The Nutritional Ecology of the Black-and-White Snub-Nosed Monkey

by

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ABSTRACT

Rhinopithecus bieti, a foregut-fermenting colobine monkey, is one of the few animals in the world that subsists largely on lichen for much of the year. Lichen is rarely consumed as the primary food source by most animals for very long because of its low protein and tendency to contain anti-nutritive compounds. Samples of *Usnea longissima* collected in *R. bieti* habitat depressed *in vitro* fermentation. In a selection trial, wild monkeys showed strong selection for one *Usnea* chemotype over a second type. The partial dry matter digestibility of *Usnea longissima* fed to captive *R. bieti* that were also fed other foods was high (81.3 ± 3.5%, n = 5). Endogenous fecal protein losses were estimated at 2.8 ± 1% of dry matter intake, and true protein digestibility was 88 ± 6%. Although typically primates select diets with high protein: fiber ratios, *R. bieti* fed high-lichen diets did not follow this trend, instead selecting against lichen and for a diet with a lower protein:fiber ratio. *R. bieti* relies on lichen as a staple fallback food during harsh winters, and is able to do so despite lichen's antimicrobial properties. *R. bieti* may be in or near negative nitrogen balance in winter if they consume a lichen-only diet, and this will be most pronounced for growing animals or pregnant or lactating females. Because lichens are so sensitive to climate change, and *R. bieti* habitat is expected to have one of the most rapidly-changing climates on earth, careful monitoring of not only lichen abundance, but also the chemotypic composition of lichens is needed in order to evaluate the sustainability of *R. bieti* in their current habitat.

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INTRODUCTION

The black-and-white snub-nosed monkey, *Rhinopithecus bieti* (Figure 1-1), is an endangered colobine monkey endemic to the Hengduan mountains of southeastern Tibet and northwest Yunnan Province, China (IUCN 2012). As one of the few "charismatic megafauna" in the region, *Rhinopithecus bieti* plays a crucial role in generating support for local conservation efforts. Concern for the monkey has spurred the establishment of the 2,820-km^2 Baimaxueshan Reserve to protect the numerous plants and animals in this designated UNESCO biodiversity hotspot (UNEP WCMC 2011; Weckerle et al. 2010). *Rhinopithecus bieti* lives at some of the highest elevations of any non-human primate (Long et al. 1996) and has a diet unique among mammals – the majority of their diet throughout much of their range is lichen (Kirkpatrick 1996; Xiang et al. 2007b). It is the only mammal that consumes lichen year-round. The aspects of *Rhinopithecus bieti*'s behavior and physiology that enable them to use lichen are key adaptations for this species to exist in this harsh environment. However, warming temperatures and increasing human impact in the region are threatening this key resource and by extension, this species. Only 1-2,000 individuals are thought to exist in the wild (IUCN 2012; Xiang et al. 2007a).

The Hengduan Mountains are a narrow range of the eastern Transhimalayas bounded to the east and west by the Mekong (Lancang) and Yangtze (Jinsha) rivers as they flow south off of the Tibetan plateau. Within this narrow range, there are approximately fourteen groups of *Rhinopithecus bieti*

located between 26°14' N (northwest Yunnan Province) and 29°20' N (southern Tibet, Figure 1-2). The terrain is rugged and steeply sloped, and the climate is harsh; snow covers the ground 4-6 months of the year, and the average monthly temperatures in winter are often below freezing. *Rhinopithecus bieti* occupies a narrow elevation band between 2,600 and 4,500 m that consists primarily of deciduous forests in the south, mixed broadleaf and conifers in the middle, and exclusively coniferous forests in the north, where the habitat is colder, drier, higher and less biodiverse (Long and Kirkpatrick 1994; Xiang et al. 2007a, 2007b; Xiao et al. 2003).

LICHEN IS A KEY FALLBACK FOOD AT HIGH ELEVATIONS

In this harsh habitat, the staple foods of other colobine monkey diets - leaves, fruits and seeds - are often rare or completely unavailable (Grueter et al. 2009b). Lichen, however, is ubiquitous and generally abundant year-round (Grueter et al. 2009b; Kirkpatrick 1996). Lichen, a symbiotic organism composed of a fungal partner and a photosynthetic bacterium or algae (Nash 2008), forms the bulk of *Rhinopithecus bieti*'s diet in winter when other diet items are unavailable, but also continues to constitute a large proportion of the diet in summer when other diet items are both available and abundant (Grueter et al. 2009b; Kirkpatrick 1996; Xiang et al. 2007b). In the northernmost extent of their range, *Rhinopithecus bieti* consumes primarily black lichen (heisongluo, *Bryoria himalayana*), while in the middle and southern part of their range, several species of lichen are available and consumed including black lichen, short lichen (duansongluo, *Bryoria spp.*) and long lichen (changsongluo, *Usnea longissima*) (Grueter et al. 2009b; Xiang et al. 2007b). It is unclear from behavioral observations whether monkeys prefer some lichen species to others when both occur in a group's range, although there is anecdotal evidence that they prefer black lichen when it is available (Long Yongcheng, personal communication).

In addition to lichen, *Rhinopithecus bieti* consumes the leaves of broadleaf trees and bamboo as well as more ephemeral flowers, buds, fruits and seeds, and they have occasionally been observed consuming bark, insects, and tubers (Grueter et al. 2009b; Xiang et al. 2007b). Unlike Rhesus macaques (*Macaca mulatta*), which share the lower elevations of *Rhinopithecus bieti*'s range, *R. bieti* is not known to forage in agricultural crops (Grueter et al. 2010). In the southern and central part of the range, leaf consumption accounts for 20-60% of the observed feeding time, even in winter (Ding and Zhao 2004; Yang and Zhao 2001).

However, in the central part of the monkey's range, seasonal, nonlichen foods are completely unavailable for six to seven months of the year [November to March (Grueter et al. 2009b)], and in the northernmost part of the range, these foods can be unavailable for over eight months each year [October to May (Xiang et al. 2007b)]. Lichen consumption can be seen in the vast majority (typically over 90%) of behavioral observations during these times (Grueter et al. 2009b; Kirkpatrick 1996; Xiang et al. 2007b). Because *Rhinopithecus bieti* relies primarily on lichen during the winter months and lichen's poor nutritional quality, lichen is considered an essential fallback food[1] for this species (Grueter et al. 2009b), particularly for the northern groups. In other words, if lichen were unavailable, or if the monkey were unable to consume it, they would not be able to inhabit most of their current range.

[1] The definition of fallback food used here is "an abundant but low quality food used during periods of low overall food availability" (A. J. Marshall et al. 2009) as well as "a food whose use is negatively correlated with the availability of preferred foods"(Altmann 1998), both of which describe the role of lichen in the diet of *R. bieti*. Marshall and Wrangham (2007), defined the term "staple fallback food" as a food that is both available and consumed year-round, sometimes comprising 100% of diet. By this definition, lichen can also be described as a "staple fallback food".

COLOBINE DIGESTIVE PHYSIOLOGY UNIQUELY ENABLES THE USE OF LICHEN AS A FEEDSTUFF

Although little is known about *Rhinopithecus bieti*'s unique relationship with lichen, our knowledge of how other foregut fermenting primates and other lichen-eating animals cope with similar challenges can shed light on the challenges faced by this monkey's unique diet.

The ability to make such extensive use of lichen is a unique and critical adaptation that allows *Rhinopithecus bieti* to inhabit these harsh regions where other foods are often unavailable, and likely gives them a competitive advantage over species without these adaptations. Despite its worldwide abundance (Nash 2008), lichen is an uncommon food source for mammals. Those that do consume lichen generally limit it to a small percentage of their diet or include lichen only seasonally in their diet. There are a number of reasons for this. Lichens have anti-feedant properties that depress intake (Dubay et al. 2008; Robbins 1987). In addition, most lichens are extremely low in protein, and animals on high-lichen diets tend to be in negative nitrogen balance (Dubay et al. 2008; Robbins 1987). Lichens also contain many unique, biologically active compounds. Some of these compounds are potent toxins that can sicken or kill animals that lack adequate detoxification mechanisms (Dailey et al. 2008; Durazo et al. 2004; Guo et al. 2008).

Rhinopithecus bieti is clearly able to handle these otherwise negative nutritional aspects of lichen. These likely stems from their colobine lineage. Colobine monkeys utilize both pregastric and postgastric fermentation (Caton 1998; Chivers 1989). This digestive strategy confers the ability to obtain energy from many otherwise indigestible carbohydrates, detoxify several types of toxins, and improve nitrogen balance, all of which offer numerous advantages in the digestion of lichens.

FOREGUT FERMENTERS GET MORE ENERGY FROM LICHEN

There are dozens of different types of carbohydrates and carbohydrate bonds, only some of which are digestible by mammalian enzymes. Microbial communities in the gut are able to digest many more of these bonds, and use the resulting catabolites for their own energy needs, while storing the excess as microbial glycogen – a carbohydrate that is highly digestible by mammals. In this way, microbial fermentation allows the energy from an otherwise undigestable carbohydrate such as cellulose to be made available to a mammal.

Although lichen doesn't contain the types of fiber most commonly associated with plants (cellulose and hemicellulose), it does contains a diverse array of non-fiber polysaccharides, including lichenin and β-glucans, which are indigestible by mammalian enzymes but can be effectively fermented by gut microbes (Svihus and Holand 2000). The gut microflora of colobine monkeys has not been well described. However, early studies of wild colobines found that the volatile fatty acid composition of stomach contents was similar to that of ruminants (Ohwaki et al. 1974; Kay, Hoppe, and Maloiy 1976). The microbiome of *R. bieti's* hindgut appears to contain many unique taxa, based on 16S rRNA analysis (Wu et al. 2013). However, gut flora, while differing in species composition, exhibit community-level similarities in function across disparate taxa (Karasov and Carey 2009). Therefore, the microbial community in *R. bieti* likely has a similar functionality as in other foregut fermenters on similar diets. For example, colobine monkeys exhibit increased dry matter and NDF digestibility compared with non-foregut fermenting primates (Edwards and Ullrey 1999), lending credence to the idea that their digestive system exhibits similarities to that of other foregut fermenters, who typically also have increased NDF and fiber digestibility (Mcnab 2002). Thus, colobine monkeys are likely better able to make use of the lichen carbohydrates that require fermentation than monogastric animals would be.

FOREGUT FERMENTERS REQUIRE LESS DIETARY NITROGEN

In addition to being able to extract more energy from lichen than non-foregut fermenting animals, foregut fermentation also enables the use of lower protein feedstuffs such as lichen. Ruminant animals, which rely on pregastric fermentation, can subsist on diets with lower dietary protein quantity and quality than most monogastric animals because the ruminal microbes have the ability to use non-protein nitrogen (NPN) sources to synthesize protein. In diets with sufficient nitrogen and energy, microbial protein can exceed 40% of the post-ruminal protein supply (Polan 1988). Lichens contain substantial amounts of NPN (Claridge et al. 1999), and *Rhinopithecus bieti* can likely use this NPN to meet some of its dietary protein needs, which non-colobine monkeys would be less able to do.

In addition to dietary sources of NPN, gut microbes can utilize urea, an otherwise unusable product of mammalian protein catabolism, to synthesize proteins. In humans, approximately 40% of the urea produced in the liver is returned to the gut, and in cattle and sheep, 60-70% is returned (Lapierre and Lobley 2001). The microbes in the gut of foregut fermenters, however, can use the urea returned in this way to synthesize protein of value to the animal. Using these mechanisms, *Rhinopithecus bieti* can likely return a substantial proportion of the urea to the gut, which can then be used by the microbes for microbial protein synthesis, reducing nitrogen losses and decreasing the requirement for high quality dietary protein.

