possible trend.

RESULTS

The comparison of biometric and biomarker averages from captive (n=18) and FR giraffe (n=11) is shown in Table 1 and the population demographics are illustrated

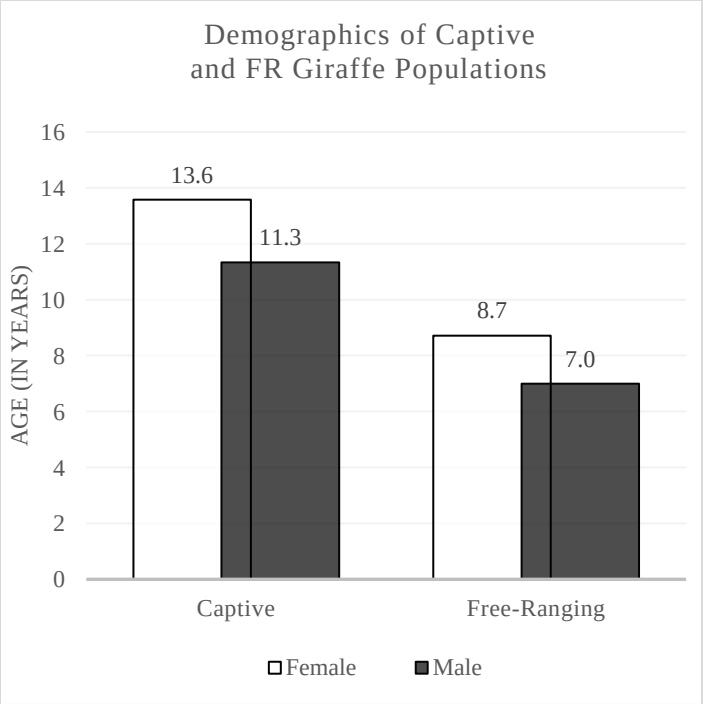

FIGURE 6. Approximate age of captive (n=18) and free-ranging giraffe populations (n=11).

in Figure 6. Of the 22 samples collected opportunistically at Rooipoort Nature Reserve, eleven were discarded due to the detection of moderate to severe hemolysis, or lysed red blood cells, suspected to have occurred from samples thawing and refreezing after transport. Samples with a hemolysis score of less than three (on a scale of four) were deemed suitable for testing. The mean age, BCS, cholesterol, and fructosamine levels were higher ($p < 0.05$) for zoo-housed giraffe in comparison to free-ranging giraffe. The opposite was true for cortisol level and NEFA status, whereas there was not a significant difference for DHEA-S in the two populations.

TABLE 1. Comparison of Biometric (Age, BCS) and Biomarker Value (Cortisol, DHEA-S, Cholesterol, NEFA, Fructosamine) Means in Captive and FR Giraffe

Item	Captive (n=18)		Free-Ranging (n=11)		95% CI for Mean Difference		p-value
	Mean	SE	Mean	SE	Lower	Upper	
Age (years)	12.8	1.8	7.9	0.8	0	9.9	0.049
BCS (1-9)	4.8	0.2	3.8	0.4	0.2	1.7	0.011
Cortisol (mg/dL)	35.7	6.3	68.6	10.1	-56	-9.8	0.007
DHEA-S (mg/dl)	46.7	6.4	52.2	4.7	-24.1	13.1	0.548
Cholesterol (mg/dL)	29.7	3.3	18.3	1.4	2.3	20.5	0.016
NEFA (mEq/L)	0.3	0	0.6	0.1	-0.5	-0.1	0.004
Fructosamine (mmol/L)	387.9	36.5	281.5	17.7	5.8	206.9	0.039

BCS, body condition score; DHEA-S, dehydroepiandrosterone-sulfate; NEFA, non-esterified fatty acids.

Stress in Captive Giraffe

As illustrated in Table 2, of the four events classified as "stressful" (immobilizations, transfers, illness, and pregnancy/birth), the level of DHEA-S in captive giraffes was correlated to instances of illness (Pearson's $r = 0.685$, $p = 0.002$) and pregnancy/birth (Pearson's $r = 0.495$, $p = 0.037$), whereas the level of fructosamine was correlated with immobilizations (Pearson's $r = 0.534$, $p = 0.022$). Additionally, there was a correlation between illness and pregnancy/birth (Pearson's $r = 0.531$, $p = 0.023$).

As captive giraffe increased in age, the number of stress events experienced also increased (Pearson's $r = 0.726$, $p = 0.001$), resulting in higher allostatic load scores for certain individuals (Pearson's $r = 0.532$, $p = 0.023$). However, there was no difference in age effect for different genders with a non-significant Age*Gender interaction of $p = 0.40$. There was not enough evidence to conclude that young male giraffes were significantly different from young female giraffes ($p = 0.54$) and the same held true for male and female giraffe older than 21 years of age ($p = 0.47$).

Stress in Free-Ranging Giraffe

Data from the samples that did not have significant degeneration or compromise in quality showed no significant correlation between biomarkers (Table 4). However, there was a correlation between certain biomarkers and comprehensive health score: BCS (Pearson's $r = -0.611$, $p = 0.046$), cortisol (Pearson's $r = 0.634$, $p = 0.036$), DHEA-S (Pearson's $r = -0.734$, $p = 0.001$), and NEFA (Pearson's $r = 0.679$, $p = 0.022$). While these objective values had no or little correlation to each other, subjective evaluation of habituation level in free-ranging giraffes did have a significant correlation to BCS (Pearson's $r = -0.072$, $p = 0.014$; Table 3). When evaluating the larger population (N=26), including those individuals that were not utilized for the biomarker data, this significant

association is maintained (Pearson's r = 0.441, p = 0.024; Table 3). Acknowledging the small number of individuals and the heavily female-skewed sex ratio, these subjective evaluations may provide insight into less quantifiable factors that influence outcomes. There were no significant associations identified in age effect for different genders. Similarly, the same held true when considering pregnancy status or age of unweaned offspring.

