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USING BIOMARKERS TO OPTIMIZE THE REHABILITATION OF WILD RAPTORS

by

MOLLY HAMAN

Under the Direction of Laura Carruth, PhD

ABSTRACT

The purpose of this study is to explore the use of stress physiology biomarkers in wildlife rehabilitation. We examined collection, extraction and enzyme immunoassay protocols in order to identify the optimal methods for testing corticosterone levels in injured raptors at a wildlife rehabilitation center. Corticosterone levels were measured periodically during the raptors' rehabilitation using noninvasive techniques. This study focuses on three species: Red-Tailed Hawks (*Buteo jamaicensis*), Red-Shouldered Hawks (*Buteo lineatus*), and Barred Owls (*Strix varia*). Fecal samples were collected from patients from all three species from May 2017 to March 2018. This information will contribute to our understanding of the stress profile of raptors in rehabilitation, and will allow for future studies to employ the methods validated by this project.

INDEX WORDS: Corticosterone, Enzyme Immunoassay, Glucocorticoids, Hormone Extraction, Raptors, Rehabilitation.

USING BIOMARKERS TO OPTIMIZE THE REHABILITATION OF WILD RAPTORS

by

MOLLY HAMAN

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of

Master of Science

in the College of Arts and Sciences

Georgia State University

2018

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Molly Haman
2018

USING BIOMARKERS TO OPTIMIZE THE REHABILITATION OF WILD RAPTORS

by

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May 2018

DEDICATION

I would like to dedicate this thesis to my partner Mike and to my family. Without your endless support I would not have been able to undertake such a daunting task. I spent many hours pondering how I would be able to complete this, and you all encouraged me when I needed it most. Even when you did not understand why I was spending so much time collecting fecal samples, you still listened to my thoughts and told me that it sounded great. Thank you for understanding that I had to move so far away for my graduate degree, and for sticking by me even when I was not there. I am sorry for the time we have been apart, and I still love you more than my work. I promise to slow down eventually.

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LIST OF ABBREVIATIONS

Atlanta Wild Animal Rescue Effort, Inc.	AWARE
Barred Owl	BDOW
Corticosterone	CORT
Enzyme Immunoassay	EIA
Georgia State University	GSU
Immunoglobulin G	IgG
Red-Shouldered Hawk	RSHA
Red-Tailed Hawk	RTHA

1 INTRODUCTION

1.1 Purpose of the Study

The purpose of this research study is to evaluate if the use of corticosterone (Cort) level monitoring, as a biomarker for stress, can contribute valuable health information for wild raptors undergoing rehabilitation at a local wildlife center. We aim to discover if monitoring Cort (a glucocorticoid secreted from the adrenal cortex) in fecal droppings across rehabilitation can be used to modify the rehabilitation of raptors to decrease morbidity caused by stress. Measuring Cort during recovery may also help to increase their survival rates post-release. Using non-invasive and minimally invasive techniques to monitor stress in animals in captivity and the wild can provide useful information for the conservation and management of different species. Stress profiles can vary greatly between different species, and there is very little information available on stress in wild raptors. Even less is known about stress in raptors undergoing rehabilitation from various injuries. Here we will investigate using Cort as a biomarker for stress in raptors to better understand how they respond to their injuries and the rehabilitation practices at the Atlanta Wild Animal Rescue Effort, Inc.

The Atlanta Wild Animal Rescue Effort Inc. (AWARE), located on the Arabia Mountain in Dekalb County, outside Atlanta, Georgia, provides care and rehabilitation for injured wildlife of all species. Of specific importance are the large number of raptors rehabilitated annually, which arrive with various injuries from natural or anthropogenic causes. Raptors in particular face an array of risks that threaten the stability of their populations, which include but are not limited to: electrocution from power lines, deforestation and habitat loss, illegal hunting and trapping, secondary poisoning, collisions with man-made structures such as windmills, vehicles, fences, etc. (Hager, 2009). The three most common raptor species that are admitted to AWARE

for rehabilitation are the Red-tailed hawk (RTHA, *Buteo jamaicensis*), the Red-shouldered hawk (RSHA, *Buteo lineatus*), and the Barred owl (BDOW, *Strix varia*). In 2014, AWARE received a total of 74 raptors of these species to be rehabilitated. Due to these risks and their frequency in being brought to AWARE, for the purpose of this study, we focused on the aforementioned species for data collection and analysis.

The use of endocrine measures in wildlife studies can provide useful information in individual and population health (Bradley & Altizer, 2007; Martin, 2009), and stress markers in particular, such as Cort, are intertwined with immunity, and correlations can be made between stress levels and the health of an individual (Martin, 2009). This is especially useful in studies of threatened and endangered species as a way to monitor the health and viability of a population (Kersey & Dehnhard, 2014), but also has applications for assessing the health of more common species. Corticosterone studies have also been used in wildlife studies as a way to measure a species' response to urbanization and habitat loss (Leyshk et al., 2013), which is one of the most pervasive threats to biodiversity. The loss of habitat can increase stress levels by decreasing food availability, increasing conspecific competition, decreasing space availability, plus a multitude of other ramifications (Leshyk et al., 2013). The use of endocrine markers to assess stress and health in wildlife populations could be invaluable, and the information that could be gained from understanding the stress levels in wild raptors could be immensely helpful in tailoring rehabilitation practices to accommodate their needs, and to potentially decrease morbidity rates during the raptors' recovery.

1.1.1 Environmental Impacts on Wildlife

The impact of humankind on the environment is pervasive. The list of man-made environmental changes is extensive. Urbanization, habitat loss, fragmentation, habitat

conversion, deforestation, pollution, climate change, and many other environmental impacts directly stem from human sources. These changes to the environment impose increasingly negative effects on ecosystems and ecosystem services, biodiversity, and population health. These negative effects can cascade down through an ecosystem and have numerous and often unforeseen effects on wildlife. Behavior, fitness, reproductive success, abundance, distribution, and population dynamics can all be altered by a disturbed environment. These fundamental issues of the Anthropocene (the current geological epoch) have contributed to the current biodiversity crisis, and it is therefore imperative for us to research new and better ways to help preserve, monitor and restore wildlife populations.

1.1.2 Urbanization

One of the greatest human impacts on wildlife populations is urbanization. The rate of people moving into urban areas is growing rapidly, and urban land conversion is a leading cause of habitat loss for wild populations (McKinney, 2002). As of 2010, approximately 80.7% of the population in the United States was living in urban areas (including cities and suburbs), while only 19.3% were residing in rural areas (USCB, 2010). Compared to numbers taken from 2000, the urban areas are continuing to grow while people are simultaneously leaving rural areas (USCB, 2010). The loss of habitat caused by urbanization and the expansion of urban centers is quite long lasting compared to other types of habitat loss (McKinney, 2002). Once a region of land is urbanized it tends to spread (McKinney, 2002), rather than be returned to its natural ecosystem. The effects that this can have on wild populations are vast. Birds, in particular, face a wide array of challenges in urban areas, and while the populations are some species are highly abundant in urban regions there is also a reduction in species richness (Chace & Walsh, 2004). Granivores and insectivores are favored in urban areas, as are resident species over those that

migrate (Chace & Walsh, 2004). This favorability results in limits of the types of birds that can live in urban areas and decreases the species diversity (Chace & Walsh, 2004). Certain species of raptors may be successful in urban regions due to greater food availability, though most raptors have a larger home range than other avian species and may not live exclusively in urban centers (Chace & Walsh, 2004). This higher availability of resources may not offset the other difficulties of living in urban areas though, such as increased interaction with humans. Bosakowski and Smith (1997) conducted a study on the distribution and species richness of raptors near urban areas in New Jersey, and they found that Red-shouldered hawks and Barred owls tended to avoid areas of their habitat that had been urbanized. Red-tailed hawks, on the other hand, were more likely to be found in regions that were disturbed by human development compared to other raptor species. The reasoning for this may lie in the fact that Red-tailed hawks are generalist predators, and therefore may be more successful in urban areas (Bosakowski & Smith, 1997). Clearly, the relationship between urbanization and the surrounding wildlife is intricate, and it does not simply incur negative effects on species in the vicinity.

1.1.3 Human Interactions

The effects of urbanization on surrounding wildlife is a multifaceted problem. It does not simply remove areas of natural habitat, but also increases human interaction with wildlife. This can come in a variety of forms and can affect the survival, fecundity, and distribution of wildlife populations among other things (Holmes et al., 1993). The greater interaction that humans have with wildlife, the more they prevent wildlife from accessing crucial resources. In the case of birds, this can be food, nesting or roosting sites, or access to mates (Gill, 2007). Additionally, greater human interaction in a natural habitat, such as on public land, can directly lead to the degradation of that habitat (Gill, 2007), further compounding the effects of this problem. The

effects of human disturbance can be measured by assessing changes in distribution, demographic factors, population size, or behavior (Gill, 2007). The response of raptors to human disturbance has been documented previously in a 1993 study by Holmes, and it measured flight response and flush distance to humans approaching on foot or by vehicle (Holmes et al., 1993). Each species showed varied responses, but all species were more inclined to flush in response to humans on foot, than by a vehicle (Holmes et al., 1993). Other studies have shown that increases in human disturbance are directly related to breeding success, such as a study conducted by Robert and colleagues (1975) which showed that human disturbance led to increased hatching failure in Western Gulls on the Farallon Islands (Robert et al., 1975). Human disturbance has also been linked to changes in feeding behavior. A study conducted on Blackbirds at three urban park regions in Madrid, Spain found that increased human disturbance lead to increased movement rate for the birds, increased vigilance, increased distance from the disturbance region towards areas with more protection, and they showed a stark decrease in foraging behavior (Fernandez-Juricic et al., 2000). These behavioral responses are typical in response to a potential predator and are correlated with increased circulatory levels of glucocorticoids such as corticosterone (McEwen et al., 2003), among other physiological responses. In addition to altered behavioral responses, wildlife may also alter their distribution in response to human development and disturbance. Bald Eagles in the Chesapeake Bay were monitored using radio telemetry, and only 4.9% of locations visited by the tagged eagles were classified as developed areas (Buehler et al., 1991). This has implications for the amount of land that is suitable for use by eagles, and Buehler speculated that in this region approximately 34% of the surrounding shoreline was suitable and the remaining 76% had little potential due to the amount of human development and activity (Buehler et al., 1991). Animals that do choose to stay in areas that have higher human activity

are more prone to experience the negative effects of human interaction, which may lead to elevated stress levels, injury, displacement or death (Bath et al., 2003). Regardless of whether these interactions were intentional harassment or not, the effects of human interaction with wildlife are still largely detrimental in nature.

1.1.4 Habitat Conversion

Alteration of land use, from the natural habitat to use for human development, is well documented as having a major impact on surrounding wildlife and biodiversity. Two of the most common types of habitat conversion, aside from the aforementioned urbanization, are logging and agriculture.

Each of these practices removes land that was previously used by native wildlife and decreases the space they have to obtain necessary resources. In a simulation study conducted by Gaston and colleagues (2003), it was estimated that the global bird population was between 39.34 to 134.04 billion individuals, with an average population of about 86.70. Of this estimate, approximately 18.5% of individuals occupy habitats that have been modified by human development as cropland or pasture (Gaston et al., 2003). All land types were taken into consideration, including tundra, ice, deciduous forest, boreal forest, temperate and tropical forest, savannah, and so on. Each type of land could be converted into cropland or pasture, and from the approximately 5000 simulations performed in this study, it is estimated that in these converted habitats approximately 22.1% density of individuals was lost in these regions (Gaston et al., 2003). The results of this study did show an increase in global bird population over time, which is associated with the habitat conversion, but it is also associated with an overall decrease in bird biodiversity because these monocultures cannot support the higher species richness (Gaston et al., 2003).

Logging also presents problems for wildlife, and it has been shown to decrease species richness of avian species in regions that undergo selective logging (Thiollay, 1997). In a study conducted in French Guiana tracking bird diversity in disturbed forests, it was found that species richness decreases from primary forest to forest selectively logged after one year, and forest selectively logged after ten years (Thiollay, 1997). Thirty-three species of raptors were also surveyed for this study, and overall it was found that their species richness increased in fragmented forest regions presumably due to ease of predation, but the total abundance of raptors decreased significantly in disturbed areas (Thiollay, 1997). In addition to the negative ecological effects that habitat conversion has on a population, there are also physiological consequences for individuals in these regions, such as increased stress levels (See Section 1.5). By expanding agriculture, logging, and other types of habitat conversion, it is clear that this will lead to a continuing loss of biodiversity, not only in avian species but for all plants, animals, and microorganisms.

1.1.5 Fragmentation

Habitat fragmentation is a consequence of urban development, agriculture, or other types of habitat destruction. The alteration in land use separates areas of native vegetation and natural habitat into disconnected fragments. These fragments are characterized by being isolated from other each other, and by possibly having a specific microclimate within and around them (Saunders et al., 1991). Land fragmentation is troublesome for many species of wildlife because it also separates them from their resources (i.e., food, shelter, mates, etc.). This has been shown to have a measurable effect on biodiversity, and the species living in areas that have been fragmented (Fahrig, 2003). Species richness, abundance, distribution and genetic diversity may all be negatively affected by habitat fragmentation (Fahrig, 2003). Additionally, the loss of

habitat associated with fragmentation may affect predation rates, foraging success, breeding success, trophic chain length, behavior, and survival (Fahrig, 2003). Raptors specifically may experience different effects compared to terrestrial animals, and these may be a good indicator species for the changes of an ecosystem because they are an apex predator (Carrete et al., 2009). Many studies have looked at the effect of fragmentation on native raptor species. One such study conducted in Argentina quantified the presence of diurnal raptors in five different biomes by conducting surveys and road counts during the breeding season and compared the estimated abundance levels across differing levels of habitat loss (natural habitat, mixture of natural and culture, culture, and urban habitat) (Carette et al., 2009). This study showed that the raptor species studied showed varying sensitivity to habitat transformation and fragmentation, but overall, the abundance and diversity of raptors in a region decreased as habitat transformation and fragmentation increased (Carette et al., 2009). These results are significant for conservation efforts because of the importance of apex predators within ecosystems (see Section 1.4).

1.1.6 Pollution

Pollution is a pervasive problem and can come in many forms. For birds, some of the most detrimental types of pollution are heavy metal pollution and endocrine disrupting chemicals (both described below). Petrochemicals are also detrimental to many species, but generally affect seabirds and other aquatic and marine species and are less likely to affect raptors.

1.1.6.1 Heavy Metal Pollution

Heavy metal pollution is found in the environment as an effect of traffic emissions, industrial emissions, weathering of buildings and pavement, agricultural sources such as pesticides and fertilizers, and so on (Wei et al., 2009). Heavy metals have been shown to accumulate in plant and animal tissues and can have negative effects on multiple organ systems,

as well as having a high level of toxicity and potentially carcinogenic effects (Tchounwou et al., 2014). As apex predators, raptors are likely to accumulate higher levels of heavy metals in their bodies from consuming prey exposed to contaminants in their environment (Kitowski et al., 2017). These elements can be deposited in the liver and eggshells of birds and can affect the survival and reproductive fitness of individuals exposed to dangerous levels (Hernandez et al., 1999). Ultimately, the effects experienced by individuals and their offspring may alter population dynamics.