In the primate literature, the protein:fiber[2] ratio is often used as a proxy for diet quality, and has been linked to colobine abundance and distribution (Chapman et al. 2002; Milton 1979). The protein:fiber ratio of lichen (0.8-6.5; Svihus and Holand 2000, Bissell unpub. data) is similar to or even much greater than that of other colobine feeds (0.3-1.1; Chapman et al. 2002). However, the overall quantity of protein and fiber is much lower in lichens (2-8% protein, 1-4% fiber; Svihus and Holand 2000, Bissell unpub. data) than in vascular plants (10-25% protein, 12-50% fiber; Chapman et al. 2002). Because of the qualitative and quantitative differences in the protein and fiber fractions of lichens and plants, the protein:fiber ratio should not be used to compare lichenivorous vs. nonlichenivorous habitats without careful consideration of the underlying values. However, the ratio may still be useful as a metric for comparisons among *Rhinopithecus bieti* habitats.

NUTRITIONAL IMPLICATIONS OF THE LICHEN-BASED DIET OF *R. BIETI*

Lichen is low in protein

Given that *Rhinopithecus bieti* subsists for lengthy periods on a diet that contains very little nitrogen or protein, it is interesting to speculate how their protein requirements are met during this time. On a hypothetical *Bryoria* or *Usnea*-only diet, the animals would only receive 0.5-1.0% nitrogen or 3-6% crude protein, well below the general primate recommendation for diets containing 6-18% high quality protein (National Research Council 2002). The monkeys may supplement their diet with insects (Xiang et al. 2007b), although in winter these are presumably difficult to find. The previously mentioned protein-sparing mechanisms likely lessen the need for protein somewhat.

[2] Measured as crude protein (CP) and acid detergent fiber (ADF).

Very few animals are known to store protein, and this mechanism has not been documented in primates. However, another lichenivorous species, reindeer (*Rangifer tarandus*), increases its lean body mass in the fall and catabolizes this protein during the winter when they feed on nitrogen-poor lichens (Barboza and Parker 2006).

Although body weight data from *Rhinopithecus bieti* are limited and body composition data are nonexistent, there are reports of some adult males weighing as much as 45 kg (Quan 2002 n=9), including one male in winter (Ren et al. 2009 n=1), but other reports, whose collection dates are unknown (but given the inaccessibility of the habitat in late winter, are likely from the spring and summer), give much lower maximum weights of only approximately 15 kg for adult males (Jablonski and Ruliang 1995; Long and Kirkpatrick 1994). With such limited data, it is mere speculation as to whether these weights simply reflect individual variations in size or are an indication that *Rhinopithecus bieti* exhibits seasonal changes in weight, as is common in many species inhabiting highly seasonal environments. Even if seasonal changes in weight occur, body composition and detailed isotopic studies would need to be conducted to determine whether the animals are changing in lean mass, or whether the changes in body weight are the more likely result of increased fat stores, a characteristic common to many seasonal animals.

Lichen is toxic

In addition to their low nitrogen and protein content, lichens also commonly produce compounds that are toxic to mammals. There are dozens of lichen genera in the eastern Himalayas, although *Rhinopithecus bieti* consumes two genera of lichen most frequently, *Usnea* and *Bryoria* (Grueter et al. 2009a; Kirkpatrick 1996; Xiang et al. 2007b). Both of these genera typically contain the hepatotoxin, usnic acid, as well as other compounds toxic to mammals including vulpinic acid, evernic acid, barbatic acid (oxidative phosphorylation decouplers and potent toxins),

fumaroprotocetraric acid (associated with allergic reactions), diffractic acid and atranorin (cytotoxins; Dailey et al. 2008; Marante et al. 2003; Perry et al. 1999). Microbial fermentation can detoxify at least some of these toxins in ruminants (Sundset et al. 2008), although only usnic acid has been studied in any detail. Although reindeer can consume large quantities of usnic acid in lichen without ill effect, approximately 400 elk near Yellowstone National Park died or had to be euthanized after consuming the lichen *Xanthoparmelia chlorochroa* (Cook et al. 2007), which contained high levels of usnic acid (Roach et al. 2006). The elk exhibited paralysis before death, and this was attributed to the usnic acid (Cook et al. 2007). However, oral administration of usnic acid to sheep resulted in a different constellation of symptoms (lethargy, anorexia, muscle weakness and abdominal discomfort), indicating that other compounds may have been involved, or that the elk microbial community metabolized the usnic acid into a different toxic compound, or that elk and sheep respond differently to usnic acid (Dailey et al. 2008). In monogastric animals including humans, usnic acid has been associated with liver damage and failure (Durazo et al. 2004; Pramyothin et al. 2004). It is thought to have anti-feedant properties, perhaps as an anti-herbivore defense (Dubay et al. 2008; Lawrey 1986). Many lichen compounds have been extensively studied for their use as antibiotics. For a foregut-fermenting animal, these compounds have the potential to interfere with the bacteria involved in the fermentation process. Indeed, samples of lichens consumed by *Rhinopithecus bieti* contained at least nine unique compounds that inhibit bovine fermentation (Bissell, unpub. data).

The development of microbial detoxification pathways is little understood. In some cases, it appears that only a single species of bacterium imparts the ability to detoxify a particular compound. Animals harboring the relevant microbial species can eat certain toxic plants, while those lacking this species sicken or die (Anjos et al. 2010; Glendinning 2007). In reindeer consuming usnic-acid

containing diets, no trace of usnic acid was found in the rumen fluid or elsewhere in the digestive tract or feces, indicating that it was rapidly degraded within the rumen (Sundset et al. 2009). *Eubacterium rangiferina*, a bacterium resistant to a number of common lichen compounds (usnic, antranoric, fumarprotocetraric, and lobaric acid) has been identified from reindeer rumens and may be a key source of reindeers' ability to cope with lichens high in these toxins. Indeed, usnic acid may even increase diet digestibility rather than decrease it in reindeer (Palo 1993). Some lichen compounds have potentially beneficial medicinal properties because of their antibacterial, antitumor, antigrowth, and even antiprion properties (Bustinza 1952; Johnson et al. 2011; Lawrey 1986).

Clearly *Rhinopithecus bieti* consumes large quantities of *Usnea* and *Bryoria* without experiencing lethal side effects despite the presence of a variety of lichen compounds that are potentially toxic to some mammals. However, detoxification can be costly to animals, and animals that eat highly toxic foods may pay the price through decreased fitness. While dietary specialists have typically evolved mechanisms to reduce these costs, they may still pay some price (DeGabriel et al. 2009; Iason 2005). In one study, a specialist beetle exhibited reduced fitness when consuming a more toxic variety of its host plant compared with a less toxic variety (Ballhorn et al. 2007). In this case, the host plant toxin was not lethal, but merely reduced weight gain, egg numbers, and time to hatching. Likewise, a lichenivorous diet may not be lethal, but it may reduce the fitness of individuals, particularly if the quantities consumed are large, such as during winter. Because the rugged habitat of *Rhinopithecus bieti* makes detailed observations difficult and few individuals can be identified, comparative data among groups that feed on different amounts of lichen are unavailable. Future studies are needed to examine whether differences in fitness exist among the groups and whether these are correlated with the groups' degree of lichenivory.

Many lichen compounds are strongly colored, and *Rhinopithecus bieti* likely can use this feature to distinguish among lichens. *Rhinopithecus bieti* have been observed carefully picking strands of the bright yellow and highly toxic *Sulcaria virens* out of clumps of *Usnea* (Bissell, pers. obs.). There can be differences in color even within a single species of lichen. *Usnea longissima*, for example, ranges in color from almost white to yellow, green, and dark gray. In one trial with semi-wild monkeys, they preferred green to white varieties of *Usnea longissima* (Bissell, unpub. data). In five samples of *Usnea longissima* collected from the habitat of four southern monkey groups, 14 separate compounds were identified using HPLC, only one of which was common to all five samples (Bissell, unpub. data). The relevance of these compounds to the health of *Rhinopithecus bieti* is still unknown.

Lichen is in decline

Lichens in the area are in danger of declining for a number of reasons. Most immediately, lichen is in danger of being overharvested. Lichens are often harvested by local residents for food, medicine, and for sale to local and national markets (Wang et al. 2001, Bissell pers. obs.). With their slow regeneration times (1-9 cm per year for *Usnea longissima*; Jansson et al. 2009; Keon and Muir 2002), lichens can become locally depleted. In addition to human harvesting, the monkeys themselves can deplete lichen in some areas. Infrastructure development in the area, primarily road building, has led to fragmentation of *Rhinopithecus bieti* habitat, isolating the otherwise wide-ranging groups into smaller patches where they also can deplete the slow-growing lichens (Xiao et al. 2003). This has already happened to one group of monkeys in Baimaxueshan Nature Reserve, where staff now must collect lichens outside the area to feed an isolated group of monkeys each day (pers. obs.).

A larger threat to the lichen, however, is climate change. Climate warming in northwest Yunnan is progressing at one of the fastest rates on earth, increasing by 3.5-9.6 °C per 100 years whereas the average rate of global temperature increase has only been 1 °C per 100 years (Haynes and Kung, *in prep*; IPCC 2007). Lichens are extremely sensitive to environmental changes and are regularly used as bioindicators for changes in temperature and water and air quality (Aptroot 2009). Large changes in lichen communities have been observed after only mild changes in the climate (Aptroot 2009). Models of climate change in these mountains predict large declines in total forest area, as well as changes in forest composition (Wong et al. 2009). Similar changes in forest cover have resulted in declines of *Usnea longissima* in Scandinavia (Esseen et al. 1981) and California (Doell and Wright 2000). *Usnea longissima*, once abundant in northern Wisconsin, was extirpated in the late 19th century (Bennett & Wetmore, 2004), roughly coinciding with greatly increased human settlement and industrialization in the area (Wisconsin Historical Society, 2014). Similar declines in *Usnea* in Yunnan could be catastrophic to the monkey because there are few other fallback foods throughout much of monkeys' range.

In addition to their sensitivity to changes in moisture and temperature, both *Usnea* and *Bryoria spp*. are sensitive to a variety of common environmental contaminants including nitrogen, sulfur dioxide and fluoride (Geiser and Neitlich 2007; Geiser et al. 2010; Thormann 2006), both of which are commonly released during metal mining operations, a major industry in many parts of northwest Yunnan. In other parts of the world, increases in air pollution have lead to rapid declines in lichen populations (Batts et al. 2004; Geiser and Neitlich 2007). Lichens also strongly absorb metals from the atmosphere and have led to heavy-metal accumulation in reindeer that feed in polluted areas (Eriksson et al. 1990; Larter et al. 2010). Increased mining operations may therefore lead to the

accumulation of heavy metals in lichens, and by extension, in *Rhinopithecus bieti*, where they are likely to have negative health effects.

Lichen's sensitivity to environmental change, and *Usnea's* sensitivity in particular, means that even if habitat destruction and fragmentation are halted, the most important food source of the monkey will almost certainly be changing. Monkeys' resilience to this change depends upon whether other species of lichens and plants will fill in the void in time, whether these new species contain compounds toxic to primates, and whether the monkeys (or their gut microbes) will be able to cope with any toxins the new species may contain. To better assess the risks to the monkey, further research is needed. Specifically, it is important to know how the Yunnan lichens (individual species as well as their chemical variants) are coping with the climate change in the area, whether they are absorbing toxins from local mining operations (and at what range from the mines), and whether plants are moving into the area at a pace to replace lichen as a winter food source. The current efforts to model the impacts of climate change on monkey habitat should be continued and expanded. If individual monkeys can be identified, examining the variation in biological fitness among groups more and less dependent on lichen would help determine the types of influence lichen has on the health and biology of these animals. Long-term monitoring of monkey groups, lichens, and other edible resources in the area is extremely difficult given the terrain and infrastructure available in the area. However, building the capacity to conduct large scale monitoring should be a priority for conservation efforts, not only to protect the monkey, but also to help conserve the numerous unique plants and animals in this biodiversity hotspot.

