A Thesis Presented to
The Faculty of Alfred University

Environmental Effects on Stress Levels Within Babydoll Southdown Sheep (*Ovis aries aries southdown*)

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In Partial Fulfillment of the Requirements for
The Alfred University Honors Program and
Biology Division Honors
April 23rd, 2019

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Abstract

Maintaining high standards of animal welfare and a positive public opinion are some of the main priorities of zoos. Zoos also serve as important educational facilities which encourage beneficial research and strive to improve conservation. One way to ensure high-quality animal welfare is to monitor any possible environmental factors which may be stressful for the animals. The purpose of this study is to quantitatively and qualitatively determine if Babydoll Southdown sheep (*Ovis aries aries southdown*) show significantly different levels of stress during different seasons. The study was conducted at the Buffalo Zoo in Buffalo, New York where changing seasons impose various environmental conditions on the sheep. The changing conditions include the number of visitors attending the zoo and changes in the weather from the summer (August of 2018) to the winter (December of 2018-Janurary of 2019). Observation was completed to qualitatively determine if the sheep were exhibiting undesirable behaviors, such as head butting, pushing, head tossing, foot stomping, or nose blowing, which can be induced by stress. Fecal samples were analyzed for cortisol levels using Arbor Assay's DetectX® Cortisol Enzyme Immunoassay Kit to quantitatively measure the sheeps' stress levels. A regression showed that there was no significant difference between the average cortisol levels between seasons (P=0.083) or between average cortisol levels and temperature (P=0.225). A negative, significant relationship was shown between the average cortisol levels and the number of visitors (P=0.01). Instances of aggressive behaviors were slightly greater in the summer months (1.20/hr) than the winter months (0.29 /hr), but there was not a significant relationship between fecal cortisol level and the number of aggressive behaviors shown (P=0.305). This suggests these behaviors occurred naturally and were not due to increased cortisol levels.

Introduction

For as long as I can remember, I have always been passionate about animals and promoting their health and well-being. This passion is what encouraged me to pursue an education in veterinary medicine, and to engage in activities that would allow me to learn more about animals and the veterinary profession in general. By becoming a veterinarian, I could be at the forefront of innovative procedures and medical research to benefit animals. I have always been amazed by the expansive skill and knowledge that veterinarians possess, which is something that further motivated me to want to achieve this goal. As I got older, I was presented with more opportunities to explore the different areas of study within the veterinary profession. During this time, I learned that some veterinarians chose to conduct research to promote and improve animal well-being, and I was encouraged to follow this path myself.

I was first introduced to the area of animal research when I assisted with a study conducted by another Alfred University student about inter- and intra-species interactions between primates at the Buffalo Zoo. This experience, along with my passion for animals, and the various laboratory and analytical skills I have acquired through taking many biology and chemistry courses, drove me to pursue my own research project. In designing my project, I knew I wanted to conduct one that included sample analysis where I would have the opportunity to also work in a laboratory. I enjoy conducting experiments in lab, and I knew having quantitative results alongside behavioral analysis would help strengthen my research.

My desire to improve animal welfare, gain valued experience in the field and in the laboratory, and to spend time around farm animals, ultimately lead me to my project design.

Being from a more rural area, I had very little experience with large or farm animals. The majority of my animal experience was with cats, dogs, and small exotic animals, and I viewed this research project as an opportunity to build my experience with farm animals. Sheep were an obvious pick for the subject of my study, since they are farm animals that can be easily found within zoos, which are often located in urban areas. Sheep are also animals that are commonly used in petting zoos. Animals within petting zoos are among those which have the most interactions with visitors, and therefore can be most affected by their presence. I decided to study the Babydoll Southdown sheep (*Ovis aries aries southdown*), housed in the Heritage Farm Children's Zoo at the Buffalo Zoo. The focus group consisted of four sheep, one female and three males, named Dolly, Charlie, Harrison, and Toby.

The overall purpose of my study is to identify if the sheep at the Buffalo Zoo showed abnormal levels of stress, through the utilization of two methods. This first method was to conduct visual observation of their behavior, specifically instances of aggressive behavior such as head butting, pushing, head tossing, foot stomping, or nose blowing. The second method was to analyze their stress levels using fecal cortisol levels. Cortisol is a hormone associated with elevated levels of stress, and can be used as an indicator for chronic or acute stress.

Stress levels were analyzed within two different seasons, the winter and the summer, to observe if the changing environmental factors affected the sheep. The two environmental factors that were specifically analyzed that could influence the sheep's stress levels were visitor attendance and weather. During the summer, there are greater numbers of people who visit the zoo, and the temperatures are generally higher. These are two things that may influence stress levels, and therefore cortisol levels, of the sheep.

The observational portion of my research was simple, but tedious. I spent several hours a day, multiple times a week, watching and recording the sheep's behaviors. This was first done by creating a list of behaviors, called an ethogram, which contained specific, defined actions for each behavior. This ensures that nothing is subjective, and the study could easily be repeated by another person. Focal sampling was used to observe each one of the sheep continuously for 15 minutes. During this time, every single behavior a sheep exhibited was recorded via an iPad containing Animal Behavior Pro software. This focal sampling was done for each of the four sheep to ensure the amount of data collected on each sheep was equal. All of this data was compiled to create a general activity budget for the sheep, specifically how much time the sheep spend engaging in each behavior. This is beneficial in determining what is normal behavior for the sheep, and if there is any variation in their behavior from one season to the next. Drastic differences in behavior can indicate a problem, one being chronic stress.

Aggressive behaviors can also be a sign of stress within animals. There are a variety of factors which can induce stress within zoo animals. Some of them are due to habitat design, visitor density, or environmental conditions, such as sound volume and artificial lighting. If the animal feels scared or that they can't move away from something that is scaring them, they may exhibit defensive behaviors. Visitor density as a stressor applies directly to animals within a petting zoo. Stress can be induced within petting zoo sheep if they are not given the opportunity to move away from visitors if interactions are undesirable. This optional space to move away from visitors is called retreat space. The required retreat space will depend on the species and on how fearful each individual is. This is why it is so important for zoos to select specific species and breeds who have a good temperament and are generally not extremely fearful, in addition to designing habitats to allow petting zoo animals the option of retreat space. For these reasons, I choose to examine specific behaviors which can be considered aggressive in sheep, in addition to tracking the visitor density of the zoo for each day that observation was completed.

For the second method, I analyzed stress levels from fecal cortisol levels. Cortisol is a hormone that helps the body react to stress. It is natural to have cortisol in the body, because it is necessary in normal metabolic functions; however, it will be released in elevated levels when the body is exposed to chronic stress. Cortisol is released from the adrenal gland, and like many other hormones, it travels through the blood until it reaches its target. Cortisol plays a role in many different processes, so it has more than one target, and therefore more than one effect on the body. Cortisol is also one of the hormones involved in the cascade of events, which occurs when our body faces a fight or flight situation. This is why chronic stress can have multiple effects on the body.

Cortisol can be found in blood, salvia, urine, and feces. Analyzing fecal cortisol levels was the best option for my project because it is noninvasive and can be collected in a manner which will not cause additional stress to the animal. For example, drawing blood can be stressful for the animal, so if being used as an indication for stress levels, it could just be caused by the sample collection. Fecal cortisol levels also provide more of a broad overview of the sheep's cortisol levels from the previous 24 hours, as opposed to the instant the sample was collected. So, for my study, using fecal cortisol levels was a perfect collection method for viewing the trends of each sheep's cortisol levels overtime.

Overall, this research is significant because studies such as these will improve animal welfare and public opinion on housing animals in zoos. Zoos are important educational facilities which encourage beneficial research, and strive to improve conservation. As long as the animals are kept in proper living conditions and are supplied with needed enrichment, nutrition, care, and treatment, they experience a high quality of life. This is valuable information that needs to be made known. This research is also relevant to the human animal bond, which is a growing area of interest within veterinary medicine. Looking at the relationship between visitors and animals within a zoo is important to determine how they are affected by crowds. High visitor densities may induce stress within some animals, but others may enjoy the enrichment the visitors provide. The results from my study showed that the sheep had lower stress levels, with respect to cortisol levels, when there was a greater visitor density.

The experiences I have had through conducting this study inspired me to pursue a career within the research field, where veterinarians are making advancements in surgical procedures, public health, disease control and prevention, and overall animal welfare. Next year, I will be attending The Ohio State University College of Veterinary Medicine to pursue a veterinary degree with a focus on research. In the future, I hope to have the opportunity to further my education to gain the knowledge and skills to be able to conduct innovative research, and the potential to improve animal welfare as a whole.

Literature Review

Current day zoos serve many beneficial purposes with respect to conservation, research, and education.[1] However, above all else, one of the main concerns within zoo management is the welfare of their animal residents. Zoos are constantly working to evaluate the physical and mental wellbeing of the animals, alongside creating a fun and stimulating environment for visitors to enjoy. However, there is still much to be learned about animal behavior, especially concerning those in captivity. This is due to the differences of environmental conditions between captive environments and natural environments in the wild. Particularly in zoo's, the effects of visitors on animals must be taken into consideration as it can be both beneficial and stress inducing.[2] How zoos improve animal welfare and encourage visitor satisfaction through conservation, research, and education will be explored more deeply throughout this review.

Various factors, other than visitors, can induce stress as well, but it can be difficult to determine what is a stressor to an animal, as they vary between species, and even between individuals within a species.[2] Acute and chronic stress can affect the behaviors and overall wellbeing of the animals.[3] Especially in petting zoos, it is important that all possible measures are taken into account to maintain the comfort of the animal, by keeping their stress levels low and their behaviors manageable. In recent years, many methods to analyze stress, and therefore wellbeing, of captive animals have emerged.[4] The ways in which zoos identify stressors will also be presented in-depth in this review. These continued efforts work to positively enhance animals' lives, because once stressed, there are actions that can be taken to rectify this and maintain high standards of animal welfare.

