ABSTRACT

ELLIS, KATIE BETH. The Examination of Fecal Cortisol in the Captive Southern White Rhinoceros (*Ceratotherium simum simum*) at the North Carolina Asheboro Zoo. (Under the direction of Dr. C. Scott Whisnant and Dr. Vivek Fellner).

Adverse physiological effects due to external and internal stimuli, such as diet and metabolism, may contribute to the low captive reproductive success rate of the Southern White Rhinoceros (*Ceratotherium simum simum*). The objective of this study was to examine fecal cortisol response in the Southern White Rhinoceros population at the North Carolina Asheboro Zoo. Seven mature Southern White Rhinoceroses, ages ranging from 10-45 years old, were divided into four groups based on behavior and rotated through a 4x4 double Latin square design incorporating four repeated periods to total eight 21-d feeding periods. The rhinoceros individuals received commercial pelleted complete feeds that varied in starch (3.4-24.0%)/fiber (11.0-27.0%) and protein (13.0-17.0%)/fat (3.0-3.9%) content. The rhinoceros population also received a bale of timothy hay, 15.9kg/herd, each day and ad libitum access to pasture bermudagrass and water during the study period. If temperatures dropped below 35ºF, the herd was brought into the boma and supplemented with nine bales of timothy hay, 143.1kg/herd. Fecal samples were collected directly from the rectum between 8:00-10:00 am on Monday or Tuesday of each week for the duration of the study, n=227 fecal samples in total. As analyzed there were no statistically significant correlations between the study pelleted complete feeds fed and cortisol values as a population. This may be due to the fact that the pelleted complete feeds only accounted for approximately 7% of the total dietary intake for most individuals, based on estimated energy intake. Fecal cortisol concentration levels showed a strong inter-individual response to each pelleted complete feed

($P<0.05$). The range of mean ± SD among individuals was found to be 5.0 ± 3.45 ng/g, DM to 17.0 ± 4.04 ng/g, DM. The overall range of cortisol values among the population was reported at 1.8-19.3 ng/g, DM. Fecal cortisol concentrations should potentially be evaluated on an individual basis and not by population. Future studies should evaluate the interactions of age, gender and captive born versus wild caught on fecal cortisol response. A captive setting that requires a higher percentage of pelleted complete feeds to low energy grasses is likely to be an ideal dietary model to evaluate potential effects of the captive diet on fecal cortisol response in the Southern White Rhinoceros.

The Examination of Fecal Cortisol in the Captive Southern White Rhinoceros (*Ceratotherium simum simum*) at the North Carolina Asheboro Zoo

by
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DEDICATION

This thesis is dedicated to my family, without whom this thesis would never have been written.

To my loving parents, Kevin and Denise, whose own strength and achievements in life gave me the courage to pursue my true passions. You taught me independence, yet I have never felt alone. I dedicate my thesis to you for being the loving parents that molded me into the person I am today.

To my brother and sister, Tyler and Holly, for reminding me that life is full of wonder and joy to be discovered every day. You are both more special to me than you could ever possibly imagine.

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To my two four-pawed family, Porter and Calie, for never ceasing to put a smile on my face and love in my heart the moment I walk through the door each day.

BIOGRAPHY

Katie Beth Ellis was born on June 9, 1988 in Havelock, North Carolina. She spent most of her childhood in Cincinnati, OH, enjoying Skyline Chili and Graeter's Ice Cream. Her family later relocated back to North Carolina where she was blessed with a beautiful baby brother and sister. After high school, she attended UNC-Greensboro for a year before transferring to North Carolina State University where she graduated with a Bachelor's degree in Animal Science. Throughout her college career she volunteered at various wildlife sanctuaries, such as Carolina Tiger Rescue in Pittsboro, NC. She always knew that wildlife and conservation were a true passion, but her study abroad trip to Belize sparked the dream of graduate school and international work with wildlife. After graduation, Katie met and married Spencer Ellis. She began working towards her Masters of Science degree in Animal Science, with a focus on reproductive physiology, at North Carolina State University under the direction of Dr. Scott Whisnant in the Fall of 2011. Katie has since been accepted and will be working on a PhD program in Conservation Sciences at North Dakota State University in the Fall of 2013.

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CHAPTER I: Literature Review

Introduction

The current threat of poaching and land conflict with humans on the Southern White Rhinoceros (*Ceratotherium simum simum*) signifies that successful captive management of the species is necessary. All aspects need to be considered; nutrition, stress-levels, health and reproduction. Focus on reproduction is a major focus of interest due to the species' low reproductive success in captivity; however, if the diet is not meeting nutritional requirements and the species is experiencing chronically elevated stress levels, proliferation of offspring is unlikely. The future populations of the southern white rhinoceros have the potential of becoming rare enough that humans will only experience this species in a captive setting; therefore, it is important to better understand their needs now as opposed to waiting until the wild populations are in greater danger of extinction from their current status of Near Threatened (Emslie, 2012).

The Southern White Rhinoceros (Ceratotherium simum simum)

The Southern White Rhinoceros is one of five remaining species of rhinoceros and is native to Africa (Emslie et al., 2010; IRF, 2005). The white rhinoceros is a massive ungulate species, second only in size to the elephant, and known for its agility and speed (Kingdon, 1997). Towards the end of the 19th century the population size had dwindled to approximately 10 individuals. Conservation efforts were quickly set in motion, by 1939 there were 120 individuals and in 2005 the population had increased to roughly 11,300 individuals

which lowered their status on the International Union for Conservation of Nature (IUCN) red list to near threatened (Caughley, 1994; Emslie, 2012; IRF, 2005; Patisaul, 2012). Despite the rapid increase in numbers, the white rhinoceros captive population still remains in a demographic crisis today (Emslie, 2012; Schwarzenberger et al., 1998). Extensive efforts to reduce inbreeding have been made by utilizing the Association of Zoos and Aquarium's (AZA) species survival plan (SSP) studbooks. Studbooks are a resource collection of information on all captive individuals, used to match breeding pairs that will ideally produce healthy offspring (Reid et al., 2012).

Along with captive population issues, the wild Southern White rhinoceros populations are continuously threatened with poaching for their horns made of solid keratin, habitat loss and the human-rhinoceros land use conflict (IRF, 2005; Kingdon, 1997). Current research studies are focusing on protection, observation and monitoring of social organization, habitat use, feeding strategies, courtship and reproductive behaviors in free-range, wild and sanctuary populations of the rhinoceros. The goal of field conservation research is to grasp an understanding of how to practice better captive management in the hopes of completely removing the Southern White rhinoceros from the IUCN red list (Emslie et al., 2010; IRF, 2005).

Reproductive Challenges within Captive Populations

In the wild, Southern White Rhinoceros males maintain a home range that generally overlaps with a small group of females. Male home ranges can vary from 2.6 km^2 to 130 km^2. Males mark their territory with dung, urine sprays, foot scuffs and rubbing against

vegetation (Kingdon, 1997). In captivity, if a male is kept within the same enclosure as the females, he will remain satellite to the group, only interacting directly with a female when she is in estrous (Hutchins & Kreger, 2006; Owen-Smith, 1975). It is not recommended to house more than one male with a group of females at any given time due to the fact that they engage in mate-guarding behavior and will potentially fight to the death to defend an estrous female for the opportunity to mate (Fouraker & Wagener, 1996). Compared to other rhinoceros species the white rhinoceros females are known to be more docile, gregarious and semi-social with other females, but extremely aggressive towards males (Hutchins & Kreger, 2006; Owen-Smith, 1975 and 2004). Females arrange themselves into loosely organized communities with stable herds of up to six females in the wild and in captivity. These groups will graze with one another in their home ranges and even cooperate to defend themselves against predators (Estes, 1991; Owen-Smith, 1975 and 2004).

The white rhinoceros can live up to fifty years in the wild and captivity, but maintain a poor reproduction rate in captivity (Kingdon, 1997; Schwarzenberger et al., 1998). They are a polygamous and polyandrous species, making mate selection important and increasing the level of competition between males (Hutchins & Kreger, 2006; Owen-Smith, 2004). Intra-sexual mating competition between males is supported by the presence of higher levels of testicular activity and androgen metabolites, such as androstenediol, in a male rhinoceros bull that is escorting a female in estrus (Hutchins & Kreger, 2006). Females only permit contact by a male while in full estrus, but even then will sometimes run a male off several times before permitting courtship behavior (Goddard, 1967). It is recommended that less dominant males are paired with dominant females to increase the chance of cooperative

mating. The period of courtship can be an extremely stressful time for a male white rhinoceros, because the possibility of sustaining an injury if he does not perceive the female's behavior correctly is high. Usually females in full estrous urinate frequently, allowing the male to detect pheromones through a Flehmen response. The male initiates courtship by approaching the female, showing dominance and possibly even threat displays (Goddard, 1967; Hutchins & Kreger, 2006). If he is not run off by this point then he will begin the long and drawn out act of copulation. The male stands behind the female and performs a chin rest much like in the horse and will rub his head up the female's spine until he is capable of using his head to pull his weight up and move his front feet into position behind her shoulders (Hutchins & Kreger, 2006). This position can last up to an hour before ejaculation occurs, which can be detected by 4-5 rapid thrusts. The female Southern White Rhinoceros has a tumultuous reproductive tract, making artificial insemination extremely challenging, and natural mating preferential (Dutta, 1991; Goddard, 1967; Hutchins & Kreger, 2006; Owen-Smith, 1975). If copulation is successful, gestation and parturition of a single calf will occur (Kingdon, 1997).

Studies have had difficulty defining a "regular" estrous cycle length, due to the fact that females have erratic luteal activity. Very little is known about the reproductive physiology of the southern white rhinoceros. Cycles can vary from 4-10 weeks, but the average, based on several studies, appears to be approximately 10 weeks long (Hindle et al., 1992; Radcliffe et al., 1997; Schwarzenberger et al., 1998). A group of females in captivity can house individuals that have short cycles, long cycles or both short and long cycles. Gestation length is 16 months to produce a single hornless calf, with an interval between

births of 2 to 5 years (Frame, 1971; Kingdon, 1997; Owen-Smith, 2004). This is one of the slowest recruitment rates among mammals, which could lead to one explanation to the difficulties in captive mating programs along with the wild population decline. Both the male and female southern white rhinoceros are capable, physiologically, of breeding at 4-5 years of age; however, females generally do not reproduce until 6-7 years of age in the wild (Kingdon, 1997). Captive-born females are displaying much
later times of reproduction and are experiencing higher numbers of false estrous cycles as well as miscarriages. In 1998 the reproductive rate of founder southern white rhinoceroses, individuals with "new" genetics to add to the breeding pool (generally from the wild), is 30%, while the first and second generation offspring have a reproduction rate of only 8% and 0% respectively, in captivity (Schwarzenberger, et al., 1998). Metrione and Harder, 2011 stated that the reproduction rate of captive females is currently at about 50% and only 38% of captive-born females have produced offspring. Males in the wild, or those housed with males in captivity, are incapable of competing with more mature and dominant males in the area; therefore, sexual maturity and mating behaviors do not occur until 10-12 years of age, delaying successful reproduction of males with needed genetics (Kingdon, 1997).

Recommended Diet Composition and Husbandry Practices

The Southern White rhinoceros is a large herbivore grazer species that feeds unselectively on low energy feed such as short grasses and roughage, comprising a high fiber (37-51% NDF) and low to moderate protein (12-14%) diet (Clauss & Hatt, 2006; Lintzenich & Ward, 1997; Perrin & Brerton-Stiles, 1999). In comparison, the black rhinoceros is a

strictly browser species that is selective in feed preferences, with higher protein, yet similar fiber intake to the grazing Southern White rhinoceros species. The black rhinoceros has a fast gut passage rate and low digestive efficiency; therefore, unlike the Southern White rhinoceros, the black rhinoceros will also eat leafy plants, shrubbery, branches and other palatable forages within their reach to meet minimum energy and nutrient requirements (Clauss & Hatt, 2006).

The digestive tract of the Southern White rhinoceros has a large cecum and colon for hind-gut microbial fermentation (Lintzenich & Ward, 1997; Stevens & Hume, 1995). Hind-gut fermentation has a high digestive efficiency for fiber and slow ingesta passage that is similar to the domestic horse (Clauss & Hatt, 2006; Field, 1968; Hutchins & Kreger, 2006; Lintzenich & Ward, 1997). Microbial fermentation of the plant fibers make up the main dietary energy source. The key to this digestive system is to maximize quantity with less focus on energy levels of grasses (Clauss & Hatt, 2006; Perrin & Brereton-Stiles, 1999; Stevens & Hume, 1995).

In the wild the Southern White rhinoceros inhabits the lowland savannah grasslands of Africa, maintaining and rotating grazing lawns with grass heights less than 5 centimeters (cm), which allows for renewal of resources (Hutchins & Kreger, 2006; Owen-Smith, 2004; Perrin & Brereton-Stiles, 1999). Grass is grazed down to 2 cm using their broad muzzle and strong lip muscles for rapid ingestion. Mature grass contains a higher fiber content which is less digestible to the white rhinoceros, but the new leaves and shoots of shorter, less mature grass, is lower in fiber and more digestible (Lintzenich & Ward, 1997).

The domestic horse, which is also a monogastric hindgut fermenter, is currently thought to be the best domesticated model for constructing a captive white rhinoceros diet that is balanced and nutritionally complete (Clauss & Hatt, 2006; Dierenfeld, 1996 and 1999; Stevens & Hume, 1995). The National Research Council (NRC) provides diet recommendations for the domestic horse that is used as a dietary model for minimum requirements for the captive white rhinoceros (Dierenfeld, 1996; NRC, 1989). Often in captivity, zoos are not able to maintain large natural grass lawns that can sustain a herd of rhinos year-round, and must supplement their individuals with dry bales of hay, especially in the winter when temperatures drop and the herds must be kept primarily indoors. Fresh cut grass is ideal and recommended to make up the bulk of the diet in captivity whenever possible, but should not be cut too short (below the selected 2 cm grazing height) to prevent constipation of the hindgut, due to elevated levels of fiber digestibility (Clauss & Hatt, 2006). Hay storage is exceptionally important, mold or dust from dry hay can cause colic and heaves as well as negative effects for keepers such as "farmer's lung" (Clauss & Hatt, 2006; Dierenfeld, 1996; Hutchins & Kreger, 2006). Colic causes abdominal pain in the rhinoceros due to a disruption of the digestive tract, for instance if fed moldy hay. Heaves in the rhinoceros and "farmer's lung" in human keepers are both similar to affects of asthma that have been triggered by an allergen or irritant, for instance dusty hay (Clauss & Hatt, 2006; Hutchins & Kreger, 2006).