TABLE 3. Correlations of Stress Accumulation (CHS) and Subjective Values (Habituation Level, BCS) in Free-Ranging Giraffe

Correlation	Individuals in Study (n=11)		Larger Population (n=26)	
	Pearson's r	p-value	Pearson's r	p-value
CHS & Habituation	-0.712	0.014	—	—
Habituation & BCS	—	—	0.441	0.024

CHS, comprehensive health score; BCS, body condition score.

TABLE 2. Correlations of Stress Variables in Captive Giraffe

		Age	BCS	Cortisol	DHEA-S	Cholesterol	NEFA	Fructosamine	AL	Immobilizations	Transport	Illness	Pregnancy/ Birth	Total Events
Age	Pearson's r	—												
	p-value	—												
BCS	Pearson's r	0.287	—											
	p-value	0.247	—											
Cortisol	Pearson's r	-0.263	-0.06	—										
	p-value	0.293	0.807	—										
DHEA-S	Pearson's r	-0.408	-0.23	0.175	—									
	p-value	0.093	0.367	0.488	—									
Cholesterol	Pearson's r	0.346	0.055	-0.212	-0.219	—								
	p-value	0.16	0.828	0.397	0.384	—								
NEFA	Pearson's r	-0.004	0.242	-0.169	-0.067	0.02	—							
	p-value	0.987	0.334	0.504	0.791	0.937	—							
Fructosamine	Pearson's r	0.193	-0.09	0.136	-0.029	0.048	0.37	—						
	p-value	0.443	0.73	0.591	0.908	0.85	0.131	—						
Allostatic Load	Pearson's r	0.182	-0.32	0.309	-0.328	0.429	0.2	0.446	—					
	p-value	0.47	0.193	0.212	0.184	0.076	0.427	0.063	—					
Immobilizations	Pearson's r	*0.558	-0.12	0.048	-0.143	0.3	0.27	*0.534	0.421	—				
	p-value	0.558	0.634	0.849	0.571	0.226	0.279	0.022	0.082	—				
Transport	Pearson's r	0.558	0.304	0.119	-0.266	-0.023	0.373	-0.242	0.279	-0.106	—			
	p-value	0.558	0.221	0.639	0.285	0.929	0.127	0.333	0.263	0.675	—			
Illness	Pearson's r	0.558	0.262	-0.232	**-0.685	0.149	0.227	0.164	0.345	-0.045	0.42	—		
	p-value	0.558	0.294	0.355	0.002	0.556	0.365	0.516	0.161	0.861	0.082	—		
Pregnancy/Birth	Pearson's r	*0.558	0.326	-0.051	*-0.495	-0.195	0.112	0.241	0.123	0.082	0.313	*0.531	—	
	p-value	0.558	0.187	0.84	0.037	0.438	0.658	0.334	0.627	0.745	0.207	0.023	—	
Total Events	Pearson's r	***0.558	0.046	-0.024	-0.435	0.289	0.366	*0.544	*0.532	***0.893	0.173	0.383	0.405	—
	p-value	0.558	0.857	0.925	0.071	0.244	0.135	0.019	0.023	< .001	0.493	0.117	0.095	—

BCS, body condition score; DHEA-S, dehydroepiandrosterone-sulfate; NEFA, non-esterified fatty acids; AL, allostatic load.
Number of asterisks indicate level of statistical significance; * p < .05, ** p < .01, *** p < .001.

Table 4. Correlations of Biometric and Biomarker Values in Free-Ranging Giraffe

		Age	BCS	Cortisol	DHEA-S	Cholesterol	NEFA	Fructosamine	CHS
Age	Pearson's r	—							
	p-value	—							
BCS	Pearson's r	0.499	—						
	p-value	0.118	—						
Cortisol	Pearson's r	-0.07	-0.314	—					
	p-value	0.837	0.347	—					
DHEA-S	Pearson's r	0.408	0.524	-0.499	—				
	p-value	0.213	0.098	0.118	—				
Cholesterol	Pearson's r	-0.15	-0.467	-0.083	-0.321	—			
	p-value	0.661	0.147	0.807	0.337	—			
NEFA	Pearson's r	-0.06	-0.534	0.301	-0.394	0.461	—		
	p-value	0.872	0.09	0.368	0.23	0.154	—		
Fructosamine	Pearson's r	0.106	-0.076	0.296	0.151	-0.473	0.117	—	
	p-value	0.756	0.825	0.376	0.657	0.141	0.732	—	
CHS	Pearson's r	-0.09	*-0.611	*0.634	*-0.734	0.501	*0.679	0.221	—
	p-value	0.789	0.046	0.036	0.01	0.116	0.022	0.514	—

BCS, body condition score; DHEA-S, dehydroepiandrosterone-sulfate; NEFA, non-esterified fatty acids; CHS, comprehensive health score.
Number of asterisks indicate level of statistical significance; * $p < .05$, ** $p < .01$, *** $p < .001$.

DISCUSSION

A large variety of biomarkers have been selected to comprise allostatic load models (Juster et al., 2010). In the development of a model for captive giraffe, biomarkers were selected that represented diverse physiological systems. Unlike other studies that have developed allostatic load models, this analysis included biomarkers that emphasized measurement of situational or environmental morbidity and mortality, rather than focusing on the strict relationship with the hypothalamic-pituitary-adrenal axis. This choice was made due to the many variables that may contribute to physiological stress and for the purpose of broader application. Comprehensive health score aims to encompass a larger scope of processes that may lead to disease or death in higher risk groups. Biometric (BCS) and biomarker values (cortisol, DHEA-S, cholesterol, NEFA, and fructosamine) were selected for the index due to stability in frozen serum and for ease and consistency of obtaining the values from free-ranging animals.

