BLACK HOWLER MONKEY (*ALOUATTA PIGRA*) NUTRITION: INTEGRATING THE STUDY OF BEHAVIOR, FEEDING ECOLOGY, AND THE GUT MICROBIAL COMMUNITY

BY

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DISSERTATION

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ABSTRACT

All animals, including primates, face the challenge of obtaining sufficient energy and nutrients despite 1) variation in food availability across habitats and seasons and 2) temporal fluctuations in nutritional requirements due to life history processes. Because variation in food availability or nutritional requirements requires animals to vary energy and nutrient intake, vary energy and nutrient expenditure, or vary digestion and assimilation of energy and nutrients to meet demands, many studies of primates examine shifts in primate activity budgets and foraging patterns across seasons and life history stages. However, few studies establish a direct relationship between activity and diet composition and energy and nutrient intake. Additionally, the mechanisms that primates use to digest and assimilate their food are largely overlooked. Mutualistic gut microbial communities impact host digestive efficiency and assimilation by breaking down otherwise indigestible material and providing hosts with energy and nutrients. Laboratory studies have demonstrated that gut microbial communities shift in response to changes in host diet and physiology, and while these shifts may allow hosts to digest food items more efficiently to meet energy and nutrient demands, no data are currently available to explore this relationship in wild primates.

This dissertation describes an integrated 10-month field study investigating the behavioral and physiological mechanisms used by non-human primates to satisfy nutritional demands in response to changes in diet and physiology. Specifically, it examines the relationship between behavior, physiology and nutrition in two groups (N = 16 individuals) of wild, black howler monkeys (*Alouatta pigra*) in Palenque National Park, Chiapas. The first chapter explores patterns in black howler monkey nutritional intake across time to determine whether howlers employ a foraging strategy that regulates energy and/or nutrient intake and whether this strategy

changes in response to the amount of ripe fruits or leaves in the howler diet. The second investigates the response of the howler monkey gut microbial community to changes in diet composition across time and the potential effects of changes in the gut microbial community on howler digestive efficiency and nutrition. Finally, the third chapter examines differences in activity, diet, and the gut microbiota among adult male, adult female, and juvenile howler monkeys to determine whether behavioral or physiological mechanisms allows adult females and juveniles to compensate for the increased nutritional demands of reproduction and growth.

The data presented in this dissertation suggest that although they are able to consume large quantities of leaves periodically, on an annual basis, black howler monkeys consume more ripe fruits than leaves. They also exhibit a protein-regulating foraging strategy similar to that of ripe-fruit-specialist spider monkeys and consume more protein energy and more total energy than spider monkeys. These results indicate that black howler monkey feeding ecology is similar to that of other primates that consume mostly fruit and that both fruits and leaves are critical to understanding howler monkey nutrition and feeding ecology. Additionally, data from this dissertation show that the impacts of the gut microbial community must be considered when discussing howler monkey ecology and evolution. The howler gut microbial community shifts in response to changes in the howler diet over time, contributing additional energy during periods of reduced energy intake. Similarly, adult female and juvenile howler monkeys are characterized by bacteria that produce more energy and vitamins compared to adult males. These differences, together with differences in nutritional intake, may play a role in allowing females and juveniles to meet the increased nutritional demands of reproduction and growth. As a result, while behavior and foraging patterns are important in understanding how howler monkeys respond to

temporal variation in food availability while maintaining activity, ranging and life history patterns, the nutritional contributions of the gut microbiota are also critical.

Understanding how primate behavior, feeding ecology, and gut microbial processes relate has important implications for the study of primate ecology and evolution. The resources provided by the gut microbial community are typically not accounted for in traditional studies of behavior and feeding ecology but are crucial for understanding primate nutrition. By pinpointing both the causes and effects of changes in gut microbial community composition and improving the understanding of how foragers adjust to changing nutritional demands in variable environments, we can approach studies of primate behavior, nutrition, and health more effectively. While the patterns and mechanisms involved may differ across primate populations in response to differences in phylogeny or habitat, improved knowledge of both nutrition and physiology is critical for primate research worldwide.

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CHAPTER 1: GENERAL INTRODUCTION

All animals, including primates, face challenges in energy and nutrient acquisition as a result of 1) variation in food availability across habitats and seasons and 2) temporal fluctuations in energy and nutrient demands due to processes such as reproduction and growth (e.g. Boinski 1988, Chapman et al 2003, Dufour and Sauther 2002, Gates 2006, Gonzalez et al 2002, Milton 1980, Rumiz et al 1986, van Schaik et al 1993a). According to mammalian bioenergetics models, if changes in food availability across habitats or seasons lead to reductions in energy and nutrient intake, individuals must reduce activity, invest fewer resources in growth and/or reproduction, or increase digestive and assimilation efficiency to compensate (McNab 2002, Peles and Barrett 2008). Likewise, as energy and nutrient demands increase during periods of growth or reproduction, individuals must increase energy and nutrient intake, reduce activity, or increase digestive and assimilation efficiency to maintain body condition and nutritional status (McNab 2002, Peles and Barrett 2008).

Compared to many other groups of mammals, primates are characterized by complex cognitive skills that may enable them to efficiently track spatial and temporal changes in food availability, as well as select or avoid food items based on changes in nutrient content across seasons (Byrne 1995, Cunningham and Janson 2007, Garber 1989, Janmaat et al 2006, Janson and Byrne 2007, Tomasello and Call 1997). These abilities may aid them in meeting energy and nutrient needs across space and time. Some primates, such as spider monkeys (*Ateles* sp.) and woolly monkeys (*Lagothrix lagotricha*), also possess locomotor specializations that allow them to quickly travel large distances to exploit ephemeral, high-energy and -nutrient foods during times of reduced food availability (Cant 1986, Cant et al 2003, Defler 1999, Parsons and Taylor 1977). Others, such as howler monkeys (*Alouatta* sp.) and indriids (e.g. *Propithecus* sp.*),* use

physiological adaptations such as high-cusped molars with pronounced shearing crests and colons or caecums with increased length, volume and surface area to improve their ability to process hard-to-digest foods such as leaves during times of reduced food availability (Campbell et al 2000, Fleagle 2013, Hemingway 1998, Lambert 1998, Lambert et al 2004).

Together, these adaptations enable primates to exploit a wide range of food resources, and primate field studies have successfully described many patterns in diet composition across primate populations and species (Di Fiore et al 2011). However, much less is understood regarding the relationship between diet composition and energy and nutrient intake. For most primates, it is unclear whether some nutrients are prioritized over others and how much intake levels vary from day to day. Although leaf-heavy diets (>50% of feeding time) are assumed to be higher in protein and lower in energy (Norconk et al 2009), and fruit-heavy diets are assumed to be lower in protein and higher in energy (Norconk et al 2009), few studies of wild primates have validated these assumptions with measures of nutritional intake (but see Felton et al 2009a, Felton et al 2009b, Rothman et al 2008, Rothman et al 2011). Data suggest that spider monkeys consuming a diet rich in ripe fruits utilize a protein-regulating foraging strategy in which they maintain a constant intake of protein energy from day to day while lipid and carbohydrate energy intake varies dramatically (Felton et al 2009a). In contrast, gorillas consuming a diet consisting of mostly leaves have been shown to utilize an energy-regulating foraging strategy in which they maintain a constant intake of overall energy across seasons while protein energy intake varies (Rothman et al 2011). However, whether fruit-heavy diets are always associated with protein-regulation and leaf-heavy diets are always associated with energy-regulation remains to be verified with studies of more primate taxa.

Furthermore, the mechanisms that primates use to digest and assimilate their food are largely overlooked (except see Lambert 1998, Lambert and Fellner 2012, Milton 1979, Milton et al 1979, Milton et al 1980, Milton and McBee 1983). Understanding these mechanisms is critical to understanding primate nutrition since digestive processes ultimately dictate the amount of energy and nutrients an individual receives. The nutritional value of a given food item may be higher or lower than suggested by nutritional analyses depending on how efficiently it is digested and assimilated (Mackie and White 1997a).

Digestive efficiency and assimilation are affected by two major factors: gut morphology and the activity of mutualistic gut microbes. Because intestinal volume and length are directly proportional to the amount of food that can be ingested per unit time and to digesta retention time (Mackie and White 1997a), larger guts allow more food to be consumed and allow more complete digestion. Larger guts also allow individuals to absorb more energy and nutrients since intestinal surface area is directly proportional to energy and nutrient absorption (Mackie and White 1997a, Sibly 1981) (although the permeability of the epithelial layer as well as the degree of paracellular absorption play a role as well; Hammond and Kristan 2000, Mcwhorter and Karasov 2007). As a result, individuals experiencing reduced energy and nutrient intake or increased energy and nutrient demands are expected to increase gut size to break down and absorb energy and nutrients more efficiently. This pattern has been reported in laboratory studies of rodents (Gross et al 1985, Hammond and Kristan 2000). For example, prairie voles (*Microtus orchrogaster*) housed at 5°C versus 23°C for 18 days exhibited an 8% increase in small intestine length and an 11% increase caecum length (Gross et al 1985). However, while changes in gut morphology are likely to improve an individual's digestive efficiency, they are also likely to

result in nutritional costs since gut tissue is expensive to produce and maintain compared to all other tissues except the brain (Aiello and Wheeler 1995, Webster 1981).

In contrast, mutualistic gut microbial communities impact digestive efficiency and assimilation by breaking down otherwise indigestible material in the diet and providing hosts with energy via the formation of short-chain fatty acids (Flint et al 2008, Goel et al 2005, Nelson et al 2003, Odenyo et al 2001). This process is unlikely to incur nutritional costs. Also, because intestinal microbes are obtained from an individual's environment (conspecifics, food, etc.), and selective pressures in the gut dynamically change due to host diet and physiology (Friswell et al 2010, Gronvold et al 2010, Knapp et al 2009, Ley et al 2006a, Ley et al 2008, Mariat et al 2009, Rudi et al 2009), gut microbial community composition shifts over time and may adapt to host needs. For example, changing the diet of 340 mice from a low-fat diet rich in plant polysaccharides to a high-fat, high-sugar diet resulted in a dramatic increase in the abundance of several classes of bacteria in the Firmicutes phylum over the course of one day (Erysipelotrichi: 3.3% to 15.9%; Bacilli: 0.1% to 13.0%) (Turnbaugh et al 2009).

Because different microbes can utilize different substrates to produce different compounds, changes in the composition of the gut microbial community lead to changes in its function and ultimately affect host nutrition (Brinkworth et al 2009, Degnan 1992, Dehority et al 1958, Donohoe et al 2011, Duncan et al 2004, Duncan et al 2007, Flint et al 2012, Fraser et al 2009, Hooper et al 2002, Macfarlane 1991, Macfarlane and Macfarlane 2003, Nicholson et al 2012, Secor 2001, Turnbaugh et al 2006). Therefore, shifts in gut microbial community composition in response to variation in host diet across habitats or seasons as well as in response to the physiological changes of reproduction and growth may allow individuals to meet nutritional needs. Studies have demonstrated that the higher the ratio of Firmicutes to

Bacteroidetes bacteria in the gut, the more energy produced by the gut microbial community (Armougom et al 2009, Ley et al 2005, Ley et al 2006b). If the Firmicutes to Bacteroidetes ratio increases during periods of low energy intake or high demand, it may allow primates to fulfill energy requirements without dramatic changes in behavior. Likewise, microbial vitamin synthesis (LeBlanc et al 2007, Santacruz et al 2010, Yatsunenko et al 2012) may provide an important supplement to primate diets during some periods of the year, such as the microbial production of folic acid during periods of female reproduction (Czeizel and Dudas 1992, Czeizel et al 2010, Lamers 2011).

This dissertation describes an integrated 10-month field study investigating the behavioral and physiological mechanisms used by non-human primates to satisfy nutritional demands in response to changes in diet and physiology. Specifically, it examines temporal variation in foraging behavior and gut microbial community composition and function in adult male, adult female, and juvenile wild black howler monkeys (*Alouatta pigra*) in Palenque National Park, Mexico. Although howlers appear to exhibit flexibility in diet and food choice in response to changing nutritional demands (Milton 1980, Milton et al 1980, Milton 1998), the relationship between dietary patterns, physiology, and gut microbial ecology and its effect on howler nutrition and health is poorly understood.

Howler monkeys are an ideal system for exploring the interaction between primate foraging strategies and digestive functions. They can dedicate up to 95% of their monthly feeding time to leaves, but also consume a large proportion of ripe fruits when available (up to 92% of monthly feeding time; Di Fiore et al 2011). This dietary flexibility allows them to occupy a wide range of arboreal habitats and to endure seasonal changes in food availability by shifting their diet composition (Di Fiore et al 2011, Rylands et al 2006). It also may allow individuals to

select the food items that they require to meet nutritional demands across time and space (Altmann 2009, Pollan 2006).

Howlers have a slightly enlarged colon with increased retention time compared to other atelines (*A. palliata:* 20.4 hrs; *A. seniculus:* 18.8-20.0 hrs; *A. guariba:* 19.0 hrs vs. *Ateles geoffroyi*: 4.7 hrs; *Ateles paniscus:* 5.25 hrs; *Ateles belzebuth:* 4.5 hrs; *Brachyteles arachnoides:*8.0-14.0 hrs; *Lagothrix lagotricha:* 2.0-14.5 hrs*;* Edwards and Ullrey 1999, Link and Di Fiore 2006, Martins 2006, Milton 1981, Milton 1984, Stevenson 2000, Yumoto et al 1999), which appears to aid them in obtaining nutrients and energy from a leaf-rich diet (Milton et al 1979, Milton 1998, Rosenberger and Strier 1989). However, they lack the specialized adaptations of the fore- and hindgut (sacculated stomach, elongated and complex caecum, etc.) present in several species of folivorous Old World monkeys (colobines) and prosimians (indriids; Bauchop and Martucci 1968, Edwards and Ullrey 1999, Lambert 1998, Milton 1998, Rosenberger and Strier 1989). Consequently, howlers are believed to depend heavily on the activity of the gut microbial community for meeting nutritional needs. They are estimated to gain as much as 31% of required daily energy from the gut microbial community (Milton and McBee 1983) and are likely to be highly sensitive to changes in its function.

Finally, compared to other atelines, howlers have an earlier age at reproduction (42-62 months vs. *Ateles:* 84-85 months, *Brachyteles:* 87-108 months, *Lagothrix:* ~87 months), a shorter gestation period (152-195 days vs. *Ateles:* 226-232 days, *Brachyteles:* 215-218 days, *Lagothrix:* 210-225 days), and a shorter interbirth interval (16-23 months vs. *Ateles:* 32-50 months, *Brachyteles:*32-41 months, *Lagothrix:* 32-41 months; Di Fiore et al 2011, Fedigan and Rose 1995). Although *Alouatta* also tends to be the smallest of the atelines (adult males: 6.1-11.4kg compared to *Ateles:* 8.2-9.1kg, *Brachyteles:* 9.4-13.8kg, *Lagothrix* 9-9.5kg), prenatal

growth rates are estimated to be higher for howler monkeys than spider monkeys (2.14-2.84g/day vs. 1.86-2.03g/day) or woolly monkeys (1.92-2.02g/day), and neonatal brain size is smaller compared to spider monkeys (53% vs. 58% of adult size; Hartwig 1996). Additionally, juvenile howlers are weaned at an earlier age compared to other atelines (11-14 months vs. *Ateles:* 24-36 months, *Brachyteles:* 18-24 months; Di Fiore et al 2011). These patterns suggest that the daily nutritional demands for growth and reproduction in adult female and juvenile howlers are likely higher than in other atelines, and adult female and juvenile howlers are likely to require pronounced changes in activity, diet, and/or gut microbial community composition and activity to meet these demands.

This dissertation examines the relationship between howler monkey behavior, physiology and nutrition in a series of three chapters. In the first, I explore patterns in howler monkey nutritional intake across time. Specifically, I aim to determine whether howler monkeys use an energy-regulating foraging strategy similar to that observed in gorillas or a protein-regulating foraging strategy similar to that observed in spider monkeys and whether this strategy changes in response to the amount of ripe fruits or leaves in the howler diet. In the second chapter, I investigate the response of the howler monkey gut microbial community to changes in diet composition across time. Assuming activity patterns are consistent throughout the year, howler monkeys should exhibit shifts in digestive efficiency via changes in gut microbial community composition and function that allow them to maintain energy and nutrient levels despite variation in the nutritional content of their diet. Finally, in the third chapter, I examine differences in activity, feeding, and gut microbial community composition and function among adult male, adult female, and juvenile howler monkeys. If adult females and juveniles have relatively higher energy and nutrient requirements than adult males due to reproduction and growth, we would

expect them to exhibit differences in either energy and nutrient intake, energy and nutrient expenditure, or digestion and assimilation of energy and nutrients. We would also expect the importance of each of these compensatory mechanisms to vary across time as diet composition shifts with food availability.

Understanding how primate behavior, feeding ecology, and gut microbial processes relate has important implications for the study of primate ecology and evolution. Although this study examines these interactions in only one population of primates, it is my hope that these data provide impetus for additional studies across a range of taxa. While the patterns and mechanisms involved may differ according to phylogeny or habitat, improved knowledge of both nutrition and physiology is critical for primate research worldwide.

CHAPTER 2: FORAGING STRATEGIES OF THE BLACK HOWLER MONKEY (*ALOUATTA PIGRA*) IN PALENQUE NATIONAL PARK, MEXICO

ABSTRACT

Because primate food resources vary dramatically in nutritional content, primates must utilize a mixture of food resources to balance energy and macronutrient intake and reduce fiber and toxin intake. To understand how primates select food resources to achieve this, many studies of primate feeding ecology describe patterns in primate diet composition across time and space. However, few studies actually measure primate energy and nutrient intake. In this chapter, I estimate energy and nutrient intake in two groups (N=16 individuals) of wild, black howler monkeys (*Alouatta pigra*) in Palenque National Park, Mexico across a 10-month period to determine how diet composition relates to nutritional intake. Because howler monkeys consume high proportions of leaves during some months of the year, and leaves tend to be lower in energy than ripe fruits, howler monkeys are generally assumed to be energy-limited. Nevertheless, during some months of the year, howler monkeys consume mostly fruits which tend to be higher in energy and lower in protein than leaves, which may change nutritional intake patterns. Data from this study suggest that black howlers meet estimated energy requirements by consuming an average of 0.57 MJ of overall energy per metabolic body weight per day and surpass protein requirements by consuming an average of 8.6g of protein per metabolic body weight per day. These estimates surpass those provided for spider monkeys. Additionally, the amount of time the black howlers spent resting was not correlated with the amount of leaves or fruit in the diet or with overall energy intake. Therefore, despite consuming a leaf-heavy diet during some periods of the year, black howlers do not appear to be energy-limited. Finally, the howlers maintained a consistent level of average daily protein energy intake regardless of diet composition, while non-protein energy intake varied dramatically in response the amount of ripe fruits consumed. This

pattern matches that observed in spider monkeys and may be a result of howler food selectivity. Additionally, because these howlers consumed fruits twice as quickly as leaves, fruit intake was higher than time-based estimates suggest. These finding suggests that howlers are similar to other fruit-eating atelines, and many common assumptions regarding howler behavior and feeding ecology must be reexamined.

INTRODUCTION

Basic discussions of primate foraging behavior commonly lump food resources into two general categories: "high" and "low" quality foods. High quality foods (e.g. ripe fruits, arthropods) are described as rich in energy or nutrients and easy to digest, but difficult to locate in time and space (Strier 2011). In contrast, low quality foods (e.g. mature leaves, bark) are described as more abundant in time and space, but more difficult to process and digest due to high amounts of fiber or plant secondary compounds (Strier 2011).

Despite their convenience, these broad generalizations underplay the complexity of primate foraging ecology. Instead of being described as "high" or "low quality," most primate food resources fall along a gradient according to extractable protein, simple sugar, and lipid content as well as fiber and plant secondary metabolite levels (Altmann 2009, Conklin and Wrangham 1994, Felton et al 2008, Felton et al 2009b, Glander 1982, Milton 1979, Milton 1991, Norconk et al 2009, Rothman et al 2011). A survey of Neotropical primate food items reports that compared to other plant parts, ripe fruit pulp generally contains higher amounts of non-structural carbohydrates (59%), lower levels of fiber (26%), and lower levels of crude protein (8%; Norconk et al 2009). Flowers and both young and mature leaves have higher levels of fiber than ripe fruit pulp (44%, 51%, 58%), but also have higher protein levels (17%, 20%, 14%;

Norconk et al 2009). Exudates provide high levels of non-structural carbohydrates (78%) but also contain soluble fiber that makes them difficult to digest, while seeds are an important source of lipids for primates that can overcome their tough mechanical defenses which include hard seed coats (Norconk et al 2009). Additionally, the nutritional content of a plant part depends heavily on the plant species from which it comes. For example, although fruit pulp is often low in protein and fiber, this is not always the case. The amount of crude protein (CP) contained in Neotropical ripe fruit can vary from 1.9% of dry weight (Hladik et al 1971) to 18.0% (Castellanos and Chanin 1996) while neutral-detergent fiber (NDF) varies from 0% to 59.2% dry weight (Felton et al 2009b). Likewise, CP levels in Neotropical young leaves can be as low as 12.8% of dry weight or as high as 24.5% (Norconk and Conklin-Brittain 2004) while NDF varies from 15.0% (Milton 1979) to 74.9% of dry weight (Felton et al 2009b).

Because the nutritional content of food items can vary so dramatically, primates must utilize a mixture of plant parts and plant species to balance energy and macronutrient intake and reduce fiber and toxin intake (Altmann 2009, Felton et al 2009a, Rothman et al 2011). As a result, primates are highly selective with regard to the food items they consume (Altmann 1998, Altmann 2009, Pollan 2006, Rozin 1976). In leaf-eating primates, several studies have shown that individuals principally consume leaves with higher protein to fiber ratios, select species with lower secondary metabolite levels, and utilize a variety of leaf species in a given day to reduce the intake of any single secondary metabolite (Calvert 1985, Freeland and Janzen 1974, Glander 1979, McKey et al 1981, Milton 1979, Oates et al 1980).

When combined with temporal variation in food item availability (Fenner 1998, Jordano 2000, van Schaik et al 1993b, van Schaik and Pfannes 2005) as well as the potential for scramble and contest feeding competition with other group members (Janson 1985, Janson 1988a, Janson

1988b, van Schaik and van Noordwijk 1988, van Schaik 1989), feeding selectivity constrains the array of food items that can be utilized by foraging primates. Therefore, many primate taxa exhibit adaptations that allow them to exploit food resources they would otherwise be unable to access. In the Neotropics, each primate genus possesses a unique suite of anatomical and behavioral specializations for obtaining and processing specific food resources (Garber 1992, Norconk et al 2009, Rosenberger 1992). The post-cranial skeletal and muscular adaptations of the *Ateles* genus allow it to use suspensory locomotion to increase travel speed and move more directly between trees when exploiting ephemeral, patchily-distributed resources such as ripe fruits (Cant 1986, Parsons and Taylor 1977). Other genera, such as *Callithrix*, *Mico,* and *Cebuella* exploit tree exudates and exhibit clawlike nails for clinging vertically to tree trunks, procumbent and elongated incisors for gouging tree bark, and an enlarged caecum and colon for processing the soluble fiber believed to be present in exudates (Garber 1992, Power 1996, Power and Oftedal 1996).

Studies of primate diets have demonstrated that these adaptations, together with temporal and spatial differences in resource availability, result in marked diversity in the plant parts and plant species consumed by different primate species, populations and individuals (Boinski 1988, Di Fiore et al 2008, Ferrari and Martins 1992, McKey 1978, Milton 1981, Overdorff et al 1997, Robbins et al 2006). For example, in a 31-month study, brown spider monkeys (*Ateles hybridus*) in Colombia were reported to consume foods from at least 123 plant species with the number of species exploited per month varying from four to 36 species (average: 15.7 species; Link et al 2012). In contrast, an 18-month study of sympatric spider monkeys (*Ateles belzebuth*) and woolly monkeys (*Lagothrix lagotricha*) indicated that spider monkeys utilized 73 species of fruits and woolly monkeys utilized 104, with approximately 27% dietary overlap (Dew 2005).

However, despite such extensive datasets examining patterns in primate diet composition across time and space, the extent to which primate diets differ in terms of nutritional content is unclear. While the ability of two primate species to specialize on different plant parts and species may result in distinct patterns of nutrient and energy intake, feeding selectivity and nutrient mixing may allow both species to consume similar amounts of nutrients and energy despite differences in diet composition (Conklin-Brittain et al 1998). Likewise, within primate species, differences in food item availability across time and space may result in temporal and spatial variation in diet composition and, ultimately, nutrient and energy intake patterns (Knott 1998, Nakagawa 1997).

Initial studies of primate nutritional intake patterns suggest that diet composition may be related to nutritional intake across species (Felton et al 2009a, Rothman et al 2011). In a nine-month study, spider monkeys (*Ateles chamek*) spent more than 70% of average monthly feeding time consuming fruits in all but one month (range: 44%-100%) and spent only 0-32% of monthly feeding time consuming leaves (Felton et al 2008). Ripe fruits generally contained more non-structural carbohydrates and lipids and less protein than all other food items (Felton et al 2009b), and calculations of nutritional intake suggested that spider monkeys were regulating protein to meet daily requirements. Regardless of the amount of fruit or leaves consumed during a given month, individuals maintained a constant intake of available protein from day to day (expressed as energy: 0.19 MJ). In contrast, lipid and carbohydrate energy intake varied dramatically in response to fruit consumption (0.7-6.2 MJ, 13.8% lipid, 86.2% carbohydrate; Felton et al 2009a). During those months in which ripe fruit intake increased, daily carbohydrate and lipid energy intake increased by 52% (Felton et al 2009b). This pattern suggests that during some periods of the year, as fruit specialists, spider monkeys had to over-consume nonprotein energy to meet protein energy requirements (Felton et al 2009a).

In a similar 12-month study of mountain gorillas (*Gorilla beringei*), leaves made up a majority of the diet in every month of the study (~55% to 96% monthly wet weight) while the proportion of fruit in the diet ranged from 4-45% wet weight (Rothman et al 2008). As reported for spider monkeys, leaves consumed by the gorillas contained fewer non-structural carbohydrates and more protein than fruits (Rothman et al 2006, Rothman et al 2007). However, in contrast to spider monkeys, an examination of the nutrient and energy content of the leaf-heavy gorilla diet revealed that total energy intake was consistent across months (adult males: ~25MJ), regardless of diet composition, while protein energy intake varied positively in response to the amount of leaves consumed (adult males: ~6-12MJ; Rothman et al 2011). The gorillas were regulating total energy intake and during periods of heavy leaf-eating, they over-consumed protein energy to meet total energy requirements (Rothman et al 2011).

Based on these studies, the consumption of a diet of mostly ripe fruit appears to be associated with a protein energy-regulating intake pattern while a diet of mostly leaves is associated with a total energy-regulating intake pattern. However, additional studies across a variety of primate taxa are needed to test the strength of this relationship. Here, I examine the relationship between diet composition and nutrient and energy intake in two groups of wild, black howler monkeys (*Alouatta pigra*) in Palenque National Park, Mexico.

Black howler monkeys belong to the family Atelidae and have been reported to consume more than 90% young and mature leaves in a given month (Table 2.1). Studies of howler monkey diets indicate that all howler species devote more than 50% of feeding time to leaves during some months of the year (Table 2.1), and one species, the brown howler monkey (*A. guariba*), has been reported to devote 56-92% of monthly feeding time to the consumption of leaves (Chiarello 1994, Mendes 1989). Howler monkeys exhibit a set of adaptations associated

with leaf-eating such as an enlarged hindgut (Chivers and Hladik 1980, Hill 1962) and an increased retention time compared to other atelines (*A. palliata:* 20.4 hrs; *A. seniculus:* 18.8-20.0 hrs; *A. guariba:* 19.0 hrs vs. *Ateles geoffroyi*: 4.7 hrs; *Ateles paniscus:* 5.25 hrs; *Ateles belzebuth:* 4.5 hrs; *Brachyteles arachnoides:*8.0-14.0 hrs; *Lagothrix lagotricha:* 2.0-14.5 hrs*;* Edwards and Ullrey 1999, Link and Di Fiore 2006, Martins 2006, Milton 1981, Milton 1984, Stevenson 2000, Yumoto et al 1999). They also depend heavily on a diverse gut microbial community to extract energy from their potentially high-fiber diet (Milton and McBee 1983). Finally, howler monkeys are reported to adopt a behavioral strategy that reduces energy expenditure (Gaulin and Gaulin 1982, Milton 1980, Rosenberger and Strier 1989, Smith 1977). Compared to other atelines, they utilize small home ranges (average: 28ha vs. 278ha in *Ateles*, 154ha in *Brachyteles* and 398ha in *Lagothrix*) and day ranges (average: 526m vs. 2,142m in *Ateles*, 1,075m in *Brachyteles*, and 1,925m in *Lagothrix*) and spend slightly more of their active hours resting (56-80% vs. 24-61% in *Ateles*, 49-61% in *Brachyteles*, and 23-36% in *Lagothrix*; Di Fiore et al 2011).

Because their leafy diet and energy-minimizing behavior imply limits on energy intake, we might expect howler monkeys to occupy a total energy-regulating foraging strategy similar to that observed in gorillas rather than the protein energy-regulating foraging strategy characteristic of spider monkeys. However, it is important to note that atelines are primarily fruit-eating primates (Di Fiore et al 2011), and the howler diet can include seasonally large amounts of fruit (Table 2.1). For example, studies of *A. palliata* by Milton (1980) show that during months of high fruit availability, fruit accounts for 66% of feeding time. Similarly, studies of *A. belzebul, A. caraya, A. palliata, and A. pigra* indicate that fruit can be responsible for 80% or more of monthly feeding time and up to 50% of total feeding time in a given year (Bonvicino 1989, Estrada 1984, Ludwig et al 2008, Pavelka and Knopff 2004, Pinto and Setz 2004, Stoner 1996).

Therefore, although most howler species consume a diet that includes more than 50% leaves during many months of the year, fruit makes up the majority of the diet during other months. If nutrient and energy intake patterns are determined by the nutritional content of the most heavily utilized plant part in the diet (Felton et al 2009a, Rothman et al 2011), we would expect howlers to exhibit monthly shifts in nutrient intake patterns from total energy-regulation to protein energy-regulation when consuming leaf-heavy versus fruit-heavy diets.

Examinations of mantled howler monkey (*A. palliata*) foraging behavior by Milton support this prediction (Milton 1979, Milton 1981). Because their small body size (females: 5.68 ± 0.63kg, males: 7.6 ± 1.13kg; Kelaita et al 2011) limits the amount of food that they can consume and their relatively unspecialized gut morphology limits digestive efficiency, Milton (1979) concluded that howler monkeys are unable to extract sufficient energy for growth, maintenance and reproduction from a leaf-heavy diet and should therefore prioritize energy intake when foraging during periods of low fruit availability. In contrast, during periods of high fruit availability, Milton (1981) postulated that because fruit is generally low in protein and long retention times limit food intake rates, howler monkeys are unable to consume enough fruit to fulfill their protein requirements and should increase protein intake by including leaves as an important component of their diet.

In this chapter, I test a series of hypotheses regarding howler monkey foraging behavior and nutrient and energy intake. **(1) Assuming leaves consumed by howlers contain more protein and less non-structural carbohydrates than ripe fruit, as leaf-eaters, black howler monkeys are expected to be characterized by a high protein, low energy diet.** Specifically, black howler monkeys should consume enough total energy daily to meet estimated energy requirements and should over-consume protein, especially during periods when leaves make up

the majority of the diet. Additionally, howler monkeys should consume less total energy and more protein energy per metabolic body weight compared to more-frugivorous spider monkeys due to the fact that fruits consumed by spider monkeys tend to have more nonstructural carbohydrates and less protein than leaves (Felton et al 2009b). **(2) Assuming that energy intake is limited on a leaf-heavy diet, howler monkeys are expected to exhibit behaviors that minimize energy expenditure.** Resting time should increase and day range length should decrease as the proportion of leaves in the diet increases and/or the amount of energy in the diet decreases. **(3) Assuming ripe fruits consumed by black howler monkeys are higher in energy and lower in protein while leaves are higher in protein and lower in energy, black howler monkeys are expected to shift their foraging strategy over time as their diet changes from fruit-heavy to leaf-heavy.** Specifically, black howlers should regulate protein energy intake during periods when fruit makes up more than 50% of the diet, and they should regulate total energy intake during periods when leaves make up more than 50% of the diet.

METHODS

Study Site: This study was conducted in Palenque National Park, Chiapas, Mexico during a ten-month period (September 2010-June 2011). The park contains approximately 900 ha of tall evergreen tropical rain forest surrounded by pasture lands (Diaz-Gallegos 1996). The mean annual temperature is 26 ºC (range 22-29 ºC), and average annual rainfall is 2200 mm (CONAGUA 2011). A rainy season occurs from June to December (avg monthly precipitation: 274 ± 79mm) while January through May are considered dry months (avg monthly precipitation: 108 ± 23mm; CONAGUA 2011).

Data describing foraging behavior were collected from two social groups of black howler monkeys inhabiting the primary evergreen rainforest in Palenque, which is dominated by tree species such as *Vatairea lundellii, Manilkara zapota, Guatteria anomala,* and *Brosimum alicastrum* (Diaz-Gallegos 1996). The Balam group consisted of two adult males, two adult females, and two juvenile males. The Motiepa group consisted of four adult males, two adult females, two juvenile males, and two juvenile females. Two of the adult males disappeared during the study and are believed to have dispersed.

Behavioral data collection: My aim in this study was to collect data describing black howler nutrient and energy intake during fruit-dominated and leaf-dominated periods of the year. Therefore, from September 2010 to June 2011, data were collected during three ten-week blocks that corresponded loosely with changes in rainfall (CONAGUA 2011) and previously documented shifts in black howler diet at Palenque (Estrada, unpublished data). Block 1 (Sept-Nov 2010) was generally associated with heavier rainfall (250-400mm) and a higher proportion of ripe fruit (30-40% of feeding time) in the howler diet (CONAGUA 2011, Estrada, unpublished data). Block 2 (Jan-Mar 2011) was generally associated with a lower proportion of fruit (0-15% of feeding time) in the diet and a higher proportion of young leaves (45-55% of feeding time), and Block 3 (Apr-June 2011) was generally associated with less rainfall (75-300mm) and a higher proportion of ripe fruit (40-65% of feeding time) in the diet (CONAGUA 2011, Estrada, unpublished data).

During each sampling block, each howler group was observed during alternating weeks. Focal individual samples of behavior were collected five days per week between sunrise and 5pm (park closing time) for a total of 1,522 hours of quantitative data (Block 1: 328 total hours,

103 feeding hours in 49 days, Block 2: 531 total hours, 139 feeding hours in 49 days, Block 3: 663 total hours, 89 feeding hours in 50 days). An equal amount of feeding data was collected for each group. The focal individual was chosen pseudo-randomly (no individual was sampled twice consecutively and priority was given to individuals that had been undersampled on previous days), and each focal sampling period lasted 20 minutes. Five activities were recorded instantaneously every two minutes: feeding (ingestion of food items), foraging (movement within a feeding tree for the purpose of acquiring food), resting (inactivity), traveling (movement within or between tree crowns whose immediate purpose was not to feed), and social activity (howling, play, sexual interaction, aggression). During feeding bouts, the plant part (ripe fruit, unripe fruit, mature leaves, young leaves, flowers, stems) and plant species being consumed was recorded, and the number of food items and grams (see below) consumed per minute was quantified when possible to provide an estimate of intake rate.

A handheld global positioning system was utilized to track black howler group movements during observations. The position of the group was recorded approximately every thirty minutes. To estimate day range, I calculated the total distance traveled between all points using ArcGIS 10.1 (ESRI 2011, Redlands, CA). The home range during each sampling block was estimated by calculating the area of the minimum convex polygon created by the data points in ArcGIS 10.1 (ESRI 2011, Redlands, CA).

Samples of the top ten food items (not plant species since howlers often consume leaves, fruits, and flowers of the same plant species) as determined by the proportion of group monthly feeding time were collected, and the average wet and dry mass of each resource was measured using five items from each of three trees. Samples of the top ten food resources used during each season also were collected and preserved in 70% methanol for metabolite profiling. Metabolites

are small molecules produced by an organism during metabolism (e.g. amino acids, alcohols, nucleotides, vitamins). In this context, plant metabolite profiles provide information regarding the nutritional value of howler monkey food items. All metabolite data were generated using gas chromatography/mass spectrometry (Poroyko et al 2011). Mass spectra were verified with authentic standards and mass spectra from a commercial database (Poroyko et al 2011).

*Data analysis***:** The behavioral data were used to calculate the average percent time the howlers spent in a given activity during a focal sample in each season. Because howler monkeys are active during all daylight hours, the average daylength during each of the three seasons was used to calculate the average number of minutes per day the howlers spent in each activity. Average ingestion rates for each plant part from each plant species, as well as average food item masses, were used to estimate the average number of grams of each food item ingested daily by each individual based on weekly and monthly data, as well as data from each entire sampling block. The average kilocalories and grams of protein ingested by each individual were calculated using published estimates for Neotropical plant parts (Table 2.2).

Metabolites extracted from the howler food resources were expressed in terms of relative concentration per gram of sample fresh weight and were categorized into amino acids, sugars, and lipids when possible. The relative concentration of each metabolite in each food resource was multiplied by the average daily grams (wet weight) of that resource consumed weekly by each individual to provide an estimate of the concentrations of metabolites consumed. Due to the method by which the metabolite concentrations were standardized, these data do not provide accurate estimates of the actual amount of each metabolite in a food item, nor can their amounts be accurately compared across categories (i.e. amino acid, sugar, lipid) within a sample.

However, relative concentrations of the same metabolite across food items and howler diets can be compared to understand feeding patterns at the metabolite level.

Feeding data were standardized by metabolic body weight (divided by body weight raised to the 0.75) using average masses for each age/sex class from a study of wild *A. pigra* in Mexico prior to analysis (Kelaita et al 2011, Kleiber 1975). Permutational multivariate analysis of variance (PERMANOVA) was used to test for the effects of sampling block on activity and diet. Data were pooled by individual for each block, and I stratified each model by howler group to control for differences between groups. Type III sums of squares were used to determine the significance of each factor in the model. All models were run for 5000 permutations.

A series of non-parametric Kruskal Wallis tests were used to test for temporal differences in the relative concentrations of each metabolite in the diet. P-values were adjusted for the 180 separate tests I conducted using a sequential Bonferroni correction with the intial $p = 0.05$ (Holm 1979, Rice 1989). Differences in literature estimates of energy and nutrients ingested were tested for significance using ANOVA, and Pearson correlations were used to identify relationships between activity and diet and nutrient intake and diet (R software).

RESULTS

Black howler diet: The percentage of time the black howler monkeys devoted to feeding on different plant parts varied across sampling blocks. In Block 1, 34% of feeding time was devoted to young leaves, 25% to ripe fruits, 18% to unripe fruits, 14% to stems, 6% to flowers, and 3% to mature leaves. During Block 2, 63% of feeding time was devoted to young leaves, 11% to ripe fruits, 13% to unripe fruits, 7% to stems, 5% to flowers, and 1% to mature leaves. In Block 3, 35% of feeding time was devoted to young leaves, 49% to ripe fruits, 2% to unripe fruits, 6% to

stems, 0% to flowers, and 8% to mature leaves. However, the time black howlers spent consuming different plant parts was not directly proportional to the amount of grams ingested. For example, the howlers consumed ripe fruit more than twice as fast as young leaves (5.87 vs. 1.60 g/min, Table 2.3). Therefore, all diet data are expressed in grams of dry weight or percent of dry weight ingested.

Overall, the average daily amount of plant material consumed by individuals in each group did not differ (Motiepa: 1057 ± 212g wet weight, 292 ± 80g/metabolic body weight; Balam: 1135 ± 485g, 320 ± 110g/metabolic body weight; $F_{2,38}$=1.40, p = 0.26). However, the grams of food consumed decreased from Block 1 (352 ± 107 g/metabolic body weight) to Block 3 (213 ± 50 g/metabolic body weight; $F_{2,38}$=8.59, p = 0.001). In each month of the study, there were some differences in the proportion of plant parts consumed between groups (Table 2.4), which resulted in significant differences in diet between groups across sampling blocks ($F_{2,38}$=4.73, p = 0.004). The most striking of these differences was the high proportion of ripe fruits in the Balam diet in February, the result of a fruiting *Ficus yoponensis* tree in the middle of their territory. Nevertheless, when I controlled for differences between groups, PERMANOVA indicated that diet composition shifted across sampling blocks for both groups ($F_{2, 38}$ = 22.06, p = 0.0002). For the Motiepa group, ripe fruits made up the majority of the diet in Block 1 (50.7% dry weight) and Block 3 (63.3% dry weight) while young leaves made up the majority of the diet in Block 2 (62.3% dry weight; Figure 2.1). The Balam group exhibited less dietary variation across sampling blocks, but the greatest proportions of ripe fruit were still consumed during Block 1 (41.3% dry weight) and Block 3 (69.5% dry weight; Figure 2.1). Young leaves and ripe fruit were consumed in similar proportions by the Balam group during Block 2 (30.8% and 34.5% dry weight, respectively; Figure 2.1).

Literature estimates of food item nutritional content suggested that ripe fruit tended to contain less protein (4.7%), and to some extent, more non-structural carbohydrates (25.3%), when compared to young leaves (protein: 12.4%, sugars: 10.0%), mature leaves (protein: 14.4%, sugars: 17.7%), flowers (protein: 16.8%, sugars: 30.1%), and stems (protein: 14.9%, sugars: 7.8%; Table 2). Although metabolite analyses revealed distinct patterns for each single metabolite across plant parts, in general, ripe fruits tended to contain lower concentrations of most amino acids and higher concentrations of most sugars compared to other plant parts (Table 2.5). Similarly, essential amino acid levels appeared to be higher in most flowers and leaves compared to ripe and unripe fruits. Like the literature estimates, these patterns suggest that ripe fruits were higher in energy and lower in protein compared to non-fruit food items. However, mature leaves tended to exhibit higher levels of lipid metabolites than ripe fruits and young leaves (Table 2.5). Therefore, because lipids have higher energy content than sugars (9 kcal/g vs. 4 kcal/g) (National Research Council 2003), differences in energy content among food items may have been less than expected based on sugar metabolites.

Nutrient and energy intake calculations using literature estimates revealed foraging patterns that were not related to the consumption of fruit or leaves. During all sampling blocks, the Motiepa group ingested fewer grams of lipids per metabolic body weight ($F_{1,38}$=7.12, p = 0.01) and included a lower average daily proportion of lipids ($F_{1,38}$=20.69, p << 0.01) and protein ($F_{1,38}$=19.70, p << 0.01; Table 2.6) in their diet. However, the differences in the proportions of lipids (3.0% vs. 3.1%) and proteins (11.9% vs. 12.3%) appeared to be biologically insignificant. Across sampling blocks, I detected significant changes in every aspect of nutritional content estimated. Both groups consumed fewer kilocalories ($F_{2,38}$=10.27, p = 0.0003), grams of protein ($F_{2,38}$=17.42, p << 0.01), grams of total non-structural carbohydrates ($F_{2,38}$=17.7, p << 0.001),

and grams of neutral detergent fiber ($F_{2,38}$=17.06, p << 0.01) per metabolic body weight in Block 3compared to Block 1 (Table 2.6). Temporal variation in lipid intake was distinct between groups ($F_{2,38}$=4.36, p = 0.02; Table 2.6). Patterns in protein energy, non-protein energy (carbohydrate and lipid), and overall energy intake were similar (Table 2.7).