Forage, here is defined as any grass or legume, mainly made up of plant leaves and stems that the rhinoceros eats while grazing in a pasture. Forage can be made up of grasses such as fescue and timothy or legumes such as alfalfa (Clauss & Hatt, 2006; Lintzenich &

Ward, 1997). Hay is a dried form of forage that is supplemented to the diet as a foodstuff for the animal (Clauss & Hatt, 2006). Forage high in neutral detergent fiber digestibility (NDFD) and balanced neutral detergent fiber (NDF) levels, should be fed to the herd. Low NDFD forages can cause impaction or colic. Forage that is too elevated in NDF and NDFD, which is too highly digestible, can lead to loose feces or colic (Dierenfeld, 1996; Hutchins & Kreger, 2006). Grass hay, water, and salt blocks should be available ad libitum (Dierenfeld, 1996; Hutchines & Kreger, 2006). The recommended dry matter intake of grass hay is 1-2.5% of body mass and alfalfa hay at 1.2-1.6% (Clauss & Hatt, 2006; Dierenfeld, 1996 and 1999; Lintzenich & Ward, 1997; Steuer et al., 2010). The white rhinoceros has an average fermentation period of 12-14 hours, an average retention time of 28 hours for fluids and 43 hours for particles as well as an advanced ability to digest Neutral Detergent Fiber (NDF) (Steuer et al., 2010).

Clauss & Hatt (2006) reported that captive diets for rhinoceros individuals often have an imbalance of carbohydrates and dietary fats, particularly essential fatty acids, potentially due to the use of grain in pelleted feeds. This imbalance is one reason that it is important to maintain an appropriate ratio of supplemented grass hay, pasture forage and pelleted complete feeds (Clauss & Hatt, 2006; Lintzenich & Ward, 1997). Dierenfeld (1996) recommends that no more than one-third of the total calories consumed come from pelleted complete feed. Diets consisting of a lower percentage of pelleted complete feeds compared to forage and supplemented dry hay are considered “maintenance,” due to the expected lower energy intake when grasses are the driving force of the overall diet. Among other concerns, feeding a high percentage of pelleted complete feeds than recommended based on body mass

can give over supplementation of energy, potentially leading to obesity issues. Grazing species are generally thought of as having a lazier eating disposition which makes them prone to obesity in captivity (Clauss & Hatt, 2006). Steuer et al. (2010) recommends a dietary average of 63.4% NDF, 32.8% Acid Detergent Fiber (ADF), 10.2% Crude Protein and 8.2% Ash. Clauss & Hatt (2006) recommend high fiber content, crude fiber of 20%, for pelleted complete feeds. Calcium levels are elevated in most captive diets compared to the calcium levels in their wild counterpart's; therefore, supplementation is generally not required. This is also true for iron, levels are usually met through the hay alone. Excess levels of iron can lead to health problems such as iron storage disease and should be monitored particularly in the Black Rhinoceros species, though iron toxicity issues are not currently seen in the Southern White rhinoceros (Clauss & Hatt, 2006). While elevated levels of calcium and iron are of concern, deficiencies in zinc can also be problematic, leading to health issues such as skin and foot lesions (Castell, 2005). Table 1 outlines the National Research Council's (NRC) nutrient recommendations for the domestic horse. These values are currently utilized for feeding the Southern White rhinoceros in captivity in an attempt to maintain a healthy weight and balanced diet (Lintzenich & Ward, 1997). Hay and pellets should be weighed before each feeding to ensure that the rhinoceros is receiving a consistent amount of food based on predetermined ration calculations for each individual (Clauss & Hatt, 2006; Lintzenich & Ward, 1997). This is exceptionally important due to the fact that a "scoop" or "handful" of food can vary in both weight and size depending on the size of someone's hand or if the "scoop" is rounded or leveled off. By weighing out feedstuff, it is ensured that individual rhinoceroses are receiving the appropriate allotment of energy intake

on a daily basis, based on their body mass. It is also important to regularly monitor body mass of the individuals to make adjustments for feed and energy intake required. After weighing, all feed should be offered on concrete pads, livestock troughs or bins to reduce the chance of gastrointestinal impaction from dirt or sand (Dierenfeld, 1996).

Table 1. Comparison of suggested nutrient concentrations needed to maintain the domestic horse as a reference for feeding the Southern White Rhinoceros (*Ceratotherium simum simum*) in captivity (90% dry matter basis)[1]

Nutrient	National Research Council[A]	Nutrition Advisory Group Handbook[B]	Low Fiber Herbivore Pellet	Grass Hay[C]
Crude Protein (%)	9.0-14.0	12.0-14.0	17.4	9.8-11.2
NDF (%)	NA*	37.0-51.0	29.3	51.0-67.4
ADF (%)	NA	NA	17.3	31.2-36.3
Vitamin A (IU/g)	1.0-3.5	1.2-2.0	5.0	NA
Vitamin D (IU/g)	0.2-0.5	0.3-0.5	1.2	NA
Vitamin E (IU/kg)	120-350	100-160	400	NA
Thiamin (mg/kg)	2.0-4.5	2.0-3.2	NA	NA
Riboflavin (mg/kg)	2.0	2.2-3.6	NA	NA
Calcium (%)	0.20-0.65	0.55-0.63	0.88	0.41-0.67
Phosphorus (%)	0.15-0.34	0.30-0.38	0.64	0.19-0.38
Magnesium (%)	0.07-0.10	0.16-0.19	0.29	0.15-0.21
Potassium (%)	0.27-0.38	1.40-1.80	1.50	1.90-2.40
Sodium (%)	0.090-0.270	0.070-0.120	0.400	0.003-0.030
Iron (mg/kg)	36-45	73-84	394	69-85
Zinc (mg/kg)	36	44-71	136	15-31
Copper (mg/kg)	9	8-14	23	5-11
Manganese (mg/kg)	36	40-55	120	25-36
Selenium (mg/kg)	0.09	0.10-0.16	NA	NA
Iodine (mg/kg)	0.09-0.54	0.20-0.40	NA	NA

[1]Source: Nutrition Advisory Group Handbook (Lintzenich & Ward, 1997), containing a compilation of recommendations for nutrient requirements in three separate tables.
*NA= information not available
[A] Nutrient concentration recommendations for the domestic horse, based on quantitative data of nutrient requirements.
[B] Nutrient profile recommendation ranges that meet or exceed the National Research Council's proposed nutrient concentrations to maintain the white rhinoceros in captivity. The suggested diet is based on a low fiber pellet to grass hay ratio.
[C] Includes timothy, coastal bermudagrass, and sudan based on the Hay Market Task Force of the American Forage and Grassland Council.

Stress as a Response

Stress is often defined as any external or internal stimulus that disturbs homeostasis of an individual. However, it is important to take into account that there is a wide variation in types of stressors and individual responses to what can be perceived as positive stress or negative stress (Metrione & Harder, 2011; Sheriff et al., 2010; Turner et al., 2002). Positive stress, also known as eustress, can come from a variety of rhinoceros stimulations such as belly rubs, maternal grooming and mating behaviors (Hutchins & Kreger, 2006). Positive stress has been shown to aid in behaviors such as learning, exploring sexual arousal and responsiveness in the white rhinoceros and many other species (Carlstead, 1994; Carlstead & Brown, 2005; Hennessy, 1979). Negative stress, or distress, is the term generally associated with "stress." This type of stressor in rhinoceroses can come from human or animal interaction, translocation, reintroduction, interspecies conflict, illness, predator-prey interaction or improper nutrition and can have physiological effects on the health, reproduction and psychological state of an animal (Carlstead & Brown, 2005; Hutchins & Kreger, 2006; Moberg, 1990). Stress is simply a biological response to a stimulus, meant to aid an individual's ability to adapt and survive by making adjustments to energy distribution throughout the system (Metrione & Harder, 2011). The white rhinoceros, while large in size, is a prey species and reacts in a "fight or flight" mentality, responding quickly and strongly to acute stressors. During acute stress, any systems that are not vital to a fight or flight response are shut down and systems such as the cardiovascular and nervous systems receive increased energy availability. The cardiovascular system increases the heart rate to optimize oxygen availability and the nervous system is stimulated in preparation for a reaction

(Widmann, 2010). Acute stressors are typically short in duration and therefore homeostasis of the system is expected to return once the stimulus has passed. However, chronic or persistent stress will have more long term effects on systems that could impair feed intake, growth and reproduction (Evans, 1977; Hutchins & Kreger, 2006; Moberg, 1990; Widmann, 2010). Persistent stress can manifest into stereotypic behaviors such as biting or licking metal bars, pacing and extensive horn rubbing (Fouraker & Wagener, 1996).

A given stimulus, such as interacting with a zoo keeper, activates the hypothalamic-pituitary-adrenal (HPA) axis which begins with the hypothalamus releasing corticotrophin-releasing factor (CRF) from the median eminence, as well as oxytocin and arginine vasopressin (AVP), both of which are hypothalamic peptides (Carlstead & Brown, 2005; Wingfield & Sapolsky, 2003). CRF is a peptide utilized for the regulation of adrenocorticotropic hormone (ACTH) and B-endorphin stimulation from the anterior pituitary. ACTH then stimulates the release of glucocorticosteroids, such as cortisol and corticosterone, from the adrenal cortex (Carlstead & Brown, 2005; Metrione & Harder, 2011; Wingfield & Sapolsky, 2003). Glucocorticosteroids are adrenal stress hormones, currently utilized as a means to study animal welfare in captivity (Hutchins & Kreger, 2006; Widmann, 2010). In most studied species, cortisol present in the blood is filtered through the liver and excreted into urine or bile byproducts. Bile enters the gut to undergo metabolism and cortisol is then excreted through feces (Möstl & Palme, 2002). The effects of stress and timing of cortisol response in the Southern White Rhinoceros is discussed in a later section.

Cortisol as an Indicator of Stress

Cortisol is the major corticosteroid in horses and thought to also be the major corticosteroid in the Southern White Rhinoceros (Carlstead & Brown, 2005; Seal et al., 1976; Turner et al., 2002). Fluctuations in the release of cortisol are both a species-specific and inter-individual stress-mediated mechanism (Carlstead & Brown, 2005; Millspaugh & Washburn, 2004). Each individual rhinoceros is capable of coping and handling external stressors with varying degrees of sensitivity. For instance, an older captive-born female, has most likely been habituated to human presence, both keepers and visitors, as well as bonded with one or more individuals in the group. This particular female would be expected to have lower mean levels of cortisol because she is more adept at maintaining homeostasis (Carlstead & Brown, 2005). Birth location could potentially make a substantial difference in a given rhinos perception of stress, if wild-born and relocated to a captive facility, he or she is likely to maintain higher basal levels of cortisol for an extended period of time while adjusting to new surroundings and fellow rhinos. This rhinoceros could also undergo desensitization of the HPA axis through adrenal hyper reactivity of cortisol if levels remained persistently elevated (Carlstead & Brown, 2005). The range and duration of elevated cortisol secretion to exhibit distress with deleterious physiological effects on an individual is still unknown (Ladewig, 2000; Millspaugh & Washburn, 2004).

Utilization of Fecal Samples for Cortisol Analysis

The use of fecal samples to measure cortisol, in place of plasma, urine and saliva collections is becoming more popular in wildlife studies due to its less invasive collection benefits (Millspaugh & Washburn, 2004; Sheriff et al., 2010). Blood based assays require a capture-restraint method for collection that can trigger a spike in cortisol levels and may not accurately reflect the data. The presence of glucocorticosteroids in urine and even more so in feces, show a time delay in the reflection of general stress. Fecal analysis will provide a look into general stress levels of the Southern White rhinoceros during a window of time 24-48 hours prior to sampling due to gut passage rate and accumulation of hormone metabolites in the gastrointestinal tract (Metrione & Harder, 2011). Glucocorticosteroids in plasma samples, on the other hand, have a pulsatile pattern in many species and only give a point in time sampling, representing an immediate spike due to a stressor in all species (Millspaugh & Washburn, 2004). In order to evaluate cortisol levels from plasma, samples should be taken daily to multiple times per day to account for circadian rhythms of the adrenal steroid. Cortisol concentrations in the horse show a circadian rhythm with cortisol levels highest in the morning, during morning feeding and the period of most activity, and was the lowest cortisol values in the evening, during evening feeding and the period of least activity or sleep (Bottoms et al., 1972; Evans et al., 1974; Hoffsis et al., 1970; Widmann, 2010). It is likely that this natural fluctuation is correlated with feeding times due to the fact that cortisol plays a key role in initiating energy metabolism and gluconeogenesis (Stull, 1988; Widmann, 2010). Other studies have indicated an elevation in cortisol levels directly before time of feeding in the mornings for horses. This increase could be occurring because of keeper

interaction, stress of group feeding at a point source or a metabolic preparation in anticipation of eating (Widmann, 2010).

In plasma, the amount of free glucocorticoids are measured for analysis, only the free form is biologically active and makes up 5-10% of the total glucocorticoids. Glucocorticoids are mostly bound to the carrier protein, corticosteroid-binding globulin (CBG) (Sheriff et al., 2010). Cortisol concentrations are determined by competitive protein binding, the bound forms will not register during analysis, cortisol must be un-bound in order to examine cortisol excretion (Seal et al., 1976). Only free glucocorticoids are degraded by the liver and excreted into the feces, ensuring that fecal glucocorticoid analysis does in fact reproduce the same free biologically active glucocorticoids as in plasma samples. Fecal glucocorticoid metabolite analysis is therefore an accurate indication of the physiological state and adrenocorticol activity stress response of an individual (Sheriff et al., 2010; Turner et al., 2002). Turner et al. (2002) reported findings that fecal glucocorticoids accurately reflected both plasma and urine levels in the White and Black Rhinoceros.

Fecal collections offer the benefit of providing an integrated hormone profile of cumulative glucocorticoid secretion over a period of time, rather than a maximum or point source response, allowing the animal to act as its own control (Möstl and Palme, 2002; Sheriff et al., 2010; Turner et al., 2002). In many species blood samples must be taken within 3 minutes of capture to provide an unbiased account of glucocorticoid levels, this can be extremely challenging and unrealistic in many situations, even in a captive setting (Sheriff et al., 2010). Use of fecal samples allows for a long term evaluation of glucocorticoid levels in a species without as much disturbance or bias to the study (Millspaugh & Washburn, 2004).

