ABSTRACT

BRAY, JESSICA. Analysis of Captive African (*Loxodonta africana*) and Asian (*Elephas maximus*) Elephant Fecal Glucocorticoid Concentrations when Compared Among Several Environmental, Management, and Health Factors. Under the direction of Dr. Kimberly Ange-van Heugten.

Identifying links between environmental, management, and health factors as they relate to physiological stress in captive elephants is crucial for the improvement of husbandry and welfare practices in North American zoos. Non-invasive fecal glucocorticoid protocols have been implemented as successful methods to examine the effects of short term and chronic elevations of cortisol in small groups of elephants; but no others have examined fecal glucocorticoids on a large scale. The study population consisted of 130 African (*Loxodonta africana*) and 104 Asian (*Elephas maximus*) elephants living within 65 different facilities across North America. Fecal samples were collected every other week over the course of 12 months and analyzed for glucocorticoid concentration. These metabolites were obtained through the use of a corticosterone enzyme-linked immunosorbent assay (ELISA). Elephant data for sex, environmental (season and climate zone), management (space experience, enrichment diversity, feed total, herd size, animal contact, and social group factors), and health factor (foot and joint scores) also were obtained through various direct and survey methods. Repeated measures analysis of fecal glucocorticoid concentrations showed significant main effects on fecal glucocorticoids for the African elephant population. Sex, feed total (number of feedings per day), and social group count were all significant predictors of fecal glucocorticoid levels ($P<0.05$); while animal contact during the day tended towards significance ($P=0.0605$). Female African elephants exhibited higher average glucocorticoid concentrations when compared to male African elephants. Increased glucocorticoid levels were positively correlated with increasing the feed count, as well as, the greater number of

social groups an African elephant experienced within a zoo. Also, African elephants experienced a decrease in glucocorticoid concentrations related to an increase in the number of animals they have contact with during the daytime.

These results were not replicated in the Asian elephant population. The Asian population showed only a single significant effect on fecal glucocorticoid concentrations. They exhibited seasonal fluctuations in glucocorticoid levels. The average glucocorticoid concentrations were higher in the Spring season in comparison to the other seasons ($P=0.0411$). Analyses showed a significant interaction effect between season and climate zone in relation to fecal glucocorticoids in Asian elephants ($P=0.0102$). These results suggest that environmental, management, and health variables are all important potential sources of stress in the captive environment, which could have an effect on elephant welfare. This research reports species specific differences in how elephants cope and adapt to their housing conditions that are not yet known and need to be further investigated to ensure their survivability in both captivity and the wild.

Analysis of Captive African (*Loxodonta africana*) and Asian (*Elephas maximus*) Elephant Fecal Glucocorticoid Concentrations when Compared Among Several Environmental, Management, and Health Factors.

by
Jessica Bray

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APPROVED BY:

Dr. Kimberly Ange-Van Heugten
Committee Chair

Dr. Janine L Brown

Dr. Charlotte Farin

Dr. David Dickey

DEDICATION

This thesis is dedicated to the two most important people in my life, my mom and my boyfriend. From a very young age, my mother pushed me to learn everything I could. She taught me so much throughout the years and always supported me in any venture I chose. Like many mothers, she wanted the best for her daughter and encouraged me to take chances and strive for the things I wanted in life. She inspired me to do the things she never got the chance to accomplish, like graduating from high school; which I completed with high honors. After high school, even though life brought many bumps in the road that did not allow me to begin college as quickly as I had hoped, she was still there in the background pushing me to keep my dreams alive. When I finally got the opportunity to attend college, she was and has always been so proud of me and tells everyone, no matter how embarrassing it may be, that her daughter is a college graduate. She will forever be my biggest fan and there isn't enough time in this life to express how grateful I am to her and everything we have been through together as mother and daughter.

For my boyfriend of nearly 9 years, I would not be where I am today if it was not for your ever consistent support and companionship. It was you that gave me the opportunity to move to a city I did not know, to a place where my family was not near; all because you wanted me to finally be able to fulfill my dreams of going to college and making my future better. The last years together have survived numerous late night study sessions and crazy stressful exam preparation, with many successes and even some failure. We have conquered spending most of the last 5 years living apart, from my living in Africa to internships in other states and now, graduate school. Thank you for always being there when I needed you to be and putting up with this life we chose for me. I couldn't have done any of this without you.

BIOGRAPHY

Jessica Bray was born in Rome, Georgia on May 30, 1984 to her mother Linda Bray (Towner). As a child she grew up in central Florida with two younger sisters, Lynnette and Samantha. Her family moved out of Florida many times to follow her step-father's career as a tool and dye mechanic, but always returned to the same area of Florida to be closer to family. Jessica spent much of her childhood playing outdoors, reading science fiction books, and working at her grandparent's produce stand and farm. She began a love for animals at a very young age and had aspirations of becoming a veterinarian one day.

Her educational career was littered with academic success; she was placed in higher education classes most of her grade school years and showed excellence in high school by being placed on the Dean's List every year while still managing to hold chair positions in both the French National Honor Society and the National Honor Society. Upon graduating from Trinity High School in 2002, Jessica worked as a veterinary assistant to save money to attend college. Unfortunately, that dream was put on hold when she moved back to Florida to help care for her grandmother who was battling colon cancer. In 2005, Jessica moved to North Carolina to then care for her mother who had been in a near fatal car accident and was unable to care for herself, by the grace of God after three years of care her mother made a nearly full recovery from her injuries.

It wasn't until 2008, when she met her boyfriend that she began again on her path towards furthering her education by attending Forsyth Technical Community College in Winston-Salem, NC. Jessica obtained her Associates in Science- Biology with high honors in 2010, then transferred to the University of North Carolina at Greensboro to complete her

Bachelor's degree in biology with a focus on conservation and genetics. While attending UNCG, Jessica discovered that she no longer felt that pursuing the veterinary school path was best suited for her interests. While tutoring students in lower level biology and conservation courses, she realized that research and wildlife conservation were her passion. This passion fueled a study abroad trip to Botswana where she attended the University of Botswana in Gaborone, Botswana. While in Botswana, Jessica spent her free time volunteering for wildlife reserves and taking courses on southern African wildlife and native languages. Her experience there only fueled her desire to continue striving for her goal to become a wildlife conservationist. Upon returning from Botswana, she graduated from UNG with her bachelor's degree in 2012 and was fortunate enough to be chosen for a laboratory management internship at the Smithsonian Conservation Biology Institute in Front Royal, VA.

During her internship under the mentorship of Dr. Janine Brown, she learned about how to manage a lab first and foremost but more importantly, she obtained copious amounts of knowledge on the field of wildlife endocrinology. It was here that, after interning for nearly 14 months, Dr. Brown so generously provided her with a research project on captive elephant stress physiology. Jessica then enrolled at North Carolina State University in the fall of 2014 under the initial mentorship of Dr. Scott Whisnant. Due to unforeseen circumstances, Dr. Whisnant stepped down which allowed Dr. Kimberly Ange-van Heugten to take over his place as her primary mentor. Jessica will graduate in the fall of 2016 with a Master of Science in Animal Science degree with a minor in statistics.

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I have to say a tremendous thank you to Dr. Kimberly Ange-van Heugten for coming in to rescue me in a really bad situation. I am so grateful for your guidance and inspiration during my graduate career. I will forever be in your debt for it was you that turned me around and helped me find my path once again. Also, a thanks belongs to Dr. Jonathan Duggins for pushing me every day in STAT 555, no matter how much I complained; and for showing me that you have to work hard to be better and I would not have been able to fix my data sets without the knowledge I gained from your course.

Also, I want to thank all of the principal investigators and analysts that participated in the larger scale project titled "Using Science to Understand Zoo Elephant Welfare". Additionally, I would like to thank all the keepers, staff, and elephants at the following zoos for their participation in this very large study: Africam Safari, Albuquerque Biological Park, Audubon Institute, Birmingham Zoo, BREC's Baton Rouge Zoo, Buffalo Zoological Gardens, Busch Gardens, Buttonwood Park Zoo, Caldwell Zoo, Cameron Park Zoo, Cheyenne Mountain Zoological Park, Cincinnati Zoo & Botanical Garden, Cleveland Metroparks Zoo, Dallas Zoo, Denver Zoo, Dickerson Park Zoo, Disney's Animal Kingdom, El Paso Zoo, Fresno Chaffee Zoo, Greenville Zoo, Honolulu Zoo, Houston Zoological Gardens, Indianapolis Zoological Society, Inc., Jacksonville Zoological Gardens, Knoxville Zoological Gardens, Lee Richardson Zoo, Little Rock Zoological Garden, Los Angeles Zoo and Botanical Gardens, Louisville Zoological Garden, Lowry Park Zoological Garden, Maryland Zoo, Memphis Zoological Garden and Aquarium, Metropolitan Toronto Zoo, Milwaukee County Zoological Gardens, Nashville Zoo, National Zoo, Niabi Zoo, North Carolina Zoological Park, Oakland Zoo, Oklahoma City Zoological Park, Oregon Zoo, Phoenix Zoo, Point Defiance Zoo and Aquarium, Riverbanks Zoological Park, Roger Williams Park Zoo, Rosamond Gifford Zoo at Burnet Park, San Antonio Zoological Society, San Diego Safari Park, San Diego Zoo, Santa Barbara Zoological Gardens, Sedgwick County

Zoo, Seneca Park Zoo, St. Louis Zoological Park, The Kansas City Zoo, Toledo Zoo, Topeka Zoological Park, Tulsa Zoological Park, Utah's Hogle Zoo, Virginia Zoological Park, Wildlife Conservation Society - Bronx Zoo, Wildlife Safari, Woodland Park, Zoo Atlanta, Zoo de Granby, and Zoo Miami.

Lastly, I have to thank my family for always supporting me even though it has taken me so long to accomplish this goal. To my mom for always being understanding even though she doesn't quite understand what my goals are. Even with all the "what are you going to do with your degree?" questions, she has still stood by me no matter what. I love you, mom! To my boyfriend and kids for being my rock when I needed something to hold onto. I can only hope that my step-sons are inspired to go after their life goals and never give up since they too stood by watching me struggle in the difficult times and till succeed in the end. Thank you to all of my friends who were there when I needed a pick me up and to make fun of me for taking so long in school, I couldn't have asked for better friends!

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CHAPTER 1: Literature Review

Introduction

Elephants belong to the order Proboscidea, from the word proboscis, which means trunk in Latin or feeder in Greek (Merriam-Webster, 2015). There are two species of elephant, the *Loxodonta africana* (African) and *Elephas maximus* (Asian). Much debate has gone into whether the species should be even more diversified to include a sub-species of Forest elephants, *Loxodonta a. cyclotis* (Brandt et al., 2012; Rohland et al., 2010). Asian elephants can grow in size up to 21 ft. long and 10 ft. in height, weighing upwards of 11,000 lbs., with the males of the species being on the larger end of those ranges (Shoshani & Eisenberg, 1982). Another feature is that the males grow ivory tusks which help in defense and foraging. Their natural habitat consists of large areas of forest including some grasslands across many countries in Asia, including Indonesia, Thailand, Laos, Nepal, and southern China (Sukumar, 2003). These areas are known to be some of the most densely populated areas by humans, which have caused a great deal of habitat loss for this species. It is common for elephants to be used in the tourism industry, as well as in the logging trade. Recent estimations show that there are approximately 39,500-47,500 Asian elephants left in the wild (Fernando & Pastorini, 2011).

African elephants do not experience the same captive working environments as their Asian counterparts, but do face complications in the wild. Some major concerns for the wild population are illegal hunting and poaching of ivory and the human conflict issues that stem from habitat loss. Female African elephants commonly stand approximately 7-9 ft. tall and can weigh anywhere between 7,000-10,000 lbs. which is small compared to the males of the species who average 13 ft. and 9,000-14,000 lbs. in height and weight, respectively (Laursen & Bekoff, 1978). These animals range over a large portion of sub-Saharan Africa, spanning over 30 countries, often referred to as the grasslands elephants. They forage on grasses, roots, fruits, and other vegetation (Dublin, 1995; Tangley, 1997; Codron et al., 2006). Known for their large body composition and ability to walk silently, both sexes grow very large ivory tusks that aid in the foraging process. Currently, there are approximately 470,000 African elephants left in the wild; with large herds existing in Kenya, Botswana, and South Africa (Elephant Database, 2013).

Both elephant species are social and they live by a strict matriarchal hierarchy. Females are polyestrous, non-seasonal breeders. They live in groups of related individuals. Typically, the dominant female is the eldest female with the subordinate females being her daughters and granddaughters. Some research has shown that there are also instances where non-related females join to form what is called a “bond group” (Moss & Poole, 1983). The dominant female will hold her position for life and after her death the eldest daughter will take over the role of matriarch. Other studies have shown that Asian elephant female groups are less matrilineal in comparison to their African counterparts, with female groups consisting independent daughter groups that are less complex in their hierarchical social structure (Fernando & Lande, 2000). Males leave the herd before the onset of puberty to form bachelor groups or to roam solitarily. It has been shown that adolescent (age 16-20 years) bulls showed higher social behaviors towards other males and kept a shorter distance between their territorial range and other bulls (Evans & Harris, 2008). It is only during musth that the males begin to seek out the females. Musth is most commonly seen in males of reproductive age and is defined as a mental and physiological state that coincides with a dramatic increase in testosterone secretion via the temporal gland, which is located between the eye and ear on either side of the head (Rajaram, 2006). A common external signal of musth other than behavioral changes is clear discharge from the eyes (Rajaram, 2006).

According to the International Union for the Conservation of Nature (IUCN), African elephants were first placed on the Red List of Threatened Species in 1986 and listed as vulnerable (IUCN, 2016b). In 1996, this population assessment was upgraded to endangered due to habitat loss and increased hunting and poaching (Michelmore et al., 1994). The 2008 population assessment by the IUCN, using data from 2007, evaluated the population to be downgraded to vulnerable once again. The data showed that the population was slightly higher in number than the previous evaluations and that it was not in current decline (Blanc, 2008). Unlike their African counterparts, Asian elephants have been listed as endangered on the IUCN Red List since 1986 and that status has not changed (IUCN, 2016a). The 2008 report stated an estimated 50% reduction in their population over the last three generations, with a generation being estimated as 25 years (Choudhury et al., 2008).