Both groups consumed fewer lipids during Block 3, but members of the Balam group consumed more grams of lipids than the Motiepa group in Block 2. This pattern suggests that the Balam group had more reliable sources of lipids during Block 2. Literature estimates of lipid content are higher for ripe fruit and flowers (Norconk et al 2009), and the Balam group consumed more of these resources than the Motiepa group during Block 2. This behavior is likely to have resulted in the higher energy intake observed for the Balam group compared to the Motiepa group during Block 2. The proportion of protein in the diet was highest during Block 2 for both groups ($F_{2,38}$=15.43, p << 0.01), and the proportion of non-structural carbohydrates was lowest ($F_{2,38}$=14.86, p << 0.01; Table 2.6). The proportion of lipids in the diet was similar across sampling periods for the Balam group but lower during Block 2 for the Motiepa group ($F_{2,38}$=13.92, p << 0.01). However, again, differences in lipid proportions across sampling blocks appeared to be biologically insignificant.

There also were significant differences between groups in the metabolites consumed ($F_{1,38}$=3.08, p = 0.035). First, pantothenic acid, or vitamin B5, was consumed in higher concentrations by the Motiepa group. It is considered an essential nutrient for many animals and is involved in co-enzyme A, protein, carbohydrate, and lipid synthesis (Bender 2003). Also, in contrast to the literature estimates, members of the Motiepa group consumed higher average daily relative concentrations of lipid metabolites per metabolic body weight compared to the Balam group ($F_{2,38}$ = 7.23, p = 0.011). Finally, during Block 1, the Motiepa group consumed a

higher relative concentration of sugar metabolites than the Balam group, while in Block 3, the Balam group consumed a higher concentration ($F_{2,38}$ = 4.8, p = 0.014; Table 2.8).

Despite these differences, both groups exhibited changes in metabolite consumption patterns across time ($F_{2,38}$ =13.34, p = 0.0002). All classes of metabolites were consumed in the highest concentrations during Block 1. When I examined the relative concentrations of single metabolites across sampling blocks, I detected no significant changes in any metabolite for the Balam group across sampling blocks. This pattern suggests that temporal patterns in the overall array of metabolites ingested were driven by small changes in the ingestion of a variety of metabolites. In contrast, temporal patterns in metabolite consumption appear to have been driven by a subset of nine metabolites in the Motiepa group (Table 2.9).

Patterns in amino acid concentration were similar to those of protein intake with both groups consuming a lower relative concentration of amino acids during Block 3 ($F_{2,38}$ = 30.36, p << 0.01; Table 2.8). Additionally, Motiepa consumed a higher concentration of sugar metabolites during Block 1 compared to both other sampling blocks while Balam consumed a lower concentration in Block 2 compared to both other sampling blocks ($F_{2,38}$ = 13.34, p = << 0.01; Table 2.8). These patterns were loosely related to those described by non-structural carbohydrate estimates. Patterns in lipid metabolites were also fairly similar to literature estimates. The average daily diet of both groups contained decreasing average daily relative concentrations of lipid metabolites per metabolic body weight from Block 1 to Block 3, but Motiepa exhibited the highest values in Block 1 with similar values in Blocks 2 and 3 while Balam exhibited the lowest values in Block 3 ($F_{2,38}$ = 25.59, p << 0.01).

Black howler activity: Across the study period the Balam group spent an average of 64.9% of daylight hours resting, 21.7% feeding, 5.0% traveling, 4.4% engaging in social behavior, and 1.8% foraging. The Motiepa group spent an average of 65.4% of daylight hours resting, 20.7% feeding, 4.5% traveling, 5.7% engaging in social behavior and 1.7% foraging. Consistent with energy-minimizing behavior, the black howler monkeys at Palenque spent the majority of their time resting during each time block (Figure 2.2).

PERMANOVA indicated that activity budget did not differ between groups ($F_{2, 38} = 1.44$, $p = 0.22$), but it did differ across sampling blocks ($F_{2, 38} = 18.07$, $p = 0.0002$). Temporal differences were driven by increased time spent resting in Block 3, which was correlated positively with daily average temperature ($r^2 = 0.47$, $p << 0.01$). All other aspects of the howler activity budget did not vary significantly across sampling blocks, and home range and day range distances also did not vary across sampling blocks (Table 2.10).

Howler activity was generally not correlated with howler energy and nutrient intake. However, protein intake per metabolic body weight was positively correlated with time spent feeding ($r^2 = 0.50$, $p = 0.003$) and negatively correlated with time spent resting ($r^2 = -0.46$, $p = 0.005$). Protein intake was also negatively correlated with daily average temperature ($r^2 = -0.64$, $p = 0.0004$).

Black howler foraging strategy: Using literature estimates to calculate the average daily ingestion of protein and nonprotein energy for each howler monkey during each week of the study revealed that average daily protein energy intake varied less than average daily nonprotein energy intake (lipids + carbohydrates, Figure 2.3a). The coefficient of variation for the daily amount of protein energy ingested was lower (59) than for the daily amount of nonprotein energy

ingested (79). During Block 1, the Balam group consumed between 0.050 and 0.48 MJ of protein energy per metabolic body weight and between 0.048 and 1.95 MJ of nonprotein energy per metabolic body weight (Figure 2.3a; Table 2.7). The Motiepa group consumed between 0.014 and 0.29 MJ of protein energy per metabolic body weight and between 0.034 and 1.83 MJ of non-protein energy per metabolic body weight (Figure 2.3c, Table 2.7).

The nutritional composition of the plant parts consumed by the howlers appeared to affect only nonprotein energy intake. Regardless of the concentration of protein in the diet in a given week, the howlers maintained a relatively constant protein energy intake. However, as the concentration of protein in the diet increased, nonprotein energy intake decreased hyperbolically (Fig 2.3b, d). Likewise, protein energy intake was not correlated to the proportion of fruit or non-fruit food items in the diet (r^2 = -0.082, p = 0.24; r^2 = 0.038, p = 0.58), while nonprotein energy intake was correlated positively to the proportion of fruit in the diet (r^2 = 0.36, p << 0.01) and negatively to the proportion of non-fruit food items (r^2 = -0.42, p << 0.01).

These patterns were identical between groups and across sampling blocks, suggesting that the howlers used the same foraging strategy throughout the year. Furthermore, examination of the average daily relative concentrations of amino acids, sugars, and lipids consumed by the howlers produced results similar to those generated using literature estimates. Relative concentrations of amino acids varied less than concentrations of sugars regardless of sampling block (Table 2.8).

DISCUSSION

In this study I tested three hypotheses concerning black howler monkey diet, behavior, and nutritional ecology. Assuming that ripe fruit is higher in energy and lower in protein than

leaves, I expected that black howler monkeys would (1) consume a high-protein, low-energy diet, (2) exhibit behaviors that minimize energy expenditure, and (3) shift their foraging strategy from energy-regulating to protein-regulating as their diet changes from leaf-heavy to fruit heavy. While the data I presented here support some of my predictions, in most cases, I found that howler behavior differed from what was expected. This finding suggests that many common assumptions regarding primate behavior and feeding ecology must be reexamined.

Energy and protein intake: Although the use of published nutritional values instead of direct nutritional analyses of howler food items requires that my results be interpreted cautiously, my overall data supported the prediction that black howlers should meet total energy requirements and surpass protein requirements. To begin with, Nagy and Milton (1979) estimated the field metabolic rate of mantled howler monkeys to be ~355 kJ/kg/day. Assuming black howler monkeys have a similar field metabolic rate, an adult black howler monkey should require 0.54 - 0.58 MJ of energy per metabolic body weight per day. My data indicate that an adult black howler monkey consumes an average of 0.52 MJ of metabolizable energy per metabolic body weight per day (range: 0.2-1.74MJ per metabolic body weight). Using data from Table 2.7, which include juveniles, this value increases to 0.57 MJ and falls in the range of what Nagy and Milton (1979) predicted (0.49-0.58 MJ).

However, I also found that the average daily intake of metabolizable energy per metabolic body weight shifted from 0.69 MJ during Block 1 to 0.52 MJ in Block 2 to 0.45 MJ in Block 3. This pattern suggests that, during some periods of the year, the howlers surpass energy demands, and, during others, they fall short. Because howler monkeys consume a large proportion of leaves, and leaves tend to have lower energy content compared to other food items

(Norconk et al 2009), I did not expect the black howlers to surpass energy demands during any period of the year. I also expected the howlers to struggle to meet energy demands during periods of heavy leaf-eating. Not only were the howlers able to surpass estimated energy demands during Block 1, but reduced energy intake during Block 3 corresponded with a ripe fruit-heavy diet, not a leaf-heavy diet. In fact, shifts in overall energy intake were not correlated with the proportion of fruit or leaves in the diet in any way.

Contrary to what I observed with energy intake, the black howlers appeared to have no problem meeting protein demands during any sampling block. Milton (1979) estimated that an adult mantled howler monkey (*A. palliata*) requires 3.26g of protein per kilogram per day. For an adult black howler monkey, this would be between 4.9 and 5.2g of protein per metabolic body weight, depending on body size. My calculations suggest that the black howlers surpassed these levels, consuming an average of 8.6g of protein per metabolic body weight. Even during Block 3 when food intake was lowest and ripe fruit proportions were high, the howlers met estimated protein requirements by consuming approximately 5.6g of protein per metabolic body weight in both groups.

In captivity, protein requirement estimates for primates are generally expressed in terms of percent dry matter intake and a minimum of 16.3% protein is recommended for most primate diets (Oftedal et al 1991). During this study the percent of protein in the black howler diet ranged from 8.0% to 19.2% of dry matter per day, with an average of 10-11% dry matter during periods of high ripe fruit intake (Block 1 and Block 3, Table 2.6) and 14-15% during periods of high young leaf intake (Block 2, Table 2.6). Although these data suggest that black howlers may have undergone periods of protein deficit, especially during Blocks 1 and 3, the total amount of protein consumed is a more informative measure than the percent protein consumed.

Additionally, data on protein requirements in primates are severely limited, and many sources overestimate requirements to ensure the nutritional well-being of captive animals (Oftedal et al 1991). In practice, animals can ingest less protein if the foods they select contain higher levels of essential amino acids (Oftedal et al 1991). The leaves consumed by primates such as howler monkeys are generally reported to be balanced in terms of essential and non-essential amino acid composition (Glander 1981), meaning that protein requirements on a leaf-heavy diet are likely to be lower. Furthermore, although my metabolite data did not allow me to calculate the actual amounts of amino acids in the food items black howlers were consuming, mature leaves contained the highest concentrations of all but one measured essential amino acid compared to other plant parts, and mature leaves were consumed in higher proportions by both howler groups in Blocks 1 (1.6% and 2.8%) and 3 (3.1% and 7.9%) compared to Block 2 (0.8% and 0.7%).

Ficus yoponensis ripe fruits also contained relatively high concentrations of several essential amino acids compared to other food items and were an important food resource year-round. The howler monkeys fed on fig fruits during all ten months of the study (*F. yoponensis* ripe fruits during 5 months--Balam group: 4 months, Motiepa group: 3 months) and figs accounted for 26.5% of the total diet (*F. yoponensis* ripe fruits: 10.6%). Therefore, the lower proportion of protein consumed during Blocks 1 and 3 does not necessarily indicate that the black howlers were protein-limited.

In general, data from this study support the prediction that in utilizing leaves as an important part of the diet, black howler monkeys meet their total energy requirements and surpass their protein requirements. However, I also predicted that if leaves are lower in non-structural carbohydrates and higher in protein than ripe fruits, black howler monkeys would consume less non-protein energy and more protein energy per metabolic body weight compared

to fruit-specialist spider monkeys. Based on my estimates of protein and non-protein energy intake, black howler monkeys consume an average of 0.13 MJ of protein energy per metabolic body weight per day (range: 0.009-0.48MJ per metabolic body weight) and an average of 0.43MJ of nonprotein energy per metabolic body weight per day (range: 0.025 MJ to 1.50 MJ per metabolic body weight). Felton et al. (2009a) estimated that Bolivian spider monkeys consume 0.037 MJ of protein energy per metabolic body weight and 0.36 MJ of nonprotein energy per metabolic body weight (range: 0.14MJ to 1.21 MJ). Black howler monkeys in this study surpassed spider monkeys in both protein and nonprotein energy intake. These results suggest that black howler monkeys are not more energy-limited than spider monkeys despite utilizing a diet with a large amount of leaves. The high relative concentrations of lipid metabolites that I detected in non-fruit food items, such as mature leaves, appear to raise howler nonprotein energy intake past what would otherwise be expected since lipids provide more than twice the energy per gram as sugars (National Research Council 2003).

Activity patterns: Like other howler monkey species (Di Fiore et al 2011), the black howler monkeys in this study exhibited energy-minimizing behavioral patterns such as utilizing small home ranges and day ranges compared to other atelines such as spider monkeys (day range: 2,142 m, home range: 278 ha) and resting for 54.2-75.0% of daylight hours (compared to 23.7-61% in spider monkeys; Di Fiore et al 2011). However, contrary to predictions for leaf-eating, energy-minimizers, the amount of time the black howlers spent resting was not correlated with the amount of leaves or fruit in the diet or with overall energy intake. Similarly, day ranges were not correlated with the plant parts or overall energy consumed. Instead, activity patterns were strongly correlated to both temperature and protein energy intake, suggesting that high

temperatures incite the howlers to rest more and feed less to lower the metabolic costs of maintaining thermal homeostasis, ultimately resulting in reduced protein intake.

The activity budget observed in howler monkeys (60-80% of time spent resting; Di Fiore et al 2011) is commonly interpreted to imply that howler monkeys are energy-limited. Although howler activity budgets may reduce active metabolic rates, thereby reducing energy requirements and allowing howlers to utilize a diet with potentially lower amounts of metabolizable energy, both my diet and activity data indicate that howler monkeys are not necessarily energy-limited on a day-to-day basis. The howlers exhibited no obvious behavioral shifts in response to changes in energy intake, even during Block 3 when energy intake was below estimated requirements. Because the howlers over-consumed energy during Block 1 and under-consumed it during Block 3, it is possible that they were able to store and utilize excess energy from Block 1 to maintain activity patterns and meet future energy demands (Dufour and Sauther 2002, Ellison 2003, Martin 2007, Oftedal 2000). In addition, energy provided by mutualistic gut microbes may have complemented howler dietary energy intake during Block 3 (Lambert and Fellner 2012, Milton and McBee 1983). The microbial fermentation of compounds found in the howler diet such as fiber or pectin provides howlers with short-chain fatty acids that can be used as an energy source, increasing the amount of energy extracted from food items (Milton and McBee 1983). Although further research is needed, it is possible that this energy source allowed howler monkeys to endure fluctuations in the nonprotein energy content of their diet without dramatically altering activity patterns.

Foraging strategy: Black howler monkeys at Palenque National Park, Mexico did not shift their nutrient and energy intake patterns temporally despite the fact that their diet included more than

50% young leaves during some months and more than 50% ripe fruit during others. Instead, they maintained a consistent level of average daily protein energy intake, regardless of the amount of ripe fruits and young leaves ingested, while non-protein energy intake varied dramatically. This pattern matches that observed in spider monkeys (Felton et al 2009a) and suggests that both spider monkeys and howler monkeys regulate protein intake despite consuming distinct diets.

To some extent, howler monkey dietary selectivity may explain the similarities I detected between howler monkey and spider monkey nutrient intake patterns (Glander 1979, Milton 1979, Silver et al 2000). Leaves consumed by howler monkeys have higher protein-to-fiber ratios than other leaves available in the same habitat (Milton 1979), and selecting food items with high protein levels should aid howlers in maintaining the consistent daily protein intake I observed in this study. Additionally, although in *A. palliata* nonprotein energy has been shown to be of limited importance in howler foraging decisions (Milton 1979), selecting food items with low fiber levels indirectly improves howler non-protein energy intake. Fiber is difficult to digest and has been shown to inhibit nutrient extraction and increase food retention time, thereby reducing both the nutritional quality of food as well as the amount of food that can be consumed (Bell 1971, Van Soest 1965, Van Soest 1967). Therefore, by optimizing the protein to fiber ratio in a leafy diet, howlers may be allowing themselves not only to meet protein requirements but also to extract other nutrients more efficiently from their diet and to consume a greater quantity of food to meet energy needs.

My data also suggest that howler monkey diets and spider monkey diets may not differ as much as commonly assumed. Black howlers in Palenque spent similar amounts of time consuming ripe fruit and leaves compared to black howler monkeys at other sites (Pavelka and Knopff 2004, Silver et al 1998) and compared to other howler species (Table 2.1). However,

black howler monkeys in this study consumed ripe fruits twice as quickly as young leaves, making ripe fruit a larger contributor to diet (57.7% fruit and 45.3% ripe fruit vs. 34.2% leaves) than time-based analyses suggest. This pattern does not appear to be unique to my study. Two separate studies of red howler monkeys also indicated higher ingestion rates for fruits compared to leaves (18.7g/min vs. 5.7g/min and 4.2g/min vs. 1.4g/min; Gaulin and Gaulin 1982, Oftedal et al 1991). If we assume, based on these data, that all howler monkeys consume fruits faster than leaves, the time-based diet analyses that dominate the published literature underestimate the amount of fruit in the howler diet. Although spider monkeys also consume fruits more quickly than leaves (Felton, unpublished data) and still include higher average annual proportions of fruits in their diets compared to howler monkeys (84.1-97.0% vs. 27.9-75.4%; Table 2.10), describing howler monkey diets in terms of grams ingested instead of feeding time results in an annual average diet of more than 50% fruits for most howler species (Table 2.11). Separate analyses of howler diets confirm this pattern (Garber et al accepted). Furthermore, the spider monkey diet can include up to 86% young leaves during some periods of the year (Chapman et al 1995), with frequent reports of seasonal diets that include 20-25% young leaves (Di Fiore et al 2008). For instance, in a study in Mexico, Chaves et al. (2011) found that, during the dry season, *Ateles geoffroyi* groups inhabiting continuous evergreen rainforest consumed 25% young leaves, 4% mature leaves, 4% flowers, 13% unripe fruit, and only 36% ripe fruit. Therefore, while differences exist between *Ateles* and *Alouatta* diets, they may not be as dramatic as commonly believed. Both genera appear to primarily consume ripe fruits but exploit a higher proportion of hard-to-digest food items during periods of reduced fruit availability.

If most howler monkey species consume a fruit-heavy diet as predicted, I would also expect them to utilize a protein-regulating foraging strategy similar to that observed in this study.

Currently no other studies of nutrient and energy intake patterns in howler monkeys exist in the literature, but a study of red howler monkeys reported that howler protein intake remains consistent regardless of whether the leaves being consumed contain 12% protein or 22% protein (Oftedal et al 1991). This pattern suggests that red howler monkeys may be regulating protein intake. However, additional studies are needed to thoroughly test predictions regarding howler monkey foraging behavior. Most studies of Central American and Amazonian howler monkeys have been conducted in evergreen rainforest habitats, and diet composition may be different in other habitats such as dry forests. Additionally, Atlantic and southern howlers in South America (*A. caraya* and *A. guariba*) appear to consume more leaves (including mature leaves) and less fruit than other howler species (Table 2.1; Garber et al accepted). Detailed studies of these populations using feeding rates in conjunction with analyses of food nutritional contents are necessary to determine how strongly the protein-regulating foraging strategy is related to the proportions of ripe fruit in the diet and how much can be attributed to dietary selectivity and microbial fermentation. If howlers consuming high proportions of leaves year-round utilize a nutrient-mixing strategy with constant protein intakes, the effects of dietary selectivity and microbial fermentation on howler nutrition must be extremely strong.

CONCLUSION

This study challenges several common assumptions regarding howler monkey behavior and foraging ecology. Despite consuming seasonally large proportions of leaves, howler monkeys do not appear to be energy-limited on a day-to-day basis and generally do not consume less overall energy than more-frugivorous spider monkeys. They also do not use an energy-regulating foraging strategy such as that reported in leaf-eating gorillas, even during periods of

heavy leaf consumption. These results suggest that examinations of howler diet and comparisons to sister taxa such as *Ateles* must change. While howler monkeys are considered "folivorous" due to their ability to exploit leaves as a major food resource, their nutritional intake and behavior are remarkably similar to those of a "frugivorous" primate and suggest the importance of fruit-eating in all atelines. Although many howler monkey species are well-studied, a deeper understanding of howler behavior, feeding ecology, and evolution depends on more detailed data collection methods and a critical investigation of many of the assumptions we take for granted.

TABLES

Table 2.1. Minimum and maximum percent time spent consuming fruit, leaves, stems, and flowers as reported across howler studies. Ranges represent minimum and maximum time spent consuming a given resource during a given month unless otherwise indicated.

Species	Location	Length of Study	Ripe Fruit	Unripe Fruit	Fruit	Young Leaves	Mature Leaves	Leaves	Stem	Flower	Source
A. belzebul	Brazil	10 months	55.0 (24.7-80.0)	0.6 (0.0-5.3)	55.6 (24.7-80.0)	19.8 (2.1-53.6)	5.0 (0.0-24.0)	75.4 (11.4-53.6)	2.6 (0.0-7.9)	5.7 (0.0-13.1)	Pinto and Setz 2004
A. belzebul	Brazil	13 months			59.0 (43.0-92.0)			13.3 (8.0-15.0)		27.6 (0.0-41.0)	Bonvicino 1989
A. caraya	Argentina	12 months			28.5 (6.0-63.0)			67.3 (37.0-86.0)		2.7 (0.0-13.0)	Bicca-Marques and Calegaro-Marques 1994
A. caraya	Argentina	7 months	17.6 (0.0-71.0)	1.0 (0.0-2.0)	18.6 (0.0-71.0)	13.0 (0.0-45.0)	51.0 (20.0-95.0)	64.0 (36.0-95.0)		12.0 (0.0-44.0)	Bravo and Sallenave 2003
A. caraya	Argentina	12 months	15.0 (3.0-31.0)	4.0 (0.0-12.0)	19.0 (5.0-34.0)	25.0 (0.0-55.0)	26.0 (4.0-77.0)	64 (22.0-65.0)		6 .0 (0.0-30.0)	Agostini et al. 2010
A. caraya	Argentina	15 months			(0.0-50.0)	39.2 (14.0-72.0)	31.3 (0.0-40.0)	70.5 (32.0-86.0)	5.0-25.0	0.0	Zunino 1989
A. caraya	Brazil	12 months			46.0 (32.0-82.0)			49.0 (17.0-61.0)		10.0 (0.0-14.0)	Ludwig et al. 2008
A. caraya	Brazil	12 months			24.0 (0.0-45.0)			65.0 (33.0-96.0)		4 .0 (2.0-23.0)	Ludwig et al. 2008
A. guariba	Argentina	12 months	21.0 (0.0-39.0)	2.0 (0.0-10.0)	24.0 (1.0-39.0)	24.0 (5.0-46.0)	27.0 (10.0-61.0)	62.0 (19.0-72.0)		6 .0 (0.0-22.0)	Agostini et al. 2010
A. guariba	Brazil	7 months			15.6 (1.0-30.0)			76.0 (64.0-88.0)		8.4 (6.0-11.0)	Mendes 1989
A. guariba	Brazil	12 months			5.0 (0.0-15.0)	43.1 (21.0-74.0)	22.6 (6.0-50.0)	73.0 (56.0-92.0)	3.0	12.0 (0.0-27.0)	Chiarello 1994
A. guariba	Brazil	12 months			8.0			80.7		7.5	Martins 2008
A. palliata	Costa Rica	24 months			28.5 (0.0-55.0)			49.0 (0.0-92.0)		22.5 (0.0-92.0)	Chapman 1987
A. palliata	Costa Rica	15 months			17 .0 (0.0-73.0)	65.0 (20.0-94.0)	6.0	71.0	1.0	11.0	Stoner 1996
A. palliata	Costa Rica	15 months			29.0 (0.0-87.0)	62.0 (0.0-90.0)	4.0	66.0		6.0	Stoner 1996
A. palliata	Costa Rica	14 months			12.5 (9.0-16.0)	44.0 (36.0-50.0)	19.0 (17.0-20.0)	69.3 (64.0-72.0)	6.0 (5.0-6.0)	18.2 (17.0-21.0)	Glander 1979
A. palliata	Mexico	12 months	41.4 (0.0-66.0)	8.5 (0.0-80.0)	51.0 (0.0-80.0)	39.3 (6.0-90.0)	10.0 (4.0-44.0)	49.0 (20.0-100.0)		0.2	Estrada 1984
A. palliata	Mexio	12 months	34.8 (0.3-69.6)	5.8 (0.4-13.1)	40.6 (2.5-79.0)	46.7 (13.2-79.3)	7.6 (0.0-18.3)	(17.2-86.8)		4.3 (0.0-10.5)	Estrada et al. 1999
A. palliata	Nicaragua	14 months	26.6	5.8	34.8 (15.9-47.6)	27.8 (11.9-56.3)	27.0 (9.6-44.2)	55.8 (32.0-82.0)	0.3	7.9 (0.4-27.7)	Williams-Guillen 2003
A. palliata	Panama	10 months			42.1 (10.0-66.0)			48.2 (26.0-84.0)		9.6 (0.0-24.0)	Milton 1980
A. pigra	Belize	12 months			41.4 (15.0-98.0)			58.6 (2.0-85.0)			Pavelka and Knopff 2004

Table 2.1 (cont.)

A. pigra	Belize	14 months	37.1 (10.1-58.2)	3.7 (0.9-5.8)	40.8 (11.0-64.0)	37.2 (17.0-65.0)	7.9 (0.0-39.0)	45.1 (32.0-66.0)		10.6 (0.0-35.0)	Silver et al. 1998
A. seniculus	Colombia	10 months	28.4	13.9	42.3	44.5	7.5	52.1	0.1	5.4	Gaulin and Gaulin 1982
A. seniculus	Colombia	10 months			52.3 (18.4-75.0)	33.9 (25.0-71.0)	1.4	35.3		1.1 (0.0-10.0)	Palacios and Rodriguez 2001
A. seniculus	French Guiana	19 months	21.5 (0.0-56.8)	4.0 (0.0-29.4)	25.5 (0.0-73.5)	54 (26.1-78.4)	3.0 (0.0-17.6)	57.0 (26.5-78.4)		12.6 (0.0-38.8)	Julliot and Sabatier 1993

Table 2.2. Plant part nutritional content based on published values.

Species	Part	Crude Protein	Available Protein	Lipids	Sugars	Source
Dendropanax arboreus	Ripe Fruit	5.4	3.0	21.8	10.6	Felton et al. 2009b
Ficus americana	Ripe Fruit	7.5	4.4		4.0	Silver 2000
Ficus aurea•	Ripe Fruit	7.1	4.0*	3.6	8.7	Milton 2008
Ficus insipida	Ripe Fruit	7.0	4.0*	5.8	14.5	Milton et al. 1980, 2008
Ficus pertusa	Ripe Fruit	5.8	2.4	1.9	38.8	Felton et al. 2009b
Ficus yoponensis	Ripe Fruit	7.5	4.2*	6.0	11.3	Milton et al. 1980, 2008
Poulsenia armata	Ripe Fruit	7.9	7.9*		56.4	Estrada et al. 1984
Other	Ripe Fruit	7.6	7.68	4.3	58.3	Norconk et al. 2009
Average		7.0	4.7	7.2	25.3	
Brosimum alicastrum	Unripe Fruit	7.2	7.28	1.2	20.7	Estrada et al. 1984, Milton 2008
Other	Unripe Fruit	7.6	7.6*	4.3	58.3	Norconk et al. 2009
Average		7.4	7.4	2.8	39.5	
Ficus insipida	Young Leaf		10.6		2.9	Milton 1979
Ficus yoponensis	Young Leaf		10.5		6.9	Milton 1979, 1981
Poulsenia armata	Young Leaf		8.5			Milton 1979
Other	Young Leaf	20.1	20.1*	1.7	20.3	Norconk et al. 2009
Average		20.1	12.4	1.7	10.0	
Other	Mature Leaf	14.4	14.4*	1.5	17.7	Norconk et al. 2009
Other	Flower	16.8	16.8*	2.3	30.1	Norconk et al. 2009
Other+	Stem	14.9	13.0		7.8	Silver 2000

+estimated using *Schizolobium parahyba* values

•estimated using *Ficus obstusifolia* values since species are similar

*used CP for AP since no AP estimate existed

Table 2.3. Average feeding rates by plant part for black howler monkeys at Palenque National Park.

Plant Part	**Average Ingestion Rate (g/min)**
Ripe Fruit	5.87 ± 5.44
Unripe Fruit	2.43 ± 0.98
Young Leaf	1.86 ± 0.92
Mature Leaf	1.60 ± 0.39
Flower	0.63 ± 0.63
Stem	2.86 ± 1.67
Seed	0.12

Table 2.4. Average diet composition (% dry weight) for Balam and Motiepa groups by month.

	Ripe Fruit		Unripe Fruit		Young Leaf		Mature Leaf		Stem		Flower	
Month	Balam	Motiepa	Balam	Motiepa	Balam	Motiepa	Balam	Motiepa	Balam	Motiepa	Balam	Motiepa
September	23.4%	61.0%	10.3%	8.5%	39.1%	24.9%	3.9%	0.9%	23.3%	4.6%	0.0%	0.0%
October	26.0%	34.1%	12.6%	26.4%	31.8%	36.3%	1.3%	0.9%	28.3%	2.2%	0.0%	0.0%
November	44.1%	48.2%	12.5%	4.8%	29.1%	13.6%	1.7%	6.3%	12.5%	9.5%	0.0%	17.5%
January	1.3%	1.2%	5.0%	3.7%	16.7%	89.5%	1.5%	2.5%	21.7%	1.5%	53.8%	1.6%
February	55.4%	14.7%	17.4%	28.1%	21.5%	51.8%	0.3%	0.2%	5.3%	5.2%	0.0%	0.0%
March	0.9%	0.0%	23.5%	27.3%	67.6%	69.8%	1.3%	0.0%	6.1%	2.3%	0.6%	0.7%
April	78.9%	55.8%	1.4%	0.2%	16.3%	24.2%	0.3%	16.1%	3.1%	0.8%	0.0%	2.8%
May	71.7%	59.7%	0.5%	6.3%	20.4%	22.8%	3.1%	9.4%	4.2%	1.7%	0.0%	0.0%
June	56.3%	53.6%	3.1%	4.1%	28.7%	35.6%	4.2%	4.4%	7.7%	2.2%	0.0%	0.0%
Average	39.8%	36.5%	9.6%	12.2%	30.1%	41.0%	2.0%	4.5%	12.5%	3.3%	6.0%	2.5%
SD	28.6%	25.0%	7.8%	11.5%	15.9%	24.9%	1.4%	5.4%	9.5%	2.7%	17.9%	5.7%

Table 2.5. Average metabolite content of plant parts consumed by black howlers at Palenque National Park. Measurements expressed in relative concentration per gram of wet weight. *Indicates essential amino acids; + indicates conditionally essential amino acids; **bold** indicates significantly different concentrations across plant parts.

		Flower (3)		**Unripe Fruit (2)**		**Ripe Fruit (7)**		**Mature Leaf (4)**		**Seed (1)**		**Stem (2)**		**Young Leaf (7)**	
Metabolite	**Class**	AVG	SD	AVG	SD	AVG	SD	AVG	SD	AVG	SD	AVG	SD	AVG	SD
2-Methylserine	Amino Acid	0	0	0	0	0	1	0	0	0	-	0	0	6	16
Alanine	Amino Acid	936	482	403	479	284	366	1435	1266	749	-	211	43	633	748
Asparagine+	Amino Acid	816	635	694	981	80	211	119	238	195	-	1933	2399	718	584
Aspartic acid	Amino Acid	1841	1478	1557	2032	155	411	1308	2093	2935	-	61	56	550	486
Glutamic acid	Amino Acid	630	604	105	17	53	121	873	924	284	-	359	426	368	285
Glutamine+	Amino Acid	24	42	0	0	0	0	29	57	0	-	0	0	0	0
Glycine+	Amino Acid	91	74	135	144	47	59	245	328	49	-	41	32	92	90
Isoleucine*	Amino Acid	122	150	56	79	20	47	76	102	16	-	14	2	107	78
Leucine*	Amino Acid	161	185	1	2	36	81	73	97	15	-	7	6	137	151
Lysine*	Amino Acid	142	247	0	0	12	32	244	338	0	-	66	84	57	75
N-Acetylglutamic acid	Amino Acid	0	0	0	0	0	0	0	0	0	-	0	0	200	639
N-methylleucine	Amino Acid	568	984	0	0	218	477	7046	14093	29738	-	1409	1431	11572	38169
Phenylalanine*	Amino Acid	107	186	36	42	38	96	135	167	0	-	0	0	120	164
Proline+	Amino Acid	1509	1878	47	66	3	5	2	3	0	-	224	313	93	111
Serine+	Amino Acid	625	474	188	188	63	119	646	1056	253	-	218	273	450	540
Threonine*	Amino Acid	214	188	69	87	35	81	347	429	141	-	104	125	189	213
Tyrosine+	Amino Acid	86	130	5	7	6	17	79	95	52	-	76	105	97	105
Valine*	Amino Acid	296	243	82	106	45	96	199	234	128	-	52	56	245	335
B-alanine	Amino Acid/Vitamin	150	194	5	7	27	38	34	39	159	-	8	9	42	38
Total		8318	5241	3381	2209	1121	1659	12890	12221	34713	-	4784	5159	15673	38120
1,2-Dipalmitoyl-glycerol	Glycerolipid	0	0	0	0	0	0	15	29	0	-	84	63	22	11
1-hexadecanoylglycerol	Glycerolipid	152	155	13	19	48	54	59	33	49	-	23	4	60	30
1-Nonadecanoylglycerol	Glycerolipid	28	48	0	0	35	63	71	134	0	-	19	10	13	19
1-octadecanoylglycerol	Glycerolipid	33	43	0	0	15	34	47	27	0	-	10	14	9	17

Table 2.5 (cont.)

1-Octedecenoylglycerol	Glycerolipid	0	0	0	0	0	0	0	0	0	-	11	0	3	0
1-Pentadecanoylglycerol	Glycerolipid	0	0	0	0	0	0	13	15	0	-	0	0	0	0
1-Tetradecanoylglycerol	Glycerolipid	25	44	0	0	0	0	65	40	177	-	2	2	1	2
2-O-Glycerol-alfa-d-galactopyranoside	Glycero-sugar	32	56	5	7	74	95	7028	10613	42	-	0	0	269	520
2-O-Glycerol-beta-D-galactopyranoside	Glycero-sugar	0	0	0	0	128	309	124	147	0	-	1	1	11	34
digalactosylglycerol	Glycero-sugar	0	0	0	0	100	264	9277	14148	0	-	8	4	247	507
1,3-Dipalmitin	Lipid	0	0	0	0	0	0	0	0	0	-	41	21	9	0
Decanoic acid	Fatty Acid Saturated	5	9	0	0	2	3	44	53	0	-	2	1	2	4
Docosanoic acid	Fatty Acid Saturated	168	62	299	383	99	64	148	106	86	-	62	21	91	59
Dodecanoic acid	Fatty Acid Saturated	15	26	0	0	0	0	44	51	0	-	2	1	4	8
Eicosanoic acid	Fatty Acid Saturated	456	95	1659	2047	118	70	326	406	202	-	105	16	223	251
Nonadecanoic acid	Fatty Acid Saturated	2	3	0	0	0	1	25	32	0	-	6	6	3	4
Octacosanoic acid	Fatty Acid Saturated	0	0	0	0	0	0	0	0	0	-	23	22	22	38
octadecanoic acid	Fatty Acid Saturated	1523	476	1111	245	1010	352	4784	4666	1023	-	1171	274	1505	892
Pentadecanoic acid	Fatty Acid Saturated	0	0	0	0	20	53	197	296	0	-	0	1	18	27
Heptadecanoic acid	Fatty Acid Saturated	19	21	9	13	9	11	162	184	23	-	26	16	30	25
Heptadecanoic acid, 16-methyl-	Fatty Acid Saturated	26	45	0	0	41	72	0	0	0	-	14	20	5	7
Hexacosanoic acid	Fatty Acid Saturated	14	25	0	0	576	1517	4	8	0	-	15	6	24	39
Hexadecanoic acid	Fatty Acid Unsaturated	2819	1582	1914	1590	2103	1478	7565	7161	1180	-	828	137	1866	1102
C16:1	Fatty Acid Unsaturated	25	23	0	0	27	70	49	58	0	-	117	39	29	7
C18:1	Fatty Acid Unsaturated	217	304	0	0	1094	2684	14	28	0	-	67	42	65	36
C18:1	Fatty Acid Unsaturated	4	6	0	0	48	82	121	242	0	-	0	0	15	40
C18:1	Fatty Acid Unsaturated	1769	1748	400	427	1006	1205	2778	3132	189	-	2050	36	1180	532
C18:2	Fatty Acid Unsaturated	2824	3804	568	3	1413	1994	2289	3022	197	-	204	19	1226	1727
C18:3	Fatty Acid	96	166	0	0	2049	3117	118	207	0	-	47	12	335	680

Table 2.5 (cont.)

C20:1	Unsaturated Fatty Acid	3	5	0	0	1	2	0	0	0	-	0	0	4	9
C20:3	Unsaturated Fatty Acid	0	0	32	46	0	1	11	22	0	-	0	0	0	0
Tetracosanoic acid	Unsaturated Fatty Acid	106	38	215	281	46	48	58	4	52	-	57	2	81	41
Tetradecanoic acid	Unsaturated Fatty Acid	37	64	0	0	5	10	339	371	0	-	57	21	90	128
Triacontanoic acid	Unsaturated Fatty Acid	4	7	56	8	8	14	83	167	16	-	82	36	31	36
Tricosanoic acid	Unsaturated Fatty Acid	5	8	2	3	9	21	11	9	0	-	3	1	9	21
Tridecanoic acid	Unsaturated Fatty Acid	0	0	0	0	0	0	2	4	0	-	0	0	0	1
Total		10403	7471	6234	4882	10086	7424	35867	39350	3236	-	5135	185	7501	3419
1,6-Anhydroglucose	Sugar	5	8	0	0	17	30	6	11	0	-	1	2	2	4
1-Ethylglucopyranoside	Sugar	118	204	0	0	20	54	650	991	0	-	0	0	75	124
1-Methyl-alpha-D-glucopyranoside	Sugar	48832	24688	11163	12724	1595	2707	13885	14722	49871	-	8010	1467	16673	26096
1-Methyl-beta-D-galactopyranoside	Sugar	143	247	0	0	7	13	0	0	0	-	0	0	0	0
Arabinofuranoside	Sugar	958	1413	0	0	7245	17876	0	0	550	-	0	0	707	1277
Arabinose	Sugar	496	547	226	182	164	163	141	109	139	-	126	154	419	667
Cellobiose	Sugar	84	142	14	20	65	127	375	481	0	-	43	11	23	26
Erythrose	Sugar	0	0	0	0	2	5	0	0	0	-	0	0	34	76
Fructose	Sugar	91388	60569	14091	977	267148	346719	34464	34570	55300	-	32600	32728	26704	31510
Galactofuranoside	Sugar	1790	3101	125	177	15063	23282	923	1730	0	-	225	294	75	68
Galactopyranose	Sugar	4176	7232	348	493	21678	43813	2686	5098	0	-	1554	1957	401	133
Galactose	Sugar	2833	4613	2585	2365	4291	8887	800	759	2269	-	1075	265	1395	2746
Galactoside	Sugar	4458	4566	819	118	23640	27766	10634	12150	3243	-	685	317	3748	4420
Glucoheptulose	Sugar	7	13	3	5	66	105	58	83	0	-	9	2	8	10
Glucopyranoside	Sugar	5076	7712	240	339	59048	70961	2582	5164	1693	-	1650	1821	712	595
Glucosamine	Sugar	317	302	20	28	0	0	0	0	162	-	0	0	0	1
Glucose	Sugar	60227	53129	38587	25347	199564	291851	46529	65686	48294	-	32652	29400	19578	14798
Maltose	Sugar	488	521	52	73	723	1241	1870	3492	140	-	88	110	55	74
Melibiose	Sugar	321	544	123	174	88	118	277	473	296	-	4	6	36	98
N-Acetyl glucosamine	Sugar	19	33	18	26	18	35	3	5	0	-	15	21	46	71

Table 2.5 (cont.)

N-Acetylglucosylamine	Sugar	112	194	18	25	6	17	0	0	0	-	0	0	55	104
Rhamnose	Sugar	274	184	16	23	243	302	93	71	6692	-	47	54	247	540
Ribose	Sugar	1453	1057	115	58	894	717	274	329	4871	-	73	35	2484	6610
Sedoheptulose	Sugar	75	48	11	16	330	707	504	472	86	-	89	89	158	286
Sorbose	Sugar	81	140	0	0	9568	19987	45	90	1282	-	12162	16939	2918	477
Sucrose	Sugar	1142	1010	87	123	10065	25275	36109	46135	17273	-	16573	9567	6797	7554
Talose	Sugar	16659	26797	6176	2950	79434	119735	7481	12549	3501	-	4341	5479	3326	3772
Trehalose	Sugar	19	17	17	24	358	843	112	160	38	-	4	6	13	32
Xylopyranoside	Sugar	17	30	0	0	178	281	513	863	103	-	14	17	149	472
Xylose	Sugar	273	147	19	27	523	595	246	255	1305	-	126	164	272	536
Xylulose	Sugar	0	0	0	0	30	78	202	239	0	-	322	448	86	31
Total		241840	157856	74873	13444	702070	861140	161459	186003	197107	-	112489	101276	87196	85908

Table 2.6. Average nutrient and energy intake for Motiepa and Balam groups across sampling periods. Calculations are based on literature estimates and are expressed in grams per metabolic body weight, energy per metabolic body weight, and percent dry weight ingested.