Considerable stress levels experienced during youth and chronic stress accumulation over time have been linked to an increased morbidity and mortality rate in humans, as well as in a few animal species including western lowland gorillas (Edes et al., 2016; Edes et al., 2018; Juster et al., 2010; McEwen, 2003). However, obtaining recorded histories of free-ranging populations is unrealistic due to economic, staff, and environmental constraints. The population studied here was well-documented because it had been used annually for research purposes, providing substantially more information than most wild populations. For example, the correlation between individual habituation level of free-ranging giraffe studied and BCS ($p = 0.024$) is interesting, but was not included in the CHS model as it cannot be replicated in most wildlife studies. In addition,

methods of monitoring these populations are limited, given that human presence may contribute to stress hormone accumulation and behavior modification (Arlettaz et al., 2015; Baker et al., 2013; Becker & Hall, 2014; Benhaiem et al., 2008; Benhaiem et al., 2013; Creel et al., 2002). One suggestion to address these limitations and insufficiencies is to implement a comprehensive health metric that composites many biomarkers dysregulated by chronic adaptation (McEwen, 1998; Edes et al., 2016; Edes et al., 2018; Juster et al., 2010; McEwen, 1998; Schultner et al., 2013; Seeman et al., 2001). However, the direct observation of some of these biomarkers may be impacted by the restraint or anesthesia required for collection (Buss et al., 2016; Maze et al., 1991). The anesthetic protocol used for free-ranging giraffe was based on clinical success from previous captures.

Body condition score has been used as a metric to estimate fitness and health status in individuals, as well as predicts response to illness, competition, and adverse environmental conditions across many species (Alzaga et al., 2008; Cook et al., 2001; Deacon et al., 2015; Gerhart et al., 1996; Hill et al., 2003; Parker & Freeman, 2011; Sánchez et al., 2018; Schultner et al., 2013). In this study, no differences ($p > 0.1$) were noted between individual captively managed giraffe in body condition score. This result is consistent with controlled environmental conditions, nutritional supplementation, and preventative medical care. However, comparison of BCS in captive giraffe provides additional context to biomarkers that can be modified by emaciated or obese body condition in free-ranging giraffe.

Free-ranging giraffe had lower ($p = 0.011$) body condition scores than captive giraffe assessed in this study with mean values of 3.8 (suboptimal) and 4.8 (nearly

optimal) fat storage, respectively. Given that low body condition scores have been associated with diminished survival for animals exposed to disease or environmental condition, free-ranging giraffe may have less robust nutritional resources (DelGiudice et al., 2011; Parker & Freeman, 2011; Romano et al., 2016). While BCS can predict a suboptimal response to environmental decline, it also may be an indicator of high interspecific competition or an ongoing disease occurrence. Interestingly, free ranging giraffe with more habituation to human presence had significantly higher BCS ($p = 0.024$) compared to peers, potentially indicating a survival advantage in this particular habitat. This value is also helpful in the evaluation of captive giraffe because there are morbidities specifically linked to over conditioned animals (cardiovascular disease, diabetes, arthritis, etc.).

Cortisol has been utilized extensively as a relative measure of health status and stress in a variety of individuals, yet the metric has been inconsistent and unreliably present across species and environmental conditions (Bashaw et al., 2016; Benhaiem et al., 2013; Bonier et al., 2009; Edes et al., 2018; Hajduk et al., 1992; Lupien et al., 1998; Mondelli et al., 2015; Pride, 2005;Wilkening et al., 2016). Although utilization of serum cortisol may not be an accurate assessment of stress, these values can be used to predict morbidity and mortality in certain circumstances (Ebrecht et al., 2004; Lupien et al., 1998; Mondelli et al., 2015; Pride, 2005).

Serum cortisol dysregulation was not correlated ($p = 0.925$) with an accumulation of stressful life events in this population of captive giraffe. However, cortisol values in free-ranging giraffe were higher ($p = 0.007$) than in captive due to short half-life and the rapid change to acute stress. The elevated values in this wild population were expected

due to capture method, which was pursuit via helicopter and darting. Comparison of fecal metabolites would be a viable option, but the collection and environmental conditions pose limitations. Identification of the specific animal source of a fecal pile is time consuming and requires direct intervention with a wild population which may impact stress levels. Additionally, there is climatic degradation due to heat, UV exposure, moisture, and other factors. However, the use of alternative tissues such as fecal metabolites, saliva, hair, and feather samples may provide opportunities to collect data non-invasively (Bashaw et al., 2016; Davenport et al., 2006; Frongia et al., 2020).

DHEA-S plays both a primary role in behavior, influencing territoriality in some species, whereas adequate serum levels can be protective against stress and secondary pathological conditions caused by stress (Boonstra et al., 2018; Gundlach et al., 2018; Lennartsson et al., 2012. Morgan et al., 2004; Prall et al., 2015). There was no significant difference of DHEA-S concentrations between captive and free-ranging populations ($p = 0.548$), nor did it correlate strongly with allostatic load in captive animals ($p = 0.184$). This stability between groups is likely secondary to the large reservoir of DHEA-S in serum. Depletion of this reservoir may require higher and more chronic stimuli perceived as stressful than what the selected individuals in the study experienced. Evaluated in conjunction with cortisol, DHEA-S potentially has an application in this metric to provide an indication of chronicity of the pressure on the HPA axis, as it represents pro-inflammatory mediators versus anti-inflammatory mediators (Almeida et al., 2008; Frongia et al., 2020; Gundlach et al., 2018).

