THE EFFECTS OF INCREASED HAY-GRAIN RATIO
ON MASAI GIRAFFE BEHAVIOR, HEALTH INDICATORS
AND FECAL MICROFLORA DIVERSITY

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The Effects of Increased Hay-to-Grain Ratio on Masai Giraffe Behavior, Health Indicators and Fecal Microflora Diversity

MICHAEL L. MONSON

ABSTRACT

We hypothesized that switching to a diet that provides a higher hay-to-grain ratio offered to four Masai giraffe (Giraffa camelopardalis tippelskirchi) at the Cleveland Metroparks Zoo would reduce oral stereotypies and increase time spent performing feeding behaviors, maintain or increase serum calcium-to-phosphorus ratio, decrease serum insulin-to-glucose ratio, and alter fecal microbiome community structure. The diet change consisted of transitioning the male from a 50:50 hay-to-grain ratio and the females from a 70:30 hay-to-grain ratio to a 90:10 ratio in even increments over eight weeks. A ration balancer was added during the 7th week of transition to ensure proper mineral and nutrient balance of the overall diet. Behavioral data, saliva, serum and fecal samples were collected during the eight weeks preceding and following the diet change. Behavioral data were collected approximately daily using instantaneous focal sampling. Serum was collected biweekly for insulin-to-glucose and calcium-to-phosphorus ratio analysis. Fecal samples were collected weekly to examine changes in microflora community structure. After the diet change, giraffe spent significantly more time feeding and significantly less time performing tongue and mouth stereotypies and people-directed and alert behaviors. The giraffe also experienced a significant decrease in salivary and serum insulin and serum insulin-to-glucose ratio. The diet manipulation did not result in a change in the serum calcium-to-phosphorus ratio, which remained >1:1 throughout the study. A significant shift in fecal microflora community structure was observed with
effects from treatment and individual animal; further studies are needed to elucidate the nature of the change in fecal microbial community structure. We believe these changes represent an overall improvement in health from feeding a higher proportion of forage in the diet, and the reduced stereotypic behaviors may indicate an improvement in the welfare of these animals.
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CHAPTER I: INTRODUCTION AND BACKGROUND

1.1 Giraffe Biology and Conservation

Conservation of a Species

The giraffe is an iconic, African ungulate. The giraffe’s taxonomy has passed through several different revisions over the years, shifting between eight distinct species, two species with three subspecies each, one species with nine subspecies, and the currently accepted four species (Seymour 2001, Groves and Grubb 2011, Dagg 1971, Fennessy et al. 2016). Masai giraffe (Table 1) is the most populous of the subspecies (Fennessy 2008). However, recent surveys have estimated that giraffe have suffered loses up to 40% since the late 1990’s (Fennessy 2012).

Table 1. Summary of current estimated giraffe subspecies status. (Fennessy 2008)

<table>
<thead>
<tr>
<th>Subspecies</th>
<th>Common name</th>
<th>Estimated Numbers</th>
<th>Subspecies Status</th>
</tr>
</thead>
<tbody>
<tr>
<td>G.c. angolensis</td>
<td>Angolan</td>
<td>18,390</td>
<td>Increasing</td>
</tr>
<tr>
<td>G.c. antiquorum</td>
<td>Kordofan</td>
<td>-</td>
<td>Possibly Extinct</td>
</tr>
<tr>
<td>G.c. Camelopardalis</td>
<td>Nubian</td>
<td>160</td>
<td>Decreasing</td>
</tr>
<tr>
<td>G.c. giraffe</td>
<td>Cape</td>
<td>13,390</td>
<td>Stable/Increasing</td>
</tr>
<tr>
<td>G.c. peralta</td>
<td>Nigerian</td>
<td>2,855</td>
<td>Decreasing</td>
</tr>
<tr>
<td>G.c. reticulate</td>
<td>Reticulated</td>
<td>27,680</td>
<td>Stable/Decreasing</td>
</tr>
<tr>
<td>G.c. rothschildi</td>
<td>Rothschild's</td>
<td>290</td>
<td>Decreasing</td>
</tr>
<tr>
<td>G.c. thornicrofti</td>
<td>Thornicroft's</td>
<td>1,160</td>
<td>Stable</td>
</tr>
<tr>
<td>G.c. tippelskirchi</td>
<td>Masai</td>
<td>40,210</td>
<td>Decreasing</td>
</tr>
</tbody>
</table>
Current threats to the Masai giraffe include illegal hunting for bushmeat, livestock disturbance, and declining woodland cover due to growing human populations destroying the giraffe’s natural habitat in favor of commercial crops (Dagg 2014). Efforts to expand this subspecies have been successful in areas in which they are not native. For example, two males and four females introduced to the Akagera National Park in Rwanda have provided a population over 100 strong in a little over 25 years (Dagg 2014, Marais et al. 2012).

Giraffe Behavior in the Wild

Dagg (2014) has described giraffe herds as having similarities to the fission/fusion groups displayed by higher-level primates such as chimpanzees and humans. Foster and Dagg (1972) observed that females would form fluid groups that continually changed in composition while adult males would generally be alone. Leuthold (1979), in a separate study, observed that the average group was 3.8 animals and subadult males would usually form separate groups and travel together. The average life span in the wild for a Masai giraffe is approximately 25 years, which is comparable to captive conspecifics (Dagg and Foster 1982). Females reach reproductive age around 4 years old and will continue to cycle every two weeks until pregnant (Bercovitch and Berry 2013). They have a gestation period between 446-470 days depending on the giraffe and subspecies; they become fertile again three weeks after giving birth (del Castillo et al. 2005).

In the wild, giraffe use their tongues extensively while gathering and consuming browse, spending from 41% to over 77% of their time foraging and feeding (Ginnett and Demment 1997, Du Toit and Yetman 2005). Large African ruminants, such as giraffe,
spend more time feeding than their smaller counterparts (Du Toit and Yetman 2005). A proposed explanation for this is that due to the properties of their food, they cannot increase food intake rate, forcing them to increase feed time to meet metabolic requirements (Du Toit and Yetman 2005). Wild giraffe consume approximately 1.6% and 2.1% for males and females respectively of their body weight daily, which is similar to other ruminants (Pellew 1984b). Studies have recorded giraffe feeding on over 65 types of plant species depending on range and season (Dagg 2014, Leuthold and Leuthold 1972). Sauer et al (1982, 1983) concluded that the giraffe choice was guided towards vegetation high in water and crude protein. Pellew (1983) found variations in the nutritional quality of items in the giraffe’s diet season to season. Pellew (1983) also discusses how wild browsing giraffe are assumed to continually adjust what food items are prioritized based on this factor and others such as presence of thorns and the time they can devote to searching and chewing.

Stereotypic behaviors are not as prevalent in wild giraffe as in captive conspecifics (Veasey et al. 1996). Studies (Fernandez et al. 2008, Reber 2008, Sato and Takagaki 1991) have reported some captive giraffe have been known to exhibit stereotypic behaviors over 15% of their time compared to the 0.8 % average for wild populations (Veasey et al. 1996). These researchers also noted that tongue-playing stereotypies occurred directly after feeding and drinking, giving rise to the idea that it may have some underlying purpose (Veasey et al. 1996).
Feeding Physiology

The giraffe is a large browsing foregut ruminant. They are known to grow up to 5.5 meters tall in the wild with sexual dimorphism of males weighing 850-1950 kg and females weighing 700-1200 kg (Fowler et al. 2003). They have many adaptations to support their browsing lifestyle such as their tall body and long tongue to reach high vegetation, a prehensile tongue, thick lips and long eyelashes to protect against thorns and sticks, and by nature of being a ruminant, a rumen.