Techniques for fecal glucocorticoid analysis have been well established and validated in numerous studies on the rhinoceros by performing a dexamethasone injection (Dex) suppression test and an adrenocorticotropic hormone injection (ACTH) stimulation test (Brown et al., 2001; Sheriff et al., 2010; Turner et al., 2002). Dex is an artificial glucocorticoid agonist that utilizes feedback inhibition on the hypothalamic-pituitary-adrenal (HPA) axis to mimic endogenous cortisol. If an animal's feedback inhibition is operating normally, not under chronic stress, plasma cortisol levels will drop down to a "normal" level after stimulation has passed. If an animal is resistant to Dex, meaning the animal is under chronic stress, the plasma cortisol levels will remain slightly elevated (Sheriff et al., 2010). A larger range of variability in fecal cortisol indicates an animal's increased sensitivity to react to a given stimulus (Carlstead & Brown, 2005). After the Dex suppression test, the ACTH stimulation test analyzes the adrenal system's responsiveness and stimulates the release of blood cortisol concentrations circulating in the blood. For an animal that is under chronic stress, the adrenal system will have an increased response to injections of ACTH (Carlstead & Brown, 2005; Metrione & Harder, 2011; Sheriff et al., 2010; Soto-Gamboa et al. 2009). Both the ACTH and Dex injections affect levels of cortisol concentrations circulating in the blood which is reflected in fecal corticoid metabolite levels (Möstl and Palme, 2002; Sheriff et al., 2010; Soto-Gamboa et al. 2009). Analysis of the fecal corticoid metabolites offers a stable indicator of the total amount of biologically active free glucocorticoids and displays an animal's physiological state (Sheriff et al., 2010). It is vital that hormones, such as cortisol, are validated within each species. Dex and ACTH are an efficient way to achieve such a validation, as has been performed for the Southern White Rhinoceros (Brown et al., 2001).

Fecal samples were utilized for this study due to the less invasive sampling technique and ability to evaluate cortisol values in a longitudinal sampling manner that provides a large window of time to view effects of potential stressors on the Southern White rhinoceros' adrenal system.

Table 2 outlines previously reported baseline cortisol levels of the Southern White rhinoceros under conditions of stress versus unstressed and habituated environments. Fecal samples that were collected from the ground could have possibly been compromised by moisture or degraded due to a time lapse from defecation to collection. The number of rhinoceros individuals utilized for cortisol analysis ranged from 2-6. Number of fecal samples ranged from 4-6 samples to calculate the average value of cortisol. Habituated to captivity is a subjective term and was not based on hormone analysis, but number of years in captivity. The analysis of three individuals over two 24 hour periods did not provide support for diurnal patterns of cortisol secretion in the Southern White rhinoceros (Turner et al., 2002).

Table 2. White Rhinoceros Published Fecal Cortisol Values Under Various Conditions and Times to Show the Variation of Cortisol Concentrations of a Stressed versus Unstressed Rhinoceros[1]

White Rhinoceros Subject	Rhinoceros	Fecal Samples	Cortisol[2] (Mean ± SE)
	n	*n*	*ng/g*
Unstressed [A]	6	6	4.1 ± 0.6
Stressed [B]	4	4	28.3 ± 3.4
Habituated/Unstressed [C]	2	5	3.4 ± 0.44
7AM-1PM [D]	3	6	3.9 ± 1.3
1PM-7PM [D]	3	5	5.4 ± 1.1
7PM-7AM [D]	3	6	3.6 ± 0.8

[1]Source: Turner et al., 2002
[2]Cortisol is measured in ng/g, DM.
[A] Unrestrained and habituated to captivity.
[B] Samples taken within 24 hours of arrival after being restrained, crated, and shipped.
[C] One male and one female that were habituated to captivity for more than 7 years and did not undergo any unusual events at time of collection. Fecal samples collected overnight for a less invasive assessment of cortisol levels.
[D] Fecal samples collected over two 24 hour periods from three individuals.

Interactions of Cortisol and Reproduction

The autonomic nervous system is involved in both the hypothalamic-pituitary-adrenal (HPA) and hypothalamic-pituitary-gonadal (HPG) axes. An environmental, physical or mental stimulus will reverberate from the HPG axis, through the hypothalamus to release gonadotropin-releasing hormone (GnRH) from the median eminence to act as a regulatory peptide on the anterior pituitary. The anterior pituitary releases two gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH), to regulate gonadal function, synthesis and release of sex steroids (Wingfield & Sapolsky, 2003). Glucocorticosteroids, secreted from stimulation of the HPA axis, oxytocin, corticotrophin-

releasing factor (CRF), arginine vasopressin (AVP), adrenocorticotropic hormone (ACTH), *B*-endorphins, and epinephrine are all known as stress hormones. Each of these stress hormones can act on various locations of the HPG axis and other reproductive structures to inhibit or stimulate the release of sex steroids (Engler et al., 1989; Tilbrook et al., 2000; Winfield & Sapolsky, 2003).

The stress induced secretion of prolactin (PRL) and glucocorticosteroids mediate the secretion of *B*-endorphins. *B*-endorphins have an inhibitory effect on the release of GnRH release, decreasing GnRH concentrations in the hypophysial-pituitary portal system within seconds of a stress induced response. The pituitary gonadotropes have a decreased sensitivity to the stimulatory effects of GnRH causing a decrease in secretion of LH (Wingfield & Sapolsky, 2003). The glucocorticosteroids act directly on the male and female gonads to decrease responsiveness to LH and reduce the number of LH receptors available for binding. In the female, a reduction in the ovarian response to LH causes an extended follicular stage, leading to longer and irregular cycle lengths. The female can also experience disruption of ovulation and impairment of uterine maturation required for implantation under conditions of stress (Winfield & Sapolsky, 2003). In the male, testosterone levels will dramatically drop below normal within minutes to hours, inhibiting the male's ability to reproduce and fertilize a female. Stress can also lead to the inhibition of hormones from the HPA axis and cause erectile dysfunction and premature ejaculation (Wingfield & Sapolsky, 2003). Males and females will both experience a drop in libido as a natural response to environmental and physiological factors signaling that reproduction is not cost efficient on the system at that given time (Tillbrook et al., 2000; Wingfield & Sapolsky, 2003).

In the female, stress induces the secretion of PRL, decreasing progesterone concentrations and antagonizing progesterone's anabolic effects on the uterus leading to the impairment of uterine maturation. Progesterone is vital to the uterine wall preparation for implantation during the luteal phase of a female's cycle. Infections in female wildlife will cause activation of the immune system to release corticotrophin-releasing hormone (CRH) and interleukin (IL-1) from the brain. The release of CRH and IL-1 induce secretion of glucocotricosteroids and a rapid reduction in the female's sexual behavior and attractiveness to a male. Males do not show the same drop in sexual behavior due to illness or infection (Wingfield & Sapolsky, 2003).

In males, the parasympathetic tone, a prerequisite for an erection, and the sympathetic tone, mediates ejaculation, activate the autonomic nervous system to cause stress-induced erectile dysfunction. If stress blocks the establishment of the parasympathetic tone, the male will be unable to form an erection. Stress can also accelerate the transition to the sympathetic tone causing premature ejaculation (Muehlenbein & Watts, 2010; Wingfield & Sapolsky, 2003). In the wild, male rhinos that can reproduce under stressful conditions are more likely to copulate and produce offspring. The sympathetic nervous system (SNS) has a direct effect on testicular function. In subordinate males an acute stressor, aggressive interaction with a dominant male, rapidly induces cortisol secretion, inhibiting the testicular axis and inducing opiate secretion. Opiates inhibit GnRH in the system leading to a decline in LH concentrations and inevitably a drop in testosterone within minutes to hours. In a dominant male a stressor can induce the release of sympathetic catecholamines, enhancing testicular parenchyma, increasing testicular blood flow and the absolute amounts of LH delivered

(Muehlenbein & Watts, 2010; Wingfield & Sapolsky, 2003). By increasing the amount of LH concentration in the system, dominant males are able to increase testosterone production and avoid a drop in testosterone which would lead to becoming subordinate to other males competing to mate. The overcompensation of the sympathetic nervous system results from a decreased sensitivity of the HPA axis to glucocorticosteroid's suppressive effects on testicular function (Muehlenbein & Watts, 2010; Wingfield & Sapolsky, 2003).

In a wild animal's natural setting, such as the African savannahs, stress induced releases of hormones leading to reproductive failure is an innate response to situations such as lack of food, water, space or safety. For example, a stress-response can provide a mating capable male with high levels of energy for the muscle and cardiovascular system to compensate for the energy requirements required for copulation. Reproducing females on the other hand are committing energy stores to the potential of abundant energy use by a fetus, taking away from her health and nutrition; therefore, it is not evolutionarily advantageous for a female to become pregnant or give birth to an offspring in poor living conditions (Carlstead & Brown, 2005; Wingfield & Sapolsky, 2003). Unfortunately, this natural response in the wild is causing a stress-induced response in captive settings due to human contact, dominance status and interspecies conflict, such as competition for food or space, even when nutrition and space appear to be overabundant by regulation standards. Stress- induced secretions of adrenal androgens, for example, can decrease proceptive and receptive behaviors in the male and female. It is vital that the male rhinoceros have strong proceptive behavior to make courtship and copulation advances towards a female or he will fail to mate and pass along his genes. Due to the fact that female rhinoceroses are extremely aggressive,

and only receptive to a male's advances during full estrous, any inhibition of receptive behavior could lead to extreme aggression and a missed opening for fertilization and pregnancy. This logic supports that male and female rhinoceroses capable of mating in spite of acute or persistent negative stressors would be naturally selected for due to their slow offspring recumbency rate (Brown et al., 2001; Schwarzenberger et al., 1998; Wingfield & Sapolsky, 2003).

Effects of Dietary Sources on Captive Rhinoceros Populations

Pelleted complete feeds are increasingly utilized in captive wildlife populations, due to the positive qualities that allow a more regularly balanced diet and to ensure that all nutritional requirements are being met via supplementing with pelleted feeds. Studying the natural diet of an exotic species in the wild is difficult; therefore, a captive domestic livestock model with a similar digestive morphology is often employed for pelleted diet formulation (McCusker et al., 2011). Research is continuously evaluating and adjusting the energy levels and quantity of feed intake for captive exotic animals in attempts to provide a diet that more readily encompasses the nutritional quality and behavioral needs of animals in captivity (McCusker et al., 2011).

A serious negative interaction of improper diet consumption on an animal's system is between obesity and reproductive success. The North Carolina Asheboro Zoo's population of seven Southern White Rhinoceroses are experiencing a lack of reproductive success which could be in part due to over conditioning. If dietary energy is provided in overabundance there can be a rapid increase in body weight and fat distribution in the rhinoceros (Bray,

1997). In the male, obesity can lead to a reduction in free testosterone, further causing issues with courtship, mounting and copulation behavior. In the female, obesity can cause cyclicity to occur earlier, later or not at all, along with phantom ovulatory cycles. Low levels of testosterone in the male and erratic levels of progesterone in the female can prevent breeding, copulation and production of an offspring (Bray, 1997).

In the mouse, several studies have shown the ob (obese) protein leptin to be defective in situations resulting in obesity (Bray, 1997). Leptin is a key protein hormone derived and secreted only from adipocytes found in adipose tissue in quantities proportional to the amount of fat present. A larger percentage of fat mass volume will result in an increased level of leptin secretion. Leptin binds to the Ob (lep) receptor (obese leptin gene) to signal the hypothalamus that fat stores have reached an adequate level for a conceptus to be carried to term in females (Bray, 1997). Obesity can result from mutations to leptin receptors, leaving larger amounts of free leptin in the system. Leptin also regulates an animal's signals for energy intake requirements, expenditure of energy, appetite, metabolic functions and can further have a role in behavior (Dryden et al., 1995).

Obesity furthermore has a strong positive correlation with neuropeptide Y (NPY) in many species such as the Southern White rhinoceros, which acts as a neurotransmitter in the brain. An increase in NPY has been shown to increase food intake in animals, especially during nocturnal feeding times (Bray, 1997; Dryden et al., 1995). Chronic stress and diets containing unnaturally high levels of fat or sugar for that species' optimal diet can signal the release of NPY, stimulating increased fat accumulation as energy storage in the abdominal region. NPY is a vasoconstrictor, in males high levels of NPY can suppress ejaculation and

mounting behaviors due to lack of blood supply to the penis. Reproductive effects can also be seen by NPY on the LH surge and GnRH production by the hypothalamic neurons. NPY has positive correlations with the animal's system to decrease anxiety under stress, perception of pain and blood pressure (Bray, 1997).

In response to a stressor, the HPA axis of the Southern White rhinoceros signals the release of glucocorticosteroids. One response that occurs due to this increase is an elevation of NPY through Type II glucocorticosteroid receptor activation and inhibition of negative feedback of Corticotropin-releasing factor (CRF) on synthesis of NPY (Kuo et al., 2007). Increased glucocorticosteroids also stimulate the gluconeogenesis pathway, increasing glucose levels in the blood. Elevated blood glucose levels trigger the release of insulin to compensate by reuptake and storage as glycogen, serving as energy storage in muscle tissue and the liver (Dryden et al., 1995). Insulin resistance develops as obesity persists or amplifies. Increased insulin resistance inhibits regulation of high glucose levels in the blood, producing potentially lethal levels of blood glucose and the inability to utilize elevated blood glucose levels as energy stores in the form of glycogen (Dryden et al., 1995; Kuo et al., 2007). Other hormones such as progesterone and estradiol are closely associated with NPY. Progesterone's levels fluctuate in parallel to levels of GnRH and NPY. Estradiol and NPY are located together in the same cells of the arcuate nucleus, causing them to interact closely (Bray, 1997).

Obese females can exhibit normal pituitary gonadotropin levels, yet have increased levels of estradiol and estrone. Adipose tissue can convert delta-4-androstenedione, produced by the adrenal gland, into estrone. Cells in muscle tissues can then convert estrone to

estradiol. In males, estradiol is the active metabolic product of testosterone. In females, estradiol is the predominant estrogenic sex hormone present throughout reproductive years (Bray, 1997). Estrone is the major estrogen derivative present during menopause and can cause erectile dysfunction in males (Sharpe & Skakkebaek, 1993). Females suffering from obesity generally show faster growth rates, with higher levels of fat percent which can trigger the pubertal process and can lead to menarche at an earlier age if the fecal reaches to minimum "critical mass" sooner. If a female surpasses the critical mass, the opposite effect can occur and the female will lack a menstrual cycle completely. Obese females generally have unnaturally heavier infants because of a decreased hydroxylation at the C2 position and increased oxidation at the 17-alpha position of estrogen derivatives. Menopause occurs earlier than natural when elevated production of follicle stimulating hormone (FSH) and ovarian failure occur (Bray, 1997). In the male, as obesity amplifies, plasma concentrations of free testosterone decreases. Sex hormone binding globulin (SHBG) is used as an indicator for elevated risk on the system due to obesity and insulin resistance. An increase in levels of insulin and insulin resistance result in decreased SHBG levels and a decrease in total testosterone present. There is a positive correlation between amount of visceral fat accumulated and levels of free testosterone, increased levels of testosterone can further amplify accumulation of visceral fat (Bray, 1997).