		Block 1		Block 2		Block 3	
		Motiepa	Balam	Motiepa	Balam	Motiepa	Balam
Available Protein	Average	10.8	10.2	8.9	10.2	5.6	5.7
	SD	3.6	2.4	2.4	2.7	1.3	1.7
% Available Protein	Average	10.7%	11.0%	13.8%	14.7%	11.4%	11.1%
	SD	1.8%	0.8%	3.4%	1.4%	0.6%	1.2%
Total Non-Structural Carbohydrates	Average	29.0	29.7	12.4	16.2	16.3	17.0
	SD	1.2	12.0	3.5	3.7	3.4	5.0
% Total Non-Structural Carbohydrates	Average	26.3%	34.3%	18.9%	22.1%	28.4%	35.2%
	SD	5.3%	5.0%	2.4%	2.8%	9.0%	6.6%
Lipids	Average	3.0	3.2	1.2	3.2	1.7	2.1
	SD	1.2	0.9	0.3	1.5	0.5	0.6
% Lipids	Average	2.9%	3.4%	2.8%	2.3%	3.3%	3.6%
	SD	0.4%	0.3%	1.1%	0.4%	0.6%	0.5%
Neutral Detergent Fiber	Average	42.3	50.3	27.2	39.9	21.9	24.7
	SD	15.9	14.5	8.8	14	4.9	8
Total Energy (Kcal)	Average	182.9	177.4	105.5	172.4	106.6	114.5
	SD	64.9	56.1	30.0	56.9	26.7	30.6

Table 2.7. Average energy intake for Motiepa and Balam groups across sampling periods. Ranges based on calculations of average daily intake for each individual during each of the five weeks of data collection during each sampling block. Calculations are based on literature estimates and are expressed in MJ per metabolic body weight.

		Block 1		**Block 2**		**Block 3**	
		Motiepa	Balam	Motiepa	Balam	Motiepa	Balam
Protein Energy (MJ)	Average	0.14	0.19	0.13	0.15	0.08	0.10
	Range	0.014-0.29	0.050-0.48	0.034-0.30	0.032-0.28	0.012-0.21	0.0088-0.27
Non-protein Energy (MJ)	Average	0.55	0.53	0.38	0.41	0.32	0.41
	Range	0.034-1.83	0.048-1.95	0.12-1.68	0.071-1.18	0.038-0.81	0.025-1.42
Total Energy (MJ)	Average	0.69	0.72	0.51	0.56	0.40	0.51
	Range	0.048-2.10	0.098-2.34	0.18-1.97	0.13-1.40	0.054-0.96	0.035-1.71

Table 2.8. Average metabolite intake for Motiepa and Balam groups across sampling periods. Metabolite values are expressed in relative concentration per metabolic body weight.

		Block 1		**Block 2**		**Block 3**	
		Motiepa	Balam	Motiepa	Balam	Motiepa	Balam
Amino Acids	Average	3,874,740	7,655,871	4,356,907	4,971,969	944,692	1,952,207
	Range	150,054-13,677,598	192,079-30,206,249	138,722-15,353,681	505,801-14,321,945	46,968-5,857,965	18,477-10,598,416
Sugars	Average	179,809,901	106,698,939	46,591,468	44,597,474	50,043,025	96,710,217
	Range	17,355,828-646,243,338	5,413,802-782,947,762	50,833,383-160,876,872	30,250,675-50,793,104	6,687,146-29,075,974	3,539,931-631,756,346
Lipids	Average	6,521,496	4,173,832	3,079,078	2,637,125	2,367,494	1,534,664
	Range	959,436-13,868,615	800,979-28,930,967	507,987-7,645,966	416,353-4,813,883	251,175-5,618,803	102,872-317,739

Table 2.9. List of metabolites that exhibited significant changes in relative concentration in the Motiepa group diet across sampling blocks. Values are expressed as average daily intake in relative concentration.

Metabolite		**Block 1**	**Block 2**	**Block 3**
2-Methylmalic acid	Average	108,477.2	14,158.5	6,016.4
	SD	83,693.9	6,438.3	3,667.8
4-Hydroxy-3-methoxyphenylethylene glycol	Average	65,038.9	11,664.2	2,643.7
	SD	33,534.0	6,884.0	1,251.2
Allantoin	Average	0.0	9,534.6	639.8
	SD	0.0	4,011.7	997.3
Beta-Amyrin	Average	225,210.2	106,049.5	24,037.8
	SD	75,190.6	39,930.4	14,130.5
Cholesterol	Average	17,379.1	4,460.3	9,438.1
	SD	6,714.6	2,433.6	2,804.1
Eicosanol	Average	95.1	217.9	498.4
	SD	36.5	72.9	149.5
Hentriacontanol	Average	5,119.6	1,200.8	308.0
	SD	2,113.8	771.2	154.4
Protocatechuic acid	Average	80,106.3	33,629.2	8,415.5
	SD	29,680.0	19,171.9	4,142.0
Xylonic acid-1,4-lactone	Average	17,655.0	23,087.8	8,593.9
	SD	5,388.4	13,906.6	3,092.8

Table 2.10. Home range and day range for each howler group during each season. ANOVA detected no significant differences in day range across seasons in either group ($F_{2,52}$= 1.55, p = 0.22; $F_{2,49}$= 0.056, p = 0.95).

	Balam		**Motiepa**	
	Home Range (ha)	Day Range (ha)	Home Range (ha)	Day Range (ha)
Rainy	9.5	309.6 ± 128.0	4.2	319.1 ± 113.7
Intermediate	9.5	236.5 ± 83.2	5.2	317.9 ± 97.0
Dry	6.1	266.2 ± 146.0	4.4	328.4 ± 106.0

Table 2.11. Average yearly proportion of fruit and leaves in the diet of howler monkeys and spider monkeys. Grams calculated using feeding rates from this study for howler monkeys and from Felton (personal communication) for spider monkeys.

Species	**Fruit (Time)**	**Leaves (Time)**	**Fruit (Grams)**	**Leaves (Grams)**
A. belzebuth	83.5	9.0	92.9	4.7
A. chamek	81.0	13.3	91.1	6.9
A. geoffroyi	67.2	16.4	84.1	9.5
A. hybridus	92.0	0.0	97.0	0.0
A. paniscus	80.0	8.0	92.0	4.0
A. belzebul	57.5	19.0	75.4	8.5
A. caraya	27.6	64.8	49.9	41.9
A. guariba	13.3	72.8	27.9	55.3
A. palliata	33.0	56.7	56.0	34.2
A. pigra	40.4	49.9	64.5	27.1
A. seniculus	40.0	48.3	62.5	27.1

FIGURES

Figure 2.1. Average daily percent of black howler diet made up of stems, young leaves, mature leaves, flowers, unripe fruit, and ripe fruit for the Balam (top) and Motiepa (bottom) group each season.

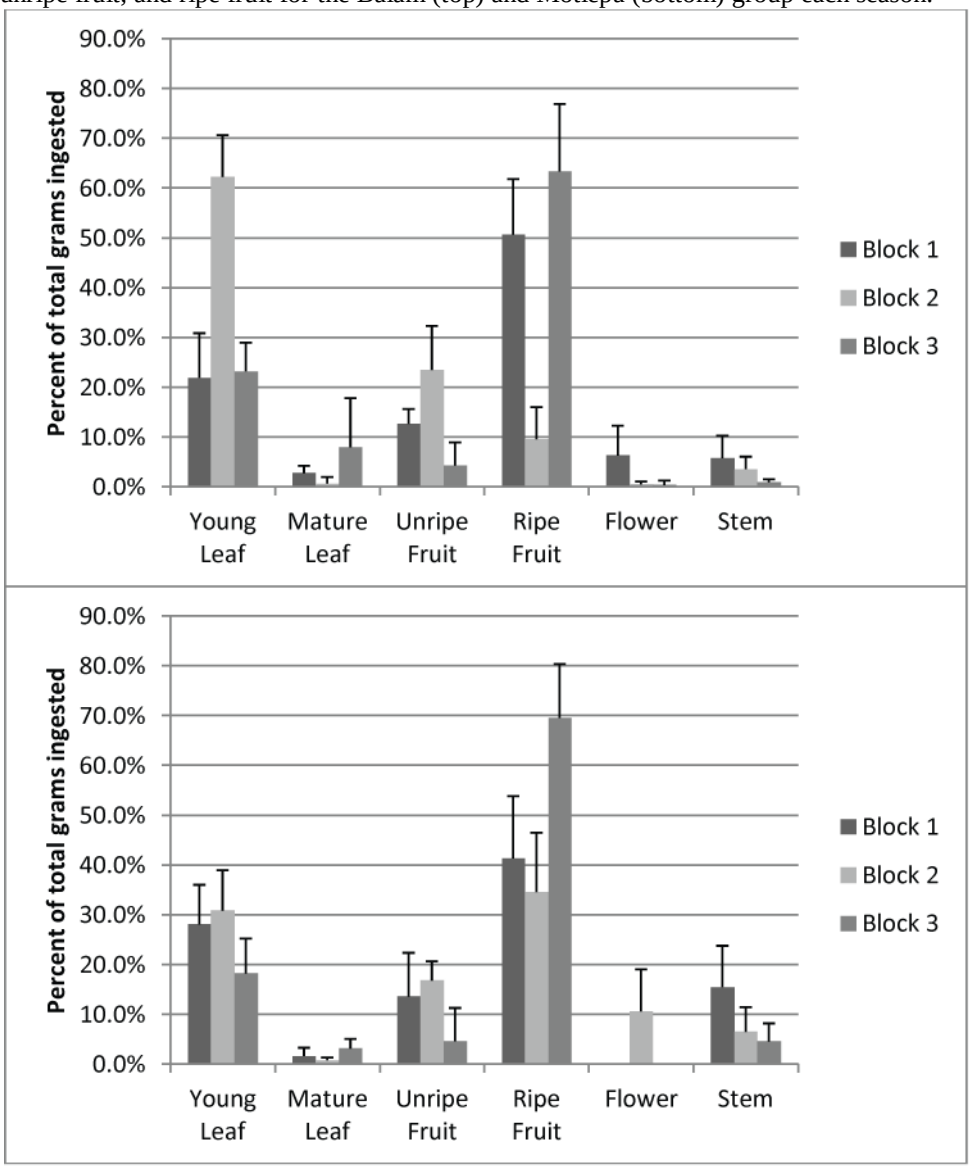


Figure 2.2. Average daily percent time spent feeding, foraging, resting, being social and traveling by black howlers in the Balam (top) and Motiepa (bottom) group each season.

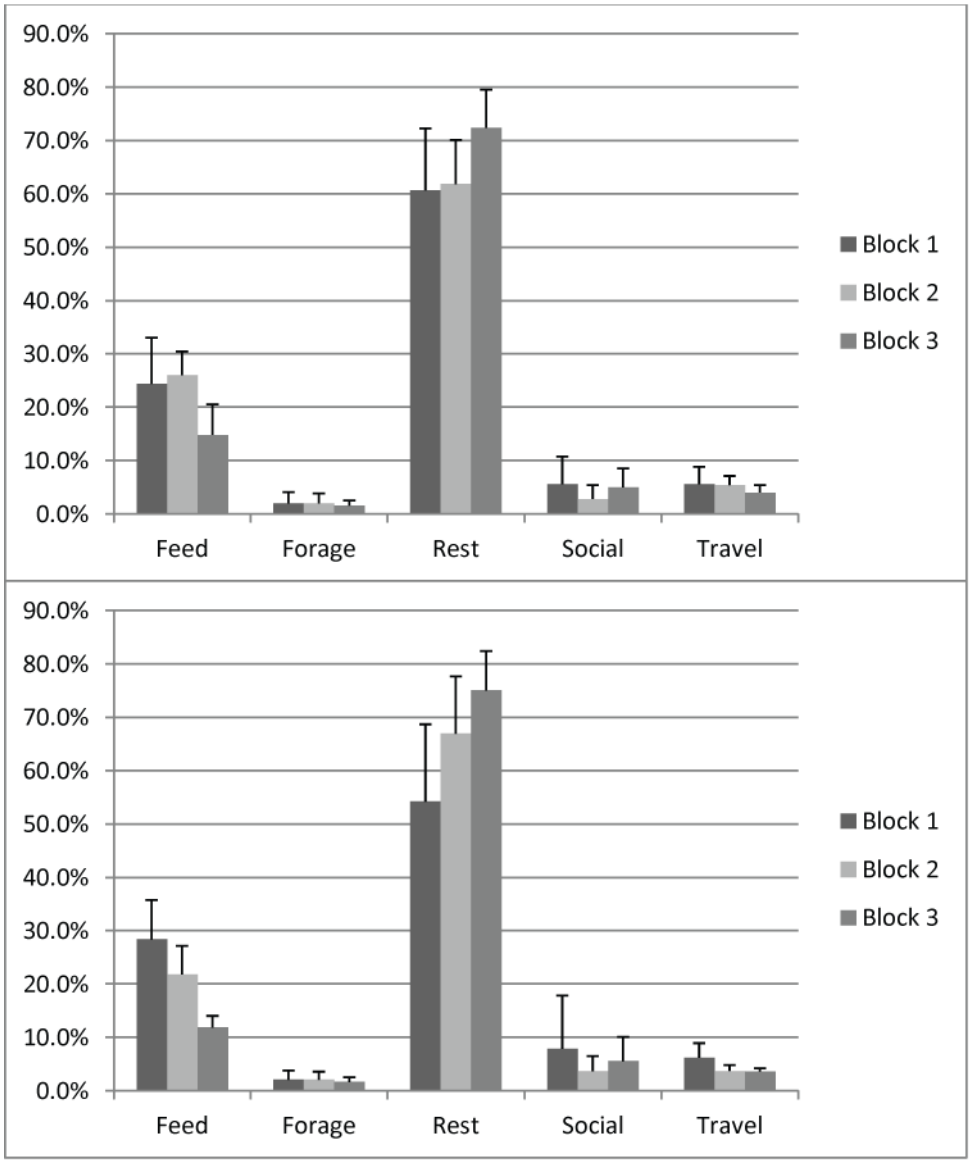
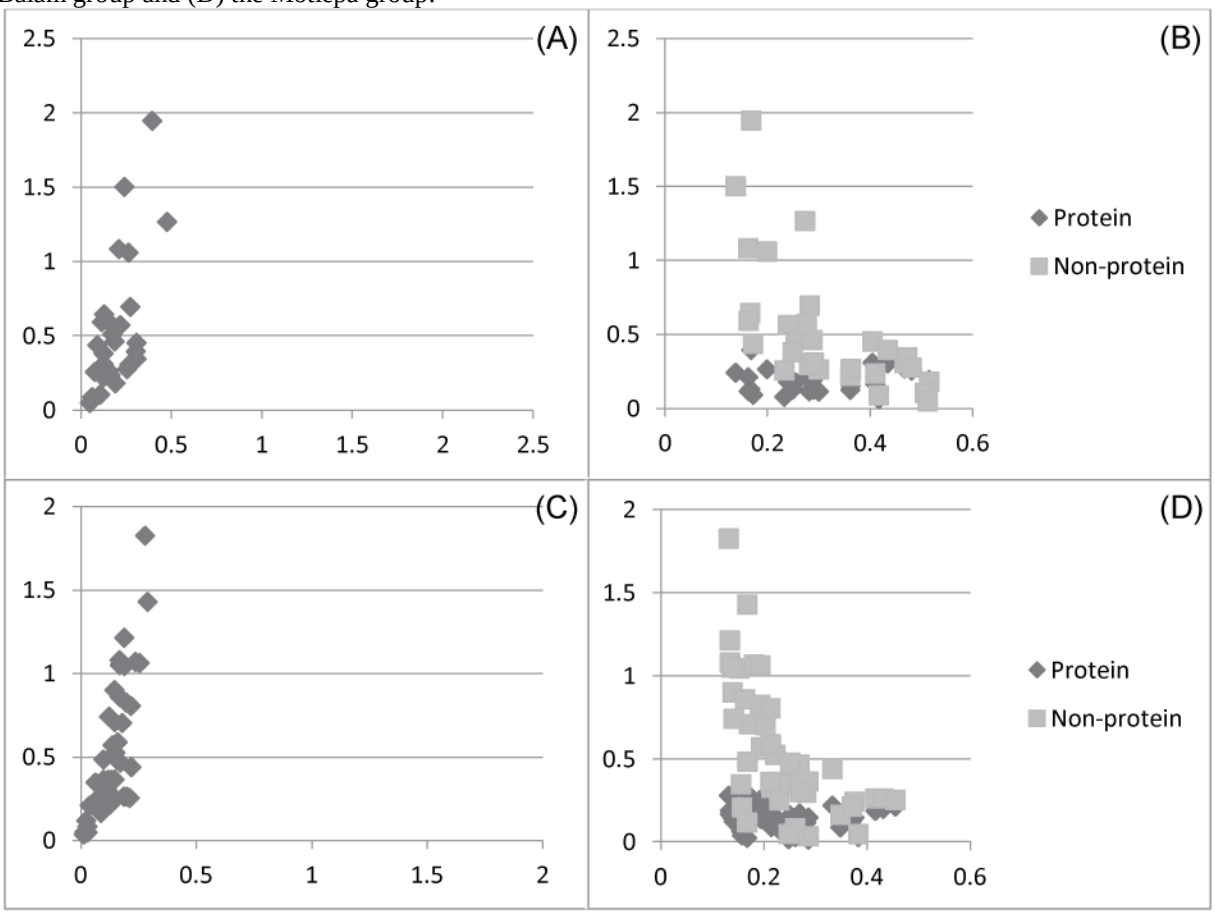


Figure 2.3. Average daily protein energy consumed per metabolic body weight plotted against the average daily non-protein energy consumed for (A) the Balam group and (C) the Motiepa group. Average daily protein and non-protein energy consumed per metabolic body weight plotted against the percentage of protein in the diet for (B) the Balam group and (D) the Motiepa group.



CHAPTER 3: THE EFFECTS OF SEASONAL DIET VARIATION ON THE GUT MICROBIOTA OF THE WILD BLACK HOWLER MONKEY (*ALOUATTA PIGRA*)

ABSTRACT

Laboratory studies have demonstrated that the mammalian gut microbial community shifts over time in response to selective pressures imposed by diet. These changes can influence host digestive efficiency since the gut microbial community provides energy and nutrients from the breakdown of otherwise indigestible materials in the host diet. For most animals, including nonhuman primates, diet composition varies temporally in response to differences in food availability. Therefore, the composition of gut microbial communities in wild animal populations should vary across seasons in response to shifts in diet. Furthermore, changes in host digestive efficiency associated with variations in the gut microbial community may make meeting nutritional demands less challenging for animals during periods of low food availability or during periods when specific nutrients required for growth and reproduction are limited. In this study, I investigate temporal variation in diet and gut microbial community composition and function in two groups (N=13 individuals) of wild, Mexican black howler monkeys (*A. pigra*) over a ten-month period in Palenque National Park, Mexico. The results show that the howler monkeys exploited a distinct diet across different periods of the study, with ripe fruits dominating during some months and young leaves and unripe fruits dominating during others. Temporal changes in the relative abundances of a range of bacterial taxa were strongly correlated with variation in components of the howler diet, which may indicate the ability of the gut microbial community to adapt to the howler diet. Additionally, the howlers exhibited increased microbial production of energy during periods of reduced energy intake. Because I observed virtually no changes in howler activity and ranging patterns during the study, these results suggest that the gut microbiota may have been providing additional energy and nutrients to the

howlers to compensate for dietary fluctuations. This study provides an important first step in understanding the host-gut microbe relationship in wild animals. Although field data are needed to verify these processes, it is likely that energy and nutrient production by the gut microbial community provides an effective buffer against seasonal fluctuations in energy and nutrient intake for a variety of primate species.

INTRODUCTION

All mammals rely upon mutualistic microbial communities in the gut to provide them with energy via the formation of short-chain fatty acids (SCFA's) from otherwise indigestible material such as cellulose (Flint et al 2008, Goel et al 2005, Nelson et al 2003, Odenyo et al 2001). Individuals are born with a sterile intestinal tract and generally acquire microbes from the environment (e.g. conspecifics, food) during the first year of life (Friswell et al 2010, Mackie et al 1999). However, even after the gut microbial community is established, its composition shifts over time in response to selective pressures imposed by diet (Arumugam et al 2011, De Filippo et al 2010, Kolida et al 2002, Turnbaugh et al 2009, Williams et al 2012, Wu et al 2011). For example, in response to a diet shift from a low-fat diet rich in plant polysaccharides to a high-fat, high-sugar diet, 340 mice inoculated with humanized gut microbiota exhibited a dramatic increase in the abundance of several classes of bacteria in the Firmicutes phylum over the course of one day (Erysipelotrichi: 3.3% to 15.9%; Bacilli: 0.1% to 13.0%; Turnbaugh et al 2009).

These changes in microbial community structure can influence host digestive efficiency (Brinkworth et al 2009, Degnan 1992, Dehority et al 1958, Donohoe et al 2011, Duncan et al 2007, Flint et al 2012, Fraser et al 2009, Macfarlane 1991, Nicholson et al 2012, Secor 2001). Increases in the relative abundance of bacteria in the Firmicutes phylum have been linked to host

obesity due to the ability of some Firmicutes to produce energy more efficiently than other taxa (Turnbaugh et al 2006). Additionally, changes in host diet can lead to differences in gut microbial community function by altering the metabolic pathways utilized by individual bacteria (Duncan et al 2004, Flint et al 2012, Hooper et al 2002, Macfarlane and Macfarlane 2003). For instance, several *Bacteroides* species cannot produce propionate from the fermentation of carbohydrates in the absence of vitamin B_{12} (Miller and Wolin 1979).

For most animals, including nonhuman primates, the availability, distribution, and nutritional content of food resources varies temporally and spatially in response to differences in microhabitat, rainfall, plant species phenology, and anthropogenic influence (e.g. Boinski 1988, Chapman et al 2003, Gates 2006, Gonzalez et al 2002, Milton 1980, Rumiz et al 1986, van Schaik et al 1993a). In the tall evergreen rainforest of Barro Colorado Island, Panama, fruit production peaks during the late dry and mid-wet seasons, leaf production peaks during the early wet season, and flower production peaks during the dry season (Milton 1980). In the semi-deciduous, northern Atlantic forest of Argentina, fruit production peaks during the wet season, and young leaf production is highest prior to the wet season when ripe fruit production is at its lowest (Kowalewski and Zunino 2004). Similarly, fiber and toxin concentrations in leaves are reported to vary in response to ecological factors such as soil nutrient levels (Campo and Dirzo 2003, Glander 1981, McKey 1978). As a result, the diets of wild animals can change dramatically across seasons and habitats (e.g. Chaves et al 2011, Goldizen et al 1988, Nakagawa 1997, Overdorff et al 1997, Rothman et al 2008). Given that the relative abundances and metabolic functions of microbial taxa in the gut are influenced by the nutritional composition of host diet, we would expect the composition and function of gut microbial communities in wild animal populations to vary across seasons and habitats in response to these changes in diet.

Recent research with howler monkeys (*Alouatta* sp.) provides preliminary evidence that the gut microbial community is impacted by changes in wild primate diet across habitats (Amato et al 2013). Groups of howler monkeys sampled at four different sites possessed distinct gut microbial communities that were strongly correlated with the array of plant species consumed, and changes in the relative abundances of microbial genes associated with SCFA production suggested shifts in gut microbial function such as increased fermentation and energy production in continuous rainforest habitats (Amato et al 2013). However, no study currently examines the impacts of temporal patterns in diet on the gut microbiota of wild animals (but see Williams et al 2012). These data are of critical importance for understanding host nutrition and health. If an individual's gut microbial community can shift over the course of days, weeks, or months in response to changes in diet (Kolida et al 2002, Turnbaugh et al 2009, Williams et al 2012, Wu et al 2011), associated shifts in host digestive efficiency may make meeting nutritional demands less challenging for animals during periods of low food availability or during periods when specific nutrients required for growth and reproduction are limited (Backhed et al 2004, Backhed et al 2007, Wostmann et al 1983). If, however, the gut microbiota fail to respond to these short-term changes in diet, or respond in a way that negatively affects digestive efficiency, the effects of limited food and nutrient availability on host health may result in reduced birth rates, reduced juvenile growth rates, increased mortality rates in juveniles, and an increased frequency in nutrient deficiencies and disease in adults (Altmann and Alberts 1987, Dunbar 1980, Gogarten et al 2012, Goldizen et al 1988, Hamilton 1985, Knott 1998, Wasser and Starling 1988).

In this study, I investigate temporal variation in diet and gut microbial community composition and function in two groups of wild, Mexican black howler monkeys (*A. pigra*) over a ten-month period. Howler monkeys respond to seasonal changes in the availability of food

items such as ripe fruit by exploiting hard-to-digest foods such as flowers, mature leaves and unripe fruits (Di Fiore et al 2011). For example, at Barro Colorado Island, howlers consume a diet with more fruit during the late dry and mid-wet seasons when it is readily available (46% of feeding time compared to 10% in the transition period), and flower consumption increases during the dry season when availability is high (18% of feeding time compared to 5% in other seasons). In contrast, during the transition period of low fruit and flower availability, howlers switch to a diet consisting of mostly leaves (85% of feeding time compared to 49% and 35% in the wet and dry seasons; Milton 1980). Because the nutritional properties of fruits, leaves, and flowers are extremely diverse, these temporal diet shifts can greatly impact howler energy and nutrient intake. While ripe fruit is generally considered an easy-to-digest food that is low in structural carbohydrates, high in energy, and low in protein, unripe fruits, young leaves, mature leaves, and flowers are considered harder-to-digest foods that are higher in protein but also higher in structural carbohydrates and toxins, and lower in energy (Table 3.1). Furthermore, as indicated in Table 3.1, within each of these categories, there is considerable species-specific variation. For example, Wrangham and Conklin (1994) report that the pulp of nine different species of African figs varies dramatically in lipid content (1.7-7.9%), crude protein content (4.3-20.7%), water-soluble carbohydrates (6.6-23.2%), and neutral detergent fiber (23.5-65.4%), and howlers at many field sites consume large amounts of fig fruits and fig leaves (Estrada 1984, Gaulin and Gaulin 1982, Glander 1981, Milton 1980, Serio-Silva et al 2002, Silver et al 1998). Therefore, changes in the consumption of both plant parts and plant species are likely to result in nutritionally distinct diets across different periods of the year.

To compensate for differences in energy and nutrient intake across time, howler monkeys are reported to employ an energy-minimizing behavioral strategy (Gaulin and Gaulin 1982,

Milton 1980, Rosenberger and Strier 1989, Smith 1977) as well as feeding selectivity (Glander 1981, Milton 1979). First, howlers maintain low active metabolic rates by resting for 62-80% of daylight hours (Di Fiore et al 2011) and utilizing small day ranges and home ranges compared to other atelines (average day range: 526m vs. 2,142m in *Ateles*, 1,075m in *Brachyteles*, and 1,925m in *Lagothrix*; average home range: 28ha vs. 278ha in *Ateles*, 154ha in *Brachyteles* and 398ha in *Lagothrix*; Di Fiore et al 2011). This behavior may allow them to endure temporary dips in energy consumption (Milton 1980). In addition, howlers preferentially consume young leaves instead of mature leaves and target a small number of tree species to reduce fiber and toxin intake and increase energy and nutrient intake (Glander 1981, Milton 1979, but see Behie and Pavelka 2012, Silver et al 2000). Mantled howlers (*A. palliata*) have been shown to consume young leaves from more tree species (nine of eleven species documented) compared to mature leaves (two of eleven species documented) and consume young leaves with a protein to fiber ratio approximately twice that of mature leaves (Milton 1979).

Shifts in gut microbial community composition and function over time may also allow howler monkeys to endure changes in diet across seasons by improving digestive efficiency and providing additional energy and nutrients cellulose (Arumugam et al 2011, De Filippo et al 2010, Flint et al 2008, Goel et al 2005, Kolida et al 2002, Nelson et al 2003, Odenyo et al 2001, Turnbaugh et al 2009, Williams et al 2012, Wu et al 2011). Although they do not possess the specialized gut morphology, such as a sacculated foregut or an enlarged caecum, utilized by leaf-eating primates such as colobines or indriids (Edwards and Ullrey 1999, Kay and Davies 1994, Milton 1980), howler monkeys have increased cecum and colon volumes (Chivers and Hladik 1980) and are characterized by relatively long food retention times compared to other atelines (*A. palliata:* 20.4 hrs; *A. seniculus:* 18.8-20.0 hrs; *A. guariba:* 19.0 hrs; *Ateles geoffroyi:* 4.7 hrs;

Ateles paniscus: 5.25 hrs, *Ateles belzebuth*: 4.5 hrs; *Brachyteles arachnoides:*8.0-14.0 hrs; *Lagothrix lagotricha:* 2.0-14.5 hrs (Edwards and Ullrey 1999, Link and Di Fiore 2006, Martins 2006, Milton 1981, Milton 1984, Stevenson 2000, Yumoto et al 1999). These characteristics suggest that howlers rely heavily on microbial fermentation in the hindgut to process the fiber and toxins found in some leaves (Milton and McBee 1983). Although it is likely that seasonal changes in diet composition affect microbial community composition and fermentation in the howler gut, we know little regarding these dynamics (Milton et al 1980). However, if temporal changes in howler diet lead to shifts in gut microbial community composition and function, they are likely to have important consequences for howler feeding ecology.

In this study, I test three hypotheses regarding the relationship between howler foraging ecology and gut microbial community composition and function. **(1) Temporal changes in howler monkey diet are associated with temporal changes in the composition of the howler monkey gut microbial community.** Assuming that ripe fruits consumed by howlers are higher in non-structural carbohydrates and lower in protein than leaves (Norconk et al 2009), during times when the majority of the diet is composed of ripe fruit (>50%), I expect to see a higher relative abundance of microbes that ferment non-structural carbohydrates such as members of the *Bacteroides* and *Prevotella* genera (Russell and Baldwin 1979, Salyers 1979). In contrast, during periods when young leaves, unripe fruit, and flowers make up the majority of the diet, I expect a higher relative abundance of cellulose-degraders such as genera of the Clostridia family and a higher relative abundance of protein metabolizers such as members of the *Propionibacterium, Bacteroides*, *Clostridium*, and *Streptococcus* genera (Attwood and Reilly 1995, Attwood et al 1996, Macfarlane et al 1986). **(2) Changes in howler monkey diet and gut microbial community composition are associated with changes in gut microbial community function.**

Non-structural carbohydrate fermentation generally leads to increased production of the SCFA, acetate, while increased fiber fermentation leads to butyrate production (Duncan et al 2003, Duncan et al 2007, Robinson et al 2001), and increased protein metabolism leads to the increased production of ammonia and branched SCFA's (Dehority et al 1958, Mackie et al 1998). Therefore, during periods of high ripe fruit consumption, I expect to detect more acetate and less ammonia and branched-chain fatty acids compared to periods of high flower, leaf, and unripe fruit consumption. During periods of high flower, leaf, and unripe fruit consumption, I expect to detect more butyrate, ammonia, and branched-chain fatty acids. **(3) Changes in gut microbial community function aid howlers in meeting nutritional requirements as their diet shifts.** I expect to detect more SCFA's during periods when howler energy intake is reduced and less ammonia and branched-chain fatty acids during periods when howler protein intake is reduced. If the gut microbiota are compensating for temporal changes in energy and nutrient intake, I also expect few differences in howler activity patterns and day ranges across time.

METHODS

To determine if howler gut microbial community composition and function vary in response to seasonal changes in diet, I collected focal samples describing feeding ecology as well as fecal samples for microbial analyses from black howlers in two neighboring social groups (N=6 adult males, 4 adult females, 6 juveniles) in Palenque National Park, Mexico across three ten-week blocks from September 2010 to June 2011. These sampling blocks corresponded loosely with changes in rainfall (CONAGUA 2011) and previously documented shifts in black howler diet at Palenque (Estrada, unpublished data). Block 1 (September-November 2010) was generally associated with heavier rainfall and a higher proportion of ripe fruit in the howler diet

(CONAGUA 2011, Estrada, unpublished data). Block 2 (January-March 2011) was associated with a lower proportion of fruit in the diet and a higher proportion of young leaves, and Block 3 (April-June 2011) was associated with less rainfall and a higher proportion of ripe fruit (CONAGUA 2011, Estrada, unpublished data).

Twenty-minute instantaneous focal individual samples were collected five days per week between sunrise and 5pm (park closing time) each day. The focal individual was chosen pseudo-randomly (no individual was sampled twice consecutively and priority was given to individuals that had been undersampled on previous days), and activity was recorded every two minutes. Five activities were recorded: feeding (ingestion of food items), foraging (movement within a feeding tree), resting (inactivity), traveling (movement between trees), and social activity (howling, play, sexual interaction, aggression, etc.). During feeding bouts, the type of food resource (e.g. ripe fruit, unripe fruit, mature leaves, young leaves, flowers, and stems) was recorded as well as the plant species. The number of food items consumed per minute was quantified when possible to provide an estimate of intake rate.

A handheld global positioning system was utilized to record the black howler group position every thirty minutes during data collection. To estimate day range, I calculated the total distance traveled between all points using ArcGIS 10.1 (ESRI 2011, Redlands, CA). The home range utilized during each sampling block was estimated by calculating the area of the minimum convex polygon created by the data points in ArcGIS 10.1 (ESRI 2011, Redlands, CA).

Samples of the top ten food items (based on the percentage of feeding time) during each sampling block time were collected for each howler group, and the average wet and dry mass of each resource was measured using five items from each of three trees. Samples of the top ten food resources used during each sampling block also were collected and preserved in 70%

methanol for metabolite profiling. Metabolites are small molecules produced by a plant during metabolism (e.g. amino acids, alcohols, nucleotides, vitamins) that influence both howler nutrition and microbial metabolism since they may be digested directly or utilized by the microbial community as substrates for fermentation or nutrient synthesis. All metabolite data were generated using gas chromatography/mass spectrometry (Poroyko et al 2011). Mass spectra were verified with authentic standards and mass spectra from a commercial database (Poroyko et al 2011).

The behavioral data were used to calculate the average percentage of time spent feeding during a focal sample in each sampling block. Because howler monkeys are active during all daylight hours, the average daylength during each period was used to calculate the average number of minutes per day the howlers fed. Average ingestion rates for each plant part of each plant species, as well as average food item dry mass, were used to estimate the average number of dry grams of each food item ingested daily by each individual. Diet composition was described in terms of estimated grams of plant parts ingested per day, grams of plant parts from each plant species, and metabolite content. Metabolites extracted from howler food resources were expressed in terms of relative concentration per gram of wet weight. The total relative concentration of each metabolite in each food resource was multiplied by the average daily grams (wet weight) of that resource consumed by each individual in each sampling period to provide an estimate of the concentrations metabolites consumed. Published literature values were used to estimate the amount of protein, non-structural carbohydrates, lipids, and metabolizable energy consumed (Norconk et al 2009). Metabolites were categorized into amino acids, sugars, and lipids when possible to compare with patterns in literature estimates. Due to the method by which the metabolite concentrations were standardized, these data do not provide accurate

estimates of the actual amount of each metabolite in a food item, nor can their amounts be accurately compared across categories (i.e. amino acid, sugar, lipid) within a sample (Poroyko et al 2011). However, relative concentrations of the same metabolite across food items and howler diets can be compared to determine their impact on howler nutrition and gut microbial communities. All feeding data were standardized by metabolic body weight (body mass raised to the 0.75) using published data on average mass for each age/sex class before analysis (Kelaita et al 2011, Kleiber 1975).

Fecal samples were collected every two weeks from each group member for microbial community composition analysis as well as measurements of volatile fatty acid (VFA) and ammonia content. VFAs are a class of SCFAs produced by microbial breakdown of fiber and some amino acids, and ammonia is a product of microbial amino acid metabolism (Mackie et al 1998). Measuring fecal VFA and ammonia content provides a proxy for VFA and ammonia production since higher production of VFAs and ammonia normally leads to higher excretion of both as waste (Mackie et al 1998). Fecal samples were stored in 96% ethanol for microbial community composition analyses, 1M NaOH for VFA analyses, and 1M HCl for ammonia analyses. They were shipped to the University of Illinois where they were kept at -80C until processing. Permits to collect and export fecal and plant samples were obtained through the Secretaria del Medio Ambiente y Recursos Naturales (SEMARNAT) and the Comisión Nacional de Areas Naturales Protegidas (CONANP), and the Secretaría de Agricultura, Ganadería, Desarollo Rural, Pesca y Alimentación (SAGARPA) in Mexico. Permits to import samples to the United States were obtained through the Center for Disease Control (CDC) and the United States Department of Agriculture Animal and Plant Health Inspection Service (USDA APHIS).

To describe microbial community composition, DNA was extracted from samples preserved in 96% ethanol using the MOBio UltraClean Soil Kit. The intergenic spacer region of the 16S ribosomal gene was amplified in all samples using polymerase chain reaction, and automated ribosomal intergenic spacer analysis (ARISA) was used to create a microbial community "fingerprint" for each sample (Kent et al 2007). PCR reactions included buffer consisting of 50 mM Tris (pH 8.0), 250 μg of bovine serum albumin per mL and 3.0 mM $MgCl_2$ (Idaho Technology, Salt Lake City, UT; cat #1770), 250 mM of each dNTP, 10 pmol of each primer, 1.25 U of *Taq* polymerase (Promega, Madison, WI), and 2 μL of extracted DNA in a final volume of 25 μL. The following primers were used to generate ARISA PCR products: 1406f, 5'- TGYACACACCGCCCGT-3' (universal, 16S rRNA gene), and 23Sr, 5'- GGGTTBCCC CATTCRG-3' (bacteria-specific, 23S rRNA gene). The 1406f primer was labeled at the 5' end with the phosphoramidite dye 6-FAM. PCR was carried out in an Eppendorf MasterCycler (Eppendorf, Hauppauge, NY) with an initial denaturation at 94°C for 2 min, followed by 30 cycles of 94°C for 35s, 55°C for 45s, and 72°C for 2 min, with a final extension carried out at 72°C for 2 min. ARISA PCR products were visualized by denaturing capillary electrophoresis using an ABI 3730xl Genetic Analyzer (Applied Biosystems, Foster City, CA) at the UIUC Keck Center for Comparative and Functional Genomics as described previously (Kent and Bayne 2010). Size-calling and ARISA profile alignment were carried out using GeneMarker version 1.95 (SoftGenetics, State College, PA). A signal detection threshold of 500 fluorescence units was used in order to exclude background fluorescence. The signal strength (i.e., peak area) of each peak was normalized to account for run-to-run variations in signal detection by dividing the area of individual peaks by the total fluorescence (area) detected in each profile, expressing each peak as a proportion of the observed community (Yannarell and Triplett 2005).

Pyrosequencing of the V1-V3 region of the 16S ribosomal RNA gene was used to generate taxonomic data for the microbial communities in a subset of samples (N=8 individuals at 15 time points; Amato et al 2013). The V1-V3 region of the 16S ribosomal RNA gene was amplified by polymerase chain reaction (20 cycles of 94°C (30s), 48°C (30s), 72°C (2 min)) using primers 27f (CGTATCGCCTCCCTCGCGCCATCAG-AGAGTTTGATYMTGGCTCAG; corresponding to nucleotides 8 – 27 of the *Escherichia coli* 16s rRNA gene) and 534r (CTATGCGCCTTGCCAGCCCGCTCAG-[MID tag 1 – 50]-ATTACCGCGGCTGCTGGCA). The amplicons were pyrosequenced using 454 FLX-Titanium technology at the J. Craig Venter Institute (Rockville, MD). 119 samples were successfully sequenced, and sequences shorter than 250nt, with homopolymers longer than 6 nucleotides, containing ambiguous base calls or incorrect primer sequences were removed. Sequences were aligned against the silva database (Pruesse et al 2007) and pre-clustered using mothur (Schloss et al 2009). Potentially chimeric sequences were detected and removed using uchime in mothur (Schloss et al 2009). The remaining reads were clustered using a custom perl script. OTUs were defined as sharing $\geq$ 97 % sequence identity. OTUs detected fewer than twice across the entire data set were removed as probable artifacts. Rarefaction data, Simpson, Shannon-Weaver and Chao1 indices were produced using mothur (Schloss et al 2009). Taxonomic profiles were generated using the RDP Classifier (Wang et al 2007).

Fecal VFA content was measured using gas chromatography (Erwin et al 1961). Samples preserved in 1M NaOH were neutralized with phosphoric acid and centrifuged to remove particulate matter before being processed. The amount of each VFA detected was expressed both in millimoles per gram of feces and as a proportion of total VFAs. Ammonia content was measured using spectrophotometry. Samples preserved in 1M HCl were processed according to

Chaney and Marbach (Chaney and Marbach 1962). Fecal ammonia content was expressed in millimoles per gram of feces. Data for both VFA and ammonia content were standardized according to sample mass and adjusted for dilution.

Permutational multivariate analysis of variance (PERMANOVA) was used to test for the effects of sampling block on gut microbial community composition, fecal VFA content, and diet. Data were pooled by individual for each sampling block, and only individuals from which data were collected during every sampling block were included in analyses (N=13). Because I detected differences across groups in all of these factors, models were stratified by group, allowing me to test for differences across sampling blocks while controlling for differences among groups. Type III sums of squares were used to determine the significance of each factor in the model. All models were run for 5000 permutations. Temporal patterns in gut microbial community composition, VFA content, and diet were visualized using partial correspondence analysis.

Depending on the distribution of data, Kruskal-Wallis tests or analysis of variance (ANOVA) were used to test for seasonal patterns in total grams of food consumed, total fecal VFA content, and total fecal ammonia content. A series of Kruskal-Wallis tests were also employed to test for temporal patterns in individual plant parts and plant species consumed, relative concentration of metabolites in the diet, concentration of individual VFAs in fecal samples, and relative abundance of bacterial taxa. P-values were adjusted for repeated tests using a sequential Bonferroni correction with the initial $p = 0.05$ (Holm 1979, Rice 1989). However, to detect bacterial taxa that differed in abundance across sampling blocks, I used a p-value of 0.05 since sequencing restrictions reduced the number of individuals for which I could generate data and therefore reduced statistical power. Indicator species analysis (R Software, labdsv package)

also was used to detect microbial genera characterizing each sampling block based on both abundance and frequency of occurrence (De Caceres and Legendre 2009). Taxa with a significant ($p < 0.05$) indicator value higher than 0.5 were considered characteristic of each sampling block.

Non-parametric Mantel tests were used to compare overall patterns in gut microbial community composition, fecal VFA content, and host diet. A series of Spearman rank correlations were used to test for relationships between the relative abundances of individual bacterial taxa and the amounts of plant parts consumed, the amounts of metabolites consumed, the amounts of protein, lipids, total non-structural carbohydrates, neutral detergent fiber, and kilocalories consumed, and the fecal concentration of individual VFAs and ammonia. Again, p-values were adjusted for repeated tests using a sequential Bonferroni correction with the intial $p = 0.05$ (Holm 1979, Rice 1989). All analyses were performed with R with the exception of non-parametric Mantel tests, which were performed using PRIMER 6 for Windows (PRIMER-E, Plymouth, United Kingdom).

RESULTS

I collected 328 hours of focal data in sampling Block 1 (103 feeding hours), 531 hours in Block 2 (139 feeding hours), and 663 hours in Block 3 (89 feeding hours). Across the study period the Balam group spent an average of 64.9% of daylight hours resting, 21.7% feeding, 5.0% traveling, 4.4% engaging in social behavior, and 1.8% foraging. Of the total grams of food ingested by Balam across the study period, 48.5% was ripe fruit, 25.7% young leaves, 11.7% unripe fruit, and 1.8%, 8.8% and 3.5% mature leaves, stems, and flowers, respectively. The Motiepa group spent an average of 65.4% of daylight hours resting, 20.7% feeding, 4.5%

traveling, 5.7% engaging in social behavior and 1.7% foraging. 41.2% of total food ingested by the Motiepa group was ripe fruits, 35.8% young leaves, 13.5% unripe fruit, 3.4% stems, 2.4% flowers, and 3.8% mature leaves.