Cholesterol has been utilized to evaluate forms of malnutrition in humans and play a role in mediating pro-inflammatory states, such as obesity or osteoarthritis

(Etukudo et al., 1999; Heliovaara et al., 1996; Kuzuya et al., 2007; Leardi et al., 2000; Tall & Yvan-Charvet, 2015). These differentiated lipoproteins have been correlated with disease states that differ in frequency between free-ranging animals and captive animals. As expected, due to dietary inconsistencies between populations, wild giraffe had a lower cholesterol ($p = 0.016$) value on average than zoo-housed giraffe. Although, atherosclerosis has not been documented in giraffe, cardiovascular inflammation and oxidation may exist at subclinical levels or exacerbate ongoing inflammatory states common in captive giraffe (arthritis, degenerative joint disease, etc.) (Dadone, 2018). Severity of nutritional inadequacy in this wild giraffe population was likely not severe enough to illicit hypocholesterolemia, as has been documented in other species (Card et al., 1985; Quiroz-Rocha et al., 2009; Schmidt et al., 2006). Additionally, while free-ranging animals are more likely to experience malnutrition, infectious sepsis, or certain other disease states, captive animals are more likely to experience pathology from obesity, advanced age, or chronic psychological stress from housing. The varying morbidities and mortalities resulting from these diverse life experiences may indicate that both low and high quartile cholesterol values have predictive values in different populations with opposing stressors.

Serum NEFA levels represent mobilization of energy from stored adipose tissue in ruminants during negative energy balance and have been used as a biomarker to evaluate cardiovascular, endocrine, reduced fertility, and gastric disorders (Adewuyi et al., 2005; Leblanc et al., 2005; Leroy et al., 2005; Paolisso et al., 1995; Pilz et al., 2007; Valckx et al., 2012). Elevated NEFA concentrations ($p = 0.004$) in free-ranging giraffe reflect a significantly higher reproductive rate and female-skewed population. This

impact is well-documented in other ruminant species (Adewuyi et al., 2005; Dijkstra et al., 2005; Valckx et al., 2012). Although the discrepancy in values is easily explained, NEFA was deemed useful due to the high number of morbidities associated with parturition. High concentrations of NEFA could indicate a decline in nutritional resources for both sexes. Furthermore, inclusion of multiple biomarkers for metabolic status is necessary due to dietary variability that may influence interpretation of these results.

Fructosamine is a negative acute phase reactant and has similar correlations to malnutrition, morbidities, and mortalities as HbA1c in humans does, regardless of sample state, fresh or frozen (Garman et al., 2018; Jamal et al., 1998; Malmstrom et al., 2014; Selvin et al., 2015; Woo et al., 1989). Inclusion of fructosamine in the comprehensive health score metric allows evaluation of nutritional adequacy over a period of several weeks prior to sampling. The suspected cause of elevated concentrations of fructosamine ($p = 0.039$) in captive giraffe serum compared to wild giraffe serum is the differences in dietary resources. Zoo-housed giraffe are provided a diet high in carbohydrates, which is readily converted to glucose, whereas wild giraffe have access to high fiber and low carbohydrate food sources. Resting fructosamine levels may be similar to cholesterol in that elevated levels are a negative health indicator for captive animals and low concentrations are a negative health indicator for free-ranging populations. Serum fructosamine may also provide insight into a stressed animal's ability to compensate to environmental or seasonal challenges, such as food shortages, poor quality forage, or excessive competition amongst peers due to high population densities.

The comprehensive health score (CHS) compiled for this population of captive giraffe was significantly associated with the number of lifetime stress events prior to

sampling. These results indicate that the cumulative impact of stressful life events may permanently alter the resting levels of certain biomarkers in giraffe, as similarly suggested in western lowland gorillas (Edes et al., 2018). There was no significant association between age and CHS, implying that deviations in the selected biomarkers are more closely related to the overall impact of stressful events, rather than deterioration of regulatory abilities with age. To confirm that this model methodology works for wild animals as it does for captive individuals, a multi-year study should be conducted that tracks stress events in free-ranging giraffe.

IMPLICATIONS

Evaluation of chronic stress in free-ranging animals poses a challenge to conservationists due to the difficulty of obtaining comprehensive histories of individuals. Continuous monitoring of individuals may be impossible and even in the most ideal of situations, is logistically and monetarily burdensome. Comprehensive health score may provide an opportunity to subsequently estimate cumulative life stress through dysregulation of biomarkers, despite an unknown history.

It has been documented in humans and numerous animal species that frequent, long-term stress causes dysregulation of many physiological systems. To the author's knowledge, this is the first documented evaluation utilizing this methodology in giraffe. As was suggested by Edes et al. (2018), evaluation of a zoo-housed species provides an opportunity to build effective scoring systems for free-ranging populations. The application of a comprehensive health score is necessarily more complex than simply compiling biomarkers. The score of a single individual will provide insight into that animal's life influences; however, it does not imply equal experience across the

population. Similarly, the average score of a population may allow inferences regarding the health or stability of the habitat but does not convey any information about the individual.

Consideration must also be given to the causes of morbidity and mortality in captivity in comparison to those variables impacting free-ranging wildlife. While environmental or psychological stress may be implied by a high allostatic load, the indication of high stress may be vastly different between two distinct populations. The causes of disease and death varies between populations and especially between captive and free-ranging animals. Accurate prediction of these outcomes require situation-appropriate, biomarker selection. This index refinement would more accurately highlight those variables that are dysregulated by the specific environmental and physiological pressures of each population. Thus, while the biomarkers selected for this study were chosen for the ability to predict morbidity and mortality caused by chronic stress, it may not accurately identify certain individuals at high risk for decline due to other factors.