The rumen complex and intestines are highly specialized to be efficient in the breakdown and fermentation of plant matter through the aid of microorganisms, and is estimated to make up approximately 12% of the giraffe’s total mass (Mitchell et al. 2015). The four chambered stomach consists of the rumen-reticulum complex, the omasum and the abomasum (Vaughan 1972). The rumen is where the food is initially stored after consumption and begins to be fermented by the microflora located in it. The giraffe will regurgitate this material in the form of a cud and chew, reswallow and repeat until the plant matter sinks down into the reticulum portion of the stomach. From here, it passes into the omasum for absorbing liquid then the abomasum, which is the most similar region to the non-ruminant stomach, before passing to the small intestine.

The giraffe, like other ruminants, rely on microorganisms to breakdown plant matter through fermentation into components they can utilize for energy. The microflora community composition in ruminants are altered from changes in diet such as low to high grain and even more subtle changes such as Bermuda grass hay to grazed winter wheat, which may represent a change in the rumen’s physiology (Pitta et al. 2010, Fernando et al. 2010).
Challenges in Maintaining Giraffe in Zoos

Giraffe in zoos are known to exhibit a number of stereotypic behaviors, including pacing, tongue playing, inedible object licking, mane biting and vacuum chewing (Seeber et al. 2012). A survey conducted by Bashaw et al. (2001) reported that 79.7% of giraffe performed at least one type of stereotypic behavior with the most prevalent being the licking of non-food objects. The majority of these stereotypies may manifest due to improper diet. Depending on the specific diet offered by different zoos, giraffe can quickly consume their needed calorie and nutrient allotment from concentrates (Figure 1), which may leave them without the necessary amount of daily oral stimulation to promote natural behaviors (Baxter and Plowman 2001). Therefore, given that the majority of observed stereotypies in giraffe are oral in nature, a change in the form or content of diet may affect the rate of stereotypic behavior in zoo-housed giraffe.

Figure 1 Mazuri Wild Herbivore Concentrate (Left) and Alfalfa Hay (Right)

Giraffe in zoos are also known to have several common health issues thought to be related to diet. The first of these is urolithiasis, a metabolic disease caused by the trauma or blockage from uroliths, which are also known as urinary calculi (Smith 2009).
Possible causes of this crystal formation are high urinary concentrations of soluble ionized minerals, dehydration, and imbalances in calcium-to-phosphorus ratio in diet. If left untreated, urethral or bladder rupture may occur. Sullivan et al. (2007) found that diets with lower amounts of concentrate and higher amounts of hay produced reduced precursors to urolith crystals in meat goats. They also found that over one third of zoos feed the majority of their diet as ADF-16 (a form of higher -starch, lower fiber concentrate), which is related to an increase in risk factors for phosphatic urolith development. Death due to obstructive urolithiasis has been reported at least seven times in captivity since 1994 (Sullivan 2007).

Rumen acidosis is another life threatening disorder that affects captive ruminants (Smith 2009). It can be caused by excessive quantities of concentrates or other feed high in quickly digested sugars, causing an increase in the amount of acid producing microbes, which change rumen microflora, decrease productivity and increase irritation in chronic rumen acidosis. Rumen acidosis, however, has been poorly documented and is a challenge to diagnose in giraffe due to the invasiveness of the methods used to collect rumen samples (rumen fistula or catheter) (Clauss et al. 2006).

Improving Giraffe Diets in Zoos

Mazuri Wild Herbivore was formulated to specifically cater to the needs of giraffe by offering a high fiber, low starch, and low phosphorus pellet. Starch is more quickly digested than fiber, which may lead to disturbances of rumen microflora and rumen acidosis (Clauss and Dierenfeld 2008). Previous studies have shown that switching from ADF-16 to Wild Herbivore decreases serum phosphorus in some ruminants (Miller et al.
Zoos cannot easily replicate the large plant diversity for giraffe to browse on. However, by offering larger amounts of forage and decreased grain in their diets, we hope to better represent the wild giraffe foraging time, and an increase in similarity of behavioral and health parameters between wild and zoo-housed counterparts.

1.2 Measuring The Effects of a Diet Change.

Measuring Animal Welfare

*Definition of Welfare*

One comprehensive and well cited definition of welfare is “Welfare refers to the measurable state of an animal in relation to its environment” (Broom 1991). Broom goes on to discuss implications of this definition as summarized below:

Welfare is a varying characteristic that is able to be measured in an unbiased manner (Broom 1991). Measures of difficulty and failure to cope give information about how poor the welfare is (Broom 1991). Preferences of an animal can give an idea of what conditions are likely to result in good welfare (Broom 1991). Animals use a variety of methods to cope and many factors must be considered when determining the state of an animal’s welfare (Broom 1991).

*Importance of Good Welfare*

Fraser et al. (1997) explained there are three primary ethical concerns of animal welfare. First, animals should be free to express natural behaviors. Secondly, the animals should not be exposed to prolonged or intense negative states such as pain and
fear and they should have the opportunity to experience pleasures. Lastly, the animals should be considered healthy both physiologically and behaviorally.

In order to discuss Fraser’s points, natural behaviors must be defined. Bracke (2006) defined natural behavior as “a behavior that animals tend to perform under natural conditions, because it is pleasurable and promotes biological functioning.” For example, Morgan and Tromborg (2007) suggest that by providing appropriate substrates to certain species, the range of behavioral opportunities can be enhanced, leading to reduced stress.

Negative states are believed to be connected with suffering, which is a subjective experience, unique and known only by the individual animal (Blackmore 2013, Dawkins 2008, Koch 2004). One approach for defining the grey area of subjective animal states that Dawkins (2008) explores is looking at what animals find positively and negatively reinforcing and then translating these to positive and negative states.

Quantifying Animal Welfare

Hill and Broom (2009) discussed that many husbandry practice changes such as enclosure relocations, diet changes, and social changes that may affect animal welfare go undocumented. Common methods of measuring these changes in state is through collecting behavioral and physiological information (Hill and Broom 2009).

One of the easiest and most cost efficient manners of assessing animal welfare is collecting behavioral data. It can be done with minimal invasion to the animal as well as not require much in terms of support personnel (animal care and veterinary staff participation). Boissy et al. (2007) suggests that many animals share similar brain structures and chemistry to humans and are therefore likely to experience positive and
negative emotions much like ourselves. Behaviors such as playing, self-grooming, and expressing affiliation between animals can generally be seen as proxies for positive emotions (Boissy et al. 2007). Conversely, emotional states such as pain, fear, and lack of control are thought to be signs of animal suffering, for which one of the most common proxies for negative welfare is the prevalence of stereotypies (Broom 1991). A stereotypy is defined as a repetitive, invariant behavior pattern with no obvious goal or function (Mason 1991). Georgia Mason (1991) stated that increased occurrence of stereotypies is related to poor welfare due to suboptimal environment or animal care. However, citing a negative relationship between corticosteroid and stereotypy levels, Mason and Latham (2004) have also explained that a presence of stereotypies can be representative of good welfare such as a “do it yourself enrichment” and that a lack of stereotypies in some instances can be representative of low welfare.