1.1.6.2 Endocrine Disrupting Chemicals

Endocrine disruptors are another detrimental pollutant that raptors and other species of wildlife can be exposed to. These compounds mimic endogenous hormones and can disturb the normal regulation of endocrine system (Casals-Casas et al., 2011). Endocrine disruptors generally act by binding to hormone receptors in the body and compete with endogenous hormones for binding to their respective receptors (Casals-Casas et al., 2011). Some common endocrine disruptors are found in pesticides, plastics (such as bisphenol-A), phthalates, flame retardants, and many other products (Casals-Casas et al., 2011). When these chemicals make their way into the environment as environmental contaminants they can have deleterious effects on wildlife, as was seen with the pesticide dichlorodiphenyltrichloroethane (DDT). In the case of raptors and other animals, endocrine disruptors can affect developmental processes, reproductive ability and success (Vos et al., 2000). Endocrine disruptors can affect the reproductive success of birds by causing eggshell thinning, which results in the eggs being unable to support the weight of the incubating mother. Additionally, these compounds can also result in decreased hatching success, behavioral feminization, reproductive failure, and other kinds of effects (Vos et al., 2000). The effect of endocrine disrupting chemicals, like that of heavy metals, can damage the

individual exposed and also their offspring, which can disturb population dynamics and threaten the stability of the species.

1.1.7 Causes of Injury and Mortality in Raptors

Urbanization and human development have altered natural habitats for wildlife species and have led to an increase in potential health threats for affected individuals. Some raptor species hunt in urban areas due to the increase in prey availability and thus face an array of health threats in this type of habitat. Some of the most common threats to raptors include electrocution from power lines, collisions, unintentional poisoning from eating poisoned prey, and illegal hunting or trapping (Hager, 2009). These threats can cause injury and result in an individual's need for rehabilitation, or possibly mortality depending on the extent of the damage. Electrocution caused by power lines primarily leads to mortality in raptors, and despite measures taken by electrical companies to increase the safety of power lines the problem still persists (Lehman, 2001). Collisions are another major cause of injury and mortality in raptors (Fix et al., 1990; Wendell et al., 2002), and often are caused by man-made structures such as vehicles, fences, windows or wind turbines. Poisoning is also a concern for raptors in urban areas because of the prevalence of rodenticides. These chemicals are a type of pesticide used to control rodents by preventing the vitamin K cycle in the liver, which results in hemorrhage and death (Lambert et al., 2007). Rodenticides can result in secondary, unintentional poisoning of raptors when they consume rodents that have ingested the poison. The secondary poisoning of raptors by rodenticides results in the same symptoms and may result in internal injury or death caused by hemorrhage dependent on the amount of poison present (Hegdal et al., 1988). Hunting and trapping also lead to injury or death in raptors, and as all birds of prey are protected under the Migratory Bird Treaty Act of 1918, any hunting or trapping of raptors is illegal. Though gunshot

trauma makes up only a small percentage of injuries in raptors admitted to wildlife centers or veterinary services (Richards et al., 2005; Deem et al., 1998), the injuries sustained from this type of trauma lead to morbidity or mortality in some cases. It is important to understand each of these threats to raptors because they are connected to human-related activities, which can cause injury or death in individuals, and also may affect population levels as a whole.

1.1.8 Loss of Biodiversity

The cumulative effects of urbanization, human interaction, loss of habitat, pollution, and all human-related threats to raptors are essential to consider for the preservation of their populations. Raptors are apex predators and play a crucial role in the ecosystems that they inhabit (See Section 1.4). Currently, biodiversity is decreasing at an alarming rate (Dirzo et al., 2014), and as humans are a driving cause for species extinctions, it is our responsibility to try to mitigate the losses. Biodiversity needs to be supported within ecosystems to ensure that all ecosystem functions are maintained at working levels (Gamfeldt et al., 2008). Generally speaking, increasing species richness also increases the sustainability of ecosystem functions (dependent on the role, or the number of roles, a species plays in an ecosystem) (Gamfeldt et al., 2008). Therefore, each species is important to preserve as they all contribute to the overall health of an ecosystem. This study aims to contribute to the preservation of raptor species by monitoring physiological markers, and ultimately to help preserve biodiversity as a whole.

1.2 Wildlife Responses to Environmental Change

The changes occurring in the environment are pervasive, and they take their toll on the surrounding wildlife. Habitat loss, fragmentation, pollution and other disturbances can affect wildlife at the community and population level by affecting abundance, distribution and species richness, and so on. Environmental change can also affect populations at the individual level by

altering survival, stress response, epigenetics, reproductive fitness, and so on. Understanding how wildlife responds to changes in the environment could be important in determining the negative effects of human development and globalization.

1.2.1 Adaptation and Rate of Extinction

The environment is constantly changing, and in order for an organism to survive, it is necessary to be able to respond to changes in the environment in a way that may increase their suitability to that environment. In the case of habitat conversion and urbanization, surrounding wild populations need to adapt to their surroundings at an unsustainable rate (Chevin et al., 2010). If adaptation to the new environment is not possible, species may migrate to new habitats or become extinct. One of the most drastic effects the environmental change may have on wild populations is an increase in the rate of extinction for the surrounding species. A species may become extinct when the rate of change in an environment happens rapidly and continuously, such that they do not have the ability to acclimate to the changes (Chevin et al., 2010). When a population becomes sufficiently small, it may begin to spiral into an extinction vortex due to environmental factors, demographic stochasticity, and genetic components such as inbreeding (Fagan & Holmes, 2006). Environmental factors that affect the stability of a population include but are not limited to variations in climate and natural disasters. These factors can lead to a stark loss of individuals, which can compound the problems of demographic stochasticity and inbreeding and may lead to a bottleneck of genetic variation (Blomqvist et al., 2010). Demographic stochasticity can also be problematic in small, finite populations (Lande, 1993). In cases where most of the individuals of a population have not reached, or have passed, their breeding capability, or if the number of males outweighs the number of females (or vice versa), a population can spiral into an extinction vortex relatively quickly if the birth rate does not exceed

the death rate. Gestation length and time to reach maturation can also affect the ability of a population to sustain its numbers. Inbreeding then causes further issues in the population, when the genetic variability cannot be maintained and can affect the survivability of individuals which may contribute to the death rate as well (Blomqvist et al., 2010).

1.2.2 Demographic Responses

Many aspects of a population may be altered in response to environmental changes, one of which is the demography of the population. Demographic stochasticity in wild populations refers to the survival and reproductive probabilities for a given developmental stage within a population (Lande, 1988), which may include: the number of individuals in a population, the birth and death rates, the sex ratio, the number of individuals of a certain age, the time to reach sexual maturity, the number of individuals that survive from one year to the next, the number of offspring produced in a breeding season, among other values. The combined effect of these values plays a role in the probability of a population to survive for a certain amount of time (Lande, 1988). One of the most detrimental effects of demographic stochasticity on the survival of a population occurs when the population is unsustainable, or when the death rate exceeds the birth rate. This means that the population is decreasing, and the number of individuals born is not able to replace the individuals lost. When a population that is sufficiently small is unsustainable, it is at risk of local, or potentially global extinction (Blaustein et al., 1994). Many factors can contribute to a population becoming unsustainable, such as habitat loss, disease, predator-prey dynamics, competition, inbreeding, and so on. It is therefore important to understand how species respond to environmental change at the population level, and how their life history is incorporated into the demography of their population, in order to better manage species that are at risk of becoming threatened, endangered or extinct.

1.2.3 Developmental and Physiological Responses

In addition to demographic changes, wildlife may experience developmental changes in response to an altered environment. Inbreeding is an example of this, and it can be challenging for a small population. Inbreeding occurs when populations become sufficiently small and isolated so that the alleles available are limited and eventually become fixed (Keller & Waller, 2002). The smaller the population becomes, the faster alleles become fixed. This loss of genetic diversity leaves a population vulnerable to genetic bottlenecks, and the population may be unable to adapt to new environmental stressors. Inbreeding also leads to an increase in deleterious mutations, which can affect the development, survival, and fecundity of an individual (Keller & Waller, 2002).

Aside from the genetic changes of inbreeding, epigenetic factors may alter developmental processes in response to environmental change as well. These factors, such as methylation and acetylation, can modify chromatin structures to make areas of the DNA accessible or inaccessible for transcription (essentially turning a gene on or off) (Feil & Fraga, 2012). Diet, environmental pollution, temperature, parental care, and other stressors can affect epigenetics, and cause long-lasting developmental changes that may be heritable for subsequent generations as well (Feil & Fraga, 2012). Epigenetic processes are essential for organisms to be able to adapt and respond to their environment, but in conditions that are too harsh, it may have deleterious effects. These changes can happen to organisms in utero, but also postnatally (Feil & Fraga, 2012). An example of a phenotypic change attributed to epigenetic alterations seen in response to environmental changes are can be seen in aphids, which can go from wingless to winged insects in response to predators or other stressors. Also, when the Agouti gene is expressed in mice, they will exhibit a yellow coat, obesity, and diabetes. Various plant species will flower early via

epigenetic signals in response to temperature changes in the environment, and so on. (Feil & Fraga, 2012). Often the epigenetic signals themselves are intertwined with and influenced by the endocrine system (Monaghan, 2008), and thus it is relevant to understand how hormones vary with the environment to avoid deleterious epigenetic changes in an organism or its offspring.

1.2.4 Behavioral Responses

Behavior can be flexible, and changing behavioral reactions in response to the environment are well documented. It is a useful mechanism for adaptation, and changes in behavior are often seen in response to urbanization and human development (Lowry et al., 2012). Many behavioral responses may change, such as foraging, reproduction, use of resources, and use of shelter (Lowry et al., 2012). Breeding seasons may be shortened or lengthened in some species near urban areas where resources (i.e., food from humans, waste, man-made water sources) are regularly available (Lowry et al., 2012). Species such as songbirds that use vocalizations during the breeding season may also be affected by the increased noise pollution in urban settings (Ditchkoff et al., 2006). Movement and foraging behaviors can also be greatly affected by urbanization and human disturbance. Some species will completely alter their activity temporally. Species that are normally diurnal may become exhibit crepuscular or even nocturnal activity in order to avoid human interaction (Ditchkoff et al., 2006), and this differs from the typical life history seen in rural counterparts. Large carnivores, such as coyotes, often show this difference in activity in urban environments, and this may affect their ability to feed based on the availability of prey at night compared to during the day (Ditchkoff et al., 2006). Home range size may be altered as well. It has been shown that coyotes and bobcats in urban areas show increased home range sizes, but in other species like raccoons and key deer, a decreased home range size has been reported (Lowry et al., 2012). This change in range is most

likely attributed to the availability of food sources or lack thereof, and the increased range in bobcats and coyotes may reflect their return to a den after foraging in an urban area (Lowry et al., 2012). Some species may exploit human-linked food resources as well, from waste sites or by direct feeding from humans (Lowry et al., 2012). These behavioral alterations associated with environmental change are distinctly different from the behaviors exhibited by rural counterparts, and this may be deleterious for urban populations.

1.3 The Role of Rehabilitation Centers

Because of the numerous threats facing wildlife today the availability of wildlife rehabilitation centers, especially near urban areas, is essential to the conservation and preservation of species. Rehabilitation centers may be useful tools for conservation as a way to combat the effects of human development on wild populations, to assess population health data, to provide educational programs for the general public, and to allow for the reintroduction of injured wildlife back to their natural habitats. There are negative aspects of rehabilitation as well, such as an increase in patient stress caused by captivity, and an increase in the potential for disease due to different species of animals being housed in close proximity. The detrimental effects of rehabilitation may lead to a decrease in reintroduction success. Rehabilitation centers may also be impacted by political or economic motivations. Many wildlife centers, such as AWARE, are non-profit organizations that rely on donations to treat their patients, and therefore may be affected by a lack of funds or supplies and volunteers which ultimately can affect the health of patients.

1.3.1 Raptors in Rehabilitation

Raptors in the wild face a wide array of threats (See Section 1.1.7). These threats may be of natural or anthropogenic origins, and many may lead to injury or death. Urban areas may be

especially dangerous for raptors, and because of these threats, wildlife rehabilitation centers are an essential resource for injured animals. As apex predators, it is essential to have rehabilitation centers available for wild raptors in order to protect these species and maintain the health of the ecosystem. Raptors are often admitted to rehabilitation centers for collision injuries, such as with vehicles, windows, wind turbines, fences, and so on. (Hager, 2009). They are also prone to electrocution from power lines, secondary poisoning from rodenticides and other pesticides, and occasionally gunshot wounds from illegal hunting (Hager, 2009). Rehabilitation centers must be equipped to handle all of these injuries to treat raptors, with the aim of releasing as many patients as possible. Birds of prey may present with difficult injuries (Burke et al., 2002). Therefore, it is necessary to understand the best methods for medical care, housing, and rehabilitation.

1.3.2 Raptor Care Guidelines

To better the treatment outcomes for raptors at rehabilitation centers it is important to understand the optimal care protocols for these species. Many aspects of a raptor's life history may determine their response to rehabilitation. Hunting style is crucial to understand with some raptors using the "sit and wait" method, in which they perch and watch the forest floor below for prey, while others use "soaring and scooping" to actively search for prey in flight. Dependent on the injury, it may be possible to release raptors that use the sit and wait method with a minor injury, but more active raptors that have not recovered the ability to maneuver swiftly to capture prey could slowly starve if they are not fully healed (Park, 2003). To mitigate post-release mortality, it is vital to test hunting ability in raptors prior to release (Park, 2003), which is an essential part of the protocol used by AWARE. Diet in the wild should also be considered for captive raptors. A typical raptor diet for in rehabilitation is dead mice or chicks. However, some species may refuse to eat mice and chicks if their diet preference is insects and reptiles (Park,

2003). Socially, raptors are solitary except during the breeding season. This may also complicate rehabilitation efforts if they are housed near, or with conspecifics (Park, 2003). This type of stress may result in further injury from raptors actively avoiding other birds and accidentally flying into the walls of their enclosures, which can cause feather damage, cracked beaks, sprains, bruising, and so on (Park, 2003). Stress in captive raptors has also been correlated with immunosuppression and lack of appetite (Park, 2003). Placing towels over the doors of enclosures is advisable to decrease stress in raptors housed in highly lit, busy areas (Park, 2003). Diet changes are advised for rehabilitating raptors that demonstrate a lack of appetite, and hunting and social characteristics should be examined to select an appropriate treatment plan. Such measures should be taken in rehabilitation centers to cater to the needs of specific species of raptors and to decrease stress in patients during treatment. This may allow for the selection of better treatment plans, and possibly more successful post-release outcomes.

1.4 Raptors Profiles

This study will focus on collecting data from three species of raptors: red-tailed hawks (*Buteo jamaicensis*), red-shouldered hawks (*Buteo lineatus*), and barred owls (*Strix varia*). These species are the most common raptors brought to AWARE, and each of them has differing life histories. They are all apex predators, but exhibit differences in geographic range, feeding behavior, reproductive behavior, and survivorship. These differences are important and may result in each species responding to rehabilitation differently. Thus, it is crucial to understand the life history of each in order to offer better care, and as a means to evaluate their stress in rehabilitation. The following sections will detail the pertinent information of each species, as well as give an overview of their role within the ecosystem.

1.4.1 Role in the Environment

Raptors are apex predators and consume a variety of different mammals and other small vertebrates. Apex predators are known to play an important role within an ecosystem and are also the source of great interest for the general public. Because of this interest, these vertebrates are often the center for conservation initiatives because they garner much support (Sergio et al., 2006). Though this may diminish the need for conservation of less charismatic species in some instances, there is still a need for the protection of vertebrates at the apex of the food chain.