At risk populations may be identified through utilization of this methodology. Through rigorous biomarker selection, it may be possible to predict specific morbidity and mortality outcomes. Strength of correlations between composites of different values and the targeted outcome may eventually allow for the development and application of a comprehensive health score to predict deaths caused from infectious disease, poaching, dietary insufficiency, or other fatal threats. Comparisons of biomarkers across populations with similar stressors can provide guidance for distribution of funding, time, and resources to intervene and prevent negative outcomes.

While allostatic load is a broadly successful metric for evaluating chronic stress and predicting negative outcomes caused by stress, the methodology has limitations that prompted a change in approach. The selection criteria for biomarker inclusion in an allostatic load metric are far narrower than comprehensive health score: an AL biomarker must be dysregulated due to conscious recognition of a stressful event, resulting in the release of cortisol and subsequent physiological adaptation. Comprehensive health score targets biomarkers dysregulated by life events associated with pathology, such as parasitism, or specific mortality events, such as poaching, without the necessary element of conscious recognition by the animal. This allows for a broader application and utilization of CHS. For example, poachers may favor hunting animals that have higher coat quality over those with poor coat quality. A study at Texas A&M University found that dogs had improved coat quality as serum phospholipid levels increased (Rees et al., 2001). In certain populations of wild canids, if poaching is a significant cause of morbidities and mortalities, serum phospholipid may be a reasonable biomarker to include as part of a CHS evaluation. Additionally, this conceptual change in biomarker selection has another benefit. While AL requires recognition by individual animals of a stressful environment or event, CHS can be utilized in less cognitively advanced species that may lack the capacity to anticipate or process stressful stimuli.

Each species and population of animal will have a different “ideal” composite of biomarkers determined by the life events and mortality causes most relevant to that group. This highlights the importance of surveying both subjective and objective measures of health and comparing those values to known life histories, morbidities, and mortalities. Psychosomatic stress is well-established as a cause of illness and

predisposition for death and it is proposed that early CHS systems will likely utilize many of the same serum hormones, inflammatory indicators, and immune measures. However, supplementation of these datasets with additional biomarkers that may have unknown significance but strong correlations with negative outcomes will still have value and application for population monitoring. Additionally, very few biomarkers have been tested to predict outcomes in non-mammalian species and this deficiency of information currently limits broader application of the CHS model.

CONCLUSIONS

To this point, no metric had been established for the assessment of chronic stress in giraffe. Through the evaluation of several biomarkers that are dysregulated secondary to stressful life events, comprehensive health score has the capacity to provide insight into the unknown history of free-ranging giraffe and may predict likely outcomes for both free-ranging and captive giraffe populations. Although refinements are necessary, there is a significant association between the composited biomarker indices and events that are perceived by humans to be stressful to giraffe. Morbidity and mortality risks are species and environment specific, thus the comprehensive health score biomarker composites should be specifically tailored to the population of primary concern for the most accurate outcome predictions. Future evaluations should construct biomarker indices based on targeted populations that account for situational negative outcomes. Though future research is warranted, comprehensive health score provides a foundation for a more applicable tool in conservation research through a conceptual shift in biomarker selection and composite design.

LITERATURE CITED

Adewuyi, A. A., Gruys, E., & van Eerdenburg, F. J. C. M. (2005). Non esterified fatty acids (NEFA) in dairy cattle. A review. *Veterinary Quarterly, 27*(3), 117-126. doi:10.1080/01652176.2005.9695192

Alzaga, V., Vicente, J., Villanua, D., Acevedo, P., Casas, F., & Gortazar, C. (2008). Body condition and parasite intensity correlates with escape capacity in Iberian hares (*Lepus granatensis*). *Behavioral Ecology and Sociobiology, 62*(5), 769-775. doi:10.1007/s00265-007-0502-3

Arlettaz, R., Nusslé, S., Baltic, M., Vogel, P., Palme, R., Jenni - Eiermann, S., Genoud, M. (2015). Disturbance of wildlife by outdoor winter recreation: allostatic stress response and altered activity–energy budgets. *Ecological Applications, 25*(5), 1197-1212. doi:10.1890/14-1141.1

Baker, M. R., Gobush, K. S., & Vynne, C. H. (2013). Review of factors influencing stress hormones in fish and wildlife. *Journal for Nature Conservation, 21*(5), 309.

Bashaw, M. J., Sicks, F., Palme, R., Schwarzenberger, F., Tordiffe, A. S. W., & Ganswindt, A. (2016). Non-invasive assessment of adrenocortical activity as a measure of stress in giraffe (Giraffa camelopardalis). *BMC veterinary research, 12*(1), 235. doi:10.1186/s12917-016-0864-8

Becker, D. J., & Hall, R. J. (2014). Too much of a good thing: resource provisioning alters infectious disease dynamics in wildlife. *Biology letters, 10*(7). doi:10.1098/rsbl.2014.0309

Benhaiem, S., Delon, M., Lourtet, B., Cargnelutti, B., Aulagnier, S., Hewison, A. J. M., Verheyden, H. (2008). Hunting increases vigilance levels in roe deer and modifies feeding site selection. *Animal Behaviour, 76*(3), 611-618. doi:10.1016/j.anbehav.2008.03.012

Benhaiem, S., Hofer, H., Dehnhard, M., Helms, J., & East, M. L. (2013). Sibling competition and hunger increase allostatic load in spotted hyaenas. *Biology letters, 9*(3), 20130040. doi:10.1098/rsbl.2013.0040

Bonier, F., Martin, P. R., Moore, I. T., & Wingfield, J. C. (2009). Do baseline glucocorticoids predict fitness? *Trends in Ecology & Evolution, 24*(11), 634-642. doi:10.1016/j.tree.2009.04.013

Boonstra, R., Dušek, A., & Flynn, R. W. (2018). DHEA and territoriality during the nonbreeding season in male American martens (*Martes americana*). *Journal of Mammalogy, 99*(4), 826-835. doi:10.1093/jmammal/gyy067

Burtis, C.A. and Ashwood, E.R. (1999). Tietz Textbook of Clinical Chemistry. 3rd Edition, W. B. Saunders Co., Philadelphia, 826-835.