CONCLUSIONS

The unique ability to use lichen as a fallback food has enabled *Rhinopithecus bieti* to occupy this high-elevation, seasonal niche. However, like all organisms dependent upon unique resources, this

reliance on a single resource that is itself at risk also puts *Rhinopithecus bieti* at a high risk of extinction (Jernvall and Wright 1998; Wright 2007). In order for *Rhinopithecus bieti* to survive in its current range, it must continue to be able to obtain sufficient food resources during the long winters. While lichen plays this role today, the changing climate and the concomitant changes in forest elevation and composition may limit *Rhinopithecus bieti*'s ability to rely on this sensitive resource in the future. Effective conservation of this species will require carefully monitoring food resource abundance throughout *Rhinopithecus bieti*'s current range to determine if lichen is declining and whether incoming species are able to fill the dietary void. With proactive planning and careful resource management, *Rhinopithecus bieti* may be able to continue inhabiting its high-elevation habitat. Fortunately, although *Rhinopithecus bieti* is by necessity currently extremely reliant on lichen, it is not an obligate lichen feeder; it can and does subsist and reproduce on lichen-free diets in captivity and on low-lichen diets in the southern part of its range. Therefore, even if various forces do eventually lead to the extirpation of this species from its current range, the possibility exists that *Rhinopithecus bieti* could survive elsewhere if suitable conditions could be found.

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Figure 1-1. Male, female and infant *Rhinopithecus bieti*. Photo by Long Yongcheng of The Nature Conservancy.

Figure 1-2. Location of *R. bieti* groups (red flags). From http://www.rhinopithecus.net/taxa.htm

Chapter 2: **Nutritional performance of colobine monkeys on a variety of diets, with special emphasis on *Rhinopithecus bieti,* the black-and-white snub-nosed monkey**

ABSTRACT

Rhinopithecus bieti is a highly-endangered colobine monkey with an unusual diet – for much of the year, many groups rely heavily on lichen, a food source relied on by few other mammals. In this study, 19 digestibility trials were conducted with four species of colobine monkeys, including *R. bieti*, *Colobus angolensis*, *C. guereza* and *Trachypithecus obscurus*, to examine similarities and differences in digestive performance across a wide range of diets. We measured endogenous fecal crude protein losses, assessed true protein digestibility explored whether *R. bieti* exhibits a negative relationship between fiber and dry matter digestibility, and whether they would select for diets with a high protein:fiber ratio as has been observed in a number of primate species. Fiber content did not depress dry matter digestibility over the range of fiber contents tested. Endogenous fecal protein losses were estimated to be 2.8 ± 1.0g/100 g of dry matter intake and true protein digestibility was 88 ± 6%. Dry matter and neutral detergent fiber digestibility was similar to previous colobine studies. While in most trials monkeys consumed diets with higher protein: fiber ratios than what they were offered, *R. bieti* fed diets containing lichen did not follow this trend, instead selecting against lichen and for a diet with a lower protein:fiber ratio.

Keywords: colobus, snub-nosed monkey, digestibility, selection, protein:fiber ratio

INTRODUCTION

Colobine monkeys are distinguished from other primate groups by their use of foregut fermentation to digest their food (Kay and Davies 1994; Caton 1998; Caton 1999). Like other foregut fermenting mammals such as macropods and ruminants, this digestive system enables colobines to obtain more energy from structural carbohydrates and improves the biological value of dietary nitrogen compared with non-colobine primates. Previous studies found that the colobine stomach has similar fermentation products (volatile fatty acids) as in the rumen of ruminants (Drawert, Kuhn, and Rapp 1962; Bauchop and Martucci 1968), and that colobines have a higher dry matter (DM) and neutral detergent fiber (NDF) digestibility than non-colobine primates on comparable diets (Dierenfeld, Koontz, and Goldstein 1992; Edwards and Ullrey 1999; Nijboer and Clauss 2006).

Although most colobine monkeys subsist on a diet of primarily leaves, *Rhinopithecus bieti*, the black-and-white snub-nosed monkey, depends for much of the year on lichen. *R. bieti* inhabits a narrow mountain range in the Eastern Himalayas between 2,600 and 4,500 meters above sea level. Throughout the northern extent of this highly seasonal habitat, lichen is the only food available during the winter, although the monkeys continue to eat large quantities of lichen in the summer when other foods are both available and abundant. Lichen is consumed by few other mammals to such an extent, largely because of the low protein content of lichen and its possession of antifedant properties. Reindeer and other animals consuming high-lichen diets are typically in negative nitrogen balance. *R. bieti's* ability to survive many months on a lichen diet may be related to the increased digestive efficiency due to its colobine lineage or as-yet-unknown mechanisms for conserving nitrogen, perhaps by reducing endogenous nitrogen losses.

This study aimed to evaluate the digestion of *R. bieti* and compare it to other colobine monkeys across a range of diets. This study leveraged the variety in diets offered to captive colobines at

multiple institutions to explore how four colobine species housed in two countries digest diets that vary across a greater range of nutrient and ingredient composition than has been used in previous studies.

In this study we tested three predictions:

(1) *R. bieti* is able to limit endogenous fecal protein losses to low levels and a has a high true digestibility of protein.

(2) The negative relationship between dietary fiber content and dry matter digestibility observed in other mammal species (Robbins 1994; Van Soest 1994) would hold true in colobines.

(3) The black-and-white snub-nosed monkey (*Rhinopithecus bieti*), which consumes a high-carbohydrate, low-nitrogen foodstuff, lichen, for much of the year in the wild (Kirkpatrick 1996; Xiang et al. 2007; Grueter et al. 2009), would exhibit similar DM and NDF digestibility as other captive colobines.

Additionally, within the limitations of this study, we tested whether the colobine trend towards selecting diets with the highest protein:fiber ratio (Davies, Bennet, and Water 1988; Chapman et al. 2002; Chapman and Chapman 2002) would hold true across the variety of diets. Overall, our study adds new data on the digestive performance of five colobine species over a wide range of ingredients and nutrients that are successfully offered to and consumed by captive animals at zoological facilities within and between two countries, and provides data that can be used to understand the diet of *R. bieti*

MATERIALS/METHODS

Eleven groups of colobine monkeys (four species, including *R. bieti*) at 7 institutions in the US and in China were acclimated to a constant diet for two weeks prior to the start of each of 19 collection periods (Table 2-1). Each monkey group was housed in single-species enclosure. The enclosures varied in size and exposure to the outdoors, but all enclosures had solid floors where all feces and orts (food provided but not consumed) could be collected. The animals were subject to neither heat nor cold stress during these trials and had access to protected indoor areas.

Each monkey group housed together eating one particular diet was considered an experimental unit (n=19), with some groups sampled more than once on different diets. The diet(s) fed to each monkey group varied by institution and were representative of items that were normally fed to the animals, except for lichen, which was new to the *R. bieti* groups. The diets all contained a manufactured primate biscuit (offered dry, soaked, or steamed), and commercial fruits and vegetables (Table 2-2). Some diets contained browse (leafy branches from trees and shrubs) and/or lichen. Diets offered were the same each day throughout the trial. Each trial contained a two-week acclimation period, followed by a three-day collection period during which all feed, orts and feces from the monkey group were weighed each day. All (100%) of orts and feces were collected, as well as samples of each feed ingredient. The samples were either dried in a forced-air oven at 60°C to constant weight immediately, or frozen and later dried to constant weight. The dried samples were ground through a Wiley Mill with a 1-mm screen except for fruit samples, which were ground through a meat grinder with dry ice to prevent gumming. Each ground sample was further analyzed for dry matter at 105°C. Final dry matter for each sample was calculated as (weight out of dryer / wet weight into dryer) x (ground weight out of 105°C / ground weight into 105°C).

Intake of each individual feed ingredient was calculated as the amount of the ingredient fed minus orts for that ingredient by weight on a dry-matter basis. Samples of each dry, ground feed ingredient sample were then recombined with other feed ingredient samples in the exact proportion that each was consumed to create a composite sample of the specific diet each monkey group consumed during each trial. These composited diets and each diet's corresponding feces were analyzed for dry matter (DM), crude protein (CP), neutral detergent fiber (NDF), and crude fat (Fat) using standard methods (Goering and Van Soest 1970; AOAC 1980; AOAC 1990; NFTA 2006) at the University of Wisconsin-Madison Soil Testing Laboratories. Acid detergent fiber (ADF) was calculated from published nutrient composition values for each feed ingredient.All values and proportions given are on a dry matter basis.

Endogenous fecal crude protein losses and true protein digestibility were estimated using the Lucas Test (Van Soest 1994; Fonnesbeck 1969) by regressing the digestible crude protein versus the dietary crude protein and examining the y-intercept (an estimate of fecal endogenous losses) and the slope (an estimate of the true protein digestibility).

All statistical analyses were performed in the R statistical package using the *stats* (R Core Team 2012) and *car* (Fox and Weisberg 2011) analysis packages. Plots were produced in R using the *ggplot2* graphing package (Wickham 2009: 2). All error terms are standard errors. Statistical significance was established at $p < 0.05$, whereas $p < 0.05 < 0.1$ was taken to reflect a trend.

RESULTS

Diet Composition

Ingredient composition and selection

Table 2-3 lists the 19 trials and the proportions of each food category as offered (AO) and as consumed (C) on a DM basis by the monkey groups during the three-day collection period. Peanuts, although not a true nut, are included with other nuts.

The monkey groups consumed diets that averaged 4.6 percentage points higher in biscuits ($t(18)=-2.4$, $p = 0.02$), and 3.0 percentage points higher in fruit ($t(18)=-2.9$, $p = 0.01$) than the diets they were fed. The diets they consumed were 6.0 percentage points lower in browse/hay than what they were offered ($t(18)=2.8$, $p = 0.03$).

The monkey groups in US zoos were, on average, offered diets 35 percentage points higher in biscuit than those at the Chinese institution ($t(8.3) = 8.5$, $p < 0.001$), 30.2 points lower in browse ($t(10.5) = -7.5$, $p < 0.001$), 13.4 points lower in fruit ($t(7.7)=-5.4$, $p < 0.001$), 8.1 points lower in greens ($t(6.2)=4.9$, $p = 0.002$), and 7.6 points higher in vegetables ($t(6.3)=3.5$, $p = 0.01$).

Nutrient composition

The diets consumed averaged 17.1 ± 0.9% crude protein, 24.4 ± 1.0% NDF and 4.0 ± 0.6% crude fat. The high level of fat consumed in trial D was largely the result of a large consumption of peanuts.

The diets consumed averaged 2.7 percentage points lower in NDF ($t(18) = p < 0.0001$) and tended to be 1.08 points lower in ADF ($t(18)=2.0$, $p = 0.06$) than the diets offered to the monkey groups.

On average, the colobine groups in the US consumed diets 10.4 percentage points higher in crude protein (22.5 ± 1.1) than the *R. bieti* groups in China (12.1 ± 0.5; t(8.7)=8.6, $p < 0.001$), 8.6 percentage points lower in NDF (23.1 ± 1.1 vs. 31.7 ± 0.4, t(7.4)=-7.2, $p < 0.01$), 4.3 points lower in ADF (23.1 ± 0.8 vs. 31.7 ± 0.9, t(16.5)=-3.4, $p = 0.003$) and 3.5 percentage points higher in crude fat (6.0 ± 1.0 vs. 2.5 ± 0.1, t(6.1)=3.6, $p = 0.03$, Table 2-4).

Fiber sources

The main source of NDF in the US colobine groups was the commercial primate biscuit (74.0 ± 2.6), whereas in the *R. bieti* groups, the browse provided the largest fraction of the NDF (51.3 ± 1.7, Table 2-5).

Protein:fiber ratio and selection

The CP:ADF ratio of the as-fed and consumed diets is given in Table 2-6. Diets consumed tended to have higher ratios than the diets fed, averaging 0.08 units higher (t(18)=-2.1, $p = 0.053$). However, four (L, M, N O) of the five trials (K, L, M, N, O) in which high amounts of lichen were fed to two Chinese groups of *Rhinopithecus bieti* did not follow this trend.

The average CP:ADF ratio in the US monkey groups (1.53 ± 0.33) was much higher than in the Chinese monkey groups (0.63 ± 0.04, t(6.3)=7.2, $p = 0.0003$).