In a 2006 article, Sergio and colleagues conducted a study that aimed at identifying the relationship between raptors as apex predators and biodiversity (Sergio et al., 2006). Six species of raptors were studied at both breeding sites and control sites in the Italian Alps, and surveys were conducted using song recognition to determine the total number of all avian species and individuals in each area. The number of avian species in each area was used as a measure of biodiversity, and compared to the presence of raptors at breeding sites or the control sites. Higher numbers of raptors were found at the breeding sites, and higher numbers of avian species, including vulnerable and non-vulnerable species, and individuals were recorded in these areas as well (Sergio et al., 2006). This indicates that the presence of apex predators is positively correlated with ecosystem biodiversity (Sergio et al., 2006).

Additionally, apex predators such as raptors may also play an important role in controlling the populations of smaller predators, or mesopredators, such that other prey populations may be able to thrive in response (Ritchie & Johnson, 2009). This definition of apex and mesopredator are context dependent (Ritchie & Johnson, 2009). An apex predator in one habitat may be a mesopredator in another dependent on the other species in that region. Though apex predators have a positive correlation with biodiversity, when apex predators decline from

an area it may allow mesopredators to move in, which can have cascading trophic effects on that ecosystem (Ritchie & Johnson, 2009). The presence of apex predators within a system is vital and has multiple roles in regulating ecosystem and food web processes through top-down control (Vidal & Murphy, 2018). Through reasonable conservation and management programs, apex predator populations should be bolstered to support ecosystem functions overall.

1.4.2 Barred Owls

In this study, we collected samples from two species of hawks and one species of owl. The barred owl (*Strix varia*) is a non-migratory species that has a wide range spanning across North America (Mazur & James, 2000). These owls can inhabit forests, swamps, riparian habitats, and has expanded into boreal forest habitats (Mazur & James, 2000). They are usually found in old-growth, mature forests that have both deciduous and coniferous trees (Mazur & James, 2000). Barred owls have a rounded head without ear tufts and a distinct facial disc. They are approximately 43-50 cm in height and weigh between 470 to 1,050 gm (Mazur & James, 2000). They are characterized by having dark eyes, a light yellow beak, and drab feather coloration that is beneficial for blending in with their surroundings (Mazur & James, 2000). The feather markings give them their name, as the coloration pattern is described as "barred" (see Figure 1), meaning they have alternating bars of dark and light coloration. While there are no sex differences in plumage, there is a slight difference in body size with the females being generally larger than males. As with other raptors, barred owls are voracious predators, but primarily nocturnal, as opposed to the two diurnal hawk species involved included in this study. They are opportunistic hunters and eat a wide variety of prey items including small mammals such as rabbits and mice, birds, amphibians, reptiles and even some invertebrates (Mazur & James, 2000). Barred owls are mostly a "sit and wait" predator, occupying high perches, and using their

acute vision and hearing to locate prey below. They are also almost entirely silent fliers because of their flight speed, and arrangement and adaptation of plumage (Sarradj et al., 2011). Barred owls are considered to be monogamous and to form permanent life-long pair bonds (Mazur & James, 2000), though not enough data is available to confirm this. Another important aspect of barred owl life history is their relationship with the great horned owl. In some regions, such as in Georgia, both barred owls and great horned owls occupy the same habitat, and barred owls are known to avoid interaction great horned owls. The great horned owl will prey on barred owl fledglings, and the two species may compete in small forest habitats because they have similar diets. These two species were known to be within 400 meters of each other only 1% of the time even when occupying the same region (Mazur & James, 2000). This could be significant to this study because AWARE often accepts great horned owls for rehabilitation, and they are kept in close quarters in the beginning stages of rehabilitation. Additionally, barred owls are known to avoid areas of human activity, and changes in physiology have been recorded in response to human interaction (Mazur & James, 2000). The total barred owl population in N. America appears to be increasing, though there are concerns associated with regions that experience vast forest clearing due to urbanization, logging, or other human activities (Mazur & James, 2000). This highlights the significance of rehabilitation centers as they pertain to the management of this species.

Figure 1: Barred Owl ambassador at AWARE. The feathers of the Barred Owl are distinctively barred with alternating light and dark coloration. Photo credit: Atlanta Wild Animal Rescue Effort, Inc.

1.4.3 Red-Shouldered Hawks

The red-shouldered hawk (*Buteo lineatus*) is another species involved in this study, which is commonly brought to AWARE for various injuries (See Section 2.3). This raptor species has a broad range in N. America, and its year-round habitats are concentrated along the east and west coasts of the United States, and to some extent within the Midwest (Dykstra et al., 2008). Red-shouldered hawks can be found in a diverse array of forests, but preference is given to mature forests with mixed deciduous and coniferous woodland (Dykstra et al., 2008). Per its name, red-shouldered hawks have distinct red plumage patches on their shoulders, as well as black and white feathers along the chest, and barred primary flight feathers (See Figure 2). This species has a weight range of 460 to 930 gm, and a wingspan of 30.9 to 35.3 cm (Johnsgard,

1990). Both males and females are similar in appearance, though females are generally larger in size (Johnsgard, 1990). They are diurnal hunters and direct search predators that will search for prey while in flight (Johnsgard, 1990), but they also use the "sit and wait" method (Dykstra et al., 2008). Red-shouldered hawks hunt a wide range of prey including small mammals and birds to snakes, frogs, and insects (Johnsgard, 1990). They are thought to form pair bonds and to maintain a high level of nest site fidelity (Johnsgard, 1990). Red-shouldered hawks build their nests in the spring and have an average of three eggs per clutch (Johnsgard, 1990). Populations of red-shouldered hawks in the northern United States are on the decline, but populations in other regions have remained stable (Dykstra et al., 2008). It has been reported that red-shouldered hawks avoid areas of their territory that have been urbanized (Bosakowski & Smith, 1997), though other studies have described that they thrive in urban areas (Dykstra et al., 2008). Because some hawks may be more sensitive to human interaction (Bosakowski & Smith, 1997; Coon et al., 2015), this could affect their rehabilitation and ability to recover from injury.

Figure 2: An adult Red-Shouldered Hawk, showing the distinctive plumage of the species. Photo credit: Atlanta Wild Animal Rescue Effort, Inc.

1.4.4 Red-Tailed Hawks

The third raptor included in this study is the red-tailed hawk (*Buteo jamaicensis*). This is the most common species in this study and the species from which the most samples were collected. The red tailed-hawk is one the most widespread raptors in N. America, with a distribution ranging from Canada south to Mexico, and even further into Central America (see Figure 3) (Preston & Beane, 2009). Hawks living in the northern regions of the range will migrate south during the breeding season, but those occupying the more southern regions are non-migratory, year-round residents (Preston & Beane, 2009).

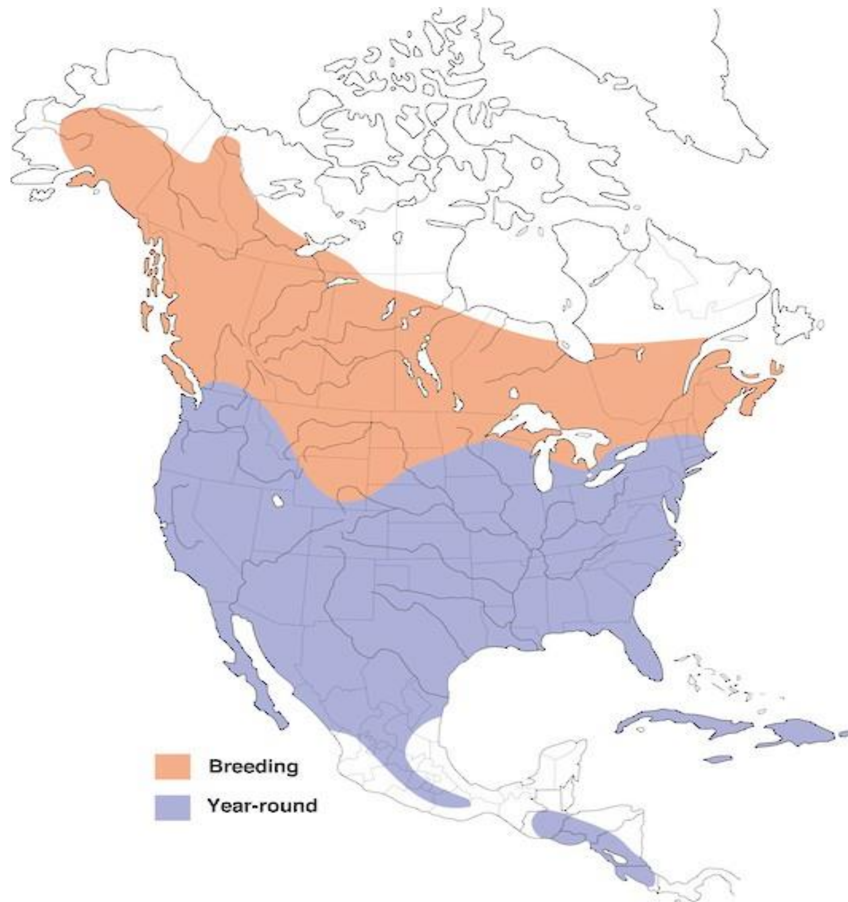

Figure 3: This map shows the range of the Red-Tailed Hawk in North and Central America. Photo credit: Cornell Lab of Ornithology (Preston & Beane, 2009).

Red-tailed hawks weigh between 710 to 1550 gm and have a wingspan of 33.7 to 42.7 cm (Johnsgard, 1990). There are minimal morphological sex differences. However, females are larger on average than the males (Johnsgard, 1990). These hawks exhibit distinctive rusty, red tail plumage have a lighter ventral surface, with varying degrees of darker feathers (Johnsgard, 1990; and see Figure 4). Their primary flight feathers are barred on the ventral side and end in an obvious dark band (Johnsgard, 1990). Red-tailed hawks are successful generalist predators that are widespread across N. and Central America and have been known to adapt their diet to suit the local habitat (Johnsgard, 1990). Like most hawks, the red-tail is a diurnal hunter and active during the day. These hawks employ the “soaring and scooping” method of prey capture (Johnsgard, 1990), and to a lesser extent, the “sit and wait” method. Red-tailed hawks are thought to form permanent pair bonds with their mates and to reuse nest sites from previous breeding seasons (Johnsgard, 1990). This species is also known to inhabit a diverse array of habitats and have been seen in hardwood forests, pastures, lowland hardwoods, wooded river bottoms, and grasslands (Johnsgard, 1990). They are incredibly tolerant of habitat variation and have been found in more urbanized regions (Johnsgard, 1990). They are also known to displace red-shouldered hawks from their habitat, as has been described from the breeding areas of the upper regions of the Midwest (Johnsgard, 1990). Because of their prevalence in N. America, and their tolerance of urban environments (Bosakowski & Smith, 1997; Preston & Beane, 2009), it is possible that the red-tailed hawks may be more tolerant of rehabilitation practices as compared to other species in the study.

Figure 4: A Red-tailed hawk ambassador at AWARE. Photo credit: Atlanta Wild Animal Rescue Effort, Inc.

1.5 Wildlife Endocrinology

The main goal of this study is to identify the feasible and repeatable methods for measuring corticosterone levels, as an index of stress, in raptors undergoing rehabilitation to better understand how they respond to captivity and treatment. The use of endocrine measures in wildlife management and conservation has increased over the past few decades (Ganswindt et al., 2012), and is accepted as a practical method for monitoring reproductive and adrenotropic function in an array of different species. This is especially useful for captive or endangered species to supplement species survival plans, and to gain valuable information about how species respond to husbandry and management practices. However, little is known about using endocrine measures in raptor rehabilitation and wildlife management. The methods for collecting, extracting, and monitoring steroids in different species occupying a wide range of habitats has

not been thoroughly validated. Here, we present the essential background information on wildlife endocrinology, as well as the reasoning for monitoring fecal glucocorticoids in raptors in rehabilitation.

1.5.1 The Function of Stress

Stress is the adaptive, physiological response of an organism to changes in the environment. Stress involves the fight or flight response and is a means for animals to deal with environmental uncertainty (Sheriff et al., 2011). The mechanism for reacting to stressful stimuli is a highly regulated cascade of physiological events that may result in a behavioral response. The stress response is multimodal and requires a concerted number of neural signals, hormonal secretions, and physiological changes to occur in the correct order. In addition to the stress response resulting in behavioral adaptations to environmental changes, this sequence of events can also alter homeostasis, control gene expression, disrupt metabolism, growth, reproduction, healing, and resource allocation (Boonstra, 2013). It is essential to understand how different species cope with environmental changes as a means to better their conservation and management programs.

1.5.2 Glucocorticoids and the HPA Axis

The hypothalamic-pituitary-adrenal (HPA) axis functions as a multi-level neuroendocrine stress response system. The structures work in synchronized action to secrete glucocorticoids from the adrenals (Herman et al., 2003). The stress response is initiated by a stimulus in the environment, which triggers the paraventricular nucleus of the hypothalamus to release corticotropin-releasing hormone (CRH). The release of CRH then stimulates the pituitary gland to release Adrenocorticotropic Hormone (ACTH), which is secreted into the bloodstream and acts on the adrenal glands to stimulate the release of glucocorticoid hormones such as

corticosterone (Herman et al., 2003). Once secreted, corticosterone binds to specific receptors in the body, such as the glucocorticoid receptor or GR, resulting in an up or down regulation of transcription (Falkenstein et al., 2000). This can lead to changes in cellular processes, brain function and behavior (Falkenstein et al., 2000). Glucocorticoids have a diverse array of actions and may serve different functions during development and maturity. Because of their wide array of biological actions and the relationship corticosterone has with stress response, it can be beneficial to measure glucocorticoids in wildlife.

1.5.3 Stress in Wildlife

There is vast amount of information available detailing stress in wildlife. It is a lucrative field, especially in light of the current and rapid loss of biodiversity that is happening as a result of anthropogenic causes (Dirzo et al., 2014). It is advantageous to be able to track stress in wildlife in captive and free-ranging environments because there are species-specific differences in the stress response, and species may respond to captivity and other conservation programs differently. Using non-invasive techniques, such as fecal glucocorticoid monitoring, to obtain this information is indispensable as well, and is undoubtedly necessary for studies involving threatened or endangered species. In addition to glucocorticoid monitoring being advantageous for threatened and endangered species, it may also provide valuable information for animals in rehabilitation. Rehabilitation can cause elevated stress in raptors (Park, 2003), which can be detrimental to treatment as it may be immunosuppressive (Sorrells et al., 2009). Below, we will present the current knowledge in the field as it relates to this study.

1.5.4 Previous Studies

Many studies have been conducted to analyze glucocorticoids in different vertebrate species, and to validate the methods for testing fecal glucocorticoids. A study by Malcolm and

colleagues (2013) examined glucocorticoids levels in fecal and hair samples from Asiatic Black Bears that were in kept in captivity on bile farms in China and compared those to samples taken after the bears were relocated to a rescue facility. Both hair and fecal samples showed a marked decrease in cortisol after the bears were moved to the rescue center, which provided them improved husbandry practices (Malcolm et al., 2013). Another study analyzed fecal glucocorticoid levels in elephants living in a conservation area in Kenya that are exposed to frequently to humans via ecotourism and local human settlements (Ahlering et al., 2013). The researchers found that despite being subjected to more human interaction, the fecal glucocorticoid levels were not elevated as compared to elephants in other conservation areas with less human traffic (Ahlering et al., 2013). An additional study conducted by Franceschini and colleagues (2008) on Grevy's zebras analyzed the stress response after translocation to a national park in Kenya. The zebras were tranquilized, captured, translocated to a new, unknown area, and held in captivity for five weeks prior to release in the national park (Franceschini et al., 2008). Fecal glucocorticoids were measured before, during, and after captivity. Glucocorticoid levels were elevated throughout captivity, but they returned to the levels before translocation after they were released into the park (Franceschini et al., 2008). Another interesting study was conducted in cheetahs, comparing the stress activity of cheetahs in a zoo to free-ranging cheetahs (Terio et al., 2004). The researchers found that cheetahs in captive environments had significantly elevated fecal cortisol levels compared to free-ranging cheetahs. Additionally, they analyzed adrenal gland morphology in cheetahs that had died, and they found that the cortical region of captive cheetah adrenal glands was also significantly larger than free-ranging cheetahs, which is indicative of chronic stress (Terio et al., 2004).