Conservationists, researchers, and governments across the globe have established protective environments in the wild for both elephant species. Unfortunately, these wild populations are still declining (Asian) and vulnerable (African), which adds greater pressure to procure self-sustaining captive populations. Therefore, it is a priority for zoos and other facilities to come up with husbandry protocols that provide the necessary captive environments to facilitate successful breeding of these species. Some of the basic needs that are addressed in these husbandry protocols include how the animals eat, sleep, and move within their environment. The thesis research presented here contributes to the current husbandry standards and welfare of captive elephants housed in North American facilities by focusing on the environmental factors; which include social group number, herd size, enrichment variability, climate, and season as indicators of adrenal function as it relates to stress.

North American Population

The North American captive elephant population consists of 470 individuals, representing both species, according to studbook numbers from 2014 (Keele, 2014; Olson, 2014). In 2014, the Asian elephant studbook, listed 252 (n=53 male, n=199 female) elephants in 55 facilities, which makes up 53.62% of the total captive population. The captive population of African elephants in 2014 was listed as 218 individuals (n=42 male, n=176 female), split up between 65 institutions; making up the other 46.38% of the captive elephant population in North America. Of the 199 Asian elephant females, 124 were between 38 and 55 years old (Keele, 2014); while over 60% of the African elephant females ranged in age from 25-40 years old with the majority of them being on the latter end of this age range (Olson, 2014). It was also shown that of the 176 total African elephant females, only 52 were able to or had potential to be bred (Olson, 2014). This data supports recent claims that the captive population is aging out due to many of the females being above or at the end of their reproductive years, which are those between 25 and 48. In an effort to boost the current captive population reproductive status, eighteen African elephants from Swaziland have recently been brought to facilities within the United States (Tapia, 2016). These elephants, although wild, have been living in a conservation reserve in Swaziland and provide hope for future successful reproductive measures in captivity.

Studbook numbers from 2014 show that 136 Asian elephants and 164 African elephants were included in the Association of Zoos & Aquariums (AZA), Elephant Taxon Advisory Group (TAG) & Species Survival Plan (SSP) (Keele, 2014; Olson, 2014). The AZA elephant TAG/SSP group is vital to the survival of captive elephants due to their continued guidance in regards to elephant care, reproductive database management, and continued research of the species. The elephant TAG/SSP also helps to manage the captive population by advising on proper husbandry protocols and advises facilities on which animals should be bred to one another in order to provide genetic diversity within the population.

It is important to take advantage of having these animals in captivity, as it is an opportunity to better understand their needs and hopefully transition this knowledge to wild elephants as well. Captive populations of at risk species are critical outreach for educating the public of their wild vulnerability and helpful in raising money for conservation efforts by giving the public a tangible source of inspiration to help their wild conspecifics. These animals also stand to provide scientific research opportunities, training of the next generation of conservationists, and help organizations, like the AZA, lobby with governments for aid in conservation efforts around the world by providing the researchers the information they need to support such change (Smith & Hutchins, 2000).

The first African elephant born in captivity in North America was in 1978, since then 44 % of the live births have died within their first year of life; 33% of those died within the first month (Olson & Wiese, 2000). Olson & Wise also looked at the life histories of captive females and developed a 50-year population projection map for female African elephants. Their map exhibited a dramatic decline in population over those 50 years, with a large number of the females being in a post-reproductive state (aged 35-50, in this study) (Olson & Wiese, 2000). They reported that if the poor reproductive rates (Ratio= birth: death) were improved over the next 5-10 years, that the African population would be self-sustaining throughout that 50-year predictor period. Similar evidence through demographic analysis, was shown when adjusting for the captive Asian population in North America, noticing that it too was not currently a self-sustaining population (Wiese, 2000). Wiese estimated that without importation of young, reproductively viable female Asian elephants or a major increase in current reproductive success of the captive population, the North American population would drop from 241 females in 1999 to 181 by 2009, with 117 of them to be older than 35 (Wiese, 2000).

The current number of female Asian elephants housed in North American zoos, according to 2014 studbook numbers is 199 individuals (Keele, 2014). These numbers show that with increased efforts to improve reproductive success in captivity and importation of individuals as needed, facilities have been able to slow the decline of the population; although it is still declining. Faust et al. (2006), found that a re-evaluation of the reproductive rates in Asian elephants being housed in North America produced no new evidence of a population that was able to increase the number of births to support a self-sustaining captive population. They found that the Asian population model simulation declined by approximately 2% every year (Faust et al., 2006).

In 2011, population modeling projections of the North American captive African elephant population showed that there was an average of 3.5 births per year between 2001-2010 and that if the current reproductive rates stayed the same, the total population would see an annual decline of 2.3% over the next 30 years (Faust & Marti, 2011). Indicating an aging population with an unsustainable birth: death ratio. Faust & Marti (2011b) also investigated the North American captive Asian elephant population with population projections supporting a 1.6% decline annually over the next 30 years. Drastic measures would be required in order to make either species population self-sustaining at near current population sizes and even with major changes to the reproductive rates, there is no guarantee for population stability.

From a reproductive standpoint, some important measures for potential captive success are the age at onset of puberty, length of reproductive viability, and average lifespan. A study conducted in 2004, showed that captive elephants in the North American captive population, the majority being female, had an average lifespan of 44.8 and 33.0 years, for Asians and Africans respectively. It was also noted that, due to lack of accurate life history data at the time, on the captive African population within North America, the average age might not be as robust as one would like to see (Wiese & Willis, 2004). A more recent study on a large wild population of African elephants in Kenya, showed that the age at first reproduction was approximately 13.8 years, with females having an average lifespan of 41 years old (Lee et al., 2016). This average lifespan for wild African elephants is longer than that of the previous data shown for captive animals. Of these wild females, 95% were non-reproductive by age 50; also, 95% had died by the age of 65 (Lee et al., 2016). Given the longevity of elephants, concerns about how to best socialize, house, and manage these animals are all important husbandry factors that need research attention if the species is to be adequately and humanely conserved.

AZA Housing and Social Guidelines

Being a social, herd species, it is vital to the well-being of captive elephants to place them in facilities with other elephants (Rees, 2000). It has been found that elephant-to-elephant interaction plays a larger positive role and is more important to them, than common forms of behavior enrichment such as food and toys (Rees, 2000). In 2005, the AZA changed their elephant housing mandates. They require that any facilities breeding or planning to breed elephants must have a herd size of 6-12 elephants, with the ability to safely house two or more bulls (Reed, 2005). Nonbreeding facilities should not house less than two individuals but could have up to six elephants within a herd group (Reed, 2005). The AZA has since revised the policy and now states that zoos must house no less than three females or at least have the space needed for three female elephants or two males, to expand their herds. Along those same group composition lines, it was stated that no AZA accredited zoo shall have mixed species herds and those zoos having both must now house them in species specific herd groups (AZA, 2012). Not only did the AZA enforce new group and housing standards, they also required facilities to switch from direct to protected human contact management systems; which also adds additional restraints to the way that these animals are cared for in captivity by their human keepers. These new regulations have caused many facilities to re-evaluate the importance and costs of having elephants at their locations. Those facilities who could not meet these new standards had to relocate their elephant(s) to other facilities. Some captive facility management teams do not feel that herd size should be a top priority for the welfare of captive elephants. According to Hutchins (2006), elephants are more flexible in captivity than we give them credit for. He criticized those who enforce herd and larger space requirements for enclosures as measures of well-being, citing that if negative behaviors such as stereotypies or aggression, are not present within these elephants; then there is no need to re-evaluate husbandry protocols. Hutchin's stance is not common among the majority of researchers and zoo management, but it does bring to light another viewpoint to consider when making welfare decisions.

Reproductive Viability

A large captive husbandry consideration is the reproductive viability of an elephant herd. Many surveys have been conducted to assess the reproductive status of captive elephants housed in North American zoos and sanctuaries. The last survey in 2008 compared the cyclicity status of both Asian and African females as a measure of reproductive viability within the entire population to that of survey results from 2002 and 2005 (Dow et al., 2011). Acyclicity rates of Asian and African females, in comparison to the two previous surveys, were (13.3, 10.9, & 11.1%) and (22.1, 31.2, & 30.5%), showing 2002, 2005, and 2008 by species, respectively (Dow et al., 2011). The acyclic rates for Asian females has stayed relatively the same from 2002-2008. However, African females experienced an increase in acyclicity from 2002-2005, but showed similar rates of acyclicity in the 2008 survey. African females have higher rates of acyclicity overall when compared to Asian females, yet the reason behind why this occurs is not fully understood. Another reproductive factor examined was whether acyclicity was present or not when a bull was in proximity verses facilities without a bull. The data showed a significant decrease (92.5% vs 58.3%) in Asian and (64.9% vs 57.8%) in African acyclicity status with a bull being present (Dow et al., 2011). These gender presence results suggest gender herd dynamic changes can potentially increase the current rates of acyclicity seen in these captive elephant groups.

Stress Response and Glucocorticoid Action

Endocrinology is the study of the endocrine system which involves organs within the body and their secretions, also called hormones. These hormones act as messengers being sent out into the blood to stimulate or inhibit specific pathways in the body (Norris & Carr, 2013). Some important areas of the body that are involved in the endocrine system are the hypothalamus, pituitary, thyroid, intestines, and reproductive organs; although the hypothalamus plays a critical role in hormone signaling within the body. The hypothalamus has many nuclei that act as communication centers in the brain. These nuclei become activated by specific stimuli and cause the secretion of hormones for target cells (Norris & Carr, 2013). An example of this cascade is the hypothalamic-pituitary-adrenal gland axis.

The adrenal gland is comprised of two parts, the adrenal medulla and the adrenal cortex. The medulla is involved in catecholamine production, while the cortex provides a variety of products including glucocorticoids. The adrenal cortex is comprised of three parts, the zona glomerulosa, fasciculata, and reticularis (Norris & Carr, 2013). The zona fasciculata is responsible for glucocorticoid production, mainly cortisol. Cortisol is an important factor in many processes within the body, although it is commonly considered the bio-marker for stress (Hellhammer et al., 2009). Cortisol production occurs in a rhythmic pattern in many animals with the highest cortisol concentrations happening in the early hours of the day, just before the individual becomes active, which is identified as a circadian (approximately every 24 hours) rhythm; although not all animals experience this exact rhythmic pattern in cortisol expression (Chung et al., 2011). However, this circadian rhythm has been shown using urine from Asian elephants (Brown et al., 2010) and saliva from African elephants (Casares et al., 2016).

Cortisol is a major factor in mediating the body's stress response. Cortisol has two types of receptors (Type 1 and Type 2) located throughout the body (Juruena et al., 2004). Type 1 (mineralocorticoid) is thought to be involved in regulation of the circadian changes in glucocorticoids, while the Type 2 (glucocorticoid) receptor is used mainly in times of stress when concentrations of cortisol can be very high (Juruena et al., 2004). Stress is a much debated term used to describe some form of reaction within the body, to a stressor. For the purpose of this paper, the term stress is by definition, the disruption of homeostasis by exposure to a stressor (Reeder & Kramer, 2005). However, not all stress is bad and the response to a stressor is different for every individual (Brown et al., 2008). There is a unique difference between "good" and "bad" stress; eustress is considered to be the positive form of stress that is identified as having a beneficial effect on the animal (Selye, 1987); while distress is defined as unhappiness or pain (Selye, 1953), which is what people commonly refer to when they use the term "stress". Analyzing adrenal hormones associated with the stress response is a common method of evaluating how each individual experiences a life stressor.

The stress response begins with stage one, which is the fight or flight condition. This stage is more immediate, consisting of catecholamine synthesis. Catecholamine expression causes many things to occur in the body; such as increased blood flow to the muscles and increased heart rate (Sabban, 2010). The second stage of stress response is glucocorticoid synthesis. Glucocorticoids cause lipolysis, increased blood pressure, and replacement of glycogen within the body (Nicolson, 2008). This process is beneficial short term (minutes to hours) but excess long term glucocorticoids or chronic stress (months to years) has detrimental effects on the body. Specifically, high levels of cortisol for long periods, can cause degradation of proteins (muscle atrophy), decreased immune system function, and decreased reproductive hormone secretions in non-rodent mammals (Munck et al., 1984; Tilbrook et al., 2000). In humans, it is known that high levels of cortisol due to chronic stress can cause depression, obesity, increased susceptibility to disease and insulin resistance (Cohen et al., 2007; Torres & Nowson, 2007).

Cortisol is one of the most studied hormones to date and yet there are many unanswered questions. Adrenocorticotropic hormone (ACTH) is the releasing hormone for glucocorticoids like cortisol. Thus, ACTH challenges in elephants have shown how and when cortisol is excreted from the body in the urine and feces (Brown et al., 1995; Wasser et al., 2000). Differences in an animal’s social status, age, and environment can also play a role in adrenal function. Sexual dimorphism has been seen in areas of the brain where females and males differ significantly in the number of hormone receptors, allowing for differing changes in the body to occur (Goldstein et al., 2001). Studying the adrenal response to stressors in captive and wild populations of animals is a complex process and the results vary within the individual, conspecifics, and among species.

Non-invasive Hormone Monitoring

Early experiments investigating what is now known as hormones, were rather invasive, involving the removal of organs of interest and blood sampling (Berthold, 1849). That sort of invasiveness is no longer ideal when dealing with easily stressed animals whose populations are already in decline. Obtaining blood is often reported as the best source of hormone collection, however, when looking at hormones involved with stress; this procedure tends to artificially increase these levels due to the handling required for blood draws (Reinhardt et al., 1990; Reinhardt et al., 1991). As a result, scientists have begun to validate non-invasive collection protocols using bodily fluids and excrement as suitable sources of hormone data.