Diet Digestibility

The Lucas Test conducted using the data from the 19 studies mentioned here as well as 16 additional studies from the published literature (Table 2-7; Figure 2-1) indicated that the endogenous fecal crude protein losses were 2.8 ± 1.0 g of crude protein per 100 g of dry matter intake ($p = 0.003$) and that the true digestibility of the crude protein in captive colobine diets was 88

± 6% (p <0.0001) . *R. bieti* did not exhibit a different slope or intercept than other species of colobine monkeys (p > 0.8).

Contrary to the prediction, neither DM nor NDF digestibility in the present study declined significantly with increasing dietary NDF ($F_{1,15}$=1.44; $F_{1,15}$=0.76, respectively, ns), over the range of dietary NDF values in the study (regression not shown). As predicted, there were no significant differences between *R. bieti* studied in China and the other colobine groups studied in the US in apparent digestive efficiency for either DM (t(7.4)=1.1, p = 0.30) or NDF (t(8.3)=0.7, p = 0.48; Table 2-8). Average digestibilities over all diets were 79 ± 1% for DM and 64 ± 2% for NDF.

DISCUSSION

Diet composition

All groups of each monkey species consumed a large proportion of their diet as a manufactured primate biscuit (49 ± 4% of dry matter consumed). However, only the *R. bieti* groups in China were fed or consumed diets containing appreciable quantities of browse (28 ± 3%). This may be in large part because the U.S. trials occurred during late fall and winter, when browse is most limited. Some zoos offered hay as a way to increase fiber levels for the animals, but this was not consumed during any of these trials.

One of the key differences between the diets consumed by the U.S. and Chinese monkeys is that the predominate source of structural carbohydrates (NDF) in the Chinese animals was from the browse, while the US monkeys' primary source was the commercial primate biscuits.

It is unclear whether or how the reliance on manufactured feeds affects the health of captive colobines. The negative influences of processed foods, particularly grains, on human and animal

health are well established (Van Soest 1984; Slavin et al. 1999; Slavin 2003). Yet, the introduction of commercial primate foods has dramatically improved the health and longevity of many captive primates, beginning with "Zoo Cake" in the early 1900s (Sue Crissey 2001), and leading to today's many primate diets, including several brands of "leaf-eater" biscuits that are intended for folivorous primates like colobines. Indeed, these leaf-eater biscuits are the biscuits that were fed to the U.S. monkey groups.

Nearly all groups in both countries had infants (see Table 2-1), indicating that adult monkeys fed these diets were in relatively good nutritional status. Fecal quality can also be an indicator of gut health. For logistical reasons, fecal quality could not be routinely assessed in these trials. However, the fecal quality of feces was opportunistically observed, and all of these were firm and fully formed. However, loose stools are reported to be a common problem in captive colobines (Nijboer 2006, personal observations, and communications with zoos).

The non-human primate NRC guide recommends diets with approximately 30% NDF for colobines, and none of the primate groups in this study met that target, although the *R. bieti* groups in China came closest. The NRC offers no guidelines for crude protein that are specific to colobines, but the recommendations for other primates suggest a minimum of 6-8% CP for maintenance. All of the diets in this study are well above this target level, with the lowest CP% being 13.1% CP. There are no specific recommendations for crude fat, although the levels of crude fat in this study are within ranges of commercial primate diets (NRC 2003; St. Louis Zoo 2003).

Diet selection

Under the right set of experimental conditions, some animals can exhibit selection towards a particular nutrient "target". In one study of domestic cats, cats that were familiar with several diets

that varied in their proportion of energy from protein, fat, and carbohydrates consistently chose food pairs that resulted in a particular proportion of protein, fat, and carbohydrate (Hewson-Hughes et al. 2011), and similar results have been seen with domestic dogs (Hewson-Hughes et al. 2012), and even in insects (Simpson and Raubenheimer 2001; Jensen et al. 2012). Although this study could not meet the stringent setup used with these laboratory animals, an attempt was made to see if the monkeys were perhaps selecting a particular nutrient target.

Because this study utilized the existing diet of the animals at each institution, the amounts offered had often been adjusted over time to reduce waste and to ensure that the animals ate the complete diet. Therefore, the animals had only a moderate opportunity to make dietary selections. In the most dramatic example, the monkey group in trial C consumed 97% of the DM they were fed, and exhibited only a 0.01 percentage point difference in the CP:NDF ratio from what they were fed. However, other groups consumed 74.3 ± 2.2% of the dry matter offered to them, allowing greater selection. Other factors, such as competition with other group members and physical characteristics of the variety of foods offered could have also influenced the results.

Despite the somewhat constrained opportunities for selection, most monkey groups in this study did indeed select diets with higher CP:ADF ratios than what they were offered. It is, however, interesting to note that there were four trials in which the monkeys selected diets with *lower* CP:ADF ratios. In these trials, high amounts (>15%) of lichen were offered, which the monkeys selected against (Table 2-3). Although selection for high CP:ADF ratios seems to hold for several primate species under a variety of conditions (Milton 1981; Chapman and Chapman 2002), under some circumstances it may not. The *Usnea longissima* lichen fed to the monkeys in these trials was low in both crude protein (< 6%) and ADF (6%). By selecting *against* lichen and *for* the other diet items, which tended to be higher in ADF than CP, the monkeys selected a diet having a lower

CP:ADF ratio than the diets they were offered. Clearly, factors other than CP and ADF or their ratio also drive selection. In the case of lichen, these factors may include the presence of possible toxins (Emmerich et al. 1993; Perry et al. 1999; Dailey et al. 2008), or the lichen's taste, texture, smell, or association with positive or negative stimuli. Because the lichen was presented in the same way as other foods in the diet, the most likely cause for the animals selecting against the lichen in this study was a factor intrinsic to the lichen, such as its taste, smell, texture, or toxicity.

Given the results of this study, it is interesting that wild *R. bieti* consume large quantities of lichen not only during winter, but also during summer, when leaves and other food items are abundantly available. Leaves are typically much higher in protein (St. Louis Zoo 2003) than lichen. Potentially, if protein requirements have already been met by other means, lichen may serve as a highly digestible source of energy for the animals. Lichens, a symbiotic organism, are identified by their fungal partner. However, they also contain a photosynthetic partner, typically cyanobacteria or algae, and these vary in chemical makeup, creating different "chemotypes" of lichen within a single lichen species (Nash 2008). In the wild,*R. bieti* may be able to discriminate among the many chemotypes of lichen to find those with the characteristics they prefer, something they were unable to do in the captive feeding trials.

Digestibility

The apparent dry matter digestibility (78.7 ± 0.7%) of the diets was within the range reported by Edwards and Ullrey (1999) (77-81%) for three species of colobines and by Kirkpatrick for *R. bieti* (71-80%) (2001). The digestibility of NDF in this study (63.8 ± 1.6%) was lower than that in foregut fermenting monkeys in the Edwards and Ullrey study (72-77%) and at the lower end of the range of that recorded by Kirkpatrick (61-80%), but still higher than that reported for non-colobine primates (45-47%;Edwards and Ullrey 1999). Like primates, ruminant foregut fermenters also

typically have higher digestibilities than non-foregut fermenting animals (Demment and Van Soest 1985).

Apparent crude protein digestibility in this study was high (70.3 ± 1.6%), as was expected, well within the range reported by Nijboer (2006), 50-88%,Table 2-7) for *Trachypithcus* and *Colobus* species and higher than that reported by Kirkpatrick (49-62%; Kirkpatrick et al. 2001) for *R. bieti*. The lower CP digestibility in Kirkpatrick's study is likely because the dietary CP in those trials was also low (10-14%), and crude protein digestibility decreases with decreasing dietary crude protein (Peters and Harper 1985; Jean et al. 2001). Indeed, the lowest apparent CP digestibilities in the present study were seen on the lowest CP diets, as expected.

Using the body weight and daily caloric intake of the one singly-housed 12-kg animal studied in these trials, endogenous crude protein losses of 2.8 g /100 g of dry matter intake would amount to approximately 20 g per day or 1.67 g/kg BW. Although definitive data are lacking, this is in line with minimum protein intakes that prevent negative nitrogen balance in other primates (NRC 2003). The observed endogenous fecal nitrogen loss (4.5 g of N per kg of dry matter intake) is within the ranges observed in other adult mammals, including leaf-eating marsupials at 4.2 ± 1.4 g N/kg DMI, and grazing ruminants,4.4 ± 0.3, and only slightly lower than observed in browsing ruminants, at 5.3 ± 1.3 g N/kg DMI (Robbins 1994). It appears that colobine monkeys, including *R. bieti*, do not exhibit a particular ability to minimize endogenous protein losses,

Although typically dry matter digestibility declines as fiber content of the diet increases (Demment and Van Soest 1985), in this case this was not seen. This may be in large part due to the fact that the NDF fraction of these diets was from very diverse sources, ranging from primarily intact plants in *R. bieti* diets to a variety of refined agricultural products in the biscuits of the US animals. These

included seed hulls, ground hay, and wheat bran, all of which exhibit different rates and extents of fermentation (Sunvold et al. 1995; Chumpawadee and Pimpa 2008), and may have led to the variation in dry matter digestibility observed.

CONCLUSIONS

Overall, the measured digestive performance features of *R. bieti* do not appear to be dramatically different from those of the other colobine species studied to date, and the apparent digestibility values for DM, CP and NDF are in line with previously reported values (Table 2-7) in *R. bieti* (Kirkpatrick et al. 2001), colobines (Edwards and Ullrey 1999), and primates (NRC, 2003). A positive relationship between dietary crude protein and crude protein digestibility was observed, although the expected negative relationship between dietary fiber and dry matter digestibility was not. Crude protein digestibility was lower in *R. bieti* than in other colobines, but this was expected given the lower CP content of the diets provided to *R. bieti* during the feeding trials. This trend followed that of other mammalian species.

The monkeys in this study did follow the colobine trend of selecting diets with higher protein:fiber ratios, except for diets containing high levels of lichen, when they selected lower protein:fiber ratios. The sampled diets met or exceeded NRC maintenance recommendations for protein, but not for fiber. The ingredients and the nutrient composition of the diets offered to colobine monkeys in both the US and China differed from each other, often in substantial ways, such as in the proportion of biscuits or browse, or the amount of protein. All of the diets exceeded NRC protein recommendations, but none met the NRC recommendations for NDF. Despite this, nearly all of the groups were successfully rearing young and all appeared healthy.

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Table 2-1. Species for which digestibility trials were conducted in the US and in China

Trial	Species	Country	# of Animals[1]
A	*Colobus guereza*	USA	5.1
B	*Colobus guereza*	USA	3.1.1
C	*Trachypithecus obscurus*	USA	1.2
D	*Colobus angolensis*	USA	0.2.2
E	*Colobus angolensis*	USA	1.0
F	*Colobus guereza*	USA	1.2
G	*Colobus guereza*	USA	2.1
K	*Rhinopithecus bieti*	China	1.1.1
L	*Rhinopithecus bieti*	China	1.2.2
M	*Rhinopithecus bieti*	China	1.2.2
N	*Rhinopithecus bieti*	China	1.2.2
O	*Rhinopithecus bieti*	China	1.2.2
P	*Rhinopithecus bieti*	China	1.2.2
Q	*Rhinopithecus bieti*	China	1.2.2
R	*Rhinopithecus bieti*	China	1.2.2
S	*Rhinopithecus bieti*	China	1.1.2
T	*Rhinopithecus bieti*	China	2.2.1
U	*Rhinopithecus bieti*	China	1.1.1
V	*Rhinopithecus bieti*	China	1.1

[1]Animal numbers are given as adult males.adult females.juveniles or unknown sex.

Table 2-2. Ingredients used in trial diet categories. Each institution fed a unique diet from among these ingredients.