Many studies have been conducted on large, endangered mammals as a component of conservation programs, but there are also studies of avian species. One study was conducted by Tempel and Gutierrez in 2001 to track corticosterone levels in the California spotted owl. The population of this species was declining, and the purpose of this study was to determine if glucocorticoid levels were chronically high, which could affect reproduction rates (Tempel & Gutierrez, 2004). The researchers found that glucocorticoid levels were elevated in non-breeding individuals at the beginning of the breeding season, but aside from that they experienced difficulties in collection and saw a high level of variation in corticosterone levels between individuals (Tempel & Gutierrez, 2004). They reported that some of the variations in corticosterone might be due to differences in the samples. Owls have a cecum, consisting of two sacs attached to their intestinal tract that they empty once a day, and this study found that the corticosterone levels between fecal and cecal samples are variable (Tempel & Gutierrez, 2004). Another study conducted in Spain at the Centro de Estudios de Rapaces Ibericas (Center of Studies of Iberian Raptors) tracked fecal glucocorticoids in the golden eagle and the peregrine falcon (Staley et al., 2007). These raptors were either bred in captivity or non-releasable due to injury. For the study, birds were treated with ACTH, and fecal samples were collected for three days after each treatment (Staley et al., 2007). ACTH stimulation tests are a standard method employed in experiments to assess stress responsiveness in different species. The researchers found that the golden eagles involved in the study were able to deal with the rigors of the ACTH tests and they gained body mass during the testing phase. However, the peregrine falcons were much more sensitive, and they lost weight in response to the ACTH tests and handling (Staley et al., 2007). This study shows evidence for species-specific differences in stress activity and

highlights the need for understanding stress responsiveness in different species especially in the context of conservation management programs and husbandry practices.

The number of studies conducted involving fecal glucocorticoid monitoring for wildlife goes far beyond those listed previously. These results indicate the utility and value of conducting such studies and provide essential information through the use of non-invasive techniques

1.6 Importance

It is essential for scientists to come up with new and better ways to solve problems. Currently, the growth of the human population is not sustainable for the environment, and it is causing a massive loss of biodiversity (Dirzo et al., 2014). It is generating more problems than solutions. Anthropogenic changes in the environment, such as habitat loss and fragmentation, disturb the surrounding wildlife and have largely negative effects on individuals and populations. Species may adapt to changes in their environment in a variety of different ways, or, if they do not have the capacity to change or find another suitable habitat, they may become extinct on a local or global scale (Blaustein et al., 1994). Wild raptors are apex predators in their habitats, which are beneficial for overall ecosystem health and to maintain biodiversity in an area (Sergio et al., 2006). Raptors face a wide array of threats in their environment, especially near urban areas. Due to these threats, it is advantageous to have wildlife rehabilitation centers available for injured raptors. In order to provide the best possible care for these patients, we propose that measuring fecal glucocorticoids can provide valuable information to better understand the species-specific stress activity and the response to rehabilitation of the Barred owl, Red-tailed hawk, and Red-shouldered hawk. Therefore, we conducted this study to validate the optimal methodology to measure fecal corticosterone is raptors undergoing rehabilitation as a means to

further the field of wildlife endocrinology and to provide adapted protocols for use in future studies.

2 METHODS

The purpose of this study is to examine the validity and feasibility of measuring corticosterone levels using non-invasive techniques of raptors undergoing rehabilitation at a wildlife center. Data was collected from three species of raptors at the Atlanta Wild Animal Rescue Effort, Inc. (AWARE), located on Arabia Mountain outside of Atlanta, Georgia, from May 2017 through March 2018. Fecal, feather and blood samples were obtained from most subjects, but this study will focus on determining the validity of measuring corticosterone from fecal samples. Fecal samples were also collected from ambassadors (raptors that are non-releasable due to the nature of their injuries and are used for educational purposes at AWARE), which will serve as a potential comparison to patient samples, and as a means to evaluate a mode of sample collection. Multiple collection and extraction methods and two hormone assays were tested to assess the feasibility of conducting this type of research.

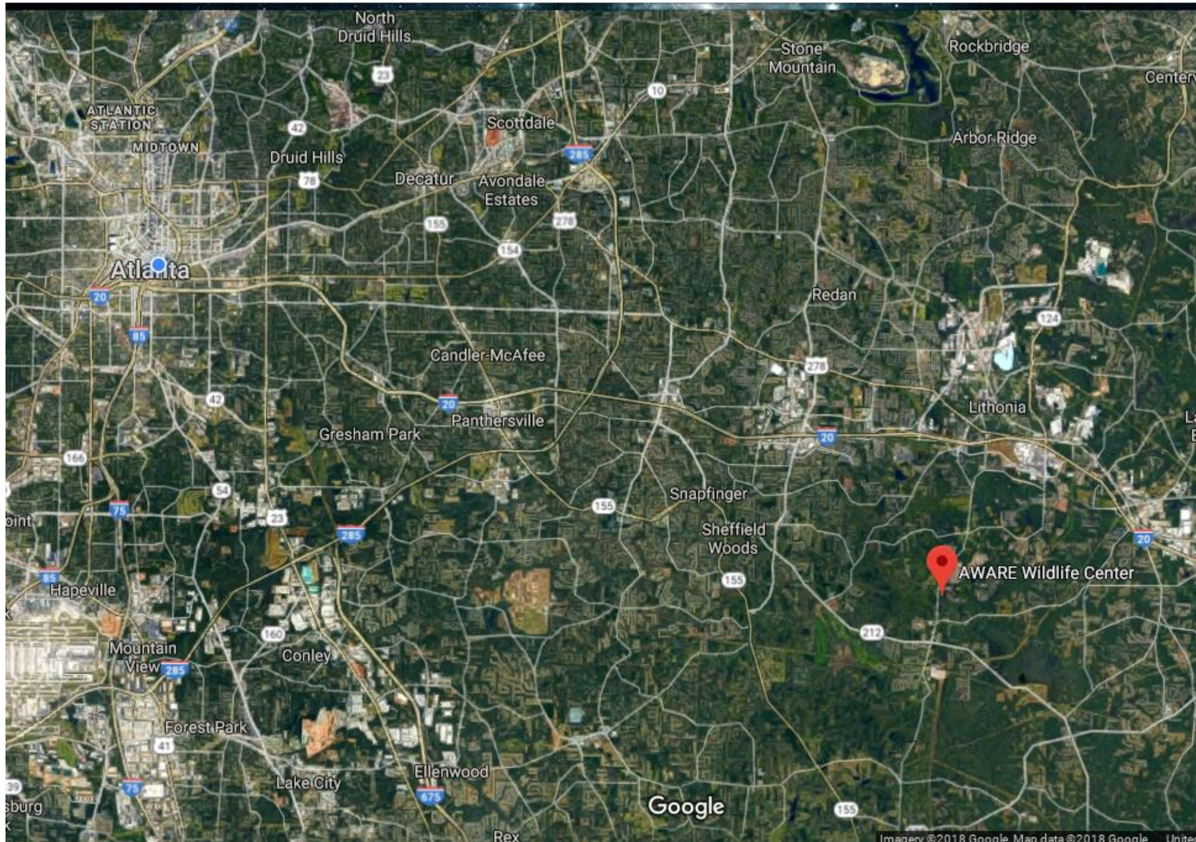

Figure 5: AWARE, and its proximity to Atlanta, Georgia. AWARE is approximately 21 miles southeast of downtown Atlanta. Map data: 2018, Google.

2.1 Ethics Statement

Approval to conduct this research was obtained from the International Animal Care and Use Committee (IACUC) of Georgia State University and with permission from the Atlanta Wild Animal Rescue Effort, Inc. Fecal samples were collected using non-invasive methods, and blood and feather samples to be used in later studies were collected by the AWARE staff according to an approved protocol.

2.2 Rehabilitation Phases and Practices

AWARE is one of the few wildlife rehabilitation centers near Atlanta that accepts all species of injured wildlife for treatment. This service is especially valuable in urban areas, and AWARE accepts a high volume of patients each year. Of particular importance are the number

of raptors admitted to AWARE annually. In 2014, 74 raptors were brought to AWARE for treatment, and the most common species seen are the red-tailed hawk (*Buteo jamaicensis*), the red-shouldered hawk (*Buteo lineatus*), and the barred owl (*Strix varia*). These species were the focus of this study, and samples were obtained from every individual of each species admitted permitting they were in stable condition and at AWARE during sample collection.

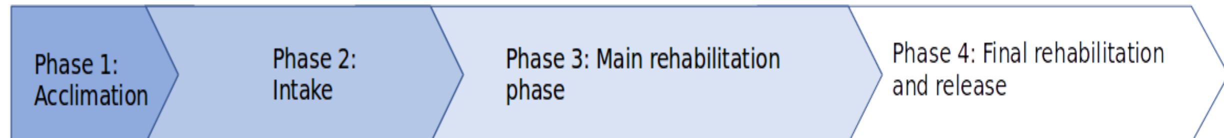

Figure 6: Phases of Rehabilitation

AWARE has a standard protocol for rehabilitation that consists of multiple phases (See Figure 6). Raptors brought to AWARE undergo standard intake processing, which involves the animal being captured and brought to the facility, and then quickly assessed for life-threatening injuries. Animals may be brought to AWARE by members of staff, or by the citizens that discovered the injured animal. If the animal is not in immediate need of attention it is allowed to acclimate to the cage it was transported in for approximately 20 minutes before treatment. If it is deemed necessary, it is provided with a heating pad as well. After this resting period, the animal is given a physical exam, a diagnosis, and then a treatment plan. At the beginning of rehabilitation, the animal will be housed in a small indoor enclosure (See Figure 7).

Figure 7: Example of indoor enclosures found at AWARE. These are from the "Mammal Room," and show the typical enclosures where ambassadors are housed when they are rotated indoors.

When the animal is well enough, it will be moved to a larger outdoor flight enclosure and may be housed with conspecifics. Towards the end of their recovery, the animal will undergo more extensive flight conditioning in a larger outdoor enclosure and will be tested for predatory ability. At this phase of rehabilitation, the raptor should be mostly healed and will be introduced to live mice and monitored for their ability to hunt. If the animal is determined to be fit for release, it is taken back to its original territory, or a suitable habitat in the same county to be reintegrated into the wild. If an animal is thriving but is unable to survive in the wild, then it is added to AWARE's non-releasable placement program to find a home in a long-term wildlife care center such as a zoo or educational program. If an animal cannot thrive or have a good quality of life, then it will be humanely euthanized per AWARE's protocol.

Figure 8: Examples of some of the outdoor enclosures at AWARE. Picture A (left) is a smaller enclosure, and Picture B (right) is a larger flight cage at AWARE. In the middle, is an arena where live rats are placed to test the predatory ability of raptors.

2.3 Patients and Ambassadors

Samples were obtained from a total of 25 raptors undergoing rehabilitation, and an additional five ambassadors. Raptors face an array of risks in urban areas, and the patients seen at AWARE arrive with a wide variety of injuries. Table 1 shows the raptors brought to AWARE for rehabilitation from whom samples were obtained. Each patient or ambassador was brought to AWARE for different injuries. Thus, each patient has a different treatment plan, and spend differing amounts of time in rehabilitation. Less severe injuries lead to shorter rehabilitation times, and more severe injuries require patients to be in rehabilitation for longer. This translates into obtaining different numbers of fecal samples from each patient as well. The number of fecal

samples obtained from each patient is also affected by the disposition outcome. This is the result of rehabilitation, which may conclude in "Delta" (i.e., the patient died during rehabilitation, as some individuals may not be able to recover from their injuries), euthanasia if it is determined to be the best course of action by AWARE, transfer to a different facility if a patient is deemed non-releasable but AWARE does not have room to admit it as an ambassador, or patients are released back to into the wild close to where they were found if they have fully recovered, and have shown the ability to hunt and feed themselves. Table 1 shows the outcome of rehabilitation for each patient in the study, and also the number of patients still undergoing rehabilitation. The injuries range from slight emaciation to more severe neurological symptoms or broken limbs. Many of the injuries that the raptors sustained before rehabilitation are likely caused by collisions (i.e., with cars, windows, fences, and so on), or from secondary poisoning when neurological symptoms are observed (Hager, 2009). Flat flies, an ectoparasite of the genus Hippoboscidae, and emaciation are also commonly seen in raptors admitted to AWARE for treatment. Collisions often result in fractures, loss of primary feathers, or other wounds, and these injuries were observed in many patients in the study. Secondary poisoning, which can be caused by a raptor ingesting a rodent poisoned with rodenticide, can present as neurological symptoms and hemorrhage as well. For the species involved in the study, we collected data from a total of twelve red-tailed hawks (RTHA), six red-shouldered hawks (RSHA), and seven barred owls (BDOW), and the details of their rehabilitation can be seen in Table 1 below. The high number of red-tailed hawks, as compared to barred owls and red-shouldered hawks, seen in the study may be attributed to the fact that red-tails are generalist predators, and may thrive better in urban areas than the other two species (Bosakowski & Smith, 1997). A total of 103 fecal samples were obtained from the patients in Table 1, and blood and feather samples were collected from

some patients as well to be used in later studies. The final disposition for patients involved in the study resulted in four Delta (died during rehabilitation), four euthanized, five transferred, two released, and ten are still undergoing rehabilitation. Rehabilitation times for each patient range anywhere from less than a month (i.e., patient 17-0426), to over a year (i.e., patient 16-0874).

Table 1: Patient Data. The following table shows the data for each individual patient involved in the study. Intake date, injuries, rehabilitation outcomes, and the number of fecal samples obtained are recorded here.

Patient ID	Species	Intake Date	Injury	Disposition Date	Final Disposition	Number of Fecal Samples
17-0253	RTHA	5/5/17	Right Eye	8/9/17	Released	5
17-0125	RTHA	3/30/17	Left Humerus Fracture, Left Eye Cloudy	11/21/17	Transferred	13
17-0097	RTHA	3/14/17	Right Humerus Injury, Tattered Right Wing	9/15/17	Transferred	6
16-1005	RTHA	12/19/16	Left Wing Mass	6/19/17	Euthanized	1
16-0851	RTHA	9/24/16	Right Humerus Injury	6/8/17	Transferred	0
16-1007	RSHA	12/20/16	Right Wing, Left Knee Injury	6/19/17	Euthanized	1
16-0968	RSHA	11/7/16	Left Femur Injury, Calcified Phalanges	8/15/17	Euthanized	6
17-0039	RSHA	2/3/17	Neurological, Blood in Nares	8/3/17	Euthanized	5
17-0036	BDOW	2/1/17	Emaciated, Blood in Nares, Neurological	7/29/17	Transferred	3
17-0426	RSHA	6/24/17	Neurological	7/14/17	Released	3
16-0874	BDOW	10/14/16	Left Humerus Crepitus, Left Elbow Fracture	10/15/17	Transferred	10
17-0682	RSHA	9/3/17	Missing Primary Feathers	10/18/17	Delta	5
17-0777	RTHA	10/12/17	Right Shoulder Injury	N/A	Still in Rehabilitation	7
17-0779	RTHA	10/13/17	Abnormal, Wounded Left/Right Legs	N/A	Still in Rehabilitation	8
17-0794	BDOW	10/28/17	Cracked Beak	N/A	Still in Rehabilitation	6
17-0849	BDOW	11/29/17	Left Foot Injury	N/A	Still in Rehabilitation	5
17-0851	BDOW	11/30/17	Humerus and Elbow Fractures, Emaciated, Flat Flies	12/5/17	Delta	1

17-0881	RTHA	12/26/17	Hematomas on Neck	N/A	Still in Rehabilitation	4
17-0884	BDOW	12/27/17	Right Wing Wound, Cere Bruised, Flat Flies	12/31/17	Delta	1
18-0010	RTHA	1/10/18	Wound on Left Foot	N/A	Still in Rehabilitation	4
18-0003	RTHA	1/6/18	Emaciated	N/A	Still in Rehabilitation	4
18-0012	RSHA	1/14/18	Neurological, Slightly Emaciated	1/17/18	Delta	1
18-0044	RTHA	2/11/18	Right Radius and Ulna Fracture	N/A	Still in Rehabilitation	1
18-0030	BDOW	1/29/18	Left Eye Wound, Wound on Cere	N/A	Still in Rehabilitation	2
18-0054	RTHA	2/20/18	Neck Wound, Flat Flies	N/A	Still in Rehabilitation	1

Table 2: Ambassador Data. Data collected from ambassadors is outlined below, and emphasizes the injuries they endured and the number of years they have spent at AWARE.