Including multiple measures of an animal’s state may produce a clearer picture of what affects the welfare state of the animal. These can include measures such as pregnancy status, metabolic hormone levels, blood nutrient levels, and body condition scoring of the animal’s body. These can be assessed in a variety of ways. Some are noninvasive such as using fecal collection for metabolic hormones or visual inspection for health. More invasive procedures include blood draws or ultrasounds, which can give a more detailed picture, but, they can only be used if the animal is trained or in an opportunistic setting such as a veterinary visit. Measuring health measures using biomaterials such as blood and saliva can be further complicated due to required time from animal care and veterinary staff, costs of running desired tests, and limit of previously available data for comparison.
Measuring Changes in Behavior

All occurrence and instantaneous sampling are the two major methods utilized to collect behavioral data in the zoological setting. In all occurrence sampling the start and end times of each behavior performed are recorded for the set observation period. It is most useful when the behaviors of interest are infrequent (Crockett and Ha 2010). In the method used in my study, instantaneous sampling, the behavior recorded is what is occurring at the exact instant of the end of a predetermined time interval. The main advantage of this method is its simplicity, resulting in being able to be learned quickly and high inter-reliability scores between observers (Crockett and Ha 2010). Instantaneous sampling can be done using focal follows, where an observer follows one animal for a set time period, or scan sampling, where an observer records multiple individuals’ behavior consecutively for each interval (Crockett and Ha 2010). An instantaneous focal follow technique was used in my study as it allowed subtle feeding and stereotypy behaviors to be accurately observed and allowed greater inter-reliability scores across volunteers.

Measuring Changes in Physiology

Insulin

Insulin secretion from the pancreas can be influenced by glucose, certain amino acids, and volatile fatty acids (Brockman 1978). When food is consumed in large quantities in a short period of time, the resulting increase in glucose availability causes an increase in insulin, which helps facilitate the movement of sugars into cells for storage or use, lowering blood sugar levels in the process. Insulin works in conjunction with
glucagon to maintain normal blood sugar levels. In monogastric species high amounts of insulin over time can lead to insulin resistance and even diabetes (Flier et al. 1996).

However, the role of insulin is less well understood in ruminants (Brockman 1978). It is suggested that feeding larger amounts of forage may improve ruminant metabolism (Zhuang et al. 2014). For example, holstein cows fed a lower amount of concentrate experienced decreased glucose and insulin levels post prandial in comparison to cows fed higher amounts of concentrate, which may reflect reduced insulin sensitivity in the latter (Jenny and Polan 1975). Offering higher levels of forage may lengthen consumption and digestion times of the giraffe leading to lower and more steady insulin levels.

*Leptin*

Leptin is a hormone made primarily by adipose tissue. It is believed to play a central role in body energy homeostasis (Chilliard et al. 2005). Leaner breeds of both beef and dairy cattle express lower plasma leptin levels and have an exponential response of leptin to increased body fat (Chilliard et al. 2005). Insulin and glucose levels are believed to affect short term regulation of leptin levels in ruminants, but body fat is the key factor in its regulation, suggesting its possible value as an indicator of body condition due to diet changes (Chilliard et al. 2005).

*Calcium-to-Phosphorus Ratio*

The recommended serum calcium-to-phosphorus ratio is 1:1 or greater, but calcium-to-phosphorus ratios below 1 are often observed in captive ruminants (Miller et al. 2010).
Staying above this ratio is believed to provide amounts of phosphorus needed for metabolic needs without putting the animal at risk to develop uroliths (Schmidt and Barbiers 2005). Giraffe experience higher urinary phosphorus levels as the dietary calcium-to-phosphorus ratio drops, which may put ruminants at greater risk to develop phosphatic uroliths (Sullivan et al. 2007). The rate of saliva secretion has been shown to be higher while feeding or ruminating than during rest (Bailey and Balch 1961). Increased feeding may result in greater phosphorus excretion in feces (rather than in urine), as saliva supplies up to 80% of endogenous fecal phosphorus (Horst 1986, Bravo et al. 2003). Thus an increase in time spent feeding may also in increase saliva secretion and phosphorus fecal excretion in giraffe, which may decrease the cases of urolithiasis in zoo-housed giraffe.

In summary, increasing the amount of forage compared to concentrates fed to giraffe may improve both their welfare and health. Specifically, we hypothesize that transitioning giraffe to an ~90:10 % hay-to-grain ratio will increase feeding related behaviors and decrease oral stereotypies. We also hypothesize increased serum calcium-to-phosphorus ratios, decreased serum insulin-to-glucose ratios, and shifts in the fecal microflora community structure after the diet change may then follow as the hay-to-grain ratio is increased.
CHAPTER II: THE EFFECTS OF INCREASED HAY-TO-GRAIN RATIO ON MASAI GIRAFFE BEHAVIOR, HEALTH INDICATORS AND FECAL MICROFLORA DIVERSITY

2.1 Introduction

More than 120 Masai giraffe (Giraffa camelopardalis tippelskirchi) and 500 giraffe total live in accredited North American zoos (ISIS-ZIMS 2014). Giraffe are iconic African animals, as well as one of the most popular and commonly exhibited species in zoos. However, some zoo-housed giraffe can be afflicted by behavioral and health issues, such as stereotypic behaviors, urolithiasis, and rumen health issues that may all relate to diet.

Stereotypies are defined as a repetitive, invariant behavior pattern with no obvious goal or function (Mason 1991). The stereotypies present in captive giraffe include pacing, tongue playing, inedible object licking, mane biting and vacuum chewing (Seeber et al. 2012). A survey conducted by Bashaw et al. (2001) reported that ~80% of zoo-housed giraffe performed at least one type of stereotypic behavior; the most prevalent was the licking of non-food objects. In the wild, giraffe use their tongues
extensively while gathering and consuming browse. By contrast in zoos, giraffe may quickly consume their needed caloric and nutrient allotment if the majority of their food is in the form of concentrates. Short feeding times can leave them short of necessary amounts of oral stimulation (Baxter and Plowman 2001), and contribute to the development of oral stereotypic behavior. Bashaw et al. (2001), suggested that oral stereotypies are more closely linked to feeding variables than to other environmental variables such as enclosure size, and changes in diet can lessen stereotypies in other zoo species (Leeds et al. 2016, Less et al. 2014). For example, oral stereotypies can be decreased and time spent ruminating increased with the addition of coarse meadow hay to giraffe diets (Baxter and Plowman 2001). Therefore, diets that increase time spent feeding may beneficially influence giraffe behavior.

In addition to behavior, diet can influence health. Urolithiasis is a metabolic disease caused by the trauma or blockage of the urethra and bladder from uroliths, or urine crystals (Smith 2009). This condition has been documented in zoo giraffe, with 10 of 41 zoos surveyed reporting conditions related to urolithiasis, and multiple known deaths due to urolith obstruction (Smith 2009, Wolfe et al. 2000, Sullivan et al. 2010). One possible cause of these crystals forming is an imbalance in calcium-to-phosphorus ratio (Ca:P) in diet (Smith 2009). While the recommended serum calcium-to-phosphorus ratio is 1:1 or greater, calcium-to-phosphorus ratios below 1 are often observed (Miller et al. 2010). Diets with higher hay-to-concentrate ratios produced a decrease in urine crystal count scores (considered a precursor to urolith formation) from 2.63 to 1.89 in meat goats (Sullivan et al. 2007). In a comparison of varying giraffe diets across seven zoos, those with lower hay intake and higher concentrate-to-hay ratios resulted in a tendency for
higher serum and urinary phosphorus levels in giraffe, which may put ruminants at greater risk to develop phosphatic uroliths (Sullivan et al. 2007). The rate of saliva secretion has been shown to be higher in cattle while feeding or ruminating than during rest (Bailey and Balch 1961). As up to 80% of endogenous fecal phosphorus originates from saliva production (Horst 1986, Bravo et al. 2003), increased feeding time may also result in greater phosphorus excretion in feces (rather than urine). Low roughage diets decrease the rate of saliva formation, which may divert this excretion of phosphorus to the urinary system (Belknap and Pugh 2002). Thus an increase in time spent feeding may also increase saliva secretion and phosphorus fecal excretion in giraffe which may decrease risk of urolithiasis in zoo housed giraffe.