Browse/Hay		Primate biscuits	Fruits	Greens	Nuts	Vegetables	
Alfalfa	*Medicago sativa*	Custom product	Apple	Bok choy	Almond	Broccoli	Green onions
Honeysuckle	*Lonicera frgrantissima*	HMS Leafeater	Banana	Cabbage	Peanut	Carrots	Potato
Mulberry	*Morus spp.*	LabDiet Monkey Diet	Cantaloupe	Collards	Walnut	Celery	Sweet potat
Sugarberry	*Caltis laevigata*	Marion Leafeater	Grape	Endive		Corn	Tomatoes
Sweet Gum	*Liquidambar styraciflua*	Mazuri Leafeater	Orange	Kale		Cucumbers	Tomatoes
Wax Myrtle	*Myrica cerifera*		Papaya	Lettuce		Frozen peas	Turnips
Willow	*Salix spp.*		Pear	Spinach		Green Beans	Zucchini
				Turnip Greens			

Table 2-3. Composition of each trial diet as a percentage of diet dry matter for the diet as-offered (AO) and as consumed (C).

Diet	Biscuits		Browse		Fruit		Greens		Lichen		Nuts		Vegetables	
	AO	C	AO	C	AO	C	AO	C	AO	C	AO	C	AO	C
A	61	65	19	0	6	9	7	6	0	0	0	0	8	20
B	78	74	0	0	0	0	10	15	0	0	3	4	8	8
C	54	39	0	0	9	14	14	19	0	0	0	1	23	27
D	70	82	0	6	1	1	6	1	0	0	14	7	9	3
E	81	75	0	4	0	0	8	0	0	0	1	18	10	2
F	64	58	0	0	14	6	15	28	0	0	0	0	7	7
G	59	71	18	0	13	13	3	5	0	0	0	0	7	11
H	31	44	43	19	23	32	2	3	0	0	0	0	1	2
I	32	42	42	24	22	29	2	3	0	0	0	0	2	2
J	37	54	36	13	23	26	2	4	0	0	0	0	2	3
K	28	36	32	26	18	25	1	2	17	8	0	0	2	3
L	28	37	32	24	20	28	1	2	17	5	0	0	2	3
M	29	39	32	22	18	25	1	2	17	9	0	0	2	3
N	23	31	27	29	16	22	1	2	31	13	0	0	2	3
O	21	32	22	25	14	24	1	1	34	15	0	0	2	3
S	38	38	36	36	22	22	0	0	0	0	0	0	5	5
T	40	40	34	34	22	22	0	0	0	0	0	0	4	4
U	34	40	45	37	18	18	0	0	0	0	0	0	4	5
V	34	34	45	45	18	18	0	0	0	0	0	0	4	4
Avg	44	49	24	18	15	18	4	5	6	3	1	2	5	6
SE	4	4	4	3	2	2	1	2	3	1	1	1	1	2
US Avg	67	66	5	1	6	6	9	11	0	0	3	4	10	11
US SE	4	5	3	1	2	2	2	4	0	0	2	2	2	3
China Avg	31	39	35	28	19	24	1	2	10	4	0	0	3	3
China SE	2	2	2	3	1	1	0	0	4	2	0	0	0	0

Table 2-4. Nutrient composition of the diet consumed during feeding trials.

Diet	CP	NDF	ADF	Crude Fat
A	19.6	16.8	11.2	4.9
B	24.4	20.9	14.5	6.7
C	17.8	15.4	12.8	4.8
D	23.2	22.3	11.7	13.1
E	23.5	22.0	13.8	5.2
F	22.8	18.9	12.9	4.2
G	21.3	22.7	13.1	4.6
H	14.8	26.6	19.4	2.6
I	14.8	28.2	19.7	2.6
J	16.6	25.8	18.7	2.7
K	13.4	29.8	16.1	2.9
L	13.1	29.4	18.6	2.8
M	13.8	29.9	18.2	2.9
N	13.4	29.7	17.8	3.0
O	13.7	27.8	16.9	3.1
S	14.7	22.0	20.3	2.5
T	15.1	24.5	19.9	2.5
U	14.8	25.1	18.9	2.5
V	14.8	25.2	21.5	2.5
Avg	17.1	24.4	16.6	4.0
SE	0.9	1.0	0.7	0.6

Table 2-5. Proportion of NDF obtained from browse and biscuit.

Trial	NDF % from browse	NDF % from biscuit
A	0.0	78.4
B	0.0	82.5
C	0.0	66.7
D	0.0	73.9
E	0.0	75.0
F	0.0	65.5
G	8.0	76.0
H	51.2	34.9
I	52.6	33.8
J	42.2	42.7
K	55.6	15.8
L	56.9	17.0
M	53.9	16.9
N	53.0	13.0
O	49.1	13.6
S	51.2	34.9
T	48.7	39.7
U	39.7	46.3
V	60.0	30.2

Table 2-6. Protein:fiber (ADF) ratio of diets consumed by colobine monkeys

Trial	As-Fed	Consumed
A	1.24	1.85
B	1.70	1.71
C	1.42	1.51
D	2.04	2.16
E	1.76	1.80
F	1.47	1.47
G	1.08	1.39
H	0.59	0.67
I	0.60	0.68
J	0.66	0.76
K	0.58	0.65
L	0.58	0.56
M	0.58	0.58
N	0.68	0.58
O	0.79	0.61
S	0.65	0.68
T	0.67	0.71
U	0.65	0.78
V	0.57	0.62

Table 2-7 Diet composition and digestibility in the literature to date. Diet values are percent of diet dry matter. Digestibility is as a percent of the amount consumed

Author	Species	Diet CP	Diet NDF	DM Digest.	CP Digest.	NDF Digest.
Bissell (2014)	*Rhinopithecus bieti*	10.6	29.2	75.9	61.0	64.1
Bissell (2014)	*Rhinopithecus bieti*	10.4	30.3	79.5	62.5	72.2
Bissell (2014)	*Rhinopithecus bieti*	10.3	30.4	79.5	62.6	72.6
Bissell (2014)	*Rhinopithecus bieti*	10.4	28.2	76.2	64.0	60.0
Bissell (2014)	*Rhinopithecus bieti*	10.6	28.8	77.5	64.4	64.8
Bissell (2014)	*Rhinopithecus bieti*	13.3	28.1	75.5	64.9	58.2
Bissell (2014)	*Rhinopithecus bieti*	14.2	28.6	77.8	66.7	58.2
Bissell (2014)	*Rhinopithecus bieti*	13.8	30.9	79.9	67.5	58.2
Bissell (2014)	*Rhinopithecus bieti*	13.4	31.3	81.2	68.1	65.9
Bissell (2014)	*Rhinopithecus bieti*	13.1	27.6	76.4	68.3	58.8
Bissell (2014)	*Rhinopithecus bieti*	14.7	26.5	79.9	69.3	63.2
Bissell (2014)	*Colobus angolensis*	24.8	20.7	73.2	71.2	50.9
Bissell (2014)	*Colobus guereza*	20.8	18.9	79.1	73.4	63.9
Bissell (2014)	*Rhinopithecus bieti*	14.1	26.2	77.1	74.0	57.4
Bissell (2014)	*Trachypithecus obscurus*	19.3	20.0	83.3	75.4	63.6
Bissell (2014)	*Colobus guereza*	19.0	18.7	81.0	78.8	70.6
Bissell (2014)	*Colobus guereza*	24.8	24.9	78.2	80.1	70.7
Bissell (2014)	*Colobus angolensis*	25.2	20.2	78.0	80.2	59.4
Bissell (2014)	*Colobus guereza*	18.2	20.6	86.7	83.2	80.5
Kirkpatrick (2001)	*Rhinopithecus bieti*	15.0	30.0	71.0	49.0	60.0
Kirkpatrick (2001)	*Rhinopithecus bieti*	10.0	37.0	80.0	58.0	81.0
Kirkpatrick (2001)	*Rhinopithecus bieti*	14.0	29.0	78.0	62.0	70.0
Nijboer et al (2006d)	*Colobus guereza*	18.4	42.7	68.0	55.0	63.0
Nijboer et al (2006d)	*Colobus guereza*	20.4	43.8	65.0	57.0	56.0
Nijboer et al (2006d)	*Trachypithecus obscurus*	20.8	26.6	77.0	70.0	74.0
Nijboer et al (2006d)	*Trachypithecus obscurus*	19.9	28.7	78.0	81.0	69.0
Nijboer et al. (2001)	*Trachypithecus françoisi*	19.9	41.1	59.0	50.0	47.0
Nijboer et al. (2001)	*Trachypithecus françoisi*	20.0	51.6	65.0	68.0	62.0

Nijboer et al. (2001)	*Trachypithecus françoisi*	20.4	41.4	74.0	70.0	68.0
Nijboer et al. (2006c)	*Trachypithecus auratus aratus*	19.7	26.0	91.0	68.0	70.0
Nijboer et al. (2006c)	*Trachypithecus auratus aratus*	21.7	27.1	84.0	71.0	77.0
Nijboer et al. (2006c)	*Trachypithecus auratus aratus*	19.5	29.3	78.0	73.0	64.0
Nijboer et al. (2006c)	*Trachypithecus auratus aratus*	20.2	28.1	78.0	88.0	74.0
Watkins et al. (1985)	*Colobus guereza*	16.0	25.2	87.0	78.0	81.0
Oftedal et al. (1984)	*Colobus guereza*	23.0	16.0	83.0	83.0	68.0

Table 2-8. In vivo apparent digestibility of diets

Trial	DM	CP	NDF
A	79.0	73.4	63.9
B	78.2	80.1	70.7
C	83.3	75.4	63.6
D	78.0	80.2	59.4
E	73.2	71.2	50.9
F	81.0	78.8	70.6
G	86.7	83.2	80.5
H	76.4	68.3	58.8
I	75.5	64.9	58.2
J	77.1	74.0	57.4
K	75.9	61.0	64.1
L	76.2	64.0	60.0
M	77.5	64.4	64.8
N	79.5	62.6	72.6
O	79.5	62.5	72.2
S	79.9	67.5	58.2
T	77.8	66.7	58.2
U	79.9	69.3	63.2
V	81.2	68.1	65.9

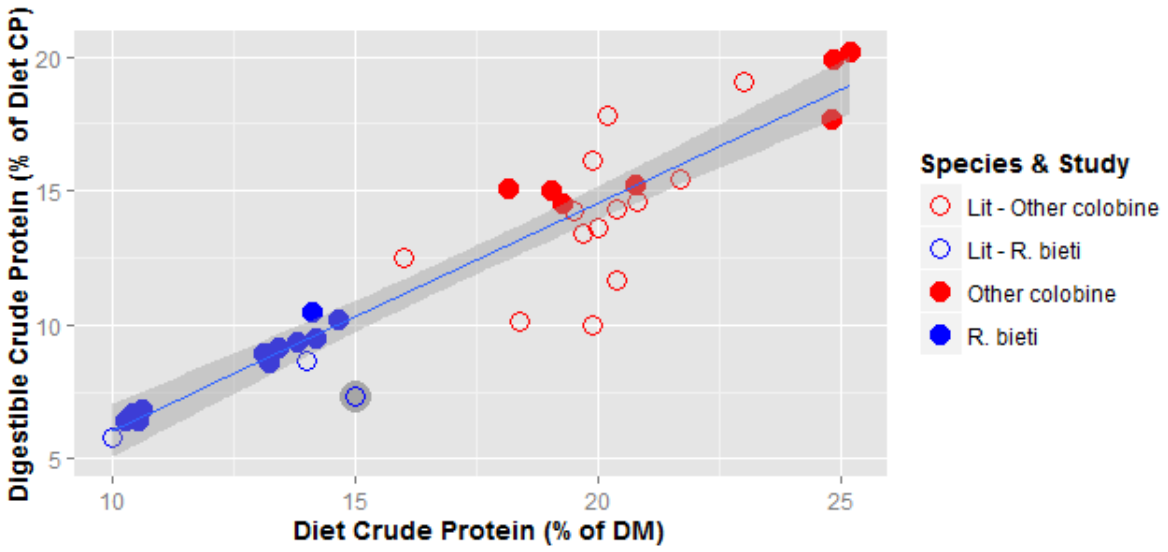

Figure 2-1. Lucas test for the determination of endogenous crude protein losses and true protein digestibility across colobine feeding trials from this study and reported in earlier literature.