Purpose of Zoos

The perception of zoos and their purpose has changed drastically throughout history. The earliest zoos date back to the era of the ancient Egyptians, Chinese, and Romans.[5] The first modern zoos were a luxury owned by wealthy citizens in Europe. These facilities were mainly seen as a status symbol, but were occasionally opened to public viewing for entertainment purposes.[5] In these situations, the comfort and quality of life of the animals was not a main priority. Conditions for the animals did not begin to improve until the Zoological Society of London was formed in the early 20th century.[5] Along with the growth of these societies was an emergence of many public zoos. However, the main purpose of these zoos was still visitor satisfaction, causing animal needs to be neglected.

Within the past three decades, zoos have changed their missions regarding the priority of animals' wellbeing and animal rights.[1] Many have transformed into education and conservation centers, as opposed to public entertainment. Today's zoos play a variety of different roles to encourage conservation, research, and education to benefit the animals while striving to maintain both visitor satisfaction and positive public opinion of zoos.[1]

i. Conservation

There are a variety of ways in which zoos aid in the conservation of species. The first is the monitoring of populations of wild species, done by measuring naturally occurring breeding success and recording population viability, which can help identify the effects of humans on wildlife.[6] Zoos also improve conservation by implementing breeding programs for endangered species, which allows more individuals to be released into the wild.[5] Through resorting and maintaining species populations, the genetic integrity of the species can maintained. In addition to this, the balance of the ecosystems in which the endangered species resides can be preserved.[5]

In the case where animals are being negatively affected due to habitat degradation, zoos can serve as a safe haven. They function as a tool to provide a safe environment for animals to survive if changes to their natural environment might threaten their lives.[1] This is particularly true for those who are losing their natural habitats from degradation due to human disturbances. Recently, a successful breeding program was implemented to increase the Arctic fox (*Vulpes lagopus*) population in Norway. The numbers of foxes were severely declining due to habitat destruction, decreasing the population of their main food source, rodents.[7] In 2000, there were about 34-40 adult individuals remaining in the wild, so a captive breeding program was initiated in an attempt to conserve the population.[7] With the help of Langedrag Familiepark Zoo and experienced farmers who created realistic, artificial environments to breed the Arctic fox pups, 295 pups were born and released across four different locations over the following decade.[7] After a few years, the populations were reestablished and by 2014, over 600 of the wild born pups were descendants of the captive born pups.[7]

Although these methods seem virtuous in theory, they can be difficult to implement successfully in reality. Certain animals, such as cheetahs (*Acinonyx jubatus*), do not survive well in captivity.[1] There are less than 10,000 cheetahs estimated worldwide, qualifying them as an endangered species.[8] This number initiated efforts to artificially manage the cheetah population through captive breeding by zoos.[8] Despite continued efforts, the increased rate of diseases and stress have led to low reproductive rates within an artificial environment.[8] Only a 20% pairing success rate, and 50% of females producing a litter after breeding have been observed.[9] These results show that implementing breeding programs within a given species does not always provide much of a benefit for the population. These programs can be expensive, complex, and could face challenges reintroducing species into their natural habitat.[1] Within breeding programs there is a lot at stake, and failures can lead to lost resources, sometimes at the expense of the species.[1] However, if a zoo can successfully implement these programs with species who have a high successful reproduction rate, such as with the Arctic fox, it is a powerful tool in which species conservation can be monitored and maintained.

ii. Research

In addition to conservation efforts implemented by zoos, further opportunities to improve animal welfare arise through the ability of scientists to learn more about each species by conducting research.[1] Zoos provide and support research efforts, which allow knowledge of animal behavior to be gained. There are endless behavioral studies to be done to help enrich the lives of animals by providing the most naturalistic environment for the animal, as well as an authentic experience for the visitor. Essentially, zoos provide an environment of scientific study to improve animal conservation and education.[1] These efforts are strengthened when zoos establish partnerships with universities, encouraging research efforts that benefit exotic species residing within the zoo.[1] Zoo research mainly focuses on animal behavior, but can also be conducted within the areas of biomedical research, nutrition, genetics, and husbandry.[10] For example, the San Diego Zoo is implementing research to understand the role of disease within wild amphibian populations, and the development of disease-control measures for captive breeding and reintroduction programs.[11] This will benefit both captive and native populations of species, such as salamanders and frogs, from deadly diseases like chytridiomycosis.[11]

iii. Education

In addition to offering the scientific community the opportunity to gain more knowledge about animals, zoos can serve as an attraction where the general public can become informed. Many people are unaware of which species are endangered and how human behaviors have influenced this. Educating the public on how they can help reduce the human impact on declining populations of endangered species has the potential to improve the situation.[6] Zoos provide an informal educational environment where they can teach the general public about what they can do to help animals survive in the wild through animal demonstrations, interactive shows or talks, and even informational signs near exhibits.[5] The more knowledge the general public learns about the animals, as well as animal conservation efforts, the more likely they are to build a connection and feel empathy towards endangered species and therefore, becoming more likely to participate in conservation efforts.[12]

Koenig (2015) studied how the presence of informational displays located around the Gorillas of African Forest exhibit at the Houston Zoo affected visitor's knowledge about gorillas.[13] A pre and post test were distributed to visitors who encountered the exhibit containing questions about gorilla taxonomy, natural history, behavior and conservation.[13] Post tests were answered with 68% accuracy, a 3% increase from pre test scores, with the highest scores occurring within the conservation and taxonomy categories.[13] This study suggests that zoos can be an important resource in successfully educating the public.

Zoos are successful in implementing informal learning because it is a passive and enjoyable experience, as opposed to a structured classroom setting.[12] Educational psychologists have spoken to the benefits of an informal educational setting, especially in which the visitor willingly participates for entertainment.[5] The appearance of an animal's enclosure plays a role in the zoo's efforts to educate visitors about exotic species, as well. More naturalistic environments can informally educate visitors about a species' environment, as well as increase the general public's understanding of how exotic species behave, without making a conscious effort to learn.[12] These naturalistic enclosures also benefit the animals because it allows them to demonstrate behaviors they would when comfortable.[12] After relocating a small group of chimpanzees from cages into a more naturalistic environment, stereotyped behavior, such as rocking, and self-directed behavior, such as self-grooming, self-directed genital contact, and self-motion play, decreased drastically.[14] Visitors also have a more positive impression of the zoo in response to the apparent high quality of care and comfort of each animal.[12]

iv. Maintaining Visitor Satisfaction

We must keep in mind that zoos do face a variety of challenges in attempt to balance these multifaced ideals. Providing the best possible care for animals, participate in conservation efforts, and encourage educational research projects to be completed, as well as incorporating visitor satisfaction into the mix, can create conflicts. All of these aspects are essential to the success of the zoo and maintained health of each animal. Zoos tend to face harsh criticism with respect to animal rights and welfare.[1] The general public has a negative response to animals kept in cages, being neglected or possibly exposed to harsh human interactions.[1] This can be combated with updating facilities to represent more naturalistic environments.[12] Maintaining strict animal care routines and monitoring nutrition and hygiene can help curve the public's harsh opinions.[5] In addition to this, encouraging positive intraspecies interactions through proper introductions and acclimation to a new environment can increase the positive wellbeing of the animals.[1] Zoos depend on revenue from visitors in order to remain open and running at an accredited level. Due to this, zoos bank on visitor satisfaction since admissions revenue is the major source of income. Therefore, within meeting demands of conservation efforts, public education and research efforts, the entertainment aspect must also be maintained, only now, the zoos main focus is on the animal's wellbeing.

Many zoos also have exhibits dedicated to fostering positive interactions between people and animals, such as petting zoos. Although, there must be a balance between providing an environment where visitors can come into contact with the animals and the animals' comfort level within their enclosure. The main goal of these attractions is to develop a positive attitude regarding contact with animals.[15] In order for this to occur, the animals involved must cooperate and avoid expressing undesirable behaviors that could create a negative experience.[15] As mentioned by Anderson et al. (2002), undesirable behaviors such as charging and headbutting, are not unnatural but they can negatively impact the interaction between a visitor and an animal.[16]

There are various ways positive experiences can be facilitated by zoos.[15] Choosing breeds that may be in contact with people that are well-tempered and less fearful.[15] The African pygmy goat is often chosen as a breed to have within petting zoos because they are very amiable.[15] There are also various behavioral techniques, such as training, which can be implemented to help acclimate these animals to being around people. Rearing conditions play a vital role in how the animals respond to people. If they grow up exposed to others of their same species and having positive experiences with human contact, they will be more likely to perform more favorably within exhibits that include physical contact with visitors.[15]

Providing animals with the ability to be in control over the space between themselves and humans or other animals, known as critical distance, can also contribute to better petting zoo interactions.[15] The inability of an animal interacting with humans to maintain their comfortable critical distance can lead to fearfulness and, therefore, undesirable behaviors due to this fear.[15] Anderson et al. (2002) found that the sheep's rate of undesirable behaviors decreased to 1.09 from 2.58 per hour when they were allowed full retreat space during times of high visitor density.[14] In theory, the more fearful an animal, the greater critical distance they will require to ensure their own safety and decrease undesirable behaviors.[15]

The ability of the animals to maintain critical distance can be affected by visitor density and enclosure design.[15] This once again brings up the issue of balancing visitor satisfaction and animal welfare. Zoos need to be mindful of how many visitors have access to an interactive exhibit, which may require limiting the number of visitors who can go into the exhibit at once. But, in doing this, there are decreased chances that the animals will become fearful due to overcrowding and the inability to maintain control over their desired critical distance. This problem can also be improved with an enclosure design that allows the animals to retreat to a space where they can be alone if they are feeling frightened.[15] This gives the animal control over their environment and therefore can decrease undesirable behaviors.[15]

Anderson et al. (2002) concluded that high volumes of visitors can possibly stress out animals in petting zoos but stressful environments can be minimized by allowing the animals room for retreat space, to distance themselves from the visitors.[15] This extra space that was given to the animals, where visitors can't access, decreases aggressive and undesirable behaviors.[15]

There are various studies which have identified factors that influence fear and determined what can be done to help manage undesirable behavior in a petting zoo. [15,17,18] Various species of animals react differently to having a high density of visitors in or by their enclosure.[19] These behavioral differences can include becoming less active, having higher stress levels and higher levels of aggression and escape behaviors.[19] None of these factors are beneficial to the welfare of the animal or in the best interest of the zoo. If precautionary measures are taken to help reduce the impact of potentially stressful environmental factors, petting zoos can be a good environment for more social farm animals, such as some species of goats and sheep.