Lack of rumination can lead to changes in rumen microflora, decreased productivity and irritation with chronic rumen acidosis, D-lactic acidosis, or possible death (Smith 2009). An increase in the amount of acid produced by organisms in the rumen can be caused by excessive quantities of concentrate feed that may fail to stimulate proper rumination and saliva production (Owens et al. 1998). Both rumen and fecal pH decreased when ewes were fed high concentrate diets, indicating that concentrates may contribute to acidic conditions in the rumen (Neumann and Dehority 2008). Saliva has qualities of a buffer and helps maintain the acid-base equilibrium of the rumen (Gonzalez et al. 2012). Therefore, feeding a diet lower in concentrates may help stimulate saliva production, stabilize rumen pH and improve long-term health.

Diet also influences metabolism. Insulin is a hormone released from the pancreas, which helps facilitate the movement of sugars into cells for storage or use (Brockman 1978). However, for many mammals, low insulin and insulin-to-glucose ratios reflect
better insulin sensitivity and thus, better metabolic health (Guerrero-Romero and Rodriguez-Moran 2001, Katz et al. 2000). Holstein cows fed a lower amount of concentrate experience decreased glucose and insulin levels post prandial in comparison to cows fed higher amounts of concentrate, which may reflect reduced insulin sensitivity in the cows fed higher amounts of concentrates (Jenny and Polan 1975). Though it is not the main objective of the present study, we hypothesize that an increase in forage and decrease in grain concentrate will lead to a decreased serum insulin and insulin-to-glucose ratio.

Leptin is a hormone made primarily by adipose tissue and is believed to play a central role in body energy homeostasis (Chilliard et al. 2005). Leaner breeds in both beef and dairy cattle express lower plasma leptin levels with an exponential response of leptin to increased body fat (Chilliard et al. 2005). Insulin and glucose levels are believed to affect short term regulation of leptin levels in ruminants but body fatness is the key factor in its regulation, suggesting its possible value as an indicator of any changes in body condition of an animal due to diet changes (Chilliard et al. 2005).

Changing from diets that are higher in concentrated pellets to those higher in forage (hay) can also alter rumen and fecal bacteria. Neumann and Dehority (2008) found that both rumen and fecal total bacteria increased when animals are fed diets higher in concentrate compared to those higher in forage. Due to difficulties in collecting rumen samples from captive giraffe, these demonstrated relationships between rumen and fecal bacterial populations indicate that changes in fecal microbiota may be an appropriate proxy for changes in rumen microbiota.
In summary, there is an abundance of information to suggest that a high forage diet will have beneficial effects on oral stereotypic behaviors and overall health in zoo-housed giraffe. The increased hay-to-grain ratio was examined to see if it has quantifiable effects on stereotypic behavior, metabolic hormones that regulate insulin sensitivity, and gut microbial communities. We hypothesize that switching to a diet that provides a higher hay-to-grain ratio will reduce oral stereotypies by increasing time spent performing feeding behaviors, maintain or increase the calcium-to-phosphorus ratio, decrease insulin-to-glucose ratios, maintain leptin levels, and result in significant changes in fecal microbiome communities.

2.2 Methods

Animals and Animal Care

The Cleveland Metroparks Zoo (CMZ) housed three adult females, one adult male, and two juvenile giraffe during this study, though we did not include the juveniles in the diet change. One giraffe was in the first trimester of a 15-month pregnancy, and the two other females were nursing calves older than 5 months of age at the start of the 6-month study (Table 2). All procedures were reviewed and approved by the Institutional Animal Care and Use Committee at Cleveland State University and the Animal Care and Use Committee at Cleveland Metroparks Zoo.

Table 2. Sex, age and maternal status for each giraffe.

<table>
<thead>
<tr>
<th>Giraffe</th>
<th>Symbol</th>
<th>Sex</th>
<th>Age</th>
<th>Status</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>∆</td>
<td>Female</td>
<td>8</td>
<td>Nursing</td>
</tr>
<tr>
<td>B</td>
<td>○</td>
<td>Female</td>
<td>7</td>
<td>Pregnant</td>
</tr>
<tr>
<td>C</td>
<td>◊</td>
<td>Male</td>
<td>8</td>
<td>NA</td>
</tr>
<tr>
<td>D</td>
<td>□</td>
<td>Female</td>
<td>6</td>
<td>Nursing</td>
</tr>
</tbody>
</table>
Experimental Design

One goal of the diet transition was to change the form and volume of the feed offered without significantly altering nutrient content or caloric intake during the baseline and post diet change periods (Table 3). Nutrient content was calculated based on information published by Mazuri Exotic Animal Nutrition (Richmond, IN) and average alfalfa hay diet analysis completed by Dairy One (Ithaca, NY). The amount of each diet item (hay and grain) was gradually shifted in evenly distributed weekly increments over a period of 8 weeks. The male was shifted from a 50:50 diet ratio of 6.81 kg Mazuri Wild Herbivore (Mazuri Exotic Animal Nutrition) pelleted grain and 6.81 kg alfalfa hay daily to a ~10:90 ratio of 1.4 kg of grain and 11.9 kg of hay per day. For the females, who are group fed, the transition was from a 30:70 baseline ratio of ~3.7 kg Mazuri Wild Herbivore pelleted grain and 9.1 kg alfalfa hay daily per female to a ~10:90 ratio of 1 kg of grain and 11.9 kg hay per female per day (Table 4). Percent daily food consumption was recorded and weekly weights measured to ensure that animal weights remained stable. Beginning with the 7th week of diet transition onward, a ration balancer (0.53 kg/female, 0.8 kg male; Tribute Alfa Essentials, Upper Sandusky, OH) was fed to all giraffe daily to ensure diets were meeting the daily vitamin and mineral requirements.
Table 3. Nutrient composition of female and male giraffe diets before (baseline) and after (post) an increase in the hay-to-grain ratio.

<table>
<thead>
<tr>
<th>Nutrient</th>
<th>Females Baseline</th>
<th>Females Post</th>
<th>Male Baseline</th>
<th>Male Post</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude Protein (%)</td>
<td>18.44</td>
<td>20.11</td>
<td>17.53</td>
<td>19.54</td>
</tr>
<tr>
<td>Crude Fat (%)</td>
<td>2.63</td>
<td>2.65</td>
<td>2.90</td>
<td>2.75</td>
</tr>
<tr>
<td>Crude Fiber (%)</td>
<td>29.22</td>
<td>28.30</td>
<td>31.19</td>
<td>28.01</td>
</tr>
<tr>
<td>NDF (%)</td>
<td>46.62</td>
<td>44.59</td>
<td>50.72</td>
<td>44.54</td>
</tr>
<tr>
<td>ADF (%)</td>
<td>33.58</td>
<td>32.61</td>
<td>35.69</td>
<td>32.21</td>
</tr>
<tr>
<td>Lignin (%)</td>
<td>6.27</td>
<td>6.82</td>
<td>5.94</td>
<td>6.60</td>
</tr>
<tr>
<td>Starch (%)</td>
<td>2.03</td>
<td>1.86</td>
<td>2.73</td>
<td>2.19</td>
</tr>
<tr>
<td>Ash (%)</td>
<td>9.92</td>
<td>10.76</td>
<td>9.70</td>
<td>10.61</td>
</tr>
<tr>
<td>Calcium (%)</td>
<td>1.29</td>
<td>1.40</td>
<td>1.24</td>
<td>1.38</td>
</tr>
<tr>
<td>Iron (%)</td>
<td>0.04</td>
<td>0.04</td>
<td>0.04</td>
<td>0.04</td>
</tr>
<tr>
<td>Magnesium (%)</td>
<td>0.33</td>
<td>0.33</td>
<td>0.35</td>
<td>0.34</td>
</tr>
<tr>
<td>Phosphorus (%)</td>
<td>0.26</td>
<td>0.34</td>
<td>0.27</td>
<td>0.37</td>
</tr>
<tr>
<td>Potassium (%)</td>
<td>1.99</td>
<td>2.17</td>
<td>1.86</td>
<td>2.10</td>
</tr>
<tr>
<td>Sodium (%)</td>
<td>0.25</td>
<td>0.24</td>
<td>0.33</td>
<td>0.27</td>
</tr>
<tr>
<td>Zinc (mg/kg)</td>
<td>52.23</td>
<td>45.58</td>
<td>76.24</td>
<td>57.44</td>
</tr>
<tr>
<td>Copper (mg/kg)</td>
<td>11.81</td>
<td>16.76</td>
<td>15.04</td>
<td>21.23</td>
</tr>
<tr>
<td>Manganese (mg/kg)</td>
<td>64.99</td>
<td>53.87</td>
<td>87.36</td>
<td>61.62</td>
</tr>
<tr>
<td>Selenium (mg/kg)</td>
<td>0.25</td>
<td>0.77</td>
<td>0.33</td>
<td>1.09</td>
</tr>
<tr>
<td>DE (kcal)</td>
<td>34,518</td>
<td>34,692</td>
<td>37,963</td>
<td>36,835</td>
</tr>
</tbody>
</table>