Chapter 3: **Lichen as a food source for *Rhinopithecus bieti,* the black-and-white snub-nosed monkey.**

ABSTRACT

Rhinopithecus bieti, a foregut-fermenting colobine monkey, is one of the few animals in the world that subsists largely on lichen for much of the year. Lichen is rarely consumed as the primary food source by most other species for very long because of its low protein and tendency to contain anti-nutritive compounds. This study examined whether the lichen *Usnea longissima* collected from different areas within *R. bieti* habitat would have different chemotypes, whether *R. bieti* discriminates among these chemotypes, whether the different chemotypes contain compounds that alter microbial fermentation, and whether *U. longissima* would depress diet digestibility when fed to captive animals. Wild monkeys showed strong preferences when presented with two chemotypes of lichen in a selection trial, and both chemotypes depressed *in vitro* gas production. Because lichens are so sensitive to climate change, and *R. bieti* habitat is expected to have one of the most rapidly-changing climates on earth, careful monitoring of not only lichen abundance, but also the chemotypic composition of lichens is needed in order to evaluate the sustainability of *R. bieti* in their current habitat.

Keywords: lichen, *Rhinopithecus bieti*, colobine, in vitro fermentation, lichen compounds

INTRODUCTION

Lichens

Lichens are a symbiotic combination of a photosynthetic organism (blue-green algae or cyanobacteria) with a fungus. They are typically long-lived, slow-growing, and can be found in some of the harshest environments on earth, including arctic tundra, exposed rock surfaces, and deserts. They are used as sensitive biological indicators of pollution and environmental change (Nash 2008). Lichens produce a plethora of chemical compounds known for their antibacterial, antitumor, antigrowth, and even antiprion properties (Bustinza 1952; Lawrey 1986; Johnson et al. 2011). Lichen compounds differ among and even within lichen species, as different lichen species (identified by the fungal member) can partner with different photosynthetic organisms, producing different chemotypes within the same species (Culberson, Culberson, and Johnson 1985; Lawrey 1986; Nash 2008).

Lichens as animal food

Lichen is primarily composed of non-fibrous polysaccharides (Stuelp et al. 1999; Svihus and Holand 2000), which are highly digestible both by lichenivorous animals *in vivo* (Robbins 1987; Wallsten 2003; Dubay, Hayward, and Martínez del Rio 2008) and in *in vitro* fermentation systems (Jenks and Leslie Jr 1988; Palo 1993).

However, despite both lichen's digestibility and its abundance in many environments, few animals consume lichen as the majority of their diet. Those that do, typically only eat it when other foods are scarce. One reason for this is that many lichens are low in protein, often less than 3% crude protein (Scotter 1965; Scotter 1972; Staaland et al. 1983; Storeheier et al. 2002), whereas vertebrate crude protein requirements are typically 6% or higher (NRC 2003; NRC 2006; NRC 2007). Mule deer, reindeer, red-backed voles, and northern flying squirrels on lichen-only diets reduce intake, lose weight and are in negative nitrogen balance (Robbins 1987; Storeheier et al. 2004; Dubay, Hayward, and Martínez del Rio 2008; Nilsson et al. 2009). Another reason is that lichens also often contain toxins such as usnic or

vulpinic acid, which may reduce intake either directly due to their bitter taste or indirectly by reducing microbial activity through lowering pH or via antibiotic properties (Lauterwein et al. 1995).

Usnic acid is a defining feature of the genus *Usnea*, one of the main lichens consumed by *R. bieti*. Usnic acid, a phenolic derivative of dibenzofuran, is well-known for its antimicrobial properties (Francolini et al. 2004). Unlike other phenolic secondary metabolites, usnic acid does not appear to work by damaging cell membranes, but is instead cytostatic – inhibiting growth and reproduction of microbes via antimitotic effects (Cocchietto et al. 2002; Cardarelli et al. 1997), which may explain why usnic acid depresses the extent of digestion in sheep (Palo 1993). At high levels, usnic acid is a potent hepatotoxin (Dailey et al. 2008; Durazo et al. 2004; Guo et al. 2008). Usnic acid was implicated in the death of over 400 elk who consumed the lichen *Xanthoparmelia chlorochroa* (Roach et al. 2006; Cook et al. 2007).

A few ruminants, though, consume usnic acid-containing lichens without apparent problems. Reindeer, for example, possess the bacteria *Eubacterium rangiferina* in their rumens, which rapidly degrades usnic acid to the point where it is undetectable in the rumen (Sundset et al. 2008; Sundset et al. 2009). Reindeer who have not consumed lichen recently, however, may lack the necessary microbes to cope with either lichen's toxins or its carbohydrates, and develop diarrhea and "wet belly", both associated with rumen malfunction (Ahman et al. 2002; Nilsson et al. 2009).

Lichens as part of Rhinopithecus bieti's diet

Unlike other lichen-consuming species, the black-and-white snub-nosed monkey, *Rhinopithecus bieti*, appears to consume lichen in high amounts (Bauchop and Martucci 1968; Kay, Hoppe, and Maloiy 1976). This foregut-fermenting colobine monkey lives at elevations that range from 2,600 to 4,500 m above sea level in the Eastern Himalayas of China's Yunnan Province and the Tibetan Autonomous Region. In this harsh habitat, the foods that form the staple of other colobine monkey diets - leaves, fruits, flowers and seeds - are often rare or completely unavailable (Grueter et al. 2009). Lichen, however, is ubiquitous and generally abundant year-round in this area (Kirkpatrick 1996; Grueter et al. 2009). Lichen forms the bulk

of *Rhinopithecus bieti*'s diet in winter when other diet items are unavailable, but also continues to constitute a large proportion of the diet in summer when other diet items are abundant (Kirkpatrick 1996; Xiang et al. 2007; Grueter et al. 2009). Because this reliance on lichen is so unusual among mammals, it raises questions about what features of the lichen and/or the animal enable this unique dietary strategy. For example, the lichens in this region may lack the toxic substances found in the same genera elsewhere, the monkeys may be able to identify and select the less toxic chemotypes of particular lichen species, or the monkeys may possess specialized microbes in their gut that allow them to consume otherwise toxic substances. This study examines the antimicrobial effects of several chemotypes of *Usnea longissima*, the monkeys' selection between two of these chemotypes, and attempts to examine the impact of individual lichen compounds in isolation on microbial communities.

In the middle and southern part of *R. bieti* range, several species of lichen are available and consumed including black lichen (duansongluo, *Bryoria spp.*) and long or beard lichen (changsongluo, *Usnea longissima*) (Xiang et al. 2007; Grueter et al. 2009). Although these lichens lack the cellulose and hemicellulose that dominate most measures of plant fiber, they do contain compounds indigestible by mammalian enzymes (which is a common definition of fiber), including glucans and other polysaccharides (Stuelp et al. 1999; Svihus and Holand 2000). This indicates that *R. bieti* likely relies on its gut microbes to extract energy from these lichens. However, the presence of antimicrobial compounds could reduce digestibility *in vivo* if the microbes cannot tolerate them. Indeed, when lichens (*Cladina spp.*) were incubated with rumen fluid extracted from cattle, rather than from lichen-consuming reindeer, the overall in vitro digestibility was greatly decreased (15% vs more than 70%) in one study (Wallsten 2003). In another study, *Usnea spp.* was approximately half as digestible when fermented in rumen fluid from cattle versus rumen fluid obtained from deer fed 25% lichen diets (Jenks and Leslie Jr 1988). Thus, having the appropriate microbial community can have a significant effect on lichen digestion.

Furthermore, different lichens can have widely different digestibility in *in vitro* systems, in one case from less than 10% to over 80% for different lichen species (Person et al. 1980). To date, only one study has

examined digestibility in *R. bieti* (Kirkpatrick et al. 2001). Although Kirkpatrick *et al.* did not measure lichen digestibility directly, they did determine that as the proportion of lichen (*Bryoria spp.*) in the diet increased in three trials, that the overall diet digestibility increased as well – an indication that the lichen was highly digestible. Overall, it is unknown how well *R. bieti* can digest lichens, in particular the *Usnea longissima* that makes up such an important part of their diet in the south, nor is it known whether toxins that so negatively impact other systems will have a similar impact in this one.

Based on what is known about lichen ingestion by other mammals, this study tests several hypotheses about the relationship between lichen and *R. bieti*:

1. Different chemotypes of *Usnea longissima* exist in *R. bieti* habitat in Yunnan Province, China and these chemotypes vary in their ability to alter fermentation
2. *R. bieti* can discriminate among different chemotypes of *Usnea.*
3. *Usnea longissima* digestibility by *R. bieti* will exceed 70%, and when added to a typical captive diet will not depress digestibility.

MATERIALS/METHODS

Lichen collection

Five samples of *Usnea longissima* were collected in Northwest Yunnan Province, People's Republic of China within the range of *R. bieti* during field studies. The samples were collected in three different locations (Table 3-1) during the course of field observations. Approximately 500 g of lichen was collected as it hung from trees at each location. Samples G and W were manually separated from larger collections by color. Sample K is a pooled sample from across over 20 bags of lichen collected by rangers throughout the Tachen area.

Nutrient Analyses

Prior to analysis, samples were ground through a 1-mm screen in a Wiley Mill and analyzed for dry matter (DM), crude protein (CP), neutral detergent fiber (NDF), crude fat (fat), minerals (P, Ca, K, Mg, Na), and ash using standard methods (Goering and Van Soest 1970; AOAC 1980; AOAC 1990a; AOAC 1990b; Soil & Plant Analysis Laboratory 2005; NFTA 2006) at the University of Wisconsin-Madison Soil Testing Laboratories.

Selection Trials

Two samples, G and W, were used in a selection trial with semi-wild monkeys living at Tachen. These monkeys live within a part of the Xiangguqing Yunnan Golden Monkey Park within the Baimaxueshan Nature Reserve. Although they are free-living, they are confined within the park by roads and natural features in all directions. There was concern that the monkeys had depleted the food resources in their area, so park rangers now provision the animals with lichen collected from outside the group's area twice daily. The lichen is spread out in a designated feeding area observable from a boardwalk.

In this study, two chemotypes of *Usnea longissima* were offered to the monkey group in four separate trials. The two types were called "Green" and "White" by the park rangers and differed slightly but clearly in color accordingly. The rangers reported that the animals showed preferences for some colors of *U. longissima* over others. For each selection trial, 10 "pile pairs" (Figure 3-1) of lichen were placed throughout the monkeys' feeding area. Each pile pair consisted of 100 g of green and 100 g of white lichen placed approximately 10 cm apart from each other. Although moisture can affect the coloration of lichen, the different lichens had been stored in bags together for at least 24 hours, so moisture content was estimated to be similar. Both lichens used were dry to the touch, pliable and not brittle. The pile pairs were separated from each other by at least 1 meter. For ease in data recording, the placement (left-right) of the two colors within each pile pair was the same during each trial, but varied among the four trials.

As the monkey group (50-100 animals) came down from the hills for their twice-daily feedings of lichen, two observers noted on a map which pile (green or white) was touched first, whether it was inspected or sniffed, and which pile was consumed first by the first monkey to approach each pile pair. Because of the difficulty in post-trial weighing of the scattered lichen piles, the samples were not weighed after the trial.

Determination of the partial digestibility of lichen

One lichen sample, K, was the composite sample of the lichen used in a series of digestibility trials conducted on two captive groups (Group 1 and Group 2) of *R. bieti* containing 1 adult male, 2 adult females, and 2 juvenile (1.2.2) animals each. Each animal group housed together was considered one experimental unit.

Each digestibility trial included a 14-20 day adaptation period followed by a 3-day collection period. There was a 7-10 day transition period in between each trial during which the animals were slowly migrated to the next trial diet (Figure 3-2). The adjustments in the proportion of lichen in the diets during the transition period did not exceed two percentage points of change in lichen proportion per day. The animals were offered diets containing different proportions of a basal diet (Table 3-2) and lichen (Table 3-3), and the animals were monitored closely several times each day for signs of digestive disturbance or other problems. Dietary lichen proportions of greater than 17% were refused by the monkeys.