Overall, the main goal of creating positive interactions between visitors and animals is to avoid causing the animal to feel threatened and fearful. When animals feel threated, it is instinctual for them to believe they are in danger and act accordingly by possibly engaging in aggressive behaviors.[15] Completing studies to identify what influences fear are helpful in improving conditions to make animals within these types of exhibits more comfortable. As previously mentioned, factors that influence fear are genetics, habitat design, visitor density and each animal's desired critical distance are all factors that can be considered when increasing the animals comfort level.

Animal Welfare Within Zoos

Working to increase the comfort of animals in zoos and decrease any possibly stressful events promotes values defined within animal welfare. Animal welfare is defined as the "conscious experiences of individual animals, what they feel, and their physical health."[20] This includes the animal's ability to cope with challenges, such as being exposed to a new environment, diet, or new members of the same species. Their care routine and exhibit should be designed to promote freedom. This can be greatly influenced by offering the ability to have control over movements within their environment and making decisions.[20] Aspects of control can include the ability to freely hide, forage for food, build nests and other biologically appropriate behaviors. By being able to carry out these activities at their own will, natural instincts are not being suppressed. The inability to perform these tasks could lead to undesirable behavior and, furthermore, physical health aliments.[20]

Suffering and poor welfare can occur when these conditions are not met, which leads to the animal becoming frustrated.[20] This can result in feelings of pain, fear or exhaustion and is why it is important for human caretakers to provide the most appropriate conditions possible for each species. There are a variety of factors that play a role in animal welfare assessments, which include the genetic background of the animals, rearing conditions, exhibit design, husbandry practices, records of key life events, and the characteristics and behavior of the individual animals.[20]

Various methods can be utilized in order to evaluate how these factors are effecting animal welfare. The first and simplest is to conduct observational studies to analyze behavior. There is a multitude of ways in which an animal behavior study can be approached. Some methods are more complex and tedious, which caused the emergence of simplified, quicker approaches. One example would be flagging species specific indicators, which can be a result of stress. Red flag indicators identify that changes needed to be made regarding diet, enrichment, enclosure design and materials or social interactions to reduce causes of stress.[20] For example, broken teeth in polar bears occurs if they are being fed an insufficient diet or are an indication that improper materials are being used for enrichment or within areas of their enclosure. Red flag indicators help provide a standard to quickly and easily assess animal welfare that is species specific. If there appears to be an increased prevalence of a red flag indicator, more in depth studies can be conducted to determine the cause of the problem and methods to fix it can then be evaluated.

Behavioral studies are simple, but can be time consuming, tedious and provide varying results. More reliable results are produced when behavioral studies are completed in combination with methods that provide more quantitative results, such as an analysis of glucocorticoid levels or a measure of heart rate and emotional states.[20] This type of analysis, however, can be time consuming, as well as complex, due to the validation required to individualize the tests to each species.[20] These methods can also be expensive, so it is difficult for zoos who may be struggling financially to gain enough revenue to care for the animals needs and facilities. In addition to these factors, there tends to be a lack of qualified. There is a high level of experience required to properly design and execute these types of.[20]

Despite these factors, there is currently ongoing research to determine how this can be improved. For example, if zoos and aquariums were to collaborate with schools to recruit students from animal welfare programs or PhD programs, there would be an increased number of experienced personnel available to do this type of work.[20] A partnership such as this can benefit both parties since this will provide students with a hands-on opportunity to complete research as well as provide them with a true perspective of how zoos are managed. Oftentimes, students have a skewed perspective of zoos and aquariums, but this opportunity would aid in the refutation of such perspectives.

Evaluating Stress and its Effects Within Zoo Animals

In order to maintain a high standard of animal care and welfare, the levels of stress experienced by each animal should be investigated to ensure they are being provided with the best possible care. Although, this can be difficult because it can be hard to determine what exactly is considered stressful for different species. Stress includes "anything that causes an animal to stray from homeostasis."[17] This is due to the fact that stressful situations can initiate a fight or flight response within animals, which is an innate instinct animals and humans are born for protection.

Stress has the ability to effect many interconnected pathways, making it hard to pinpoint one universal reaction as a result of stress.[3] The reactions to stress differ among and within species.[3] Chronic stress can lead to decreases in immune and reproductive function, which is why it is important for animal caretakers to avoid exposing animals to stressors.[3] Due to this, there is an increased chance of arteriosclerosis, osteoporosis, diabetes, changes in blood pressure, body temperature, heart rate, leukocytosis, metabolism, respiratory rates, sleep patterns, and weight gain or loss.[3] All of these negatively affect quality of life because the animal becomes more susceptible to diseases. Also, if reproductive function is suppressed, any breeding program that includes that animal may fail unexpectedly, leading to the inability to regenerate the animal's population and maintain its gene pool.[3]

There are a variety of causes of stress that most zoos try to avoid, but sometimes they still occur. Due to the nature of the zoo setting, the animals are constantly exposed to people they are unfamiliar with, the visitors.[3] A common cause of stress is imposing unnatural social situations, such as removing an individual from group or placing an individual with a new group abruptly without a proper introduction period.[3] Behavioral studies of golden lion tamarins (*Leontopithecus rosalia*) revealed that they have strict monogamy and are pair bonded with one mate.[10] Introducing a new individual can cause intraspecific competition between females causing injury and death.[10] Other housing limitations, such as a small cage size or isolating a normally social species, and environmental conditions, such as appropriate temperatures, lighting, or noises, can also affect chronic stress levels.[3] It is important to know about a species' natural habitat and the conditions normally encountered in the wild in order to best mimic that environment in captivity. Providing the most realistic conditions possible with respect to the species native environment will allow the animals to engage in behaviors and activities they would instinctively participate in the wild.

All of these factors can be adjusted in an attempt to remove or lessen the effect of the stressor. However, there are unavoidable events that could be stressful. An example would be capturing animals for medical evaluations, relocations, or visits by a veterinarian.[3] Especially within a zoo setting, animals will be moved from their exhibit to an examination area regularly for a physical exam. Routine husbandry procedures, such as feeding, medical procedures, palpation, pregnancy exams and weighing, are also necessary instances where an animal might feel stressed.[3] Even though stressful situations try to be avoided, these are considered necessary because of the great importance regarding the animal's health.

What can be done to evaluate stress levels? Quantitatively analyzing physiological activities controlled by hormones, such as cortisol, within an animal's body in response to both chronic and acute stress allows us to gauge the wellbeing of the animal. A sign of consistent stress can be identified by high cortisol levels, which is a type of glucocorticoid steroid.[15] In response to stressors, the anterior pituitary gland releases adrenocorticotropic hormone (ATCH), which begins a chain of events in various parts of the body, including the stimulation of the production of cortisol in the cortex of the adrenal glands.[21] Cortisol travels through the blood from the adrenal gland to receptors located inside cells throughout the body.[22] Responses to cortisol occur when the hormone binds to its corresponding cytoplasmic receptors, which usually act as transcription factors and have the ability to control the expression of genes. This results in various types of reactions depending on which gene is being acted upon. Increased cortisol levels can promote gluconeogenesis, the formation of glycogen, the degradation of fat and protein, and inhibit the inflammatory response.[23] Cortisol is a beneficial hormone when an individual is placed in a life or death situation but high long-term levels can be harmful.[22]

How exactly can we measure cortisol within an animal? When cortisol is released into the body, it is likely to bind to proteins, such as corticoid binding globulin or albumin.[23] Only about 4% of cortisol is free floating in blood and not bound, so when analyzing cortisol levels, it is best to analyze cortisol plasma level, which is the cortisol attached to the various proteins.[23] The free floating cortisol level in blood will remain the same when plasma levels are actually elevated and therefore will not produce accurate results.[23] There are various bodily fluids that can be used to analyze plasma cortisol levels, such as a blood sample, bile cortisol metabolite levels from stomach acid, fecal cortisol metabolite levels and salivary cortisol.[23]

The methods used to analyze cortisol levels, and therefore stress, can be one of the causative factors of stress within an animal because of the invasive techniques required to collect the sample. Therefore, determining fecal cortisol levels is a way to indirectly analyze an animal's stress level without performing an invasive procedure. Fecal cortisol levels have been shown to be correlated with plasma cortisol levels over time.[4] Some other advantages to using fecal samples as the matrix to analyze cortisol levels are that metabolites can accurately detect stressors over time and the method is noninvasive, which avoids generating stress from collecting a sample[17]

There are some disadvantages to using fecal samples however, an acute stressor may have a delayed increase in cortisol level within a fecal sample as opposed to the instant increase that appears in plasma corticosterone levels.[3] The inability to detect acute stressors may lead one to believe the animal is not under any stress but in reality, this may be untrue.[3]

Another means of analyzing stress would be through behavioral observations. This is done by addressing particular behaviors that have previously been linked to stress. Within sheep, there are certain types of aggressive behaviors that could be a sign of stress. However, the same behaviors could also be expressed in order to establish dominance. Head butting can be a result of a sheep feeling threatened, attempting to establish dominance, or even playing. This would be an example of a behavior classified as aggressive but is not necessarily stress related.[24] Aggressive behaviors are observed naturally, beginning within lambs as play fighting.[25] Considering another scenario, sometimes when there is a shortage in food, aggressive behaviors escalate and the dominance hierarchy becomes more prevalent.[25] Overall, within a heard of sheep, a consistent, unilateral dominance relations between individuals is likely to occur.[24]

Environmental factors can affect any type of animal whether they are captive or wild. For example, the day to day change in weather, including temperature extremes and precipitation, can induce a physiological response to maintaining homeostasis.[26] Joksimovic-Todorovic et al. (2001) assessed the effects of heat stress on dairy production. Heat stressed cows have a much lower milk production and a lower quality of milk.[26] This was due to expending energy in order to maintain their body temperature, and therefore homeostasis, as opposed to using energy for milk production.[26] In addition to this, seasonal variations were observed within Nguni cows with higher cortisol levels occurring within the summer months.[27] It is also possible that genetic traits are linked to cortisol levels. Nguni cows with darker coat color had higher cortisol levels.[27] This could possibly be due to the fact that darker coat colors retain more heat and could attract more parasites, such as ticks.