Name (ID)	Species	Intake Date	Injury	Years at AWARE	Number of Fecal Samples
Beakers (15-1199)	RTHA	11/5/15	Broken Maxillary Beak	3	10
Koko (Unknown)	RTHA	Before 2003	Broken Toe, Feather Follicle Damage Left Wing	Unknown	4
Tappy (12-0879)	BDOW	11/2/12	Head Trauma, Cracked Beak	6	2
Gazer (10-0933)	BDOW	10/8/10	Right Eye Corneal Abrasion	8	1
Owlbert (Unknown)	BDOW	Before 2003	Left Wing Injury	Unknown	3

2.4 Collection Methods

Multiple collection methods were tested for this project in order to find the optimal protocol to collect and store fecal samples prior to extraction and measurement. Fecal samples were collected from patients between May 2017 and March 2018, and from ambassadors for a nine-day period in March 2018. Fecal samples were collected at two-week intervals for patients, and ambassador samples were collected daily, if possible, by the researchers or AWARE staff until 20 samples were obtained.

2.4.1 Ambassador Samples

Per AWARE protocol, we were unable to collect samples from ambassadors while they were in their outdoor enclosures, so all of the ambassador samples were obtained from indoor cages. The ambassadors are brought indoors on a rotational basis and are housed in a row of small enclosures in the "Mammal Room" at AWARE. This is a temperature-controlled room with the fluorescent lighting, and it has high levels of activity during the day. Both diurnal and nocturnal species are kept here when they are rotated inside. The indoor enclosures allowed for ease of collection compared to the outdoor patient samples. These were obtained from metal cages, which are lined with newspaper and occasionally bath towels. Ambassador samples were taken with assistance from AWARE staff to restrain the raptor during collection as a safety precaution for the researchers and the raptors. Tongue depressors were used to scoop samples from the enclosure, and they were immediately transferred to small centrifuge tubes filled with one mL of 80% methanol. This collection method was employed as a means to try to better preserve the fecal glucocorticoids in the samples. Other researchers have stored fecal samples in methanol to preserve samples in the field (Pappano et al., 2010), and ethanol has been documented for use before extraction as well (Khan et al., 2002). Samples were labeled, and stored in a refrigerator (4ºC) at AWARE until they were transported to GSU. Samples were kept in a centrifuge tube storage box and were transported in a standard Styrofoam cooler. Once the samples were brought to GSU, they were again stored in a refrigerator (4ºC) until they were extracted and assayed using an enzyme immunoassay. A log of the sample collection for ambassadors can be seen in the Appendix.

One additional ambassador sample was taken using the standard collection methods for patients, obtaining samples with a tongue depressor and storing in a plastic bottle or bag in a

freezer (-20 ºC), which is described in more detail in Section 2.4.2. This sample, which can be seen in the Appendix, was tested using enzyme immunoassay and was compared to samples from patients.

2.4.2 Patient Samples

The patient samples were collected between May 2017 and March 2018 at two-week intervals. According to the phases of rehabilitation (Figure 6), raptors are housed in either indoor or outdoor enclosures dependent on their progress with the treatment plan. At the beginning of rehabilitation, patients are housed alone in small indoor enclosures, and as patients heal they are moved outside to larger enclosures where they can fly for small distances, and finally they are moved to large flight cages where their predatory abilities are tested. Samples were taken from patients at every stage if possible, and different collection methods had to be used for different enclosures.

Samples collected inside followed similar protocol to the ambassador sample collection. Patients kept inside at the start of their treatment were held in the "Raptor Room" (RR) at AWARE, and some patients were kept in the "Mammal Room" (MR) if the enclosures in RR were full. Each metal cage was lined with newspaper and a bath towel, and the doors for each cage were covered with towels as well to try to decrease stress in patients because of the high level of activity that occurs during the day at AWARE. Indoor samples were obtained using either a tongue depressor or a plastic spoon to scoop fecal samples from the newspaper or towel lining the cage. These samples were collected by one researcher without restraining the animals, unless a bird was known to be aggressive or of high stress in which case assistance from the AWARE staff was utilized. To collect samples without causing undue stress in the patients, it was necessary to move slowly, keep one's hands in view of the raptor, and to keep the door to the

cage only open as far as necessary to reach the sample to prevent any escapes. After scooping the sample onto the plastic spoon or tongue depressor, they were transferred into either a plastic bottle or bag, labeled with the appropriate information, and then stored in a freezer (-20ºC) at AWARE. Samples were moved to GSU periodically and were stored in a cooler with ice during transport. Once at GSU, the samples were returned to a freezer (-20ºC).

Samples taken from the outdoor enclosures followed a different protocol by necessity. Researchers were permitted to enter each enclosure and to stay with the patients until samples were collected. More than one raptor is usually kept in each outdoor enclosure. Because of this, it was necessary for the researchers to monitor every patient in an outdoor enclosure, wait for the patients to defecate, and to be able to identify each patient to ensure each sample was recorded correctly with the correct Patient ID. The outdoor enclosures are lined with gravel, and this can cause difficulties in the collection and extraction of the fecal samples. To try to prevent gravel from being incorporated into samples during collection, the outdoor enclosures were lined with newspaper. Due to the size of each enclosure, it was not possible to completely line each enclosure, nor to predict where each patient would defecate. Some samples did not land onto newspaper, in which case it was necessary to collect the gravel using a tongue depressor because it was not possible to separate the two substances, and these samples were transferred to an appropriately labeled plastic bag or bottle. Samples that did land onto the newspaper were folded up into the newspaper and put into an appropriately labeled plastic bottle or bag. The newspaper was kept with the samples to try to preserve as much as possible, rather than transferring the sample from the paper into the bag or bottle. All sample bags or bottles were labeled and stored in a freezer at AWARE until they were to be transported to GSU. Samples were transported in a cooler with ice and were returned to a freezer (-20ºC) upon arrival. These samples were kept

frozen until they were assayed. Table 4 shows a complete log of all the patient samples collected during the study in chronological order. Included in the table are the Patient IDs for each test subject, the species, which enclosure they were in during that collection, and the date of collection. The enclosures labeled with “RR” (Raptor Room) or “MR” (Mammal Room) followed by a number are the indoor enclosures and are a part of Phase 2 of rehabilitation (See Figure 6). OE1B and OE2 are both small outdoor enclosures and are a part of the third phase of rehabilitation. RSF, LSF, and NFL are all flight cages and part of the fourth and final phase of rehabilitation.

2.5 Extraction Methods

In addition to employing multiple modes of collection methods, we also tested two extraction protocols for the patient samples and an additional method for the ambassador samples. Extractions are a necessary step in the preparation of samples for steroid hormone analysis using enzyme immunoassay or radioimmunoassay. Hormone extraction is required for solid samples, such as fecal, hair or feather samples, to isolate the steroid hormones from the background organic matter. In this study, we test both a wet and dry extraction protocols for the patient fecal samples, and the ambassador samples were extracted using the dry protocol exclusively. The wet extraction protocol was provided by the Saint Louis Zoo Endocrinology Lab, and the dry extraction protocol was furnished by Arbor Assays as a part of their Cortisol and Corticosterone DetectX Enzyme Immunoassay kits.

2.5.1 Wet Extraction Protocol

The wet extraction protocol, provided by the Saint Louis Zoo, is an avian specific extraction for testing fecal steroid hormones. Because birds have a cloaca, urine and feces are excreted together, and to accurately measure steroid hormones from such samples it is necessary

to separate the urates out. This protocol uses β-glucuronidase to cleave the urea from the fecal matter. Of the patient samples collected, 36 were extracted using this protocol. Samples were removed from the freezer and allowed to thaw. After thawing, samples were mixed to distribute the feces and urates evenly. Between 0.25 and 0.5 gm of the sample were to be added to a scintillation vial. After samples were added, the vials were weighed to obtain the wet weight of the sample. Then 2.5 mL of phosphate-buffered saline (PBS) was added to the first samples that were run, and those that met the 0.5 gm requirement. These samples include Vial number 1 through 10, as seen in Table 5 in the Appendix. For the samples that only met the 0.25 gm requirement, 1.25 mL of PBS was added, and these include vial number 11 through 36. Then 12.5 or 25 μg of β-glucuronidase was added to each vial according to the weight of the sample (0.25 or 0.5 gm). After the addition of the PBS, the vials were incubated in an oven overnight at 37° C. The following day, samples were removed from the oven, and 1.25 or 2.5 mL of methanol was added to each vial based on the weight of the sample. After the addition of methanol, the vials were put on a shaker for 4 hours. Then the liquid from each sample was decanted into a new centrifuge vial, and centrifuged for one hour at 4,000 RPM, decanted into new vials again and then frozen until they were to be assayed. The solid matter left over in the vials after decanting was then dried in the oven at 80°C overnight and was weighed to obtain the dry weight of each sample.

Due to difficulties with collection methods not all of the samples met this weight requirement. Additionally, there were troubles with conducting the extraction of samples that contained gravel. Some of the samples were pooled, which used multiple samples from the same patient to try to meet the sample weight requirement for this protocol. Even after pooling, some of the samples were not heavy enough for this extraction protocol and thus would not provide an

accurate reading from the enzyme immunoassay (Discussed in Section 3). This ultimately resulted in the decision to use the dry extraction method.

2.5.2 Dry Extraction Protocol

The dry extraction protocol was obtained from Arbor Assays. For the first round of dry extractions, seven samples were ran using the following protocol to troubleshoot its efficacy. These samples were removed from the freezer and allowed to thaw at room temperature. 15 mL falcon tubes were used for the extraction, and each was labeled with the appropriate Patient ID. After samples had thawed, any non-digested material was removed using wooden sticks, if possible, and samples were thoroughly mixed. For samples with gravel that could not be removed, the rocks were crushed and evenly mixed with the feces. Between 0.1 and 0.5 grams of sample were weighed out, and added to each Falcon tube. Five mL of 80% aqueous methanol was added to each 0.5 gram sample, and one mL of methanol per 0.1 gram of sample was added to the samples that did not meet the 0.5 gram weight requirement. After the addition of methanol, the samples were vortexed for 20 minutes and then transferred to centrifuge tubes. Samples were then centrifuged at 3000 RPM for 15 minutes at room temperature. Then, 500 µL of the supernatant from the centrifuge tubes was added into new tubes, and the liquid was evaporated off using a SpeedVac set at 60° C for 3 hours. The dried samples were then frozen at -20°C until they were to be assayed.

There were multiple issues detected with this extraction, and modifications were made after the first cortisol assay was run. The protocol was conducted in the same manner, but the samples containing gravel were not crushed. The results of the first EIA indicated that the crushed gravel in the samples created some interference, and skewed the results of the assay. Another step was added to the extraction protocol as well. Instead of vortexing samples

immediately after the addition of the methanol, they were allowed to sit at room temperature for 30 minutes, and then they were vortexed for 30 minutes instead of 20 minutes. All steps after this were kept the same, but some differences were made to the assay preparations as well (Covered in Section 2.6).

Some modifications were made to the extraction protocol to extract glucocorticoids from the ambassador samples stored in 80% methanol as well. The centrifuge tubes were removed from the -20ºC freezer, and the samples were transferred into 15 mL Falcon tubes. Room temperature 80% methanol was then added to samples to equal 1 mL of methanol per 0.1 gram of sample. Some samples were spiked to run the extraction efficiency, and some were prepared in a 1:2 dilution. After the addition of methanol, the samples were then vortexed for 20 minutes and centrifuged for 15 minutes at 3000 RPM. Then a 500 µL aliquot of supernatant from each Falcon tube was pipetted into a new, labelled tube. The tubes were then evaporated to dryness in a SpeedVac at 60º C for 3 hours. The dried samples were then stored in a -20ºC freezer until they were to be assayed.

2.6 Enzyme Immunoassay Protocol

Two enzyme immunoassay kits from Arbor Assays (Ann Arbor, MI) were tested to analyze the corticosterone in the fecal samples. Both a DetectX Cortisol Multi-Species (K003-H1) and Corticosterone Multi-species (K014-H1) Enzyme Immunoassay kits were used to measure corticosterone in the extracted samples. The protocols for each of these assays are similar but use different conjugates and antibodies specific to cortisol and corticosterone. These hormones have nearly identical molecular structures, and thus it is possible to test for corticosterone using either EIA.

Prior to running the assay, extracted samples were removed from the freezer and brought to room temperature for 15 to 30 minutes. The dried samples were reconstituted in 100 µL of ethanol and 400 µL of assay buffer. Then, this solution was vortexed and allowed to sit for five minutes, and this step was repeated two additional times to ensure the hormone sample was completely reconstituted. Next, the samples were diluted to a 1:10 concentration to ensure that the ethanol would not interfere with the assay. To do this, 450 µL of assay buffer was added to 50 µL of sample. These sample dilutions were then run immediately with the cortisol EIA. The first round of cortisol assays did not yield optimal results, so the protocol was modified to use a 1:5 dilution of the sample rather than a 1:10. This was used for the second cortisol EIA, and 400 µL of assay buffer was added to 100 µL of sample to create the 1:5 dilution. The ambassador samples collected in 80% methanol were prepared in the same manner to be assayed with the corticosterone EIA, and these samples were diluted to a 1:10 concentration. Some of the ambassador samples were also prepared in a 1:2 dilution to obtain the recovery percentage.

The standard samples and assay plates were prepared according to each EIA protocol. The standards measured in each plate are used to make a standard curve, and to calibrate the assay. Each well was filled according to the provided protocol, and sample wells were run in duplicate. The Cortisol EIA plates are coated with a goat anti-mouse Immunoglobulin-G (IgG), while the Corticosterone EIA plates contained a donkey anti-sheep IgG. These are the specific antibodies that plates are coated with, which react and bind the steroid hormone. After the addition of the standards or samples in each well, a cortisol or corticosterone-peroxidase conjugate is added, as well as an additional antibody. The cortisol kit includes a mouse monoclonal antibody, while the corticosterone kit has a sheep polyclonal antibody that is specific for corticosterone. After the peroxidase and antibody are added to each well, the assay plate is

placed on a shaker and incubated for one hour at room T. Then the plate is washed with a wash buffer, and the provided substrate added, which reacts with the bound peroxidase conjugate to cause a visible color change in the solution that can be detected by a photometer. The plate is incubated again for 30 minutes and then is analyzed with a microtiter plate reader at 450 nm. The wavelength detected in each well corresponds to the amount of corticosterone found in solution. The concentration of corticosterone is then measured by the software associated with the plate reader and is generated into a data table for further analysis.