Samples of feed, and all of the feces and orts were dried in a forced air oven at 60°C to constant weight and ground through a manual meat grinder. Intake of each feed ingredient was calculated as feed minus orts by weight on a dry-matter basis. Each dry, ground feed ingredient was then recombined with other feed ingredients in the proportion that each was consumed to create a composite diet sample of the diet the animals consumed for each trial. The nutrient content of composite diets and their corresponding feces were analyzed as above.

The partial digestibility of lichen was determined by the following equation: $D_L = (D_D – (D_B*P_B))/P_L$, where D_L = lichen digestibility, D_D = total diet digestibility, D_B= basal diet digestibility, P_B = the proportion of the total diet that is the basal diet, and P_L= the proportion of the total diet that is lichen (Van Soest 1994). This model assumes that the digestibility of the basal diet portion remains constant.

In vitro fermentations

In order to examine the antimicrobial activity of *Usnea* from *R. bieti* habitat, the impact of Usnea and Usnea compounds was tested in a bovine *in vitro* fermentation system. Furthermore, to determine whether the lichen itself was poorly fermentable versus whether there were specific lichen compounds that inhibited fermentation, lichen compounds were removed via an acetone extraction and tested separately from the compound-free lichen material.

The method of Feige et al. (1993) was used to extract lichen compounds from the lichen. In this method, subsamples weighing 0.1 g of each ground lichen sample were mixed with acetone for 60 minutes on a laboratory shaker. The acetone supernatant was transferred to a clean vial, and then evaporated in a fume hood, leaving behind a residue of the extracted compounds. The resulting residue was resuspended in 50 µl acetone and then added to a hay standard and allowed to evaporate.

The substrates tested included:

1. Hay alone (positive control)
2. Hay with 50 µl acetone added and then allowed to evaporate (to test whether the solvent itself contained residue that negatively impacted the *in vitro* system).
3. A 50/50 mixture of hay standard and ground lichen (*Usnea longissima*) from lichen samples G and W
4. Hay standard mixed with the lichen material (pellet) left behind after the extraction (which should be free of the lichen compounds)

5. Hay standard with 50 ml acetone-soluble extracts from G and W

Fermentations were conducted in 20-ml serum vials that had known weights and volumes. The fermentations including the preparation of the rumen fluid inoculum followed the methods of Weimer et al. (2004), except that amounts were adjusted to use 20-ml serum vials. Specifically, each vial received 0.10 g of substrate, 6.7 ml of buffer, 300 μl of reducing agent (Goering and Van Soest 1970) and 3 ml of rumen fluid inoculum . Gas pressure readings were recorded at 48 h. The final gas volume produced was calculated by converting from PSI recorded using the known volume of each vial and subtracting the volume of air lost in the syringe at each measurement point.

Samples were run in triplicate using a single batch of rumen fluid. Differences were analyzed using an ANOVA followed by a one-sided Dunnet's test to determine whether the mean gas production in samples with lichen extract was less than the mean of those without lichen extract (solvent blanks). All analyses were conducted using the R statistical package (R Core Team 2012) with the *multcomp* (Hothorn, Bretz, and Westfall 2008) and *car* (Fox and Weisberg 2011) libraries. The selection trials were analyzed using a X^2 test.

RESULTS

Nutrient Analyses

The nutritional composition of the five lichen samples is given in Table 3-4.

Selection Trials

Between three and five animals participated in each of the four selection trials, although many more passed through the trial area and did not stop at any pile (Figure 3-3). Although the animals were not generally individually identifiable, there was one distinct male that was the first to arrive and select from the piles during each trial. The other 2-4 animals (adult females and juveniles of unknown sex) were unidentifiable and could have been the same or different animals in each trial. In total, 18 piles were

selected from the 40 total pile pairs across four trials (22 pile pairs were not approached, Table 3-5). In all cases, the animals selected sample G and ignored sample W, regardless of the position of the piles ($\chi^2(1, N=18)$, $p < 0.0001$). In no case did the animals inspect, sniff, or otherwise interact with the W pile, and in every case the animals consumed pile G either immediately (14 out of 18 selections) or after sniffing or inspecting it first (4 out of 18 selections). The male never inspected or sniffed the lichen before consuming it.

Lichen intake and partial digestibility

The captive monkeys initially eagerly consumed the lichen when it was first presented at the initial level of 5% of the diet. However, after three days, none of the animals would eat lichen again for over a week, even thought it was offered daily. Finally, at day 10, they began consuming small quantities of lichen each day. As the proportion of lichen in the diet increased, lichen consumption increased, although it never equaled the as-offered proportion. Fecal quality was maintained throughout – no instances of diarrhea or loose stools were observed in the studied groups. All weaned juvenile and adult animals in each group were observed consuming lichen. The youngest animals appeared to eat the most, although individual intakes could not be measured.

The average partial digestibility of lichen was 81.3 ± 3.5% (Table 3-6).

In vitro fermentations

There were marked reductions in *in vitro* gas production when whole lichen samples G and W or extracts containing acetone-soluble lichen compounds from G and W were added to the standard, but not when those compounds were absent. The solvent alone (added, then allowed to evaporate) did not impact fermentation. Lichen material with the lichen compounds removed had a high in vitro digestibility, equal to or higher than hay alone. (Figure 3-4). G and W did not differ from one another ($p > 0.7$).

DISCUSSION

Rhinopithecus bieti appears to be selective when consuming lichen. Within a given patch of trees or single tree full of lichen, they will eat some clumps while passing over others, or pick up a clump of lichen and select and consume only certain portions (pers. obs.). The animals may be selecting for or against particular flavors, textures, toxins, or nutrients, and appear to use both appearance and smell. Old World Cercopithecine primates have tri-chromatic vision (Jacobs 1996). Many lichen compounds are strongly colored, and *R. bieti* likely can use this feature or other aspects of the lichens' appearance to distinguish among lichens. For example, *R. bieti* have been observed carefully picking strands of the bright yellow and highly toxic *Sulcaria virens* out of clumps of *Usnea* (pers. obs.). There can be differences in color even within a single species of lichen. *Usnea longissima*, for example, ranges in color from almost white to yellow, green, and dark gray. Smell may provide additional information, and the monkeys have been observed sniffing lichen as they forage (pers. obs.). It is unclear from behavioral observations whether monkeys prefer some lichen species to others when both occur in a group's range, although there is anecdotal evidence that they prefer black lichen when it is available (Long Yongcheng, personal communication).

The animals showed a clear preference for the green *Usnea* (G) in the selection trials over the white *Usnea* (W). It remains unclear what factors influenced the animals to select between these two chemotypes. Given that the animals passed over the white samples without any close inspection, it seems likely that visual cues are an important means that *R. bieti* can use to distinguish among chemotypes. Both samples G and W inhibited in vitro gas production when added to fermentation vials. Extraction of the acetone-soluble lichen compounds rendered the remaining lichen as digestible as the hay standard, indicating that the *Usnea* fed to *R. bieti* at Tachen was intrinsically highly digestible, and that it can contain compounds soluble in acetone that negatively influence fermentation.

Kirkpatrick et al. (2001) fed captive *R. bieti* a diet containing approximately three times more lichen than leaf material on a dry matter basis. Although the authors of that study did not assess lichen digestibility directly, they found that approximately three times more of the fecal particles were lichen than leaf, and that the proportions of lichen and leaf consumed were similar to the proportions of leaf and lichen fecal particles, concluding that lichens and leaves had similar digestibility. The dry matter digestibility of that trial diet was 80%. Likewise, diets with increasing amounts of lichen in this study had similar digestibility (c.f. Table 3-6).

Negative impacts of lichen on digestibility *in vivo* were not seen in this study. There may be several reasons for this. First, the digestibility trials spanned several months, with steadily increasing quantities of lichen to prevent any sudden diet changes that animal managers felt might compromise the captive monkeys' health. This may have allowed the development of a microbial community that could tolerate or even break down any lichen compounds with toxic or antimicrobial effects that existed. It is also possible that the levels in the diet may have never risen to the level where a noticeable physiological impact occurred. Several animals in the group were wild-caught, and had been captured over a decade before the study began. It is possible that they maintained microorganisms adapted to lichen detoxification for many years. The pattern of their lichen intake behavior is suggestive of an adaptation to the lichen, either physiologically by the induction of detoxification pathways in the animal, or by selection for stomach flora with the ability to detoxify the compounds (Freeland & Janzen, 1974). The drop in lichen consumption from days 3-10 of the study, after an initially enthusiastic consumption, could be an indication that the animals were experiencing some alterations in their internal or microbial physiology, and avoided the lichen as a result. As the microbial community adjusted, they may have resumed their intake, a pattern seen in other animals exposed to low doses of toxic compounds (Freeland & Janzen, 1974; Oates, Swain & Zantovska, 1977).

The digestibility of *Usnea longissima* determined in this study (86%) was very similar to the digesibility of a different arboreal lichen, *Alectoria sarmentosa*, measured in mule deer (85.2%) by Robbins (1987).

In another study, the digestibility of lichen *Bryoria* fed to southern red-backed voles was 87.8% ± 5.5%, and to northern flying squirrels, 89.0% ± 4.3% (Dubay, Hayward, and Martínez del Rio 2008), although both the deer and the voles were in negative nitrogen balance. The values in this study are in accord with these other, similar studies.

Lichen, composed mainly of soluble carbohydrates such as lichenin, chitin, and other forms of soluble fiber, requires time for microbial fermentation. In seven species of lichen consumed by reindeer, the peak rate of in vitro gas production occurred at 13 hours (Svihus & Holand, 2000), and then dropped off rapidly, producing one third as much gas by approximately 25 hours. However, in another study where the investigator added urea (a nitrogen source) to the fermentation, the rate of lichen fermentation remained high for over 48 hours, leading the author to suggest that lichens may need 100 hours or more for complete fermentation (Wallsten, 2003). The low nitrogen content of many lichens may depress the rate or extent of fermentation when they are the sole source of nutrients. The mean transit time (time to first appearance of an indigestible marker) in *R. bieti* was estimated to be 27 hours by Kirkpatrick (2001), and the mean retention time (a weighted average of when the markers appear in the feces) was 47 hours. This indicates that, if nitrogen is available in sufficient quantities, lichen may not be completely fermented before it passes through the gut, and that if nitrogen is low, such as it would be on winter diets, fermentation may be incomplete.

On a hypothetical lichen-only diet (crude protein estimated to be 6.12% and 88% digestible, metabolizable energy (ME) estimated to be 3 kcal/g) consumed by *R. bieti* during winter, a 12 kg animal would consume approximate 770 kcal, or 257 g of lichen dry matter per day, containing approximately 13.82 g of digestible CP consumed, equivalent to 2.14 g digestible CP/kg metabolic body weight (MBW) or 7.2% of ME intake. With endogenous losses of 3.08 g of CP/day, this would leave approximately 10 g of protein for maintenance, growth, or production.

Minimum protein requirements for primates are not well-defined. The lowest reported requirement is for adult *Callithrix jacchus* where at least 1.43 g/kg MBW (adapted from NRC 2003) is needed to avoid negative nitrogen balance. Expressed in similar terms for the hypothetical lichen-only diet, the total CP intake of *R. bieti* would be 2.44 g total CP/kg MBW, which exceeds the minimum requirements for some other adult primates (including *Homo sapiens, Cebus albirons, Saguinus fuscicollis*), but is deficient when compared with the estimated minimum requirements for adult *Macaca spp.* and the requirements for all growing primates (NRC, 2003). This indicates that *R. bieti* on a lichen-only diet could be at risk of being in negative nitrogen balance, and very likely is in negative nitrogen balance when eating this diet during growth and reproductive phases, when daily requirements are highest.