Temporal activity patterns: Activity budget did not differ between groups ($F_{2, 38} = 1.44$, $p = 0.22$), but it did differ across sampling blocks ($F_{2, 38} = 18.07$, $p = 0.0002$). These differences were the result of increased time spent resting in Block 3, which was correlated positively with daily average temperature (Balam: $r^2 = 0.50$, $p << 0.01$, Motiepa: $r^2 = 0.46$, $p << 0.01$). All other aspects of the howler activity budget did not vary across sampling blocks, and home range and day range distances also did not vary across sampling blocks (Table 3.2).

Temporal diet patterns: The average daily amount of plant material consumed by individuals in each group did not differ (Motiepa: 1057 ± 212g, 292 ± 80g/metabolic body weight; Balam: 1135 ± 485g, 320 ± 110g/metabolic body weight; $F_{2,38}=1.40$, $p = 0.26$). However, the howlers consumed more grams of food in Blocks 1 (1,294 ± 395g) and 2 (1,069 ± 341g) compared to Block 3 (785 ± 183g; $F_{2,38} = 9.15$, $p = 0.0007$). The proportions of plant parts in the diet differed between groups depending on sampling period ($F_{2,38}=4.73$, $p = 0.003$). Specifically, the Motiepa group consumed a higher percentage of young leaves (62.3% of total grams ingested) during Block 2 than did the Balam group (30.8%), while the Balam group included more flowers (10.6% vs. 0.5%) and ripe fruit (34.5% vs. 9.5%) in their diet during this period (Figure 3.1). When I controlled for this difference in my PERMANOVA model, I detected significant differences in the plant parts consumed by the howlers across sampling periods (Table 3.3, Figures 3.1 and 3.2). A higher proportion of young leaves (62.3% and 30.8%) and unripe fruits (23.5% and 16.8%) were consumed during Block 2 while more ripe fruits were consumed during Block 3 (63.3% and 69.5%, Figure 3.2).

Dietary diversity in terms of the plant species consumed was similar between groups (Motiepa: Shannon = 2.25 ± 0.27, Gini-Simpson = 0.86 ± 0.05; Balam: 2.23 ± 0.27, 0.83 ± 0.07) and marginally lower during Block 2 compared to Blocks 1 and 3 (Block 1: Shannon = 2.38 ± 0.22, Gini-Simpson = 0.86 ± 0.04; Block 2: 2.08 ± 0.24, 0.82 ± 0.06; Block 3: 2.27 ± 0.26, 0.85 ± 0.06). Although both howler groups used many of the same top ten plant species (Table 3.4), the relative importance of individual plant species consumed differed between howler groups ($F_{2,38}$ = 2.69, p = 0.003). For example, the Balam group consumed more *Ficus yopensis* mature fruits (3.5 ± 4.5g/metabolic body weight, 19.1 ± 16.1% vs. 0.01 ± 0.02g, 5.3 ± 8.0%; χ^2 = 12.98, df = , p <<0.001) and *Cojoba arborea* stems (21.4 ± 22.2g/metabolic body weight, 3.2 ± 3.0% vs. 2.4 ± 4.2g, 0.11 ± 0.39%; χ^2 = 28.87, df = , p <<0.001) than the Motiepa group. However, in both groups, the Moraceae and Fabaceae families dominated the diet, and *Ficus* was an important genus both in terms of ripe fruits and young leaves (Table 3.4). Additionally, in both groups, the total array of food items and plant species consumed differed across sampling blocks (Table 3.3, Figure 3.3). In the Balam group, I detected no single food item or plant species that drove these patterns. Instead, small changes in the consumption of all food items appear to have resulted in overall diet shifts across sampling blocks. In the Motiepa group, nine food items drove the observed shifts in overall diet across sampling blocks (Table 3.5). Of these food items, six were species of fruit.

Literature estimates of the amount of protein and energy contained in the howler diet observed in this study suggest that the Motiepa group ingested fewer grams of lipids per metabolic body weight ($F_{1,38}$=7.12, p = 0.01) and included a lower average daily proportion of lipids ($F_{1,38}$=20.69, p << 0.01) and protein ($F_{1,38}$=19.70, p << 0.01; Table 6) in their diet. However, the differences in the proportions of lipids (3.0% vs. 3.1%) and proteins (11.9% vs.

12.3%) appeared to be biologically insignificant. Despite some differences in nutrient intake among groups, there were similar differences in nutrient intake across time in both groups. Across sampling blocks, I detected significant changes in every aspect of nutritional content estimated. Both groups consumed fewer kilocalories ($F_{2,38}$=10.27, p = 0.0003), grams of protein ($F_{2,38}$=17.42, p << 0.01), grams of total non-structural carbohydrates ($F_{2,38}$=17.7, p << 0.001), and grams of neutral detergent fiber (NDF, $F_{2,38}$=17.06, p << 0.01) per metabolic body weight in Block 3compared to Block 1 (Table 3.6). Patterns in protein energy, non-protein energy (carbohydrates and lipids), and total energy intake were the same (Table 3.7). Across seasons, protein intake was positively correlated with the amount of unripe fruit consumed (Spearman's ρ = 0.56, p = 0.0002). Total non-structural carbohydrate intake was positively correlated with the amount of ripe fruit consumed (Spearman's ρ = 0.53, p = 0.0005), and NDF intake was positively correlated with unripe fruit (Spearman's ρ = 0.49, p = 0.002) and stem intake (Spearman's ρ = 0.48, p = 0.002).

Temporal variation in lipid intake was distinct between groups ($F_{2,38}$=4.36, p = 0.02; Table 3.6). Both groups consumed fewer lipids during Block 3, but members of the Balam group consumed more grams of lipids than the Motiepa group in Block 2. This pattern suggests that the Balam group had more reliable sources of lipids during Block 2. Literature estimates of lipid content are higher for ripe fruit and flowers (Norconk et al 2009), and across seasons, lipid intake was positively correlated with ripe fruit (Spearman's ρ = 0.48, p = 0.002). The Balam group consumed more ripe fruit than the Motiepa group during Block 2. This behavior is likely to have resulted in the higher energy intake observed for the Balam group compared to the Motiepa group during Block 2.

The proportion of protein in the diet was highest during Block 2 for both groups ($F_{2,38}$=15.43, p << 0.01), and the proportion of non-structural carbohydrates was lowest ($F_{2,38}$=14.86, p << 0.01; Table 3.6). The proportion of lipids in the diet was similar across sampling periods for the Balam group but lower during Block 2 for the Motiepa group ($F_{2,38}$=13.92, p << 0.01). However, like differences between groups, differences in lipid proportions across sampling blocks appeared to be biologically insignificant.

Across seasons, individual howler monkey energy and nutrient intake was correlated to activity. Energy (kcal; Spearman's ρ = -0.50, p = 0.001), protein (Spearman's ρ = -0.68, p << 0.01), and NDF intake (Spearman's ρ = -0.68, p << 0.01) were negatively correlated with time spent resting and positively correlated with time spent feeding (Spearman's ρ = 0.76, p << 0.01; Spearman's ρ = 0.89, p << 0.01; Spearman's ρ = 0.87, p << 0.01). Total non-structural carbohydrate (Spearman's ρ = 0.54, p = 0.0004) and lipid intake (Spearman's ρ = 0.64, p << 0.01) were also positively correlated with time spent feeding. However, time spent resting was also positively correlated with average daily temperatures (r^2 = 0.47, p << 0.01), and time spent feeding was negatively correlated with average daily temperatures (r^2 = -0.45, p << 0.01). These results suggest howler activity was influenced by daily average temperatures and that variations in howler dietary intake were an effect of increased resting time and decreased feeding time. Variations in howler dietary intake did not cause changes in the activity budget.

There were significant differences between groups in the metabolite profiles consumed ($F_{1,38}$=3.08, p = 0.035). Pantothenic acid, or vitamin B5, was consumed in higher concentrations by the Motiepa group, and these individuals also consumed higher average daily relative concentrations of total lipid metabolites per metabolic body weight ($F_{2,38}$ = 7.23, p = 0.011; Table 3.8). Patterns in lipid consumption may be related to slightly higher proportions of leaves

in the Motiepa diet as well as the occasional ingestion of seeds (Figure 3.1). Also, during Block 1, members of the Motiepa group consumed a higher relative concentration of sugar metabolites than those in the Balam group, while in Block 3, Balam howlers consumed a higher concentration of sugar metabolites ($F_{2,38} = 4.8$, $p = 0.014$; Table 3.7). This pattern could result from differences in ripe fruit consumption between the two groups.

Despite these differences, both groups exhibited changes in metabolite consumption patterns across time ($F_{2,38} = 11.12$, $p = 0.0002$, Table 3.3, Figure 3.4). The relative concentration of all metabolite classes differed across sampling blocks. In general, both groups consumed a lower relative concentration of amino acids during Block 3 ($F_{2,38} = 30.36$, $p << 0.01$; Table 3.8), and the average daily diet of both groups contained decreasing average daily relative concentrations of lipid metabolites per metabolic body weight from Block 1 to Block 3 ($F_{2,38} = 25.59$, $p << 0.01$). These patterns were generally similar to those seen in protein and lipid content based on literature estimates. Also, Motiepa group members consumed a higher concentration of sugar metabolites during Block 1 compared to both other sampling blocks while Balam group members consumed a lower concentration in Block 2 compared to both other sampling blocks ($F_{2,38} = 13.34$, $p = << 0.01$; Table 3.8). However, as with total non-structural carbohydrates, the most sugar metabolites were generally consumed during Block 1. For the Balam group, these patterns were not driven by any metabolite in particular, but the Motiepa group showed significant temporal changes in nine metabolites (Table 3.9). This may reflect the temporal patterns observed in the plant species being consumed by each group.

Overall, the concentrations of plant metabolites consumed were correlated to the grams of plant species consumed (Spearman's $\rho = 0.56$, $p = 0.001$) and the grams of plant parts consumed (Spearman's $\rho = 0.32$, $p = 0.001$). This pattern suggests that the concentrations of

metabolites ingested by the howlers do not depend solely on the plant part or species being ingested but that differences in the nutritional composition of each individual plant part and species contribute to the array of compounds available to howlers in a given diet.

Gut Microbial Community Composition: Sequencing data indicated no differences in Chao1 estimates of OTU (operational taxonomic unit) richness for the howler gut microbial community between social groups, but community richness changed significantly across sampling blocks ($F_{2,24} = 9.62$, $p = 0.0013$). Estimates at 1600 reads per sample decreased from Block 1 to Block 3 (Block 1: 2,337.8 ± 192.3, Block 2: 2,052.4 ± 308.3, Block 3: 1,785.8 ± 233.7). Microbial diversity was also similar between howler groups (Motiepa: Shannon = 4.72 ± 0.37, Simpson = 0.033 ± 0.017; Balam: 4.93 ± 0.27, 0.045 ± 0.030). Across the study period, Shannon diversity was higher during Block 1 compared to both Blocks 2 and 3 (Block 1: 5.17 ± 0.31; Block 2: 4.83 ± 0.44; Block 3: 4.67 ± 0.32; $F_{2,24} = 4.47$, $p = 0.024$) while Simpson diversity did not change across seasons (Block 1: 0.035 ± 0.031; Block 2: 0.029 ± 0.013; Block 3: 0.041 ± 0.0025; $F_{2,24} = 0.51$, $p = 0.61$).

Community fingerprinting (ARISA) data indicated differences in gut microbial community composition across groups at the OTU level ($F_{1,38} = 2.19$, $p = 0.0004$), and in the subset of samples I sequenced, there were differences in gut microbial community composition at the genus level ($F_{1,24} = 2.61$, $p = 0.0046$). There was no interaction between sampling block and group in either data set. Across sampling blocks, the community fingerprinting data indicated differences in microbial community composition at the OTU level while my taxonomic data indicated differences at the Family and genus levels (Table 3.10, Figure 3.5). I detected six bacterial Families and eight bacterial genera that varied in relative abundance across sampling

blocks. For example, the relative abundance of bacterial sequences assigned to the genus *Butyricicoccus* was highest during Block 2, and the relative abundances of bacterial sequences assigned to the *Coprobacillus* and *Streptococcus* genera were highest during Blocks 1 and 3 (Table 3.11). Sequences assigned to the Lachnospiraceae family such as *Oribacterium* decreased in relative abundance from Block 1 to Block 3 while bacteria in the Ruminococcaceae family and the genus *Papillibacter* increased in abundance (Table 3.11). Indicator species analysis reported that howler gut microbial communities were characterized by *Streptococcus, Mogibacterium, Gordonibacter, Xylanibacter, Akkermansia, Coprobacillus,* and *Oribacterium* during Block 1, *Anaerotruncus, Hallela* and *Prevotella* during Block 2, and *Dialister, Helicobacter, Papillibacter*, TM7, and *Solobacterium* during Block 3.

Non-parametric Mantel tests indicated a weak correlation between overall microbial community composition and plant species consumed (Spearman's $\rho = 0.28$, $p = 0.008$), and no significant correlation between microbial community composition and dietary metabolite profiles (Spearman's $\rho = 0.11$, $p = 0.10$). However, there were significant correlations between individual bacterial taxa and individual diet components (Table 3.12, 3.13). For example, the relative abundance of *Acetivibrio* was positively correlated with the amount of unripe fruit in the diet. The relative abundance of *Butyricicoccus* was positively correlated to young leaf and unripe fruit consumption, and the relative abundance of *Oscillibacter* was positively correlated with NDF intake (Table 3.12). Similarly, Firmicutes bacteria such as *Butyricicoccus, Coprobacillus, Oribacterium*, and *Dialister* were positively correlated with the relative concentration of a variety of metabolites, including 2-methylcitric acid, 2-methylsuccinic acid, 3-deoxy-arabino-hexaric acid, glucaronic acid, guanine, isoleucine, N-acetylglucosamine, octaconasol, and xylitol (Table 3.13). *Prevotella*, a Bacteroidetes, showed negative correlations with these metabolites

(Table 3.13), suggesting that the Firmicutes genera were able to outcompete *Prevotella* during periods when the relative concentrations of these metabolites were high. *Hallella* (Phylum: Bacteroidetes) and *Akkermansia* (Phylum: Verrucomicrobia) showed patterns similar to those of the Firmicutes genera (Table 3.13).

Gut Microbial Community Function: The amount of total VFAs detected in fecal samples differed significantly across sampling blocks (χ^2=17.8, df = 2, p = 0.0001). Fecal VFA concentrations were highest during Block 3 compared to Blocks 1 and 2 for both groups (Table 3.14). VFA profiles differed across groups in terms of molar proportions ($F_{2,38}$ =11.47, p = 0.0004), not concentrations ($F_{2,38}$ =0.75, p = 0.51). When group differences were controlled for, sampling block accounted for the majority of the variation in VFA profiles (Table 3.15, Figure 3.6). Kruskal-Wallis tests indicated significant differences in the molar proportions of all VFAs except isopentanoic acid across sampling blocks (Figure 3.7). The least acetic acid and the most butanoic and propanoic acid were detected during Block 2 (Figure 3.7).

There was a weak correlation between patterns of overall microbial community composition and VFA profiles across time (Spearman's $\rho = 0.24$, p = 0.001). The concentration of acetic acid was positively correlated to the relative abundance of *Coprobacillus* and negatively correlated to the relative abundances of *Butyricicoccus* and *Streptophyta*, and the concentrations of isobutanoic and isopentanoic acid were positively correlated to the relative abundances of *Xylanibacter* and *Acetivibrio*, respectively (Table 3.16).

Overall diet described in terms of plant parts was weakly correlated to fecal VFA profiles (Spearman's $\rho = 0.16$, p = 0.01). When I examined individual metabolites, though, the concentration of acetic acid was negatively correlated to the amount of young leaves

(Spearman's $\rho = -0.65$, $p << 0.001$) and unripe fruit (Spearman's $\rho = -0.58$, $p << 0.001$) in the diet and positively correlated to the amount of mature leaves (Spearman's $\rho = 0.50$, $p = 0.001$). Acetic acid also was positively correlated to the amount of *Ficus aurea, F. americana* and *Poulsenia armata* mature fruit ingested (Spearman's $\rho = 0.69$, $p << 0.001$; Spearman's $\rho = 0.44$, $p = 0.005$; Spearman's $\rho = 0.64$, $p << 0.001$) and negatively correlated to the amount of *Schizolobium parahyba* stems consumed (Spearman's $\rho = -0.51$, $p << 0.0009$). Butanoic acid was positively correlated to the amount of young leaves ingested (Spearman's $\rho = 0.43$, $p << 0.007$) and isopentanoic acid was negatively correlated (Spearman's $\rho = 0.42$, $p << 0.008$). There was no overall correlation between VFA profiles and metabolite profiles, and only fecal acetic acid and butanoic acid concentrations exhibited correlations with single metabolites (Table 3.17). Acetic acid was also negatively correlated to the amount of protein in the diet (Spearman's $\rho = -0.51$, $p = 0.001$), and isobutanoic acid was positively correlated with NDF ingestion (Spearman's $\rho = 0.46$, $p = 0.004$).

Fecal ammonia content did not differ by group ($\chi^2=0.0008$, df = 1, $p = 0.98$) but differed significantly by season in both groups ($\chi^2=24.49$, df = 2, $p << 0.001$) with higher levels detected during Block 1 compared to Blocks 2 and 3 (Figure 3.8). Fecal ammonia concentration was positively correlated to the relative abundances of *Coprobacillus, Oscillibacter,* and *Streptococcus* (Table 3.16). In terms of diet, ammonia was positively correlated protein (Spearman's $\rho = 0.43$, $p = 0.007$, total-nonstructural carbohydrate (Spearman's $\rho = 0.50$, $p = 0.001$), and neutral-detergent fiber (Spearman's $\rho = 0.51$, $p = 0.009$) intake as well as 42 different metabolites (Table 3.17). The concentration of isobutanoic acid varied in relation to ammonia as well (Spearman's $\rho = 0.42$, $p = 0.008$).

DISCUSSION

In this study I tested a series of hypotheses regarding the degree to which seasonal variation in howler monkey diet appears to influence gut microbial community composition and function. Specifically, I hypothesized that (1) temporal changes in howler monkey diet would be associated with temporal changes in the composition of the howler monkey gut microbial community, (2) changes in howler monkey diet and gut microbial community composition would be associated with changes in gut microbial community function, and (3) changes in gut microbial community function would aid howlers in meeting nutritional requirements. My data provide evidence supporting each of these hypotheses, suggesting the importance of the gut microbiota to howler monkey nutrition and health in a variable environment.

Gut Microbial Community Composition: Research on gut microbial communities and diet in humans and laboratory mice indicate that patterns in host diet over periods of years determine the predominant genera in the gut microbial community (Arumugam et al 2011, Wu et al 2011). However, short-term changes in dietary patterns over the course of days or weeks can lead to rapid shifts in gut microbial community composition (Turnbaugh et al 2009). Based on this ten-month study, I detected temporal shifts in the overall composition of the howler monkey gut microbial community that were only weakly correlated with temporal changes in the howler diet. This relationship indicates that the composition of the howler gut microbial community is stable and resists major shifts in diet that occur over a timescale of months. These results mirror a study of human gut microbial community composition which demonstrated that short-term diet shifts lasting ten days did not greatly affect overall microbial community composition (Wu et al 2011). Therefore, like humans, it appears that howler monkeys may possess gut microbiome

"enterotypes" that vary little once the adult gut microbial community is established (Arumugam et al 2011, Wu et al 2011).

Because the overall gut microbial community exhibits limited temporal variation in response to seasonal diet shifts in both black howler monkeys and humans, it is likely that most, if not all, primate species also exhibit only limited seasonal variation in gut microbial community composition. Furthermore, because primate gut microbial communities differ in composition across primate species (Yildirim et al 2010), it is possible that each primate species possesses a distinct enterotype that has evolved in response to host diet and physiology. Although further comparative studies that incorporate temporal data are necessary to test this hypothesis, this information could be crucial for understanding primate life history processes. By allowing primates to extract more energy and nutrients from their diets, gut microbial communities may reduce the severity of trade-offs in resource allocation for life history processes (Leigh and Blomquist 2011). For example, females may be able to reduce interbirth intervals without reducing their investments in each individual offspring. Additionally, the development of a gut microbial community specialized for breaking down a particular diet is likely to lower the metabolic risks some juveniles are believed to face from low foraging efficiency and feeding competition with adults (Janson and van Schaik 1993, Leigh 1994), thereby improving the potential for increased growth rates. This process would be especially important in species in which mothers invest limited resources in prenatal brain growth, and offspring support the metabolic costs of brain growth after birth (Leigh 2004). In these species, prenatal gastrointestinal development may be crucial for allowing offspring to utilize an adult diet and develop the gut microbial community quickly. Although data describing the development of the

gastrointestinal tract and the gut microbial community are currently scarce, the age of weaning is reported to be earlier for primates undergoing more postnatal brain development (Leigh 2004).

Despite relative temporal stability in the black howler gut microbial community, the existence of some overall temporal changes associated with diet as well as a unique subset of bacterial genera characterizing each sampling block suggest that howler diet influences at least a subset of the wild, howler gut microbiota on a timescale of months. Correlations between the relative abundances of single bacterial taxa and single diet components also imply an adaptation of the gut microbial community to howler diet. Variation in the consumption of general diet categories such as unripe fruit was associated with variation in the relative abundances of bacterial genera such as *Acetivibrio,* and macronutrient intake appeared to influence the relative abundances of *Acetivibrio, Hallella, Oscillibacter,* and *Streptophyta.* Additionally, when I used metabolite profiling to describe diet composition in more detail, I uncovered a large number of strong correlations between howler diet and gut microbial community composition. For example, *Papillibacter* relative abundances were positively correlated to the amount of the flavenoid, kaempferol, consumed by the howlers. Because *Papillibacter* is related to a flavenoid-degrading anaerobe (Schleifer 2009), it may possess the pathway necessary to degrade kaempferol, and increases in its abundance in response to kaempferol suggest an adaptation of the gut microbial community to howler diet.

In cases where I did not detect significant correlations between microbes and diet, positive associations between the relative abundances of bacterial taxa and the relative concentrations of metabolites in the diet also suggested an adaptive response of the gut microbial community to diet. *Xylanibacter* breaks down simple sugars including xylan (Krieg et al 2010) and was characteristic of Block 1 when relative concentrations of glucose (Block 1: 36,098,421,

Block 2: 23,980,016, Block 3: 43,808), fructose (Block 1: 56,317,334, Block 2: 25,895,079, Block 3: 21,809,496), and xylose (Block 1: 259,461, Block 2: 113,983, Block 3: 43,808) were highest. Simple-sugar degraders *Coprobacillus* and *Oribacterium* also were characteristic of this period. In contrast, *Prevotella* requires peptides as a nitrogen source as well as iron to stimulate growth (Krieg et al 2010). *Prevotella* was characteristic of Block 2 when young leaves dominated the howler diet. Leaves consumed by howlers are generally assumed to contain more tannins than fruits (Glander 1979, Glander 1982, Milton 1979), and since tannins bind protein and reduce its digestibility (Rhodes and Cates 1976), a leaf-heavy diet may have resulted in more undigested peptides reaching the howler hindgut for breakdown by *Prevotella.* Additionally, leaves consumed by howler monkeys have been shown to have a higher iron content than fruits (young leaf: 155.4 μg/g; fruit: 77.2 μg/g; Silver et al 2000), suggesting that increased iron content in the howler diet may also have stimulated *Prevotella* growth during Block 2.

Finally, while genera of the Firmicutes phylum were positively influenced by 2-methylcitric acid, 2-methylsuccinic acid, 3-deoxy-arabino-hexaric acid, glucaronic acid, guanine, isoleucine, N-acetylglucosamine, octaconasol, and xylitol, *Prevotella* (Phylum: Bacteroidetes) was negatively influenced by the same metabolites. Studies of the human gut microbiota suggest an inverse relationship between the relative abundances of Firmicutes and Bacteroidetes that is impacted by diet (Ley et al 2006b, Ley 2010, Turnbaugh et al 2009). Diets high in fat and sugar increase the relative abundances of some Firmicutes bacteria and decrease the relative abundances of Bacteroidetes while low-fat, plant-based diets have the opposite effect (Turnbaugh et al 2009). Although my dataset contained Bacteroidetes genera that exhibited patterns similar to those of Firmicutes genera, it appears that in some cases, shifts in howler diet

may have differential effects on bacteria of these two phyla. A closer examination of these interactions would further inform discussions of both howler and human nutrition and health.

Gut Microbial Community Function: The proportions of VFAs produced by the gut microbiota are determined by the interaction between the composition of the microbial community and the substrates available to the community for fermentation from the host diet (Macfarlane and Macfarlane 2003, Mackie and White 1997a, Tremaroli and Backhed 2012). Therefore, by examining fecal VFA profiles it is possible to infer basic information regarding microbial fermentation processes in the gut. Compared to studies of other primates and mammals, my black howler monkey fecal samples contained high molar proportions of acetic acid (Table 18; Lambert and Fellner 2012). This observation matches reports for mantled howler monkeys (*Alouatta palliata*; Milton and McBee 1983), suggesting that high proportions of acetic acid may be typical of howler monkeys in general. Although it is possible that howler monkey genetics and digestive physiology play a role in the production of similar VFA profiles by influencing gut microbial community composition (Buhnik-Rosenblau et al 2011, Nelson et al 2003, Spor et al 2011, Zoetendal et al 2001), it more likely that this pattern is the result of similar diets across the genus. High proportions of acetic acid are indicative of a high-fiber diet and a slow, efficient fermentation process (Hume 1997). Although howler monkeys are not reported to consume more fiber compared to other animal species, high proportions of acetic acid in howler monkey fecal matter may imply that soluble components of the howler monkey diet are more completely digested in the stomach and small intestine, and therefore, only fibrous, insoluble components are fermented in the hindgut to produce acetic acid (Hume 1997).

Furthermore, acetic acid is commonly produced by the fermentation of pectin (Degnan 1992), and pectin has been detected in the diet of the mantled howler monkey (*A. palliata*) in Panama. The leaves and fruits of *Ficus insipida* and *F. yoponensis* contain especially high levels of pectin (Milton 1991), and similar to data reported for other howler species (Estrada 1984, Gaulin and Gaulin 1982, Julliot 1996, Milton 1980, Serio-Silva et al 2002), both of these species were an important part of the black howler diet in Palenque year-round. Although the consumption of these species was not correlated with acetic acid concentrations, the consumption of *F. americana* and *F. aurea* was. Therefore, assuming these fig species also have high pectin content, high amounts of pectin in the black howler diet in Palenque may facilitate the microbial production of high proportions of acetic acid.

Subtle changes in host fecal VFA and ammonia concentrations across time indicated small shifts in microbial community function in response to changes in howler monkey diet and gut microbial community composition. For example, fecal acetic acid concentrations were highest for both groups when exploiting a diet consisting of mostly ripe fruit (Block 3), and across the study, fecal acetic acid concentrations were positively correlated with the relative abundance of bacteria from the Anaeroplasmataceae family and negatively correlated with the consumption of young leaves and unripe fruits. Although there is variation among plant species, studies of mantled howler food items report that pectin levels are lower in young leaves (4.1%) compared to ripe fruit (5.6%)(Milton 1991), and in a laboratory study of eight rats, the consumption of a 7% pectin diet for four weeks resulted in an increase in the relative abundance of the *Anaeroplasma* genus (Licht et al 2010). Because members of the Anaeroplasmataceae family have the ability to produce acetate, it is possible that the black howler diet is more readily

fermented by Anaeroplasmataceae to produce acetic acid during periods when there is more potentially pectin-rich fruit in the diet.

Differences in fecal butanoic acid concentrations across sampling blocks also indicate subtle shifts in howler gut microbial community function. The molar proportion of butanoic acid was significantly higher and the molar proportion of acetic acid was significantly lower in Block 2 for Motiepa group members compared to the other sampling periods, and in Blocks 1 and 2 for Balam group members. During Block 2, the howlers consumed the highest proportions of young leaves (62.3% and 30.8% dry weight) and unripe fruits (23.5% and 16.8%), and over the study period, butanoic acid concentrations were positively correlated to the amount of young leaves consumed while acetic acid concentrations were negatively correlated to the amount of unripe fruit consumed. *Butyricicoccus* degrades fiber and resistant starch to produce butanoic acid (Eeckhaut et al 2008), and *Butyricicoccus* relative abundances were positively correlated to young leaf and unripe fruit consumption. As a result, although my data did not show a relationship between the amount of NDF in the howler diet and either butanoic acid concentrations or the relative abundance of *Butyricicoccus,* NDF intake was positively correlated to unripe fruit intake, and it seems that fiber levels of 41.8% and 51.4% in unripe fruit and young leaves, together with increased relative abundances of *Butyricicoccus,* resulted in increased production of butanoic acid and reduced production of acetic acid during Block 2. One of the most common pathways for the production of butyrate in the gut is that which utilizes acetate as a substrate (Duncan et al 2004, Louis et al 2004). *Butyricicoccus* can utilize this pathway (Eeckhaut et al 2008), and high *Butyricicoccus* relative abundances were associated with high butanoic acid concentrations and low acetic acid concentrations, supporting the hypothesis that *Butyricicoccus* may have been converting acetate to butyrate. Furthermore, when acetic acid

concentrations were the lowest, NDF intake was the highest (Spearman's $\rho = -0.30$, $p = 0.06$). Therefore, it appears that the combination of high relative abundances of *Butyricicoccus* with a diet characterized by young leaves and unripe fruit provided the howler monkeys with more energy-rich butyrate from the fermentation of fiber and the conversion of acetate to butyrate.

Finally, protein metabolism by the howler monkey gut microbiota appears to have been affected by host diet. Howler monkeys exhibited a higher concentration of fecal ammonia during Block 1, and ammonia was positively correlated to protein, total non-structural carbohydrate, and NDF intake. Ammonia is produced when undigested protein reaches the hindgut and is fermented by the bacterial community to produce energy (Hungate 1966). Most bacteria can use ammonia as a nitrogen source when energy is not limiting, but if ammonia is produced in excess of bacterial requirements, it builds up and can be toxic to the host (Cotta and Russell 1997). Therefore, increased protein intake generally results in increased fecal ammonia excretion (Cummings et al 1979, Mackie et al 1998), and it is likely that increased protein intake during Block 1 resulted in higher fecal ammonia concentrations. Both energy and protein intake surpassed estimated howler monkey requirements during this time (~0.49-0.58 MJ of energy per metabolic body weight, ~3.26g of protein per metabolic body weight, Chapter 1), and while excess energy (carbohydrates and lipids) can be stored in adipose tissue for later use (Dufour and Sauther 2002, Ellison 2003, Martin 2007, Oftedal 2000), excess must be converted to ammonia and excreted. The positive correlation between fecal isobutanoic acid, another product of microbial protein breakdown, and ammonia support the hypothesis that excess protein was being excreted by the howlers during Block 1. However, the relationships between ammonia and total non-structural carbohydrates and NDF may be an effect of covariation in macronutrient intake.

During Block 1, the howler monkeys consumed the most food and therefore consumed the highest quantities of all macronutrients measured.

Role of Gut Microbiota in Howler Nutrition: An examination of black howler foraging behavior and activity patterns suggests that the shifts I observed in gut microbial community function contributed to howler nutritional ecology. Although I observed shifts in howler diet composition, I also detected shifts in gut microbial community composition and gut microbial community function, and across sampling blocks, I observed virtually no changes in howler activity and ranging patterns. These results suggest that the gut microbiota may have been providing additional energy and nutrients to the howlers to compensate for dietary fluctuations and allow the howlers to maintain activity levels.

To begin with, increases in butanoic acid production during Block 2 may have supplemented the metabolizable energy in the howler diet. Overall energy intake in Block 2 (0.52 MJ per metabolic body weight) was higher than that of Block 3 (0.45 MJ per metabolic body weight), but it fell very close the lower limit of estimated requirements for howler monkeys (0.49-0.58 MJ per metabolic body weight, Chapter 2), compared to energy intake in Block 1 (0.69 MJ per metabolic body weight, Table 3.7). Additionally, non-protein energy intake was lowest for both groups during Blocks 2 and 3 (Table 3.7). However, despite these differences in energy intake, the amount of time the black howlers spent resting and traveling did not change significantly from Block 1 to Block 2, and day ranges and home ranges were essentially the same. Butanoic acid is the main energy source utilized by host colonocytes (Flint et al 2012, Roediger 1980), and, with the exception of the brain, the gut is the most energy-expensive tissue in the body (Aiello and Wheeler 1995, Webster 1981). Therefore, increased butanoic acid

production may have aided the howler monkeys in offsetting some of the basal metabolic costs of an enlarged hindgut during periods of moderately reduced energy intake (Block 2), allowing them to utilize more metabolizable energy for activities such as traveling, foraging, and social interactions.

During Block 3, VFA production also appears to have been critical to howler nutrition. Block 3 was characterized by high maximum daily temperatures during Block 3 (30°C in Block 1, 32°C in Block 2, 35°C in Block 3), and high daily temperatures were correlated with a significant increase in time spent resting. Resting generally occurred between 10am and 2pm, when temperatures are typically highest in Palenque (CONAGUA 2011), and resting postures during this sampling block tended to mimic those associated with high temperatures in other studies (lying down with limbs spread; Bicca-Marques and Calegaro-Marques 1998), suggesting that the howlers were attempting to decrease metabolic rates during periods of high temperatures through inactivity. However, the more time the howlers spent resting, the less time they spent feeding ($r^2 = -0.56$, $p << 0.01$), and, in the absence of increased feeding rates, overall food intake, and therefore protein and energy intake, was lowest during Block 3. Therefore, despite high proportions of ripe fruit in their diet, the howlers may have been the most nutrient- and energy-limited during Block 3. Because overall fecal VFA concentrations were highest in Block 3 compared to Blocks 1 and 2, is seems likely that increased VFA production during Block 3 aided the howlers in meeting energy requirements despite reduced food intake. In particular, higher amounts of acetic acid, which can be used by the host for fatty acid synthesis, lipogenesis, and muscle metabolism (Flint et al 2012), and propanoic acid, which can be used to produce blood glucose (Flint et al 2012), may have provided howlers with the energy necessary to

maintain time spent traveling and engaging in social behavior as well as travel similar distances compared to Blocks 1 and 2.

CONCLUSION

Overall, my data suggest that a combination of howler energy-minimizing behavioral strategies, food selectivity, and flexible gut microbial community functions allowed the howler monkeys to meet energy and nutrient demands despite major diet shifts over time. With the exception of Block 3 when resting time increased, I saw very little variation in the howler monkey activity budget across time while the amount of energy and nutrients ingested changed dramatically from day to day as well as across sampling blocks. Although additional studies are needed to more accurately quantify howler energy and nutrient intakes as well as energy and nutrient demands, it appears that small home ranges and short day ranges combined with long periods of inactivity each day reduce howler energy and nutrient demands while selective feeding allows howlers to extract more protein from fruit-heavy diets and more energy from leaf-heavy diets than would be expected based on the general nutritional properties of ripe fruits and leaves (Chapter 2). In addition, the ability of the howler gut microbial community to shift in response to changes in the howler diet enhances howler digestive efficiency by providing energy-rich SCFA's through the fermentation of otherwise indigestible plant carbohydrates. These contributions appear to be most important during periods when feeding selectivity alone cannot provide the howlers with sufficient energy to meet demands.

The gut microbial community is likely to provide similar nutritional benefits to other primates during periods of fluctuating resource availability. A review of primate responses to seasonality reports that over 70% of responses involve dietary shifts while less than 10% involve

changes in home range, day range or activity levels (Hemingway and Bynum 2005). For example, a seven-month study of moustached and saddle-back tamarins (*Saguinus mystax, S. fuscicollis)* indicated that activity levels and foraging patterns remained similar despite changes in the plant species and, to some extent, the plant parts being consumed during different periods of the year (Garber 1993). In these cases, primates must be offsetting changes in energy and nutrient intake physiologically. Although field data are needed to verify these processes, it is likely that energy and nutrient production by the gut microbial community provides an effective buffer against seasonal fluctuations in energy and nutrient intake for these primates.

Metagenomic examinations of the howler gut microbiome as well as other primate gut microbiomes are necessary to more precisely describe the metabolic processes responsible for diet-associated shifts in fermentation and to more thoroughly investigate differences in amino acid and vitamin synthesis. However, because investigations of host-gut microbe relationships are currently dominated by laboratory studies, this study provides an important first step in understanding the host-gut microbe relationship in wild animals experiencing natural temporal fluctuations in diet. The gut microbial community is not a static entity with a fixed function. It adapts to variations in host diet and provides the host with critical nutritional resources. These resources are typically not accounted for in traditional studies of behavior and feeding ecology, but they may be the key to understanding the effects of seasonality on wild animal behavior, nutrition and health.

TABLES

Table 3.1. Nutritional content of Neotropical plant species and plant parts based on published literature. CP: crude protein; AP: available protein; TNC: total non-structural carbohydrates; NDF: neutral detergent fiber. All values expressed as percent of dry weight. *Values may have been calculated with alternative methodology.

Plant Species	Plant Part	%CP	%AP	%TNC	%Lipids	%NDF	Source
Ampelocera edentula	Flower	29.07	na	43.12	1.68	9.52*	Castellanos and Chanin 1996
J. spinosa	Flower	19.1	9.7	7.6	4.5	55.8	Felton et al. 2009b
P. laevis	Flower	10	3.6	14.7	3.2	51.8	Felton et al. 2009b
Pithecellobium sama	Flower	19.8	na	na	na	47.4	Oftedal 1991
Pyrostegia dichotoma	Flower	16.1	na	58.5	1.4	21.5	Norconk and Conklin-Britain 2004
Syagrus sancona	Flower	15	4.7	32	3.6	55.3	Felton et al. 2009b
B. guadichaudii	Mature Leaf	12.4	4.8	11.2	3.6	56.6	Felton et al. 2009b
Cecropia eximia	Mature Leaf	na	10.11	na	na	60.33	Milton 1979
Ceiba pentandra	Mature Leaf	na	9.09	na	na	41.81	Milton 1979
Ficus boliviana	Mature Leaf	9.7	2.6	20.1	7.1	51.4	Felton et al. 2009b
Ficus insipida	Mature Leaf	na	7.46	na	na	36.28	Milton 1979
Ficus yoponensis	Mature Leaf	na	12.36	na	na	33.26	Milton 1979
Heliocarpus americanus	Mature Leaf	16.2	7.1	10.3	4.9	65.5	Felton et al. 2009b
J. spinosa	Mature Leaf	14.5	6.1	9.8	4.4	65.1	Felton et al. 2009b
Machaerium purpuracens	Mature Leaf	na	11.11	na	na	45.84	Milton 1979
Marsdenia macrophylla	Mature Leaf	14	10.1	8.4	5	50.6	Felton et al. 2009b
Melloa quadrivalvis	Mature Leaf	19.2	14.3	3.3	2.5	67.8	Felton et al. 2009b
Platypodium elegans	Mature Leaf	na	11.66	na	na	39.62	Milton 1979
Poulsenia armata	Mature Leaf	na	7.44	na	na	44.68	Milton 1979
Protium panamense	Mature Leaf	na	6.37	na	na	41.99	Milton 1979
Tetragastris panamensis	Mature Leaf	na	8.09	na	na	43.66	Milton 1979
Urera baccifera	Mature Leaf	17	10.4	5.1	3	5.3	Felton et al. 2009b
Alibertia latifolia	Ripe Fruit	28.8	na	29.7	1.3	31.9	Norconk and Conklin-Britain 2004
Amaioua corymbosa	Ripe Fruit	6.5	na	11.9	13.6	58.6	Norconk and Conklin-Britain 2004
Anomospermum reticulatum	Ripe Fruit	13.39	na	48.29	1.52	10.1*	Castellanos and Chanin 1996
Astrocarium murumuru	Ripe Fruit	3	2.4	58.1	3.4	20.9	Felton et al. 2009b
Astrocaryum standleyanum	Ripe Fruit	4.8	na	45.5	1.4	Na	Milton 2008
Batocarpus amazonicus	Ripe Fruit	4.6	4	65.6	2.9	12.9	Felton et al. 2009b
Beilschmiedia pendula	Ripe Fruit	6.2	na	11.9	25.4	Na	Milton 2008
Brosimum alicastrum	Ripe Fruit	9.3	na	20.7	1.2	Na	Milton 2008
Brosimum guadichaudii	Ripe Fruit	7	4.7	56.3	1.6	25.6	Felton et al. 2009b
Brosimum guianense	Ripe Fruit	7.6	na	na	2.3	Na	Norconk and Conklin-Britain 2004
Capparis muco	Ripe Fruit	18.7	na	39.4	3.1	11.9	Norconk and Conklin-Britain 2004
Catostema commune	Ripe Fruit	6.97	na	70.35	1.73	4.7*	Castellanos and Chanin 1996

Table 3.1 (cont.)

Cecropia concolor	Ripe Fruit	11	6.4	17.3	7	59.2	Felton et al. 2009b
Cecropia sciadophilla	Ripe Fruit	7.18	na	41.66	10.28	30.14*	Castellanos and Chanin 1996
Celtis iguanea	Ripe Fruit	7.8	7.1	71.2	0	2.6	Felton et al. 2009b
Chiococca alba var. purple	Ripe Fruit	6.7	na	49	7.8	31.7	Norconk and Conklin-Britain 2004
Clarisia racemosa	Ripe Fruit	9.95	na	70.44	1.87	3.5*	Castellanos and Chanin 1996
Coccoloba striata	Ripe Fruit	6.1	na	40.1	0.9	34.9	Norconk and Conklin-Britain 2004
Cordia alliodora	Ripe Fruit	3.8	1.8	67.4	1.3	26.8	Felton et al. 2009b
Cordia nitida	Ripe Fruit	13.9	na	5.7	0.2	Na	Hladik et al. 1971
Couma macrocarpa	Ripe Fruit	2.18	na	54.32	9.41	1.94*	Castellanos and Chanin 1996
Coussapoa asperifolia	Ripe Fruit	5.28	na	54.62	8.19	16.43*	Castellanos and Chanin 1996
Dacroides peruviana	Ripe Fruit	13.68	na	34.18	1.75	39.54*	Castellanos and Chanin 1996
Dendropanax arboreus	Ripe Fruit	5.4	3	10.6	21.8	53.5	Felton et al. 2009b
Dialium guianensis	Ripe Fruit	7.59	na	65.52	3.9	6.08*	Castellanos and Chanin 1996
Didymopanax morototoni	Ripe Fruit	6.2	6.8	19.4	33.2	14.5	Felton et al. 2009b
Dipteryx odorata	Ripe Fruit	4.87	na	54.37	5.19	12.93*	Castellanos and Chanin 1996
Doliocarpus major	Ripe Fruit	4.5	na	21.1	3.8	Na	Milton 2008
Erythroxylum steyermarkii	Ripe Fruit	3.5	na	50.3	11.7	34.5	Norconk and Conklin-Britain 2004
Eugenia monticola	Ripe Fruit	5.1	na	52.9	1.2	37.5	Norconk and Conklin-Britain 2004
Faramea occidentalis	Ripe Fruit	4.1	na	38.8	0.1	Na	Milton 2008
Ficus boliviana	Ripe Fruit	8.1	2.2	24	2.7	41.6	Felton et al. 2009b
Ficus bullenei	Ripe Fruit	7.1	na	3.7	3.4	Na	Milton 2008
Ficus cf. guianensis	Ripe Fruit	5.33	na	55.9	4.76	28.05*	Castellanos and Chanin 1996
Ficus costaricana	Ripe Fruit	6.9	na	6.4	3.9	na	Milton 2008
Ficus eximia	Ripe Fruit	1.3	2.6	53.1	2.6	na	Felton et al. 2009b
Ficus insipida	Ripe Fruit	7	na	14.5	5.8	na	Milton 2008
Ficus obtusifolia	Ripe Fruit	4.1	na	8.7	3.6	na	Milton 2008
Ficus pertusa	Ripe Fruit	5.8	2.4	38.8	1.9	na	Felton et al. 2009b
Ficus trigona	Ripe Fruit	4.1	0.9	24.8	2.2	na	Felton et al. 2009b
Ficus trigonata	Ripe Fruit	5.6	na	10.5	6.4	na	Milton 2008
Ficus yoponensis	Ripe Fruit	7.5	na	11.3	6	na	Milton 2008
Garcinia macrophylla	Ripe Fruit	8.16	na	na	na	14.44*	Castellanos and Chanin 1996
Gnetum urens	Ripe Fruit	18.02	na	53.57	1.1	22.08*	Castellanos and Chanin 1996
Guazuma ulmifolia	Ripe Fruit	4.4	1.7	25.8	1.1	57.7	Felton et al. 2009b
Gustavia superba	Ripe Fruit	15.2	na	5.1	42.3	Na	Milton 2008
Heliocostylis tomentosa	Ripe Fruit	7.77	na	50.37	5.9	17.21*	Castellanos and Chanin 1996
Hirtella racemosa	Ripe Fruit	7.1	na	0.9	2.3	72.6	Norconk and Conklin-Britain 2004

Table 3.1 (cont.)