Body conditioning of males and females is especially important if reproduction of offspring is desired. In females, unhealthy body conditions, overweight or underweight, can be directly associated with numerous reproductive events and systems that can be negatively affected by the diet. Nutrients to pay close attention to, especially when attempting to prevent

obesity in the Southern White Rhinoceros, are energy and protein intake, with a focus on increasing low energy forage intake, such as bermudagrass with balanced NDFD levels (Smith, 2005). The number one goal of captive white rhinoceros management is the promotion of animal welfare to maintain a healthy and physiologically balanced population with the hopes of future breeding and reintroduction success when necessary (Hutchins & Kreger, 2006).

In summary, for the reasons shown, focus on the captive management techniques for the Southern White Rhinoceros are increasingly necessary in order to understand how to protect their wild counterparts. Due to the fact that the natural diet of the Southern White Rhinoceros consists of pasture grasses in bulk with high NDF and moderate NDFD levels, it is assumed that an overall diet comprising of pasture grasses, balanced quantities of pelleted complete feeds and supplemented hay that are high in digestible fiber will be the most suitable and readily digestible. The digestive tract and adrenal gland system work closely together; therefore, if the diet is balanced and closely resembles the natural diet, the animal's system should remain in homeostasis.

CHAPTER II: The Examination of Fecal Cortisol in the Captive Southern White Rhinoceros (*Ceratotherium simum simum*) at the North Carolina Asheboro Zoo

Introduction

The Southern White Rhinoceros (*Ceratotherium simum simum*) originates in the savannah grasslands of South Africa, Namibia, Zimbabwe and Kenya, with smaller populations in Uganda, Botswana and Swaziland. Reports in 2010 declared a population of approximately 20,170 southern white rhinoceros individuals in the wild and in 2008 there were a reported 750 individuals in captivity. The species is currently considered Near Threatened due to a consistent rise in poaching threats (Emslie, 2012). The white rhinoceros is a hindgut-fermenter and a strictly grazing species with a natural diet made up of short grasses in the savannahs of Africa (Kingdom, 1997). Zoological institutions typically feed a mixture of pasture grasses, dry hays and pelleted complete feeds to meet the estimated nutritional requirements, based on the NRC domestic horse model, in captivity. Due to the white rhinoceros's low reproductive rate of less than 50% in captivity it is important to establish a well balanced diet and captive management practice.

A validated method for assessing animal welfare is to analyze levels of corticosteroids as a measure of stress response by an individual. There is an abundance of definitions for the term stress, but many still encompass the earliest known explanation of Claude Bernard in 1878 (von Borell, 2001). Stress is a broad term to describe a biological adjustment in response to an unpredictable external or internal stimulus in order to adapt to new conditions or perceived threats (Metrione & Harder, 2011). Rapid changes occur to the

cardiovascular, immune, nervous and endocrine systems to increase energy availability in the event that a fight or flight response will be required. Increased secretion of glucocorticoids, such as cortisol, by the hypothalamic-pituitary-adrenal (HPA) axis is elicited in response to a physical or physiological stimulus (Carlstead & Brown, 2005). Feed-intake, metabolism, reproductive function and growth are impaired in response to a chronic stressor (Turner et al., 2002). The mobilization of metabolic resources by increased glucocorticoid secretion in the event of a perceived stressor is a normal adaptive response and is only thought of as potentially harmful if glucocorticoid levels remain elevated for a prolonged period of time (Carlstead & Brown, 2005). The system of a healthy individual will return to a normal and productive state in response to an acute stressor; however, prolonged states of stress can have detrimental effects on the physiological system and behavior of an individual. Possible stressors affecting the captive southern white rhinoceros include, but are not limited to, dominance status, herd size, enclosure size, exposure to humans including keepers, mating and diet (Metrione & Harder, 2011).

Cortisol levels, used as an indicator of stress response, can be analyzed in blood plasma, saliva, urine and feces (Turner et al., 2002). In free-ranging animals, the collection of blood, saliva and urine as a measure of animal welfare can be a stressor in itself. Animals that are not individually housed can make it challenging for an unbiased look at cortisol levels when utilizing feces and urine. Fecal collections attempt to achieve an unbiased and remote assessment view of each individual's cortisol levels. Due to the fact that cortisol degradation and contamination can be an issue, it is ideal to take a direct rectal collection; however, this fecal collection method requires keeper interaction which is known to be a

potential stressor for the Southern White rhinoceros (Carlstead & Brown, 2005). A significant correlation between plasma cortisol levels and fecal glucocorticoids has been shown in mammals. Fecal cortisol analysis shows a cumulative window, most concentrated at 48 hours and still present at elevated levels up to one week, of secretion versus a point in time reference as is seen in plasma analysis (Mostl & Palme, 2002; Turner et al., 2002). This supports that the use of fecal analysis is sufficient and equivalent to the use of plasma, saliva and urine as a means for evaluating cortisol levels (Carlstead & Brown, 2005; Metrione & Harder, 2001; Millspaugh & Washburn, 2004; Turner et al., 2002).

The captive diet of the southern white rhinoceros can have a variety of impacts on the physiological system, such as gut passage rate, reproductive success and immune system function (Clauss & Hatt, 2006; Hutchins & Kreger, 2006; Wingfield & Sapolsky, 2003). Elevated levels of fiber and starch can affect weight of the animal and retention time of the gastrointestinal tract (Lintzenich & Ward, 1997). High levels of dietary starch and fat may lead to increased stimulation of the sympathetic nervous system and release of cortisol in mammals (Seematter et al., 2005). Cortisol in turn has a key role in initiating gluconeogenesis and energy metabolism, making it a major factor in digestion and utilization of nutrients (Stull & Rodiek, 1988). Elevated levels of cortisol can lead to decreased muscle tissue through protein degradation (Getty et al., 1988). Modifications to nutrient composition of the southern white rhinoceros diet could induce an increase in cortisol levels. The objective of the current study was to investigate the fecal cortisol concentrations of the southern white rhinoceros in relation to nutrient composition in pelleted complete feeds. It was hypothesized that the diet highest in fiber would be associated with lower fecal cortisol

levels, due to the fact that the Southern White rhinoceros's natural dietary intake is high in fiber. Therefore, a high fiber diet in captivity would more naturally resemble the wild diet.

Materials and Methods

Animal use protocols were approved by the North Carolina Asheboro Zoo's animal use committee.

Animal Housing and Management

Seven mature Southern White Rhinoceroses (*Ceratotherium simum simum*) of varying ages (10-45 years), five females and two males, were used in this study. Five of the seven rhinoceroses (#1759, 1760, 1761, 1763 and 1925) were housed on the main pasture exhibit with a variety of hoofstock species. The main pasture exhibit is made up of approximately 40 acres, 37 of which are considered usable pasture. The other two, geriatric rhinoceroses (#941 and 942), were housed separately in "the annex," each with access to their own fenced pasture and barn stalls (Table 1).

Table 1. Background Information of the Seven North Carolina Asheboro Zoo (NCAZ) White Rhinoceroses[1]

Rhino ID #	**1763**	**1925**	**1761[2]**	**1760[3]**	**1759[4]**	**942**	**941**
Rhino Name	Abby	Duma	Kit	Linda	Natalie	Olivia	Stan
Gender	F	M	F	F	F	F	M
Birth Year	2003	1986	1997	1988	1992	1968	1970
Reproductive status	Reproductive	Reproductive	Reproductive	Reproductive	Reproductive	Non-reproductive	Non-reproductive
Captive Born vs. Wild Caught	Captive Born[5]	Captive Born[6]	Wild Caught[7]	Wild Caught[7]	Wild Caught[7]	Wild Caught[7]	Wild Caught[7]
Arrival at NCAZ	May 2007	November 2011	May 2007	May 2007	May 2007	November 1987	November 1987
Weight (lbs.)[8]	4,914	4,850	4,312	4,755	3,768	3,508	4,074
Concentrate Feed (lbs.)	3.5	7.0	3.5	3.5	3.5	10.5	14.0
Housing Location	Main Pasture	Main Pasture	Main Pasture	Main Pasture	Main Pasture	The Annex	The Annex

[1] Information provided by the plains keeper staff of NCAZ.
[2] Rhino #1761 was a nursing calf to rhino #1760 when they were both brought into captivity. She had three pregnancies and two live births in captivity.
[3] Rhino #1760 gave birth to three calves in captivity, including #1761 and #1763, rhino #1763 was her last calf.
[4] Rhino #1759 has had three calves in captivity.
[5] Born at San-Diego Wild Animal Park.
[6] Born at White Oak Conservation Center.
[7] Wild caught from Kruger National Park, South Africa.
[8] Last known weight as of October, 2012.

All animals had access to indoor and outdoor exhibits during the study period. If temperatures dropped below 35ºF all rhinos were brought into the barn. The duration of this study was over a period of time in which all individuals were only required to be brought indoors a 3-4 times towards the end of the trial. The four females housed on the main pasture (#1759, 1760, 1761, and 1763) ate together in the boma, an animal enclosure, where keepers distributed the pelleted complete feed. Rhinos #942 and #941 had individual barn areas and were fed separately in the annex.

Diet Rotation and Fecal Collection

The seven rhinos were grouped based on behavior and randomly assigned four diets that varied in fiber/starch and protein/fat composition. The amount of concentrate feed and dry hay fed to each rhinoceros was kept consistent with amounts pre-trial. Each rhinoceros was given Sand Clear™, a high soluble fiber supplement to clear out the gut, for five days before the first diet transition began. The day before each diet transition, weights were taken for the five rhinos housed on the main pasture. Day one of diet transition, each rhinoceros was fed 75% of their previous complete feed mixed with 25% of their new complete feed. Day two through four, rhinos were fed a complete feed mix of 50% previous with 50% new. Day five of transition, the complete feed mix was 75% previous with 25% new. On day six through twenty-one, for a total of sixteen days, 100% of the assigned complete feed for that rotation period was fed. Each rhinoceros group underwent eight concentrate feed rotations and ate four different formulated concentrate feeds (Table 2). Water and fresh pasture, bermudagrass on exhibit, were available ad libitum.

Table 2. Southern White Rhinoceros (*Ceratotherium simum*) Feed Rotation Schedule in a Double-Latin Square Model[1]

	Group 1 #941 & #1925	**Group 2 #1761 & #1759**	**Group 3 #942**	**Group 4 #1763 & #1760**
Dates				
Rotation 1A 6/13/12-7/3/12	ADF 16[2]	ADF 25[3]	Wild Herbivore (WH)[4]	LiFe WH[5]
Rotation 1B 7/4/12-7/24/12	ADF 25	Wild Herbivore (WH)	LiFe WH	ADF 16
Rotation 1C 7/25/12-8/14/12	Wild Herbivore (WH)	LiFe WH	ADF 16	ADF 25
Rotation 1D 8/15/12-9/4/12	LiFe WH	ADF 16	ADF 25	Wild Herbivore (WH)
Rotation 2A 9/5/12-9/25/12	ADF 16	ADF 25	Wild Herbivore (WH)	LiFe WH
Rotation 2B 9/26/12-10/16/12	ADF 25	Wild Herbivore (WH)	LiFe WH	ADF 16
Rotation 2C 10/17/12-11/6/12	Wild Herbivore (WH)	LiFe WH	ADF 16	ADF 25
Rotation 2D 11/7/12-11/27/12	LiFe WH	ADF 16	ADF 25	Wild Herbivore (WH)

[1] Feed supplied by Mazuri® Exotic Animal Nutrition, Land O' Lakes, Inc.
[2] ADF 16 contains wheat midds, alfalfa, corn, soybean meal, and molasses.
[3] ADF 25 contains alfalfa, wheat midds, and molasses.
[4] Wild Herbivore (WH) contains soy hulls, soybean meal, beet pulp, oat hulls, aspen, molasses, Na sesquicarb, and flax.
[5] LiFe Wild Herbivore (WH) contains oat hulls, soybean meal, timothy hay, wheat midds, beet pulp, soy hulls, and aspen.

The rhinoceros herd was divided based on behavior and assigned into one of the two collection days, Monday or Tuesday. The Monday group included rhinoceroses #941, #1760, #1763, and #1925. The Tuesday collection group included rhinoceros #942, #1759, and #1761. Rhinoceros #1925 was a potentially mating male that chose to come in to eat and have feces collected with whichever rhinoceros female he was courting at the time. He was almost solely collected with Monday's group. Diet transitions began on Wednesdays of each week. Extra large samples of rhinoceros feces (about one ball of feces) were taken on the Monday and Tuesday before each new diet transition for nutrient analysis and placed in quart size ziplock bags. Fecal samples for nutrient analysis were freeze dried in a VirTis Freeze Dryer (SP Scientific, PA) on the forage sample setting. The freeze dried samples were then taken to the North Carolina Department of Agriculture and Consumer Service's (NCDA) Farm Feed Testing Service Laboratory for a complete analysis of protein, Neutral Detergent Fiber (NDF), Acid Detergent Fiber (ADF), fat, and ash.

Sample Collection for Cortisol Assay

Fecal samples for cortisol assays were taken once a week between 8:00-10:00 am, either on the Monday or Tuesday collection day, for the duration of the study. Fecal samples were collected directly from the rectum of the rhinoceros in order to obtain a fresh and uncontaminated sample. Feces was then placed into 30 mL semen collection plastic tubes and labeled with the animal's ID number, date and time of collection. The tubes were then frozen and stored at -20ºC until ready for shipment. Samples were transported in a cooler with ice packs for an hour and a half drive to North Carolina State University, Raleigh, NC where all

samples were immediately stored at -20ºC until ready for assay.