3 RESULTS

3.1 Collection Method Results

The collection methods for this project resulted in several unanticipated problems, and to some extent, they affected the results of the extraction and assay protocols. The samples collected from indoor enclosures were largely uncomplicated to extract and measure, but those used for the wet extraction did not all meet the weight requirements for the protocol (Discussed in 3.2). The samples that were collected in the outdoor enclosures were more troublesome. The outdoor enclosures are lined with gravel, and a large proportion of samples taken from these enclosures (OE1A, OE1B, OE2, LSF, RSF, NFL) inevitably had gravel in the sample. Newspaper was used to line the enclosures during collection, but it is impossible to predict where a fecal sample is going to fall so often they were not collected on the newspaper. The presence of gravel in the sample caused difficulties in the wet extraction because it skewed the weight of the fecal sample. We were unable to remove the gravel to obtain an accurate sample weight, so samples containing gravel were not extracted using the wet extraction protocol. Some of the

samples containing gravel were crushed to extract the steroid hormones, but an accurate Cort measure could not be obtained by the EIA using this method (See Section 3.3). It was more beneficial to reconstitute the gravel samples in 80% methanol to obtain an accurate reading with the EIA. The samples that were collected on a newspaper from outdoor enclosures were problematic as well. Though more of the sample could be captured on the newspaper without interference from gravel, much of the sample would be absorbed into the paper during storage. This also led to difficulties with extraction because it led to lower sample weights, and samples that were sufficiently small could not be scraped off of the paper. Changes were made to the extraction protocol to deal with these issues, and they did allow for these samples to be extracted and assayed with greater success (See Section 3.2). Ambassador samples were also collected and were stored in centrifuge tubes with one mL of 80% methanol. This method was employed because of the difficulties encountered with samples stored on newspaper and because it easily allows the gravel to be separated from the sample. Additionally, this collection method may help to preserve corticosterone in samples before they can be transferred to the -80 ºC freezer. It also allows for more successful extractions and assays to be conducted (See Section 3.2 and 3.3).

Figure 9: This is a picture taken from inside one of the outdoor enclosures showing the gravel substrate that lines each enclosure.

3.2 Extraction Method Results

This study tested multiple extraction protocols. The wet extraction method was obtained from the Saint Louis Zoo Endocrinology Lab, where it is used to analyze a variety of avian fecal samples. This method of extraction required that a specific weight of sample be used to obtain an accurate concentration of Cort from the EIA. We were able to extract 36 samples using this method, but not all of the samples met the weight requirement (See Appendix I, Table 6). This protocol required between 0.25 and 0.5 grams of fecal matter. Most of the samples we collected weighed much less than this, possibly due to stress in rehabilitation or changes in diet, and the samples that contained gravel prevented an accurate weight from being obtained so they could not be extracted with this protocol. Only 36 samples out of 103 were extracted in this manner, and due to the difficulties with this protocol, we sought out other methods to analyze the

remainder of the samples. Because of the inconsistencies associated with this protocol, none of the samples extracted in this manner were analyzed with the cortisol or corticosterone EIA.

The dry extraction protocol was more flexible with the sample weight requirement and allowed for better extraction results from samples containing gravel. Modifications had to be made to this protocol after the first cortisol EIA was run. Rather than doing a 1:10 dilution of the sample to assay buffer, a 1:5 dilution was used instead. It is advisable to use a 1:10 dilution to prevent interference from the methanol in the EIA. However, it was more advantageous to increase the amount of sample used in the dilution for these samples to obtain a better result for the corticosterone concentration. Comparisons of the EIA results from the different dilutions can be seen in Section 3.3.

3.3 Enzyme Immunoassays Results

To analyze our samples we used both a Cortisol and Corticosterone EIA. All of the samples we analyzed we extracted using the dry extraction protocol. The first Cortisol EIA tested seven samples that were extracted and reconstituted with a 1:10 dilution of the sample to assay buffer. The second Cortisol assay compared sample reconstituted with a 1:10 dilution to samples reconstituted with a 1:5 dilution to determine which was better for detecting corticosterone. The third assay we ran was a Corticosterone EIA, and we ran samples that had already been tested with the Cortisol EIA to determine which was best for the purpose of this project. For the Cortisol DetectX, the recovery rate was between 73 to 98%, and the detection limit was 45.4-pg/ml. Intra-assay & inter-assay coefficient variations were 14.7% and 10.9%, respectively. For the Corticosterone DetectX, the recovery rate was between 78 to 99%, and the detection limit was 6.9 pg/mL. Intra-assay & inter-assay coefficient variations were 4.8% and 9.9%,

respectively. Table 3 below describes all independent samples that were run in each assay, and the details of the sample preparation.

Table 3: This table shows the data for the samples run on each assay. The weight (g), extraction and dilution type, assay, and corticosterone concentration (pg/mL) is listed for each sample that was tested.

Patient ID	Sample Weight	Extraction	Assay	CORT Concentration (pg/mL)
18-0010	0.5	Dry, 1:10 Dilution	Cortisol 1	24.59
17-0777	0.5	Dry, 1:10 Dilution	Cortisol 1	38.43
18-0003	0.2	Dry, 1:10 Dilution	Cortisol 1	297.90
17-0794	0.5	Dry, 1:10 Dilution	Cortisol 1	18.67
BEAKERS	0.3	Dry, 1:10 Dilution	Cortisol 1	26.88
17-0849	0.5	Dry, 1:10 Dilution	Cortisol 1	26.63
18-0030	0.1	Dry, 1:10 Dilution	Cortisol 1	42.30
17-0881	5.0	Dry, 1:5 Dilution	Cortisol 2	271.00
17-0881	5.0	Dry, 1:5 Dilution	Cortisol 2	24.81
17-0881	4.0	Dry, 1:5 Dilution	Cortisol 2	32.28
18-0003	1.0	Dry, 1:5 Dilution	Cortisol 2	69.91
17-0794	Reconstitued From Assay 1	Dry, 1:5 Dilution	Cortisol 2	27.50
BEAKERS	Reconstitued From Assay 1	Dry, 1:5 Dilution	Cortisol 2	76.69
17-0849	Reconstitued From Assay 1	Dry, 1:5 Dilution	Cortisol 2	45.49
18-0010	0.5	Dry, 1:5 Dilution	Corticosterone	Below Curve
17-0777	0.5	Dry, 1:5 Dilution	Corticosterone	16.24
17-0794	Reconstitued From Assay 1	Dry, 1:5 Dilution	Corticosterone	46.04
BEAKERS	0.3	Dry, 1:5 Dilution	Corticosterone	522.10
17-0849	0.5	Dry, 1:5 Dilution	Corticosterone	1.55
17-0881	5.0	Dry, 1:5 Dilution	Corticosterone	67.39
18-0003	1.0	Dry, 1:5 Dilution	Corticosterone	53.26

3.3.1 *Cortisol Assay #1*

The first Cortisol Assay we ran used samples that were extracted with the dry extraction protocol and had been reconstituted in a 1:10 dilution. The standard curve shown below (Figure 10) shows the percent bound (%B/B0), or the percent standard sample bound in the well compared to the maximum binding ability of the well. Because the maximum bound is fixed for each well, the more cortisol standard that is added to the well then the less corticosterone is

bound to the antibody in the well, thus the more cortisol is free in the solution of the well. This unbound cortisol will alter the absorbance in the well, and will be measured by the assay plate reader at 450 nm. Therefore, as can be seen in the standard curve for the first assay, there is a negative relationship between the %B/B0 and the concentration of steroid hormone.

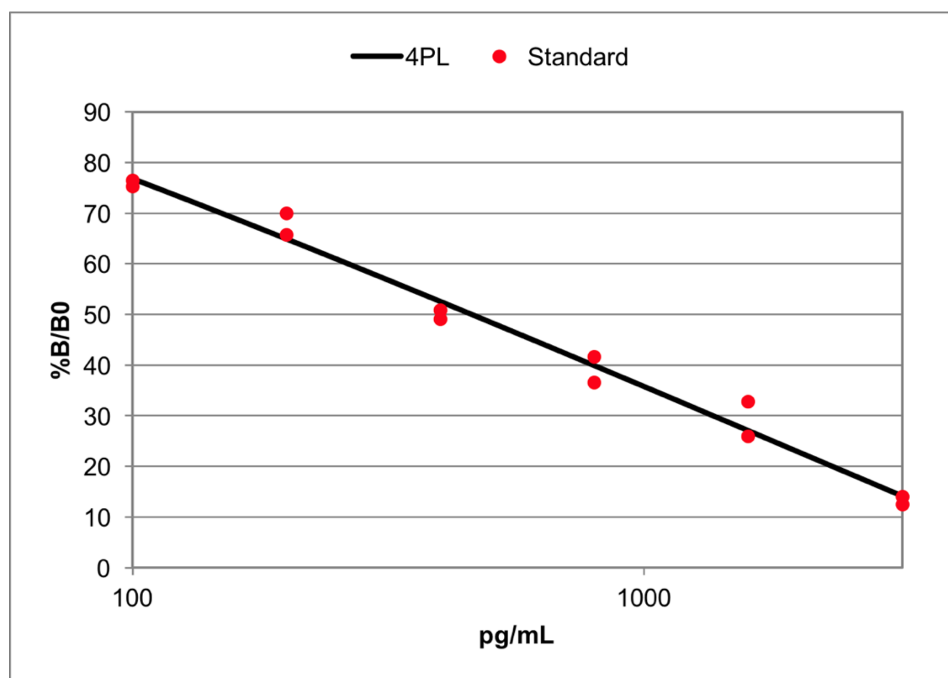

Figure 10: Standard Curve generated for the first Cortisol EIA.

The samples of the first assay were ran independently, and were compared to samples spiked with known concentrations of cortisol to determine the extraction efficiency and the percent recovery for this assay. The extraction efficiency was measured, dependent on the concentration of the known standard added to the test sample, and the recovery percentage for these samples was 62 to 93%. The results for the first Cortisol EIA are shown below (Figure 11), where the concentrations for the independent and spiked samples are shown in the graph. Additional data is available in Table 3, which outlines the Cort concentration for all independent

samples in each assay, and the individual Cort concentration values for each sample can be found in Table 8 of the Appendix.

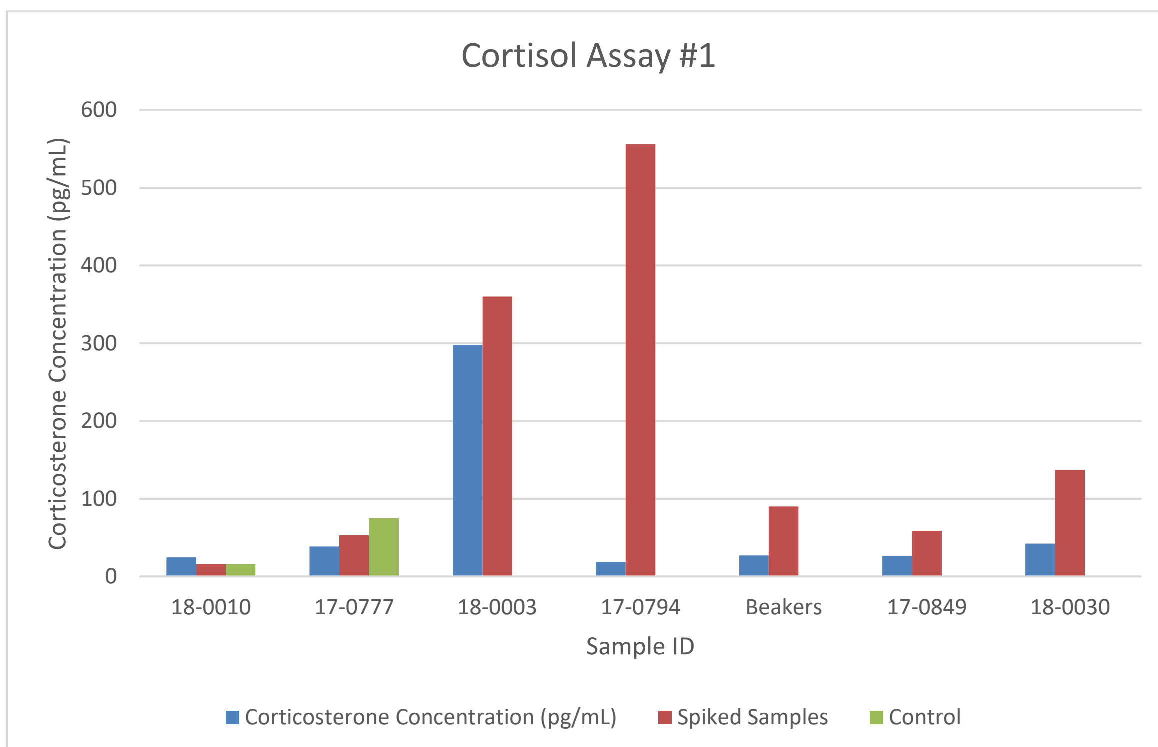

Figure 11: This graph shows the results of the first Cortisol EIA. The corticosterone concentration (pg/mL) is shown for the independent samples and samples spiked with known cortisol concentrations.

3.3.2 Cortisol Assay #2

The second Cortisol assay we ran was used to compare the Cort concentrations of samples prepared in a 1:10 dilution with assay buffer versus samples prepared with a 1:5 dilution. Also, as seen in the standard curve below (Figure 12), two additional standards were added to the curve to optimize the detection of samples with lower Cort concentrations.

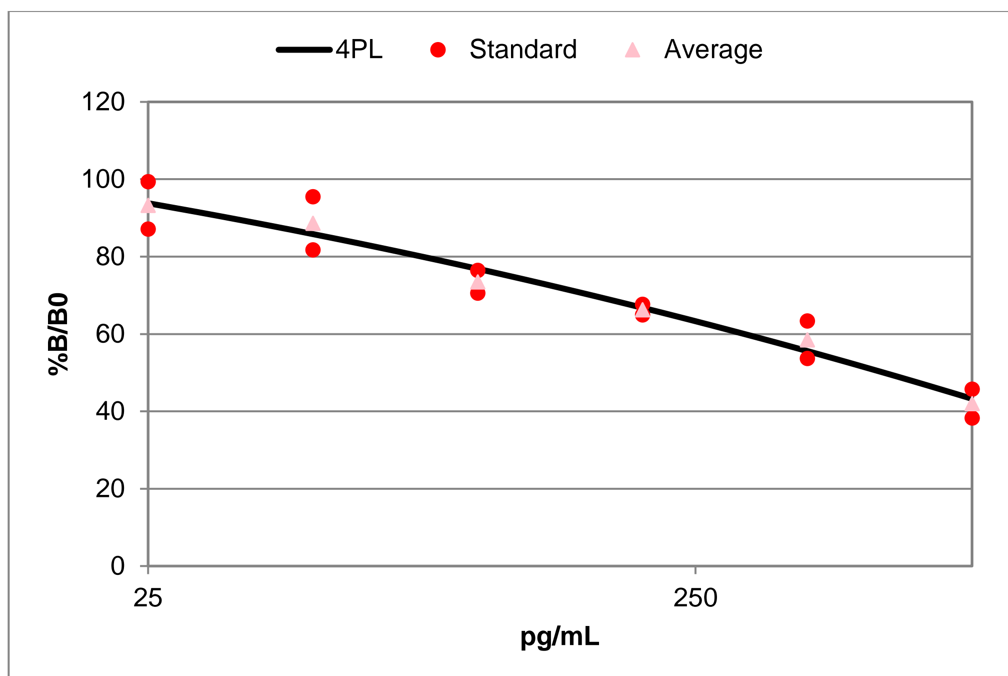

Figure 12: Standard Curve generated for the second Cortisol EIA.