Buss, P., Miller, M., Fuller, A., Haw, A., Wanty, R., Olea-Popelka, F., & Meyer, L. (2016). Cardiovascular effects of etorphine, azaperone, and butorphanol combinations in chemically immobilized captive white rhinoceros (*Ceratotherium simum*). *47*(3), 834-843. doi:10.1638/2015-0298.1

Card, W. C., Kirkpatrick, R. L., Webb, K. E., & Scanlon, P. F. (1985). Nutritional Influences on Nefa, Cholesterol, and Ketones in White-Tailed Deer. *The Journal of Wildlife Management, 49*(2), 380-385. doi:10.2307/3801537

Cefalu, W. T., Bell-Farrow, A. D., Petty, M., Izlar, C., & Smith, J. A. (1991). Clinical validation of a second-generation fructosamine assay. *Clinical chemistry, 37*(7), 1252. doi:10.1093/clinchem/37.7.1252

Chung, H. F., Lees, H., & Gutman, S. I. (1988). Effect of nitroblue tetrazolium concentration on the fructosamine assay for quantifying glycated protein. *Clinical chemistry, 34*(10), 2106. doi:10.1093/clinchem/34.10.2106

Cook, R., Cook, J., Murray, D., & Zager, P. (2001). Nutritional condition models for elk: Which are the most sensitive, accurate, and precise? *Journal of Wildlife Management, 65*(4), 988-997. doi:10.2307/3803047

Creel, S., Fox, J. E., Hardy, A., Sands, J., Garrott, B., & Peterson, R. O. (2002). Snowmobile Activity and Glucocorticoid Stress Responses in Wolves and ElkActividad de Vehículos para Nieve y Respuestas de Stress Glucocorticoide en Lobos y Alces. *Conservation Biology, 16*(3), 809-814. doi:10.1046/j.1523-1739.2002.00554.x

Dadone, Liza. (2018). Lameness Diagnosis and Management in Zoo Giraffe. 10.1016/B978-0-323-55228-8.00088-6.

Davenport, M. D., Tiefenbacher, S., Lutz, C. K., Novak, M. A., & Meyer, J. S. (2006). Analysis of endogenous cortisol concentrations in the hair of rhesus macaques. *General and Comparative Endocrinology, 147*(3), 255-261. doi:10.1016/j.ygcen.2006.01.005

Deacon, F., Nel, P. J., & Bercovitch, F. B. (2015). Concurrent pregnancy and lactation in wild giraffes (*Giraffa camelopardalis*). *African Zoology, 50*(4), 331-334. doi:10.1080/15627020.2015.1120643

Dijkstra, J., Forbes, J. M., & France, J. (2005). *Quantitative aspects of ruminant digestion and metabolism* (2nd ed.. ed.). Wallingford, Oxfordshire, UK ; Cambridge, MA: Wallingford, Oxfordshire, UK ; Cambridge, MA: CABI Pub.

Edes, A. N., Wolfe, B. A., & Crews, D. E. (2016). Rearing history and allostatic load in adult western lowland gorillas (Gorilla gorilla gorilla) in human care. *Zoo Biology, 35*(2), 167-173. doi:10.1002/zoo.21270

Edes, A. N., Wolfe, B. A., & Crews, D. E. (2018). Evaluating allostatic load: a new approach to measuring long-term stress in wildlife. *49*(2), 272-282. doi:10.1638/2016-0070.1

Etukudo, M. H., Agbedana, E. O., Akinyinka, O. O., & Osifo, B. O. (1999). Plasma electrolytes, total cholesterol, liver enzymes, and selected antioxidant status in protein energy malnutrition. *African journal of medicine and medical sciences, 28*(1-2), 81.

Frongia, G., Peric, T., Leoni, G., Satta, V., Berlinguer, F., Muzzeddu, M., Comin, A. (2020). Assessment of Cortisol and DHEA Concentrations in Griffon Vulture (*Gyps fulvus*) Feathers to Evaluate its Allostatic Load. *Annals of Animal Science, 20*(1), 85-96. doi:10.2478/aoas-2019-0051

Garman, E., Chadburn, A. J., Abbas, R., Modupe, A., Thomas, O. L., Chugh, S., Gama, R. (2018). Fructosamine: A Negative Acute Phase Reactant. *Journal of Diabetes Science and Technology, 12*(1), 234-235. doi:10.1177/1932296817712527

Gerhart, K. L., White, R. G., Cameron, R. D., & Russell, D. E. (1996). Estimating Fat Content of Caribou from Body Condition Scores. *The Journal of Wildlife Management, 60*(4), 713-718. doi:10.2307/3802369

Gundlach, N. H., Schmicke, M., Ludes-Wehrmeister, E., Ulrich, S. A., Araujo, M. G., & Siebert, U. (2018). New approach to stress research in phocids--potential of dehydroepiandrosterone and cortisol/dehydroepiandrosterone ration as markers for stress in harbor seals (*Phoca vitulina*) and gray seals (*Halichoerus grypus*). *Journal of Zoo Wildlife Medicine.* 49: 556-563. doi:10.1638/2017-0191.1

Hajduk, P., Copland, M. D., & Schultz, D. A. (1992). Effects of capture on hematological values and plasma cortisol levels of free-range koalas (*Phascolarctos cinereus*). *Journal of wildlife diseases, 28*(3), 502. doi:10.7589/0090-3558-28.3.502

Heliövaara, M., Aho, K., Knekt, P., Reunanen, A., & Aromaa, A. (1996). Serum cholesterol and risk of rheumatoid arthritis in a cohort of 52 800 men and women. *British journal of rheumatology, 35*(3), 255.