Cortisol Assay

For analysis, 0.5 g of thoroughly mixed wet feces was mixed with 4.5 mLs of 90% methanol in deionized water by shaking for 40 minutes on an automatic shaker. The mixture was then centrifuged at 2500 x g for 15 minutes at 4ºC. The supernatant was collected and transferred to a 6 ml glass culture tube and evaporated to dryness under nitrogen gas (99.9% purity). The dried sample was then reconstituted in 0.15 ml of cortisol zero calibrator (25COZ, Siemens Medical Diagnostics, Los Angeles, CA) (Ange-van Heugten et al., 2009; Huber et al., 2003). Cortisol concentrations were measured by radioimmunoassay (RIA; Figure 1), Coat-A-Count® cortisol assay kit (Siemens Medical Diagnostics, Los Angeles, CA) according to the instructions provided by the manufacturer. All samples were assayed in duplicate. Unknown values were doubled in amount to fifty µl and re-analyzed if the sample ran above 90% binding. The final concentrations were adjusted by a factor of 0.5 for the unknowns pipetted at fifty µl. Additional points of 0.00 and 0.50 were added to the low end of the standard curve for both runs, by diluting the manufacturers supplied standards with the zero sample reagent from the kit. The inter-assay coefficient of variation was 15.3%. Sensitivity of the assay was 0.04 µg/dl (approximately 90% binding). This assay has been proven to be consistent and reliable (Ange-van Heugten et al., 2009; Brown et al., 2001; Panzani et al., 2009).

A 1.0 g wet representative sample was taken and dried to completion at 100-105ºC for approximately 24 hours to calculate percent dry matter. A 0.5 g wet sample was used for

the cortisol assay and adjusted to 100% dry matter. The fecal cortisol data given in µg/dl was converted and reported as ng/g measurements.

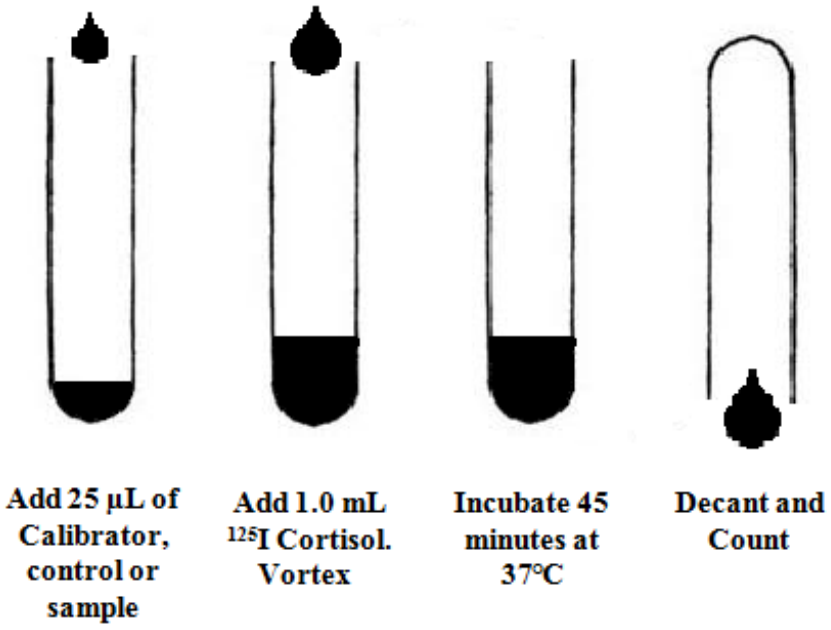

Figure 1. RIA Procedure for Fecal Samples. Adapted from Coat-A-Count® Cortisol (Siemens Medical Solutions, Los Angeles, CA).

Statistical Analysis

Statistical analyses were conducted using the PROC GLM procedure of SAS 9.1 to analyze fecal cortisol means and SEM, as well as the standard error of dietary nutrients (SAS Institute Inc., Cary, NC, USA). The effect of the study treatments on cortisol values were analyzed by individual rhinoceros. To better control for variation due to the subjects

(rhinoceros), each individual was subjected to all four treatments (diets) following the guidelines of the experimental design. Latin Square with Period was used as a column factor and Pair (of animals) as the row factor. There were as many periods and pairs as treatments (t=4). Each period (p= 8 total) received all treatments and the animal within each pair received the same treatment at any given period. A number of measurements were collected during the assigned time period for a total of approximately 16 observations per Latin square. Two Latin square rotations were performed, giving a total of 32 observations on average for each individual over the entire study of 238 days. Statistical significance was considered to be $P< 0.05$.

For each Latin Square, a general linear model was fitted for the cortisol response values and analyzed statistically by population. Period, Pair and Treatment were considered fixed effects in the model. Period by Pair and Animal within Pair were considered random effects. Repeated measures at each Period and Animal within the Pair combination (an corresponding treatment) were considered as sub-samples and source of variability (random effect). A combined analysis of both Latin Square rotations included the following fixed effects in the model: 1. Latin square rotation, 2. period with Latin square rotation, 3. pair, 4. interaction of Latin square rotation and pair, 5. treatment, 6. interaction of Latin square rotation and treatment, 7. interaction of period, treatment, and pair within the Latin square rotation. Animal within each pair was considered a random effect, as well as the interaction of Latin square rotation and animal within the pair. Repeated measures at each Latin Square rotation, Period and Animal within pair combination (and corresponding treatment) were considered as subsamples and source of variability (random effect).

Tukey's range test for multiple comparisons was used for mean separation. Statistical significance level was $\alpha = 0.05$. The area under the curve (AUC) and the average AUC value per unit-time interval was calculated for each individual rhinoceros to show the individual response for that animal.

Results

As analyzed there was no overall significance in effect between the four commercial pelleted complete feeds on fecal cortisol concentrations among the North Carolina Asheboro Zoo rhinoceros population (Table 3). However, there was a range in cortisol concentrations among the pelleted complete feeds as both an average and by rotation. Dietary nutrient values between the four study diets vary, particularly between the Wild Herbivore #5ZF1 diet and ADF 16 #5648. Wild Herbivore #5ZF1 can be considered the highest fiber, lowest starch study diet. The ADF 16 #5648 has the lowest fiber and highest starch levels of all four study diets.

An inter-individual cortisol concentration response to each of the pelleted complete feeds was noted between diets for individuals #942 ($P = 0.0188$), #1761 ($P = 0.0054$), and #1763 ($P = 0.0011$) (Table 4). The pre-study analysis of cortisol concentrations while on the original diet of Wild Herbivore #5ZF1 tended to be significantly different between the individuals ($P = 0.0012$), but not over time ($P = 0.6839$). The individual #1760 had significantly elevated fecal cortisol concentrations values over the entire study compared to the other individuals, but one of the lowest ranges (±2.13 ng/g). Individual #1763 had the largest range of fecal cortisol concentration values over the course of the study (±8.44 ng/g).

When evaluating significance between latin square rotations one and two, inter-individual cortisol concentration response was noted for individuals #942 (P= 0.0104), #1761 (P= 0.0061), #1763 (P= 0.0248), and #1925 (P= 0.0031) (Table 5). This supports an effect of time and environment on cortisol concentration values for these four rhinoceros. The same four individuals expressed significant impacts of the latin square rotations one and two by treatment on cortisol concentrations values (Table 6). When analyzed for lowest cortisol concentrations values by rhinoceros it appears that there is a range of dietary effects among individuals and that no one pelleted complete feed can be definitively selected for at this percent concentration of the overall diet. Tables 7-10 exhibit the overall dietary composition of total feed intake by individual to show the expected intake of nutrients across the diets. Due to the large amount of pasture intake, the pelleted complete feeds provide only a small variation between diets.

The Area Under the Curve (AUC) represents the change in cortisol levels through time, measured up to the last time point (238 days) to show an individual response for each Southern White Rhinoceros at the North Carolina Asheboro Zoo (Figures 2-8). The AUC calculation ignores changes in diet over the period of time. The P-value= 0.14 for the paired t-test on average AUC per day, indicating that the two rotations of the study did not show significant differences in average AUC per day for the set of animals in this study. The mean ± SD values for this herd of Southern White Rhinoceros ranged from 5.0 ± 3.45 to 17.0 ± 4.04, with a total of 227 samples taken (~32 samples/rhinoceros). AUC values ranged from 1,130 to 4,055 (ng/g)* (days) by individual. The average AUC values ranged from 4.8 to 17.0 (ng/g)*(1 day).

Table 3. Fiber, starch, protein, fat and iron levels of four commercial pelleted complete feeds specially formulated for the Latin-square rotation dietary study[1] on cortisol levels of the Southern White Rhinoceros (*Ceratotherium simum simum*) population at the North Carolina Asheboro Zoo

	Wild Herbivore #5ZF1[2,3]	LiFe Wild Herbivore #5Z0X	ADF 16 #5648	ADF 25 #5649
Dietary Nutrients				
Protein, %	13.0	13.8	17.0	15.0
Fat, %	3.25	3.90	3.30	3.00
Crude Fiber, %	27.0	20.7	11.0	19.0
NDF, %	49.0	43.4	26.0	36.0
ADF, %	32.0	25.5	15.0	24.0
Starch, %	3.4	6.0	24.0	7.0
Iron, ppm	315	185	290	350
Fecal Cortisol, ng/g, DM[4]				
Rotation One	9.1±1.08	9.1±0.85	10.2±0.86	9.6±0.83
Rotation Two	10.73±1.07[a]	10.1±0.79[a,b]	9.2±0.87[a,b]	7.9±1.07[b]
Average	9.9±1.08	9.6±0.82	9.7±0.87	8.75±0.95

[1] Feed supplied by Mazuri® Exotic Animal Nutrition, Land O' Lakes, Inc.

[2] Analytes of the study diets were standardized to: Ash,0.5%; Biotin,0.45%; Ca,0.9%; Choline,15%; Co,1.75%; Cu,20%; Fe,185-350%; Folic Acid, 1.6%; I,1.6%; K,0.3-0.35%; Linoleic Acid,0.5%; Mg,0.3-0.35%; Mn,125%; Na,0.5%; Niacin,55%; Omega-3 FA,0.5%; Omega-6 FA,0.5%; P,0.45-0.55%; Panacid,30%; Pyridoxine,10%; Riboflavin,20%; S,30%; Se,0.3%; Thiamin,5%; Vit. A, 2.95%; Vit. B12,10.45%; Vit. D3,1.2%; Vit. E,250%; Vit. K,5%; Zn,125% respectively.

[3] Original Southern White Rhinoceros zoo diet was Wild Herbivore Hi-Fiber #5ZF1 without standardized levels of vitamins and minerals.

[4] Significant differences in cortisol levels of each rhinoceros, evaluated by row, between the four study pelleted complete feeds ($P < 0.05$) are indicated by superscript [a,b]. Cortisol values are given in Mean ± SEM.

Table 4. Fecal cortisol level comparisons between pelleted complete feeds of seven Southern White Rhinoceros (*Ceratotherium simum simum*) at the North Carolina Asheboro Zoo while consuming four specially formulated study pelleted complete feeds[1]

	#941	#942	#1759	#1760	#1761	#1763	#1925
Cortisol, ng/g, DM[2,3,4]							
Original Diet[5]	11.98	9.94	7.27	17.71	4.37	11.40	15.97
ADF 16	5.98	10.08[a]	5.42	17.84	10.42[a]	6.21[a]	9.89
ADF 25	8.01	10.19	2.58	15.71	4.44[b]	7.90[a]	9.14
Wild Herbivore(WH)	7.19	13.13[b]	3.75	16.38	2.71[b]	14.65[b]	7.19
LiFe WH	6.98	10.84	3.81	16.67	6.72	9.78[a]	10.64
SEM	1.02	0.84	1.01	1.81	1.34	1.24	1.24

[1] Feed supplied by Mazuri® Exotic Animal Nutrition, Land O' Lakes, Inc.
[2] Fecal cortisol values are expressed on a 100% DM basis.
[3] Values are expressed as Mean ± SEM.
[4] Significant differences in cortisol levels of each rhinoceros, evaluated by column, between the four study pelleted complete feeds ($P < 0.05$) are indicated by superscript [a,b].
[5]The Original Diet is Wild Herbivore without standardized nutrients. Cortisol values were evaluated on this pelleted complete feed pre-study as a control period and is not evaluated for significance with the study pelleted complete feeds.

Table 5. Fecal cortisol level comparisons between Latin square rotation one and Latin square rotation two for the seven Southern White Rhinoceros (*Ceratotherium simum simum*) at the North Carolina Asheboro Zoo while consuming four specially formulated pelleted complete feeds[1]

	Rotation 1	Rotation 2	SEM
Cortisol ng/g, DM[2,3,4]			
#941	5.96	8.12	0.72
#942	12.66[a]	9.46[b]	0.60
#1759	3.48	4.30	0.72
#1760	16.10	17.20	1.28
#1761	3.96[a]	8.19[b]	0.95
#1763	8.05[a]	11.22[b]	0.87
#1925	11.47[a]	6.96[b]	0.88

[1] Feed supplied by Mazuri® Exotic Animal Nutrition, Land O' Lakes, Inc.
[2] Fecal cortisol values are expressed on a 100% DM basis.
[3] Values are expressed as Mean ± SEM.
[4] Significant differences in cortisol levels of each rhinoceros between latin square rotations, evaluated by row, of pelleted complete feeds ($P < 0.05$) are indicated by superscript [a,b]. Different letters indicate that cortisol concentrations differed between rotation one and rotation two of the pelleted complete feeds for that given Southern White Rhinoceros.

Table 6. Fecal cortisol level comparisons between the Latin square rotations of pelleted complete feeds of seven Southern White Rhinoceros (*Ceratotherium simum simum*) at the North Carolina Asheboro Zoo while consuming four specially formulated study pelleted complete feeds[1]

	#941	#942	#1759	#1760	#1761	#1763	#1925
Cortisol, ng/g, DM[2,3,4,5,6]							
ADF 16							
Rotation 1	6.28	13.84	5.99	16.37	5.39	3.01	15.18
Rotation 2	5.68	6.31	4.85	19.30	15.46	9.41	4.59
ADF 25							
Rotation 1	6.69	12.95	1.82	17.15	5.04	6.82	11.94
Rotation 2	9.33	7.43	3.34	14.26	3.83	8.98	6.34
Wild Herbivore							
Rotation 1	5.13	12.21	2.31	13.87	2.12	14.17	8.58
Rotation 2	9.24	14.05	5.19	18.90	3.30	15.14	5.80
LiFe Wild Herbivore							
Rotation 1	5.74	11.64	3.82	17.02	3.28	8.20	10.18
Rotation 2	8.22	10.03	3.81	16.32	10.16	11.36	11.10
SEM	1.45	1.17	1.43	2.55	1.90	1.74	1.75

[1] Feed supplied by Mazuri® Exotic Animal Nutrition, Land O' Lakes, Inc.