NDF = neutral detergent fiber, ADF = acid detergent fiber, DE = estimated ruminant digestible energy.

Table 4. Outline of diet change. Female amounts given on a group fed bases (n=3 females).

<table>
<thead>
<tr>
<th>Timeline</th>
<th>BASELINE</th>
<th>DIET CHANGE</th>
<th>POST</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Month</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Nov</td>
<td>Dec</td>
<td>Jan</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Females</td>
<td>4</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>8</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>3</td>
<td>4</td>
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<td>5</td>
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<td>7</td>
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<td>8</td>
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<td>6</td>
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<td>7</td>
<td>8</td>
<td>1</td>
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<td>2</td>
<td>3</td>
<td>4</td>
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<tr>
<td></td>
<td>5</td>
<td>6</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td></td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Ration Balancer</td>
<td>.5kg per female .8kg male</td>
<td></td>
</tr>
<tr>
<td></td>
<td>.8kg</td>
<td>1.4</td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Females</td>
<td>Hay (kgs)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>27.3</td>
<td></td>
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<tr>
<td></td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>35.7</td>
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<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Females</td>
<td>Grain (kgs)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>11.4</td>
<td></td>
</tr>
<tr>
<td></td>
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</tr>
<tr>
<td></td>
<td></td>
<td>2.8</td>
<td></td>
</tr>
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<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Male</td>
<td>Hay (kgs)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>6.8</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>11.9</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Male</td>
<td>Grain (kgs)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>6.8</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.4</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Behavioral Data Collection

Behavioral data were collected from November 16, 2014 to April 29, 2015 with 58 observation periods per an animal collected for the baseline and 64 per animal for the post diet change. Observations were made approximately once a day, seven days a week, spread evenly into six, 90 minute time frames (0800-0930, 0930-1100, 1100-1230, 1230-1400, 1400-1530, 1530-1700). Baseline and post observations were similarly balanced in AM/PM observations and day of the week. Observations were balanced to ensure that baseline and post observations were nearly equivalent and spread as equally throughout the week and time of day as possible. Before the study began, giraffe were habituated to the presence of an observer over a period of several weeks. Baseline data collection began when giraffe started to spend the majority of time in their indoor enclosure due to declining temperatures. Volunteers were trained to collect data using an exhaustive ethogram (Table 5). The ethogram had two concurrent, exhaustive channels of feeding behaviors, and other behaviors, such that two behaviors were marked at each observation point. In order to collect data for the study, observers were required to achieve greater than 90% inter-rater reliability with the primary investigator. Data were collected using 80 minute observations using randomly created starting orders of the giraffe to be observed ([www.Random.org/list](http://www.Random.org/list)). We completed 5 minutes of instantaneous focal follow per an animal, where the behavior was recorded according to the behaviors outline by the ethogram each minute before moving on to the next animal. A total of four, 5-minute focal follow sets were completed per 80 minute observation so that each animal was observed for a total of 20 minutes per observation.
Table 5. Ethogram of behaviors used in study.

<table>
<thead>
<tr>
<th>Channel 1 Behaviors</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Feeding</strong></td>
</tr>
<tr>
<td>Hay - Subject is putting hay in mouth or chewing in close proximity to hay trough when hay is present</td>
</tr>
<tr>
<td>Concentrate - Subject is putting grain in mouth or chewing in close proximity to grain feeder when grain is present</td>
</tr>
<tr>
<td>Browse - Subject is putting browse in mouth or chewing in close proximity to provided browse when browse is present</td>
</tr>
<tr>
<td>Chew - Subject is chewing without evidence of what is in mouth in a consistent circular motion. Proxy for ruminating. Mark R if ruminating is observed.</td>
</tr>
<tr>
<td>Drinking - Subject is drinking water</td>
</tr>
<tr>
<td>Other - Subject is consuming another substance not listed above such as lettuce or salt block. Please note what is being consumed</td>
</tr>
<tr>
<td>Not Feeding - Subject is not performing a feeding behavior</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Channel 2 Behaviors</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Undesirable</strong></td>
</tr>
<tr>
<td>Pace - Subject is moving around enclosure for no apparent goal or reason following an almost identical path for more than two repetitions</td>
</tr>
<tr>
<td>Tongue/Mouth - Subject is moving tongue outside of mouth without apparent reason for longer than 2 seconds or moving mouth/chewing in an erratic fashion. Mark both Feeding-Chew and Undesirable-Tongue/Mouth if there is an indication of food material in mouth or subject is also ruminating.</td>
</tr>
<tr>
<td>Lick - Subject is seen repetitively (&gt;3x) licking or mouthing non-edible items</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Social</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agonistic - Aggressive Behaviors: threat, leaning, sparring. Recorded when subject is initiator.</td>
</tr>
<tr>
<td>Affiliative - Behaviors: Nosing, licking briefly, rubbing, nursing. Recorded when subject is initiator.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Movement</th>
</tr>
</thead>
<tbody>
<tr>
<td>Locomotion - Movement of subject that results in change of location by more than one body length</td>
</tr>
<tr>
<td>Stationary - Subject is standing or laying and eyes either open or closed, not seemingly focused at a particular point and/or object.</td>
</tr>
<tr>
<td>Alert - Standing or laying still with eyes open and focused on one point.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Directed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Self Directed - Subject is investigating, or touching self, or scratching on an object.</td>
</tr>
<tr>
<td>People Directed - Animal is clearly focused on or interacting with a human.</td>
</tr>
<tr>
<td>Object Directed - Investigating, touching or manipulating an object</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Other</th>
</tr>
</thead>
<tbody>
<tr>
<td>Animal is performing a behavior not described by any other category. Please note the behavior.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Not Visible</th>
</tr>
</thead>
<tbody>
<tr>
<td>Not Visible</td>
</tr>
</tbody>
</table>
Biomaterials Collection

Whole blood was collected approximately biweekly by venipuncture in animals conditioned for voluntary blood draws. Serum was isolated from the blood per routine protocols and placed in a polypropylene tube labeled with animal ID, and date collected. Serum samples were then stored frozen in a manual defrost freezer at (-20° C) until analysis. Saliva was collected weekly by stationing the giraffe voluntarily at the side of their enclosure and offering a 4-inch synthetic salivette (Salimetrics, Carlsbad, CA) to manipulate until it became saturated with saliva. All serum and saliva were collected before the first morning feed. The sample was then placed in the supplied 15 ml tube with collection chamber (Salimetrics), labeled with animal ID and date, and stored frozen (-20 °C) until analysis. Immediately prior to analysis, samples were thawed at room temperature for 20 minutes and centrifuged (2500 x g, 15 minutes) in supplied 15 mL tubes with collection chamber (Salimetrics) to collect liquid saliva. Fecal samples (approximately 50 g) were collected weekly into bags individually labeled with animal ID and date, and stored frozen (-20 °C) until used for DNA extraction.