Hill, M. R. J., Alisauskas, R. T., Ankney, C. D., & Leafloor, J. O. (2003). Influence of body size and condition on harvest and survival of juvenile Canada geese. *The Journal of Wildlife Management, 67*(3), 530-541. doi:10.2307/3802711

Jamal, S. A., Stone, K., Browner, W. S., Ensrud, K. E., & Cummings, S. R. (1998). Serum fructosamine level and the risk of hip fracture in elderly women: a case-

cohort study within the study of osteoporotic fractures. *The American Journal of Medicine, 105*(6), 488-493. doi:10.1016/S0002-9343(98)00331-3

Juster, R.-P., McEwen, B. S., & Lupien, S. J. (2010). Allostatic load biomarkers of chronic stress and impact on health and cognition. *Neuroscience and Biobehavioral Reviews, 35*(1), 2-16. doi:10.1016/j.neubiorev.2009.10.002

Kuzuya, M., Izawa, S., Enoki, H., Okada, K., & Iguchi, A. (2007). Is serum albumin a good marker for malnutrition in the physically impaired elderly? *Clinical Nutrition, 26*(1), 84-90. doi:10.1016/j.clnu.2006.07.009

Leardi, S., Altilia, F., Delmonaco, S., Cianca, G., Pietroletti, R., & Simi, M. (2000). Blood levels of cholesterol and postoperative septic complications. *Annali italiani di chirurgia, 71*(2), 233.

Leblanc, S. J., Leslie, K. E., & Duffield, T. F. (2005). Metabolic Predictors of Displaced Abomasum in Dairy Cattle. *Journal of Dairy Science, 88*(1), 159-170. doi:10.3168/jds.S0022-0302(05)72674-6

Lennartsson, A.-K., Kushnir, M. M., Bergquist, J., & Jonsdottir, I. H. (2012). DHEA and DHEA-S response to acute psychosocial stress in healthy men and women. *Biological Psychology, 90*(2), 143-149. doi:10.1016/j.biopsycho.2012.03.003

Leroy, J. L. M. R., Vanholder, T., Mateusen, B., Christophe, A., Opsomer, G., de Kruif, A., Van Soom, A. (2005). Non-esterified fatty acids in follicular fluid of dairy cows and their effect on developmental capacity of bovine oocytes in vitro. *Reproduction (Cambridge, England), 130*(4), 485. doi:10.1530/rep.1.00735

Lupien, S., Mony De, L., Susan De, S., Antonio, C., Chaim, T., Nair, N. P. V., Michael, J. M. (1998). Cortisol levels during human aging predict hippocampal atrophy and memory deficits. *Nature Neuroscience, 1*(1), 69. doi:10.1038/271

Malmström, H., Walldius, G., Grill, V., Jungner, I., Gudbjörnsdottir, S., & Hammar, N. (2014). Fructosamine Is a Useful Indicator of Hyperglycaemia and Glucose Control in Clinical and Epidemiological Studies – Cross-Sectional and Longitudinal Experience from the AMORIS Cohort (Fructosamine and Hyperglycaemia - AMORIS Cohort). *PLoS One 9*(10), e111463. doi:10.1371/journal.pone.0111463

Maze, M., Virtanen, R., Daunt, D., Banks, S. J., Stover, E. P., & Feldman, D. (1991). Effects of dexmedetomidine, a novel imidazole sedative-anesthetic agent, on adrenal steroidogenesis: in vivo and in vitro studies. *Anesthesia and analgesia, 73*(2), 204. doi:10.1213/00000539-199108000-00015

McEwen, B. S. (1998). Protective and damaging effects of stress mediators. (Seminars in Medicine of the Beth Israel Deaconess Medical Center)(Review Article). *The*

New England Journal of Medicine, 338(3), 171. doi:10.1056/NEJM199801153380307

McEwen, B. S. (1998). Stress, adaptation, and disease. Allostasis and allostatic load. *Annals of the New York Academy of Sciences, 840*, 33.

Miksa, I. R., Buckley, C. L., & Poppenga, R. H. (2004). Detection of Nonesterified (Free) Fatty Acids in Bovine Serum: Comparative Evaluation of Two Methods. *Journal of Veterinary Diagnostic Investigation, 16*(2), 139-144. doi:10.1177/104063870401600208

Mizuno, K., Toyosato, M., Yabumoto, S., Tanimizu, I., & Hirakawa, H. (1980). A new enzymatic method for colorimetric determination of free fatty acids. *Analytical Biochemistry, 108*(1), 6-10. doi:10.1016/0003-2697(80)90686-7

Mondelli, V., Ciufolini, S., Belvederi Murri, M., Bonaccorso, S., Di Forti, M., Giordano, A., Dazzan, P. (2015). Cortisol and Inflammatory Biomarkers Predict Poor Treatment Response in First Episode Psychosis. *Schizophrenia bulletin, 41*(5), 1162. doi:10.1093/schbul/sbv028