Trichilia quadrijuga	Ripe Fruit	6.04	na	65.08	2.74	5.35*	Castellanos and Chanin 1996
Virola elongata	Ripe Fruit	6.09	na	31.13	34.72	21.45*	Castellanos and Chanin 1996
Virola nobilis	Ripe Fruit	4.5	na	18.6	42.5	na	Milton 2008
Virola sebifera	Ripe Fruit	2.7	3	40.6	22.1	8.8	Felton et al. 2009b
Virola surinamensis	Ripe Fruit	4.87	na	41.64	38.56	6.75*	Castellanos and Chanin 1996
Unid epiphyte	Stem	3.5	3.2	27.5	2.1	34.2	Felton et al. 2009
Casearia sylvestris	Unripe Fruit	18.4	na	6.6	29.9	41.7	Norconk and Conklin-Britain 2004
Dipteryx panamensis	Unripe Fruit	4.4	na	41.5	1.4	5.9*	Hladik et al. 1971
Ficus boliviana	Unripe Fruit	6.5	4.3	9.9	3.3	50.5	Felton et al. 2009
Ficus insipida	Unripe Fruit	6.6	na	8.8	4.2	8.6*	Hladik et al. 1971
Ficus trigona	Unripe Fruit	5.5	1.8	11.5	3.4	na	Felton et al. 2009b
Hura crepitans	Unripe Fruit	11.9	6.6	19.8	1.7	28.8	Felton et al. 2009b
P. laevis	Unripe Fruit	10.4	3.5	12.1	3.2	47.2	Felton et al. 2009b
Pouteria nemorosa	Unripe Fruit	2.6	2	63	3.7	5.8	Felton et al. 2009b
Ampelocera ruizii	Young Leaf	23.4	22.3	6.3	3.1	48.9	Felton et al. 2009b
Anacardium excelsum	Young Leaf	na	12.74*	na	na	36.56	Milton 1979
B. amazonicus	Young Leaf	16.5	5.6	10.4	3.8	63.3	Felton et al. 2009b
Capparis flexuosa	Young Leaf	14.3	na	20.6	2.1	51.8	Norconk and Conklin-Britain 2004
Cecropia eximia	Young Leaf	na	7.11	na	na	39.34	Milton 1979
Cecropia sp.	Young Leaf	5	na	36.4	2.3	5.8*	Hladik et al. 1971
Ceiba pentandra	Young Leaf	na	14.1*	na	na	22.98	Milton 1979
Ceiba pentandra	Young Leaf	23.8	15.4	6.7	5.6	67.5	Felton et al. 2009b
Coccoloba fallax	Young Leaf	15.4	Na	33	0.8	42.8	Norconk and Conklin-Britain 2004
Ficus boliviana	Young Leaf	16.2	8	6.4	3.3	55	Felton et al. 2009b
Ficus insipida	Young Leaf	na	10.59*	na	na	23.33	Milton 1979
Ficus yoponensis	Young Leaf	na	9.36*	na	na	36.77	Milton 1979
H. americanus	Young Leaf	19	9.5	8.7	5	70.1	Felton et al. 2009b
J. spinosa	Young Leaf	18.2	6.5	7.4	4.2	59.1	Felton et al. 2009b
Jacaranda copaia	Young Leaf	na	13.75*	na	na	30.24	Milton 1979
M. quadrivalvis	Young Leaf	28	25.8	8	3	38.7	Felton et al. 2009b
Machaerium oblongifolium	Young Leaf	19.1	13.5	7.2	5.4	74.9	Felton et al. 2009b
Machaerium purpuracens	Young Leaf	na	15.89*	na	na	20.7	Milton 1979
Maytenus guianensis	Young Leaf	12.8	na	30.6	2.6	35.2	Norconk and Conklin-Britain 2004
Platypodium elegans	Young Leaf	na	20.69*	na	na	33.82	Milton 1979
Poulsenia armata	Young Leaf	na	8.5*	na	na	36.48	Milton 1979
Protium panamense	Young Leaf	na	13.1*	na	na	18.7	Milton 1979
Tabebauia serratifolia	Young Leaf	24.5	na	20.9	1.6	48.6	Norconk and Conklin-Britain 2004
Tetragastris panamensis	Young Leaf	na	12.08*	na	na	15.02	Milton 1979

Table 3.1 (cont.)

Hyeronima alchoneoides	Ripe Fruit	3.83	na	42.73	5.98	38.27*	Castellanos and Chanin 1996
I. edulis	Ripe Fruit	9.7	6.2	35.9	1.9	52.1	Felton et al. 2009b
Inga edulis	Ripe Fruit	4.2	3.8	72.3	0.8	13	Felton et al. 2009b
Iranthera laeva	Ripe Fruit	9.15	na	46.87	19.94	16.42*	Castellanos and Chanin 1996
Jacaratia spinosa	Ripe Fruit	7.8	6.1	64.4	1.7	18.6	Felton et al. 2009b
Lacmellea edulis	Ripe Fruit	3.7	na	34.4	3.6	na	Hladik et al. 1971
Laetia procera	Ripe Fruit	10.44	na	22.13	21.38	33.42	Castellanos and Chanin 1996
Mangifera indica	Ripe Fruit	4.1	na	na	na	21	Oftedal 1991
Micranda minor	Ripe Fruit	4.04	na	52.87	0.54	37.96	Castellanos and Chanin 1996
Micropholis cf. eggensis	Ripe Fruit	6.99	na	74.15	0.97	6.81	Castellanos and Chanin 1996
Micropholis melinomeana	Ripe Fruit	6.33	na	61.54	6.11	6.79	Castellanos and Chanin 1996
Morinda tenuiflora	Ripe Fruit	14.2	na	19.2	7.4	46.6	Norconk and Conklin-Britain 2004
Oryctanthus alveolatus	Ripe Fruit	9.6	na	28.8	38.3	19.9	Norconk and Conklin-Britain 2004
Ouratea castaneaefolia	Ripe Fruit	5.75	na	na	38.57	Na	Castellanos and Chanin 1996
P. laevis	Ripe Fruit	7.1	3	48.4	1.9	34.7	Felton et al. 2009b
P. nemorosa	Ripe Fruit	3.2	1.8	39.2	4.4	9.9	Felton et al. 2009b
Paullinia elegans	Ripe Fruit	2.2	1.4	32	1.4	13.7	Felton et al. 2009b
Perebea xanthochyma	Ripe Fruit	11	na	26.2	2.3	7.6*	Hladik et al. 1971
Protium crenatum	Ripe Fruit	2.72	na	76.8	2.41	4.9*	Castellanos and Chanin 1996
Protium tenuifolium	Ripe Fruit	8.51	na	69.45	1.96	2.72*	Castellanos and Chanin 1996
Pseudolmedia laevis	Ripe Fruit	5.2	2.6	69	0.9	17.4	Felton et al. 2009b
Psidium guajava	Ripe Fruit	4.8	na	18.2	3.4	na	Hladik et al. 1971
Quararibea asterolepis	Ripe Fruit	5.4	na	31	0.2	na	Milton 2008
Quiina florida	Ripe Fruit	2.9	1	49.1	2	38.8	Felton et al. 2009b
Rollinia herzogii	Ripe Fruit	7.2	3.6	24.7	2.3	55.2	Felton et al. 2009b
Sacoglottis guianensis	Ripe Fruit	4.4	na	69.8	3.05	13.72*	Castellanos and Chanin 1996
Sapium glandulosum	Ripe Fruit	8.7	12.1	19.7	34.4	0	Felton et al. 2009b
Scheelea zonensis	Ripe Fruit	3.6	na	15.1	22.3	na	Milton 2008
Simarouba amara	Ripe Fruit	5.87	na	78.36	0.2	2.15*	Castellanos and Chanin 1996
Socratea exhorriza	Ripe Fruit	6.8	3.1	35.9	0.4	42.3	Felton et al. 2009b
Spondias mombin	Ripe Fruit	3.3	1.3	57.2	2.5	9.9	Felton et al. 2009b
Spondias mombin	Ripe Fruit	4.3	na	40	1.3	na	Milton 2008
Spondias mombin	Ripe Fruit	2.8	na	57.4	0.7	3.8*	Hladik et al. 1971
Spondias mombin	Ripe Fruit	9.61	na	52.37	1.98	5.2*	Castellanos and Chanin 1996
Spondias radlkofera	Ripe Fruit	11.7	na	24.6	3.9	na	Milton 2008
Tetragastris panamensis	Ripe Fruit	3.2	na	31	0.2	na	Milton 2008

Table 3.1 (cont.)

Thevetia nitida	Ripe Fruit	1.9	na	32.5	4	5*	Hladik et al. 1971
Trichilea tuberculata	Ripe Fruit	7.8	na	15.6	38.3	na	Milton 2008

Table 3.2. Home range and day range for each howler group during each season. ANOVA detected no significant differences in day range across seasons in either group ($F_{2,52}$= 1.55, p = 0.22; $F_{2,49}$= 0.056, p = 0.95).

	Balam		**Motiepa**	
	Home Range (ha)	Day Range (ha)	Home Range (ha)	Day Range (ha)
Rainy	9.5	309.6 ± 128.0	4.2	319.1 ± 113.7
Intermediate	9.5	236.5 ± 83.2	5.2	317.9 ± 97.0
Dry	6.1	266.2 ± 146.0	4.4	328.4 ± 106.0

Table 3.3. PerMANOVA results for the effect of time on black howler monkey diet when controlling for differences between groups. Diet was expressed in average grams of plant parts or plant species ingested daily per metabolic body weight and average daily concentration of metabolites ingested daily per metabolic body weight. SS = sums of squares; MS = mean squares

	df	**SS**	**MS**	**F value**	**R^2**	**P value**
Plant Parts (%)						
Individual	12	0.48	0.04	1.12	0.16	0.39
Sampling Block	2	1.57	0.78	22.06	0.54	0.0002
Residuals	24	0.85	0.036		0.29	
Total	38	2.9			1.00	
Plant Species (%)						
Individual	12	3.12	0.26	2.02	0.31	0.011
Sampling Block	2	3.86	1.93	14.99	0.38	0.0002
Residuals	24	3.09	0.13		0.31	
Total	38	10.07			1.00	
Plant Metabolites (conc)						
Individual	12	1.17	0.1	1.9	0.31	0.022
Sampling Block	2	1.36	0.68	13.34	0.36	0.0002
Residuals	24	1.23	0.051		0.33	
Total	38	3.76			1.00	

Table 3.4. Top ten plant species and parts consumed by the howler each season in terms of percent of total grams of food ingested.

Motiepa Block 1				**Balam Block 1**			
Plant Species	**Plant Part**	**Avg %**	**SD**	**Plant Species**	**Plant Part**	**Avg %**	**SD**
Dendropanax arboreus	Ripe Fruit	12.3	11.2	*Brosimum alicastrum*	Ripe Fruit	24.2	15.9
Guatteria anomala	Ripe Fruit	9.6	10.1	*Schizolobium parahyba*	Stem	14.7	10.6
Ficus americana	Ripe/Unripe Fruit	9.1	4.1	*Dendropanax arboreus*	Ripe Fruit	8.7	8.6
Ficus americana	Ripe Fruit	8.3	5.4	*Ficus yoponensis*	Ripe Fruit	8.0	4.0
Poulsenia armata	Young Leaf	7.9	5.6	*Guatteria anomala*	Ripe Fruit	6.0	7.5
Schizolobium parahyba	Stem	4.4	6.0	*Cojoba arborea*	Young Leaf	5.6	1.5
Ficus yoponensis	Unripe Fruit	4.2	5.5	*Brosimum alicastrum*	Unripe Fruit	3.9	2.9
Ficus insipida	Ripe Fruit	3.5	3.9	*Cojoba arborea*	Stem	3.7	3.5
Ficus aurea	Ripe Fruit	2.5	3.0	*Ficus yoponensis*	Young Leaf	2.7	1.1
Ficus pertusa	Ripe Fruit	2.3	2.4	*Ficus yoponensis*	Unripe Fruit	2.1	2.0
Motiepa Block 2				**Balam Block 2**			
Plant Species	**Plant Part**	**Avg %**	**SD**	**Plant Species**	**Plant Part**	**Avg %**	**SD**
Poulsenia armata	Young Leaf	19.5	10.6	*Ficus yoponensis*	Ripe Fruit	36.4	11.1
Schizolobium parahyba	Stem	11.2	8.8	*Schizolobium parahyba*	Stem	9.1	8.2
Brosimum alicastrum	Unripe Fruit	10.9	5.7	*Brosimum alicastrum*	Unripe Fruit	6.2	3.7
Ficus insipida	Ripe Fruit	7.5	6.0	*Poulsenia armata*	Young Leaf	6.1	3.7
Platymiscium dimorphandrum	Young Leaf	4.2	4.6	*Cojoba arborea*	Young Leaf	5.9	5.2
Brosimum alicastrum	Ripe Fruit	3.4	9.0	*Dialium guianense*	Flower	5.2	5.0
Poulsenia armata	Unripe Fruit	3.3	4.2	*Fabaceae* sp.	Young Leaf	4.2	4.6
Brosimum alicastrum	Young Leaf	2.6	3.2	*Platymiscium dimorphandrum*	Young Leaf	4.0	2.6
Fabaceae sp.	Young Leaf	2.5	5.1	*Ficus yoponensis*	Unripe Fruit	3.5	2.3
Ficus yoponensis	Young Leaf	1.7	1.8	*Brosimum alicastrum*	Young Leaf	2.2	2.5
Motiepa Block 3				**Balam Block 3**			
Plant Species	**Plant Part**	**Avg %**	**SD**	**Plant Species**	**Plant Part**	**Avg %**	**SD**
Poulsenia armata	Ripe Fruit	24.5	8.5	*Poulsenia armata*	Ripe Fruit	23.2	17.3
Ficus americana	Ripe Fruit	15.1	5.2	*Ficus yoponensis*	Ripe Fruit	14.1	8.5
Ficus aurea	Ripe Fruit	10.4	4.1	*Ficus aurea*	Ripe Fruit	12.3	3.2
Ampelocera hottlei	Ripe Fruit	8.2	6.6	*Compsoneura* sp.	Ripe Fruit	9.8	7.4
Ficus yoponensis	Ripe Fruit	7.2	6.6	*Cojoba arborea*	Young Leaf	4.9	3.5
Poulsenia armata	Young Leaf	6.7	3.3	*Brosimum alicastrum*	Ripe Fruit	3.8	7.0
Brosimum alicastrum	Unripe Fruit	1.6	2.1	*Cojoba arborea*	Stem	2.9	2.8
Brosimum alicastrum	Young Leaf	1.1	1.3	*Poulsenia armata*	Young Leaf	2.7	1.9
Cojoba arborea	Young Leaf	1.1	1.3	*Ficus maxima*	Ripe Fruit	1.3	2.1
Ficus yoponensis	Stem	0.9	1.6	*Ficus aurea*	Unripe Fruit	1.2	1.6

Table 3.5. Plant species and plant parts consumed in different proportions by the Motiepa group across sampling blocks. All values are expressed in percent of total dry grams of food ingested.

		Block 1		**Block 2**		**Block 3**	
Plant Species	**Plant Part**	Average	SD	Average	SD	Average	SD
Brosimum alicastrum	Flower	1.4	1.0	0.0	0.0	0.0	0.0
Brosimum alicastrum	Unripe Fruit	0.0	0.0	10.9	5.7	1.6	2.1
Ampelocera hottlei	Ripe Fruit	0.0	0.0	0.0	0.0	8.3	6.6
Dendropanax arboreus	Ripe Fruit	12.3	11.2	0.0	0.0	0.3	0.9
Ficus americana	Unripe/Ripe Fruit	9.1	4.1	0.0	0.0	0.0	0.0
Ficus aurea	Ripe Fruit	2.5	3.0	0.0	0.0	10.3	4.1
Platymiscium dimorphandrum	Seed	0.0	0.0	0.4	0.3	0.0	0.0
Platymiscium dimorphandrum	Young Leaf	0.0	0.0	4.2	4.6	0.3	0.4
Poulsenia armata	Ripe Fruit	0.0	0.0	0.0	0.0	24.5	8.5

Table 3.6. Average nutrient and energy intake for Motiepa and Balam groups across sampling periods. Calculations are based on literature estimates and are expressed in grams per metabolic body weight, energy per metabolic body weight, and percent dry weight ingested.

		Block 1		**Block 2**		**Block 3**	
		Motiepa	Balam	Motiepa	Balam	Motiepa	Balam
Available Protein	Average	10.8	10.2	8.9	10.2	5.6	5.7
	SD	3.6	2.4	2.4	2.7	1.3	1.7
% Available Protein	Average	10.7%	11.0%	13.8%	14.7%	11.4%	11.1%
	SD	1.8%	0.8%	3.4%	1.4%	0.6%	1.2%
Total Non-Structural Carbohydrates	Average	29.0	29.7	12.4	16.2	16.3	17.0
	SD	1.2	12.0	3.5	3.7	3.4	5.0
% Total Non-Structural Carbohydrates	Average	26.3%	34.3%	18.9%	22.1%	28.4%	35.2%
	SD	5.3%	5.0%	2.4%	2.8%	9.0%	6.6%
Lipids	Average	3.0	3.2	1.2	3.2	1.7	2.1
	SD	1.2	0.9	0.3	1.5	0.5	0.6
% Lipids	Average	2.9%	3.4%	2.8%	2.3%	3.3%	3.6%
	SD	0.4%	0.3%	1.1%	0.4%	0.6%	0.5%
Neutral Detergent Fiber	Average	42.3	50.3	27.2	39.9	21.9	24.7
	SD	15.9	14.5	8.8	14	4.9	8
Total Energy (Kcal)	Average	182.9	177.4	105.5	172.4	106.6	114.5
	SD	64.9	56.1	30.0	56.9	26.7	30.6

Table 3.7. Average energy intake for Motiepa and Balam groups across sampling periods. Ranges based on calculations of average daily intake for each individual during each of the five weeks of data collection during each sampling block. Calculations are based on literature estimates and are expressed in MJ per metabolic body weight.

		Block 1		**Block 2**		**Block 3**	
		Motiepa	Balam	Motiepa	Balam	Motiepa	Balam
Protein Energy (MJ)	Average	0.14	0.19	0.13	0.15	0.08	0.10
	Range	0.014-0.29	0.050-0.48	0.034-0.30	0.032-0.28	0.012-0.21	0.0088-0.27
Non-protein Energy (MJ)	Average	0.55	0.53	0.38	0.41	0.32	0.41
	Range	0.034-1.83	0.048-1.95	0.12-1.68	0.071-1.18	0.038-0.81	0.025-1.42
Total Energy (MJ)	Average	0.69	0.72	0.51	0.56	0.40	0.51
	Range	0.048-2.10	0.098-2.34	0.18-1.97	0.13-1.40	0.054-0.96	0.035-1.71

Table 3.8. Average metabolite intake for Motiepa and Balam groups across sampling periods. Metabolite values are expressed in relative concentration per metabolic body weight.

		Block 1		**Block 2**		**Block 3**	
		Motiepa	Balam	Motiepa	Balam	Motiepa	Balam
Amino Acids	Average	3,874,740	7,655,871	4,356,907	4,971,969	944,692	1,952,207
	Range	150,054-13,677,598	192,079-30,206,249	138,722-15,353,681	505,801-14,321,945	46,968-5,857,965	18,477-10,598,416
Sugars	Average	179,809,901	106,698,939	46,591,468	44,597,474	50,043,025	96,710,217
	Range	17,355,828-646,243,338	5,413,802-782,947,762	50,833,383-160,876,872	30,250,675-50,793,104	6,687,146-29,075,974	3,539,931-631,756,346
Lipids	Average	6,521,496	4,173,832	3,079,078	2,637,125	2,367,494	1,534,664
	Range	959,436-13,868,615	800,979-28,930,967	507,987-7,645,966	416,353-4,813,883	251,175-5,618,803	102,872-317,739

Table 3.9. List of metabolites that exhibited significant changes in relative concentration in the Motiepa group diet across sampling blocks. Values are expressed as average daily intake in relative concentration.

Metabolite		**Block 1**	**Block 2**	**Block 3**
2-Methylmalic acid	Average	108,477.2	14,158.5	6,016.4
	SD	83,693.9	6,438.3	3,667.8
4-Hydroxy-3-methoxyphenylethylene glycol	Average	65,038.9	11,664.2	2,643.7
	SD	33,534.0	6,884.0	1,251.2
Allantoin	Average	0.0	9,534.6	639.8
	SD	0.0	4,011.7	997.3
Beta-Amyrin	Average	225,210.2	106,049.5	24,037.8
	SD	75,190.6	39,930.4	14,130.5
Cholesterol	Average	17,379.1	4,460.3	9,438.1
	SD	6,714.6	2,433.6	2,804.1
Eicosanol	Average	95.1	217.9	498.4
	SD	36.5	72.9	149.5
Hentriacontanol	Average	5,119.6	1,200.8	308.0
	SD	2,113.8	771.2	154.4
Protocatechuic acid	Average	80,106.3	33,629.2	8,415.5
	SD	29,680.0	19,171.9	4,142.0
Xylonic acid-1,4-lactone	Average	17,655.0	23,087.8	8,593.9
	SD	5,388.4	13,906.6	3,092.8

Table 3.10. PerMANOVA results for the effect of time on gut microbial community composition as determined by ARISA and 454 pyrosequencing. SS = sums of squares; MS = mean squares

	df	SS	MS	F value	R^2	P value
ARISA - OTU						
Individual	12	2.67	0.22	1.52	0.37	0.0002
Sampling Block	2	1.04	0.52	3.56	0.14	0.0002
Residuals	24	3.51	0.15		0.49	
Total	38	7.22			1.00	
454 - Family						
Individual	8	0.26	0.032	2.34	0.40	0.006
Sampling Block	2	0.18	0.092	6.74	0.29	0.0002
Residuals	14	0.19	0.014		0.30	
Total	24	0.63			1.00	
454 - Genus						
Individual	8	1.56	0.19	2.4	0.50	0.0002
Sampling Block	2	0.41	0.2	2.51	0.13	0.0004
Residuals	14	1.13	0.08		0.37	
Total	24	3.1			1.00	

Table 3.11. Bacterial taxa that exhibited significant changes in relative abundance across sampling blocks ($p<0.05$) or characterized a particular sampling block. Analyses were performed on combined data. Values are expressed in percent of total sequences.

	Block 1				**Block 2**				**Block 3**			
	Balam		Motiepa		Balam		Motiepa		Balam		Motiepa	
Taxon	Average	SD	Average	SD	Average	SD	Average	SD	Average	SD	Average	SD
Anaeroplasmataceae	2.18E-04	2.15E-04	9.07E-04	5.75E-04	9.28E-05	1.36E-04	1.09E-04	1.33E-04	2.82E-04	1.54E-04	3.72E-04	3.10E-04
Erysipelotrichaceae	0.017	0.007	0.017	0.003	0.004	0.002	0.004	0.001	0.008	0.005	0.016	0.012
Incertae Sedis XIII	0.006	0.004	0.002	0.001	0.002	0.000	0.003	0.002	0.001	0.000	0.001	0.002
Lachnospiraceae	0.326	0.041	0.395	0.056	0.308	0.024	0.340	0.057	0.223	0.082	0.278	0.024
Ruminococcaceae	0.152	0.007	0.142	0.015	0.209	0.057	0.170	0.061	0.298	0.097	0.223	0.044
Streptococcaceae	6.66E-04	2.95E-04	1.56E-03	1.12E-03	2.21E-04	1.79E-04	4.32E-04	3.41E-04	1.06E-04	8.65E-05	1.47E-04	1.01E-04
Acetivibrio	1.55E-04	1.51E-04	1.01E-04	1.00E-04	1.10E-04	4.64E-05	1.68E-05	3.37E-05	1.47E-05	2.95E-05	0.00E+00	0.00E+00
Akkermansia	0.014	0.017	0.002	0.002	0.001	0.003	0.001	0.000	0.002	0.003	0.003	0.002
Anaerotruncus	4.36E-04	4.67E-04	7.56E-05	9.08E-05	8.54E-04	1.34E-03	4.96E-04	2.41E-04	3.92E-04	2.23E-04	1.58E-04	7.82E-05
Butyricicoccus	0.003	0.001	0.003	0.001	0.004	0.002	0.004	0.000	0.003	0.001	0.002	0.001
Coprobacillus	0.011	0.007	0.011	0.003	0.002	0.001	0.001	0.001	0.004	0.002	0.012	0.010
Dialister	7.90E-05	9.64E-05	3.87E-05	4.51E-05	1.49E-05	2.98E-05	1.07E-04	3.82E-05	7.07E-05	2.65E-05	3.13E-03	5.93E-03
Gordonibacter	1.51E-03	1.33E-03	2.76E-04	7.87E-05	6.71E-04	4.54E-04	2.68E-04	1.78E-04	2.28E-04	2.37E-04	3.21E-04	1.81E-04
Hallella	0.014	0.012	0.017	0.006	0.007	0.005	0.063	0.052	0.014	0.013	0.026	0.016
Helicobacter	4.41E-04	8.82E-04	3.80E-05	7.59E-05	9.65E-06	1.93E-05	5.04E-05	6.11E-05	4.75E-05	7.05E-05	1.24E-03	2.37E-03
Mogibacterium	0.004	0.003	0.001	0.001	0.001	0.000	0.001	0.002	0.001	0.000	0.001	0.001
Oribacterium	1.88E-04	1.30E-04	6.68E-05	5.12E-05	3.56E-05	4.90E-05	4.40E-05	8.79E-05	1.87E-05	3.73E-05	1.44E-05	2.88E-05
Oscillibacter	0.028	0.022	0.014	0.003	0.023	0.018	0.008	0.002	0.006	0.004	0.008	0.003
Papillibacter	0.002	0.001	0.003	0.002	0.004	0.001	0.005	0.006	0.009	0.001	0.006	0.001
Prevotella	0.016	0.015	0.017	0.012	0.046	0.057	0.027	0.033	0.021	0.027	0.019	0.015
Streptococcus	6.49E-04	3.20E-04	1.56E-03	1.12E-03	1.69E-04	1.61E-04	4.32E-04	3.41E-04	7.26E-05	6.11E-05	1.47E-04	1.01E-04
Streptophyta	1.58E-03	1.99E-03	5.54E-04	4.95E-04	7.03E-04	3.09E-04	1.25E-03	1.14E-03	3.56E-04	5.23E-04	2.70E-04	1.33E-04
Solobacterium	6.63E-04	5.34E-04	1.25E-03	9.04E-04	2.98E-04	1.31E-04	7.34E-04	4.25E-04	1.84E-03	1.35E-03	1.05E-03	2.47E-04
TM7	0.004	0.005	0.000	0.000	0.002	0.002	0.001	0.001	0.004	0.004	0.002	0.001
Xylanibacter	0.007	0.007	0.003	0.003	0.003	0.003	0.002	0.002	0.002	0.002	0.001	0.002

Table 3.12. Spearman's ρ values for significant correlations between microbial taxa and amounts of different diet components consumed.

Taxon	Kcal	Protein	Lipid	NDF	TNC	Young Leaf	Mature Leaf	Ripe Fruit	Unripe Fruit	Stem	Flower
Acetivibrio	0.70	0.75	0.69	0.75	0.61				0.58		
Butyricicoccus						0.54			0.57		
Coprobacillus							0.53				
Dialister										-0.53	
Hallella			0.57	-0.60						-0.55	
Mogibacterium										0.55	
Incertae Sedis XIII		0.64									
Oscillibacter			-0.60	0.72						0.56	
Streptococcus											
Streptophyta		0.59				0.68					

Table 3.13. Spearman's ρ for significant correlations between microbial taxa and relative concentration of diet metabolites.

Metabolite	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1-hexadecanoylglycerol			0.95	0.68	0.66	0.89			0.88							
1-Methyl-alpha-D-glucopyranoside										0.99			0.99			
1-Methyl-beta-D-galactopyranoside			0.68	0.76	0.72	0.66										
1-Octedecenoylglycerol			0.68					0.67								
1,6-Anhydroglucose												0.78				
2-hydroxyglutaric acid							0.66									
2,3-Dihydroxybutanedioic acid			0.71	0.78	0.8	0.67										
2,3-dihydroxysuccinic acid								0.67								
24-Methylenecycloartanol				0.74	0.75											
2-Methylcitric acid			0.75	0.74	0.7	0.73		0.71	0.74			-0.69				
2-Methylglutamic acid			0.69	0.72	0.7	0.68			0.73							
2-Methylmalic acid			0.76	0.72	0.73	0.75		0.71	0.74							
2-methylsuccinic acid			0.73	0.65	0.66	0.68		0.79	0.69			-0.68				
2-Piperidinecarboxylic acid				0.68	0.67											
3,4-Dihydroxybutanoic acid			0.71	0.84	0.8	0.69			0.67							
3,4-Dimethoxycinnamic acid			0.71	0.7	0.7											
3,4,5-Trihydroxypentanoic acid								0.68								
3-Deoxy-arabino-hexaric acid			0.72	0.82	0.79	0.69			0.69			-0.68				
3-methyl-2-hydroxypentanoic acid			0.69			0.68		0.68	0.67							
4,5-dimethyl-2,6-dihydroxypyrimidine				0.66												
4-Caffeoylquinic acid			0.68	0.69	0.67	0.68		0.7	0.67							
4-Hydroxy-3-methoxyphenethyleneglycol				0.7	0.69		0.68									
4-Hydroxyproline				0.66												
Adenine				0.7												
Aspartic acid			0.66	0.68	0.66			0.74								
C16:1											-0.77				-0.77	-0.75
Diethyleneglycol			0.72			0.74		0.75	0.76							
Eicosanol					0.66		0.67									
Ethanol, (2-(3,4-dihydroxyphenyl)-							0.66									

Table 3.13 (cont.)

Erythritol			0.65	0.79	0.74										
Ferulic acid			0.69			0.67		0.78	0.67						
Gallic acid							0.68								
Glucaric acid			0.73	0.68	0.66	0.74		0.76	0.74						
Glucuronic acid			0.7	0.65	0.65	0.69	0.66		0.7		-0.72				
Glycolic acid			0.67	0.68	0.66		0.7								
Guanine			0.73	0.77	0.74	0.72			0.72		-0.67				
Hentriacontanol			0.81	0.67	0.65	0.75		0.7	0.74						
Heptacosanol			0.75	0.71	0.67	0.71		0.68	0.7						
Hexacosanoic acid							0.73								
Isoleucine			0.75	0.76	0.77	0.74			0.74		-0.69				
Kaempferol										0.72			0.73	0.72	0.71
Lactic Acid		-0.67													
Leucine							0.68								
Monomethylphosphate			0.73	0.75	0.75	0.73			0.72						
N-Acetyl glucosamine			0.7	0.75	0.68	0.69		0.7	0.69		-0.67				
N-Acetylglucosylamine							0.7								
Nicotinic acid							0.78								
Octacosanol			0.67			0.66		0.75	0.66		-0.67				
p-hydroxyCoumaric acid	-0.7														
Protocatechuic acid			0.7	0.65	0.67	0.73		0.71	0.72						
Quinic acid				0.67	0.71		0.71								
Ribose			0.67					0.73							
Shikimic acid							0.67								
Sorbitol							0.75								
Tocopherol-a			0.67	0.7	0.67	0.67	0.71	0.7	0.68						
Uridine															
Xylitol			0.74	0.81	0.8	0.7			0.69		-0.67				

1- Ruminococcaceae, 2 – *Acetibvibrio,* 3 – *Akkermansia*, 4 – *Butyricicoccus,* 5 – *Coprobacillus*, 6 – *Dialister*, 7 – *Gordonibacter*, 8 – *Hallella,* 9 – *Oribacterium,* 10 – *Oscillibacter,* 11 – *Papillibacter,* 12 – *Prevotella,* 13 – *Solobacterium,* 14 – *Streptophyta,* 15 – TM7, 16 - *Xylanibacter*

Table 3.14. Fecal concentrations (mmol) of volatile fatty acids for the Balam and Motiepa group across sampling blocks. *Indicates significant changes across time ($p < 0.05$).

Sampling Block	**Group**	**Acetic***		**Propanoic**		**Butanoic***		**Pentanoic**		**Isobutanoic**		**Isopentanoic***		**Total***	
		Average	SD	Average	SD	Average	SD	Average	SD	Average	SD	Average	SD	Average	SD
Block 1	Balam	43.0	8.2	3.4	1.1	1.9	1.0	0.3	0.1	0.2	0.1	0.2	0.1	49.0	10.3
	Motiepa	36.9	4.8	3.0	0.5	1.8	0.5	0.3	0.1	0.2	0.1	0.2	0.1	42.4	4.9
Block 2	Balam	33.0	2.1	3.6	0.4	2.4	0.4	0.4	0.2	0.1	0.0	0.1	0.0	39.7	2.4
	Motiepa	36.1	2.8	3.7	0.3	2.8	0.6	0.4	0.1	0.1	0.0	0.1	0.1	43.2	3.2
Block 3	Balam	46.5	3.0	3.7	0.5	1.7	0.2	0.3	0.1	0.1	0.0	0.2	0.1	52.5	3.1
	Motiepa	48.4	4.5	4.0	0.7	2.1	0.3	0.4	0.1	0.1	0.0	0.2	0.0	55.2	5.4

Table 3.15. PerMANOVA results for the effect of season on fecal VFA content expressed in terms of millimoles per gram of fecal material as well as percent of total VFA production. SS = sums of squares; MS = mean squares

	df	SS	MS	F value	R^2	P value
Millimoles						
Individual Sampling	12	0.07	0.0065	0.85	0.19	0.6
Block	2	0.14	0.073	9.56	0.36	0.0006
Residuals	24	0.18	0.0077		0.45	
Total	38	0.41			1.00	
Proportion						
Individual Sampling	12	0.0061	0.00051	1.22	0.18	0.35
Block	2	0.017	0.0086	20.72	0.52	0.0002
Residuals	24	0.008	0.00042		0.30	
Total	38	0.033			1.00	

Table 3.16. Spearman's ρ values for significant correlations between microbial taxa and fecal VFA and ammonia concentration.

Taxon	Acetic Acid	Isobutanoic Acid	Isopentanoic Acid	Ammonia
Anaeroplasmataceae	0.55			
Erysipeltotrichaceae				0.53
Streptococcaceae				0.61
Acetivibrio			0.56	
Butyricicoccus	-0.61			
Coprobacillus	0.54			0.54
Oscillibacter				0.53
Streptococcus				0.63
Streptophyta	-0.61			
Xylanibacter		0.53		

Table 3.17. Spearman's ρ for correlations between the relative concentration of ingested metabolites and fecal VFA and ammonia concentration.

Metabolite	Acetic	Butanoic	Ammonia
1,2,3-trihydroxybenzene			0.79
1,2,3-trihydroxybutane	-0.21		
1,6-Anhydroglucose			0.7
1-Methyl-beta-D-galactopyranoside	-0.71		
1-Octedecenoylglycerol			0.65
2,3-Dihydroxybutanedioic acid	-0.67		
2,3-dihydroxysuccinic acid			0.66
2,4,5-Trihydroxypentanoic acid			0.55
2,5-dihydroxybenzoic acid			0.56
2,4-Methylenecycloartanol			0.57
2-Methylcitric acid			0.56
2-Methylmalic acid			0.76
2-Oxoisocaproic acid			0.61
2-Piperidinecarboxylic acid	-0.71		
3,4,5-Trihydroxypentanoic acid			0.73
3,4-Dimethoxycinnamic acid			0.63
3-Deoxy-arabino-hexaric acid			0.63
3-hydroxybenzoic acid			0.57
3-methyl-2-hydroxypentanoic acid			0.63
3-methyl-2-oxobutanoic acid			0.65
4-Caffeoylquinic acid	-0.7		
4-Hydroxy-3-methoxyphenethyleneglycol			0.65
4-Hydroxyproline	-0.55		
Allantoin	-0.62	0.55	
Beta-Amyrin			0.62
C16:1			0.62
C18:1			0.69
Chlorogenic acid	-0.71		
Citric acid	0.61		
Diethyleneglycol			0.53
Erythronic acid			0.63
Ethanol, (2-(3,4-dihydroxyphenyl)-			0.6
Galacturonic acid	-0.63		
Gallic acid			0.59
Glucaric acid			0.64
Glucuronic acid			0.62
Hentriacontanol			0.71
Inositol, myo-			0.56
Itaconic acid			0.64
Kaempferol			0.62

Table 3.17 (cont.)

Mannitol		0.55
N-Acetylglucosylamine	-0.36	
N-Acetyl glucosamine		0.55
Neochlorogenic acid	-0.73	
Nonacosanol		0.58
Octacosanol		0.71
p-hydroxybenzaldehyde		0.59
Protocatechuic acid		0.63
Shikimic acid		0.59
Sorbitol	-0.63	
Thymine		0.58
Vanillic acid		0.67
Xylopyranoside		0.56
Xylose		0.57

Table 3.18. Volatile fatty acid concentrations and profiles in nonhuman primates and mammals. Table modified from Lambert (2012).

WILD	**Total VFA Concentration (mM)**	**% Acetate**	**% Butyrate**	**%Propionate**	**Source**
Procolobus verus	230				Ohwaki et al. 1974
Colobus guereza	107–434				Ohwaki et al. 1974
Cercopithecus aethiops	190–229				Brourton et al. 1991
Cercopithecus mitis	122–199				Brourton et al. 1991
	138–180				Clemens and Phillips 1980
Papio cynocephalus	95–170				Clemens and Phillips 1980
Pan troglodytes	44	31	10	3	Ushida et al. 2006
Alouatta palliata		94	6	0.4	Milton and McBee 1983
Alouatta pigra	40-55	83-89	3-7	7-9	this study
Homo sapiens	100	57	22	21	Cummings et al. 1987
CAPTIVE					
Trachypithecus cristatus	95–133	47–56	24–26	10–18	Bauchop and Martucci 1968
Semnopithecus entellus	89–233	46–50	22–23	14–23	Bauchop and Martucci 1968
Colobus guereza	53–65				Kay et al. 1976
Colobus guereza	79	61	23	10	Lambert and Fellner 2012
Cercopithecus neglectus	65	47	21	26	Lambert and Fellner 2012
Papio hamadryas	87	48	36	11	Lambert and Fellner 2012
Pan troglodytes	81	55	25	12	Lambert and Fellner 2012
Gorilla gorilla	89	58	25	11	Lambert and Fellner 2012
Pongo abelii	62-67	22-27	10-14		Schmidt et al. 2005
Bos taurus	96–210	48–74	14–28	7–18	van Soest 1994
Ovis aries	70–140	40–66	19–40	9–15	Blaxter et al. 1956

FIGURES

Figure 3.1. Partial correspondence analysis illustrating patterns in the grams of plant parts consumed by the howlers each season with the effect of individual removed. Each point represents the diet of one individual.

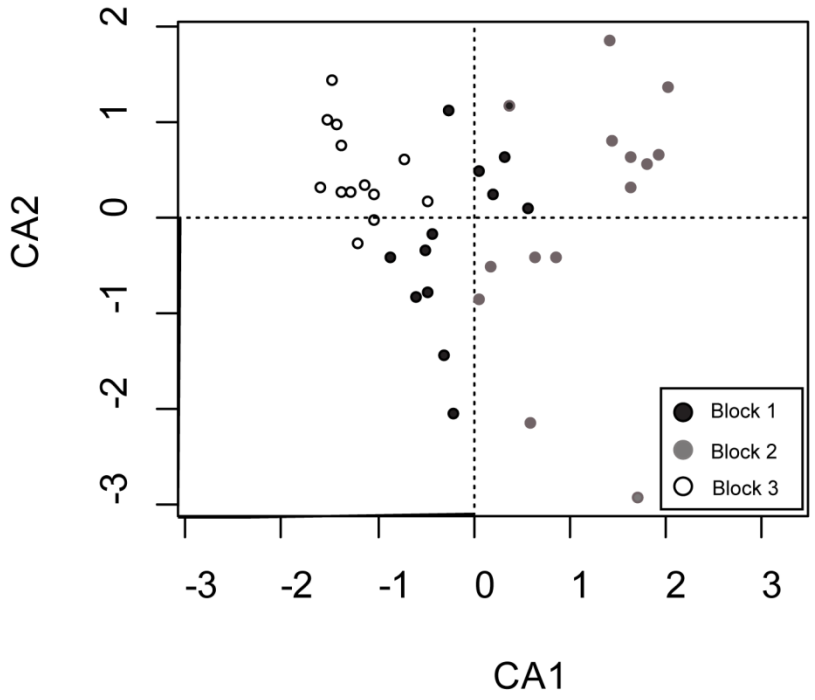


Figure 3.2. Plant parts consumed by the Motiepa group (top) and the Balam group (bottom) across sampling blocks. Values are expressed in percent of total dry grams consumed.