Validation of non-invasive hormone monitoring assays has proven to be a great use to those studying both captive and wild animal populations. Assays to test for reproductive hormones such as testosterone, progesterone and estradiol have been used to monitor a range of species such as the giant panda (*Ailuropoda melanoleuca*), the bell frog (*Litoria raniformis*), tropical bats (*Saccopteryx bilineata*), and African elephants (Foley et al., 2001; Germano et al., 2009; Kersey et al., 2010; Voigt & Schwarzenberger, 2008). Sample collections of urine, feces, and saliva have been used to study various hormones in both captive and wild elephants (Brown et al., 2010; Dathe et al., 1992; Foley et al., 2001). By using non-invasive strategies, researchers can evaluate whole populations of animals simply by waiting for them to defecate, urinate, or chew on a mouth swab. These techniques are especially important when looking at 'stress' hormones, because they typically do not cause the animal(s) the stress of being handled or captured. However, non-invasive samples can have potential negatives. For example, fecal corticoid measurements are complicated because they represent a pooled value of the amount of cortisol or cortisol metabolites that were in circulation over a long period of hours to days (which is different from blood samples that show only the current concentration), depending on the gastro-intestinal excretion rate of the animal. It has been shown that after ACTH challenge in elephants the excretion rates are approximately 4-8 hours for urine and approximately 36 hours post injection for feces (Brown et al., 1995; Wasser et al., 2000).

Environmental Factors

Captive and wild populations experience their environment in different but similar ways. In the wild, an animal is already adapted to many of the environmental factors that it will encounter in its lifetime. The same cannot be said for the vast majority of captive elephants, as captivity is not their natural habitat. Evaluating how animals respond to their environment is no easy task and the number of variables involved can be overwhelming; some examples are environmental temperature, exhibit type and space, social verses non-social species aspects, and enrichment effect.

Social support plays an important role in how humans respond to stress. People with social support networks are less likely to have significantly increased cortisol levels as a response to psychological stress than those who did not have a social support (Heinrichs et al., 2003). Therefore, if social animals, such as humans and elephants, are kept alone in a space without other conspecifics, the likelihood that their stress response will be much greater, is higher. There are other situations based on social interactions that relate back to social stress on an individual, such as status within a group. Studies of dominance hierarchies in the animal world have shown differing results for and against the theory that high cortisol levels suppress reproduction. Two studies on adrenal function in wild canids have shown positive relationships between high dominance status and increased fecal corticoid levels in Ethiopian wolves (*Canis simensis*) and African wild dogs (*Lycaon pictus*) (Creel et al., 1997; Van Kesteren et al., 2012); with similar findings in breeding pairs of Florida scrub jays (*Aphelocoma c. coerulescens*); (Schoech et al., 1991). However, the relationship between dominance and adrenal activity in a group of 81 captive female African elephants was shows to be unrelated (Proctor et al., 2010). A different research study found high levels of fecal glucocorticoids to be correlated to lower ranking individuals in a free-ranging wild population of African elephant females (Foley et al., 2001). Subordinate females could be exhibiting higher cortisol levels due to interactions with the dominant females around resources that are considered important or scarce, such as food and water. A study of five female captive African elephants showed that the more dominant females were found to occupy the watering pool for longer periods of time when compared to the more subordinate females of the herd group and exhibited dominance over the subordinates by occupying the narrower, restricted flow areas for longer periods within the exhibit (Leighty, Soltis, & Savage, 2010). This could lead to increased glucocorticoids in subordinates when tasked to interact with their dominant counterparts for access to those vital areas of their shared enclosure. Other social aspects can also have an effect on cortisol, such as the introduction of an elephant to a new herd or changes in social grouping patterns. For example, a research report showed that daily salivary cortisol before, during, and after the introduction of an Asian elephant to an established herd increased three fold for the young newcomer, upon introduction to the existing herd members and decreased to near baseline within 24 hours after the initial introduction event (Dathe et al., 1992). An increase in cortisol as a response to the new environment is often thought to be arousal based. The use of saliva as a replacement for blood sampling has become more popular because it represents a close estimation of the free cortisol in the blood (Wood, 2009); which gives an accurate "point in time" value of the circulating cortisol within the body.

A review of the housing and herd sizes of wild and captive elephants, found that captive elephant herd sizes are often smaller (less than four) than those of wild herds and that they lack calf presence (Schulte, 2000). In 2006, Veasey reported research to show that the potential lack of space allotted and management's poor social integration are some of the concerns for captive elephant welfare (Veasey, 2006). Similarly, enclosure size and animal density of Père David's deer (*Elaphurus davidianus*) stags showed that decreased enclosure size and higher population density within the enclosures caused increased fecal cortisol. Twelve deer stags shared approximately 100 hectares which was considered off display or "free-range", when those deer were moved into a display pen of only 0.5 hectares, they exhibited significantly higher cortisol than when they were in the "free-range" pen (Li et al., 2007). A comparison of captive brown brocket (*Mazama gouazoubira*) and marsh deer (*Blastocerus dichotomus*) found quite different effects to changes in enclosure type, preferring to be housed in individual stalls rather than a large outdoor enclosure during the night hours (Christofoletti et al., 2010). This cortisol difference in species preference for a specific enclosure type helps to illuminate the complications facilities face when trying to best house them in captivity. Rhesus monkeys (*Macaca mulatta*) exhibited lower levels of cortisol when housed in outdoor enclosures when compared to those housed in indoor rooms, neither enclosure type showed significant effects from inanimate environmental enrichment or social housing changes (Schapiro et al., 1993). Repetitive behaviors, also referred to as stereotypies, of six African elephants increased in frequency in the on-exhibit animals just before they were to be moved indoors at the end of the day and in those elephants who were housed off-exhibit all day (Hasenjager & Bergl, 2015). The authors stated that the increased repetitive behaviors of the on-exhibit group were due to the transition into a more confined space or possibly the anticipation of being fed; with the explanation of the off-exhibit group behavior being attributed to the lack of exhibit complexity and confinement in the off-exhibit space. The anticipation of being in a more confined space, as evaluated as a potential stressor, has not been studied much in elephants but a recent study looked at the amount of resting behavior shown in captive elephants being housed indoors at night. It was shown that elephants who are housed within two body lengths of a conspecific spent a significantly ($p<0.01$) greater amount of time resting than those who were not housed near another elephant (Williams et al., 2015). This could be related to the social support point addressed previously. The way an individual animal perceives its space is an important factor when trying to define appropriate husbandry protocols for captivity. Therefore, the more husbandry professionals know about each specific animal, the more they can accommodate them with the most effective stress reducing techniques. Clouded leopards (*Neofelis nebulosa*) exhibited significantly ($p<0.05$) lower fecal corticoid concentrations when given taller enclosures that allowed them to not only perform their natural hiding instincts but allow them to position themselves away from stressors of their environment like zoo visitors and neighboring predators (Wielebnowski et al., 2002).

Weather is another important factor when studying animal species because changes in the weather patterns throughout the seasons could have significant effects on animals by reducing or improving access to much needed resources within their environment. For wild animals, seasons bring the possibility for variations in food and shelter resources, mating, hibernation, and even migration for some. Captive environments may cause some of those seasonal changes to be void, given the regular access to food, shelter, and medical resources. However, depending on the captive location, seasonality may still significantly alter captive animal livelihood (food type can vary, water and rain or snow fluctuations, and temperature related appropriate housing changes). For example, an undisturbed group of captive red deer (*Cervus elaphus*) housed on 45 hectares, exhibited much higher fecal cortisol levels during the winter months than any other part of the year (Huber et al., 2003). Several factors were taken into consideration when looking at how the environment effected fecal glucocorticoid levels, the only ones that were significant predictors of fecal corticoid levels were lower ambient temperatures and snow cover of those months (Huber et al., 2003). Similar effects were seen in a group of Asian elephants, with a strong negative relationship between low temperatures and stereotypies (Rees, 2004). In Rees' 2004 study, eight captive elephants were studied; of the elephants who exhibited stereotypies, higher frequencies of stereotypic behavior were seen when the ambient temperatures were coldest. Although both of these species are housed in captivity, climate and seasonal changes can still act as potential stressors.

When evaluated, cortisol secretion patterns in the saliva of eight captive Asian elephants showed seasonal variation, with the highest levels being between September and December (Menargues Marcilla et al., 2012). Seasonal changes in cortisol were also found in wild Pyrenean Chamois (*Rupicapra pyrenaica pyrenaica*), using fecal corticoids to show highest cortisol levels in the winter months, which left the authors to speculate about a circannual (about a year) rhythm in cortisol secretion (Dalmau & Manteca, 2007). These natural variations in cortisol concentration are important to keep in mind when evaluating cortisol levels longitudinally. In a more recent study on climate and seasonality on semi-captive Asian elephants in Myanmar, they found there to be significant differences in the fecal corticoid concentrations between seasons, with the monsoon season (June - August) having the highest concentrations when compared to January which is a dry, cooler period (Mumby et al., 2015). A two-year study investigating the effects of poaching on 218 wild African elephants found that the elephants exhibited increased fecal glucocorticoid levels in relation to periods of low rainfall, which was evaluated to be a seasonal component (Gobush et al., 2008).

Environmental factors like social interactions, enclosure size, and climate could all play a role in how an animal experiences its captive environment. Another factor to evaluate is how the animals are responding to enrichment programs. The use of enrichment is often used to help alleviate or prevent negative behaviors by giving the animals items such as toys or food that help to encourage natural behaviors such as foraging. A meta-analysis of 54 studies on enrichment and its effects on stereotypies in multiple captive species showed that providing species specific enrichment items to captive animals reduced the frequency of stereotypic behaviors (Shyne, 2006). In leopard cats (*Felis bengalensis*), four individuals were placed in varying enclosures that differed in the amount of enrichment (branches and hiding places) and adrenal function was evaluated using urine sampling. Urinary cortisol concentrations and stereotypic behaviors were reduced when the cats were in the more enriched enclosures (Carlstead et al., 1993). Unfortunately, enrichment and how it affects negative behaviors in elephants is not well understood. Some studies show that providing browse during the day as enrichment showed an increase in feeding and decrease in inactivity (Stoinski et al., 2000); others show that food enrichment such as hiding peanuts in the outdoor enclosures did not encourage natural behaviors like foraging beyond the enrichment itself (Wiedenmayer, 1998). In 2004, a study found there to be no correlation between stereotypies and blood cortisol concentrations in three African elephants, but did express that individual elephant cortisol patterns showed that each experienced the change in environment differently (Wilson et al., 2004). It is difficult to determine the impact of specific environmental changes when it comes to physiological and behavioral measures because every individual is different in how they cope with a specific stressor. This is the challenge we face when attempting to evaluate the welfare of animals in captivity. Captive environmental variables are complex and it is often best to include several different factors in a study to try to get a complete picture of what sorts of potential stressors the animal is experiencing. Elephant environments can be affected by anything ranging from sex of the animal and their conspecifics, to species differences, herd number, individual health, enrichment, and climate.

CHAPTER 2: Analysis of Captive African (*Loxodonta africana*) and Asian (*Elephas maximus*) Elephant Fecal Glucocorticoid Concentrations when Compared Among Several Environmental, Management, and Health Factors.

Introduction

There are currently two known species of extant elephants, the African (*Loxodonta africana*) and Asian (*Elephas maximus*) elephant species. Both species are listed on the International Union for the Conservation of Nature (IUCN) Red List of Threatened Species. According to the 2008 IUCN report, African elephant status in the wild was marked as vulnerable (Blanc, 2008). A status report from 2008 reported the wild Asian elephant population is listed as endangered, with population estimations showing a decrease in population size by nearly 50% over three generations (Choudhury et al., 2008). Due to increased habitat loss, human conflict, and poaching of both species, recent wild population numbers are estimated between 39,500 and 47,500 Asian elephants and approximately 470,000 African elephants (Elephant Database, 2013; Fernando & Pastorini, 2011). At these rates, elephants are facing potential extinction in the wild, with the Asian elephant population being at a more critical status than their African counterpart.

The pressure for zoos, conservation programs, and researchers to identify and solve the problems facing elephants is immense. Elephants are a challenging species to house in captivity given their size and longevity although they are considered a flagship conservation species and thus a favorite species to exhibit (Bowen-Jones & Entwistle, 2002). To be successful, it is important that captive elephant programs evaluate the basic husbandry needs of these animals, as well as, the more complex factors that may affect welfare in a captive environment. As part of a large multi-institutional captive elephant welfare project funded by the Institute of Library and Museum Services (IMLS), results from multiple studies have shown that environmental and social variables have both positive and negative impacts on stereotypic behavior, reproductive cyclicity status and hyperprolactinemia in females, and housing experiences in both elephant species (Brown et al., 2016; Greco et al., 2016; Meehan et al., 2016).

The research presented here contributes to the existing husbandry standards for captive elephants in North American zoos by comparing several of the IMLS environmental and social factors that were previously determined to have a significant impact on aspects of elephant health and welfare, by examining the variables potential effect on adrenal function (glucocorticoids) as a biomarker of stress. The primary factors examined in the current elephant research project were season, climate, animal and social contact, enrichment diversity, and herd size.

Elephants in the Wild

Wild African (*Loxodonta africana*) and Asian (*Elephas maximus*) elephants live in two very different climates which have very different environmental challenges. The majority of the wild African elephant population live in savannahs, deserts, and grasslands covering more than thirty countries in sub-Saharan Africa. There also are some forest dwelling African elephants (*Loxodonta a. cyclotis*) which are technically classified as a sub-species of the African elephant. This classification, however, is heavily debated as many scientists believe they should be listed as their own species due to research on the ecological and DNA differences (Brandt et al., 2012; Rohland et al., 2010). Temperatures and rainfall amounts, among other environmental factors, differ dramatically across the natural elephant home-ranges throughout Africa. The animals have been known to range from the northern tropics all the way down to the southern temperate zone (between 16.5° North and 34°South) (Blanc, 2008). To compare, Cameroon (in the northern home range) can experience between 60cm and 900cm of rainfall per year; while Namibia (in the southern home range) experiences up to 600mm of rainfall annually (Info-Namibia, 2016; Nations Encyclopedia, 2016).