Because this assay was used to compare different dilutions, there are no percent recovery values associated with extraction efficiency wells. As can be seen in Figure 13, and Table 9 in the Appendix, the concentration detected by the plate reader is higher in every 1:5 dilution compared to the detection in the 1:10 dilution, except in one instance (the second sample of 17-0881). Because of this, we have determined that using a 1:5 dilution of sample to assay buffer is preferable over using a 1:10 dilution.

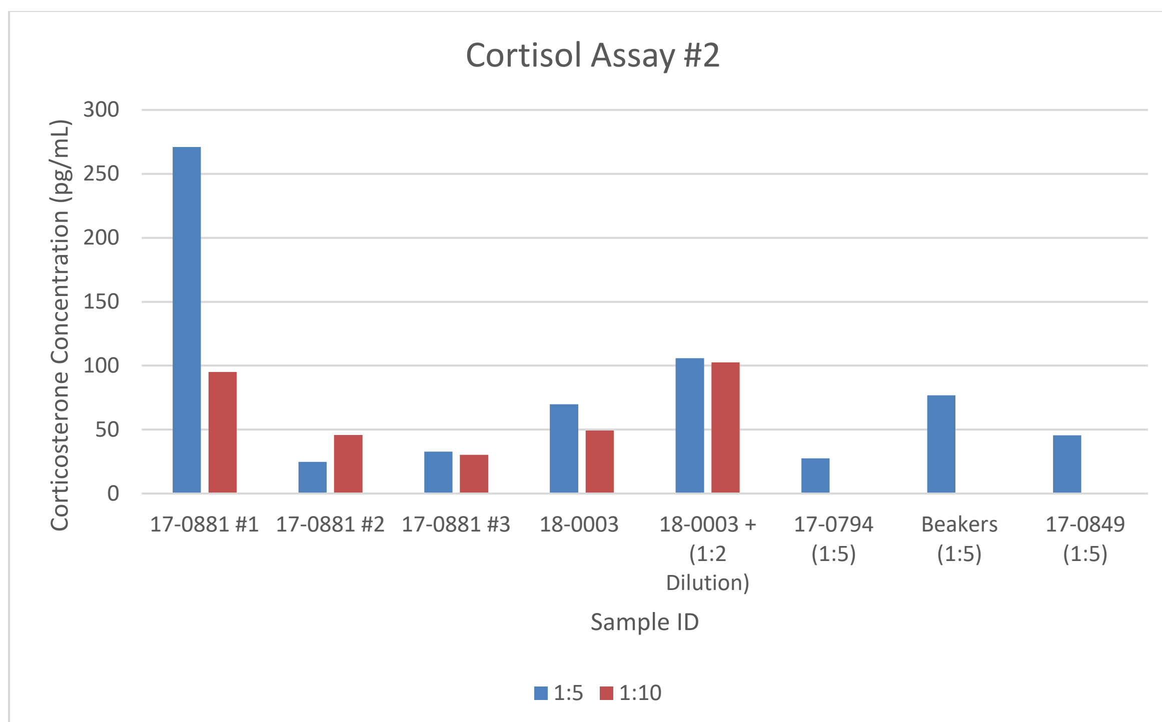

Figure 13: This graph shows the results of the second Cortisol EIA. Samples were prepared using a 1:5, or a 1:10 dilution with assay buffer, and the corticosterone concentrations (pg/mL) were compared.

3.3.3 Corticosterone Assay #1

The first Corticosterone EIA we ran used some of the samples previously tested with the first and second Cortisol EIAs. The samples had been extracted with the dry extraction protocol, and were prepared in a 1:5 dilution with the assay buffer. The standard curve shown in Figure 14 has data for 9 standard samples ranging from 39.06 to 10,000 pg/mL of corticosterone. It was necessary for these samples to have a greater number of standards that have a lower concentration of Cort to be able to accurately detect low Cort values in the samples.

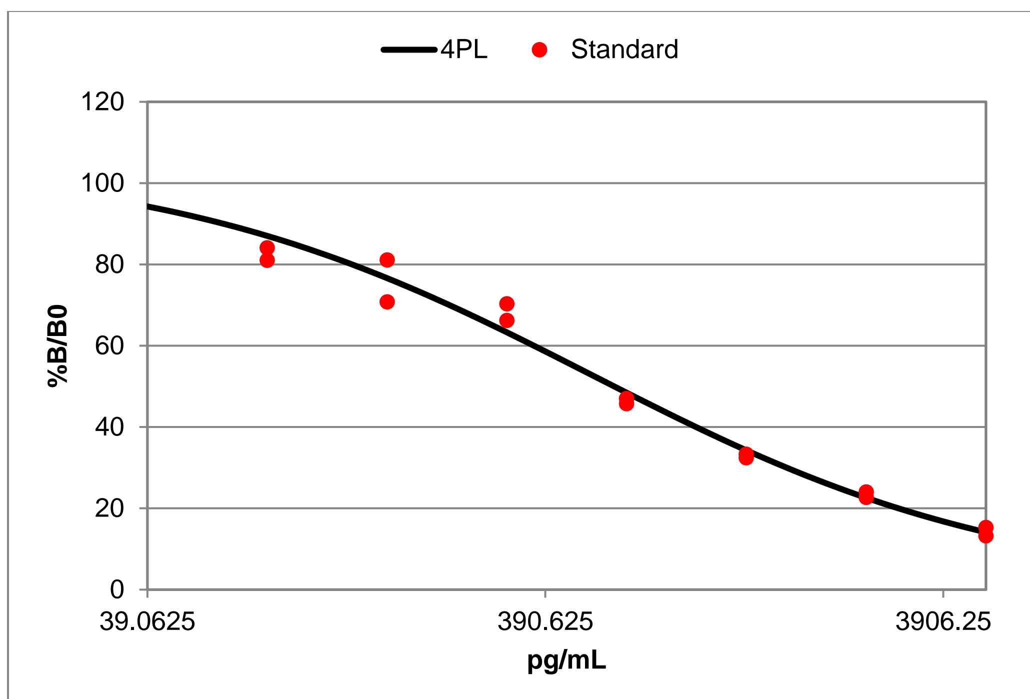

Figure 14: Standard Curve generated from the first Corticosterone EIA.

The results of the first corticosterone assay that we conducted measured the extraction efficiency values, and the recovery rate was 78 to 99%. Figure 15 below shows the Cort concentrations for each sample tested independently, the spiked samples, and it included the Cort concentrations for samples analyzed using the Cortisol EIA. Additional data is available in Table 3 for the samples tested independently, and Table 10 in the Appendix details the exact Cort concentrations for all samples tested with this EIA. We found that the Corticosterone EIA showed a better detection of Cort in the samples than the Cortisol EIA did, presumably due to more specific binding by the antibodies. Additionally, the Corticosterone EIA had a higher recovery percentage than the Cortisol EIA for the extraction efficiency wells containing spiked samples. Because of this, we suggest the use of the Corticosterone EIA kits over that of the Cortisol EIA kits to test Corticosterone concentration in raptor fecal samples.

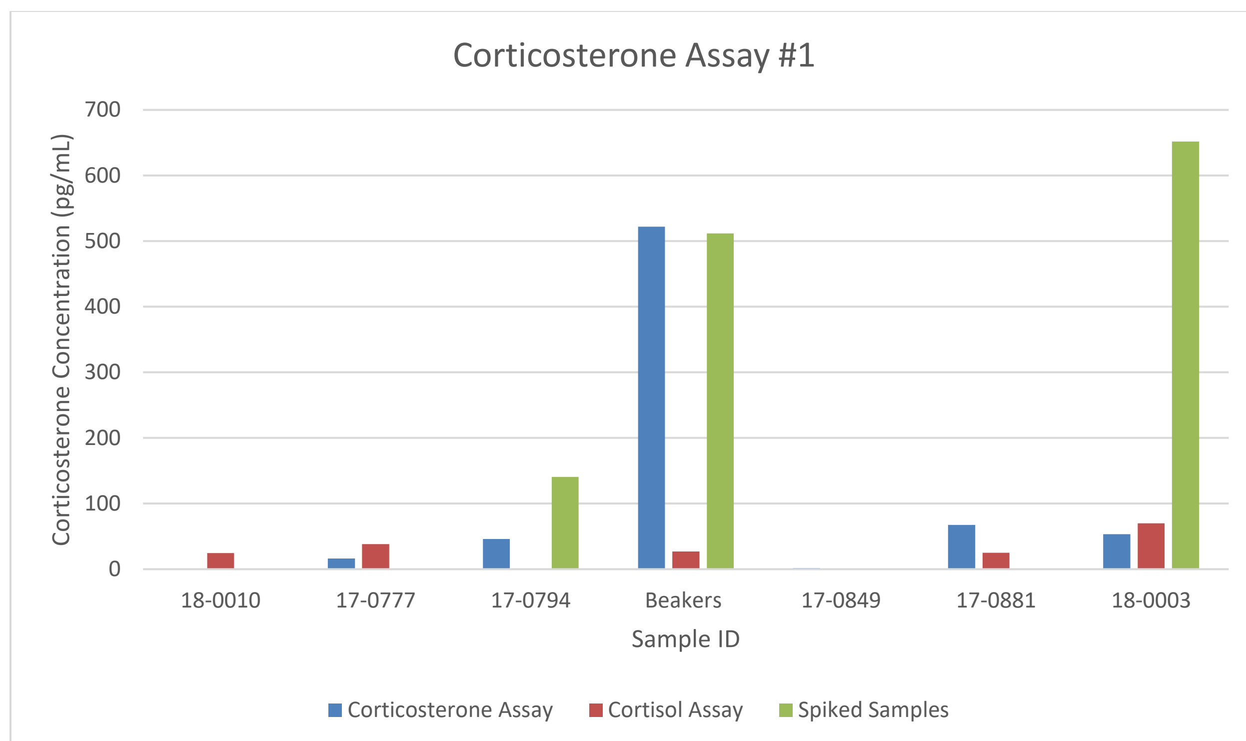

Figure 15: This graph shows the results of the first Corticosterone EIA. The corticosterone concentrations (pg/mL) are shown for the independent samples, as well as the samples spiked with known concentrations of corticosterone.

3.3.4 Corticosterone Assay #2

The second Corticosterone EIA we ran used some of the ambassador samples collected in 80% methanol. These samples were extracted using the dry extraction protocol, and were prepared in a 1:10 dilution of sample to assay buffer. The samples were compared to samples in a 1:2 dilution, and spiked samples to measure the extraction efficiency, and a recovery percentage was determined to be between 66 and 100%. Figure 16 below shows the standard curve generated for the second corticosterone assay, and has a comparable range to the first corticosterone standard curve with a higher %B/B0 at the low end of the curve. Figure 17 shows the data obtained from this assay, and additional information can be found in Table 11 in the Appendix. The results of this assay show that collecting the fecal samples in 80% methanol is an

adequate method for storage, and that the 1:10 dilution preparation worked well for the detection of corticosterone in these samples.

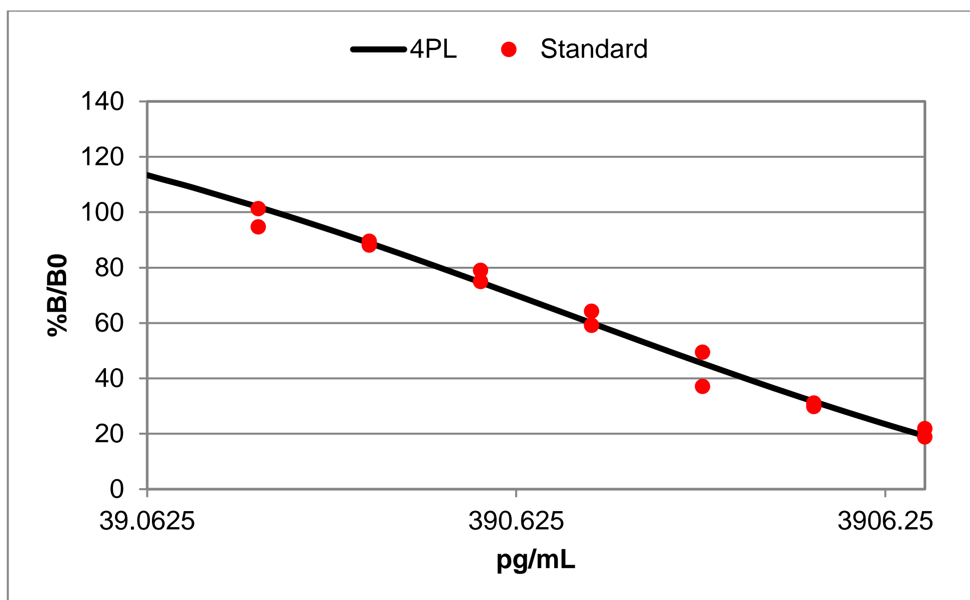

Figure 16: Standard curve generated for the second Corticosterone enzyme immunoassay that analyzed ambassador samples.

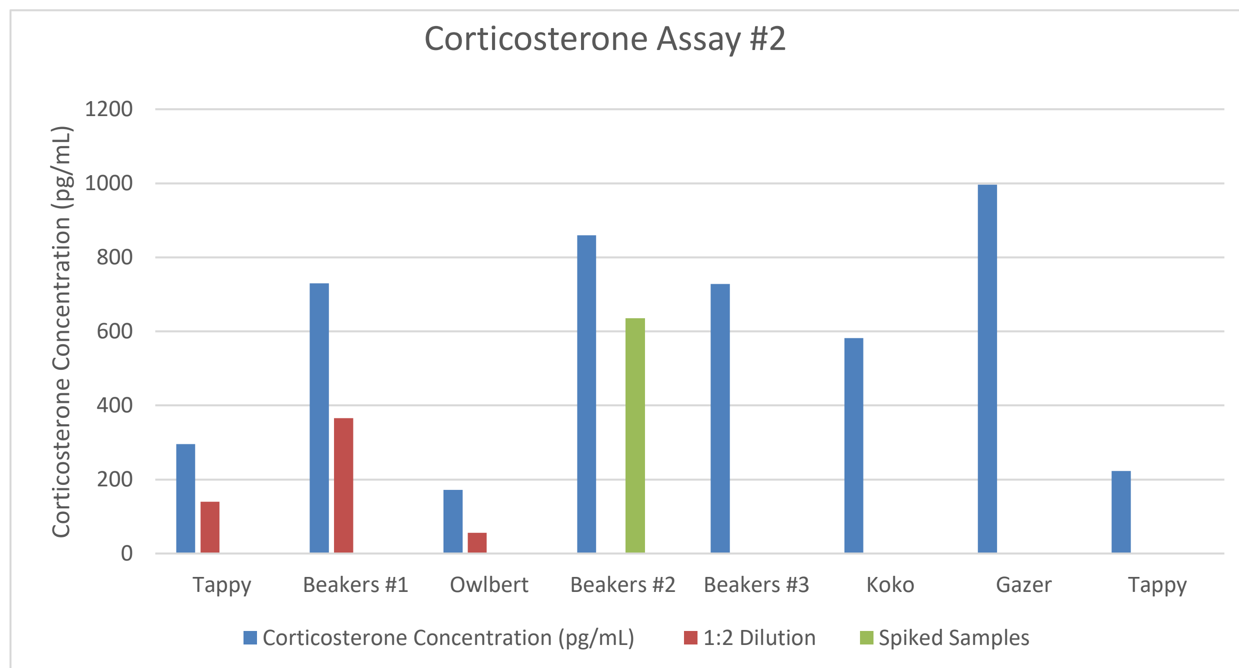

Figure 17: This t shows the data for the second Corticosterone EIA that measured the Cort concentrations of the ambassador samples collected in 80% methanol.