Serum and Saliva Analysis

Insulin was quantified using a commercial bovine insulin enzyme immunoassay (EIA; Mercodia, Winston Salem, NC) according to the manufacturer’s instructions. Serum and saliva serially diluted with supplied assay buffer demonstrated parallelism with the standard curve \((t = -0.016, p = 0.98 \text{ serum}, t = 2.44, p = 0.47 \text{ saliva})\). Recovery of samples spiked with known amounts of standard curve averaged 88% and 97%, for serum and saliva respectively. Assay sensitivity was 0.03 ng/ml and intra- and inter-assay
coefficients of variation for saliva were 4.83% and 2.46%, respectively. For serum insulin, all samples were run within a single assay, and the intra-assay CV was 3.84%. Serum leptin was measured using a commercial EIA with the provided Mouse standards (B-Bridge International, Inc, Santa Clara,CA). Serially-dilated serum exhibited parallelism with the standard curve (t = - 0.20, P > 0.05). Recovery of samples spike with known amounts of standard curve averaged 93%. Assay sensitivity was 100 pg/ml. All samples were run within a single assay and the intra-assay coefficient of variation was 2.96%. Serum calcium, phosphorus and glucose were quantified using a standard serum chemistry analyzer (Vet Test 8008; IDEXX Laboratories, Inc., Westbrook, Maine, USA).

Fecal Microflora DNA Extraction and Purification

Extraction of DNA was performed using a bead beating extraction protocol (Burke 2008). Fecal material (0.25 g) was added to a bead beating tube with sterile forceps along with 700 µl of cetyl-trimethyl ammonium bromide (CTAB) and put on a Precellys 24 Homogenizer (Bretin Technologies, Montigny-le Bretonneux, France) on the 2 x 20 setting. The tubes were then centrifuged at 13,200 RPM for one minute. Under the fume hood, 300 µl of the supernatant was pipetted into a sterile 1.5ml tube for phenol-chloroform extraction of nucleic acids. Phenol/chloroform/isoamylalcohol (300 µl) was added to the supernatant and mixed via manual agitation. Under the fume hood, the solution was spun for 5 minutes at 13,200 RPM. The top aqueous layer (approximately 200 µl) was removed and placed in a new sterile 1.5 ml tube, and the phenol chloroform addition and centrifugation steps were repeated. The top aqueous layer (approximately
150 µl) was removed and placed in a new sterile 1.5 ml tube. An equal volume of polyethylene glycol 8000 in 2.5 M NaCl (approximately 150 µl) was added to precipitate nucleic acids while the solution was incubated at 37 °C for 15 minutes. This tube was then centrifuged for 5 minutes at 13,200 RPMs and the supernatant discarded. The tube was then filled with 1.5 ml of 80% ice cold ethanol and centrifuged for 5 minutes at 13,200 RPMs. The ethanol supernatant was aspirated and discarded. The nucleic acids were dried in an Eppendorf Vacufuge (Hauppauge, NY, USA). The nucleic acids were resuspended in 100 µl of Tris-EDTA buffer, allowed to sit for 5 minutes, and being transferred to siliconized tubes and stored at -25 °C to use as a template for PCR.

Polymerase Chain Reaction- Terminal Restriction Fragment Length Polymorphism (PCR-TRFLP)

Primer sets (338f, 926r) were selected that amplified the 16s rRNA gene region of the bacterial DNA (Burke et al. 2005, Martin and Rygiewicz 2005). The reverse primer was labelled with a 6FAM label and the forward primer labelled with Hex label (Burke et al. 2005). PCR was completed in 50 µl reactions as previously described with 35 rounds of amplification in a PTC-200 thermocycler (GMI, Ramsey, MN, USA; (Burke et al. 2005)). Labelled amplicon product was used to generate TRFLP profiles using the endonuclease MBO1 at the Life Sciences Core Laboratories Center (Cornell University) and analyzed using GS600LIZ size standards and Peak Scanner™ Software (Version 2.0, Applied Biosystems, Foster City, CA, USA).
Behavior and Physiology Statistical Analysis

Time spent feeding on hay, concentrate and browse as well as time spent chewing were combined into an additional category called feeding. All behaviors were analyzed using a generalized linear mixed model (PROC GLIMMIX; SAS Institute, Cary, North Carolina). For PROC GLIMMIX, individual animal was included as a random factor. All models except time spent feeding on browse had a random intercept. A random slope was included when it contributed to a better model fit as follows; day of week for people directed, and alert behavior models, and time of day for pacing, feeding, object-directed, self-directed and stationary behavior models. A negative binomial distribution with a log link function was used for all behaviors with an offset for time spent visible. Degrees of freedom were calculated using the between/within method. All behavior data are presented as % of time visible.

Serum insulin, glucose, insulin-to-glucose ratio (a proxy for insulin sensitivity), leptin, calcium, phosphorus, and calcium-to-phosphorus ratio were log transformed to approximate a normal distribution and then analyzed using a general linear mixed model (PROC MIXED; SAS Institute, Cary, North Carolina, USA). A random intercept and the random factor of individual animal was included in all models. Degrees of freedom were calculated using the between/within method. Models were built using maximum likelihood estimation and final models were computed using restricted maximum likelihood (REML).

For both PROC GLIMMIX and PROC MIXED, models with the lowest \(-2 \log \text{likelihood}\) estimates were chosen as the final model. Treatment (Baseline vs. Post diet change) was always included in the model regardless of whether it was significant,
because it related to the main hypothesis. All other factors were removed from the model at p > 0.10 unless they contributed to a significantly lower -2 log likelihood. Post-hoc results are presented as the least squared means ± SEM.

Weights of the giraffe from baseline and post diet change were analyzed using a paired t-test with all baseline and post diet changes weights for each giraffe averaged and paired for the analysis.

TRFLP Statistical Analysis

TRFLP profiles were processed using the TRFLPR package in R (Petersen et al. 2015, R Core Team 2014). Only peaks which accounted for greater than 1% of the relative peak area were included in sample analyses. TRFLP profiles were arcsine-square root transformed prior to analysis. Nonmetric multi-dimensional scaling analysis (NMDS) was used to assess bacterial community structure across the treatment (before vs. after diet change) using the metaMDS function in the Vegan package in R (Oksanen et al. 2007, R Core Team 2014). The number of dimensions was selected using a scree plot and distances were determined using the Bray-Curtis method. Differences in centroid location between treatment and animal were tested via PERMANOVA using the adonis function in the Vegan package (Oksanen et al. 2007). For all statistical analyses, p < 0.05 was considered statistically significant.
2.3 Results

Behavior

Increasing the hay-to-grain ratio fed to the Masai giraffe was associated with a significant increase in the percent of time spent performing feeding behaviors (F\(1,3\) = 15.4, \(p = 0.03\); Figure 2), and time stationary (F\(1,3\) = 30.9, \(p = 0.01\); Figure 2). The amount of time spent performing tongue and mouth stereotypies also decreased by approximately 50% post diet change (4.3 ± 2.3 baseline vs 2.1 ± 1.1 post % time; F\(1,3\) = 11.60, \(p = 0.04\); Figure 3), and decreased in all four giraffe. People-directed (F\(1,3\) = 34.63, \(p = 0.01\); Figure 3) and alert behaviors (F\(1,3\) = 17.97, \(p = 0.02\); Figure 3) also decreased following the diet change in all four giraffe. One giraffe (B) spent close to twice as much time performing alert (7.9%) and people-directed behaviors (10.10%) compared to averages for the other giraffe (4.46%, 6.04%, respectively) during the baseline period. During the post period, the amount of time spent performing these behavior was more comparable to others (2.5% of time alert giraffe B vs. 1.2% average of other giraffe, 2.0% of time people-directed for giraffe B vs. 2.0% average of other giraffe respectively). An increase in the hay-to-grain ratio fed to giraffe had no significant effect on time spent licking, pacing, locomotion, object-directed behaviors, or self-directed behaviors. The average time not visible for all giraffe was 0.94% during the baseline and 1.54% during the post period.
Figure 2. Least squared mean ± standard error of the mean (LSM ± SEM) % of time spent performing behaviors during the baseline (grey bars) and post (white bars) diet change periods. Feeding behaviors were analyzed independently of the other behaviors. * denotes a significant difference (p < 0.05) between baseline and post diet change.