[2] Fecal cortisol values are expressed on a 100% DM basis.

[3] Values are expressed as Mean ± SEM.

[4]Effect of rotation (R1 versus R2) within each pelleted complete feed by individual: 1. #942: ADF 16-R1 and ADF 16-R2 (P= 0.0011), ADF 25- R1 and ADF 25- R2 (P= 0.0150), 2. #1761: ADF 16-R1 and ADF 16-R2 (P=0.0017), LiFe Wild Herbivore- R1 and LiFe Wild Herbivore- R2 (P= 0.0208), 3. #1763: ADF 16-R1 and ADF 16-R2 (P= 0.0176), 4. #1925: ADF 16-R1 and ADF 16-R2 (P= 0.0011), ADF 25- R1 and ADF 25- R2 (P= 0.0290).

[5]Effect of each pelleted complete feed by individual for rotation one: 1. #1763: ADF 16 and Wild Herbivore (P= 0.0003), ADF 16 and LiFe Wild Herbivore (P= 0.0471), ADF 25 and Wild Herbivore (P= 0.0152), Wild Herbivore and LiFe Wild Herbivore (P= 0.0250), 2. #1925: ADF 16 and Wild Herbivore (P= 0.0225), ADF 16 and LiFe Wild Herbivore (0.0470)

[6]Effect of each pelleted complete feed by individual for rotation two: 1. #942: ADF 16 and Wild Herbivore (P= 0.0005), ADF 16 and LiFe Wild Herbivore (P= 0.0308), ADF 25 and Wild Herbivore (P= 0.0058), Wild Herbivore and LiFe Wild Herbivore (P= 0.0129), 2. #1761: ADF 16 and ADF 25 (P= 0.0005), ADF 16 and Wild Herbivore (P= 0.0003), ADF 25 and LiFe Wild Herbivore (P= 0.0315), Wild Herbivore and LiFe Wild Herbivore (P= 0.0212), 3. #1763: ADF 16 and Wild Herbivore (P= 0.0305), ADF 25 and Wild Herbivore (P= 0.0214), 4. #1925: ADF 16 and LiFe Wild Herbivore (P= 0.0242).

Table 7. Dietary intake and analyte composition of the total diet consisting of pelleted complete feed, timothy hay and pasture bermudagrass for the North Carolina Asheboro Zoo Southern White Rhinoceros (*Ceratotherium simum simum*) #941, based on reported daily intake

	#941			
Intake (kg DM/d)				
Pellets[2]	6.4	6.4	6.4	6.4
Timothy Hay[3]	2.3	2.3	2.3	2.3
Pasture[4]	15.9	15.9	15.9	15.9
Study Diet	**ADF 16**	**ADF 25**	**WH[5]**	**LiFe WH**
Analyte (%, DM)				
Crude Protein, Diet[6]	15.31	14.79	14.27	14.48
Crude Protein, Feces[7]	10.99	13.02	10.33	10.35
NDF, Diet	48.79	51.39	54.78	53.32
NDF, Feces	51.27	53.97	58.80	57.18
ADF, Diet	29.58	31.92	34.00	32.31
ADF, Feces	45.52	48.00	50.25	48.14
Fat, Diet	2.70	2.62	2.68	2.86
Fat, Feces	5.07	8.22	6.52	6.19
Ash, Diet	6.66	6.66	6.66	6.66
Ash, Feces	17.82	13.85	20.33	14.51

[1] Feed supplied by Mazuri® Exotic Animal Nutrition, Land O' Lakes, Inc.

[2]Daily pellet intake based on weight per scoop from feed intake records for each individual rhinoceros.

[3]Daily timothy hay intake, while on exhibit pasture, is based on an allotment of one bale per day (15.9 kg bale) divided amongst the seven rhinoceroses in the herd and assuming equal intake per rhinoceros.

[4]Daily pasture intake calculated based on reported timothy hay supplementation of nine bales of hay per day (143.1 kg) divided amongst the seven rhinoceroses in the herd when off of the pasture exhibit. It is assumed that there is equal intake for each individual rhinoceros.

[5]WH= Wild Herbivore

[6]Diet analytes are based off of the combined reported pelleted complete feed values, analyzed timothy hay and analyzed pasture bermudagrass. Analysis performed by the North Carolina Department of Agriculture and Consumer Services, Farm Feed Testing Services Laboratory, Raleigh, NC.

[7]Feces was analyzed by the North Carolina Department of Agriculture and Consumer Services, Farm Feed Testing Services Laboratory, Raleigh, NC to determine fecal analyte values per individual rhinoceros for each of the four study diets.

Table 8. Dietary intake and analyte composition of the total diet consisting of pelleted complete feed, timothy hay and pasture bermudagrass for the North Carolina Asheboro Zoo Southern White Rhinoceros (*Ceratotherium simum simum*) #942, based on reported daily intake

	#942			
Intake (kg DM/d)				
Pellets[2]	4.8	4.8	4.8	4.8
Timothy Hay[3]	2.3	2.3	2.3	2.3
Pasture[4]	15.9	15.9	15.9	15.9
Study Diet	**ADF 16**	**ADF 25**	**WH[5]**	**LiFe WH**
Analyte (%, DM)				
Crude Protein, Diet[6]	15.19	14.77	14.36	14.53
Crude Protein, Feces[7]	11.29	10.43	10.23	10.40
NDF, Diet	50.42	52.49	55.19	54.03
NDF, Feces	55.77	NA[8]	50.60	59.15
ADF, Diet	30.62	32.49	34.14	32.80
ADF, Feces	46.42	44.60	44.00	46.39
Fat, Diet	2.66	2.60	2.64	2.78
Fat, Feces	7.97	5.95	9.50	7.45
Ash, Diet	7.10	7.10	7.10	7.10
Ash, Feces	15.45	NA	12.32	10.87

[1] Feed supplied by Mazuri® Exotic Animal Nutrition, Land O' Lakes, Inc.

[2]Daily pellet intake based on weight per scoop from feed intake records for each individual rhinoceros.

[3]Daily timothy hay intake, while on exhibit pasture, is based on an allotment of one bale per day (15.9 kg bale) divided amongst the seven rhinoceroses in the herd and assuming equal intake per rhinoceros.

[4]Daily pasture intake calculated based on reported timothy hay supplementation of nine bales of hay per day (143.1 kg) divided amongst the seven rhinoceroses in the herd when off of the pasture exhibit. It is assumed that there is equal intake for each individual rhinoceros.

[5]WH= Wild Herbivore

[6]Diet analytes are based off of the combined reported pelleted complete feed values, analyzed timothy hay and analyzed pasture bermudagrass. Analysis performed by the North Carolina Department of Agriculture and Consumer Services, Farm Feed Testing Services Laboratory, Raleigh, NC.

[7]Feces was analyzed by the North Carolina Department of Agriculture and Consumer Services, Farm Feed Testing Services Laboratory, Raleigh, NC to determine fecal analyte values per individual rhinoceros for each of the four study diets.

[8]NA= Information not available due to insufficient sample size.

Table 9. Dietary intake and analyte composition of the total diet consisting of pelleted complete feed, timothy hay and pasture bermudagrass for the North Carolina Asheboro Zoo Southern White Rhinoceros (*Ceratotherium simum simum*) #1759, based on reported daily intake

	#1759			
Intake (kg DM/d)				
Pellets[2]	1.6	1.6	1.6	1.6
Timothy Hay[3]	2.3	2.3	2.3	2.3
Pasture[4]	15.9	15.9	15.9	15.9
Study Diet	**ADF 16**	**ADF 25**	**WH[5]**	**LiFe WH**
Analyte (%, DM)				
Crude Protein, Diet[6]	14.90	14.74	14.58	14.64
Crude Protein, Feces[7]	11.84	11.36	12.89	15.06
NDF, Diet	54.32	55.13	56.18	55.73
NDF, Feces	50.57	60.42	52.48	50.36
ADF, Diet	33.11	33.84	34.49	33.96
ADF, Feces	40.1	42.11	45.97	48.22
Fat, Diet	3.02	2.53	2.54	2.60
Fat, Feces	7.75	6.04	8.54	9.17
Ash, Diet	8.16	8.16	8.16	8.16
Ash, Feces	22.23	16.25	22.49	22.26

[1] Feed supplied by Mazuri® Exotic Animal Nutrition, Land O' Lakes, Inc.

[2]Daily pellet intake based on weight per scoop from feed intake records for each individual rhinoceros.

[3]Daily timothy hay intake, while on exhibit pasture, is based on an allotment of one bale per day (15.9 kg bale) divided amongst the seven rhinoceroses in the herd and assuming equal intake per rhinoceros.

[4]Daily pasture intake calculated based on reported timothy hay supplementation of nine bales of hay per day (143.1 kg) divided amongst the seven rhinoceroses in the herd when off of the pasture exhibit. It is assumed that there is equal intake for each individual rhinoceros.

[5]WH= Wild Herbivore

[6]Diet analytes are based off of the combined reported pelleted complete feed values, analyzed timothy hay and analyzed pasture bermudagrass. Analysis performed by the North Carolina Department of Agriculture and Consumer Services, Farm Feed Testing Services Laboratory, Raleigh, NC.

[7]Feces was analyzed by the North Carolina Department of Agriculture and Consumer Services, Farm Feed Testing Services Laboratory, Raleigh, NC to determine fecal analyte values per individual rhinoceros for each of the four study diets.

Table 10. Dietary intake and analyte composition of the total diet consisting of pelleted complete feed, timothy hay and pasture bermudagrass for the North Carolina Asheboro Zoo Southern White Rhinoceros (*Ceratotherium simum simum*) #1760, based on reported daily intake

	#1760			
Intake (kg DM/d)				
Pellets[2]	1.6	1.6	1.6	1.6
Timothy Hay[3]	2.3	2.3	2.3	2.3
Pasture[4]	15.9	15.9	15.9	15.9
Study Diet	**ADF 16**	**ADF 25**	**WH[5]**	**LiFe WH**
Analyte (%, DM)				
Crude Protein, Diet[6]	14.90	14.74	14.58	14.64
Crude Protein, Feces[7]	13.15	13.21	11.35	8.90
NDF, Diet	54.32	55.13	56.18	55.73
NDF, Feces	54.45	54.02	51.35	59.39
ADF, Diet	33.11	33.84	34.49	33.96
ADF, Feces	47.48	46.73	49.07	46.31
Fat, Diet	3.02	2.53	2.54	2.60
Fat, Feces	8.84	9.81	9.09	5.86
Ash, Diet	8.16	8.16	8.16	8.16
Ash, Feces	21.29	21.66	18.69	17.78

[1] Feed supplied by Mazuri® Exotic Animal Nutrition, Land O' Lakes, Inc.

[2]Daily pellet intake based on weight per scoop from feed intake records for each individual rhinoceros.

[3]Daily timothy hay intake, while on exhibit pasture, is based on an allotment of one bale per day (15.9 kg bale) divided amongst the seven rhinoceroses in the herd and assuming equal intake per rhinoceros.

[4]Daily pasture intake calculated based on reported timothy hay supplementation of nine bales of hay per day (143.1 kg) divided amongst the seven rhinoceroses in the herd when off of the pasture exhibit. It is assumed that there is equal intake for each individual rhinoceros.

[5]WH= Wild Herbivore

[6]Diet analytes are based off of the combined reported pelleted complete feed values, analyzed timothy hay and analyzed pasture bermudagrass. Analysis performed by the North Carolina Department of Agriculture and Consumer Services, Farm Feed Testing Services Laboratory, Raleigh, NC.

[7]Feces was analyzed by the North Carolina Department of Agriculture and Consumer Services, Farm Feed Testing Services Laboratory, Raleigh, NC to determine fecal analyte values per individual rhinoceros for each of the four study diets.

Table 11. Dietary intake and analyte composition of the total diet consisting of pelleted complete feed, timothy hay and pasture bermudagrass for the North Carolina Asheboro Zoo Southern White Rhinoceros (*Ceratotherium simum simum*) #1761, based on reported daily intake

	#1761			
Intake (kg DM/d)				
Pellets[2]	1.6	1.6	1.6	1.6
Timothy Hay[3]	2.3	2.3	2.3	2.3
Pasture[4]	15.9	15.9	15.9	15.9
Study Diet	**ADF 16**	**ADF 25**	**WH[5]**	**LiFe WH**
Analyte (%, DM)				
Crude Protein, Diet[6]	14.90	14.74	14.58	14.64
Crude Protein, Feces[7]	12.56	11.31	11.20	12.92
NDF, Diet	54.32	55.13	56.18	55.73
NDF, Feces	49.44	59.89	52.39	51.01
ADF, Diet	33.11	33.84	34.49	33.96
ADF, Feces	46.79	42.74	49.39	49.43
Fat, Diet	3.02	2.53	2.54	2.60
Fat, Feces	NA[8]	4.95	7.33	8.75
Ash, Diet	8.16	8.16	8.16	8.16
Ash, Feces	23.64	14.72	25.28	24.28

[1] Feed supplied by Mazuri® Exotic Animal Nutrition, Land O' Lakes, Inc.

[2]Daily pellet intake based on weight per scoop from feed intake records for each individual rhinoceros.

[3]Daily timothy hay intake, while on exhibit pasture, is based on an allotment of one bale per day (15.9 kg bale) divided amongst the seven rhinoceroses in the herd and assuming equal intake per rhinoceros.

[4]Daily pasture intake calculated based on reported timothy hay supplementation of nine bales of hay per day (143.1 kg) divided amongst the seven rhinoceroses in the herd when off of the pasture exhibit. It is assumed that there is equal intake for each individual rhinoceros.

[5]WH= Wild Herbivore

[6]Diet analytes are based off of the combined reported pelleted complete feed values, analyzed timothy hay and analyzed pasture bermudagrass. Analysis performed by the North Carolina Department of Agriculture and Consumer Services, Farm Feed Testing Services Laboratory, Raleigh, NC.

[7]Feces was analyzed by the North Carolina Department of Agriculture and Consumer Services, Farm Feed Testing Services Laboratory, Raleigh, NC to determine fecal analyte values per individual rhinoceros for each of the four study diets.

[8]NA= Information not available due to insufficient sample size.