4 CONCLUSIONS

This descriptive project was designed to determine the feasibility of using non-invasive techniques to test the corticosterone levels in raptors undergoing rehabilitation. Because we conducted our study in coordination with a non-profit wildlife rehabilitation center, we had no control over the patients that were brought to AWARE, their species, sex, age, ability to thrive, injuries sustained, and the number of patients that could participate in the study. When we started this project, our goal was to measure Cort in injured raptors brought to AWARE during rehabilitation in order to optimize rehabilitation practices for each species in the study. Due to the unanticipated problems we encountered, we altered the premise of the project to focus our efforts on validating the methods required to measure Cort in wild raptors undergoing rehabilitation. We faced challenges with the collection, extraction, and assay protocols. Per the results detailed in Section 3, we recommend that future studies collect samples and immediately submerge them into 80% methanol to prevent the hormone in the sample from being degraded or from being absorbed by newspaper, and to aid in the separation of the fecal samples from gravel or other inorganic material. We advise that a dry extraction protocol be used for samples collected from raptors. The wet extraction protocol may be useful for birds that are not injured or that are acclimated to their environment, but because these raptors are injured and presumably stressed their fecal samples did not often meet the weight requirements for this protocol. The dry extraction offers more flexibility in the weight requirement, and is allows for an easier extraction of samples containing gravel. We also recommend that future studies use the Corticosterone EIA to measure Cort in raptors, rather than the Cortisol EIA. According to Arbor Assays, the Cortisol EIA can be used to measure Cortisol and Corticosterone in a wide array of animals including avian species, but we found that the recovery rate was better in the Corticosterone EIA. Samples

not submerged in 80% methanol and tested with the Corticosterone EIA should also be prepared in a 1:5 dilution with assay buffer, as opposed to a 1:10 dilution, for better results. Though, for samples collected in the 80% methanol the 1:10 dilution worked well for corticosterone detection. This methodology should streamline future studies that aim to measure Cort in raptors undergoing rehabilitation, and it may be applicable for studies conducted in the field as well. The protocols used in this study could be advantageous for future studies focused on measuring stress in avian species. Due to the differences in avian fecal samples compared to those of other animals such as terrestrial vertebrates, this methodology may not be applicable or necessary for studies measuring fecal glucocorticoids in non-avian species.

Because of the current and rapid loss of biodiversity occurring on a global scale it is essential to invent new and better ways to conserve wildlife. The use of fecal glucocorticoid monitoring in captive and wild species is an accepted method for a number of management programs, and it provides useful information that may aid in the creation of better husbandry protocols for different species. We propose that measuring Cort in injured raptors is a valid technique for the conservation of raptors, and it provides valuable information about stress activity in an individual or population. The data we have collected, and the results of this study indicate that this protocol may streamline future studies that aim to measure fecal glucocorticoids in avian species. However, this methodology does not preclude the possibility of other complications. The feasibility of future studies may depend not only on the methodology, but also on the availability of funding, access to test subjects, personnel, equipment, and so on. Many factors contribute to the success of a project. This study merely contributes one aspect of utility to future studies, though we hope that is enough. Additional information is needed to determine if Cort concentrations measured in raptors undergoing rehabilitation can be used to predict their

reactions to specific rehabilitation practices. Studies following this research should focus on this to assess the value of this data. By validating the methods required to measure Cort in injured raptors at AWARE, we hope to allow future studies to understand the stress activity of raptors as a means to create species-specific practices that may optimize their care and decrease stress.

APPENDIX

Table 4: Log of fecal samples collected from patients at AWARE during the study.

Patient ID	Species	Enclosure	Date Collected
17-0253	RTHA	RR9	5/22/17
16-0874	BDOW	RR10	5/23/17
17-0039	RSHA	RSF	5/23/17
16-0968	RSHA	OE2	5/23/17
16-1007	RSHA	OE2	5/23/17
17-0125	RTHA	OE1B	5/23/17
17-0253	RTHA	RR9	6/6/17
16-0874	BDOW	RR10	6/6/17
16-0968	RSHA	OE2	6/13/17
16-1005	RTHA	OE1B	6/19/17
17-0125	RTHA	OE1B	6/19/17
17-0036	BDOW	LSF	6/19/17
17-0125	RTHA	OE1B	6/25/17
17-0097	RTHA	OE1B	6/25/17
17-0253	RTHA	RR9	6/26/17
16-0874	BDOW	RR10	6/26/17
17-0253	RTHA	NFL	7/11/17
16-0968	RSHA	OE2	7/11/17
17-0426	RSHA	RSF	7/11/17
17-0039	RSHA	RSF	7/11/17
17-0125	RTHA	OE1B	7/11/17
17-0036	BDOW	RR12	7/11/17
16-0874	BDOW	RR10	7/13/17
16-0874	BDOW	RR10	7/25/17
17-0036	BDOW	RR12	7/25/17
17-0253	RTHA	NFL	7/27/17

16-0968	RSHA	OE2	7/27/17
17-0125	RTHA	OE1B	7/27/17
17-0097	RTHA	OE1B	7/27/17
17-0039	RSHA	OE2	7/31/17
17-0039	RSHA	OE2	8/15/17
16-0968	RSHA	OE2	8/15/17
17-0125	RTHA	NFL	8/15/17
17-0097	RTHA	OE1B	8/15/17
16-0874	BDOW	RR10	8/22/17
17-0039	RSHA	OE2	8/27/17
17-0125	RTHA	OE1B	8/27/17
17-0097	RTHA	OE1B	8/27/17
16-0874	BDOW	RR10	9/4/17
17-0682	RSHA	RR11	9/4/17
16-0968	RSHA	OE2	9/4/17
17-0097	RTHA	OE1B	9/4/17
17-0125	RTHA	OE1B	9/5/17
17-0426	RSHA	RR9	9/18/17
16-0874	BDOW	RR10	9/18/17
17-0682	RSHA	RR11	9/18/17
17-0125	RTHA	RSF	9/18/17
16-0874	BDOW	RR10	10/1/17
17-0682	RSHA	NFL	10/1/17
17-0426	RSHA	RR9	10/1/17
17-0125	RTHA	RSF	10/1/17
17-0125	RTHA	RSF	10/3/17
17-0682	RSHA	NFL	10/3/17
16-0874	BDOW	RR10	10/10/17
17-0779	RTHA	RR7	10/16/17
17-0777	RTHA	RR8	10/16/17
17-0682	RSHA	NFL	10/16/17
17-0125	RTHA	RSF	10/16/17
17-0125	RTHA	RSF	10/22/17
17-0777	RTHA	RR8	10/22/17
17-0125	RTHA	RSF	11/12/17
17-0777	RTHA	OE1B	11/12/17
17-0779	RTHA	OE1B	11/12/17
17-0794	BDOW	RR10	11/12/17
17-0779	RTHA	OE1B	11/29/17
17-0794	BDOW	RR10	11/29/17
17-0777	RTHA	OE1B	12/4/17

17-0849	BDOW	RR11	12/5/17
17-0851	BDOW	RR8	12/5/17
17-0849	BDOW	OE1B	12/27/17
17-0794	BDOW	OE1B	12/27/17
17-0777	RTHA	RSF	12/30/17
17-0779	RTHA	RSF	12/30/17
17-0884	BDOW	RR7	12/30/17
17-0881	RTHA	RR11	12/30/17
18-0003	RTHA	MR1	1/14/18
17-0881	RTHA	RR11	1/14/18
18-0010	RTHA	RR7	1/14/18
18-0012	RSHA	RR3	1/15/18
17-0849	BDOW	OE1B	1/15/18
17-0779	RTHA	RSF	1/14/18
17-0794	BDOW	OE1B	1/15/18
17-0779	RTHA	RSF	1/31/18
17-0881	RTHA	RR11	1/29/18
18-0010	RTHA	RR7	1/29/18
17-0849	BDOW	OE1B	1/30/18
17-0794	BDOW	OE1B	1/29/18
17-0777	RTHA	RSF	1/29/18
18-0003	RTHA	RR9	1/29/18
17-0777	RTHA	RSF	2/13/18
18-0030	BDOW	RR12	1/30/18
18-0044	RTHA	RR11	2/13/18
17-0779	RTHA	RR9	2/13/18
18-0030	BDOW	RR12	2/12/18
17-0794	BDOW	OE1B	2/13/18
17-0849	BDOW	OE1B	2/13/18
17-0881	RTHA	RSF	2/14/18
18-0003	RTHA	OE2	2/13/18
18-0010	RTHA	OE2	2/13/18
18-0003	RTHA	RSF	2/14/18
18-0010	RTHA	RSF	2/14/18
17-0779	RTHA	RR9	3/6/18
18-0044	RTHA	RR11	3/6/18
18-0054	RTHA	RR10	3/6/18
17-0881	RTHA	NFL	3/7/18
17-0777	RTHA	NFL	3/7/18
18-0010	RTHA	NFL	3/7/18
17-0794	BDOW	OE1B	3/7/18

18-0030	BDOW	OE1B	3/7/18

Table 5: Log of fecal samples collected from ambassadors at AWARE using the 80% methanol collection method.

Name	Species	Date Collected
Koko	RTHA	3/18/18
Beakers	RTHA	3/18/18
Tappy	BDOW	3/18/18
Gazer	BDOW	3/18/18
Beakers	RTHA	3/19/18
Beakers	RTHA	3/19/18
Koko	RTHA	3/20/18
Beakers	RTHA	3/20/18
Owlbert	BDOW	3/20/18
Owlbert	BDOW	3/21/18
Koko	RTHA	3/21/18
Beakers	RTHA	3/21/18
Owlbert	BDOW	3/22/18
Koko	RTHA	3/22/18
Beakers	RTHA	3/22/18
Beakers	RTHA	3/23/18
Beakers	RTHA	3/24/18
Beakers	RTHA	3/25/18
Tappy	BDOW	3/25/18
Beakers	RTHA	3/26/18

Table 6: Log of fecal samples collected from ambassadors at AWARE using the standard collection method.

Name	Species	Date Collected
Beakers	RTHA	2/13/18

Table 7: Log of samples extracted using the Wet Extraction Protocol, and the accompanying sample weights.

Vial Number	Patient ID	Collection Date	Extraction Date	Sample Weight
1	16-0874	5/23/17	12/12/17	0.54
2	16-0968	5/23/17	12/12/17	0.09
3	16-0874	6/26/17	12/12/17	0.366

4	17-0253	6/26/17	12/12/17	0.279
5	16-0874	7/25/17	12/12/17	0.2503
6	17-0036	7/11/17	12/12/17	0.3316
7	17-0097	7/27/17	12/12/17	0.2066
8	16-0968	7/27/17	12/12/17	0.4809
9	17-0039	8/27/17	12/12/17	0.2206
10	16-0874	8/22/17	12/12/17	0.5897
11	17-0039	7/11/17	1/10/18	0.2534
12	17-0036	7/25/17	1/10/18	0.2529
13	16-0874	9/4/17	1/10/18	0.2523
14	17-0097	9/4/17	1/10/18	0.2584
15	17-0682	9/4/17	1/10/18	0.2527
16	16-0874	9/18/17	1/10/18	0.2586
17	17-0426	9/18/17	1/10/18	0.2588
18	17-0682	9/18/17	1/10/18	0.2579
19	17-0125	7/27/17	1/10/18	0.2551
20	16-0874	6/6 and 7/13/2017	1/20/18	0.205
21	16-0874	10/1/17	1/20/18	0.2501
22	16-0874	10/10/17	1/20/18	0.2578
23	16-0968	6/13 and 7/11/2017	1/20/18	0.1592
24	17-0097	6/25 and 7/11/2017	1/20/18	0.1793
25	17-0097	8/15 and 8/27/2017	1/20/18	0.2616
26	17-0777	10/16/17	1/20/18	0.2618
27	17-0777	11/12/17	1/20/18	0.2561
28	17-0779	10/23/17	1/20/18	0.2647
29	17-0779	11/12/17	1/20/18	0.2584
30	17-0779	11/29/17	1/20/18	0.2591
31	17-0851	12/5/17	1/23/18	0.2243
32	17-0125	10/22/17	1/23/18	0.2587
33	17-0125	10/3/17	1/23/18	0.2663
34	17-0426	10/1/17	1/23/18	0.2659
35	17-0794	11/12/17	1/23/18	0.2596
36	17-0794	11/29/17	1/23/18	0.2589

Table 8: Corticosterone concentration values (pg/mL) for all samples tested in the first Cortisol EIA.

Sample Preparation	Corticosterone Concentration (pg/mL)

1N 18-0010	24.59
1N + 100 µL 800 pg/mL	15.78
1N + 100 µL Buffer	16.11
2N 17-0777	38.43
2N + 100 µL 1600 pg/mL	52.8
2N + 100 µL Buffer	74.61
3N 18-0003	297.9
3N + 800 pg/mL (1:2)	360
4N 17-0794	18.67
4N +1600 pg/mL (1:2)	556.4
5N Beakers	26.88
5N + 200 pg/mL (1:2)	89.86
6N 17-0849	26.63
6N + 100 pg/mL (1:2)	58.86
7N 18-0030	42.3
7N + 400 pg/mL (1:2)	137.1

Table 9: Corticosterone concentration values (pg/mL) for the samples run on the second Cortisol EIA.

Sample ID	Corticosterone Concentration (pg/mL)
17-0881 (1:5)	271
17-0881 (1:10)	94.98
17-0881 (1:5)	24.81
17-0881 (1:10)	45.82
17-0881 (1:5)	32.84
17-0881 (1:10)	30.28
18-0003 (1:5)	69.91
18-0003 (1:5) + (1:2 Dilution)	105.8
18-0003 (1:10)	49.27
18-0003 (1:10) + (1:2 Dilution)	102.7
17-0794 (1:5)	27.5
Beakers (1:5)	76.69
17-0849 (1:5)	45.49

Table 10: Corticosterone concentration values (pg/mL) for samples run on the first Corticosterone EIA.

Sample ID	Corticosterone Concentration (pg/mL)
18-0010	0
18-0010 (Cortisol Assay)	24.59
17-0777	16.24
17-0777 (Cortisol Assay)	38.43

17-0794	46.04
17-0794 + 312.50 pg/mL	140.4
Beakers	522.1
Beakers + 625 pg/mL	512
Beakers (Cortisol Assay)	26.88
17-0849	1.55
17-0881	67.39
17-0881 (Cortisol Assay)	24.81
18-0003	53.26
18-0003 + 1250 pg/mL	651.9
18-0003 (Cortisol Assay)	69.91

Table 11: This table shows the data for the second corticosterone assay, and includes the recovery percentages for samples compared to the 1:2 dilution or the spiked sample.

Name	Corticosterone Concentration (pg/mL)	1:2 Dilution	Spiked Samples	Recovery Percentage
Tappy	295.7	140	N/A	95%
Beakers #1	729.7	366	N/A	100%
Owlbert	172	56.33	N/A	66%
Beakers #2	859.4	N/A	635.4	86%
Beakers #3	727.7	N/A	N/A	N/A
Koko	582.2	N/A	N/A	N/A
Gazer	996.3	N/A	N/A	N/A
Tappy	223.1	N/A	N/A	N/A

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