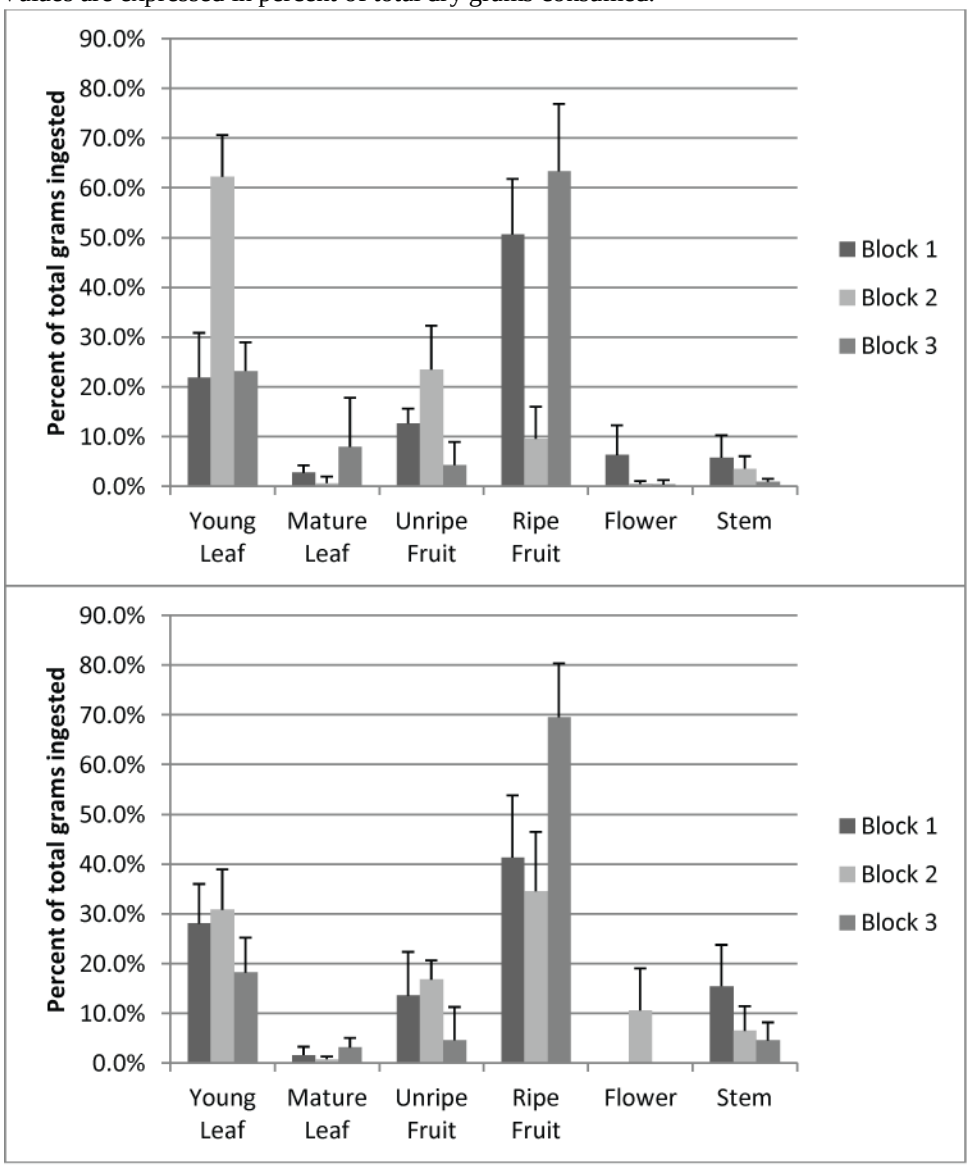


Figure 3.3. Partial correspondence analysis illustrating patterns in the grams of plant parts from each plant species consumed by the howlers each season with the effect of individual removed. Each point represents the diet of one individual.

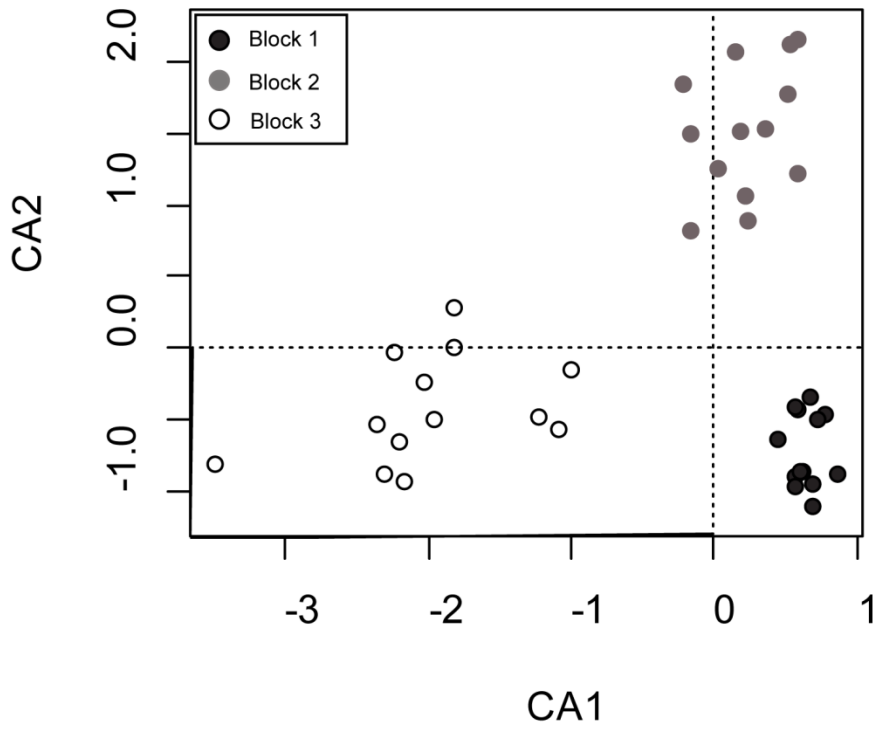


Figure 3.4. Partial correspondence analysis illustrating patterns in the plant metabolites consumed by the howlers each season with the effect of individual removed. Each point represents the diet of one individual.

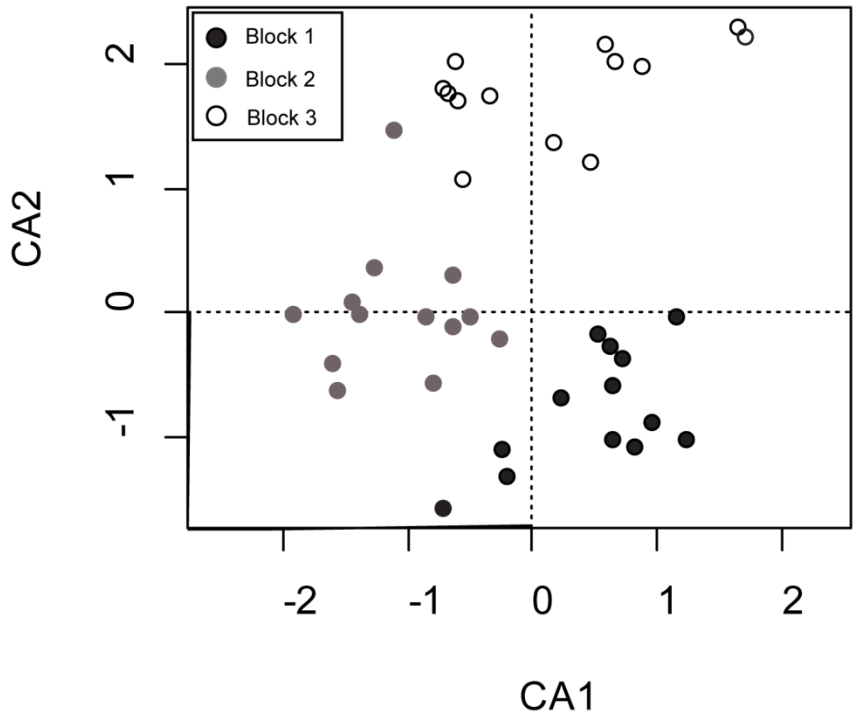


Figure 3.5. Partial correspondence analysis illustrating patterns in microbial community composition at the OTU level each season as determined using ARISA with the effect of individual removed. Each point represents the gut microbial community of one individual.

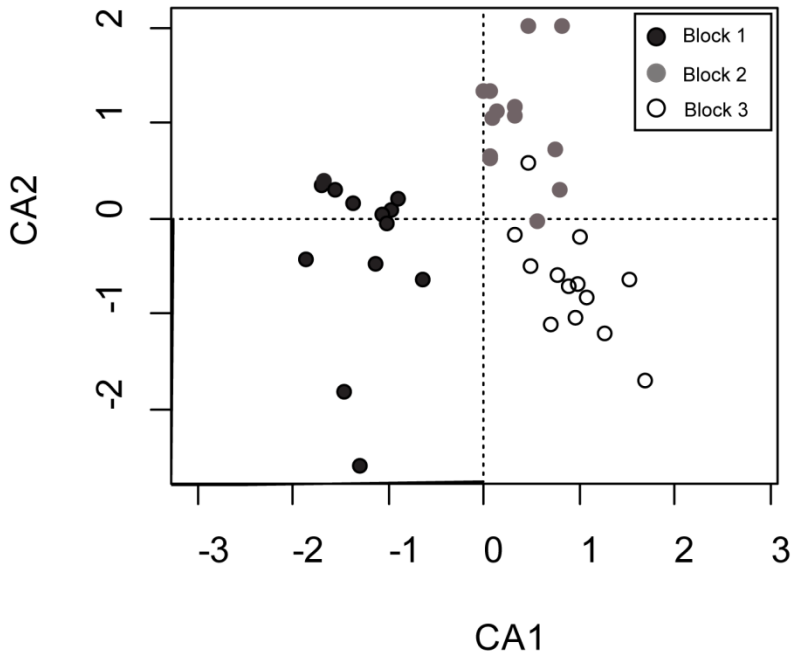


Figure 3.6. Partial correspondence analysis illustrating patterns in the millimoles of VFA's excreted by the howlers each season with the effect of individual removed. Each point represents the VFA profile of one individual.

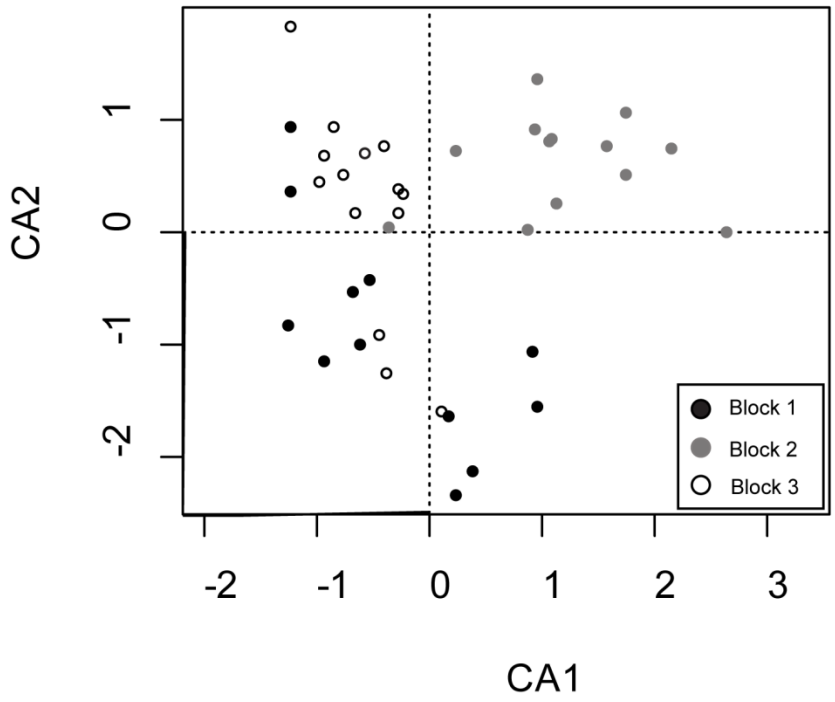


Figure 3.7. (A) The proportion of acetic acid detected in fecal samples from the Balam and Motiepa groups across sampling blocks. The proportion of other VFA's detected in fecal samples from (B) the Balam group and (C) the Motiepa group.

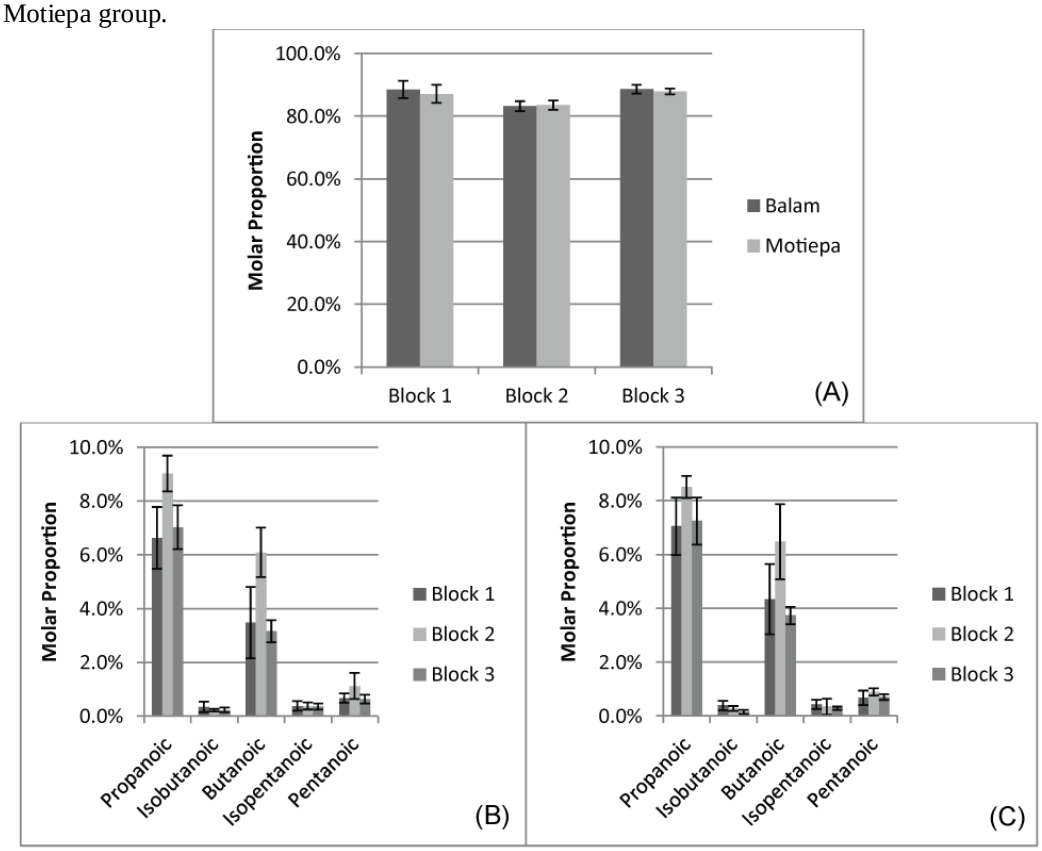
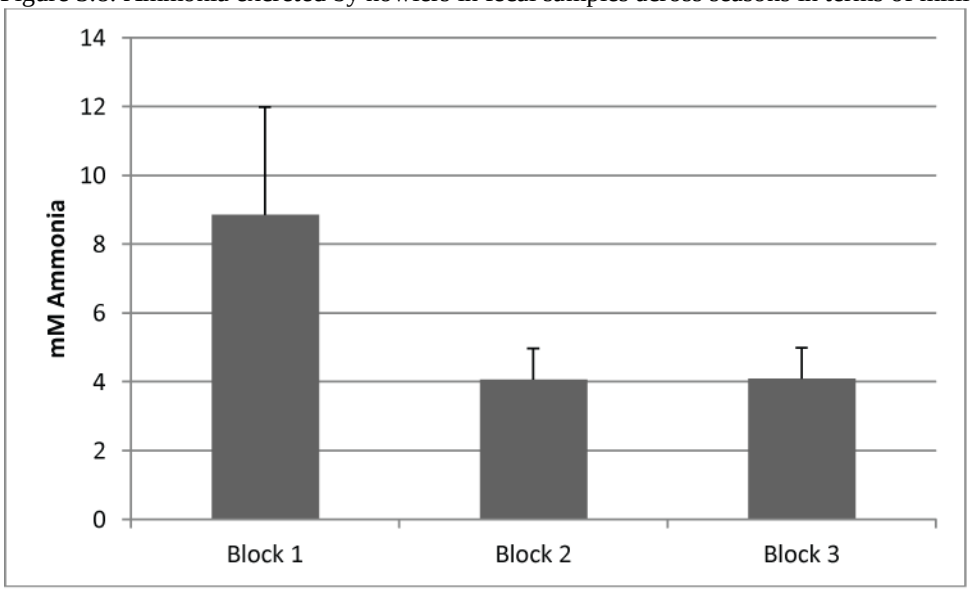


Figure 3.8. Ammonia excreted by howlers in fecal samples across seasons in terms of millimoles per gram of feces.



CHAPTER 4: BEHAVIORAL AND PHYSIOLOGICAL MECHANISMS FOR SATISFYING NUTRITIONAL DEMANDS IN ADULT MALE, ADULT FEMALE, AND JUVENILE WILD BLACK HOWLER MONKEYS (*ALOUATTA PIGRA*)

ABSTRACT

In all mammals, including primates, growth, pregnancy, and lactation result in increased nutritional demands for juveniles and adult females. These demands are expected to lead to changes in activity, diet, and/or digestion and energy and nutrient assimilation. Because studies of female and juvenile primates do not consistently provide evidence of differences in activity or diet that would suggest they are compensating for increased nutritional demands, changes in digestive efficiency are likely to be important. Mutualistic gut microbial communities have been shown to contribute to host digestive efficiency and nutrition by breaking down indigestible compounds. Because host physiology can affect gut microbial community composition, it is possible that changes in host physiology during reproduction and growth trigger shifts in the gut microbiota that improve host digestive efficiency. In this study I examine differences in activity budget, diet, and the gut microbial community in adult male (N=4), adult female (N=4), and juvenile (N=5) wild black howler monkeys (*Alouatta pigra*) across a ten-month period in Palenque National Park, Mexico to determine how adult females and juveniles compensate for increased nutritional demands. Results indicate that activity budgets were similar among age and sex classes, but adult females and juveniles consumed more protein, lipids and total non-structural carbohydrates per metabolic body weight than adult males as well as more overall energy across the entire study. Adult males, adult females, and juveniles also possessed distinct gut microbial communities, irrespective of diet. Juveniles were characterized by energy-producing bacteria from the phylum Fimicutes such as *Roseburia* and *Ruminococcus* while adult females were characterized by *Lactococcus,* which has been associated with folate biosynthesis.

Adult females also possessed a higher Firmicutes to Bacteroidetes ratio, suggesting an increased capacity for energy harvest. Finally, fecal volatile fatty acid content was higher in juveniles than adults, which implies increased microbial fermentation and energy production. Based on this study, it appears that female and juvenile black howler monkeys increase energy and protein intake and exhibit distinct gut microbial communities to meet the nutritional demands of reproduction and growth. Additional studies are needed to better understand the dynamics of the gut microbiota during these periods, but these processes are likely to be important for most primate taxa.

INTRODUCTION

In all mammals, including primates, metabolic requirements vary among individuals due to life history processes such as reproduction and growth (Brown et al 2004, Dufour and Sauther 2002, Ginnet and Demment 1997, Woolley et al 2009). Pregnancy and lactation are estimated to increase female primate daily energy requirements by 20-30% and 37-39%, respectively (Aiello and Wells 2002), and lactation is estimated to increase maternal protein requirements by more than a third (Oftedal et al 1991). During certain phases of these periods, primate mothers are responsible for supplying the energy and nutrients for brain development in offspring, a process which requires almost twice as much energy as normal growth (Aiello and Wheeler 1995, Aiello and Wells 2002). For weaned juveniles, growth is also nutritionally costly. For example, a two-year old yellow baboon (*Papio cynocephalus)* weighing four kilograms needs 100 kJ of energy daily to grow five grams per day, which represents a 150% increase over daily metabolic requirements (Altmann and Alberts 1987, Altmann and Samuels 1992). When body size is considered together with growth, the same juvenile baboon would require 1.94 times more energy than an 11-kg adult per kilogram per day. Likewise, for captive capuchins (*Cebus*

albifrons) fed a highly digestible protein source, it has been shown that, relative to body size, even the lowest protein requirements for weaned juveniles are approximately 183% greater than those for adults (Oftedal et al 1991). These requirements surpass those estimated for gestating and lactating females (Aiello and Wells 2002, Oftedal et al 1991).

According to bioenergetics models (McNab 2002, Peles and Barrett 2008), increased nutritional demands in juvenile and reproductively active female primates are expected to lead to changes in activity, diet, and/or digestion and energy and nutrient assimilation. Data from some primate taxa such as gelada baboons, chimpanzees and titi monkeys (*Theropithecus gelada*, *Pan troglodytes*, *Callicebus cupreus*) suggest that gestating or lactating females spend more time feeding, spend less time traveling, and consume more potentially protein-rich insects than other group members (Bates and Byrne 2009, Dunbar and Dunbar 1988, Herrera and Heymann 2004). This behavior enables them to increase energy and nutrient intake and/or decrease energy expenditure. However, in many cases these differences are subtle. For example, lactating female chimpanzees spend less time traveling than other group members but travel the same distances (Bates and Byrne 2009). In these cases, the nutritional costs of reproduction are either lower or are being fulfilled in another manner.

For juveniles, behavioral patterns appear to increase energy expenditure instead of decrease it. Several studies report that, especially during periods when adults are resting, juvenile primates tend to play more than adults (Baldwin and Baldwin 1974, Fagen 1993, Oliveira et al 2003, Prates and Bicca-Marques 2008, Stevenson et al 2005, Watts and Pusey 1993). Play is considered essential for primate juveniles to develop social and locomotor skills (Fagen 1993, Poirier et al 1978), and in general, play represents only 1-10% of most primate and mammal activity budgets (Beckoff and Byers 1992, Fagen 1971). However, in addition to reducing resting

time, play requires energy (Beckoff and Byers 1992). In a study of three juvenile pronghorns, play accounted for 2% of the activity budget but 20% of energy expenditure not associated with metabolism, and individuals that engaged in play were estimated to weigh approximately 7% less than if they had been resting (Miller and Byers 1991). In primates, no estimates of play-related energy expenditure are currently available, but studies of juvenile captive squirrel monkeys, wild langur monkeys and wild gelada baboons report that less time is spent playing when less food is available or when food is more difficult to process (Baldwin and Baldwin 1976, Barrett et al 1992, Sommer and Mendoza-Granados 1995). This relationship suggests that the energetic costs of play are large enough to impact host energy balance and nutrition. When combined with the nutritional demands of growth, we would expect these costs to result in increased energy and nutrient intake by juveniles, but in most cases differences between adult and juvenile diets are nonexistent or subtle (Harrison 1983, Johnson and Bock 2004, MacKinnon 2006, Stone 2006, Stone 2007). This pattern suggests that either juvenile energy needs are not as high as believed or that juveniles are compensating for those needs in another way.

For reproductively active females and growing juveniles that do not exhibit changes in diet or activity to compensate for increased nutritional demands, physiological changes that increase digestive efficiency and assimilation may play an important role in providing extra energy and nutrients (Peles and Barrett 2008). Increasing the volume of the gut improves digestive efficiency while increasing the surface area of the gut as well as the permeability of the epithelial layer improves nutrient and energy assimilation (Chivers and Hladik 1980, Mackie and White 1997a, Sibly 1981). Therefore, we would expect individuals with increased nutritional demands such as females and juveniles to possess larger, more permeable guts. These patterns have been documented in studies of rodents with increased energy needs and/or decreased

energy intake (Green and Millar 1987, Hammond and Kristan 2000, Naya et al 2007, Naya et al 2008). However, changes in gut morphology incur nutritional costs since gut tissue is energetically expensive to produce and maintain relative to all other body tissues except the brain (Aiello and Wheeler 1995, Webster 1981). Additionally, dramatic changes in gut morphology have not been observed in large-bodied mammals such as primates (Milton 1999).

In contrast, mutualistic microbial communities in the large intestine have been shown to contribute to host digestive efficiency and nutrition by breaking down fiber and other undigested compounds (Mackie and White 1997b). Gut microbial community composition shifts in response to changes in host diet and can affect host metabolism and digestive efficiency (Martinez et al 2009, Muegge et al 2011, Turnbaugh et al 2009), and host physiology has also been shown to affect gut microbial community composition (Bailey and Coe 1999, Suzuki et al 1983). For example, in a study of macaque infants (*Macaca mulatta*), increased plasma cortisol levels were associated with a reduced abundance of fecal lactobacilli (Bailey and Coe 1999). Therefore, it is possible that changes in host physiology resulting from reproduction and growth could trigger shifts in the composition of the gut microbiota that improve host digestive efficiency. In human and nonhuman primates, gestating females undergo a hormone-mediated reduction in natural killer cell cytotoxicity and a local and systematic shift from cell-mediated immunity to humoral immunity to prevent rejection of the fetus (Gabrilovac et al 1988, Marzi et al 1996, Raghupathy 1997, Wegmann et al 1993). Because signals from the gut microbiome also are thought to decrease the cell-mediated immune response and increase the humoral response (Zaph et al 2008), such pregnancy-induced hormone shifts are likely to encourage microbial colonization in the gut and change community composition. The associated changes in digestive efficiency may be less costly than those induced by modifications to gut morphology since gut microbes utilize

the undigested portion of the host diet which the host cannot otherwise exploit (Mackie and White 1997a).

If shifts in the gut microbiota result in improved digestive efficiency and energy and nutrient assimilation, they may allow females and juveniles to meet nutritional requirements without major shifts in activity or diet. Higher relative abundances of microbial genera from the Firmicutes phylum can increase the production of short-chain fatty acids, an important energy source for the host (Ley et al 2005, Turnbaugh et al 2006). Additionally, *Bacteroides* have been associated with increased HDL-cholesterol and folic acid in pregnant women while *Bifidobacterium*, *Enterobacteriaceae*, and *E. coli* are associated with increased folic acid, ferritin and reduced transferrin, respectively (Santacruz et al 2010). Folate plays a role in DNA synthesis, and deficiencies can cause birth defects such as neural-tube defects (Czeizel and Dudas 1992, Czeizel et al 2010, Lamers 2011). Reduced ferritin is associated with iron deficiencies which can cause low birth weight and reduce neonatal health (Allen 2000, Romslo et al 1983, Taylor et al 1982). Therefore, the production of these compounds by members of the gut microbial community is likely to improve female nutritional status during gestation.

Shifts in the gut microbiota that affect host nutrition are likely to be especially crucial to primates living in seasonal environments with dramatic changes in food availability. In these situations, because primate life histories are slow compared to other mammals (Case 1978, Sugiyama 2004), juveniles and females must meet the nutritional demands of growth and reproduction across periods of both high and low food availability. When diet is constrained during periods of low food availability, changes in gut microbial community composition and metabolic activity may be an effective mechanism by which hosts can achieve the nutritional status necessary for reproduction and growth.

Howler monkeys (*Alouatta* sp.) are an ideal system for exploring primate behavioral and physiological strategies for meeting nutritional demands. Compared to other atelines, they have an earlier age at reproduction (42-62 months vs. *Ateles:* 84-85 months, *Brachyteles:* 87-108 months, *Lagothrix:* ~87 months), a shorter gestation period (152-195 days vs. *Ateles:* 226-232 days, *Brachyteles:* 215-218 days, *Lagothrix:* 210-225 days), and a shorter interbirth interval (16-23 months vs. *Ateles:* 32-50 months, *Brachyteles:*32-41 months, *Lagothrix:* 32-41 months; Di Fiore et al 2011, Fedigan and Rose 1995). Although *Alouatta* also tends to be the smallest of the atelines (adult males: 6.1-11.4kg compared to *Ateles:* 8.2-9.1kg, *Brachyteles:* 9.4-13.8kg, *Lagothrix:*9-9.5kg), prenatal growth rates (2.14-2.84g/day) are estimated to be higher than in *Ateles* (1.86-2.03g/day) and *Lagothrix* (1.92-2.02g/day), and neonatal brain size (53% of adult size) is smaller compared to *Ateles* (58%; Hartwig 1996). Juvenile howlers also are weaned at an earlier age compared to other atelines (11-14 months vs. *Ateles:* 24-36 months, *Brachyteles:* 18-24 months; Di Fiore et al 2011). These patterns suggest that adult female howler monkeys invest more metabolic resources in prenatal offspring growth than other adult female atelines while juvenile howlers invest more resources in brain growth after birth and weaning than other juvenile atelines (high investment over a shorter growth period). As a result, the daily nutritional demands for growth and reproduction in *Alouatta* juveniles and adult females may be higher than in other atelines, and adult females and juveniles are likely to require pronounced changes in activity, diet, and/or gut microbial community composition and activity to meet these demands.

As energy-minimizers and leaf-eaters, howler monkeys spend more than 50% of their active hours resting (Di Fiore et al 2010) and may have constrained activity budgets that cannot be markedly altered in response to changes in nutritional needs. Furthermore, howler monkeys live in socially cohesive groups which may limit inter-individual variation in behavior and diet

(Pavelka 2011). However, their diets can change dramatically across seasons from principally leaves and flowers, which are commonly considered to be relatively higher in protein and lower in energy compared to ripe fruit, to mostly ripe fruit, which is assumed to be lower in protein but richer in energy (Behie and Pavelka 2012, Estrada 1984, Gaulin and Gaulin 1982, Milton 1979). This dietary flexibility may allow females and juveniles to compensate for the demands of reproduction and growth through dietary selectivity and the exploitation of food items that are higher in energy or nutrients. In addition, howlers depend heavily on their gut microbiota for the breakdown of fiber and the production of energy (Milton and McBee 1983). Therefore, shifts in the composition of the gut microbial community may be important for allowing howlers to maintain nutritional balances during reproduction and growth, particularly during periods of low ripe fruit availability.

In this study I describe the mechanisms by which female and juvenile Mexican black howler monkeys (*A. pigra*) meet nutritional demands across seasons. I focus on activity budget, diet, and gut microbial community composition and function. Studies of black howler monkey behavior have provided evidence for changes in female activity patterns and diet during gestation and lactation (Dias et al 2011, Serio-Silva et al 1999), but no study of black howler monkeys has directly compared activity and diet among adult males, adult females, and juveniles. Additionally, although a recent study of black howlers provides preliminary evidence that gut microbial community composition differs in gestating females compared to other group members (Amato and Righini accepted), data spanning seasons is necessary to confirm these patterns.

Black howler activity budgets differ minimally across seasons despite dramatic variations in diet composition (Chapter 2). Therefore, I expect adult females, adult males and juveniles to demonstrate differences in diet, gut microbial community composition, and microbial metabolic

activity to compensate for nutritional demands. In this study, I test three hypotheses examining these factors. **(1) Adult females and juveniles consume more energy and protein per metabolic weight than adult males.** I expect these differences to be most notable during periods when energy and protein intakes surpass estimated requirements for howler monkeys. Assuming energy and protein are readily available in the diet during these periods, adult females and juveniles should be able to simply consume more food per metabolic body weight to meet increased nutritional demands. Additionally, because growth is more nutritionally costly than reproduction (Aiello and Wells 2002, Altmann and Alberts 1987, Oftedal et al 1991), I expect juveniles to exhibit stronger dietary differences than adult females when compared to adult males. **(2) Adult females and juveniles possess distinct gut microbial communities compared to adult males.** I expect that adult females and juveniles will exhibit higher relative abundances of microbes that are known to produce energy more efficiently, such as Firmicutes (Turnbaugh et al 2006), especially during periods of reduced energy and protein intake. Assuming energy and protein are not readily available in the diet during these periods, adult females and juveniles will rely more heavily on microbial metabolism for energy and nutrients to meet increased nutritional demands. Specifically, females and juveniles are expected to exhibit higher relative abundances of microbes associated with protein synthesis such as members of the *Helicobacter* and *Proteus* genera, as well as *Streptococcus, Bacteroides, Coprococcus,* and *Roseburia* (Reitzer and Magasanik 1987, Yatsunenko et al 2012), especially during periods of reduced protein intake. Adult females and juveniles are also expected to exhibit more bacterial taxa involved in vitamin production such as *Streptococcus, Lactobacillus,* and *Lactococcus,* which are associated with an increased number of genes for folate biosynthesis (Yatsunenko et al 2012), and *Bacteroides, Eubacterium, Propionibacterium,* and *Fusobacterium,* which are associated with K and B

vitamin production (Albert et al 1980, Hill 1997). Again, I expect stronger differences in juveniles than adult females. **(3) Gut microbial community function should differ in adult males, adult females, and juveniles.** If adult females and juveniles are producing more energy and protein via gut microbial processes than adult males, I should observe a higher concentration of volatile fatty acids (VFA) and a lower concentration of ammonia in their feces. Ammonia and branched-chain VFAs are products of microbial protein metabolism while other VFAs such as acetate, butyrate, and propionate are produced by fermentation of fiber (Mackie et al 1998). Levels of these compounds in feces provide an estimate of microbial activity since excretion is generally proportional to production (Mackie et al 1998). I expect adult females and juveniles to exhibit higher fecal VFA concentrations and lower fecal ammonia concentrations compared to adult males. Juveniles should have higher VFA concentrations and lower ammonia concentrations compared to adult females.

METHODS

To detect age- and sex-based differences in the howler gut microbiota over time and to determine whether shifts in gut microbial community composition are associated with changes in microbial activity and digestive efficiency, I collected behavioral data and fecal samples for microbial analyses from howlers in two neighboring social groups (N=16) in Palenque National Park, Mexico. The Balam group consisted of two adult males, two adult females, and two juvenile males (approximately one and three years of age, respectively). The Motiepa group consisted of four adult males, two adult females, two juvenile males (approximately two and three years of age, respectively), and two juvenile females (approximately one year and five months of age, respectively). Two of the adult males disappeared during the study and are

believed to have dispersed. These males, as well as the youngest juvenile female, were not included in analyses since data were not collected from them across the entire study period.

Data were collected across three ten-week blocks from September 2010 to July 2011. Block 1 lasted from September to November 2010. Block lasted from January to March 2011, and Block 3 lasted from April to June 2011. Sampling blocks corresponded loosely to seasonal shifts in rainfall (CONAGUA 2011) as well as previously documented temporal shifts in black howler diet (Estrada, unpublished data). More rain tends to fall during the months sampled in Block 1, and less rain falls in months corresponding to Block 3 (CONAGUA 2011) while fruit-eating tends to be higher during the months sampled in Blocks 1 and 3 and lower in Block 2 (Estrada, unpublished data).

Twenty-minute focal individual samples were collected five days per week between sunrise and 5pm (park closing time) each day. I collected a total of 1,522 hours of quantitative data (Block 1: 328 total hours, 103 feeding hours in 49 days, Block 2: 531 total hours, 139 feeding hours in 49 days, Block 3: 663 total hours, 89 feeding hours in 50 days). The focal individual was chosen pseudo-randomly (no individual was sampled twice consecutively and priority was given to individuals that had been undersampled on previous days), and activity was recorded every two minutes. Five activities were recorded: feeding (ingestion of food items), foraging (movement within a feeding tree), resting (inactivity), traveling (movement between trees and within a feeding tree), and social activity (howling, play, sexual interaction, aggression, etc.). During feeding bouts, the plant part (e.g. ripe fruit, unripe fruit, young leaves, mature leaves, flowers, stem) and plant species being consumed was recorded. The number of food items consumed per minute was quantified when possible to provide an estimate of intake rate. Samples of the ten food resources consumed most often by each black howler group (as

determined by proportion of monthly feeding time) were collected, and the average wet and dry mass of each resource was measured using five items from each of three trees.

A handheld global positioning system was utilized to track black howler monkey group movement patterns during observations. The position of the group was recorded every thirty minutes. To estimate day range, I calculated the total distance traveled between all points using ArcGIS 10.1 (ESRI 2011, Redlands, CA). The home range during each sampling block was estimated by calculating the area of the minimum convex polygon created by the data points in ArcGIS 10.1 (ESRI 2011, Redlands, CA).

Samples of the top ten food resources used by each group during each season were collected and preserved in 70% methanol for metabolite profiling. Metabolites are small molecules produced by metabolism such as amino acids, alcohols, nucleotides, and vitamins. Metabolites present in the host diet influence both host nutrition and microbial metabolism since they may be digested directly or utilized by the microbial community as substrates. All metabolite data were generated using gas chromatography/mass spectrometry (Poroyko et al 2011). Mass spectra were verified with authentic standards and mass spectra from commercial database (Poroyko et al 2011).

The behavioral data were used to calculate the average percent of time spent feeding during a focal sample in each season. Because howler monkeys are active during all daylight hours, the average daylength during each season was used to calculate the average number of minutes per day the howlers fed. Average ingestion rates for each plant part of each plant species, as well as average food item dry mass, were used to estimate the average number of dry grams of each food item ingested daily by each individual. Diet was described in terms of grams of plant parts ingested per day, grams of plant parts from each plant species, and relative

concentration of metabolites. Metabolites extracted from the howler food resources were expressed in terms of relative concentration (units) and were categorized into amino acids, sugars, and lipids when possible. The total relative concentration of amino acids, sugars, and lipids in each food resource was multiplied by the average daily grams (wet weight) of that resource consumed by each individual in each sampling period to provide an estimate of the concentrations of amino acids, sugars, and lipids consumed. Due to the method by which the metabolite concentrations were standardized, these data do not provide accurate estimates of the actual amount of each metabolite in a food item, nor can their amounts be accurately compared across categories (i.e. amino acid, sugar, lipid) within a sample. However, relative concentrations of the same metabolite across food items and howler diets can be compared to understand feeding patterns at the metabolite level and their impact on the howler gut microbiota. Published literature values (Estrada 1984, Felton et al 2009b, Milton 1979, Milton 2008, Norconk et al 2009, Silver et al 2000) were used to estimate the amount of protein, non-structural carbohydrates, lipids, and metabolizable energy consumed. All feeding data were standardized by metabolic body weight (divided by body weight raised to the 0.75) using published average masses for each age/sex class prior to analysis (Kelaita et al 2011, Kleiber 1975).

Fecal samples were collected every two weeks from each individual for microbial community composition analysis as well as measurement of volatile fatty acid (VFA) and ammonia content. Fecal samples were stored in 96% ethanol for microbial community composition analyses, 1M NaOH for VFA analyses, and 1M HCl for ammonia analyses. They were shipped to the University of Illinois where they were kept at -80C until processing. Permits to collect and export fecal and plant samples were obtained through the Secretaria del Medio Ambiente y Recursos Naturales (SEMARNAT) and the Comision Nacional de Areas Naturales

Protegidas (CONANP), and the Secretaria de Agricultura, Ganaderia, Desarollo Rural, Pesca y Alimentacion (SAGARPA) in Mexico. Permits to import samples to the United States were obtained through the Center for Disease Control (CDC) and the United States Department of Agriculture Animal and Plant Health Inspection Service (USDA APHIS).

To describe microbial community composition, DNA was extracted from samples preserved in 96% ethanol using the MOBio UltraClean Soil Kit. The intergenic spacer region of the 16S ribosomal gene was amplified in all samples using polymerase chain reaction, and automated ribosomal intergenic spacer analysis (ARISA) was used to create a microbial community "fingerprint" for each sample (Kent et al 2007). PCR reactions included buffer consisting of 50 mM Tris (pH 8.0), 250 μg of bovine serum albumin per mL and 3.0 mM $MgCl_2$ (Idaho Technology, Salt Lake City, UT; cat #1770), 250 mM of each dNTP, 10 pmol of each primer, 1.25 U of *Taq* polymerase (Promega, Madison, WI), and 2 μL of extracted DNA in a final volume of 25 μL. The following primers were used to generate ARISA PCR products: 1406f, 5'- TGYACACACCGCCCGT-3' (universal, 16S rRNA gene), and 23Sr, 5'-GGGTTBCCC CATTCRG-3' (bacteria-specific, 23S rRNA gene). The 1406f primer was labeled at the 5' end with the phosphoramidite dye 6-FAM. PCR was carried out in an Eppendorf MasterCycler (Eppendorf, Hauppauge, NY) with an initial denaturation at 94°C for 2 min, followed by 30 cycles of 94°C for 35s, 55°C for 45s, and 72°C for 2 min, with a final extension carried out at 72°C for 2 min. ARISA PCR products were visualized by denaturing capillary electrophoresis using an ABI 3730xl Genetic Analyzer (Applied Biosystems, Foster City, CA) at the UIUC Keck Center for Comparative and Functional Genomics as described previously (Kent and Bayne 2010). Size-calling and ARISA profile alignment were carried out using GeneMarker version 1.95 (SoftGenetics, State College, PA). A signal detection threshold of 500 fluorescence

units was used in order to exclude background fluorescence. The signal strength (i.e., peak area) of each peak was normalized to account for run-to-run variations in signal detection by dividing the area of individual peaks by the total fluorescence (area) detected in each profile, expressing each peak as a proportion of the observed community (Yannarell and Triplett 2005).

Pyrosequencing of the V1-V3 region of the 16S ribosomal RNA gene was used to generate taxonomic data for the microbial communities in a subset of samples (N=8 individuals at 15 time points; Amato et al 2013). The V1-V3 region of the 16S ribosomal RNA gene was amplified by polymerase chain reaction (20 cycles of 94°C (30s), 48°C (30s), 72°C (2 min)) using primers 27f (CGTATCGCCTCCCTCGCGCCATCAG-AGAGTTTGATYMTGGCTCAG; corresponding to nucleotides 8 – 27 of the *Escherichia coli* 16s rRNA gene) and 534r (CTATGCGCCTTGCCAGCCCGCTCAG-[MID tag 1 – 50]-ATTACCGCGGCTGCTGGCA). The amplicons were pyrosequenced using 454 FLX-Titanium technology at the J. Craig Venter Institute (Rockville, MD). 119 samples were successfully sequenced, and sequences shorter than 250nt, with homopolymers longer than 6 nucleotides, containing ambiguous base calls or incorrect primer sequences were removed. Sequences were aligned against the silva database (Pruesse et al 2007) and pre-clustered using mothur (Schloss et al 2009). Potentially chimeric sequences were detected and removed using uchime in mothur (Schloss et al 2009). The remaining reads were clustered using a custom perl script. OTUs were defined as sharing $\geq$ 97 % sequence identity. OTUs detected fewer than twice across the entire data set were removed as probable artifacts. Rarefaction data, Simpson, Shannon-Weaver and Chao1 analyses were performed using mothur (Schloss et al 2009). Taxonomic profiles were generated using the RDP Classifier (Wang et al 2007).

Fecal VFA content was measured using gas chromatography (Erwin et al 1961). Samples preserved in 1M NaOH were neutralized with phosphoric acid and centrifuged to remove particulate matter before being processed. The amount of each VFA detected was expressed both in millimoles per gram of feces and as a proportion of total VFA's. Ammonia content was measure using spectrophotometry. Samples preserved in 1M HCl were processed according to Chaney and Marbach (Chaney and Marbach 1962). Fecal ammonia content was expressed in millimoles. Data for both VFA and ammonia content were standardized according to sample mass and adjusted for dilution.