In contrast, Asian elephants typically live in rainforest and low woodland/grass environments. Many countries in Asia are home to the wild Asian elephant population, including Thailand, Nepal, and Indonesia (Sukumar, 2003). Temperatures throughout these areas also vary dramatically. For example, the temperatures in Nepal at night in the winter can reach below zero degrees Celsius (C) with summer temperatures climbing up to 30°C; while the average temperature in Thailand ranges 22-27°C all year-round (WeatherOnline, 2016).

Elephants are the largest land mammals. Free-ranging African elephants typically range between 7-9 ft. in height for females and average 13 ft. for males; having weights ranging 7,000-14,000 lbs. with the males commonly being on the larger end of the weight range (Laursen & Bekoff, 1978). Free-ranging Asian elephants are smaller in height and weight, with a maximum height of 10 ft. and weights topping out around 11,000 lbs.; again with the males covering the larger ends (Shoshani & Eisenberg, 1982).

North American Population

There are 470 elephants housed in captive facilities across North America, according to 2014 studbook numbers (Keele, 2014; Olson, 2014). The most recent edition of the Asian elephant studbook reports that there are 252 elephants in 55 facilities, with 136 of them being listed on the Association of Zoos & Aquariums (AZA), Elephant Taxon Advisory Group (TAG) and Species Survival Plan (SSP); (Keele, 2014). Also, of the 252 elephants, 199 are female and of those, 124 were 38-55 years old (Keele, 2014). The remaining 218 elephants represent the captive African elephant population in North America; these animals are housed in 65 different facilities (Olson, 2014). The 2014 African elephant studbook showed that 164 of the 218 African elephants were listed in the AZA elephant TAG and SSP; of which most of them were aged between 25 and 40 years old (Olson, 2014). The importance of the AZA elephant TAG and SSP groups are to set appropriate captive husbandry guidelines in relation to captive elephant care, population management, reproduction, and continuing research.

Housing elephants in captivity is important for research into species survival techniques and for the education of the next generation of elephant conservationists. Studying them in captivity allows for a greater understanding of how elephants live and reproduce, in hopes to apply that knowledge to not only the current captive populations, but also to the wild elephant herds. The first African elephant to be born in North America was in 1978, however, since then 44% of all live births have ended in death (Olson & Wiese, 2000). An update on the captive North American elephant population conducted in 2006 reported that neither elephant species was self-sustaining at that time (Faust et al., 2006). Due to the continued captive elephant population decline and the extreme drought situation in southern Africa, an importation of elephants from the wild was conducted to help support the current captive population. This was completed earlier this year by importing 18 African elephants from Swaziland (Tapia, 2016) into North America in a rare attempt to boost the reproductive success of the captive herds; while saving the 18 animals from being culled in the wild.

Non-invasive hormone monitoring and stress

Given the sensitive nature of housing wild animals in captive environments, new methods of research have been implemented to gather necessary information while not causing undue stress to the individual. Previous research in hormone monitoring used invasive techniques such as venipuncture, however, it is now known that when studying glucocorticoid hormones, this method can artificially increase these hormones due to the stress of capture and sampling (Reinhardt et al., 1990; Reinhardt et al., 1991). Therefore, invasive methodology is no longer common place when evaluating adrenal function in wild and captive animals. Scientists have discovered that non-invasive methods of collection, such as using saliva or excrement, are a more suitable source for hormone analysis (Proctor et al., 2010; Wood, 2009).

Validation of hormone assays designed for feces, urine, and saliva have been conducted so that researchers are able to gather the necessary information to answer many questions regarding an animal's health. Sex hormones such as testosterone, estradiol, and luteinizing hormone have been extracted and used in many exotic species as diverse as African and Asian elephants, bell frogs (*Litoria raniformis*), giant pandas (*Ailuropoda melanoleuca*), and tropical bats (*Saccopteryx bilineata*) (Foley et al., 2001; Germano et al., 2009; Kersey et al., 2010; Voigt & Schwarzenberger, 2008; Yon et al., 2007).

One of the most common hormones studied is cortisol, which is stimulated in the adrenal gland via adrenocorticotropic hormone (ACTH). Cortisol, a glucocorticoid, is used as a bio-marker for stress levels in animals and humans (Hellhammer et al., 2009). Stress is a term used to explain a biological reaction within the body, to a stressor. For the purpose of this paper, the term stress is by definition, the disruption of homeostasis by exposure to a stressor (Reeder & Kramer, 2005). There is a difference between what is considered to be "good" and "bad" stress. Eustress is the positive form of stress that is identified as having a beneficial effect on the well-being of the animal (Selye, 1987); while distress (often called "stress") is considered a negative reaction to a life stressor (Selye, 1953). However, the ability to determine eustress versus distress hormonally is not currently possible, although it has been shown that chronically elevated levels of cortisol have detrimental effects to an animal's health, regardless of why they are elevated (Torres & Nowson, 2007). Investigating adrenal hormones associated with the stress response is a common practice when analyzing how each individual experiences a potential targeted life stressor. ACTH challenges in elephants show cortisol metabolites are excreted from the body in both urine and feces (Brown et al., 1995; Wasser et al., 2000).

The use of fecal hormone concentrations is popular for wild animals, as it does not require the collector to interact with the animals in order to obtain the samples. This same method is used in captive animals to help alleviate unnecessary stress during the collection process for both the animal and the person. It also allows the collection to happen at any time thus allowing more time for collection. The glucocorticoids in feces represent a pooled value of circulating adrenal hormones over time, which is different for all species and can vary among individuals, primarily due to the differing gastro-intestinal excretion rates of each. In elephants, the excretion rate in urine is 4-8 hours post ACTH injection and approximately 36 hours for feces (Brown et al., 1995; Wasser et al., 2000). It should be noted that unlike salivary and blood cortisol concentrations (which are single, point in time values; see Wood, 2009), fecal glucocorticoids represent an accumulated concentration value over a longer time frame; which is often a better method of analysis when evaluating chronic stress in animals. These cumulative cortisol estimations provide a less acutely sensitive evaluation of circulating hormone levels within the animals and thus one small stressor during a single time point will not dilute the research data.

Environment

The environment plays a big role in how captive and wild animals experience their daily lives. For social species, aspects of social structure such as status, has been shown to have an effect on fecal cortisol levels. For example, two separate studies conducted on Ethiopian wolves (*Canis simensis*) and African wild dogs (*Lycaon pictus*) showed that higher dominance status was correlated to higher fecal cortisol levels (Creel et al., 1997; Van Kesteren et al., 2012). Although there is conflicting evidence in regards to elephants and social structure effects; a study of eighty-one captive African elephants showed no correlation between dominance status and serum cortisol (Proctor et al., 2010). However, a study of free-ranging animals showed that lower ranking individuals exhibited higher fecal corticoids when compared to the more dominant African elephants (Foley et al., 2001). It is possible that the variation seen between the two elephant studies can be attributed to differences in social bonds, ages, and group numbers when comparing captive and wild herd groups. Additionally, Foley et al. reported increased fecal cortisol levels related to larger group size and the dry season of the free- ranging African elephant herds studied (Foley et al., 2001).

Two captive environmental variables of interest to investigate are herd size and housing differences. This area of study is of particular importance due to the new AZA husbandry guidelines that set strict housing parameters based on the need for increasing animal welfare in captivity (AZA, 2012). Not all husbandry staff within captive institutions agree that these variables alone hold the potential to be stressors for the animals. Some believe these measures are not beneficial areas of concern in regards to elephant welfare practices (Hutchins, 2006) and that it may be more about how these variables are related instead of evaluating isolated parameters. Considering the large number of elephants being housed in captivity in North America, there is likely a facility (zoo/ conservation center) specific effect on fecal glucocorticoids due to the inherent differences in management factors between sixty different facilities. A study comparing free contact and protected contact management strategies of captive elephants showed that these two management types were not significant predictors of serum cortisol concentrations, however, facility was (Proctor & Brown, 2015). These data indicated a likelihood that facility specific differences such as space experience, enrichment, and social factors may affect cortisol levels in captive elephants. Existing research on herd size, housing and space differences in relation to fecal glucocorticoids for captive elephants is not available, which is why these variables were included in the present research study. Given the large scale of the current research population and variable set, these data more accurately represent measures for future comparison of the larger captive elephant population in North America as a whole.

Weather and climate are important factors to consider when evaluating environmental stressors, as each species and each individual experiences their physical environment differently. For many species, living in captivity has the benefits of a stable and climate controlled living space. For elephants, there often is a significant amount of time spent in outdoor enclosures with others within their herd, which allows greater amounts of time for these animals to be exposed to different climate and seasonal variations. Clubb and Mason (2002), reported that captive elephants in European zoos experience lower temperatures than their wild counterparts. Although, a study of wild African elephants in Kenya showed that elephants were known to live near the snowy edges of Mt. Kenya; which would mean they experienced temperatures as low as -14.4°C (Spinage, 1994). The reasoning behind such an anomaly herd could be related to habitat destruction, which has forced these animals to adapt to new (cooler) environments. Seasonality in fecal glucocorticoid concentrations was found within a large study (n=218) of wild African elephants in Tanzania, with higher glucocorticoid concentrations being positively correlated to periods of low rainfall in the dry season (Gobush et al., 2008). Another elephant study looking at seasonal fluctuations in cortisol found that the captive Asian elephants being housed at a zoological park in Spain (also in the Northern hemisphere), exhibited a seasonal change in cortisol levels, with the highest levels between September and December (Menargues Marcilla et al., 2012). Climate related changes in glucocorticoid concentrations during the seasons were seen in these published studies, even though all animals were housed in very different captive environments and locations. A semi-captive study also showed there to be a significant relationship between season and fecal glucocorticoid concentrations in the Asian elephants studied; elephants had the highest cortisol levels during the monsoon season (July-August) (Mumby et al., 2015). It is possible that elephants have species specific temperature and climate preferences that have not yet been identified and that they may adapt to certain climatic preferences.

The management factor of enrichment has been evaluated as having potential environmental effects on glucocorticoid levels. A group of captive leopard cats (*Felis bengalensis*) was given different enclosure types with varying enrichment items in each type of enclosure; it was found that the cats had lower fecal corticoid levels when given highly enriched, vertical enclosures (Carlstead et al., 1993). The cats in this study were able to climb up and hide from visitors in order to better cope with the stress of being on exhibit, this behavior relates to their natural instincts to be hidden and out of sight, as well as, it gives the animals more options for coping with stressors in their environment. The effect that increased enrichment plays on decreasing negative behaviors and glucocorticoid secretion in captive elephants is not understood, although there have been several studies that have attempted to explain it. Wilson et al. (2004) found there to be no correlation between blood cortisol and stereotypic behaviors in three African elephants. Other studies have shown that hiding peanuts in an elephant exhibit as an enrichment, did not encourage foraging behaviors beyond the initial finding of the peanuts (Wiedenmayer, 1998). Elephants are very intelligent animals with the ability to form long term memory and spatial recognition (Hart et al., 2008). Therefore, while complex enrichment strategies are encouraged in the captive setting, creating beneficial strategies can be time consuming and difficult for the limited time available to the care staff.

It is a complex task to analyze all the possible environmental factors that have the potential to have a negative impact on fecal glucocorticoid concentrations in captive elephants. Any negative impact can contribute to poor reproductive performance and lower success rates within these species. This current research study consists of 49.8% of the total captive elephant population in North America and focuses on the basic demographic differences (age, sex, and species) and the varied environmental factors (Season, Climate, Space Experience, Enrichment and Management, Social, and Health) that hold the potential to be stressors within the species. Many of the variables presented in this research are novel to the study of captive elephant welfare (climate zone and space experience); not only has it not been evaluated in this way, nor has it been evaluated on such a large scale population.

Materials and Methods

Study Animals and Sample Collection

This study includes 234 elephants living at 65 different AZA institutions across North America (see Appendix A); consisting of both the African (n=130; 103 females and 27 males) and the Asian (n=104; 83 females and 21 males) species. Of the 65 total facilities, 32 of them housed African herds, 28 had strictly Asian herds, and the remaining 5 facilities housed both African and Asian elephants. Study individuals ranged in age between infant and 64 years old. All animals were housed in indoor-outdoor enclosures that meet the AZA elephant TAG and SSP husbandry guidelines for facilities accredited by the AZA (AZA, 2012). Fecal samples were collected semimonthly, every 2 weeks, over the course of 12 months. The facilities involved in this study began sampling between December 2011 and June 2012, with all facilities collecting for 12 consecutive months.

Fecal sampling protocols called for samples to be collected fresh from the ground, samples could be collected 1-3 days after the twice monthly, morning serum collection (serum data is not included in this research study). The fecal collection protocol called for keepers to mix the feces to obtain homogeneity, then place a fist sized (egg shaped) sample in a small fecal bag. Samples were stored frozen (-20°C) and shipped to the Smithsonian Conservation Biology Institute (SCBI) where they were stored for approximately 6-10 months before processing. All samples were lyophilized (Lyophilizing machine; Labconco, Kansas City, MO) and crushed to separate fecal powder from larger particulates, such as hay, then stored frozen (-20°C) in polypropylene tubes (Perfector Scientific; Atascadero, CA) for approximately 1-2 months until processing. Fecal corticoids were extracted using the method employed by Ganswindt et al. (2002) with slight modifications. Briefly these modifications include: 0.1g (+/- 0.02) of lyophilized fecal powder was placed in 16 x 125mm glass tubes (Fisher Scientific; Pittsburgh, PA) and vortexed on a multi-tube vortex (Glas-Col; Terre Haute, IN) for 30 minutes in 5ml of 80% methanol then centrifuged (20min at 2200rpm) (Sorvall RC 3C Plus; Thermo Fisher Scientific, Waltham, MA). Once the supernatant was recovered, the pellet was re-suspended in 5ml of 80% methanol and centrifuged again (15min at 2200rpm). Both supernatants were combined in clean 16 x 125mm glass tubes and dried completely under forced air in a fume hood using fecal tube drying racks, overnight (7pm-7am). The samples were reconstituted in 1ml of 100% methanol and dried again. Dilution buffer (1ml, 0.149M NaCl, 0.1M $NaPO_4$; with pH 7.0) was added to each sample before placing them in a sonicator (Part# 08895-60; Cole-Parmer, Vernon Hills, IL) for 30 seconds to free and dissolve any remaining particulate within. Finally, all samples were diluted (1:8) in dilution buffer and frozen (-20°C) in preparation for enzyme-immunoassay (EIA) corticosterone analyses.