Figure 3. LSM ± SEM % of time performing various behaviors during the baseline (grey bars) and post diet change (white bars) periods. * denotes a significant difference of p < 0.05 between baseline and post diet change.
Physiological Changes

Increasing the hay-to-grain ratio fed to the giraffe was associated with a decrease in average serum \( F(3) = 24.71, p = 0.02 \) and salivary insulin \( F(3) = 16.56, p = 0.03 \) concentrations, serum glucose concentrations \( F(1,3) = 12.91, p = 0.04 \), serum insulin-to-glucose ratios \( F(3) = 10.47, p = 0.048 \), and serum leptin concentrations \( F(3) = 15.01, p = 0.03 \). Least squared mean serum concentrations before and after the diet change are depicted in Table 6. It is interesting to note that the male giraffe had approximately three-fold higher average serum insulin concentrations (26.95 uIU/mL) and at least three fold higher average serum leptin concentrations (880.72 ng/ml) compared to the average for the three females (Insulin 7.95, 13.07 and 7.15 uIU/mL; Leptin 254.77, 59.69 and 85.95 ng/ml, respectively). The male also experienced the most dramatic decrease in salivary insulin post diet change (Figure 4). However, all four animals experienced a decrease in salivary and serum insulin, and a progressive decrease in insulin-to-glucose ratio (Figure 5) throughout the study. The diet change had no significant observed effect on serum blood calcium levels, phosphorus levels, or calcium-to-phosphorus ratios (Table 6). All giraffe finished their hay over 75% of the time. On days when they left a portion of their hay, the average amount left over was less than 6% (range 3.7-29.4%) of total hay intake during the baseline and post-treatment. All concentrate was consumed every day in baseline and post. Giraffe weight did not change significantly between baseline and post diet change (Paired T-Test; \( T(3) = -0.26, p=0.81 \); Figure 6).
Table 6. LSM±SEM salivary and serum biomarkers before and after an increase in hay-to-grain ratio in four Masai giraffe.

<table>
<thead>
<tr>
<th>Source</th>
<th>Factor</th>
<th>Baseline LSM±SEM</th>
<th>Post-Diet Change LSM±SEM</th>
<th>p-value&lt;sup&gt;1&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Salivary</td>
<td>Insulin (ng/mL)</td>
<td>0.82 ± 0.24</td>
<td>0.43 ± 0.05</td>
<td>0.03*</td>
</tr>
<tr>
<td>Serum</td>
<td>Insulin (ng/mL)</td>
<td>0.58 ± 0.09</td>
<td>0.41 ± 0.08</td>
<td>0.02*</td>
</tr>
<tr>
<td>Serum</td>
<td>Glucose (ng/mL)</td>
<td>85.63 ± 1.55</td>
<td>77.48 ± 2.05</td>
<td>0.04*</td>
</tr>
<tr>
<td>Serum</td>
<td>Insulin/Glucose (mU/mmol)</td>
<td>3.55 ± 0.44</td>
<td>2.75 ± 0.46</td>
<td>0.048*</td>
</tr>
<tr>
<td>Serum</td>
<td>Leptin (ng/mL)</td>
<td>204.17 ± 6.96</td>
<td>181.97 ± 8.32</td>
<td>0.03*</td>
</tr>
<tr>
<td>Serum</td>
<td>Ca (mg/dL)</td>
<td>9.62 ± 0.11</td>
<td>9.43 ± 0.15</td>
<td>0.14</td>
</tr>
<tr>
<td>Serum</td>
<td>P (mg/dL)</td>
<td>5.01 ± 0.28</td>
<td>5.19 ± 0.29</td>
<td>0.73</td>
</tr>
<tr>
<td>Serum</td>
<td>Ca/P</td>
<td>1.9 ± 0.11</td>
<td>1.8 ± 0.12</td>
<td>0.51</td>
</tr>
</tbody>
</table>

<sup>1</sup>an asterisk denotes a significant change of p < 0.05 between baseline and post diet change.

Figure 4. Salivary insulin concentrations before (baseline) and after (post) an increase in hay-to-grain ratio in an adult male Masai giraffe (giraffe C).
Figure 5. Changes over time in serum insulin-to-glucose ratio in four Masai giraffe. Samples taken during the baseline period are to the left of the vertical line while those taken during the 8 weeks post-diet change are to the right of the vertical line. Serum was collected approximately bi-weekly during both baseline and post periods. Giraffe A, B, C, and D are represented by triangles, circles, diamonds, and squares respectively. Linear regression lines were fit to each individual’s set of serum samples and are represented by the dotted lines.

Figure 6. Giraffe weights from baseline to post data collection. Individual animals A, B, C, and D are represented by triangles, circles, diamonds, and squares, respectively. Pre and post diet change are represented by the first and second grouping of black markings respectively while grey markings represents the transition period weights. Note that the Y axis does not start at zero to better visualize changes in weight over time.
Microflora

A three-dimensional ordination solution for NMDS analysis revealed a significant change in fecal microfloral community structure due to the diet change (PERMANOVA, stress = 0.12 F = 22.69, p = 0.001; Figure 5) and individual animal (PERMANOVA, stress = 0.12, F = 2.377, p = 0.009). Furthermore, there was a significant interaction between treatment (diet change) and animal (PERMANOVA, stress = 0.12, F = 3.31, p = 0.001).

Figure 7. NMDS ordination plot of mean axis score ± SEM individual Masai giraffe fecal microflora community structures from baseline (open shapes) and post diet change (closed shapes). Individual animals A, B, C, and D are represented by triangles, circles, diamonds, and squares respectively. Permanova analysis revealed a significant change in community structure due to diet change and individual animal with an interaction between diet change and animal (p < 0.01 for all).
2.4 Discussion

The increased hay-to-grain ratio diet given to the four giraffe was associated with increased time spent feeding along with a decrease in time spent performing tongue and mouth stereotypies, people-directed behaviors, and alert behaviors. The giraffe also experienced a decrease in serum insulin-to-glucose ratio and leptin, while serum calcium-to-phosphorus ratio was maintained before and after the diet change. A shift in the giraffe fecal microflora community structure was also observed with differences explained by both treatment effects and by individual animal. Taken together, we believe these results represent an improvement in giraffe health.

The observed increase from 44% to 51% of time spent feeding over the course of the diet change better approximates the time spent feeding and foraging by wild giraffe that generally range from 50% to 70% (Du Toit and Yetman 2005, Ginnett and Demment 1997, Pellew 1984a). This was also an increase over the 27.5% of time performing feeding related behaviors observed in a 2006-2007 study at the same institution (Reber 2008). While increased feeding time was a desired outcome for this study due to potential health benefits, further studies are needed to elucidate any relationship between increased forage and direct health outcomes.