Permutational multivariate analysis of variance (PERMANOVA) was used to test for the effects of age and sex on gut microbial community composition, fecal VFA content, diet, and activity. For gut microbial community composition as described by ARISA, I ran two PERMANOVA models. The second model included additional ARISA data generated from black howler individuals in two groups at a separate site (El Tormento Experimental Forest, Campeche, Mexico) during the same time period (N=33 total individuals) and was used to verify patterns in gut microbial community detected in the subset of samples with contextual data presented in this study. For all models, data were pooled by individual for the entire study period as well as by season. Unless no group differences were detected, models using these data were stratified by group, allowing me to test for differences in diet and gut microbial community composition and function across variables while controlling for differences among groups. Type III sums of squares were used to determine the significance of each factor in the model. All models were run for 5000 permutations.

Age- and sex-based patterns in gut microbial community composition, VFA content, and diet were visualized using partial correspondence analysis. Kruskal-Wallis tests were used to test

for age and sex differences in total grams of food consumed, total fecal VFA concentration, and in individual plant parts consumed, individual fecal VFAs and ammonia, and microbial taxa. Non-parametric Mantel tests were used to compare patterns in gut microbial community composition, fecal VFA content, and host diet. A series of Spearman rank correlations were performed to detect relationships between single components of diet, single VFA's, and single microbial taxa. For all analyses probability was set at $p < 0.05$, but p-values were adjusted for repeated tests using a sequential Bonferroni correction (Holm 1979, Rice 1989). I did not adjust p-values for tests examining differences in the relative abundances of individual microbial taxa across sampling blocks since the small number of sequenced samples reduced statistical power. All analyses were performed with R with the exception of non-parametric Mantel tests, which were performed using PRIMER 6 for Windows v 6.1.10 (PRIMER-E, Plymouth, UK).

RESULTS

Activity and diet patterns: Analysis of 328 hours of behavioral data in the rainy season (103 feeding hours), 663 hours in the dry season (89 feeding hours), and 531 hours in the intermediate period (139 feeding hours), indicated that overall activity patterns during the study were similar between groups ($F_{2, 38} = 1.44$, $p = 0.22$) but differed according to howler age (Table 4.1, Figures 4.1, 4.2). Juveniles consistently spent less time resting than both adult males and females and more time foraging, engaging in social behavior and traveling. These differences were mainly an effect of juveniles playing during periods that adults spent resting, and they did not change across seasons ($F_{1, 38}=2.4$, $p = 0.12$).

On average, male and female adult howlers did not consume more grams of food per day (1,114 ± 277g; wet weight) than juvenile howlers (960 ± 353g; Kruskal-Wallis $\chi^2 = 1.37$, df = 1,

p = 0.24), but adult males (978 ± 214g) consumed less food than adult females (1251 ± 340g; Kruskal-Wallis χ^2 = 5.3, df = 1, p = 0.02). When metabolic body weight was accounted for, adults (205 ± 75g) and juveniles (232 ± 85g) continued to consume similar amounts of food (Kruskal-Wallis χ^2 = 2.14, df = 1, p = 0.14) while adult females (104 ± 28g) consumed less than adult males (193 ± 42g; Kruskal-Wallis χ^2 = 5.3, df = 1, p = 0.02).

Because the average diet composition across the study period differed between groups in terms of the proportion of plant parts ($F_{2,38}$=3.15, p = 0.05) and plant species consumed ($F_{2,38}$ = 5.98, p = 0.002), data for both groups were not combined for these categories. For all other categories, I combined the data for both groups for analyses. In general, the howler diet differed in terms of the plant parts ($F_{2,38}$ = 22.06, p = 0.0002), plant species ($F_{2,38}$ = 14.99, p = 0.0002), and plant metabolites ($F_{2,38}$ = 13.34, p = 0.0002) consumed across sampling blocks. The grams of plant parts consumed per metabolic body weight by the howlers also differed by age and adult sex (Table 4.2, Figure 4.3, 4.4). Adult males consumed less young leaves, ripe fruit, and stems per metabolic body weight compared to adult females and juveniles (Figure 4.4). These differences did not vary with season ($F_{2, 38}$=0.96, p = 0.53; $F_{2, 38}$=0.67, p = 0.66). The grams of plant species consumed per metabolic body weight differed by age and adult sex classes within each social group (Table 4.2, Figure 4.5). This pattern was not driven by changes in any single plant species, and there were no significant differences detected in the proportions of plant species consumed (Table 4.2). Patterns in plant species consumption across age and sex classes did not change across time ($F_{2, 38}$=0.94, p = 0.45; $F_{2, 38}$=0.67, p = 0.80).

Plant metabolite profiles provided evidence of age and sex differences (Table 4.2, Figure 4.6) that did not vary across time ($F_{2, 38}$=1.15, p = 0.35). These differences were not driven by patterns in any subset of metabolites. Dietary patterns described using metabolite profiles of food

resources were moderately correlated with plant parts consumed (Spearman's $\rho = 0.27$, $p = 0.034$) and plant species consumed (Spearman's $\rho = 0.26$, $p = 0.05$). These data suggest that although describing diet in terms of plant parts and plant species may partially describe its nutritional content, patterns of plant metabolite intake vary in response to nutritional composition of plant parts or plant species utilized. This is likely due to the fact that each plant part and plant species exhibits a unique metabolite composition depending on its metabolic and physiological processes (Schwab 2003).

Based on literature estimates of food resource nutritional content (Table 4.3), adult females and juveniles consumed more overall energy, protein, total non-structural carbohydrates, lipids and neutral detergent fiber per metabolic body weight than adult males (Table 4.4). When I examined nutritional intake within each sampling block, only protein intake was significantly different among age and sex classes, and this pattern was consistent across sampling blocks. Metabolite analyses of the howler diet provided somewhat different results. These analyses indicated that juveniles consumed a higher concentration of amino acids per metabolic body weight compared to adults while adult females consumed a lower concentration of sugar metabolites compared to males (Table 4.5). These patterns differed in significance across sampling blocks, but the overall trend was the same (Table 4.5). Females also consumed a lower concentration of lipid metabolites than males and juveniles during Blocks 2 and 3 (Table 4.5). Based on these data, it appears that juveniles exhibit an increased protein intake compared to adults while adult females exhibit a reduced carbohydrate energy intake compared to juveniles and adult males.

Gut Microbioal Community Composition: After controlling for group differences, community fingerprinting data indicated that microbial community composition was distinct for adult and juvenile howlers at the operational taxonomic unit (OTU) level (Table 4.6, Figure 4.7). When I included samples from another site in Campeche, Mexico (El Tormento, 5 adult females, 8 adult males, 5 juveniles), these differences were maintained, and differences between adult males and adult females also were detected (Table 4.6). These results suggest that the patterns in the Palenque National Park dataset were not an effect of small sample size. In the Palenque dataset, differences among adult and juvenile howler monkeys were the same regardless of sampling block ($F_{2, 38}$=0.90, p = 0.65).

Chao1 estimates of gut microbial community richness based on sequencing data at the OTU level did not differ according to age or sex (adult male: 2027 ± 51OTUs; adult female: 2153 ± 145 OTUs; juveniles: 1987 ± 91 OTUs; χ^2 = 1.78, df = 1, p = 0.18; χ^2 = 0.86, df = 1, p = 0.35). Shannon (adult male: 4.81 ± 0.12; adult female: 5.02 ± 0.17; juveniles: 4.78 ± 0.13; χ^2 = 1.78, df = 1, p = 0.18; χ^2 = 0.86, df = 1, p = 0.35) and Simpson (adult male: 0.04 ± 0.002; adult female: 0.03 ± 0.01; juveniles: 0.04 ± 0.02; χ^2 = 0.11, df = 1, p = 0.74; χ^2 = 0.86, df = 1, p = 0.35) diversity indices were similar across age and sex classes as well.

Sequencing data from a subset of the sampled individuals (N=8) revealed a trend for differences in overall gut microbial community composition among age and sex classes at the bacterial Class, Order, and Family level (Table 4.6). When I examined individual microbial genera for trends across age and sex classes, I found significant differences in the relative abundances of two Classes, six Orders, four Families, and seven genera. For instance, the relative abundances of Bacillales, Solirubrobacterales, and *Brevundimonas* were higher in adult males compared to adult females and juveniles (Table 4.7). *Anaerovorax* and *Sphingobacteria*

were present in higher abundances in adult females compared to adult males and juveniles (Table 4.7), and *Brachyspira*, *Paraprevotella*, and *Roseburia* were detected in higher abundances in juveniles compared to adults while Opitutaceae and *Anaerotruncus* were detected in lower abundances (Table 4.7). Indicator species analysis reported 23 bacterial genera that characterized adult male howler monkeys, including *Brevundimonas*, *Desulfovibrio* and *Opitutus* (Table 4.8). Twelve genera, including *Enterococcus, Helicobacter,* and *Lactococcus,* characterized adult females, and 17 genera, including *Faecalibacterium, Roseburia,* and *Ruminococcus,* characterized juveniles (Table 4.8). The ratio of Firmicutes to Bacteroidetes, a measure of energy harvest potential by the microbiota, tended to be higher in adult females (7.85 ± 1.61) compared to adult males (5.73 ± 0.57) and juveniles (5.35 ± 2.56).

Non-parametric Mantel tests indicated a correlation between patterns of microbial community composition and the plant species consumed by individual howlers (Spearman's ρ = 0.49, p = 0.006). There was a significant correlation between the relative abundance of *Ruminococcus* and the amount of kilocalories consumed (Spearman's ρ = 0.90, p = 0.005) and between the relative abundance of *Caulobacterales* and NDF (Spearman's ρ = -0.88, p = 0.004), but I detected no other significant correlations between any microbial taxa and individual components of the diet. No significant relationship between microbial community composition and metabolite profiles was detected, and there was no correlation between any single bacterial taxa and any metabolite.

Gut microbial community function: The total amount of volatile fatty acids detected in fecal samples did not differ significantly across age and sex classes (χ^2= 1.74, p = 0.19; χ^2= 0.08, p = 0.77), but fecal VFA profiles differed by host age (Table 4.9, 4.10, Figure 4.8). This pattern was

not driven by differences in any single VFA concentration, and the proportions of VFAs produced did not vary across age and sex classes (Table 4.9, Figure 4.9). I detected no differences in fecal ammonia content between age and sex classes (Adult Female: 5.5 ± 0.9, Adult Male: 5.4 ± 1.8, Juvenile: 6.3 ± 1.6; χ^2= 0.54, p = 0.46; χ^2= 0.24, p = 0.62). Sampling block did not affect patterns in fecal VFA or ammonia concentrations. No significant correlation between VFA profiles and any component of diet was detected across age and sex classes. Microbial community composition and VFA profiles also were not correlated overall, and no individual bacterial taxa demonstrated a correlation with ammonia or any individual VFA.

DISCUSSION

In this study, I tested several hypotheses regarding the behavioral and physiological mechanisms howler monkeys might use to meet changing nutritional demands. Specifically, I predicted that if no differences were detected in activity budget across age and sex classes, adult female and juvenile howler monkeys would (1) consume more protein and energy than adult males, (2) exhibit gut microbial community composition distinct from that of adult males, and (3) exhibit a gut microbial community function distinct from that of adult males. I expected these differences to vary depending on diet and energy and protein intake, and I also expected juveniles to exhibit more dramatic changes compared to females due to the relatively higher costs of growth compared to reproduction. My data indicated that, as observed in studies of many other primate taxa (Baldwin and Baldwin 1974, Fagen 1993, Oliveira et al 2003, Prates and Bicca-Marques 2008, Stevenson et al 2005, Watts and Pusey 1993), juvenile howlers spent more time being social and less time resting than adults. However, I detected no other activity differences among age and sex classes that would suggest compensation for increased energy

and nutrient needs. Therefore, I expected to find differences in both diet and gut microbial community activity and function across howler age and sex classes.

Diet: My data provide evidence that howler monkey diet differs across age and sex classes. Differences in the plant parts and plant species consumed by adult male, adult female, and juvenile black howler monkeys resulted in differences in the arrays of plant metabolites consumed. However, because I detected no significant differences in the consumption of any single metabolite among age and sex classes, it appears that the overall variation in metabolite intake across age and sex classes was a result of adult male, adult female, and juvenile howlers consuming slightly different amounts of a wide variety of metabolites. This pattern suggests that female and juvenile howler monkeys may differentially exploit the same foods as adult males to alter overall metabolite intake patterns. It is possible that the additive effect of subtle differences in a variety of single metabolites aids females and juveniles in meeting the nutritional demands of reproduction and growth.

Additionally, published nutritional data for the food items consumed suggest that adult females and juveniles consumed more protein, lipids and total non-structural carbohydrates per metabolic body weight than adult males as well as more overall energy across the entire study (Table 4.4). Within each season, adult females and juveniles also consumed more protein per metabolic body weight than males and exhibited a non-significant trend of increased energy intake. Metabolite analyses also indicated that juvenile howlers consumed a higher relative concentration of amino acids per metabolic body weight compared to adults, especially during Block 2 (Table 4.5). Therefore, it appears that females and juveniles consumed both more energy

and more protein than adult males despite relatively limited age- and sex-based variation in general dietary categories such as ripe fruits, young leaves, and flowers.

The differences I observed in terms of nutritional intake among adult female, adult male, and juvenile howler monkeys generally did not change across sampling blocks despite strong variation in the plant parts, plant species, and metabolites consumed. This pattern suggests that individuals maintain similar foraging strategies regardless of the types of food being consumed. Although the amount and origin of total energy and protein consumed changes across time females and juveniles always consume more energy and protein than males.

Because the estimated costs of juvenile growth are higher than the estimated costs of female reproduction (Aiello and Wells 2002, Altmann and Alberts 1987, Oftedal et al 1991), I expected juvenile howlers to consume more energy and protein than adult females. I did not observe this trend in my nutritional data. Either the relative costs of growth and reproduction differ from estimates, or juveniles are using physiological mechanisms such as shifts in the gut microbial community to make up for nutritional requirements not met by diet.

Gut microbial community composition: My microbial data suggest that juveniles possessed a distinct gut microbial community from adults. Overall gut microbial community composition differed according to howler age, and taxonomic data indicated that juveniles were characterized by higher relative abundances of *Paraprevotella* and *Roseburia* compared to other group members and were also characterized by *Faecalibacterium, Oribacterium, Oscillibacter, Robinsoniella,* and *Ruminococcus*. Most of these genera belong to the Firmicutes phylum and have been shown to efficiently produce VFAs that benefit the host (Cotta et al 2009, Khan et al 2012, Schleifer 2009, Turnbaugh et al 2006). For example, *Roseburia* is a known butyrate-

producer, and some strains also have urease activity (Duncan et al 2002, Duncan et al 2007, Pryde et al 2002, Yatsunenko et al 2012). Because butyrate is the primary energy source for colonocytes (Flint et al 2012, Roediger 1980) and urease contributes to host and microbe protein balances (Langran et al 1992, Meakins and Jackson 1996), increased relative abundances of *Roseburia* in the juvenile howler gut may provide metabolic benefits for growing juvenile howlers. Likewise, VFA production by other Firmicutes characteristic of the juvenile howler microbiota may contribute to colonocyte energy balances, as well as host lipogensis and muscle metabolism (Flint et al 2012).

Gut microbial surveys of humans indicate that infants have a simpler gut microbial community than adults, dominated by *Bifidobacterium* (Benno and Mitsuoka 1986, Kurokawa et al 2007, but see Palmer et al 2007, Rinne et al 2005, Yatsunenko et al 2012), which plays an important role milk metabolism and vitamin biosynthesis (LeBlanc et al 2012, Rossi et al 2011, Sela et al 2008, Sela and Mills 2010, Yatsunenko et al 2012). I identified no *Bifidobacterium* in either adult or juvenile howlers, and although I saw a trend for lower microbial richness in juvenile howler monkeys, there were no significant differences in this measure of microbial community complexity. Because the juveniles in this study foraged independently on a diet very similar to that of adults and appeared to be completely weaned, comparisons to infant humans may be inappropriate. In human infants, there is evidence that the gut microbiota has the ability to break down plant-derived compounds before weaning (Koenig et al 2011, Kurokawa et al 2007), and weaned juvenile howler monkeys in this study were likely even more well-adapted to a plant-based diet. Therefore, we would not expect to see high abundances of microbial taxa associated with a milk-heavy diet (Koenig et al 2011). Furthermore, the adult gut microbiota is

generally established in humans by the time children reach one year of age (Mackie et al 1999), and the juvenile howler monkeys that I included in this study were all at least one year old.

Although overall microbial community composition did not appear to differ across adult sex classes, increasing the sample size with data from El Tormento revealed distinctions between adult males and adult females. Furthermore, adult females from Palenque possessed a distinct microbial community compared to adult males and juveniles from Palenque. Adult females at Palenque had a higher Firmicutes to Bacteroidetes ratio than adult males and juveniles suggesting higher fermentation efficiency and increased production of VFAs (Schleifer 2009, Turnbaugh et al 2006). As was the case with juveniles, potentially higher VFA production from Firmicutes bacteria may aid females in meeting increased energy requirements. Additionally, females were characterized by *Lactococcus,* which has been associated with folate biosynthesis in humans (Yatsunenko et al 2012). Folate is a crucial vitamin for pregnant woman since it plays a role in DNA synthesis, and deficiencies can result in neural-tube defects and other developmental complications (Czeizel and Dudas 1992, Czeizel et al 2010, Lamers 2011). *Lactococcus* in the female howler gut may be an important source of folate. Finally, while many strains of *Helicobacter* are thought to be pathogenic, the urease activity of *Helicobacter* allows the creation of ammonia and carbon dioxide from urea (Reitzer and Magasanik 1987, Yatsunenko et al 2012). Ammonia is an important substrate for amino acid production by microbes, and urease also plays an important role in nitrogen recycling when host diets are low in protein (Langran et al 1992, Meakins and Jackson 1996, Reitzer and Magasanik 1987). Therefore, higher abundances of *Helicobacter* in female howlers may lead to a more robust protein source for other energy- and nutrient-producing microbes (Yatsunenko et al 2012).

Few human studies have investigated the relationship between the gut microbiota and host nutrition in women, especially during pregnancy and lactation (Collado et al 2008, Koren et al 2012, Santacruz et al 2010). However, pregnancy has been associated with increased numbers of gut bacteria (Collado et al 2008), decreased bacterial richness, and increased relative abundances of Proteobacteria and Actinobacteria (Koren et al 2012). Additionally, studies investigating obesity in pregnant women have reported increased levels of *Bacteroides, Clostridium, Staphylococcus,* and Enterobacteraceae associated with weight gain (Collado et al 2008, Santacruz et al 2010). *Bifidobacterium* has been associated with increased folic acid and transferrin levels and reduced ferritin while *Bacteroides* has been associated with increased HDL-cholesterol and folic acid (Santacruz et al 2010). In the black howler monkey dataset, there were no differences in in microbial richness across age and sex classes, and I did not observe high relative abundances of any of the bacteria identified in human studies in female howler monkeys, with the exception of *Enterococcus* (Family: Enterobacteraceae). Most of the bacterial taxa identified in studies of pregnant women were either absent or present in very low levels in howler monkeys.

It is also interesting to note that while female howlers possessed gut microbial communities that appear to have some nutritional benefits, many of the genera that I found characterizing female howler microbial communities contain species or strains that have been associated with disease in humans, including *Catonella, Corynebacterium, Helicobacter, Mogibacterium, Mycobacterium,* and *Pseudomonas* (Chen et al 2012, Chichlowski et al 2008, Fox et al 2001, Hermon-Taylor 2009, Madi et al 2010, Prescott et al 1980). Although it is impossible to know whether the bacteria I detected are pathogenic due to the relatively broad taxonomic resolution of my analyses, a high number of pathogenic bacteria characterizing the

female howler gut microbial community could be a result of hormone-induced changes in the immune system and its interaction with the gut microbiota (Gabrilovac et al 1988, Marzi et al 1996, Raghupathy 1997, Wegmann et al 1993, Zaph et al 2008). However, it is also entirely possible that these genera represent normal, non-pathogenic residents of the female gut (e.g. Woolcock and Mutimer 1980). Further research is necessary to understand the role of these genera in the female gut microbiota.

Although diet and microbial community composition and function varied in both howler groups across sampling blocks, the differences among age and sex classes did not. Changes in howler diet across time have been correlated to shifts in gut microbial community composition and function (Chapter 3). Therefore, since adult male, female, and juvenile howler diets consistently differed from each other in terms of the metabolites consumed despite changes in the group diet composition, we would also expect adult male, female, and juvenile howler gut microbial community composition and function to differ from each other in similar ways across time. However, because the relative abundances of microbial taxa that differed among age and sex classes in this study were not strongly correlated to variations in diet, it is possible that the microbial taxa that vary across age and sex classes are responding to differences in host physiology (e.g. immune system, sex hormones, growth hormones) and not diet. If we compare the results from this study to the results from a study describing differences in gut microbial taxa across time in response to diet, only the order, Bacillales, varies in relative abundance in response to both diet and age and sex class (Chapter 3). Therefore, it seems likely that the bacterial taxa identified in each study are responding to distinct selective pressures.

Gut microbial function: Despite differences in microbial community composition, no strong differences were detected among adult male, adult female, and juvenile howlers in microbial activity. Fecal VFA and ammonia concentrations across individuals were virtually identical throughout the study. For adult males and females, this suggests that microbial protein metabolism and fiber fermentation did not differ despite differences in the Firmicutes:Bacteroidetes ratio. Therefore, to benefit host nutrition, differences in the gut microbiota must be linked to vitamin production or other processes that were not measured in this study (Koenig et al 2011, LeBlanc et al 2012, Yatsunenko et al 2012). However, it also is possible that differences in the rates of VFA and ammonia absorption existed among individuals, obscuring my ability to detect changes in production by measuring net VFA and ammonia excretion (Vogt and Wolever 2003).

In contrast, although juveniles consumed fewer grams of food than adults and, due to body size, presumably possessed smaller guts with potentially shorter retention times and therefore less time for microbial fermentation (Demment and Van Soest 1985, Parra 1978), they generally produced equal amounts of ammonia and VFA's as adults. If measures of fecal VFA and ammonia concentration are proportional to production (Mackie et al 1998), these data suggest that juvenile howler microbial communities were more active in terms of protein metabolism and energy production than those of adults. My observation of a high number of VFA-producing bacteria from the Firmicutes phyla characterizing the juvenile gut microbiota supports this hypothesis.

Reproduction and growth: Due to limited sample sizes, the influences of distinct stages of the female reproductive cycle on female behavior, diet, and gut microbial community composition

and function could not be explored statistically. Additionally, based on the timing of infant births during the study, all females cycled through pregnancy and lactation in relative synchrony, making it difficult to separate the effects of time and reproductive stage. All four females appeared to be pregnant during Block 1, and three gave birth and began lactating during Blocks 2 and 3. Black howlers have been reported to have birth peaks during the dry season in Belize (Brockett et al 2000), and Block 3 loosely corresponded with the dry season. Timing births to coincide with seasons in which nutrient intake is high can be a strategy for overcoming the increased nutritional demands of lactation and juvenile growth (Bitteti and Janson 2000). However, low energy and protein intake during lactation in Block 3 do not support this hypothesis, and 3.5 years of data do not suggest a strong pattern of birth seasonality in black howlers at Palenque (Van Belle, unpublished data).

Regardless of the sample size, if female reproductive stages influenced activity, diet, or gut microbiota, we would expect to see changes in these variables as the females cycled through reproduction, but sampling period was not a significant factor influencing these variables across age and sex classes. Instead, it seems that, compared to adult males, adult female howlers maintained small differences in diet characterized by increased energy and protein intake as well as differences in microbial community composition year-round compared to adult males. This result suggests that female energy and nutrient demands are constantly elevated above adult male levels. Periods in which female black howlers are neither gestating nor lactating are very short since interbirth intervals have been estimated at 16-22.5 months for the genus *Alouatta*, gestation lasts approximately 6 months, and juvenile howlers are weaned at about 11-14 months (Di Fiore et al 2011). There are also reports of black howler females conceiving while offspring are still dependent (Van Belle et al 2009). Therefore, it seems likely that female howlers rarely reach a

physical state with reduced nutritional demands that allow them to adopt the male diet or exhibit a gut microbiota similar to that of males.

Similarly, for juveniles older than one year I found no evidence that diet or gut microbial community composition and function varied with age. Juvenile howler monkeys exhibited the same differences in diet and gut microbial community composition and function when compared to adults regardless of the sampling block, and therefore their age. This pattern suggests that the nutritional costs of growth in juveniles do not change over time. Although primate juveniles experience growth peaks (Altmann and Alberts 1987, Garber and Leigh 1997), growth in howler monkeys occurs relatively linearly over a period of about 3-5 years until individuals reach adult body size (Leigh 1994). As a result, juveniles are likely to have constantly high energy and nutrient needs until they reach maturity, and require consistent differences from adults in diet and gut microbial community composition and function to compensate over this period.

Although they were constant, the differences I detected in howler diet, gut microbial composition, and microbial activity that could compensate for increased demands were subtle. Overall, age and sex only explained 10-30% of the variation in diet and gut microbiota, and a relatively small number of metabolites and bacterial taxa were driving the patterns I observed. These data suggest that the daily energy and nutrient demands associated with primate reproduction and growth may not be as great as generally estimated (Aiello and Wheeler 1995, Aiello and Wells 2002, Altmann and Alberts 1987, Altmann and Samuels 1992, Oftedal et al 1991). Compared to many mammals, primate gestation is long, fetal and postnatal growth slow, and primate milk is energetically dilute (Case 1978, Hinde and Milligan 2011, Martin 2007, Oftedal et al 1991), meaning daily needs for reproduction may be lower for primates than for many other mammals. Howler monkeys, in particular, have lower milk protein content and fat

concentrations than other New World primate taxa (e.g. *Cebus, Saimiri, Cebuella, Leontopithecus*; Hinde and Milligan 2011), making it less costly to produce the same quantity of milk in a given time period. Likewise, slow life histories, characterized by an extended juvenile period (Case 1978) may result in lower daily nutritional demands in juvenile primates compared to other juvenile mammals and reduce the amount of behavioral and physiological adjustment necessary to meet daily nutritional demands (Case 1978, Janson and van Schaik 1993). It is also important to note that as relatively large-bodied New World primates, howler monkeys may have an increased capacity to store energy and nutrients for later use (Oftedal 2000), making them less nutritionally susceptible to temporal fluctuations in food availability and diet (Dufour and Sauther 2002, Ellison 2003, Martin 2007). Increased nutrient and energy intake one day may allow howlers to endure decreased nutrient and energy intake on subsequent days, and energy and nutrient stores from one season may provide resources during another. As a result, subtle changes in behavior and physiology may be sufficient to allow hosts to obtain the energy and nutrients they require.

Because the nutritional demands of reproduction and growth vary across primate taxa depending on body size, brain size, interbirth intervals and age at first reproduction, and each taxa's ability to compensate for these demands depends on physiology and social systems as well as food availability and diet (Dufour and Sauther 2002, Ross 1998, Ross 2003), we would expect both the behavioral and physiological strategies for compensation to vary across primate taxa. For example, lactating gelada baboons spend more time feeding than other group members, and pregnant and lactating female titi monkeys consume more insects than other individuals (Dunbar and Dunbar 1988, Herrera and Heymann 2004). In these species we might expect changes in microbial community composition and function to be weaker than those observed in howler

monkeys since reductions in host activity and/or increases in energy and nutrient intake may provide sufficient compensation for the demands of reproduction. In contrast, if changes in diet composition, such as increased insect consumption, alter the selective pressures on microbial taxa in the gut, we would expect to see more dramatic changes in gut microbial community composition and function than those observed in black howler monkeys. Additionally, it is possible that variation in diet metabolite content across age and sex classes in these species is similar to that observed in howler monkeys but more strongly correlated to patterns of plant part or plant species intake. In this case, variation in nutritional intake would be more easily detectable using focal data describing the plant parts and plant species consumed, but we would expect to observe microbial shifts similar to those in howler monkeys.

CONCLUSION

The results of this study suggest that female and juvenile black howler monkeys experience constant increases in energy and nutrient demands due to reproduction and growth. To meet these demands, they use both changes in diet including increased energy and protein intake as well as shifts in their gut microbiota including increased relative abundances of energy-producing bacteria from the Firmicutes phylum. However, further research is necessary to understand the influence of age- and sex-based differences in gut microbial community composition on the function of the gut microbiota and the supply of energy and nutrients to the host. Similarly, evaluations of host nutritional status are crucial for understanding trade-offs between energy requirements, energy intake, and the utilization of energy stores (Dufour and Sauther 2002, Ellison 2003). The gut microbiota are likely to impact primate nutrition and health

across a variety of taxa, but the type and magnitude of that impact is likely to change depending both on host physiology and host diet and/or habitat.

TABLES

Table 4.1. PERMANOVA results for the effect of host age and sex on activity. SS = sums of squares; MS = mean squares

	df	**SS**	**MS**	**F value**	**R^2**	**P value**
Age	1	0.074	0.074	49.17	0.77	0.0002
Sex	1	0.0065	0.0065	4.31	0.07	0.06
Age*Sex	1	0.0057	0.0057	3.77	0.06	0.06
Residuals	9	0.013	0.0015		0.14	
Total	12	0.096			1.00	

Table 4.2. PERMANOVA results for the effect of host age and sex on diet. SS = sums of squares; MS = mean squares

	df	SS	MS	F value	R^2	P value
Plant Part (g)						
Age	1	0.069	0.069	4.73	0.21	0.009
Sex	1	0.05	0.05	3.43	0.15	0.029
Age*Sex	1	0.097	0.097	6.58	0.30	0.002
Residuals	9	0.13	0.015		0.40	
Total	12	0.33			1.00	
Plant Part (%)						
Age	1	0.025	0.025	2.00	0.13	0.09
Sex	1	0.016	0.016	1.28	0.09	0.23
Age*Sex	1	0.039	0.039	3.08	0.21	0.04
Residuals	9	0.11	0.013		0.60	
Total		12				1
Plant Species (g)						
Age	1	0.25	0.25	1.68	0.13	0.018
Sex	1	0.14	0.14	0.9	0.07	0.2
Age*Sex	1	0.24	0.24	1.62	0.12	0.017
Residuals	9	1.36	0.15		0.69	
Total	12	1.97			1.00	
Plant Species (%)						
Age	1	0.19	0.19	1.52	0.12	0.21
Sex	1	0.12	0.12	0.96	0.08	0.14
Age*Sex	1	0.15	0.15	1.19	0.1	0.06
Residuals	9	1.14	0.13		0.73	
Total	12	1.58			1.00	
Plant Metabolite (conc)						
Age	1	0.089	0.089	3.26	0.17	0.06
Sex	1	0.056	0.056	2.04	0.11	0.14
Age*Sex	1	0.16	0.16	5.77	0.3	0.01
Residuals	9	0.25	0.03		0.47	
Total	12	0.52			1.00	

Table 4.3. Plant part nutritional content based on published values.

Species	Part	Crude Protein	Available Protein	Lipids	Sugars	Source
Dendropanax arboreus	Ripe Fruit	5.4	3.0	21.8	10.6	Felton et al. 2009
Ficus americana	Ripe Fruit	7.5	4.4		4.0	Silver 2000
Ficus aurea•	Ripe Fruit	7.1	4.0*	3.6	8.7	Milton 2008
Ficus insipida	Ripe Fruit	7.0	4.0*	5.8	14.5	Milton et al. 1980, 2008
Ficus pertusa	Ripe Fruit	5.8	2.4	1.9	38.8	Felton et al. 2009
Ficus yoponensis	Ripe Fruit	7.5	4.2*	6.0	11.3	Milton et al. 1980, 2008
Poulsenia armata	Ripe Fruit	7.9	7.9*		56.4	Estrada et al. 1984
Other	Ripe Fruit	7.6	7.68	4.3	58.3	Norconk et al. 2009
Average		7.0	4.7	7.2	25.3	
Brosimum alicastrum	Unripe Fruit	7.2	7.28	1.2	20.7	Estrada et al. 1984, Milton 2008
Other	Unripe Fruit	7.6	7.6*	4.3	58.3	Norconk et al. 2009
Average		7.4	7.4	2.8	39.5	
Ficus insipida	Young Leaf		10.6		2.9	Milton 1979
Ficus yoponensis	Young Leaf		10.5		6.9	Milton 1979, 1981
Poulsenia armata	Young Leaf		8.5			Milton 1979
Other	Young Leaf	20.1	20.1*	1.7	20.3	Norconk et al. 2009
Average		20.1	12.4	1.7	10.0	
Other	Mature Leaf	14.4	14.4*	1.5	17.7	Norconk et al. 2009
Other	Flower	16.8	16.8*	2.3	30.1	Norconk et al. 2009
Other+	Stem	14.9	13.0		7.8	Silver 2000

+estimated using *Schizolobium parahyba* values

•estimated using *Ficus obstusifolia* values

*used CP for AP since no AP estimate existed

Table 4.4. Average nutrient and energy intake by howler age and sex class. *Indicates significant differences between age and sex classes ($p < 0.05$). TNC: total non-structural carbohydrates; NDF: neutral detergent fiber; NPE: non-protein energy (kcal); PE: protein energy (kcal). All non-proportion values are standardized by metabolic body weight.

	Male		**Female**		**Juvenile**	
	Average	SD	Average	SD	Average	SD
Kcal*	113.4	21.0	162.5	26.9	149.4	36.9
Protein(g)*	6.6	1.2	9.9	0.8	9.0	1.4
% Protein*	12.3%	0.6%	11.5%	1.6%	11.4%	1.8%
Lipid (g)*	1.8	0.4	2.9	0.8	2.4	0.7
% Lipid*	3.3%	0.2%	3.2%	0.4%	3.0%	0.4%
TNC (g)*	16.5	3.6	21.7	2.6	21.5	6.7
% TNC	30.7%	3.1%	24.9%	3.4%	26.3%	3.7%
NDF (g)*	24.0	4.6	40.4	8.3	37.1	8.4
NPE (MJ)*	346.4	74.6	447.0	51.3	432.5	129.9
PE (MJ)*	105.0	18.8	150.4	9.4	140.2	23.0
NPE:PE	3.3	0.4	3.0	0.2	3.0	0.6

Table 4.5. Relative concentration (per metabolic body weight) of amino acids, sugar metabolites, and lipid metabolites consumed by adult male, adult female, and juvenile howler monkeys across sampling blocks. *Indicates significantly different values ($p < 0.05$).

		Amino Acids		**Sugars**		**Lipids**	
		Average	SD	Average	SD	Average	SD
Block 1	Male	5,430,365	3,734,189	181,834,665	68,492,356	6,089,311	23,41,556
	Female	4,265,977	1,961,781	75,706,208*	48,237,210	4,525,948	854,078
	Juvenile	7,251,397	1,958,045	158,202,457	101,103,148	5,685,388	2,471,488
Block 2	Male	4,412,642	1,824,821	55,460,000	4,604,992	3,053,480	22,0865
	Female	3,136,060	1,462,232	26,741,120*	4,978,255	1,617,971*	36,6094
	Juvenile	6,020,296*	1,360,691	52,510,641	9,479,299	3,833,126	725,416
Block 3	Male	1,483,248	1,085,996	76,277,998	58,559,201	2,071,785	586,066
	Female	583,804	199,440	59,409,201	29,200,172	1,213,066*	407,155
	Juvenile	2,006,055	1,396,324	80,736,707	39,073,383	2,546,451	670,870

Table 4.6. PerMANOVA results for the effect of host age and sex on gut microbial community composition after controlling for group differences. ARISA (+) indicates the model with individuals from El Tormento, Campeche used to verify patterns in the Palenque dataset. SS = sums of squares; MS = mean squares

	df	SS	MS	F value	R^2	P value
ARISA - OTU						
Age	1	0.12	0.12	1.19	0.10	0.019
Sex	1	0.09	0.09	0.88	0.07	0.68
Age*Sex	1	0.10	0.10	1.00	0.08	0.34
Residuals	9	0.92	0.10		0.75	
Total	12	1.23			1.00	
ARISA (+) - OTU						
Age	1	0.35	0.35	1.58	0.05	0.002
Sex	1	0.17	0.17	0.79	0.02	0.52
Age*Sex	1	0.25	0.25	1.15	0.04	0.025
Residuals	29	6.40	0.22		0.89	
Total	32	7.16			1.00	
454 - Class						
Age	1	0.002	0.002	1.41	0.13	0.25
Sex	1	0.002	0.002	1.88	0.18	0.16
Age*Sex	1	0.003	0.003	2.97	0.28	0.07
Residuals	4	0.004	0.001		0.38	
Total	7	0.011			1.00	
454 - Order						
Age	1	0.001	0.001	1.17	0.11	0.31
Sex	1	0.002	0.002	1.99	0.19	0.16
Age*Sex	1	0.003	0.003	3.09	0.29	0.07
Residuals	4	0.004	0.001		0.38	
Total	7	0.01			1.00	
454 - Family						
Age	1	0.001	0.001	0.88	0.11	0.52
Sex	1	0.006	0.006	0.80	0.1	0.55
Age*Sex	1	0.023	0.023	3.15	0.38	0.06
Residuals	4	0.03	0.008		0.49	
Total	7	0.06			1.00	
454 - Genus						
Age	1	0.056	0.056	0.72	0.1	0.65
Sex	1	0.051	0.051	0.66	0.1	0.73
Age*Sex	1	0.11	0.11	1.39	0.2	0.22
Residuals	4	0.31	0.08		0.58	
Total	7	0.54			1.00	

Table 4.7. Relative abundances of microbial taxa that differed significantly ($p < 0.05$) across age and sex classes in a subset of the sampled individuals (N = 8). *Indicates taxa that differed significantly among adult males and females. Other taxa differed among adults and juveniles.

		Male		**Female**		**Juvenile**	
		Average	SD	Average	SD	Average	SD
Epsilonproteobacteria	Class	2.4E-05	2.7E-06	5.5E-05	3.5E-05	7.1E-06	1.0E-05
Opitutae	Class	2.2E-03	2.2E-03	1.7E-03	1.2E-03	3.8E-05	1.9E-05
Bacillales*	Order	2.5E-05	1.1E-07	0.0E+00	0.0E+00	0.0E+00	0.0E+00
Campylobacterales	Order	2.4E-05	2.7E-06	5.5E-05	3.5E-05	7.1E-06	1.0E-05
Caulobacterales*	Order	2.8E-05	5.5E-07	6.1E-06	1.2E-05	6.1E-05	8.6E-05
Opitutales	Order	1.1E-03	1.2E-03	5.9E-04	5.6E-04	7.1E-06	1.0E-05
Rhizobiales	Order	2.6E-04	1.3E-04	4.1E-04	2.5E-04	1.4E-04	2.4E-05
Solirubrobacterales*	Order	1.0E-04	1.3E-04	0.0E+00	0.0E+00	0.0E+00	0.0E+00
Brachyspiraceae	Family	2.2E-04	9.7E-05	2.4E-04	4.7E-05	2.9E-04	1.3E-06
Caulobacteraceae*	Family	2.8E-05	5.5E-07	6.1E-06	1.2E-05	6.1E-05	8.6E-05
Helicobacteraceae	Family	2.4E-05	2.7E-06	5.5E-05	3.5E-05	7.1E-06	1.0E-05
Opitutaceae	Family	1.1E-03	1.2E-03	5.9E-04	5.6E-04	7.1E-06	1.0E-05
Anaerotruncus	Genus	4.4E-04	1.0E-04	5.1E-04	4.2E-04	1.6E-04	5.3E-05
Anaerovorax	Genus	3.9E-04	6.3E-05	6.5E-04	2.3E-04	1.6E-04	2.8E-05
Brachyspira	Genus	2.0E-04	7.4E-05	2.4E-04	4.7E-05	2.9E-04	1.3E-06
*Brevundimonas**	Genus	6.1E-05	4.6E-05	0.0E+00	0.0E+00	4.7E-05	6.6E-05
Paraprevotella	Genus	0.0E+00	0.0E+00	0.0E+00	0.0E+00	3.7E-05	6.9E-06
Roseburia	Genus	3.7E-03	1.9E-03	5.1E-03	1.5E-03	9.5E-03	4.6E-03
Sphingobacteria	Genus	0.0E+00	0.0E+00	6.2E-06	1.2E-05	0.0E+00	0.0E+00

Table 4.8. Average relative abundance (± SD) of microbial genera characterizing adult male, adult female (*), and juvenile (+) howler monkeys.

	Male		**Female**		**Juvenile**	
	Average	SD	Average	SD	Average	SD
*Acetanaerobacterium**	0.0E+00	0.0E+00	2.4E-05	2.8E-05	0.0E+00	0.0E+00
Aeromicrobium	1.6E-05	2.3E-05	0.0E+00	0.0E+00	0.0E+00	0.0E+00
Allobaculum	3.5E-04	4.9E-04	0.0E+00	0.0E+00	0.0E+00	0.0E+00
*Anaerovorax**	3.9E-04	6.3E-05	6.5E-04	2.3E-04	1.6E-04	2.8E-05
Asaccharobacter	5.7E-06	8.1E-06	0.0E+00	0.0E+00	0.0E+00	0.0E+00
Asteroleplasma+	1.8E-05	7.9E-06	5.6E-05	7.1E-05	2.0E-04	2.4E-04
Asticcacaulis+	0.0E+00	0.0E+00	0.0E+00	0.0E+00	1.4E-05	1.9E-05
Bacillus	1.3E-05	1.8E-05	0.0E+00	0.0E+00	0.0E+00	0.0E+00
Barnesiella+	7.5E-05	1.1E-04	3.1E-04	2.8E-04	4.2E-04	5.8E-04
Brevundimonas	6.1E-05	4.6E-05	0.0E+00	0.0E+00	4.7E-05	6.6E-05
*Catonella**	0.0E+00	0.0E+00	1.1E-05	1.3E-05	0.0E+00	0.0E+00
Chryseobacterium+	0.0E+00	0.0E+00	0.0E+00	0.0E+00	5.4E-06	7.6E-06
Conexibacter	5.4E-06	7.6E-06	0.0E+00	0.0E+00	0.0E+00	0.0E+00
*Corynebacterium**	0.0E+00	0.0E+00	2.1E-05	2.7E-05	0.0E+00	0.0E+00
Desulfovibrio	2.0E-03	8.9E-04	7.5E-04	7.3E-04	4.4E-04	3.5E-04
Dialister	2.1E-03	2.8E-03	6.2E-05	3.6E-05	7.2E-05	6.8E-05
*Enterococcus**	0.0E+00	0.0E+00	7.1E-05	8.5E-05	0.0E+00	0.0E+00
Erwinia+	0.0E+00	0.0E+00	0.0E+00	0.0E+00	4.5E-06	6.3E-06
Faecalibacterium+	5.6E-03	4.6E-03	1.1E-02	1.0E-02	1.9E-02	2.3E-03
Gemmatimonas	1.4E-05	2.0E-05	0.0E+00	0.0E+00	0.0E+00	0.0E+00
Gp1	2.8E-05	4.0E-05	0.0E+00	0.0E+00	0.0E+00	0.0E+00
Hallella	4.4E-02	2.7E-02	1.4E-02	1.2E-02	2.2E-02	1.4E-02
*Helicobacter**	1.7E-05	6.4E-06	6.0E-04	7.3E-04	7.1E-06	1.0E-05
Hespellia	1.5E-05	2.1E-05	0.0E+00	0.0E+00	0.0E+00	0.0E+00
Kofleria	6.5E-06	9.1E-06	0.0E+00	0.0E+00	0.0E+00	0.0E+00
*Lactococcus**	0.0E+00	0.0E+00	8.5E-06	1.1E-05	0.0E+00	0.0E+00
Marmoricola	2.0E-05	2.9E-05	0.0E+00	0.0E+00	0.0E+00	0.0E+00
Microbacterium	2.5E-05	3.5E-05	0.0E+00	0.0E+00	0.0E+00	0.0E+00
*Mogibacterium**	7.1E-04	8.9E-04	2.2E-03	1.0E-03	6.4E-04	3.9E-04
*Mycobacterium**	1.4E-05	2.0E-05	3.9E-05	4.2E-05	5.4E-06	7.6E-06
Opitutus	2.9E-04	1.7E-04	6.2E-05	5.4E-05	7.1E-06	1.0E-05
Oribacterium+	1.8E-05	7.8E-06	5.9E-05	6.0E-05	1.1E-04	1.5E-05
Oscillibacter+	9.2E-03	2.1E-03	1.2E-02	3.0E-03	2.6E-02	2.0E-02
Paraprevotella+	0.0E+00	0.0E+00	0.0E+00	0.0E+00	3.7E-05	6.9E-06
Parasporobacterium	6.0E-06	8.5E-06	0.0E+00	0.0E+00	0.0E+00	0.0E+00
Phyllobacterium	6.5E-06	9.1E-06	0.0E+00	0.0E+00	0.0E+00	0.0E+00
*Pseudomonas**	0.0E+00	0.0E+00	2.7E-05	2.0E-05	0.0E+00	0.0E+00
Rhizobium	1.8E-04	2.1E-04	2.1E-05	2.6E-05	2.7E-05	3.9E-05
Robinsoniella+	1.1E-05	1.6E-05	3.7E-05	3.4E-05	7.4E-05	6.0E-05

Table 4.8 (cont.)