Morgan, C. A., Southwick, S., Hazlett, G., Rasmusson, A., Hoyt, G., Zimolo, Z., & Charney, D. (2004). Relationships Among Plasma Dehydroepiandrosterone Sulfate and CortisolLevels, Symptoms of Dissociation, and Objective Performance in Humans Exposedto Acute Stress. *Archives of General Psychiatry, 61*(8), 819-825. doi:10.1001/archpsyc.61.8.819

Möstl, E., Maggs, J. L., Schrötter, G., Besenfelder, U., & Palme, R. (2002). Measurement of Cortisol Metabolites in Faeces of Ruminants. *Veterinary Research Communications, 26*(2), 127-139. doi:10.1023/A:1014095618125

Paolisso, G., Tataranni, P., Foley, J., Bogardus, C., Howard, B., & Ravussin, E. (1995). A high concentration of fasting plasma non-esterified fatty acids is a risk factor for the development of NIDDM. *Clinical and Experimental Diabetes and Metabolism, 38*(10), 1213-1217. doi:10.1007/BF00422371

Parker, V. J., & Freeman, L. M. (2011). Association between Body Condition and Survival in Dogs with Acquired Chronic Kidney Disease. *Journal of Veterinary Internal Medicine, 25*(6), 1306-1311. doi:10.1111/j.1939-1676.2011.00805.x

Pilz, S., Scharnagl, H., Tiran, B., Wellnitz, B., Seelhorst, U., Boehm, B. O., & März, W. (2007). Elevated plasma free fatty acids predict sudden cardiac death: a 6.85-year follow-up of 3315 patients after coronary angiography. *European Heart Journal, 28*(22), 2763-2769. doi:10.1093/eurheartj/ehm343

Prall, S. P., Ambu, L., Nathan, S., Alsisto, S., Ramirez, D., & Muehlenbein, M. P. (2015). Androgens and innate immunity in rehabilitated semi-captive orangutans (*Pongo*

pygmaeus morio) from Malaysian Borneo. *American Journal of Primatology, 77*(6), 642-650. doi:10.1002/ajp.22387

Pride, E. (2005). High faecal glucocorticoid levels predict mortality in ring-tailed lemurs (*Lemur catta*). *Biology letters, 1*(1), 60. doi:10.1098/rsbl.2004.0245

Quiroz-Rocha, G. F., Leblanc, S., Duffield, T., Wood, D., Leslie, K. E., & Jacobs, R. M. (2009). Evaluation of prepartum serum cholesterol and fatty acids concentrations as predictors of postpartum retention of the placenta in dairy cows. *Journal of the American Veterinary Medical Association, 234*(6), 790. doi:10.2460/javma.234.6.790

Rees, C. A., Bauer, J. E., Burkholder, W. J., Kennis, R. A., Dunbar, B. L., & Bigley, C. E. (2001). Effects of dietary flax seed and sunflower seed supplementation on normal canine serum polyunsaturated fatty acids and skin and hair coat condition scores. *Veterinary Dermatology, 12*(2), 111-117. doi:10.1046/j.1365-3164.2001.00234.x

Romano, F. R., Heinze, C. R., Barber, L. G., Mason, J. B., & Freeman, L. M. (2016). Association between Body Condition Score and Cancer Prognosis in Dogs with Lymphoma and Osteosarcoma. *Journal of Veterinary Internal Medicine, 30*(4), 1179-1186. doi:10.1111/jvim.13965

Schmidt, D. A., Ellersieck, M. R., Cranfield, M. R., & Karesh, W. B. (2006). Cholesterol Values in Free-Ranging Gorillas (*Gorilla gorilla gorilla* and *Gorilla beringei*) and Bornean Orangutans (*Pongo pygmaeus*). *Journal of Zoo and Wildlife Medicine, 37*(3), 292-300. doi:10.1638/05-040.1

Schultner, J., Kitaysky, A. S., Welcker, J., & Hatch, S. (2013). Fat or lean: adjustment of endogenous energy stores to predictable and unpredictable changes in allostatic load. *Functional Ecology, 27*(1), 45-55. doi:10.1111/j.1365-2435.2012.02058.x

Seeman, T., McEwen, B., Rowe, J., & Singer, B. (2001). Allostatic load as a marker of cumulative biological risk: MacArthur studies of successful aging. *Proceedings of the National Academy of Sciences of the United States of America, 98*(8), 4770. doi:10.1073/pnas.081072698

Selvin, E., Rawlings, A. M., Lutsey, P. L., Maruthur, N., Pankow, J. S., Steffes, M., & Coresh, J. (2015). Fructosamine and Glycated Albumin and the Risk of Cardiovascular Outcomes and Death. *Circulation, 132*(4), 269. doi:10.1161/CIRCULATIONAHA.115.015415

Sánchez, C., Becker, D., Teitelbaum, C., Barriga, P., Brown, L., Majewska, A., Altizer, S. (2018). On the relationship between body condition and parasite infection in wildlife: a review and meta-analysis. *Ecology Letters,* 21 (12), 1869-1884.

Tall, A., & Laurent, Y.-C. (2015). Cholesterol, inflammation and innate immunity. *Nature Reviews Immunology, 15*(2), 104. doi:10.1038/nri3793

Valckx, S. D. M., De Pauw, I., De Neubourg, D., Inion, I., Berth, M., Fransen, E., Leroy, J. L. M. R. (2012). BMI-related metabolic composition of the follicular fluid of women undergoing assisted reproductive treatment and the consequences for oocyte and embryo quality. *Human Reproduction, 27*(12), 3531-3539. doi:10.1093/humrep/des350

Woo, J., Chan, S. M., Mak, Y. T., & Swaminathan, R. (1989). Biochemical predictors of short term mortality in elderly residents of chronic care institutions. *Journal of Clinical Pathology, 42*(12), 1241. doi:10.1136/jcp.42.12.1241