Animal Perception on Human Interaction

Animals in a zoo are constantly exposed to unfamiliar people, visitors, but cared for by a small group of people they see frequently. Animals do have the ability to distinguish between people, and therefore different keepers.[24] In response to this, they may act differently around certain individuals.[23] Keepers spending time with animals for training can help improve the animal's welfare because these interactions between the animal and keepers can lead to a positive feeling towards all visitors.[24] This is due to the fact that animals can make the association between humans and a positive interaction.[24] However, if the number of keepers taking care of one animal is too high, chances are, each keeper spends less time with the animal, which leads to less time to create a positive human-animal interaction. This is supported by Hosey et al. (2014) that found that clouded leopards had higher cortisol levels when exposed to a greater number of keepers.[24]

Zoos depend on visitor support, but how does this constantly changing demographic effect the animals? The Visitor Effect is defined as "the influences of visitors on the behavior of zoo animals which includes visitor crowd size, volume of sound made, proximity of animal to the visitors, gender of visitor and level of interaction with the animal.[29] Considering this aspect of a zoo is constantly changing, it can be difficult to determine an exact number of visitors around an animal's exhibit, how the animal is behaving at that same time and if these two factors are related.[29] Complex correlation methods are often used to help evaluate this to determine if the Visitor Effect truly plays a role on the animal's behavior.[29]

One of the first studies completed of this nature reported that fluctuating large numbers of visitors affected the behaviors of primates.[30] This was determined through observing and recording four behavioral factors: number of interactions with the audience, number of interactions with cage-mates, levels of locomotory activity, and spatial dispersion in the cage.[31] A more recent study conducted with western lowland gorillas reported to see an overall more negative rather than positive response to large crowds.[29] The presence of a large crowd can lead to a decrease of species specific behaviors and increase in aggressive behaviors, abnormal behaviors and cortisol levels.[30] However, Stoinski et al. (2012) produced contradictory results when the majority of behaviors within studied gorillas did not change with varying crowd size.[30]

The Visitor Effect makes sense in theory, but it can be very difficult to statistically support. The ability to identify the influence of visitors' effect on each animal's behavior differs by species and would require a lot of time and dedication to create a feasible experiment to assess this, due to all the varying factors.[30] For example, if it was believed that large cats spend less time resting and paced more when visitors were present, it would be difficult to show that the visitors were the result of the animal's increased frequency of pacing. There is a possibility that the visitors were more interested in the cat when it was pacing so therefore, more visitors were present when the cat was displaying this behavior. Consequently, it is difficult to determine if the visitors influenced the cat's behavior or if the cat's behavior influenced the visitor reactions.

Another hypothesis, referred to as the Visitor Attraction hypothesis, addresses visitor's responses to an animal's actions. It predicts that active animals attract more attention from visitors.[29] Therefore, animals might be persuaded to become more active when more people are around in order to gain attention. Also, the way animals perceive people can also affect their cortisol levels based on how comfortable they feel. For example, animals can view humans as caretakers, predators, competition or prey which can affect their behaviors towards them.[30] Due to these differing reactions based on how people are viewed, the animal's response can make the stimulus of visitors as stressful, neutral or enriching.[28]

This leads to debate of whether crowd size and the amount of visitors present truly effects animal behavior. Aggressive and undesirable behavior change was not noted to significantly occur consistently and universally for a group of gorillas.[30] These behaviors were also not significantly correlated to visitor density.[30] It appeared that each gorilla was influenced differently by visitor density, which shows that reactions to this type of stimulus are specific to the individual. Males had a higher correlation to aggressive behavior than females in response to a large crowd, even though neither were significant.[30] Overall, there was no significant change in agression due to a large crowd within gorillas of different ages.[30] Considering all of this and how reactions of animals differ per individual within the same species, general claims speaking for the whole of the species, or even population, can not be made with respect to crowd density.

In analyzing large crowd size does have an effect on behavior, the way the animals can view visitors based on their enclosure design should be taken into account. The animals' experience of sound volume, and visitory activity can be effected by the type of enclosure each species has.[28] Choo et al. (2011) analyzed the behavior of orangutans who reside in an interactive treetop, 'free-ranging' exhibit in the presence and absence of large crowds.[28] This exhibit design allows visitors to easily view the orangutans and have the ability to get fairly close to them. There was little effect of visitor crowd size on orangutan behavior.[28] This could possibly be due to the fact that these orangutans are habituated to higher visitor density because of the location of enclosure.[28] However, visitors within close proximity, less than ten meters, caused a decrease in playing and socializing amoung the orangutans.[28] These results suggest that the orangutans are normally unaffected by the visitor density but when visitors become too close, it causes them to act more cautious.

Allowing animals in exhibits to see and interact with visitors doesn't always cause negative effects.[29] Some species, such as many primates, are very social and visitors serve as a form of enrichment due to the constant variability.[29] However, testing on this theory has not been done as extensively as other enrichment methods. Most behavior studies conducted in attempts to correlate visitor density and crowd size to animal behavior have been with primates, such as in Stoinski (2012), Choo (2011) and Hosey (1987), so there is no way to tell if other species will react similarly.

Overall, zoos face the struggle of juggling multiple priorities in order to remain a reputable center for offering education for the public about exotic animals, promoting conservation efforts, and encouraging beneficial research all the while maintaining the health and wellness of the animals residing there. Studies within animal welfare are constantly improving and utilizing new methods to evaluate their quality of life. Through these evaluations, improvements within enclosure design, care routines and enrichment materials can be made to provide the best quality of life for all species residing within the zoo.

Experimental Introduction

Petting zoos can be an excellent educational opportunity to introduce the public to different animals, aspects of conservation efforts, and to create a positive interactive experience between visitors and the animals.[19] Although, there must be a balance between providing an environment where visitors can come into contact with the animals and the animals' comfort level within their enclosure. Especially in a petting zoo, the animals can be easily stressed out from their constant interaction with a variety of visitors, particularly if they don't have a place to escape to when frightened.[15] Anderson et al. (2002) concluded that high volumes of visitors can possibly stress out animals in petting zoos but, stressful environment can be minimized by allowing the animals room for retreat space, to distance themselves from the visitors.[15] Extra space given to the animals, where visitors can't access, decreases aggressive and undesirable behaviors.[15] Training to help acclimate the animals to the environment of a petting zoo to become more used to interacting with visitors has also been proven to be beneficial.[19] Showing less of these behaviors benefits the zoo, the visitors' experience and, most importantly, the animals' comfort level.

Sheep are prey animals from the *Bovidae* family.[15] They tend to engage in behaviors that correspond with their natural ability to avoid predators.[15] Due to this, it is common for sheep to quickly run away or charge when threatened.[19] Especially in a petting zoo setting, this behavior is not desirable. When considering the stressors placed on a sheep in a petting zoo, such as loud noises or quick movements of surrounding visitors, these behaviors occur frequently due to the fearfulness of the sheep. Zoo visitors have been identified as a possible stressor for the animals.[32] Although, it is hard to quantify the overall impact this stress has on everyday life and behaviors. More research on this topic can improve animal welfare along with creating more positive interactions between the animals on zoo visitors.

Species of animals react differently to having a high density of visitors in or by their enclosure.[19] These behavioral differences can include becoming less active, having higher stress levels, and higher levels of aggression and escape behaviors.[19] These factors are not beneficial to the welfare of the animal or in the best interest of the zoo. If precautionary measures are taken to help reduce the impact of potentially stressful environmental factors, petting zoos can be a good environment for more social farm animals, such as some species of goats and sheep.

Another factor that can cause stress is weather extremes, particularly long periods of very hot temperatures or exposure to direct sunlight.[33] Exposure to warm weather in the summer months can make maintaining internal body temperature more energetically intensive, especially for ruminants, and therefore possibly stressful.[34] Although, a chronic exposure to these high temperatures, or other altered environmental factors, can lead to acclimation, in which the animal alters their physiology to be more apt to living within the new environmental conditions.[34]

Stress can be quantitatively analyzed through indirect physiological responses. Cortisol levels extracted from feces have been shown to correlate to an animals current physiological state and provide an easy, non-invasive way to determine the stress levels of the animal.[35] Cortisol is a beneficial hormone when an individual is placed in a life or death situation but long-term high levels can be harmful.[22] In humans, prolonged stress leads to physical ailments, such as weight gain, lethargy, sleeplessness and a compromised immune system, that can be induced by the continuous supply of cortisol in the body. Animals also experience physical ailments when placed in high stress situations, such as in traveling zoos and petting zoos.[15] This is why it is so important to ensure measures are taken to keep animals in a low stress environment.

This study seeks to determine if there is a relationship between aggressive behaviors and cortisol levels within sheep. In addition, the effects of the environmental factors the sheep experience, such as the weather and visitor attendance, could affect the observed cortisol levels and behaviors of sheep who reside within a zoo. In order to view one at a time, experiments must be done to isolate a single factor. This goes beyond the scope of this research, which is based on observation of animal's natural behaviors within their habitat. This qualitatively and quantitively assessed animal behavior and stress levels within the petting zoo in the summer months compared to the winter months through observational study and sample analysis. I would expect that high visitor attendance and temperatures during the summer is related to higher levels of cortisol. Higher cortisol levels evidentially lead to a more stressed sheep and therefore possibly causing the sheep to exhibit higher instances of aggressive behavior.

Methods

Study Subjects

Babydoll Southdown sheep (*Ovis aries aries southdown*) who reside within the Heritage Farm Petting Zoo at the Buffalo Zoo were the subjects of this study. This group consisted of one female, Dolly, and three males, Charlie, Harrison and Toby. All of the sheep are eight years old and were born during the same spring. Their diet consists of four bales of timothy hay throughout the day, unlimited straw and one cup of grain split between the sheep daily. In the summer, all of the sheep were slightly overweight, therefore a new exercise regimen was instituted where they were taken on two walks every other day. Harrison began to have a limp, so he was exempt from walks. In the winter, Toby also developed a limp due to old age and arthritis. Due to this, the exercise was reduced to one walk twice a week. Both Toby and Harrison were on anti-inflammatory medication during the winter months to alleviate discomfort due to their arthritis.