Table 12. Dietary intake and analyte composition of the total diet consisting of pelleted complete feed, timothy hay and pasture bermudagrass for the North Carolina Asheboro Zoo Southern White Rhinoceros (*Ceratotherium simum simum*) #1763, based on reported daily intake

	#1763			
Intake (kg DM/d)				
Pellets[2]	1.6	1.6	1.6	1.6
Timothy Hay[3]	2.3	2.3	2.3	2.3
Pasture[4]	15.9	15.9	15.9	15.9
Study Diet	**ADF 16**	**ADF 25**	**WH[5]**	**LiFe WH**
Analyte (%, DM)				
Crude Protein, Diet[6]	14.90	14.74	14.58	14.64
Crude Protein, Feces[7]	12.24	13.02	10.52	10.17
NDF, Diet	54.32	55.13	56.18	55.73
NDF, Feces	56.37	NA[8]	53.42	57.74
ADF, Diet	33.11	33.84	34.49	33.96
ADF, Feces	48.24	45.23	46.94	44.90
Fat, Diet	3.02	2.53	2.54	2.60
Fat, Feces	8.08	8.39	9.79	6.70
Ash, Diet	8.16	8.16	8.16	8.16
Ash, Feces	23.70	NA	17.89	17.45

[1] Feed supplied by Mazuri® Exotic Animal Nutrition, Land O' Lakes, Inc.

[2]Daily pellet intake based on weight per scoop from feed intake records for each individual rhinoceros.

[3]Daily timothy hay intake, while on exhibit pasture, is based on an allotment of one bale per day (15.9 kg bale) divided amongst the seven rhinoceroses in the herd and assuming equal intake per rhinoceros.

[4]Daily pasture intake calculated based on reported timothy hay supplementation of nine bales of hay per day (143.1 kg) divided amongst the seven rhinoceroses in the herd when off of the pasture exhibit. It is assumed that there is equal intake for each individual rhinoceros.

[5]WH= Wild Herbivore

[6]Diet analytes are based off of the combined reported pelleted complete feed values, analyzed timothy hay and analyzed pasture bermudagrass. Analysis performed by the North Carolina Department of Agriculture and Consumer Services, Farm Feed Testing Services Laboratory, Raleigh, NC.

[7]Feces was analyzed by the North Carolina Department of Agriculture and Consumer Services, Farm Feed Testing Services Laboratory, Raleigh, NC to determine fecal analyte values per individual rhinoceros for each of the four study diets.

[8]NA= Information not available due to insufficient sample size.

Table 13. Dietary intake and analyte composition of the total diet consisting of pelleted complete feed, timothy hay and pasture bermudagrass for the North Carolina Asheboro Zoo Southern White Rhinoceros (*Ceratotherium simum simum*) #1925, based on reported daily intake

	#1925			
Intake (kg DM/d)				
Pellets[2]	3.2	3.2	3.2	3.2
Timothy Hay[3]	2.3	2.3	2.3	2.3
Pasture[4]	15.9	15.9	15.9	15.9
Study Diet	**ADF 16**	**ADF 25**	**WH[5]**	**LiFe WH**
Analyte (%, DM)				
Crude Protein, Diet[6]	15.35	15.05	14.75	14.87
Crude Protein, Feces[7]	9.81	7.76	12.74	12.03
NDF, Diet	54.38	55.88	57.82	56.99
NDF, Feces	57.96	51.65	51.96	50.06
ADF, Diet	33.15	34.50	35.70	34.72
ADF, Feces	47.95	49.30	48.98	47.29
Fat, Diet	2.70	2.65	2.70	2.79
Fat, Feces	5.33	3.93	7.14	7.40
Ash, Diet	7.82	7.82	7.82	7.82
Ash, Feces	17.97	40.87	23.13	22.26

[1] Feed supplied by Mazuri® Exotic Animal Nutrition, Land O' Lakes, Inc.

[2]Daily pellet intake based on weight per scoop from feed intake records for each individual rhinoceros.

[3]Daily timothy hay intake, while on exhibit pasture, is based on an allotment of one bale per day (15.9 kg bale) divided amongst the seven rhinoceroses in the herd and assuming equal intake per rhinoceros.

[4]Daily pasture intake calculated based on reported timothy hay supplementation of nine bales of hay per day (143.1 kg) divided amongst the seven rhinoceroses in the herd when off of the pasture exhibit. It is assumed that there is equal intake for each individual rhinoceros.

[5]WH= Wild Herbivore

[6]Diet analytes are based off of the combined reported pelleted complete feed values, analyzed timothy hay and analyzed pasture bermudagrass. Analysis performed by the North Carolina Department of Agriculture and Consumer Services, Farm Feed Testing Services Laboratory, Raleigh, NC.

[7]Feces was analyzed by the North Carolina Department of Agriculture and Consumer Services, Farm Feed Testing Services Laboratory, Raleigh, NC to determine fecal analyte values per individual rhinoceros for each of the four study diets.

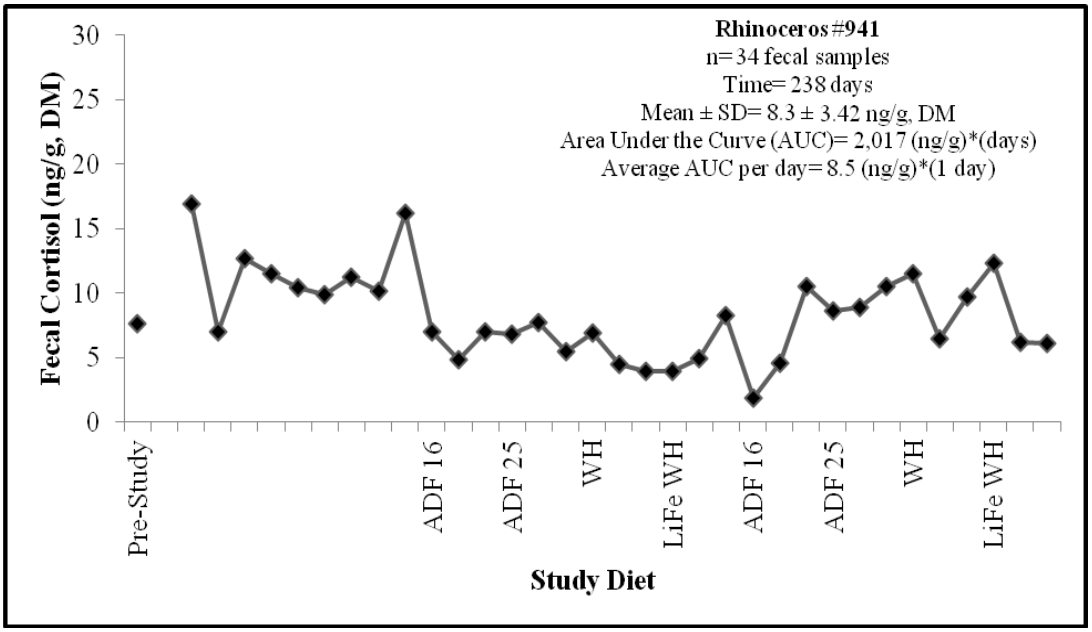

Figure 2. Cortisol levels for the Southern White Rhinoceros (*Ceratotherium simum simum*) individual #941 at the North Carolina Asheboro Zoo, while on four study pelleted complete feeds.

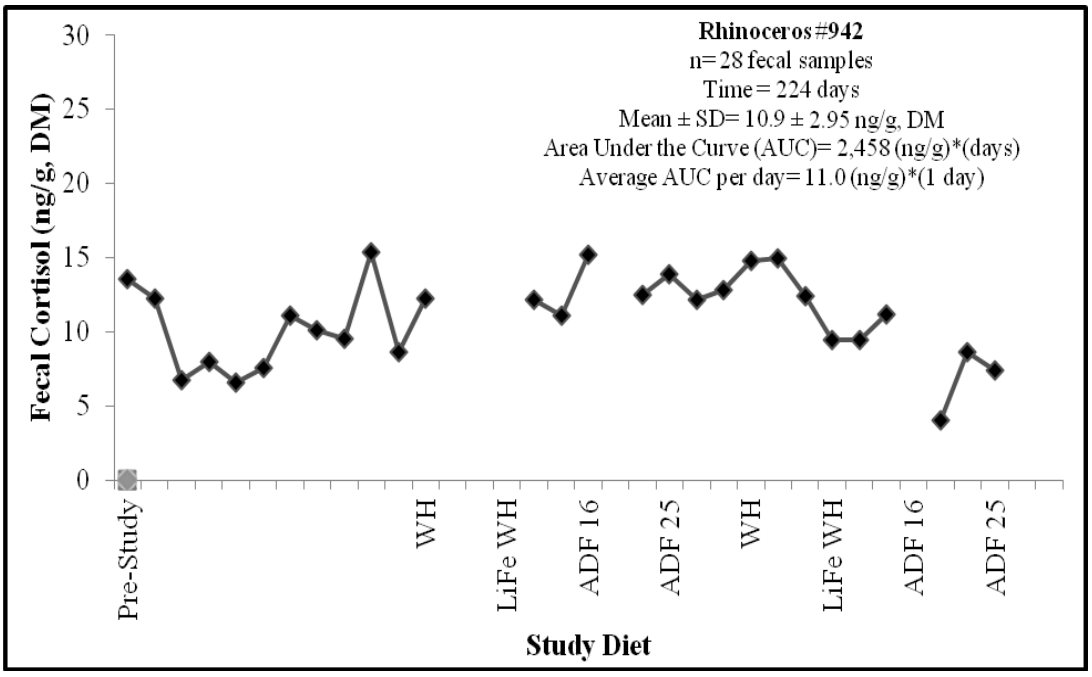

Figure 3. Cortisol levels for the Southern White Rhinoceros (*Ceratotherium simum simum*) individual #942 at the North Carolina Asheboro Zoo, while on four study pelleted complete feeds.

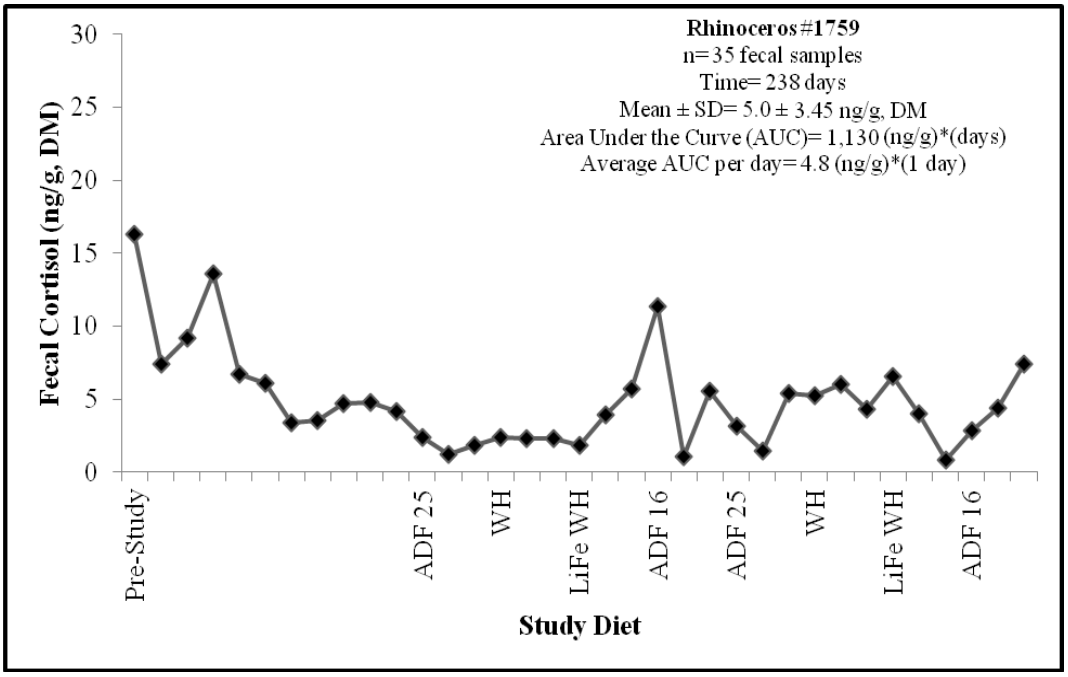

Figure 4. Cortisol levels for the Southern White Rhinoceros (*Ceratotherium simum simum*) individual #1759 at the North Carolina Asheboro Zoo, while on four study pelleted complete feeds.

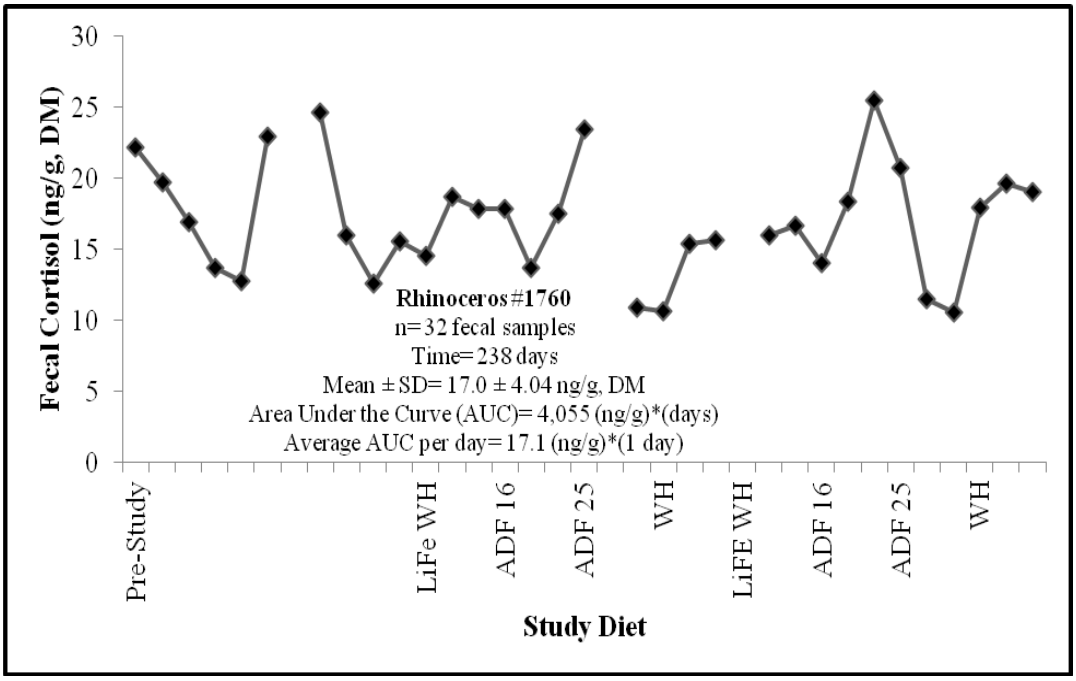

Figure 5. Cortisol levels for the Southern White Rhinoceros (*Ceratotherium simum simum*) individual #1760 at the North Carolina Asheboro Zoo, while on four study pelleted complete feeds.