Though not measured in this study, saliva production increases during feeding and ruminating in other species (Bailey and Balch 1961), which may promote a more balanced rumen pH. In addition, increased feeding may result in greater phosphorus excretion in feces (rather than urine), as saliva supplies up to 80% of endogenous fecal phosphorus (Horst 1986, Bravo et al. 2003, Bailey and Balch 1961). Thus, an increase in
the hay-to-concentrate ratio in giraffe may lead to an increase in saliva production and fecal phosphorus excretion lowering the potential for urolithiasis.

A positive correlation exists between oral stereotypies and proportion of concentrate fed to giraffe, heifers and other ungulates (Bergeron et al. 2008, Baxter and Plowman 2001, Redbo and Nordblad 1997). Correspondingly, increasing forage was associated with tongue and mouth stereotypies decreasing by half in the present study from 4.3% to 2.1% of time. This is probably due to the increased time it took to consume a larger volume of forage, thereby providing a greater degree of oral stimulation (Luginbuhl et al. 1989). Our results show a lower percentage of oral stereotypies than data from a 2006-2007 study at the same zoo in which time spent performing stereotypies varied, but reached as high as 35% of time (Reber 2008). The 2.1 ± 1.1 % of time performing stereotypies observed in this study is similar to the 0.8% of time spent performing tongue playing stereotypic behaviors in wild populations (Veasey et al. 1996). Baxter and Plowman (2001) concluded that stereotypic behavior in giraffe can be viewed as a proxy for lower welfare and therefore, the reduction of tongue and mouth stereotypies during this study may be viewed as a positive change in welfare.

Increased alert behaviors have been observed in stressed individuals that showed increased cortisol levels in other species of mammals (Carlstead et al. 1993) and both alert (also called vigilance) and stereotypic behaviors have been shown to increase from environmental stressors (Chamove et al. 1988). Welp et al. (2004) suggested that cows adjust their alert (vigilance) behaviors according to their fearfulness of objects or environments, which may make alert behavior a possible welfare indicator. However, the decrease in people-directed and alert behaviors may have also been a byproduct of the
reduction of looking for keepers to deliver food items, since food was more readily available and in increased quantities throughout the day.

The observed decrease in salivary and serum insulin, serum glucose and serum insulin-to-glucose ratios post diet change may be an outcome of approximately the same nutrients being fed in a larger volume of forage, decreased consumption rate, and increased time fermenting and digesting the larger volume of food. Larger particles are retained in the reticulo-rumen for a longer amount of time than small particles with the same density (Kaske and Engelhardt 1990), altering fermentation rate in the rumen and nutrients available to the abomasum and small intestine between different hay-to-concentrate ratios. Greater digestive time may contribute to the differences observed in glucose and insulin levels. Our findings are supported by reports of lower insulin and glucose when cows were fed high forage, low concentrate diets compared to those fed high concentrate, low forage diets (Schoonmaker et al. 2003, Jenny et al. 1974). Based on reports in other species including dairy cows, the observed decrease may indicate an overall improvement in insulin sensitivity (Guerrero-Romero and Rodriguez-Moran 2001, Katz et al. 2000, Holtenius and Holtenius 2007).

Despite giraffe maintaining body weight throughout the study, we observed an approximately 10% decrease in serum leptin from baseline to post diet change, which may indicate an overall decrease in body fat. This change was contrary to our expectations given that approximately the same nutrients and calories were fed in the baseline and post diets. Anecdotally, a gradual and slight decline in serum leptin was observed in the three females throughout the study, but not the male. Whether this disparity is due to inherent sex differences, differences in baseline leptin concentrations
due to the male having a 3-fold higher concentrations, or due to the pregnant and lactating status of the females is not known. Contrary to the pattern observed in the pregnant female in our current study, early pregnancy is associated with progressively increasing serum leptin concentrations in humans and primates (Hardie et al. 1997). Serum and plasma leptin levels are known to be suppressed during early lactation in ruminants and bats (Block et al. 2001, Kunz et al. 1999), but are reported to start increasing gradually again near 8 weeks postpartum in dairy cows (Lacasse et al. 2011). The two lactating females in the current study were more than 20 weeks postpartum by the beginning of the 6-month study, making the potential relationship between the observed decrease in leptin and lactation less clear. Thus, future studies are needed to elucidate the relationship between increased forage and serum leptin in giraffe.

Although we observed increased feeding time, there was no significant change in the serum phosphorus, calcium levels or calcium-to-phosphorus ratio in our zoo-housed animals. However, our giraffe were above the 1:1 serum calcium-phosphorus ratio recommended for ruminant browsers (Miller et al. 2010) prior to the diet change. This ratio is believed to provide amounts of phosphorus needed for metabolic needs without putting the animal at risk to develop rumen acidosis and uroliths (Miller et al. 2010). Our giraffe also had a seemingly higher average calcium-to-phosphorus ratio after the diet change than a small subset of free ranging giraffe from Schmidt et al (2011) at 1.8 vs 1.1, respectively. The dietary phosphorus supplied to our giraffe both before and after the diet change falls within the recommended 0.35 to 0.5% dry matter basis range recommended by the Giraffe Nutrition Workshop (Schmidt and Barbiers 2005). Phosphorus absorption is directly related to dietary intake while calcium absorption is on
an “as needed basis” by the animal in a well-controlled homeostasis (Reinhardt et al. 1988). Therefore, given the similar levels of phosphorus in baseline and post diets, and the fact that calcium-to-phosphorus ratios were not inverted prior to the diet change, the lack of significant change in the calcium-to-phosphorus ratio is not surprising.

For this study, we explored whether changes in the form, but not nutrient content, of a diet induced changes in fecal microbial community structure. TRFLP allows for detection of such changes but does not identify specific bacterial family or species differences. Very little work has been done with giraffe microbial communities. AlZahal et al. (2015) recently reported TRFLP and 454-pyrosequencing analysis completed on single fecal samples from five different giraffe at a different zoo and found low intrasample variation and high inter-sample similarity attributed to uniformity of their husbandry and feeding management. However, no work has been done looking at the influence of diet of giraffe gut microflora. De Menezes et al. (2011) found differences in rumen microbial communities via TRFLP in dairy cows fed pasture or a total mixed ration composed of maize silage, concentrate blend, grass silage, molasses and straw. Many have postulated that different diets result in different rumen metabolites available for lower gastrointestinal activity, altering the lower gastrointestinal bacterial community structure (Stewart et al. 1997, Ellis et al. 2008). In future studies, further genetic sequencing of the bacterial DNA will allow a more detailed look at the microflora community structure changes due to increased hay-to-grain ratio.

There are no reports of insulin/glucose measurements or leptin in either free ranging or captive giraffe. Thus, this study represents the first information for salivary or serum metabolic indicators for giraffe in zoos or the wild. Future studies to establish reference
ranges for salivary and serum markers of insulin, glucose and leptin would provide valuable additional information. In addition, comparison of gut microbial communities between zoo-housed and free-ranging individuals would provide groundwork for studying the influence of various diets on health in these specialized browsers. While more studies are needed to establish ideal diets for giraffe in zoos, we believe the behavioral and physiological changes observed following an increase in hay-to-grain ratio in the current study represent an incremental improvement in health. In addition, the reduction in stereotypic behaviors may indicate an improvement in the welfare of this group of Masai giraffe.
REFERENCES


REBER, L., 2008. The relationship of feeding frequency and feeding behaviors to ruminal PH of Masai Giraffe(Giraffa camelopardalis tippelskirchi) housed at the Cleveland metroparks Zoo, West Virginia University-Department of Animal and Nutritional Sciences.