During the summer, the sheep were alternated between spending the day in their "contact pen" where they can be pet by visitors and in their indoor pen, where they cannot be touched by visitors. Care was taken to ensure samples were collected equally between days the sheep had contact with visitors and the days they did not to avoid error. Occasionally this schedule varied due to the sheep's behavior; if they appeared to be tired, stressed or uncomfortable due to arthritis, they were kept inside even if scheduled to be in their petting pen. Occasionally, about once every two weeks, the sheep would be put into the pasture where they have a much larger area to reside in and can graze freely, although this depended on the weather, the sheep's schedule, and the other animals whom they shared the pasture.

During the winter, the sheep were mainly in their heated, indoor pen. They were offered access to their contact pen every third day but also had the option to come inside. The sheep did not have access to the pasture in the winter because the grass was normally covered by snow. Every night, during both the summer and winter, the sheep are brought inside and locked in the barn.

Observational Data Collection

Observations were completed a day prior to fecal sample collections. A total of 42 hours of observational data was collected. Observations consisted of 15-minute focal sampling for each of the four sheep via Animal Behavior Pro software on an iPad[36]. All behaviors listed within the ethogram (Table 1) are mutually exclusive, except for when the sheep are being pet by visitors, in order to create an accurate activity budget. Data were collected for two-hour intervals per day. Observations were completed over two seasons, the summer, from August 4th to August 14th, 2018 and the winter, from December 20th, 2018 to January 6th, 2019. An average activity budget was made for each season, and a Friedman test was used to determine if there was a significant difference in the behaviors exhibited during each season. This test was chosen due to the small sample size making the data nonparametric. A Mann-Whitney U test was used to determine if there was a significant relationship between instances of aggressive behaviors and cortisol levels. All statistical tests were completed via Minitab.

Fecal Samples

Collection

Collection of fecal samples occurred between 8:00 AM and 10:00 AM before visitors arrived by zookeepers, labeled and placed into a freezer at -20°C. The Samples were then transferred to Dr. Susan Margulis's lab at Canisius College.

Extraction

All samples were defrosted and mixed prior to weighing. A 0.5 (+/- 0.05) gram portion of each sample was combined with 80% ethanol solution. Each sample was then vortexed and placed horizontally onto racks on a rotator. Samples were left to mix for 16 hours overnight. After mixing, each tube was centrifuged for 15 minutes at 15,000 RPM. A 1mL portion of the supernatant from each sample was removed and combined with 1 mL of cortisol assay buffer, creating a 1:10 dilution. The samples were temporarily stored frozen until analysis. Exact extraction protocol is listed in Appendix A.

Validation (Appendix C)

Parallelism/Linearity

A random selection of samples, called a pooled sample, was created solely for validation purposes. The sample pool was created by mixing 100 µl of 22 of the 41 samples, ensuring samples from each sheep were present. The pooled sample was used to create a serial two-fold dilution in assay buffer. Dilutions ranged from 1:20 to 1:5120 producing a total of 10 samples within the serial dilution. A graph was created of the percent binding of the standards to the antibody against cortisol present in the sample. Parallelism was ensured between the samples and the standards by receiving R-value close to one. The dilution at which the samples would be run was determined through observing where the curve crosses the 50% binding. This processed determined the samples would each be run at a 1:20 dilution.

All the samples were initially analyzed at a dilution of 1:20. However, four samples had an unusually high cortisol level, so they were analyzed again at a 1:50 dilution to achieve more accurate results. My results ran parallel to the standard (R^2 = 0.9695, Figure 1).

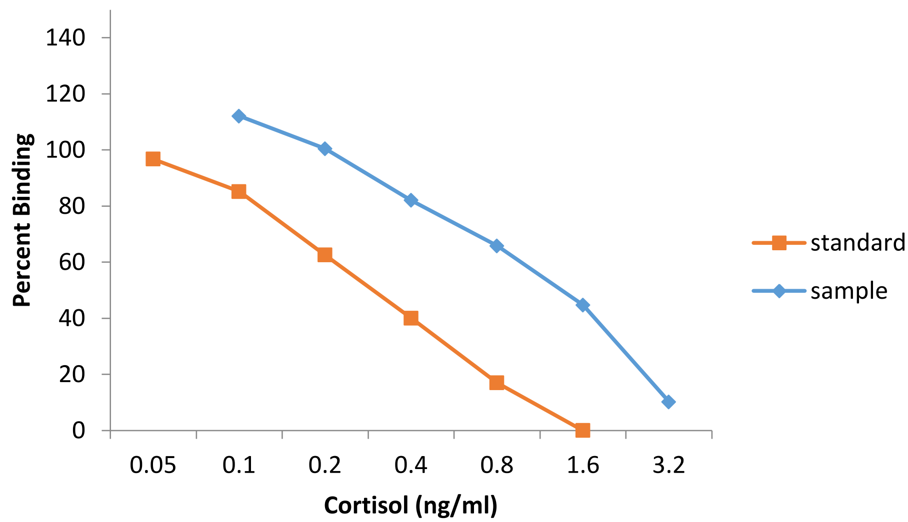


Figure 1: Test for linearity by analyzing the percent binding of the assay against a pooled sample containing know cortisol standards (R^2 = 0.9695, y = -19.797x + 158.3).

Recovery

A sample was selected at random to complete the recovery validation. A solution (800µL) of this sample was made at a 1:20 dilution and 100µL was distributed between six test tubes. A set of standards containing known cortisol concentrations ranging from 3,200 pg/mL to 50 pg/mL was created via a serial dilution. Sample (100µL) was then added to each standard (100µL) and vortexed. The samples were assayed, and their values were used to determine the percentage of cortisol recovery and whether there was interference in binding in the assay due to the sample matrix.

It was determined that there was an average recovery rate of 112%. Having a value over 100% of this can be due to cross-reactivity to other hormones within the matrix of the sample (Appendix D). These hormones can include dexamethasone (18.8%), prednisolone (1-dehydrocortisol) (7.8%), and corticosterone (1.2%). Regardless, the same error occurs within each sample, which eliminates the variation due to this.

Intra- and Inter-Assay Variability

To test intra-assay variability, the same sample was analyzed six times on the same plate. Differences across wells of a plate can be due to plate reader or plate washer errors. It was determined that these values had a coefficient of variance of 8.95%. Arbor Assays reports that a value below 15% is acceptable and means there is little variation among samples ran within the plate. Inter assay variability was also tested to determine variability between plates. Samples were chosen at random and run again on a separate plate to do this. It was determined that these values had a coefficient of variance of 4.25%. Arbor Assays reports that a value below 10% is acceptable and means there is little variation among samples ran within different plates. However, to avoid inter-assay variability within this study, all the samples were analyzed on the same plate.

Data Analysis

The strip-well plates were run in accordance with the procedures listed in Arbor Assay's DetectX® Cortisol Enzyme Immunoassay Kit (Appendix B). Slight variations were made to the sample preparation. The wet weight was used for sample analysis, as the samples were not dried in a SpeedVac or under nitrogen because there was not access to the instruments and materials needed to complete this.

For each day, the four sheeps' cortisol levels were averaged together. These values where used in the comparison between cortisol levels and environmental factors. These values for each day were than averaged again to produce one average value for the summer and winter season. A series of regressions was then used to determine if there is a significant relationship between fecal cortisol levels and visitor attendance, instances of aggressive behavior or the weather. Fecal cortisol levels represent the average cortisol levels within the sheep 12-24 hours before the sample was collected.[37,38] To account for this, when comparing any factor to cortisol level, the data from the day before was paired with the fecal sample. This ensures the cortisol level that is recorded matches the environmental conditions that may have influenced it.

Table 1: Sheep ethogram based from Anderson et al (2002).[14]

BEHAVIOR		DEFINITION
GENERAL	Walking	Lifting at least two legs simultaneously and moving the body in any direction
	Pushing	Pressing the front or side of the head or with the shoulder on the other lamb
	Locomotive	Making a jump so that no body part has contact with the ground or performing a quick run with no apparent destination
	Resting	Standing Still- Fully upright position, not moving its body in any direction while observing its environment or other lamb and elimination or urination may be performed Lying- Body weight supported by belly, and legs folded under the body
	Engaging with Enrichment	Interacting with toy object by rubbing or pushing the object with paw or head
	Eating	Head held in or above food trough. Also includes switching side of food through to continue feeding
	Scratching	Rubbing part of body against pen wall or with leg or mouth
	Social	Keeping nose in contact with or within three centimeters of any part of other lamb
	Vocalizing	Making any noise
	Other	Participating in a behavior not listed
	Out of Sight	Subject is not currently visible
UNDESIRABLE BEHAVIORS	Head Butting	Hitting head against the other lamb's head, taking a stance in the direction toward the other lamb, often with ears folded back, resulting in either a butt or not
	Head Tossing	Moving head quickly from side to side for no apparent reason
	Foot Stomping	Lifting foot and harshly stomping down, without the intention of movement
	Nose-Blowing	Loudly exhaling air from nose
	Leaving	Turning and moving in opposite direction of intended target of interaction

Results

Aggressive Behaviors

There is not a significant difference between the activity budgets observed in the summer and winter (χ^2= 3.00, P=0.083). The most common behavior is resting (61% in the winter and 49% in the summer) and aggressive behaviors account for less than 1% of the total activity budget for both seasons. Other behaviors include defecating, engaging with enrichment, pushing, foot stomping, head butting, head tossing, and scratching. These behaviors occurred so infrequently compared to resting, eating or drinking and walking, they were grouped together (Figure 2 and Figure 3).