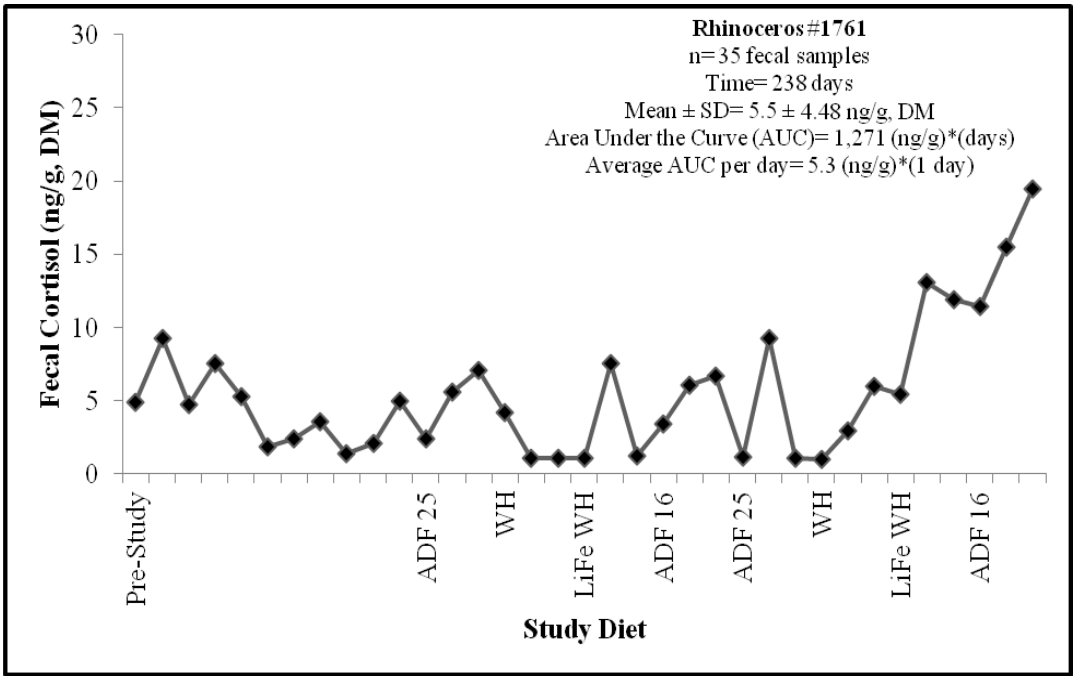

Figure 6. Cortisol levels for the Southern White Rhinoceros (*Ceratotherium simum simum*) individual #1761 at the North Carolina Asheboro Zoo, while on four study pelleted complete feeds.

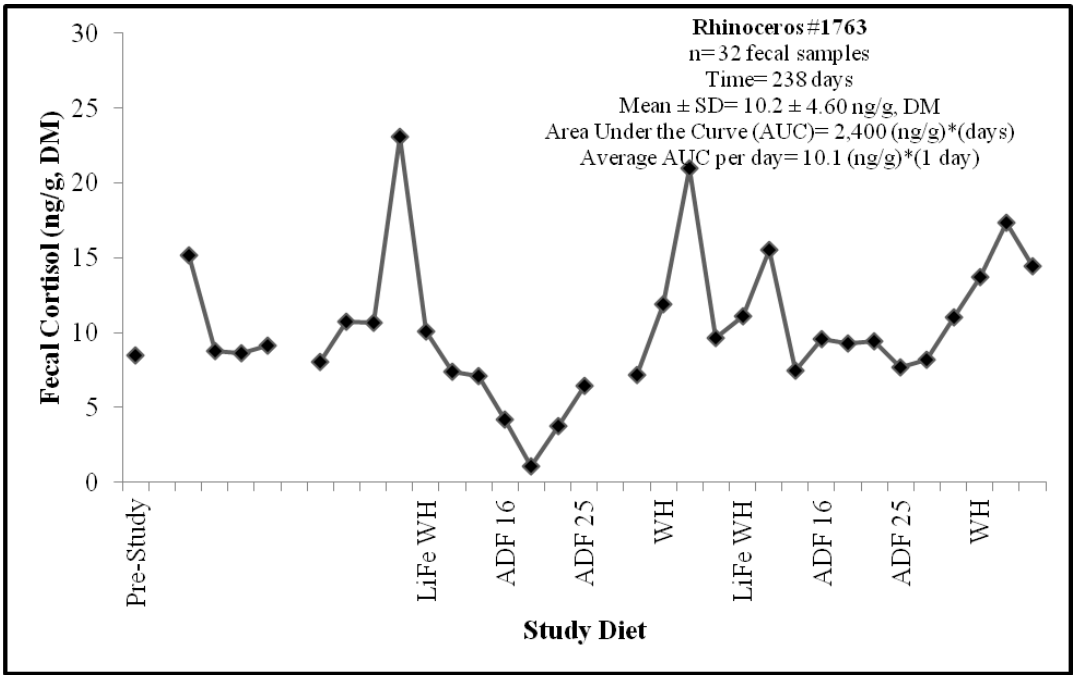

Figure 7. Cortisol levels for the Southern White Rhinoceros (*Ceratotherium simum simum*) individual #1763 at the North Carolina Asheboro Zoo, while on four study pelleted complete feeds.

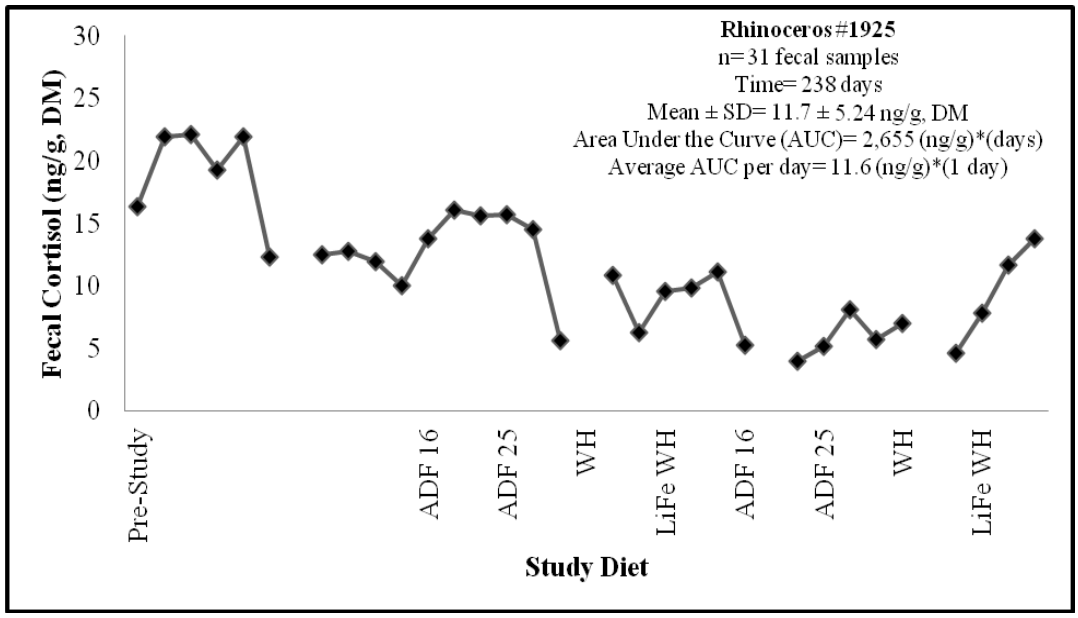

Figure 8. Cortisol levels for the Southern White Rhinoceros (*Ceratotherium simum simum*) individual #1925 at the North Carolina Asheboro Zoo, while on four study pelleted complete feeds.

Discussion

The Southern White Rhinoceros (*Ceratotherium simum simum*) is classified as a grazer, with hindgut fermentation, that strictly feeds on grasses (Lintzenich & Ward, 1997). In the wild habitat they are able to maintain grazing lawns and consume grasses according to availability; however, in captivity, many rhinoceros populations are restricted to smaller pastures and grass availability (Perrin & Brerton-Stiles, 1999). Pasture drives the majority of nutrient intake in this trial , but the diet is supplemented with a pelleted complete feed in order to meet recommended nutritional requirements to maintain the Southern White Rhinoceros in captivity based on the National Research Council's (NRC) domestic horse nutrient recommendations (Clauss & Hatt, 2006; Dierenfeld, 1999; National Research Council, 1989). Analyses of the composition of wild white rhinoceros diets for comparison are scarce; therefore, the horse, which has a similar hindgut fermentation digestive tract as the Southern White Rhinoceros is used as a nutritional model to make dietary recommendations in captivity (Dierenfeld, 1999). As a large, non-ruminant herbivore, this species does not experience daily food intake limitations due to a slow passage rate of fiber, because they digest it less completely than ruminant species. Therefore, they should be able to obtain minimum nutrient requirements from poorer energy diets than ruminant species and can have a diet made up in bulk of grasses (Shrader et al., 2006).

The aim of this study was to determine if the different diet formulations, specifically if the amount of fiber, would have an effect on the amount of cortisol in the feces. Variations in cortisol concentrations were widely ranging due to the inter-individual affects of perceived stress (Carlstead & Brown, 2005). Cortisol has both a species-specific and inter-individual

response to the stress-mediated mechanism (Carlstead & Brown, 2005; Millspaugh & Washburn, 2004). This study evaluated a total of 227 samples, approximately 32 samples per individual rhinoceros, making it one of the largest collections of cortisol data to date for the Southern White Rhinoceros. Average fecal cortisol values by individual ranged from 5.0 ± 3.45 ng/g to 17.0 ± 4.04 ng/g, DM, spanning 12.0 ng/g. This range in averages supports previous literature stating that cortisol response is inter-individual specific, especially within the same captive setting, while likely being exposed to the same acute and chronic stressors as all other individuals in the herd (Carlstead & Brown, 2005). All fecal samples were taken via direct rectal collection to prevent contamination or degradation of the cortisol metabolites in the feces. There is a possibility that keeper interaction during fecal collection had a small effect of the stress response of the individual. It is unlikely that the sample was compromised, due to the fact that the keeper interaction would be considered an acute stressor, allowing homeostasis to return to the individual rhinoceros's system once the stimulus was over. Fecal cortisol levels are an image of chronic stress over a long-term period (up to a week); therefore, it would be expected that once habituated to fecal collection, this stimulus would have little to no effect on fecal cortisol levels (Brown et al., 2001; Turner et al., 2002).

There did not appear to be a clear relationship for cortisol concentrations of the population (n=7) and the pelleted complete feed treatments in this particular study. This is likely due to the fact that the pellets were only a small percentage (6.5- 21.9%) of the overall diet and therefore did not have a significantly large enough impact on the individual animal's cortisol values. The North Carolina Asheboro Zoo is fortunate to have an abundant pasture

for grazing and therefore can feed a smaller percentage of pellets to supplement the overall diet in order to meet the minimum recommended nutritional requirements. No correlation between percent of pellets in the overall diet consumptions and elevated cortisol concentrations were noted. Future studies should be performed at locations that require a larger percentage of pelleted complete feeds to make up the overall diet in order to determine if there is a true affect of the pellets on cortisol concentration levels. These findings are supported by previous research that reported no correlation between cortisol concentration and diet in the domestic horse (Stull, 1988).

On average as a population (n=7), cortisol concentration values (range= 8.13-9.49 ng/g, DM) were higher than previously reported averages for unstressed and habituated white rhinoceroses, but fall within the range of previously reported fecal cortisol concentration values (range= 3.4-28.3 ng/g, DM) (Turner et al., 2002). Among individual's average the range for this study was 1.82-19.30 ng/g. Thus, results from the current work appear valid.

Increased cortisol concentrations may lead to decreased metabolism, increased visceral fat deposition, decreased immune response, gastrointestinal dysfunction, feed-intake effects and suppression of ovarian function or testosterone production (Evans et al., 1977; Möstl & Palme, 2002; Seematter, 2005; Turner et al., 2002). These possible deleterious physiological effects are the results of chronic activation of the hypothalamic pituitary adrenal axis that results in the increased cortisol levels (Möstl & Palme, 2002). Cortisol also plays a major role in digestion by initiating gluconeogenesis and energy metabolism in the digestive system, through activation of the HPA axis (Stull, 1988). If this process is hindered, the animal will be unable to mobilize the appropriate systems for proper digestions and

metabolism of the feed.

Several studies have previously measured cortisol concentrations in feces to determine stress levels of the Southern White Rhinoceros (Brown et al., 2001; Metrione & Harder, 2011; Turner et al., 2002). Fecal cortisol is an extremely beneficial method for less invasive measurements of stress in wild animals that gives an integrated evaluation of secretion and metabolism over a 24-48 hour period (Sheriff et al., 2010; Turner et al., 2002; Whitten et al., 1998). Other methods for evaluating cortisol, such as serum and saliva, give an acute picture of cortisol concentrations in response to stress and can easily miss a reaction if not taken in the appropriate window of time. These collection methods are also challenging to acquire from wild animals without having a direct affect on their stress levels (Whitten et al., 1998).

There are numerous other possible external and internal factors that can impact cortisol concentration levels of an animal including gender, age, weight, natal vs. non-natal institution, captive vs. wild born, social dominance, cyclic vs. acyclic, exploration, training, human interaction, and maternal stress effects (Metrione & Harder, 2011; Sheriff et al., 2010). Many of these stressors could be interpreted as good stress, or eustress; however, it is important to note that when analyzing cortisol as a measure of stress, negative and positive stressors are measured the same way without the ability to differentiate between the two, as cortisol effects remain the same. Effects of physical stress and obesity and been reported to have direct influences on elevated cortisol concentrations (Seematter et al., 2005). The North Carolina Asheboro Zoo held the Southern White Rhinoceros species from both genders and a wide age range or 10-45 years old. There were not enough representatives to statistically

analyze for the effect of location, gender or age on cortisol concentrations in this study. Future studies further evaluating effects of animal housing and management are recommended in correlation with a captive setting that utilizes a larger percentage of pelleted complete feeds in the diet before making a conclusive decision on the effects of diet on cortisol concentrations in the captive Southern White Rhinoceros.

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