Hormone Analysis

Fecal glucocorticoid metabolites were analyzed by a double antibody enzyme immunoassay protocol developed by SCBI (unpublished). The corticosterone antibody (CJM006), a polyclonal that was produced in rabbits, was obtained from Coralie Munro (University of California, Davis, CA), and the conjugated horseradish peroxidase (HRP) was produced at SCBI. The CJM006 antibody has been validated for elephants by Watson et al.

(2013) and SCBI (unpublished). Antibody cross-reactivity is 100% corticosterone, 14.25% desoxycorticosterone, 2.65% progesterone, <1% testosterone, cortisol, cortisone, and estradiol (Watson et al., 2013).

For this study, samples, standards (3.9-1000 pg /well; Sigma Diagnostics, St. Louis, MO), and controls were added in duplicate (50uL per well) to a pre-coated goat anti-rabbit IgG, 96 well plate at room temperature. Then 25 uL of diluted conjugated HRP (1:45,000) was added to all wells, immediately followed by 25 uL of the diluted corticosterone antibody (1:60,000) to all but non-specific binding (NSB) wells. Plates were then covered with a microplate sealer and incubated at room temperature on an agitator (Model E6121; Eberbach Corporation, Ann Arbor, MA) for one hour. After the incubation period, the plates were washed thoroughly five times with wash solution (1:20 dilution, 20X Wash Buffer part no. X007; Arbor Assays, MI) and blotted dry. Once dry, 100 mL of Moss TMB (3, 3', 5, 5' – Tetramethylbenzidine); (Moss Inc., Pasadena, Maryland) was added to the entire plate and allowed to incubate on the bench for thirty minutes; then stopped by adding 50 uL of the 1N HCl (hydrochloric acid) stopping solution. Immediately after applying the stop solution, optical density was read in a plate reader at 450 nm (OPsys MR; Dynex Technologies, Chantilly, VA). The inter-assay coefficient of variation (CV %) for the high control was 8.129%, while the low control CV% was 15.1% (n=200). The intra-assay CV% was maintained to be less than 10% for all duplicates; with an assay sensitivity at 0.2ng.

Research Variables

To better explain how the environment plays a role in potential adrenal stress responses, fecal glucocorticoids were measured in captive elephants, across numerous raw data and created variables as representations of potential factors involved in captive animal management. For the purposes of this study, these variables were grouped into the following categories: Demographics, Environmental, Space Experience, Enrichment and Management, Social, and Health (Table 1).

Demographics

The age variable was created using the age that all study elephants were in July 2012, this was considered a mid-point within the study. The age category variable was created to categorize the individuals into the following life stages: Infant (0-3 yrs. old), Juvenile (4-11 yrs. old), Sub-Adult (12-20 yrs. old), and Adult (21+ yrs. old). These categories were important when considering how life stages effect fecal glucocorticoid concentrations. To investigate gender differences in fecal corticoids, a sex variable was created that used the common terms of female and male.

Environmental Variables

Season

To investigate the potential seasonal influences on fecal glucocorticoids, the study was divided into the four seasons exhibited across the Northern Hemisphere; winter, spring, summer, and fall (Encyclopaedia Britannica, 2016). Fecal glucocorticoid concentrations were averaged into single representative values for each of the seasons for all research statistical analyses; samples between December and February were combined to give a winter value with March-May, June-August, and September-November representing spring, summer, and fall; respectively.

Climate

The climate variable was created to further investigate the potential relationship between climate diversity and fecal glucocorticoids. The climate variable used the nine established climate zones from the National Oceanic and Atmospheric Administration (NOAA) that have been identified through historical climate analysis (NOAA, 2016). The zones are groupings of states within the lower 48 states of the United States and are named as follows: Central, East North Central, Northeast, Northwest, South, Southeast, Southwest, West, and West North Central (Appendix B). Each facility was matched to their corresponding climate zone by identifying the physical location of each. Africam Safari is located in northern Mexico nearest to Texas, so it was placed in the South climate zone due to its proximity to the climate zone cutoff. The West North Central climate zone did not contain any elephants in the current study group (Table 2).

Independent Environmental Variables

Additional environmental variables were chosen based on their potential to have an impact on the fecal glucocorticoid concentrations of the study elephants. These variables included: enrichment (type, frequency, and effect), housing (type and experience), and social (status, contact, and experience). Definitions of all variables used can be found in Table 1.

Collection of these data was conducted through management and keeper surveys that were electronically distributed to all participating facilities. The management surveys required monthly logs be kept and reported for each of the variables, especially pertaining to housing and social time budgets (Meehan et al., 2016).

Space Experience

A housing variable was created in order to understand how an elephant experiences the space they are provided in their enclosure. The space experience variables were defined as the average total space an elephant has the ability to use during both the day and night, weighted by the amount of time the elephant actually spent in that provided space (Meehan et al., 2016). Exhibit size was calculated using the total amount of space (sq. ft.) provided to the elephants within a facility. The amount of time the elephants spent within each area was reported in monthly percentages via the management surveys. The use of the total space experience variable for this study was to evaluate how each elephant experiences all possible environments combined (Indoor, Outdoor, Choice of In / Out).

Enrichment and Management

Enrichment diversity is an indexed value that was made to represent the type of enrichment combined with the frequency of its application as an enrichment item, as defined by Greco et al. (2016). This score can range from 0 (meaning only one type of item was used every time) to 3.4 (representing equal usage of all 30 listed enrichments). To see a listing of all 30 enrichment items from the survey, see Appendix C.

Management factors of feed total and herd size were also included. Feed total represents the total number of feedings in a 24-hour period while herd size is defined as the total number of animals at a facility, regardless of housing or social grouping differences. These were zoo specific variables, which means they did not vary between individuals being housed at the same facility.

Social

The social variables listed in Table 1 and further described by Meehan et al. (2016) were used to examine how daytime animal contact and social groupings impact fecal glucocorticoid concentration. Animal contact by day is defined as the total number of elephants that each individual elephant has contact with during the day. In relation to animal contact, social group contact during the day was defined as the number of social groupings an individual experienced during the daytime. Finally, social group count which is defined as a zoo level variable, is the maximum number of social groups at a facility over the course of the 12-month study period.

Health

The two health variables listed in Table 1, foot score and joint score, were included because they are a measure of elephant health in captivity that might indicate chronic stress. Foot health is a common issue related to the overall health and wellness of captive elephants (Veasey, 2006). Foot and joint health evaluations were conducted on each of the elephants within the study by veterinary professionals and reported in the respective elephant health records (Miller et al., 2016). Foot scores were 0-3 for each of the four feet giving an overall score between 0 and 12, with 0 being completely healthy. Joint health, or musculoskeletal, scores ranged from 0-3, with 0 being an individual with healthy joints.

Statistical Analysis

The software used to run the statistical analysis was SAS version 9.4 (SAS Institute, Cary, NC). To better identify species specific differences, the study population was divided into separate African and Asian elephant models. Proc Freq and Proc Means were used to obtain descriptive data on all variables for both species models. A multivariate, mixed effects model (Proc Mixed) was employed to determine which of the environmental variables (see Table 1) were potential predictors of fecal glucocorticoid metabolite concentration. Using mixed models allow for the use of the individual animal as a random effect. During the initial model building process, it was discovered that facility was a statistically significant factor for both the African and Asian elephant models ($P<0.05$). To better account for facility specific variability that variable, (zoo_ID, as seen in Table 1), was included into both models as a random intercept. Repeated measures by season (levels =4) were also included within the mixed modeling analysis. Chi-square tests were also run to establish spread of species over climate zones. For all analytical purposes, significance was established as any P-value of <0.05.

Table 1. Description of variables used in the analysis of environmental factors affecting fecal glucocorticoid concentrations in African (*Loxodonta africana*) and Asian (*Elephas maximus*) elephants in AZA zoos.

Variable	Unit	Description
Demographics		
Age	Elephant	Age of elephant as of July 2012 (mid-study).
Age Category	Elephant	Four age related categories: Infant (0-3 yrs.), Juvenile (4-11 yrs.), Sub-Adult (12-20 yrs.), and Adult (21+ yrs.).
Sex	Elephant	Female or Male.
Species	Elephant	African or Asian.
Environmental		
Climate	Zoo	Climate designation for North America established by the National Oceanic and Atmospheric Administration (NOAA), 9 zones overall.
Season	N/A	Winter, Spring, Summer, and Fall.
Space Experience		
Total Space (day & night)	Elephant	Average value of total space an elephant is housed in during the day and night, weighted by the amount of time spent in that space.
Enrichment & Management		
Enrichment Diversity	Zoo	The number of enrichment types and their frequency, determined using the Shannon-Weaver index. (0-3.4)
Feed Total	Zoo	Number of feedings provided in a 24-hour day.
Herd Size	Zoo	Total number of elephants at a facility, regardless of housing differences.
Social		
Animal Contact (day)	Elephant	Number of elephants an elephant shared enclosure space with during the day.
Social Group Contact (day)	Elephant	Maximum number of social groups an elephant experienced of during the day.
Social Group Count	Zoo	Total number of social groups at a facility.
Health		
Foot Score	Elephant	A veterinarian determined foot abnormality score based foot pad, nails, and interdigital space health, ranging 0-3 per foot with an overall score ranging 0-12 per elephant (0 being healthy).
Joint Score	Elephant	A veterinarian determined musculoskeletal score of 0-3 based on range of motion and/or joint abnormalities (0 being healthy).

References to variable creation and further explanation can be found in Miller et al. (2016), Meehan et al. (2016), and Greco et al. (2016).

Table 2. Number of African (*Loxodonta africana)* and Asian (*Elephas maximus*) elephants across eight climate zones in the U.S.[1]

Climate Zone[1*]	Central	East North Central	North-east	North-west	South	South-east	South-west	West
Species								
African (n)	26	2	16	6	21	38	4	17
Asian (n)	20	0	13	11	28	11	10	11

[1*]Climate zones established by the National Oceanic and Atmospheric Administration (NOAA); the West North Central climate zone was not represented in this research project.

Results

Demographics

Age

The study population ranged in age from 0 (infant)-64 years old. The average ages were (27.7 ±1.06 years) for Africans and (34.3 ±1.51 years) for Asians. The oldest elephant in the African population was 52 years old, with the oldest Asian being 64 years old. There were six Asian elephants that were older than the maximum age for the African population of 52 years old. Each elephant was placed into a life stage category based on age. Of the 130 African elephants, there were nine infants, 14 juveniles, two sub-adults, and 105 adults. The majority (80%) of the individuals in this study were classified as adult (21+ yrs. old). Similarly, the Asian population had 81 of the 104 elephants being listed in the adult life stage. The Asian elephant population had fewer infants and juveniles than the African group at six and six, respectively. Overall, 84% of the sub-adults in the population were Asian, at 11 individuals compared to two African. Age was not a significant factor for either African or Asian models; however, differences in age ranges between species populations are noteworthy. Correlative data between age and average glucocorticoid concentration for all elephants is provided in Appendix D, which shows no definitive relationship between the two variables (r = 0.10).

Sex

In the African model, sex was a significant predictor of fecal glucocorticoid concentrations (P=0.0498). Female African elephants exhibited higher glucocorticoid (~18ng/g) values when compared to those of male African elephants. The same was not true of the Asian population in this study, even though both populations have a representative male population of approximately 20%.

Environmental Variables

Season

Fecal glucocorticoid concentrations averaged by season and species are shown in Table 3. Although there was variability among seasonal means for African elephants, it was not a significant factor in the univariate analysis of fecal glucocorticoid concentrations (Table 4). Fecal glucocorticoids were highest during the spring season when compared to the other seasons in Asian elephants (Table 3). Season was the only significant main effect in the Asian elephant multivariate model (P=0.0220). The univariate differences between seasons are shown in Table 5, where spring exhibited higher fecal corticoid values when compared to the fall season, as a reference (P=0.0411).

Table 3. Mean (±SEM) seasonal fecal glucocorticoid concentrations (ng/g) in African (*Loxodonta africana*) and Asian (*Elephas maximus*) elephants in North American zoos.

	African (n=130)			Asian (n=104)		
Season	**Mean ± SEM[1]**	**Min.**	**Max.**	**Mean ± SEM[1]**	**Min.**	**Max.**
Winter	108.5 ± 11.92	31.8	222.5	146.9 ± 17.33[b]	43.4	317.7
Spring	107.15 ± 11.87	37.6	266.2	156.8 ± 17.30[a*]	57.8	286.7
Summer	105.0 ± 11.89	28.8	229.7	146.2 ± 17.30[b]	49.7	324.2
Fall	110.0 ± 11.88	26.8	292.4	147.8 ± 17.30[b]	37.8	310.6

[1]SE= Standard error
[*a,b] Row means within a column with different superscripts differ (P=0.0411).

Climate

Chi-squared analyses resulted in a significant, $\chi 2$ ((7, n=234) =21.68, P=0.0029), difference between the two species and the designated climate zones. The differences in spread across the different climate zones can be seen in Table 2. Nearly 30% of the African captive population in this study reside in the southeast climate zone. In comparison, the largest grouping (27%) of the Asian captive population lives in the south climate zone. The East North Central zone has very few elephants, being that only two Africans and zero Asians are represented there. Neither species is represented in the West North Central zone.

Multivariate analysis was conducted to examine the effect of climate and season on elephant fecal glucocorticoids. There was a significant interaction between the effect of climate and season for Asian elephants (P=0.0102). Univariate main effect analysis showed that Asian elephant fecal glucocorticoids were higher in the spring season in both the Northeast and Northwest climate zones when compared to the fall (P=0.0400 and P=0.0058, respectively); with the West climate zone as reference. An interaction effect was not seen in the African elephant model.