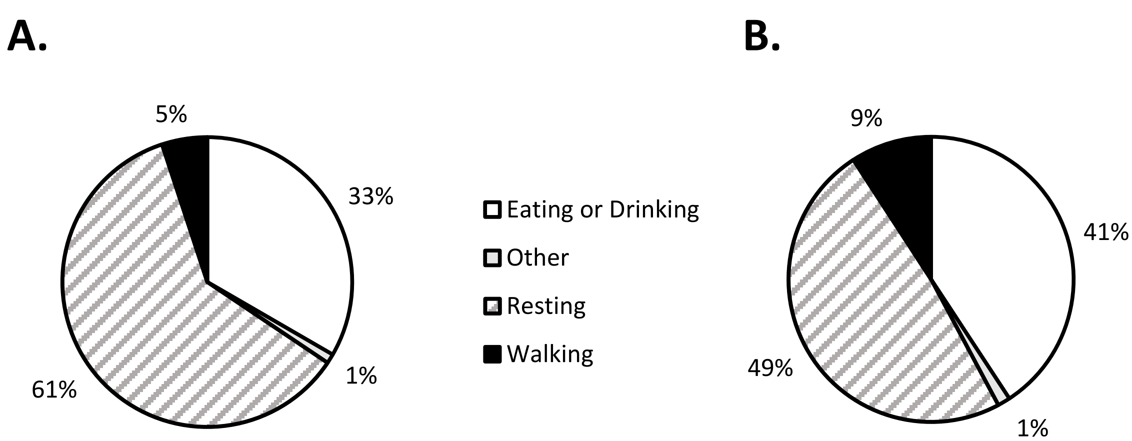


Figure 2: Average activity budget for the sheep during the winter (A) and summer (B).

Instances of aggressive behaviors, including head butting, pushing, head tossing, foot stomping, or nose blowing, were recorded to assess stress. During the summer, there was an average occurrence of 1.20 ±0.79 aggressive behaviors shown per hour. Within the winter, there were 0.29 ±0.14 aggressive behaviors exhibited per hour on average. There is not a significant difference in instances of aggressive behaviors between seasons (W=35, P=0.729).

Occurrences of aggressive behaviors, with respect to cortisol levels, are not related (R^2=0.25, P=0.305; Figure 3).

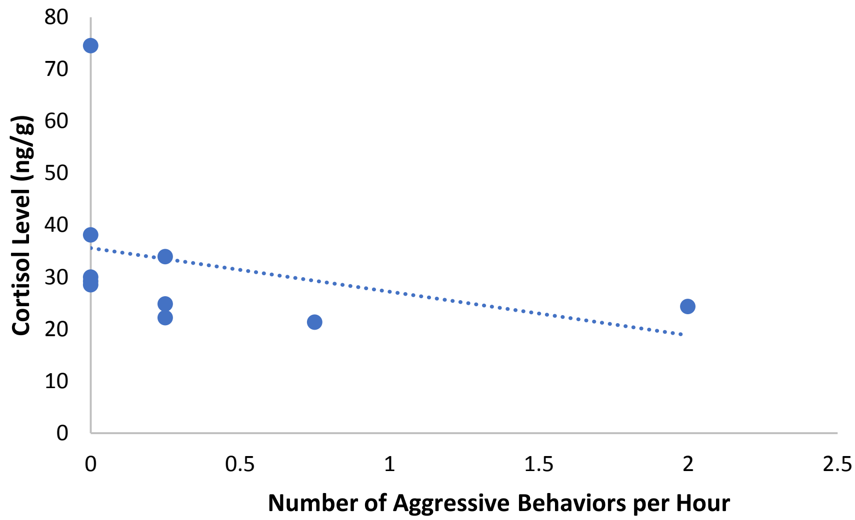


Figure 3: Average cortisol levels per day compared to instances of aggressive behaviors per hour.

Cortisol Levels

In general, higher cortisol values are seen in the winter than the summer. It was determined that the summer average cortisol level was 25.91 ±5.16 ng/g (Figure 4) while the winter average cortisol level was 42.82 ±20.16 ng/g (Figure 5). However, there is no significant difference between average cortisol levels between seasons (W=24.0, P=0.07). It must be noted that on December 23rd, the sheep experienced unusually high cortisol levels. This is believed to be a result of a recent veterinary visit for an annual check-up. However, the data point is not more than two standard deviations away from the mean, so it is not statistically shown to be an outlier. When this data point is omitted from the data set of the winter time frame, the average cortisol level observed becomes 32.27 ±7.17 ng/g.

Individual differences in cortisol level can be viewed in Figure 4 and 5. During the summer, Toby's cortisol levels are generally higher from day to day compared to the other sheep with an average of 29.44 ng/g. Dolly's fecal cortisol levels were the lowest out of the sheep with an average of 22.76 ng/g. However, all the sheeps' fecal cortisol levels are more variable in the winter and one is not consistently higher or lower than the rest from day to day. Overall during the winter, Dolly had the highest average cortisol levels (44.75 ng/g) and Charlie had the lowest average cortisol levels (41.34 ng/g).

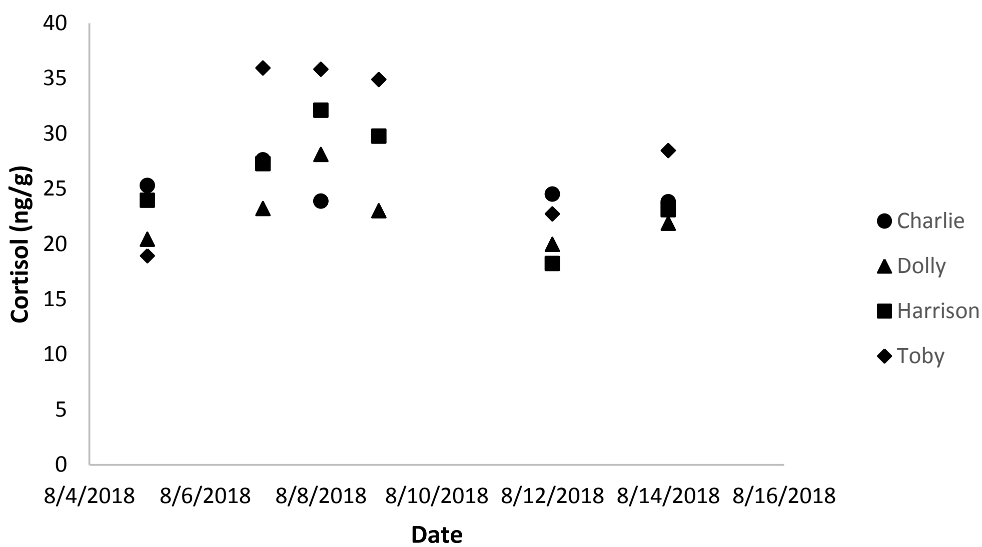


Figure 4: Each Sheep's cortisol levels within the summer time frame (August 5th-August 14th).

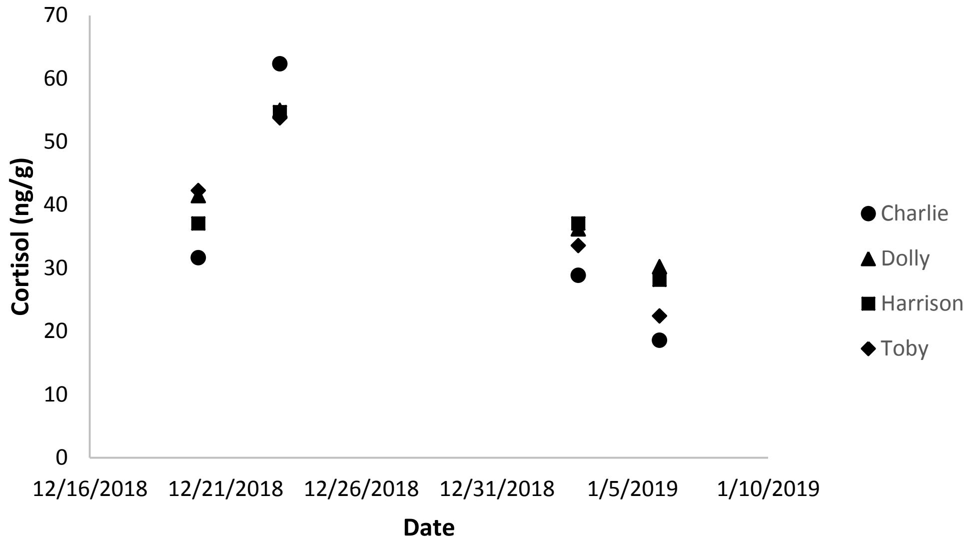


Figure 5: Each Sheep's cortisol levels within the winter time frame (December 20th-Janurary 6th).

Environmental Impacts

Guest Attendance

The sheep's cortisol levels were compared to visitor attendance records to see if increased visitors caused stress. The average number of guests during the summer was 2,280 people per day while during the winter timeframe was 362.7 people per day. There is a significant, negative relationship between visitor attendance records and average cortisol levels (R^2=0.79, P=0.01; Figure 6). It must be noted that general attendance records were used for this analysis and not necessarily everyone who visits the zoo will go into the petting zoo area by the sheep.

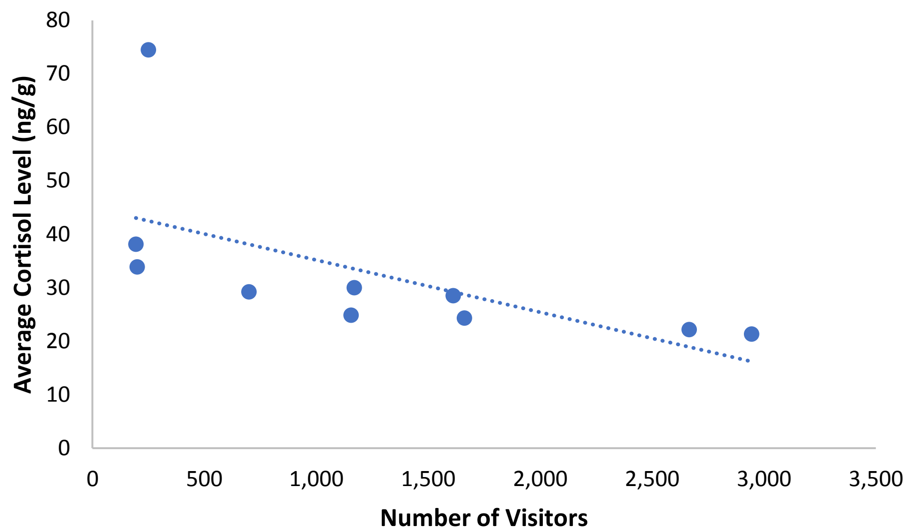


Figure 6: Average cortisol levels per day compared to visitor attendance records per day.