Roseburia+	3.7E-03	1.9E-03	5.1E-03	1.5E-03	9.5E-03	4.6E-03
Ruminococcus+	1.2E-03	1.5E-04	1.4E-03	6.3E-04	2.7E-03	2.7E-03
Serratia	1.5E-05	2.1E-05	0.0E+00	0.0E+00	0.0E+00	0.0E+00
Shuttleworthia	1.3E-05	1.9E-05	0.0E+00	0.0E+00	0.0E+00	0.0E+00
Sphingomonas+	0.0E+00	0.0E+00	0.0E+00	0.0E+00	7.1E-06	1.0E-05
Streptomyces	6.5E-06	9.1E-06	0.0E+00	0.0E+00	0.0E+00	0.0E+00
*Streptophyta**	3.0E-04	1.1E-04	1.2E-03	6.6E-04	4.6E-04	8.9E-05
Subdivision 3 genera incertae sedis+	0.0E+00	0.0E+00	0.0E+00	0.0E+00	4.5E-06	6.3E-06
*Succiniclasticum**	3.0E-03	9.1E-04	7.1E-03	9.9E-03	3.7E-05	5.2E-05
Synergistes+	0.0E+00	0.0E+00	0.0E+00	0.0E+00	2.5E-05	3.5E-05
Tannerella+	0.0E+00	0.0E+00	0.0E+00	0.0E+00	1.7E-05	2.4E-05
TM7 genera incertae sedis+	1.4E-03	4.8E-04	1.2E-03	7.9E-04	5.1E-03	5.2E-03
Treponema	7.7E-04	1.1E-03	0.0E+00	0.0E+00	0.0E+00	0.0E+00

Table 4.9. PerMANOVA results for the effect of host age and sex on fecal VFA content in terms of millimolar concentration and molar proportions (%). SS = sums of squares; MS = mean squares

	df	SS	MS	F value	R^2	P value
Millimoles						
Age	1	0.018	0.018	11.79	0.41	0.007
Sex	1	0.006	0.005	3.3	0.11	0.1
Age*Sex	1	0.012	0.012	7.43	0.26	0.05
Residuals	9	0.014	0.002		0.31	
Total	12	0.045			1.00	
%						
Age	1	0.0001	0.0002	0.58	0.06	0.39
Sex	1	0.00004	0.00004	0.21	0.02	0.68
Age*Sex	1	0.00003	0.00003	0.18	0.017	0.66
Residuals	9	0.002	0.0002		0.88	
Total	12	0.002			1.00	

Table 4.10. Fecal VFA concentrations across age and sex classes. Patterns did not differ across sampling blocks.

		Acetic		Propanoic		Butanoic		Pentanoic		Isobutanoic		Isopentanoic		Total	
		AVG	SD	AVG	SD	AVG	SD	AVG	SD	AVG	SD	AVG	SD	AVG	SD
Male	**Balam**	38.6	0.4	3.5	0.3	2.3	0.7	0.3	0.1	0.1	0.0	0.2	0.1	45.0	1.6
	Motiepa	40.1	1.0	3.2	0.0	1.8	0.1	0.3	0.0	0.1	0.0	0.1	0.0	45.7	0.8
Female	**Balam**	40.9	0.3	3.8	0.1	2.4	0.0	0.4	0.0	0.1	0.0	0.2	0.0	47.8	0.4
	Motiepa	37.8	1.9	3.1	0.4	1.9	0.2	0.3	0.0	0.1	0.0	0.2	0.1	43.4	2.3
Juvenile	**Balam**	42.8	3.6	3.7	0.7	2.1	0.8	0.3	0.1	0.1	0.0	0.1	0.0	49.2	5.2
	Motiepa	48.0	9.6	4.0	0.8	2.2	0.4	0.4	0.1	0.1	0.0	0.1	0.0	54.9	10.9

FIGURES

Figure 4.1. Partial correspondence analysis illustrating patterns in the activity budget of adult male, adult female, and juvenile howlers with the effect of group removed. Each point represents the activity budget of one individual.

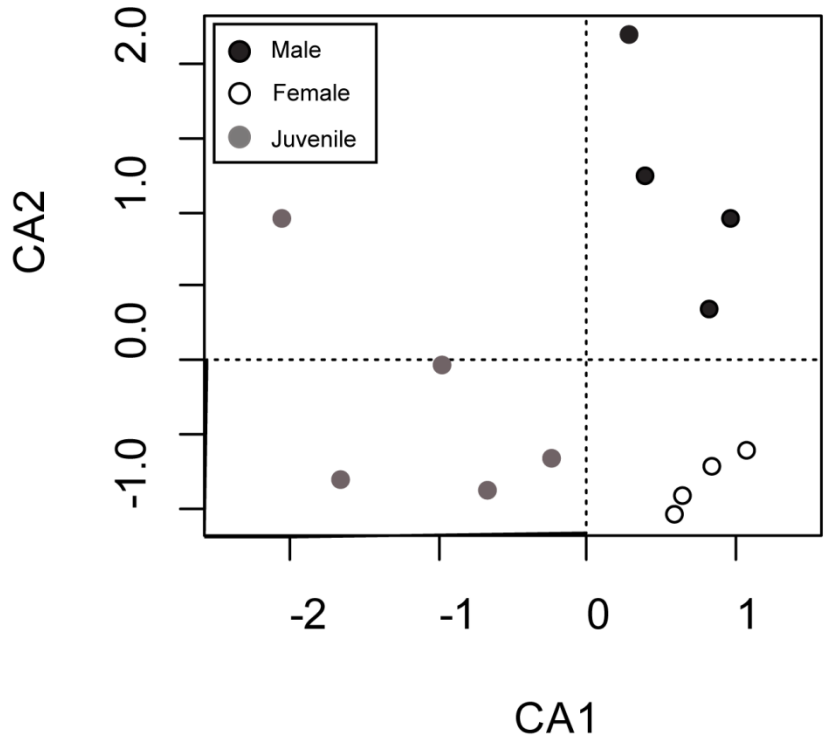


Figure 4.2. Activity budget of adult male, adult female, and juvenile howlers in terms of proportion of total minutes active. No differences were detected between groups or across time so data are combined.

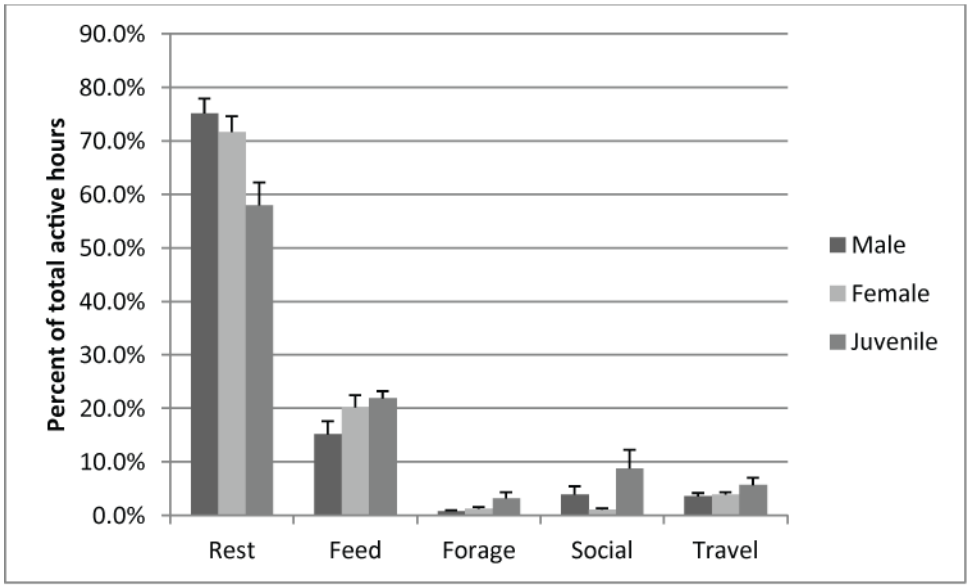


Figure 4.3. Partial correspondence analysis illustrating patterns in the plant parts consumed by adult male, adult female, and juvenile howlers with the effect of group removed. Each point represents the diet of one individual.

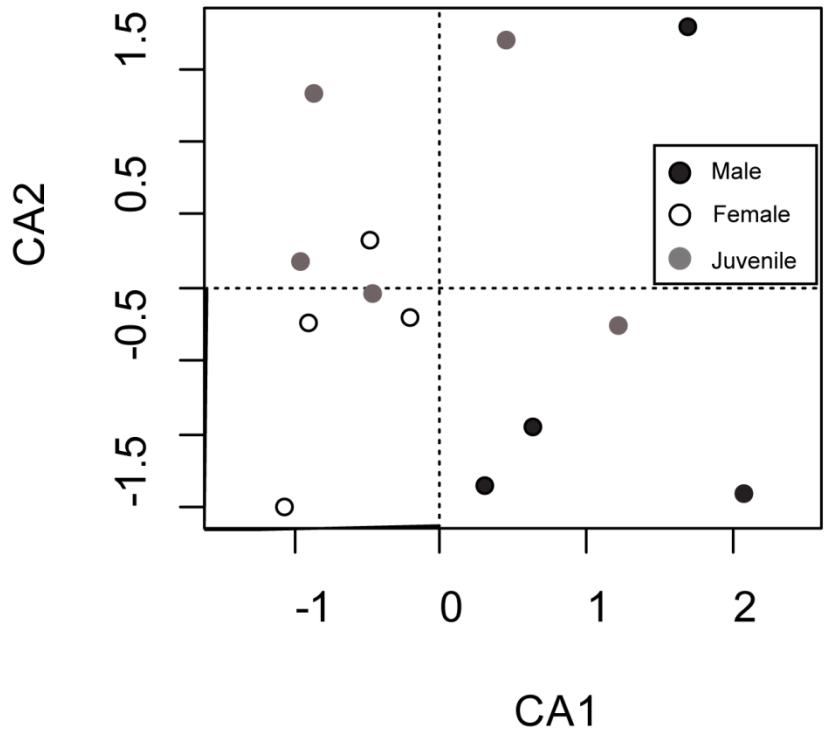


Figure 4.4. Plant parts consumed by howlers of different age and sex classes in terms of (A) grams per metabolic body weight and (B, C) proportion of total grams consumed. Proportions differed between groups so data are presented separately.

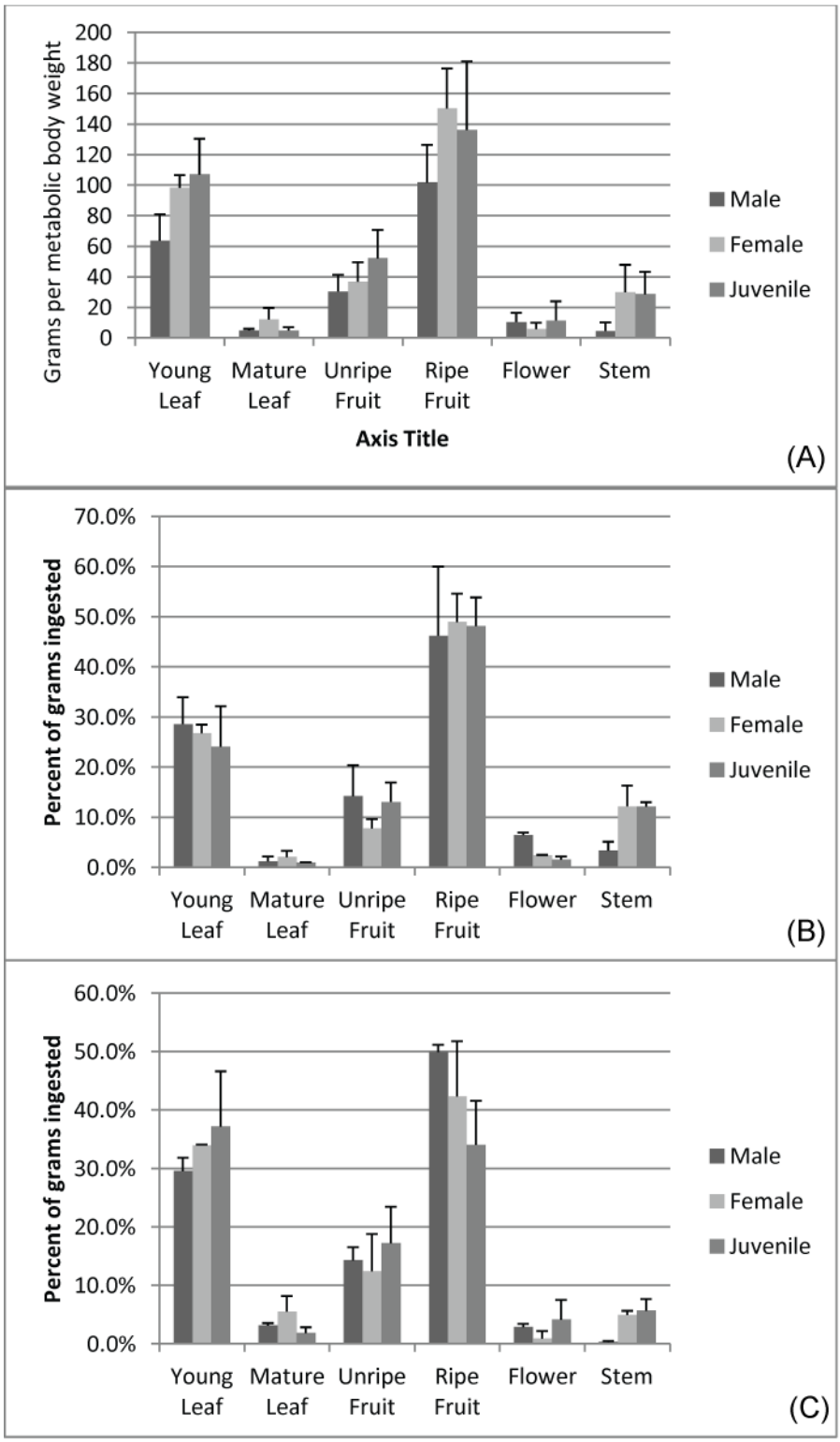


Figure 4.5. Partial correspondence analysis illustrating patterns in the grams of each plant species consumed by adult male, adult female, and juvenile howlers per metabolic body weight with the effect of group removed. Each point represents the diet of one individual.

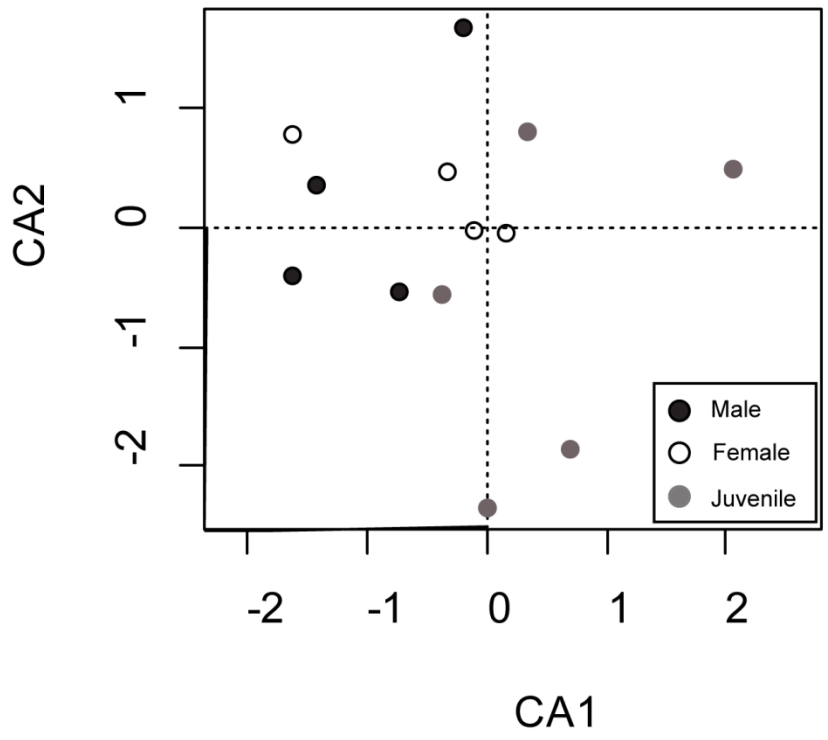


Figure 4.6. Partial correspondence analysis illustrating patterns in the plant metabolites consumed by adult male, adult female, and juvenile howlers with the effect of group removed. Each point represents the diet of one individual.

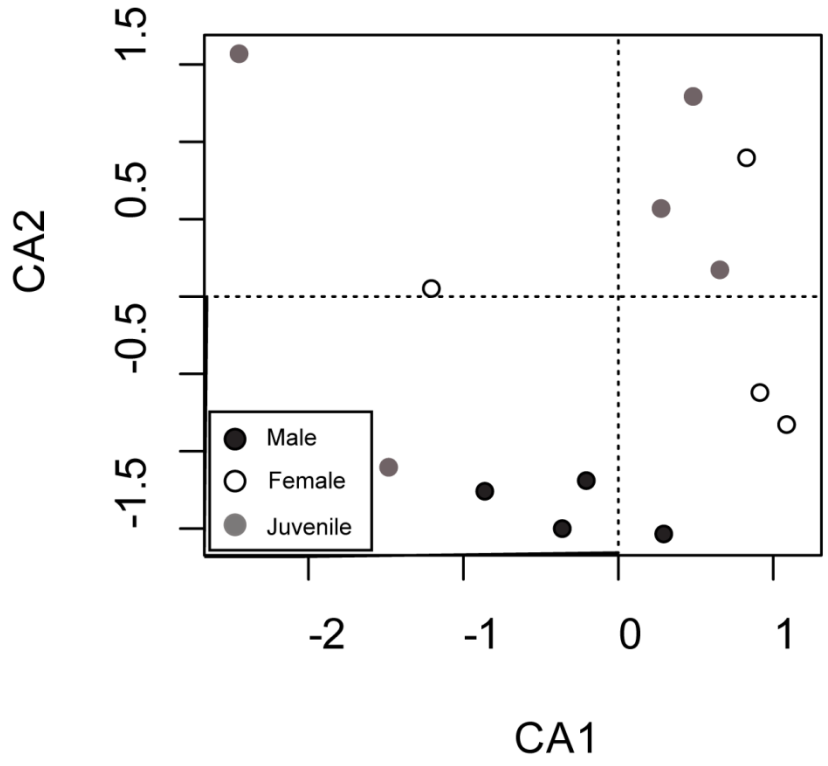


Figure 4.7. Partial correspondence analysis illustrating patterns in gut microbial community composition at the OTU level measured using ARISA for adult male, adult female, and juvenile howlers with the effect of group removed. Each point represents the microbial community of one individual.

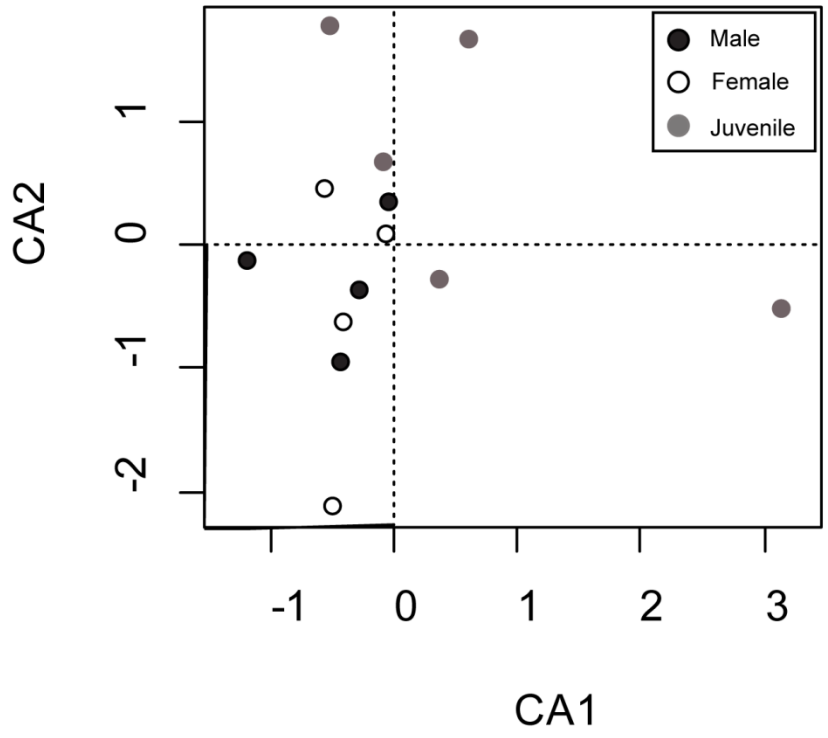


Figure 4.8. Partial correspondence analysis illustrating patterns in the VFA profiles (mM) of adult male, adult female, and juvenile howlers with the effect of group removed. Each point represents the VFA profile of one individual.

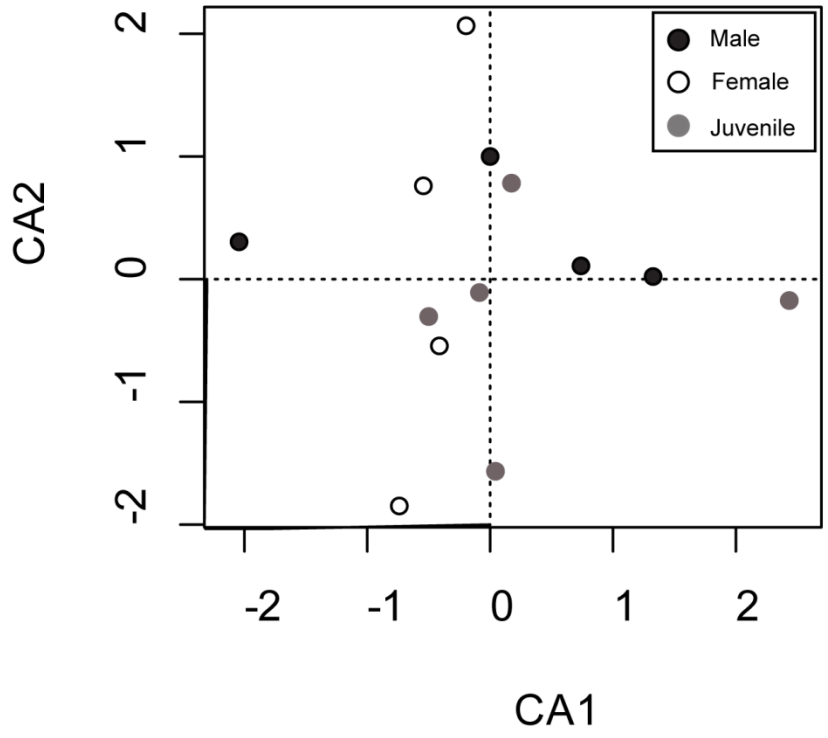
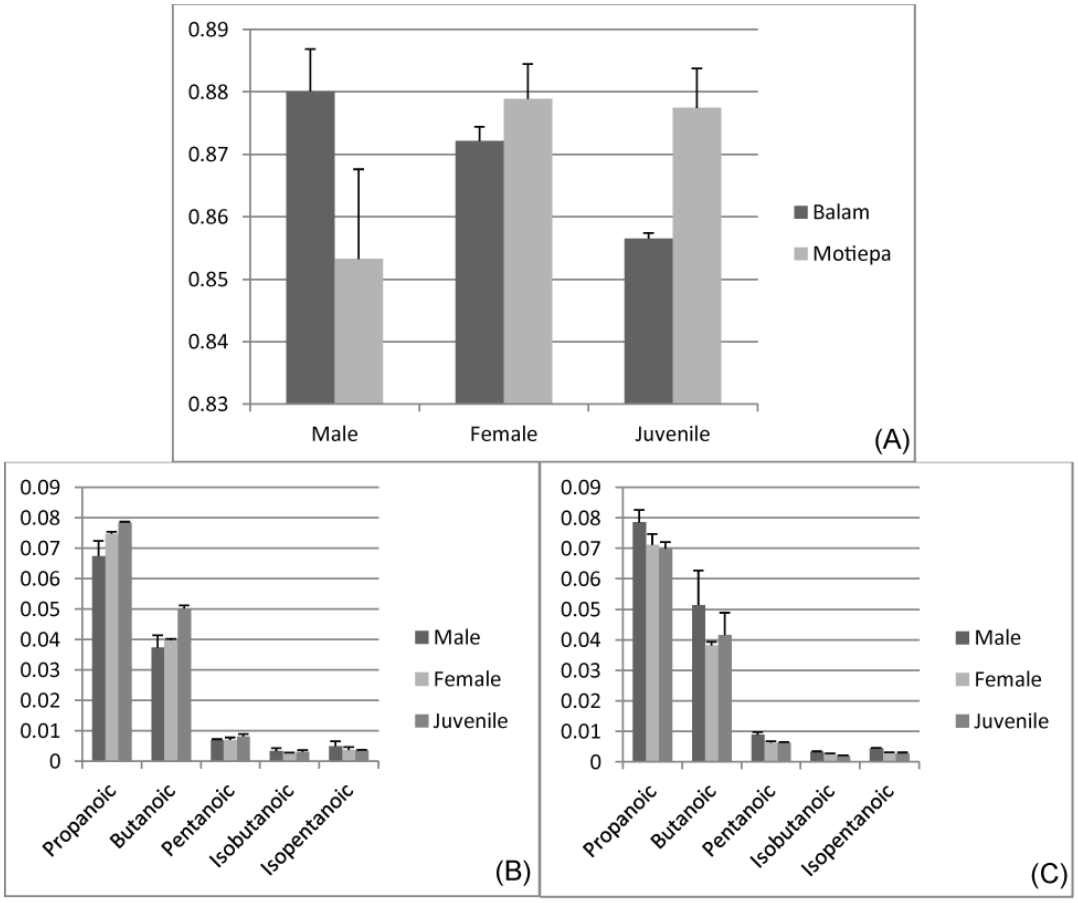


Figure 4.9. (A) Fecal acetic acid content of adult and juvenile howlers in terms of proportion of total millimoles in fecal material. Millimoles of other VFA's in fecal samples from the (B) Balam group and (C) Motiepa group.



CHAPTER 5: GENERAL CONCLUSION

The data presented in this dissertation provide a variety of new perspectives from which to approach studies of howler monkey foraging ecology and nutrition. Although howlers are able to consume large quantities of leaves, my data demonstrate that, on an annual basis, black howler monkeys consume more ripe fruits than leaves. They also exhibit a protein-regulating foraging strategy previously observed in ripe-fruit specialist spider monkeys and consume both more protein energy and more total energy than spider monkeys (Felton et al 2009a, Felton et al 2009b). These results suggest that black howler monkeys are more similar to other fruit-eating primates than commonly believed. Additionally, when feeding rates are taken into account, it appears that most howler species, with the exception of *A. caraya* and *A. guariba* , incorporate large proportions of fruit in their diets (Garber et al accepted).Therefore, it appears that although leaves are an important part of the howler diet, especially during periods of low fruit availability (Estrada 1984, Glander 1981, Milton 1980, Rumiz et al 1986), both fruits and leaves represent critical components of the howler diet, and each needs to be considered in assessing howler monkey feeding ecology.

A dietary emphasis on both fruits and leaves appears to be shared by all genera of the ateline radiation. Howler monkeys and muriquis (*Brachyteles* sp.) are reported to spend less average annual feeding time consuming fruits (5-59% and 12-73%, respectively) compared to spider monkeys (*Ateles*, 54-92%) and woolly monkeys (*Lagothrix*, 67-79%; Di Fiore et al 2011). However, assuming all atelines consume fruits more quickly than leaves, we would expect the annual percentage of the diet devoted to fruit to be larger for howler monkeys and muriquis than time-based estimates suggest. If this is the case, the greatest proportion of plant tissues consumed by all atelines is fruit, and it is likely that all genera exhibit similar protein-regulating foraging

strategies (although some variation may exist due to insect consumption in *Lagothrix*; Di Fiore et al 2011). Additionally, each ateline genus exhibits seasonal variation in diet composition and exploits hard-to-digest resources during periods of fruit scarcity (Di Fiore et al 2011). For example, on an annual basis, spider monkeys and woolly monkeys have been observed to spend an average of up to 17% and 16% of annual feeding time consuming leaves, respectively (Di Fiore et al 2011). Although howlers and muriquis consume more leaves annually (81% and 67%, respectively), these data suggest that hard-to-digest resources are an important component of all ateline diets. Consequently, it may be best to reframe investigations and discussions of the behavior of all atelines to highlight the importance of both leaves and fruits to nutritional balances.

Data from my dissertation also suggest that the impacts of the gut microbial community must be considered when investigating or discussing the ecology and evolution of howler monkey behavior and dietary patterns. The howler gut microbial community shifts in response to changes in the howler diet over time, enhancing howler digestive efficiency by breaking down undigested plant carbohydrates and providing energy-rich volatile fatty acids. These contributions appear to be most important during periods of reduced energy and nutrient intake (Block 3). In addition, adult female and juvenile howler monkeys are characterized by bacteria that produce more energy (Turnbaugh et al 2006) and vitamins (LeBlanc et al 2007) compared to adult males. These differences, together with differences in nutritional intake, may play a role in allowing females and juveniles to meet the increased nutritional demands of reproduction and growth, especially since highly cohesive social groups may not permit dramatic changes in energy and nutrient intake or expenditure among individuals (Pavelka 2011). As a result, while behavior and foraging patterns are important in understanding how howler monkeys respond to

temporal variation in food availability while maintaining activity, ranging and life history patterns, the nutritional contributions of the gut microbiota are also critical. The gut microbial community is not a static entity with a fixed function. It adapts to variations in host diet from season to season and provides the host with nutritional resources including energy and vitamins. These resources are typically not accounted for in traditional studies of behavior and feeding ecology but are crucial for understanding howlers' ability to utilize diets characterized by potentially high amounts of fiber and toxins as well as the effects of seasonality on howler monkey behavior, nutrition and health.

The nutritional contributions of the gut microbial community are also likely to be crucial for understanding the ecology and evolution of other primates as well. All primate habitats can be considered seasonal in that they undergo temporal variation in the availability of particular animal and plant tissues (Fenner 1998, Jordano 2000, van Schaik et al 1993a, van Schaik and Pfannes 2005). Additionally, an examination of 130 studies of 100 species and subspecies of primates revealed that over 70% of the primate responses to seasonality involve changes in diet composition (Hemingway and Bynum 2005). Therefore, the ability of the gut microbial community to adapt to and efficiently extract energy and nutrients from a variety of food items likely impacts the nutrition and health of all primates. Furthermore, many instances of dietary switching in primates involve the use of hard-to-digest resources during at least some months of the year (Hemingway and Bynum 2005). For example, sakis are known to utilize seeds with hard coats and potentially high levels of toxins (Di Fiore et al 2011, Norconk et al 2009). Orangutans can include up to 37% bark in their diets during periods of low fruit availability (Knott 1998), and African colobines consume large amounts of both mature leaves and seeds (Di Fiore et al 2011). Even small-bodied primates such as Goeldi's monkeys include large proportions of

soluble fiber-heavy exudates and fungus in their diets during some months (Di Fiore et al 2011, Porter and Garber 2004, Porter et al 2009, Power 1996). The gut microbial community is likely to play an important role in breaking down toxins and structural carbohydrates in all of these food items.

For primates occupying fragmented habitats with altered resource availability, the ability of the gut microbial community to adapt to the diet and break down toxins and structural carbohydrates is likely to be especially critical. In fragmented and anthropogenically-disturbed habitats howlers have been reported to consume food items from a distinct, and sometimes reduced, array of plant species, depend more on lianas as food sources, and utilize diets with higher year-round proportions of hard-to-digest resources such as leaves and stems (Arroyo-Rodriguez and Dias 2010, Cristobal-Azkarate and Arroyo-Rodriguez 2007, Dunn et al 2009). These patterns are especially strong in small, isolated forest fragments with high amounts of edge habitat (Arroyo-Rodriguez and Dias 2010, Cristobal-Azkarate and Arroyo-Rodriguez 2007, Dunn et al 2009, Dunn et al 2010), and in these situations, the nutritional contributions of the gut microbial community are likely to be critical to howlers. However, changes in the plant species utilized in degraded habitats may lead to shifts in the composition of the gut microbial community (Amato et al 2013). These changes have the potential to negatively affect howler nutrition and health by reducing the amount of energy produced by the gut microbial community and increasing the production of toxic microbial byproducts (Amato et al 2013). Therefore, to be successful, conservation efforts must address both the direct impacts of habitat degradation on howler nutrition and health as well as the indirect impacts regulated through the gut microbial community.

Since habitat degradation and anthropogenic impacts have similar effects on many primate diets (e.g. Chancellor et al 2012, Chaves et al 2012, Martins and Setz 2000, Riley 2007, Tesfaye et al 2013, Tutin 1999, Wong et al 2006), the dynamics of the gut microbial community are likely to be important to conservation efforts for all primate species. For example, spider monkeys (*Alouatta geoffroyi*) in Mexico have been shown to use a more diverse diet with a higher proportion of leaves in forest fragments compared to continuous forest, and they depend less on trees and more on lianas (Chaves et al 2012). Similarly, colobus monkeys (*Colobus vellerosus*) in a forest fragment in Ghana consumed more leaves and utilized lianas more often compared to colobus monkeys in a nearby continuous forest (Wong et al 2006). These diet changes across habitats are likely to exert distinct selective pressures on primate gut microbial communities, and the nutrition and health consequences of these shifts in gut microbial community composition must be determined and addressed. An understanding of whether shifts in the gut microbial community induced by habitat and diet change improve primates' ability to utilize hard-to-digest resources or whether they negatively affect health is vital to primate conservation in habitats worldwide.

Although our knowledge of host-gut microbe interactions is currently dominated by data from laboratory studies, this study provides an important first step in understanding the host-gut microbe relationship in wild animals experiencing natural temporal fluctuations in diet. Although howler monkeys have a somewhat elongated hindgut (Chivers and Hladik 1980), they lack a specialized foregut and consume a diet of mostly ripe fruit on an annual basis (Chapter 2). Therefore, they provide an excellent model for examining nutrition and gut microbial community dynamics in other fruit-eating primates. Additionally, the ability of howler monkeys to consume large amounts of hard-to-digest leaves (over 80% of monthly feeding time; Estrada 1984,

Glander 1981, Milton 1980, Rumiz et al 1986) makes them an excellent model for primates such as mountain gorillas, sakis, orangutans, marmosets, and Goeldi's monkeys that include leaves and other hard-to-digest food items such as seeds, bark, exudates, and fungus in their diet (Di Fiore et al 2011, Knott 1998, Porter and Garber 2004, Rothman et al 2008). However, subsequent studies must explore the effects of different food items both on nutritional intake patterns as well as on spatial, temporal, and age- and sex-based shifts in primate gut microbial community composition and function. Both primates that consume large quantities of protein-rich insects with hard-to-digest chitin exoskeletons and primates that consume large quantities of soluble fiber-rich exudates may need to regulate total energy intake, but the microbial processes necessary to break down the otherwise indigestible portions of each of these diets are likely to differ. Similarly, for primates with less variation in the food items being utilized across seasons compared to black howlers in Palenque, seasonal changes in gut microbial community composition and function are likely to be weaker than those we detected. For example, titi monkeys and gibbons exhibit low coefficients of variation for overall diet composition (in terms of plant parts) compared to atelines, and primate populations that utilize a wide variety of plant species year-round show fewer dramatic seasonal changes in the plant species being consumed (Hemingway and Bynum 2005).

The strength of age- and sex-based differences in gut microbial community composition and function also is likely to vary across primate species in response to differences in life history processes and social structure. Compared to other atelines, howler monkeys exhibit a shorter gestation period (152-195 days vs. *Ateles:* 226-232 days, *Brachyteles:* 215-218 days, *Lagothrix:* 210-225 days), a shorter interbirth interval (16-23 months vs. *Ateles:* 32-50 months, *Brachyteles:*32-41 months, *Lagothrix:* 32-41 months), higher prenatal growth rates (2.14-

2.84g/day vs. *Ateles:*1.86-2.03g/day, *Lagothrix:*1.92-2.02g/day), and smaller neonatal brain size (53% of adult size vs. *Ateles:* 58%; Di Fiore et al 2011, Fedigan and Rose 1995, Hartwig 1996), and juvenile howlers are weaned at an earlier age compared to other atelines (11-14 months vs. *Ateles:* 24-36 months, *Brachyteles:* 18-24 months; Di Fiore et al 2011). Consequently, adult female howler monkeys likely invest more metabolic resources in prenatal offspring growth than other adult female atelines while juvenile howlers likely invest more resources in brain growth after birth and weaning than other juvenile atelines. These life history patterns suggest that howler monkeys experience higher daily nutritional costs than other atelines from growth and reproduction. Based on data collected in the present study, it appears that howler monkeys use shifts in both diet and the gut microbiota to meet these costs. However, in a primate with lower daily nutritional costs from growth and reproduction, such as spider monkeys (Fedigan and Rose 1995), shifts in diet and microbiota may be less dramatic or shifts may occur only in diet or only in gut microbial community composition.

In addition, howler monkeys live in socially cohesive groups (Pavelka 2011). As a result, activity patterns and diets generally do not differ among individuals of the same social group (Pavelka 2011). For primates in social groups that experience fission-fusion dynamics such as chimpanzees and spider monkeys as well as solitary or pair-bonded primates such as orangutans or gibbons (Di Fiore et al 2011), individuals or subgroups are more likely to exhibit distinct activity patterns and diets. In these cases, the ability of adult females and juveniles to shift their behavior to meet nutritional demands may result in fewer age- and sex-based differences in the gut microbiota. Conversely, if individuals are consuming different diets, they may be imposing alternative selective pressures on the gut microbial community that might result in more age- and sex-based differences in the gut microbiota.

The research presented in this dissertation provides important baseline data regarding host-gut microbe dynamics in wild primates, but much remains to be learned. Moreover, there are several limitations in this dataset which must be considered in subsequent studies. First, because observational data limit the ability to control variables, the relationships we detected between gut microbial community dynamics and host behavior are correlational. To better establish causation, data are needed from natural experiments in which an aspect of host diet, the gut microbiota or host health is systematically altered as a result of natural processes or field-based experimental studies (see for example, Garber et al 2009). Studies of captive primates in which diet can be controlled can also complement studies of wild populations and provide further evidence for causation.

Similarly, it is important to understand in more detail the specific factors that dictate gut microbial community dynamics and determine the impact of those dynamics on the host. For example, the array of plant species being consumed by the howlers was associated with patterns in gut microbial community composition across time. However, it is unclear whether the consumption of a single plant species was driving these patterns or the entire array since the consumption of multiple plant species shifted at the same time, making the isolation of the effects of a single plant species difficult. More longitudinal data from additional primate populations will be useful in pinpointing these influences as well as the combination of both traditional nutritional analyses of food items (e.g. acid detergent fiber, crude protein, lipids) and metabolite analyses. Using both types of analyses of food nutritional content is also important to clarify the descrepancies we found between the literature estimates of energy and nutrient intake and metabolite analyses. Additionally, gut microbial community composition across age and sex classes was generally not strongly correlated to diet. Studies measuring fluctuations in sex

hormones such as estradiol and progesterone as well as growth hormones are necessary to determine if some of the differences in the gut microbiota are associated with the physiological shifts of reproduction and growth (Gabrilovac et al 1988, Marzi et al 1996, Montague et al 1997, Power and Schulkin 2006, Raghupathy 1997, Strobel et al 1998, Wegmann et al 1993, Zhang et al 1994).

Finally, direct measures of host nutrition and health are necessary to understand the ultimate effects of the gut microbial community on the host fitness. Additional studies must incorporate assessments of host nutritional status and health using measurements of factors such as body mass, c-peptides, ketones, white blood cells, antibodies, and parasite abundances (Deschner et al 2008, Gillespie 2006, Girard-Buttoz et al 2011, Harris et al 2009, Lantz et al 2011, Sheriff et al 2011, Sherry and Ellison 2007, Thompson and Knott 2008, Thompson et al 2008). Ultimately, the use of long-term datasets to examine birth and death rates in primate populations with distinct gut microbial community dynamics are also necessary to discuss the effects of the gut microbial community on primate fitness.

With this project, I aim to strengthen connections between microbiology and behavioral ecology and to transform studies of primate feeding ecology by encouraging the spread of new research techniques. Although the importance of the gut microbiota to host nutrition and, therefore, foraging behavior has been acknowledged since the ground-breaking work of Milton beginning in the early 1980's (Milton 1979, Milton et al 1979, Milton et al 1980, Milton and McBee 1983), genetic techniques such as DNA fingerprinting (Kent et al 2007, Osborn et al 2000, Yanarell and Triplett 2005) and pyrosequencing (Ronaghi et al 1998) provide large amounts of microbial community data which make it possible to describe host-gut microbe relationships in more detail. By pinpointing both the causes and effects of changes in gut

microbial community composition and improving the understanding of how foragers adjust to changing nutritional demands in variable environments, we can approach studies of primate ecology and evolution more effectively. This knowledge also can be utilized to expand conservation efforts and enrich captive primate populations by increasing the availability of foods critical to shaping the gut microbial community and benefitting primate health. Only by using interdisciplinary tools to examine the interactions of behavior and physiology can we truly begin to reveal the complexities of primate nutrition and health.

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