Independent Environmental Variables

Space Experience

The average total space experience during the day for Africans was more than twice the average total daytime space experience for Asians (Tables 6 & 7). Also, the night time average was less for Asians when compared to the average total space experience at night for

Africans, about 6500 sq. ft. and 17,800 sq. ft., respectively. Space experience during the day for African elephants tended toward significance (P=0.062) in relation to fecal glucocorticoid concentrations within the multivariate model. Space experience was not significant in the Asian elephant model, for day or night.

Enrichment and Management

In relation to fecal glucocorticoids, enrichment diversity was not significant for either species model (see Tables 4 and 5). The mean diversity index score for each group was approximately the same with nearly identical deviations from the mean (Tables 6 and 7).

Feed total, defined as the number of feedings in a 24-hour period, was a significant main effect on fecal glucocorticoid concentration for African elephants (P=0.0255, see Table 4), but was not a significant predictor for Asian elephants (see Table 5). Fecal glucocorticoid levels increased with the number of feeds per day in African elephants. African elephants had an average number of feeds per day of (6.6 ± 0.37), with a maximum number of 23 feedings in a 24-hour period. Asian elephants had a greater average number of feedings of (8.0 ± 0.56), with a maximum number of 32 feedings but no significant glucocorticoid difference despite the greater average.

The average herd size for African elephants was (5.6 ± 0.32), with the largest herd consisting of 13 individuals. Asian elephant herds averaged (4.7 ± 0.24 elephants) with a maximum herd size of 9 elephants. The multivariate models showed that herd size was not a significant effect on fecal glucocorticoids for either elephant species.

Social

For African elephants, animal contact tended towards significance with an associated reduction in glucocorticoid levels as the number of contact animals increased during the day (P=0.0605). The same relationship was not observed in the Asian model (see Table 5). The average number of individuals African elephants came in contact with during the day was about 3, whereas this number was lower for Asian elephants (Table 7).

Neither Social Group Contact nor Social Group Count was significant in relation to Asian elephant fecal glucocorticoids (see Table 5); however, Social Group Count was significant for African elephants (P=0.0201; Table 4). Increasing number of social groups at a zoo was positively associated with increasing fecal glucocorticoid concentrations for African elephants. Zoos housing Asian elephants reported a maximum of 22 social groups, while one zoo that housed African elephants reported 43 different social groups over the study period (see Tables 6 and 7).

Health

Foot and joint health variables were not significantly related to fecal glucocorticoid concentrations for either species (Tables 4 and 5). Foot scores for African and Asian elephants in this study averaged in the healthiest top 17% of the study population and joint scores for both species averaged in the top 13% for the entire study population.

Table 4. Univariate assessment of factors affecting fecal glucocorticoid concentrations in African (*Loxodonta africana*) elephants.

Variables	Age Category	Sex	Climate	Season	Beta	P value
Demographics						
Age					0.2393	0.6509
Age Category	Adult				-11.1509	0.5708
	Infant				-0.3483	0.9890
	Juvenile				7.6011	0.7558
	Sub-Adult =ref				0	.
Sex		Female			18.2055	0.0498*
		Male=ref			0	.
Environmental						
Climate			Central		-25.7038	0.2758
			East North Central		-3.6671	0.9277
			Northeast		-32.9990	0.2084
			Northwest		35.9843	0.2810
			South		-8.0742	0.7526
			Southeast		-12.9699	0.5969
			Southwest		-34.3448	0.3344
			West =ref		0	.
Season				Winter	-1.4604	0.7058
				Spring	-2.8057	0.4251
				Summer	-4.9250	0.1093
				Fall=ref	0	.
Space Experience						
Total Space Experience (day)-sq.ft.					0.0002	0.0620
Total Space Experience (night)-sq.ft.					-6.96 E-6	0.9017
Enrichment & Management						
Enrichment Diversity (0-3.4 score)					-1.8733	0.9563
Feed Total (times fed per day)					3.4821	0.0255*
Herd Size (#of animals at zoo)					-5.0871	0.1942
Social						
Animal Contact (day)					-6.7764	0.0605
Social Group Contact (day)					-4.4488	0.2596
Social Group Count					5.2477	0.0201*
Health						
Foot Score (0-12 health score)					0.7093	0.6681
Joint Score (0-3 health score)					2.8560	0.4908

*Represents statistically significant (P<0.05). References to variable creation and further explanation can be found in Miller et al. (2016), Meehan et al. (2016), and Greco et al. (2016).

Table 5. Univariate assessment of factors affecting fecal glucocorticoid concentrations in Asian (*Elephas maximus*) elephants.

Variables	Age Category	Sex	Climate	Season	Beta	P value
Demographics						
Age					0.4233	0.5175
Age Category	Adult				-26.4028	0.2803
	Infant				23.5650	0.3025
	Juvenile				24.6877	0.3766
	Sub-Adult =ref				0	.
Sex		Female			-13.2447	0.3470
		Male=ref			0	.
Environmental						
Climate			Central		6.0221	0.8268
			Northeast		1.1734	0.9719
			Northwest		49.9159	0.1758
			South		3.4291	0.8915
			Southeast		34.8739	0.3207
			Southwest		7.5810	0.8600
			West =ref		0	.
Season				Winter	-0.8727	0.8554
				Spring	8.9901	0.0411*
				Summer	-1.5611	0.6936
				Fall=ref	0	.
Space Experience						
Total Space Experience (day)- sq.ft.					-0.00002	0.9264
Total Space Experience (night)- sq.ft.					-0.00025	0.2321
Enrichment & Management						
Enrichment Diversity (0-3.4 score)					-60.7339	0.3614
Feed Total (times fed per day)					2.4350	0.2011
Herd Size (# of animals at zoo)					-16.4531	0.1073
Social						
Animal Contact (day)					0.5490	0.9274
Social Group Contact (day)					2.4747	0.7393
Social Group Count					6.2022	0.1488
Health						
Foot Score (0-12 health score)					0.1953	0.9366
Joint Score (0-3 health score)					2.7225	0.6911

*Represents statistically significant (P<0.05). References to variable creation and further explanation can be found in Miller et al. (2016), Meehan et al. (2016), and Greco et al. (2016).

Table 6. Descriptive statistics of independent environmental variables used to analyze the fecal glucocorticoid concentrations in captive African (*Loxodonta africana*) elephants.

Variable	n	Mean	Std. Error	Min.	Max.
<u>Space Experience</u>					
Total Space Experience-day (sq.ft)	130	43065.5	4235.84	1089.8	171460.0
Total Space Experience-night (sq.ft)	130	17788.7	3357.34	404.0	227356.0
<u>Enrichment & Management</u>					
Enrichment Diversity (0-3.4 score)	128	2.8	0.01	2.5	3.3
Feed Total (times per day)	128	6.6	0.37	2.0	23.0
Herd Size (# of animals at zoo)	130	5.6	0.32	1.0	13.0
<u>Social</u>					
Animal Contact (day)- (# of animals an elephant has contact with)	130	3.2	0.25	0.0	11.0
Social Group Contact (day)- (# social groups an animal experiences)	130	3.7	0.40	1.0	18.0
Social Group Count (# social groups at a facility over 12 months)	130	10.2	1.02	1.0	43.0
<u>Health</u>					
Foot Score (0-12 health score)	100	2.0	0.20	0.0	8.0
Joint Score (0-3 health score)	94	0.3	0.07	0.0	2.0

References to variable creation and further explanation can be found in Miller et al. (2016), Meehan et al. (2016), and Greco et al. (2016).

Table 7. Descriptive statistics of independent environmental variables used to analyze the fecal glucocorticoid concentrations in captive Asian (*Elephas maximus*) elephants.

Variable	n	Mean	Std. Error	Min.	Max.
Space Experience					
Total Space Experience-day (sq.ft)	102	17390.6	1221.38	1316.0	49022.2
Total Space Experience-night (sq.ft)	104	6530.6	922.04	287.0	46066.0
Enrichment & Management					
Enrichment Diversity (0-3.4 score)	91	2.9	0.02	2.5	3.2
Feed Total (times per day)	93	8.0	0.56	2.0	32.0
Herd Size (# of animals at zoo)	104	4.7	0.24	2.0	9.0
Social					
Animal Contact (day)- (# of animals an elephant has contact with)	104	2.1	0.17	0.0	7.0
Social Group Contact (day)- (# social groups an animal experiences)	104	2.4	0.19	1.0	11.0
Social Group Count (# social groups at a facility over 12 months)	104	7.4	0.63	1.0	22.0
Health					
Foot Score (0-12 health score)	89	2.1	0.24	0.0	12.0
Joint Score (0-3 health score)	90	0.4	0.08	0.0	3.0

References to variable creation and further explanation can be found in Miller et al. (2016), Meehan et al. (2016), and Greco et al. (2016).

Discussion

Glucocorticoid concentration as an indicator of adrenal activity is often used as an indicator of physiological stress and consequently humane welfare in elephants (Laws et al., 2007). The novelty of the current glucocorticoid elephant study is that no other before it has had such a robust sample size that represents nearly half of the captive elephant population in North America. The potential effects of both seasonality and climate on fecal glucocorticoid concentrations were of particular interest for this study. An additional area of interest in this project, investigated how the environmental factors of housing, enrichment, social groupings, and foot and joint health evaluations impacted fecal glucocorticoids in elephants. The results from these three separate research investigations form a more complete picture of the current welfare and management practices of captive elephants in North America by examining how all of these variables play a role in influencing fecal glucocorticoid fluctuations and potential animal stress.

Demographics

The study population represented 49.8% of the entire captive population in North America, according to studbook numbers for both species in 2014 (Keele, 2014; Olson, 2014). Age distributions for both species show that 81% of the African elephant population and 78% of the Asian elephant population are 21 years or older. Life stage classifications were arbitrarily assigned to all elephants regardless of species in the following manner: Infant (0-3 yrs.), Juvenile (4-11 yrs.), Sub-Adult (12-20 yrs.), and Adult (21+ yrs.). Although age was found to be associated with several welfare indicators in this population of elephants within the greater IMLS study [stereotypy (Greco et al., 2016), ovarian acyclicity and hyperprolactinemia (Brown et al., 2016)], it was not associated with fecal glucocorticoid secretion. The lack of significant variation in fecal glucocorticoids in relation to age for this study only further confirms similar results found in herds of wild African elephants (Ganswindt et al., 2005; Viljoen et al., 2008) and semi-captive Asian elephants (Mumby et al., 2015). It is possible that age related changes in glucocorticoids were not seen due to the fact that the majority (79.4%) of the study population was already in adulthood; which could skew any potential deviations due to a lack of younger animals within the population.

The average ages for each population were 27.7 and 34.3 years old for the African and Asian species, respectively. It was expected that the average age would be well within the adult (21+ yrs. old) age range given their longevity as a species; but surprisingly, the average age for the current study population was on the younger side of adulthood when compared to age pyramids presented in studbooks for the entire captive elephant populations by species (Keele, 2014; Olson, 2014). Studbook number analyses showed that nearly two-thirds of both the African and Asian captive elephant populations ranged from 25-48 years of age and 38-55 years of age, respectively (Keele, 2014; Olson, 2014). The oldest African elephant in this study population was at least 12 years younger than the oldest Asian elephant. Asian elephants have been reported to live longer than their African counterparts in captivity (Wiese & Willis, 2004); which is further supported in this study given the differences in maximum age and the fact that there are six Asian elephants who were older than the maximum age for the African population. Thus, further justifying a species specific difference in the lifespans of captive elephants.

Examination of sex related differences in fecal glucocorticoid concentrations showed that there was a significant main effect of sex among African elephants. Female African elephants had higher mean fecal glucocorticoid concentrations when compared to those of the males in the population. Both study populations had a 4:1 ratio of females to males, yet glucocorticoid differences in relation to sex were not found to be significant within the Asian elephant population. A study conducted on captive caribou (*Rangifer tarandus granti*) and reindeer (*R. t. tarandus*) following ACTH injection found that females of both species exhibited higher fecal glucocorticoid concentrations than males of their species (Ashley et al., 2011), suggesting that females may have greater sensitivity in their stress response. In contrast, Mumby et al. (2015) found no sex related differences in fecal glucocorticoids in a large group of Asian elephants living in Myanmar.

A major physiological event for many male (bull) elephants is the period of musth. Previous research on the topic has shown conflicting results on the effect musth has on glucocorticoid levels in male elephants. A study of eight Asian and twelve African captive bulls showed strong correlations between the increase in testosterone concentrations, a common result during musth, and increased blood cortisol levels (Brown et al., 2007). Other studies related to musth in elephants show no relationship between musth and cortisol levels in both African and Asian elephants (Ganswindt et al., 2003; Yon et al., 2007). It might be anticipated that musth in male elephants has the potential to increase glucocorticoid levels, which could alter how they perceive their environment during such a stressful event. However, in our dataset this was not the case. Our study had a disproportionate number of females to males, with not all males being recorded during a musth cycle due to timing and the potential danger involved in sample collection for their care staff. Future work should be conducted to better isolate sex-specific differences in fecal glucocorticoid secretion in captive elephant species.