Weather

The sheep's cortisol levels where then compared to temperature recorded at time of observation or sample collection to see if there is a relationship between temperature and stress (Figure 7). The average temperature during the summer timeframe was 83.4°F while average temperature during the winter timeframe was 32.8°F. There is no significant relationship between temperature and cortisol levels (R^2=0.18, P=0.225). It must be noted that during the winter time frame, the sheep were normally inside the barn, which is heated. Therefore, they were not always exposed to the cold temperatures for the whole duration of the day.

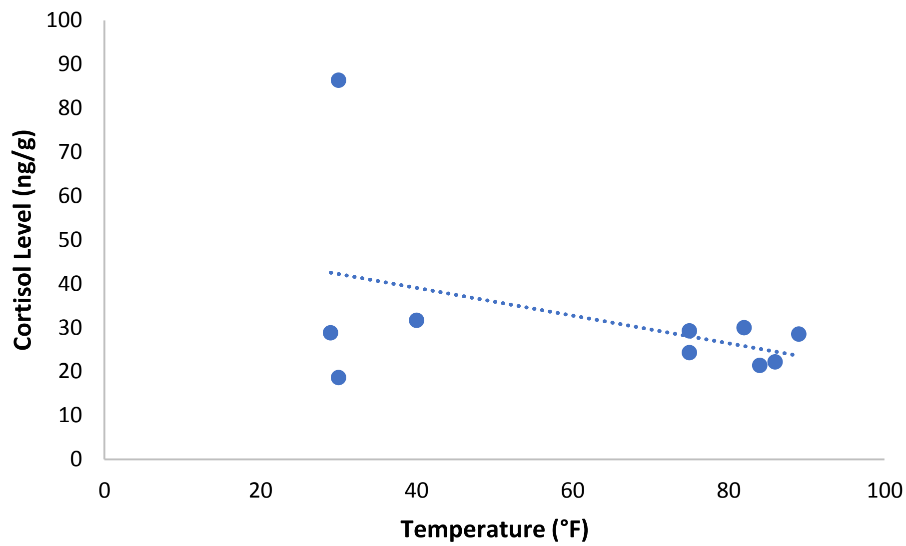


Figure 7: Average cortisol levels per day compared to the temperature per day.

Discussion

Stress was analyzed in sheep through a combination of observations of aggressive behaviors and fecal cortisol levels. These factors were analyzed to determine their effects on each other, as well as the effect of two environmental conditions on fecal cortisol levels. It was expected that higher cortisol levels would correlate positively with higher instances of aggressive behaviors, higher temperatures and higher visitor attendance records. The results produced in this study supported some of these hypotheses, but not all.

Samples were collected from each individual to ensure consistency throughout the duration of the study. Individuals will have naturally varying cortisol levels due to a slightly different physiological makeup (Figures 4 and 5), so it wouldn't be ideal to average cortisol levels of different individuals together. This is why it was vital to collect samples from each individual for each collection day. Averaging values together for each day will contain the same amount of variability that results from different individuals, eliminating this source of error.

Stoinski et al. (2012) reported higher cortisol levels in male gorillas than female gorillas. My results supported this trend. The average cortisol level of the male sheep was 34.45 ng/g. While Dolly's average cortisol level was 33.75 ng/g. However, there is only one female in the sample size so this must be taken into consideration when viewing these results. Harrison also was placed on antiinflammitory medication during the summer and continued to be treated with it through to help with a limp he developed and arthritus. Toby was then also placed on the medication during the winter for the same reasons. However, these medications did not appear to have drastic effects on their cortisol levels since the levels still varied from day to day.

Since the occurrences of aggressive behaviors did not have a significant relationship with cortisol levels, it is suggested that all of these behaviors occurred normally as part of the sheep's natural behaviors of repertoire. The aggressive behavior that was observed most often within the summer was foot stomping and in the winter was headbutting. However, with regards to the increased amount of foot stomping during the summer, it often occurred when the sheep were in the pasture, so there were more bugs around their feet and legs than there normally would be if they were in the barn. The unusual amounts of foot stomping could be attributed to the sheep's attempts to removed bugs who had landed on the feet or leg area. This would also result in these aggressive behaviors occurring naturally, or other reasons than as a result of stress.

It was expected that higher fecal cortisol levels would have been observed during the summer months, when there are higher temperatures and higher number of visitors within the zoo. However, this trend was not seen, as the sheep had higher cortisol levels in the winter when there are lower temperatures and lower numbers of visitors at the zoo. This could have occurred naturally due to regular seasonal physiological changes from winter stress and metabolic demands. Similar results were found within Pyrenean chamois, a mountain ruminate, that higher average fecal cortisol levels were observed in the winter (3,000ng/g) compared to the summer (500 ng/g) due to seasonal endogenous adaptation.[39] The cortisol levels observed from sheep were not nearly this high in either season, but a similar trend was observed.

With respect to the relationship between cortisol levels and visitor attendance records, it was expected that there would be a positive relationship between the two, but this did not hold true. A strong negative relationship was observed between visitor attendance records and sheep cortisol levels (Figure 6). Sheep had lower cortisol levels when there was a greater amount of visitors present. Over the summer, there is drastically more people the sheep are exposed to each day, but their cortisol levels remain consistent, if not lower. This is due to acclimation of the sheep to their changing surroundings. They become used to the larger influx of people over time, so it is no longer considered a stressor for them during this time period. However, the same is true for when there are few visitors. The sheep become used to having little interaction with visitors, and this becomes their new normal. When this changes suddenly without an acclimation period, there can be a sudden increase in cortisol levels due to stress.

On December 23rd, the sheep experienced extremely high cortisol levels. Speaking with the head keeper, it was determined that this can be attributed to a combination of two factors. The first being the sudden influx of visitors due to the holiday seasons and the timing of the sheep's annual veterinary checkup. The second, and more likely, cause is the veterinarian visit on December 20th, where the sheep received a normal physical exam including eye, nose and mouth exams. It has been noted that a drastic change in environmental conditions can induce stress within animals.

Temperature does not seem to be a significant factor on cortisol levels in sheep (Figure 7). The higher cortisol levels observed during the winter time period could also be attributed to physiologically adjustments; however, the current results showed the sheep are not significantly more stressed due to lower temperatures. Guerrini et al. (1982) conducted a similar study aimed to determine the effect of temperature and humidity on sheep plasma cortisol levels.[40] The highest cortisol levels within sheep were recorded during cold-humid conditions (43.64 ± 9.86 nmol/l) and the lowest in hot-humid conditions (19.03 ± 6.23 nmol/l).[40] The current results follow a similar trend, with highest average fecal cortisol levels being observed in the winter.

Through an analysis of behavior and fecal cortisol levels, it was determined that any instances of aggressive behaviors exhibited by the sheep are not due to high stress levels. Temperature was not a significant factor in influencing fecal cortisol levels whereas visitor attendance did significantly, positivity influence fecal cortisol levels. Further analysis would benefit from a yearlong longitudinal study to observe continuous trends between visitor attendance and cortisol levels. In doing this, the gradual transition of acclimation to visitor fluctuations could be better monitored. This would help certify whether acclimation to the visitor attendance is truly the cause of the observed trends in cortisol levels.

Acknowledgments

Thank you to Dr. Zimbler-DeLorenzo, for your help and guidance that allowed me to complete this research. I would also like to thank Dr. Garrett McGowan and Dr. Geoff Lippa for being a part my committee. I am very grateful for the generosity of Dr. Margulis at Canisius College, for helping me analyze fecal samples and allowing me to use her lab space. Thank you to Ellen Pritschau, the head keeper of the Heritage Farm Children's Zoo at the Buffalo Zoo, for allowing me to observe the sheep and aiding with fecal sample collection. I would also like to express my thanks to my sister, Rebecca Pera, for taking the time to proofread the majority of this paper. Lastly, thank you to the APEX program for funding this research!

Appendix A-Acquired from Dr. Susan Margulis

Extraction Protocol

1. Be sure you have enough 80% ethanol and assay buffer available for the number of extractions you will be doing (5ml ethanol per sample; 1ml assay buffer per sample)
2. Sort samples chronologically, starting with the oldest, and record dates on an extraction sheet.
3. Label 16x125mm polypropylene tubes with extraction #'s, always starting at 1.
4. If the project is ongoing, check the sample record book for sample numbers, otherwise start at #1.
5. Prepare a set of labels with animal name, sample # and date, and put on 12x75 polypropylene tubes.
6. Measure 0.5g (+/- 0.05) of mixed, defrosted fecal sample into the corresponding extraction (16x125mm) tube, being careful to remove any litter or debris.
7. Record exact weight on fecal extraction sheet, and record any comments (unusually wet or dry, hairy, etc.).
8. Fill the labeled 12x75mm polypropylene tube with leftover sample and freeze for future use, if required.
9. Continue until all samples in the set are weighed out.
10. Add 5 ml of 80% EtOH in dH20 to the weighed fecal samples and cap tubes quickly.
11. Vortex all tubes well, making sure samples are broken up.
12. Place racked tubes in baggies (to hold tubes in rack) in a horizontal position on a rotator and mix well for 14-18 hours (overnight).
13. Centrifuge tubes for 15 minutes at 1500 rpm. The centrifuge is on the 3rd floor, room 302, door code 9998#. If there aren't enough racks for the centrifuge, you can borrow some from the common equipment room (room 310, door code 1355#); return the racks when you are done.
14. Pipette 1 mL assay buffer into labeled 12x75mm polypropylene test tubes, add 1 mL, of supernatant from each corresponding tube, cap tightly, mix and store frozen. Dilution tube labels should include animal ID (name), sample #, date and 1:2.
15. Fecals and dilutions are stored in gridded boxes. Box labels should include species, animal ID (name or #) and sex (0.1 or 1.0), fecals or 1:2's, zoo, start and finish sample # and dates.