Environmental Variables

It has been shown in several species that glucocorticoid patterns change with the seasons they experience in their habitat. These include wild Pyrenean Chamois, *Rupicapra pyrenaica pyrenaica*, (Dalmau & Manteca, 2007); wild Red deer, *Cervus elaphus*, (Huber et al., 2003); captive Goral, *Naemorhedus griseus*, (Khonmee et al., 2014); and wild & captive Spider monkeys, *Ateles geoffroyi*, (Rangel-Negrin et al., 2009). Seasonality of glucocorticoids in Asian elephants has been studied in both captive and semi-captive environments. Menargues Marcilla (2012), found that the highest salivary cortisol levels in a group of captive Asian elephants being housed at Terra Natura Zoological Park in Spain, were seen in the month of October, with the lowest values in April. However, a semi-captive population of Asian elephants in Myanmar, exhibited highest fecal glucocorticoid concentrations during the monsoon season (July-August), with a short spike in activity in November, during the cool season (Mumby et al., 2015). These studies show that Asian elephants do exhibit seasonal fluctuation in glucocorticoids, which may relate directly to the appropriateness of environment among facility locations and captivity types. The current research showed no significant effects of season on fecal glucocorticoid concentrations within the African population but did show a significant main effect for the Asian population. The Asian elephant results support the previous research on seasonal fluctuations of glucocorticoid levels in elephants. Mean fecal glucocorticoid concentrations in the Asian elephant population were highest in the spring when compared to winter, summer, and fall. In addition to being a significant main effect, there was an interaction effect of season and climate for Asian elephants. The interaction effects showed that Asian fecal glucocorticoids were higher in the spring season for both the Northeast and Northwest climate zones. These results could be attributed to the changing of the seasons from winter to spring in the northern climate regions as they tend to have greater variety in temperatures during this transitional season.

Interestingly, the seasonal fecal glucocorticoid means for all four seasons were larger for the Asian elephant population when compared to those seen in the African population, suggesting that Asian elephants have higher overall glucocorticoid levels when compared to the African elephants (Table 3); which contrasts results from a similar captive elephant welfare study conducted in the UK that showed African elephants exhibited higher overall fecal glucocorticoid concentrations when compared to Asian elephants (Harris et al, 2008). Unlike the Asian population results reported above, the African results do not currently support a seasonal effect on glucocorticoid concentrations; which could be related to the different climates each is housed in. Even though there were more African elephants than Asian elephants in this current study, both populations were not evenly spread throughout areas of North America; which could be why seasonality was seen in one species and not the other. The variation in fecal glucocorticoids between the species shows that there is still much we do not know about how these animals experience their environments and whether or not the elephants exhibit some form of seasonal, species specific, preference due to things like changes in ambient temperature.

There was a difference between the distribution of the two elephant species over the different climate zones in North America. Assumptions based on a climate zone effect on glucocorticoids are unclear given that the climate zones are represented differently between the two species. This can be partially explained by the variation seen in the population distributions, as well as demographic distributions, for both captive elephant populations. The use of NOAA climate zones to investigate how climate impacts captive species is a new area of study. A more in depth examination of how those climatic differences effect animals in captivity, especially those species that spend large amounts of time outdoors, is required to better understand this topic.

Evaluation of fecal glucocorticoid means by facility when compared among facilities within a particular climate zone showed that some zoos had much higher overall glucocorticoid levels when compared to the others within that climate zone (Appendices E & F). When displayed by facility means within NOAA zones, glucocorticoids varied widely among each zone, which could be attributed to the differing climates experienced. The most notable points for these charts is that there are some facilities that have much higher glucocorticoid concentrations when compared to others. For example, a facility housing African elephants in the Northwest climate zone had an average glucocorticoid concentration that was twice as high as the next highest facility for that area. Distinct differences also were seen in several climate zones for the Asian elephant population (Appendix F). Some zones showed very little fluctuation in glucocorticoids for both species, as well. These notable differences between zoos could be due to a facility effect related to management differences as suggested by Proctor & Brown (2015) or by other uncontrolled variables (such as uneven age and gender) within these elephant populations. The effect of facility was to be expected provided the large number of zoos in this study, therefore, it was important to identify facility specific differences that may impact glucocorticoids in captive elephant species.

Independent Environmental Variables

The data collected on the independent environmental variables was through surveys distributed to each facility. Not all of those facilities submitted completed surveys and the detail included within the surveys varied. Some of the more specific data, such as foot and joint health evaluations, came directly from analyses of facility health records on the elephants. Therefore, not all individuals have complete data sets for every variable tested in this study. This had a reducing effect on the final models for both the African and Asian elephant populations, as the modeling process eliminated any individuals with missing data. As noted in Tables 6 and 7, the number of elephants per variable varied among the input variables listed. Even with missing data on some of the elephants, the majority of the original 234 elephants were accounted for in the data analysis.

There were interesting comparisons in the average space experience between the two elephant species. The space experience during the day for African elephants tended toward significance, although when evaluating the results, it is unlikely that this variable is biologically significant (Table 4). Meaning that, biologically speaking, there is not a way to relate a nearly zero square foot variance to fecal glucocorticoid concentrations. The space experience variable worked when evaluating space usage by elephant (Meehan et al., 2016), but does not appear to be relevant to the current research.

Enrichment diversity was not a significant factor related to fecal glucocorticoids for either elephant species. Of the zoos that reported information based on enrichment, data suggests that there is a moderate amount of enrichment diversity for elephants being housed in captivity. According to Greco et al., (2016), a diversity score of 0 meant that one type/item of enrichment was used every time, while a maximum score of 3.4 meant that all 30 enrichment items listed on the survey were used with equal frequency. Therefore, mean scores represented that enrichment diversity for both captive elephant populations was adequate however, there is still room for improvement. According to Greco et al. (2016), the most frequently used enrichment items in the larger IMLS study were dirt piles, pools, logs, and scratching posts; which were provided nearly every day for the majority of zoos. The lesser used enrichment items were those aimed at problem solving. It is thought that the reason for their infrequent usage is the time involved in preparing and implementing such items.

Shyne (2006) investigated the efficacy of enrichment to reduce stereotypic behaviors in a meta-analysis of many captive species including African and Asian elephants and concluded that enrichment is a critical part of reducing negative behaviors commonly associated with stress in the environment. Enrichment effects on captive elephants are not well known but some studies show that food enrichment in the form of browse increases feeding behaviors and activity budgets; while others stated that foraging enrichment did not encourage foraging behaviors beyond the enrichment itself (Stoinski et al., 2000; Wiedenmayer, 1998). Furthermore, a more recent examination of how enrichment effects stereotypic behavior in captive elephants found there was no significant relationship between the two (Greco et al., 2016). Although there seems to be no definitive answer to how enrichment affects the welfare of captive elephants, it is important to provide varied environmental enrichment to these captive animals in an attempt to curb the potential for negative behaviors to occur as well as reduce stress-related hormone fluctuations.

Another management factor investigated was the number of times an elephant is fed in a 24-hour day. This variable has the potential to impact fecal glucocorticoids due to competition for food and/or arousal. Arousal could be related to excitement to be fed, anticipatory reactions to being moved indoors or out, and potentially even caused by keeper related interactions. In the multivariate analysis for African elephants, feed total was found to be a significant main effect in relation to fecal glucocorticoid levels. This result suggests that fecal glucocorticoid levels increase as the number of feedings increases. Results similar to these were found in a study of eight African elephants at the North Carolina Zoo, as frequency of stereotypic behavior was related to increased glucocorticoid values. The researchers suggested that the increased stereotypic behaviors were likely due to anticipation of being fed (knowing they will be fed at a certain time) and the associated arousal (Hasenjager & Bergl, 2015). Similarly, a study of eight Asian elephants at Chester Zoo in the United Kingdom, showed increased stereotypic behavior near times of being fed; which was again attributed to the anticipation of routine feeding times (Rees, 2009). Therefore, increased number of feedings may not be a negative effect, but are a consequence of providing more unpredictable feedings to lower the frequency of food related stereotypic behaviors in zoo elephants (Rees, 2009).

It is possible that elephant diet nutrient variability (fiber and sugar differences) or percentages of diet from nutritionally complete feeds affected fecal glucocorticoid results as it has been postulated in other mammalian species (Ange-van Heugten et al., 2009). Factors related to feed diversity and feed total have been found to have significant effects on the likelihood of captive elephants being obese (Morfeld et al., 2016). Both species of captive elephants are reported to be overweight and / or obese when compared to their wild counterparts (Ange et al., 2001). Unfortunately, this study was not able to evaluate diet type or elephant body condition in relation to fecal glucocorticoids, although they would have been interesting additional components.

The social structure for elephants is an important factor to investigate not only when forming new herds but also when evaluating how those interactions impact stress. For African elephants, the number of animals an individual comes into contact with during the day tended toward significance. Meaning that fecal glucocorticoid concentrations decreased as the number of contact animals increased. The same was not found for the Asian model; however, the social information was based on keeper surveys. Due to the large scale of this current study, it is possible that the Asian elephant data was skewed due to the inherent variability in keeper survey responses. Studies on social support of many species show that individuals with social support tend to have decreased cortisol secretion during acutely stressful events; while regular social group changes can have an activating effect on cortisol secretion (Heinrichs et al., 2003; Levine, 1993).

Of the two other social factors evaluated in this current study, social group count was found to be a significant main effect in relation to glucocorticoid concentrations in African elephants. There was variability in the number of social groups reported at each facility over the 12-month study duration. One zoo in particular reported 43 different social groups in that time period. Further evaluation of this facility showed that it houses twelve elephants which is a large herd for a captive facility. Due to the size, issues among the elephants are more likely to occur, thus forcing management to move the elephants between different social groups more frequently when compared to a smaller herd. Therefore, it is unlikely that there was an error in the data collection for this facility given that it houses a larger than average herd size. Omitting this facility from the model removed the significance of the social group count variable, however, since it was assumed that this data is likely not an error, the facility remained in the final model for African elephants.

Previously within the greater IMLS study, social group contact was found to be a significant factor related to the higher risk of female hyperprolactinemia (Brown et al., 2016). Thus, the likelihood of hyperprolactinemia was increased by the increased number of social groups a female elephant had contact with or spent time in. Social experience also was found within the IMLS study to have a beneficial effect on stereotypic behavior; showing that individuals who spent more time in suitable social groups had reduced rates of stereotypy

(Greco et al. 2016). Therefore, social factors may pose as significant predictors in relation to fecal glucocorticoids in elephants although not noted currently.

Lastly, two health measures were included in this study to examine whether or not chronic foot and joint problems could be a contributing factor to glucocorticoid concentrations. According to the data presented above, both elephant populations are relatively healthy in regards to foot and joint health. Given this information, increases in fecal glucocorticoid levels were not related to chronic conditions in either the foot or the joints for most of the captive elephant population. However, there were instances for both species where there was an individual who met the most extreme (worst) evaluation values, which could mean that a small few of the individuals would be experiencing some sort of pain or discomfort due to these issues. For this study population, foot and joint scores were not related to fecal glucocorticoid concentrations for either species. Previous evaluation of foot and joint scores within this IMLS population showed a significant correlation between age and joint scores; which is expected as many species experience joint issues with age including elephants (Miller et al., 2016).

The lack of significance exhibited for these variables could be related to the fact that much of the population was in early adulthood. Therefore, these issues would not be as common. Although, even if we were able to isolate a relationship, any results could potentially be confounded by the likelihood that the animal(s) tend to be obese according to body condition scoring of these individuals (Morfeld et al., 2016).

Conclusion

Using glucocorticoids as an indicator of stress has been shown to be useful, however, it is important to consider the naturally occurring fluctuations of cortisol and its metabolites before making any statements with regard to the welfare of the animals. Based on our data, fecal glucocorticoids exhibited seasonal fluctuations in one species but not the other; which may be a species difference previously unknown in captivity. Ideally, future research on seasonal changes in glucocorticoid concentrations would include sampling from a facility that housed both species in order to eliminate causes of error such as facility location, climatic differences, diet, and keeper interactions. This may be difficult to do, as many facilities specialize in single species herds as prescribed by the husbandry protocols set in place by the AZA (AZA, 2012).

As the first study to document the fecal glucocorticoid concentrations of such a large proportion of the captive elephant population in North America, it is important that these data set a starting point for future research focusing on glucocorticoid concentrations in captive elephants. Providing the largest fecal glucocorticoid dataset of its kind for both the African and Asian elephant species, this current research is an invaluable tool for future captive elephant comparison studies. There is much to learn about what factors within the individual and in the environment play a role in affecting stress measures such as glucocorticoids. Given this study was conducted using feces as a method of hormone monitoring, I think the next step would be to evaluate the diets of the elephants during a large scale glucocorticoid study. Differences in diet play a role in affecting the absorption and excretion rates of fecal hormone metabolites as seen in North American red squirrels (*Tamiasciurus hudsonicus*) where changes in dietary fiber varied glucocorticoid concentrations depending on dosage and food types available (Dantzer et al., 2011). Dietary fiber increased the amount of dropping mass in European stonechats (*Saxicola torquata rubicola*) which translated to changes in the fecal androgen and corticosteroid concentrations for those birds (Goymann, 2005). Another study conducted on wild Alaskan brown bears (*Ursus arctos horribilis*) also showed a significant interaction between diet type and variations in fecal glucocorticoid concentrations, suggesting that dietary changes do play a role in how glucocorticoids are eliminated from the body (von der Ohe et al., 2004).

This thesis demonstrates the importance of looking at multiple variables as potential factors that can impact glucocorticoid levels. Wild elephant populations are in crisis and face inevitable extinction if things stay the way they are currently; therefore, it is vital for scientists and animal care facilities to work together to provide the best care and welfare standards possible for those housed in captivity. When animals are housed appropriately, we can continue to learn more from them so that we can apply this knowledge to bolster captive populations and potentially address the declining wild populations. Therefore, it is necessary to continue these research efforts to determine the specific environmental factors that detrimentally affect glucocorticoid secretion in African and Asian elephants.