Appendix B

ASSAY PROTOCOL

We recommend that all standards and samples be run in duplicate to allow the end user to accurately determine cortisol concentrations.

1. Use the plate layout sheet on the back page to aid in proper sample and standard identification.

2. If you are using the 1 by 8 well strip plate version of the kit, K003-H1 or -H5, determine the number of wells to be used and return unused wells to foil pouch with desiccant. Seal the ziploc plate bag and store at 4˚C.

   Pipet standards or samples down the plate strip columns (A to H) to ensure maximum use of the strip wells.

   The use of any wells in the whole plate versions of the kit, K003-H1W and K003-H5W will not allow use of unused parts of that plate in a later assay.

3. Pipet 50 µL of samples or standards into wells in the plate.

4. Pipet 75 µL of Assay Buffer into the non-specific binding (NSB) wells.

5. Pipet 50 µL of Assay Buffer into into the maximum binding (B0 or Zero standard) wells.

6. Add 25 µL of the DetectX® Cortisol Conjugate to each well using a repeater pipet.

7. Add 25 µL of the DetectX® Cortisol Antibody to each well, **except the NSB wells**, using a repeater pipet.

8. Gently tap the sides of the plate to ensure adequate mixing of the reagents. Cover the plate with the plate sealer and shake at room temperature for 1 hour.

9. Aspirate the plate and wash each well 4 times with 300 µL wash buffer. Tap the plate dry on clean absorbent towels.

10. Add 100 µL of the TMB Substrate to each well, using a repeater pipet.

11. Incubate the plate at room temperature for 30 minutes without shaking.

12. Add 50 µL of the Stop Solution to each well, using a repeater pipet.

13. Read the optical density generated from each well in a plate reader capable of reading at 450 nm.

14. Use the plate reader's built-in 4PLC software capabilities to calculate cortisol concentration for each sample.

Appendix C

NOTE: If you are using only part of a strip well plate, at the end of the assay throw away the used wells and retain the plate frame for use with the remaining unused wells.

CALCULATION OF RESULTS

Average the duplicate OD readings for each standard and sample. Create a standard curve by reducing the data using the 4PLC fitting routine on the plate reader, after subtracting the mean OD's for the NSB. The sample concentrations obtained, calculated from the %B/B0 curve, should be multiplied by the dilution factor to obtain neat sample values.

Or use the online tool from MyAssays to calculate the data:

MyAssays

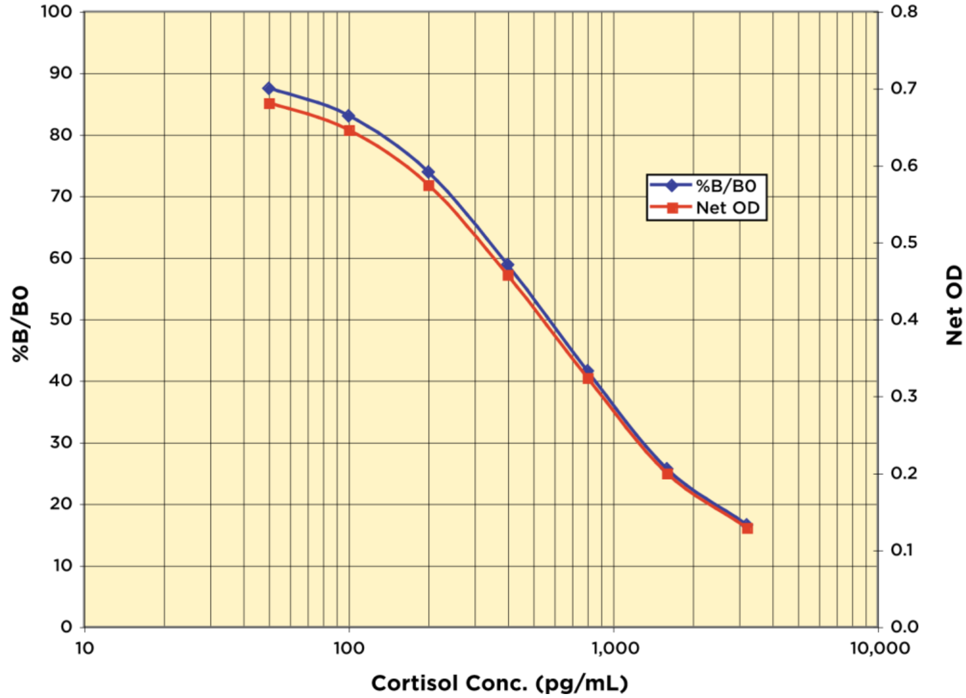
www.myassays.com/arbor-assays-detectx-cortisol-(extended-range).assay

TYPICAL DATA

Sample	Mean OD	Net OD	% B/B0	Cortisol Conc. (pg/mL)
NSB	0.080	0	-	-
Standard 1	0.209	0.129	16.58	3,200
Standard 2	0.280	0.200	25.71	1,600
Standard 3	0.404	0.324	41.65	800
Standard 4	0.538	0.458	58.87	400
Standard 5	0.655	0.575	73.91	200
Standard 6	0.726	0.646	83.03	100
Standard 7	0.761	0.681	87.53	50
B0	0.858	0.778	100	0
Sample 1	0.318	0.238	30.53	1,974.9
Sample 2	0.639	0.559	71.79	163.9

Always run your own standard curve for calculation of results. Do not use this data.
Conversion Factor: 100 pg/mL of cortisol is equivalent to 275.9 pM.

Typical Standard Curves



Always run your own standard curves for calculation of results. Do not use this data.

VALIDATION DATA

Sensitivity and Limit of Detection

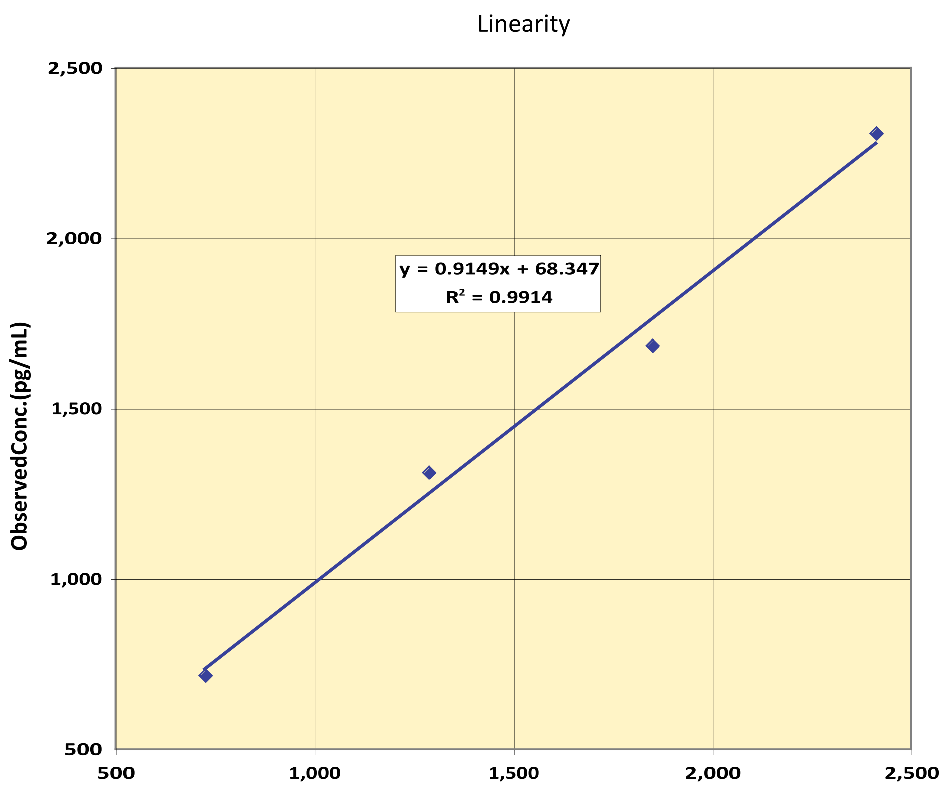
Sensitivity was calculated by comparing the OD's for twenty wells run for each of the B0 and standard #7. The detection limit was determined at two (2) standard deviations from the B0 along the standard curve. **Sensitivity was determined as 27.6 pg/mL.**

The Limit of Detection for the assay was determined in a similar manner by comparing the OD's for twenty runs for each of the zero standard and a low concentration human sample. **Limit of Detection was determined as 45.4 pg/mL.**

Linearity

Linearity was determined by taking two human urine samples diluted 1:140, one with a low diluted cortisol level of 163.9 pg/mL and one with a higher diluted level of 2,974.9 pg/mL and mixing them in the ratios given below. The measured concentrations were compared to the expected values based on the ratios used.

Low Urine	High Urine	Observed Conc. (pg/mL)	Expected Conc. (pg/mL)	% Recovery
80%	20%	715.7	726.1	98.6
60%	40%	1,311.5	1,288.3	101.8
40%	60%	1,683.3	1,850.5	91.0
20%	80%	2,306.3	2,412.7	95.6
			Mean Recovery	**96.7%**



Intra Assay Precision

Three human samples were diluted with Assay Buffer and run in replicates of 20 in an assay. The mean and precision of the calculated Cortisol concentrations were:

Sample	Cortisol Conc. (pg/mL)	%CV
1	1,174.3	6.0
2	475.9	5.6
3	177.4	14.7

Inter Assay Precision

Three human samples were diluted with Assay Buffer and run in duplicates in ten assays run over multiple days by four operators. The mean and precision of the calculated Cortisol concentrations were:

Sample	Cortisol Conc. (pg/mL)	%CV
1	1,188.1	7.2
2	508.7	6.3
3	199.7	10.9

Appendix D

CROSS REACTIVITY

The following cross reactants were tested in the assay and calculated at the 50% binding point.

Steroid	Cross Reactivity (%)
Cortisol	100%
Dexamethasone	18.8%
Prednisolone (1-Dehydrocortisol)	7.8%
Corticosterone	1.2%
Cortisone	1.2%
Progesterone	<0.1%
Estradiol	<0.1%
Cortisol 21-Glucuronide	<0.1%

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