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APPENDICES

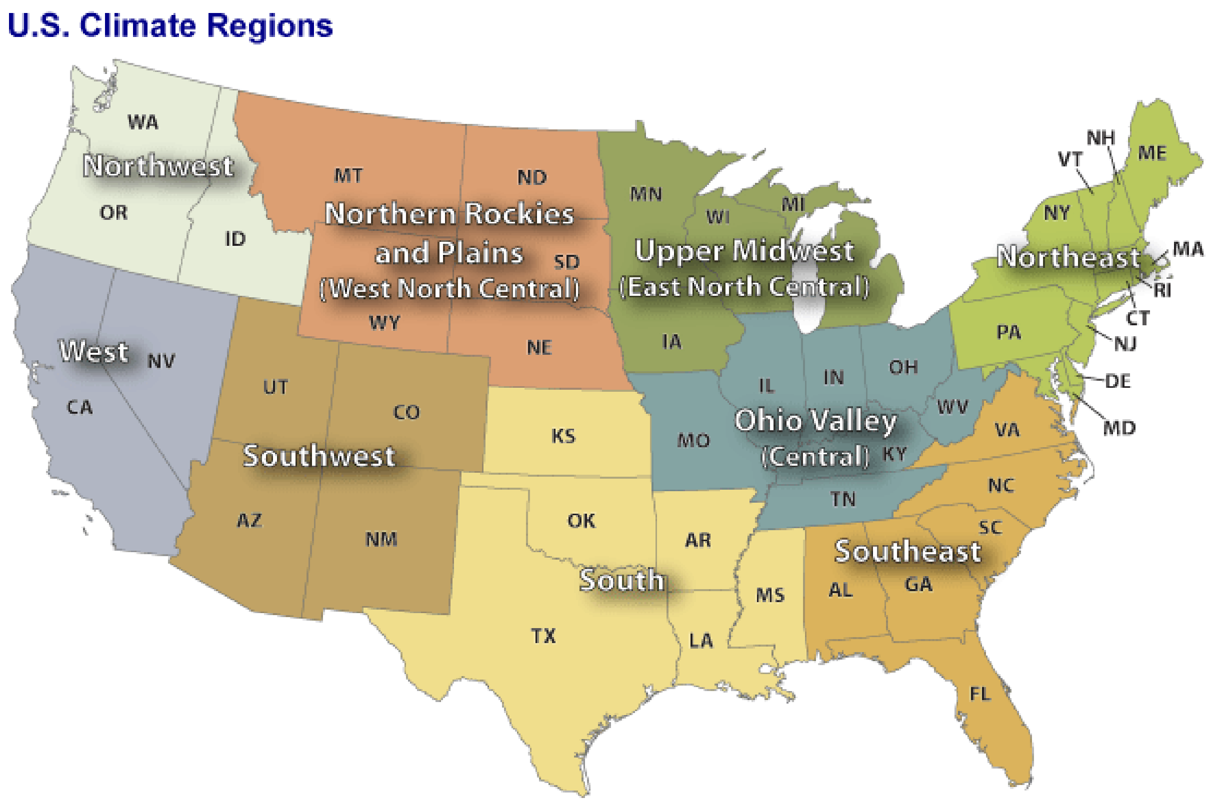
Appendix A

Participating North American zoos. Below is a listing of all 65 facilities that participated in this research study. All facilities have been separated into climate zones based on their physical locations across North America.

Climate	Facility
Central	Cincinnati Zoo & Botanical Garden
Central	Cleveland Metroparks Zoo
Central	Dickerson Park Zoo
Central	Indianapolis Zoological Society, Inc.
Central	Knoxville Zoological Gardens
Central	Little Rock Zoological Garden
Central	Louisville Zoological Garden
Central	Memphis Zoological Garden and Aquarium
Central	Nashville Zoo
Central	Niabi Zoo
Central	St. Louis Zoological Park
Central	Toledo Zoo
East North Central	Milwaukee County Zoological Gardens
Northeast	Buffalo Zoological Gardens
Northeast	Buttonwood Park Zoo
Northeast	Maryland Zoo
Northeast	Metropolitan Toronto Zoo
Northeast	Roger Williams Park Zoo
Northeast	Rosamond Gifford Zoo at Burnet Park
Northeast	Seneca Park Zoo
Northeast	Wildlife Conservation Society - Bronx Zoo
Northeast	Zoo de Granby
Northwest	Oregon Zoo
Northwest	Point Defiance Zoo and Aquarium
Northwest	Utah's Hogle Zoo
Northwest	Wildlife Safari
Northwest	Woodland Park
South	Africam Safari *located in Mexico
South	Audubon Institute
South	BREC's Baton Rouge Zoo
South	Caldwell Zoo
South	Cameron Park Zoo
South	Dallas Zoo
South	El Paso Zoo

South	Houston Zoological Gardens
South	Lee Richardson Zoo
South	Oklahoma City Zoological Park
South	San Antonio Zoological Society
South	Sedgwick County Zoo
South	The Kansas City Zoo
South	Topeka Zoological Park
South	Tulsa Zoological Park
Southeast	Birmingham Zoo
Southeast	Busch Gardens
Southeast	Disney's Animal Kingdom
Southeast	Greenville Zoo
Southeast	Jacksonville Zoological Gardens
Southeast	Lowry Park Zoological Garden
Southeast	National Zoo
Southeast	North Carolina Zoological Park
Southeast	Riverbanks Zoological Park
Southeast	Virginia Zoological Park
Southeast	Zoo Atlanta
Southeast	Zoo Miami
Southwest	Albuquerque Biological Park
Southwest	Cheyenne Mountain Zoological Park
Southwest	Denver Zoo
Southwest	Phoenix Zoo
West	Fresno Chaffee Zoo
West	Honolulu Zoo
West	Los Angeles Zoo and Botanical Gardens
West	Oakland Zoo
West	San Diego Safari Park
West	San Diego Zoo
West	Santa Barbara Zoological Gardens

Appendix B



North American climate zone divisions by region provided by the National Oceanic and Atmospheric Administration (NOAA)- https://www.ncdc.noaa.gov/monitoring-references/maps/images/us-climate-regions.gif

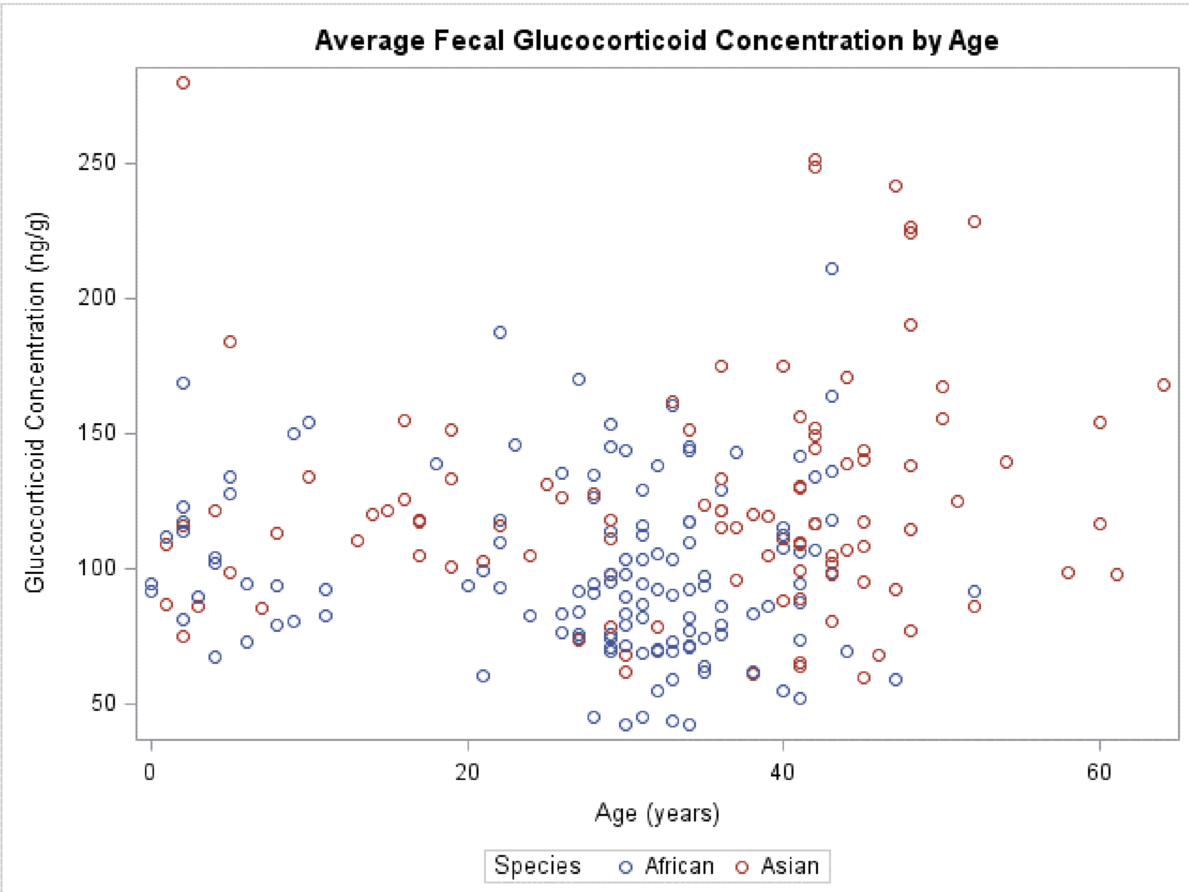
Appendix C

Enrichment items. Below is the listing of all 30 enrichment items from the management surveys provided to each facility (Greco et al., 2016).

1. Sand or dirt pile
2. Browse
3. Logs
4. Hanging objects
5. Problem-Solving items
6. Ice
7. Pool filled with water
8. Scratching post
9. Mud wallow
10. Balls
11. Scents
12. Wild animals
13. Tire
14. Keg
15. Treat boxes/bags
16. Dry wallow
17. Painting
18. Sprinklers
19. Music
20. Problem solving w/o food
21. Trees
22. Movable rocks
23. Pool filled with other
24. Snow pile
25. Waterfall
26. Nature sounds
27. Stream
28. Zoo birds
29. Zoo mammals
30. Zoo reptiles

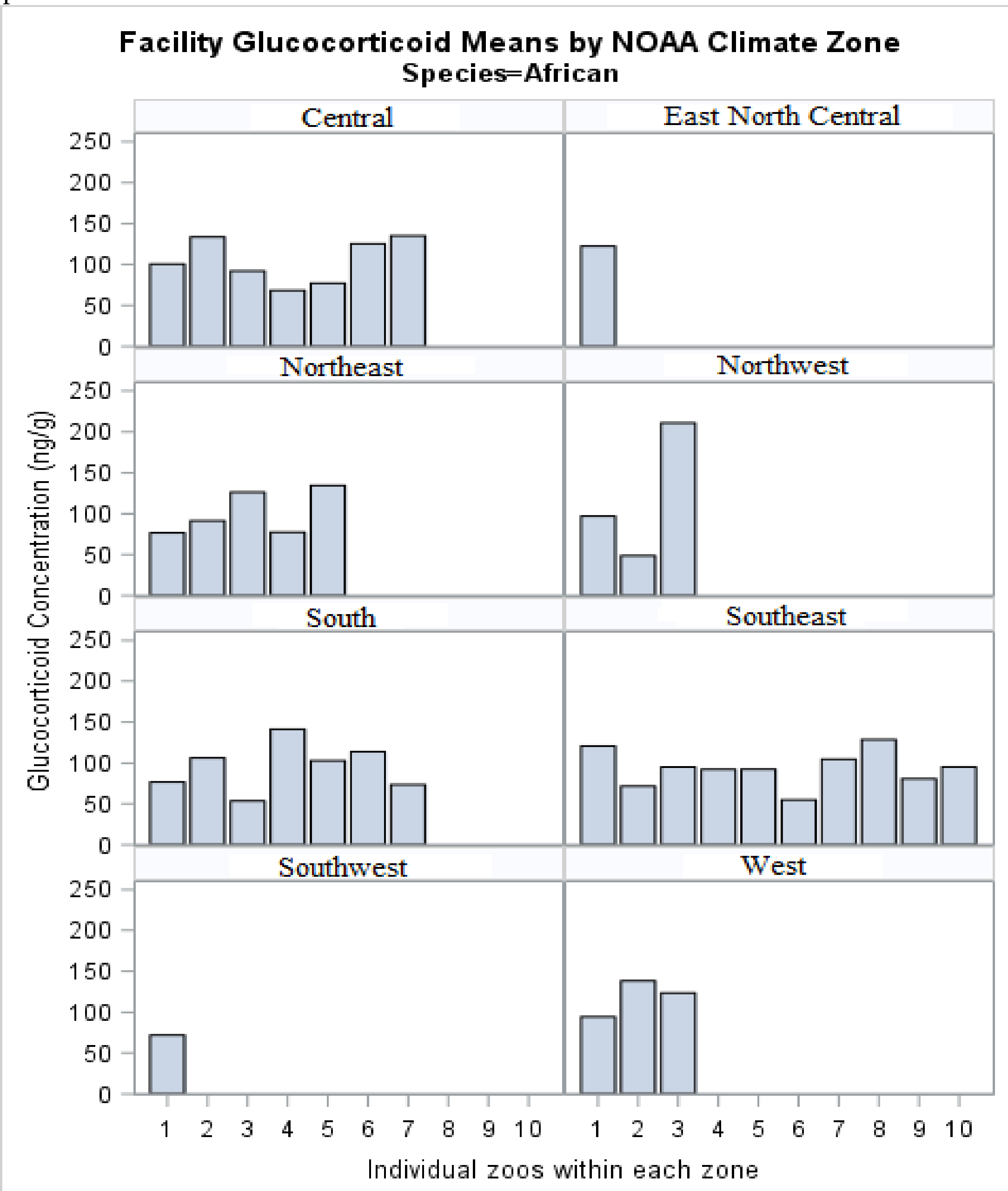
Appendix D

Scatterplot of mean glucocorticoid levels by age. African (*Loxodonta africana*) and Asian (*Elephas maximus*) elephant fecal glucocorticoid concentrations were plotted with age at time of study. Correlative analysis showed no relationship between age and glucocorticoid concentration, r = 0.10.



Appendix E

Facility glucocorticoid means by National Oceanic and Atmospheric Administration (NOAA) climate zone for African (*Loxodonta africana*) elephants. Mean fecal glucocorticoid concentrations for each facility (n=37) were obtained for all facilities housing African elephants. Facilities are separated by their respective NOAA climate zone for comparison.



Appendix F

Facility glucocorticoid means by National Oceanic and Atmospheric Administration (NOAA) climate zone for Asian (*Elephas maximus*) elephants. Mean fecal glucocorticoid concentrations for each facility (n=33) were obtained for all facilities housing Asian elephants. Facilities are separated by their respective NOAA climate zone for comparison.