On an energy basis, however, the situation is not as grim. The value of 7.2% for crude protein as a percent of ME in lichen compares favorably with values reported for other non-human primates of all life stages including adult *Saguinus fuscicollis* (6.2% of ME), adult *Callithrix jacchus* (6.0% of ME), infant *Macaca mulatta* (1.7% of ME), and adult *Macaca mulatta* (minimum of 6.7% of ME; NRC 2003).

However, these estimated requirements were set using protein sources with a high biological value (near 100%). The protein available to a lichen-consuming *R. bieti* monkey, on the other hand, will be a mixture of the protein available from lichen itself as well as microbial protein produced in the gut. Microbial protein has a biological value of approximately 60-70% (McDonald 1954; FAO 2014). The biological value of lichen protein is unknown, but may resemble that of other edible fungi – mushrooms. In one study of rats, diets containing two species of mushrooms led to sharply reduced growth rates (Longvah and Deosthale 1998), and in another, the biological value of the protein in the studied mushrooms ranged from 31-50% (Mukiibi 1971). The biological value of the protein available to *R. bieti* on the hypothetical lichen-only diet is likely less than 70%, which would increase their minimum requirements. Thus, *R. bieti* is likely in or near a state of negative nitrogen balance in winter when consuming a predominately lichen diet, particularly for females that are pregnant or nursing, and for growing young animals.

Lichen has several other aspects that make it challenging as a food source. It is very low in sodium, calcium, and phosphorus compared with NRC requirements (Table 3-4). Sodium deficiency can affect bone mineralization, growth, and reproduction. Sodium is one of the few nutrients that, when deficient, generates a specific "hunger" for it. Salt licks become valuable resources in sodium-deficient habits, such as mountains and regions far from the ocean, both of which apply to northwest Yunnan province. Salt mining is present in the area, and so salt licks may exist, although none were identified by field biologists or local livestock herders in the area when questioned. Guereza and gorillas in Africa actively seek out sources of sodium (Oates, 1978; Rothman, Van Soest & Pell, 2006; Fashing, Dierenfeld & Mowry, 2007), and it is tentatively assumed that *R. bieti* must also seek out such sources, although this has not been observed.

Calcium and phosphorus are also essential for bone metabolism and growth, and low levels can impede reproduction. Consuming vertebrate prey may mitigate some of these issues. *R. bieti* has been observed in several isolated incidents scavenging or even killing prey such as squirrels and birds (Ren *et al.*, 2010), but this behavior apprears to be extremely rare, and indeed, such carnivory has rarely been observed in any colobine monkey (Kay and Davies 1994).

It remains unclear whether *R. bieti* is under nutritional stress in winter and whether there are as-yet-unknown resources that it can draw upon to meet protein and mineral needs during winter.

CONCLUSIONS

The five samples of *Usnea longissima* lichen collected from the southern half of *R. bieti*'s range in this study varied greatly in their chemical composition. The animals showed clear selection between two chemotypes of this lichen in a small selection trial, and they were observed selecting and discarding certain clumps or strands of lichen at other times in captivity and in the wild. They appear able to digest lichen containing antimicrobial compounds at a level comparable to a captive diet and comparably to

other lichen-consuming mammals, and appear able, if not particularly willing, to consume diets containing at least 15% lichen.

The level of protein intake on a hypothetical lichen-only diet, such as is likely during winter months, particularly in the northern part of their range, is probably marginally adequate for adult, non-reproductive animals, and is likely deficient for growing, pregnant, or lactating animals. The animals may select for lichens with higher available protein content (although they did not do so in one selection trial here), have an as-yet-unknown source of protein available to them in winter, or may indeed be in negative nitrogen balance. It is possible that, like reindeer, they may catabolize body protein stores to maintain pregnancy or lactation during this time.

If the animals are truly averse to particular chemotypes of lichen, or must select for lichens with the highest protein content, then the determining the carrying capacity of habitat based only on abundance of lichen in *R. bieti* habitat may exaggerate the available resources for the monkeys because only some of the lichen may be usable by the monkeys. Because lichens are so sensitive to climate change (Geiser and Neitlich 2007; Aptroot 2009), the impending changes to the Himalayan climate will likely dramatically change the composition and availability of lichens in the area. Careful monitoring of not simply lichen abundance, but also the nutritional and chemotypic composition of lichens is needed in order to assess the sustainability of *R. bieti* in their current habitat.

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the Institute of Eastern-Himalaya Biodiversity Research at Dali University or the support of Long Yongcheng of The Nature Conservancy and the team working with *R. bieti* in Tachen. I'd like to thank Paul Weimer of the USDA Dairy Forage Research Center for his advice and assistance running the in vitros.

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Table 3-1. Samples of *Usnea longissima* used in this study

Sample	Collection Year	Collection Site	# of harvest locations at each collection site	Use of samples in this study
H	2008	Mt. Lasha	One	*In vitro* fermentation (IVF)
M	2008	Mt. Longma	One	IVF
G	2009	Tachen	Many	Selection Trials, IVF
W	2009	Tachen	Many	Selection Trials, IVF
K	2010	Tachen	Many	Digestion Trial, IVF

Figure 3-1. A lichen "pile-pair". Green *U. longissima* is on the left, White *U. longissima* is on the right. Each pile is approximately 15 cm across.

Trial 1			Trial 2			Etc.
Adaptation Period	Collection Period	Transition Period	Adaptation Period	Collection Period	Transition Period	
14-20 d	3 d	7-10 d	14-20 d	3 d	7-10 d	

Figure 3-2. Time frame of trials (only first two trials shown).

Table 3-2. Composition of basal diet (dry matter basis)

Feed	Proportion of basal diet DM
Apples	26%
Cabbage	4%
Cucumbers	2%
Lichen	0%
Mulberry leaves	23%
Pears	5%
Primate biscuit	56%
Tomatoes	1%

Table 3-3. Proportions of diet items consumed during each trial.

Trial	Animal Group	Basal Diet %	Lichen %
1	Group 1	100%	0%
2	Group 2	100%	0%
3	Group 1	92%	8%
4	Group 2	90%	10%
5	Group 1	83%	17%
6	Group 2	85%	15%

Table 3-4. Nutrient composition of *Usnea longissima* samples collected in Yunnan Province, China. Dry matter (DM) is given as a percentage of the fresh sample. All others are given in units of DM. NRC requirements are listed for colobines when available, and as as the lowest estimated nutrient requirements for other primate species otherwise.

Sample	DM	CP	NDF	Fat	P	Ca	K	Mg	Na	Ash
		%	%	%	%	%	%	%	ppm	%
H	92.58	5.86	29.38	6.20	0.12	0.30	0.23	0.05	52.6	1.38
M	93.01	6.21	31.18	6.41	0.13	0.29	0.25	0.05	44.3	1.00
G	92.29	6.07	29.91	5.02	0.08	0.36	0.26	0.05	22.0	1.99
W	92.72	6.33	31.71	4.90	0.13	0.45	0.15	0.06	66.6	2.24
K	93.56	6.11	30.94	5.91	0.12	0.44	0.23	0.05	54.8	1.72
Avg	92.83	6.12	30.62	5.69	0.12	0.37	0.22	0.05	48.06	1.67
SE	0.22	0.08	0.43	0.31	0.01	0.03	0.02	0.00	7.43	0.22
NRC primate requirements		6-18	30	NA	0.33	0.55	0.24	0.04	2000	NA

Figure 3-3. Adult female and juvenile (sex unknown) consuming the Green lichen pile from a pile pair. In the photo on the left, the adult female is actually sitting on the White pile.

Table 3-5. Selection trial results (raw counts) for *R. bieti* selecting between two suspected chemotypes of *U. longissima* lichen, green and white.

Trial	Day	Time	Green side	No. of animals	Sniff/ Inspect	Number of piles consumed (10 of each color per trial)
1	1	AM	Left	5	2	7 Green, 0 White
2	1	PM	Right	4	0	4 Green, 0 White
3	2	AM	Right	3	1	4 Green, 0 White
4	2	PM	Left	3	1	3 Green, 0 White

Table 3-6. Summary of trials done to determine the partial digestibility of lichen.

Monkey group	Lichen offered	Basal diet	Lichen	DM digestibility	Calculated lichen digestibility
1	0.0	100.0	0.0	77.0	
2	0.0	100.0	0.0	78.9	
1	15.0	90.4	9.6	76.9	76.2
1	15.0	93.5	6.5	77.3	82.1
2	15.0	90.2	9.8	78.7	77.3
1	30.0	84.8	15.2	80.6	100.9
2	30.0	82.6	17.4	80.7	89.6

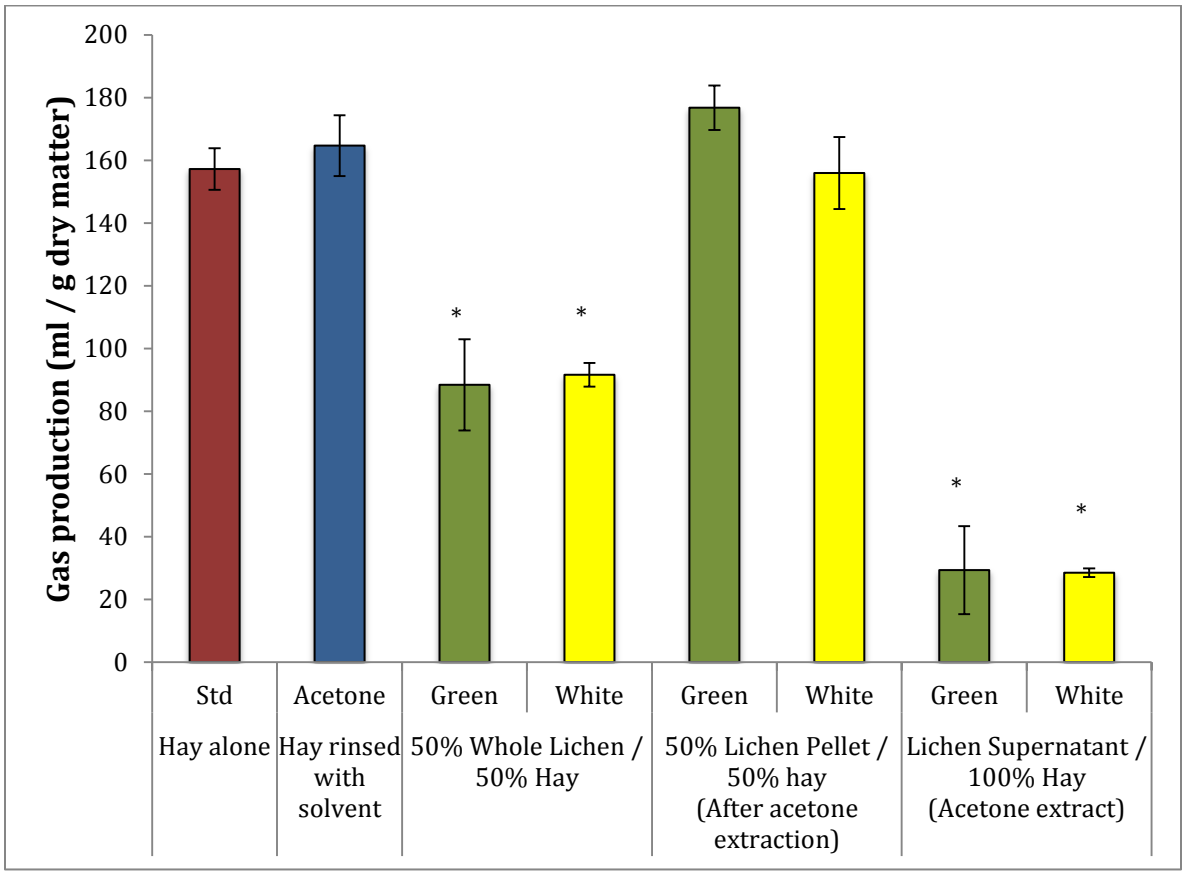

Figure 3-4. Influence of lichen components on *in vitro* gas production (n=3 for each bar). Samples that differed ($p < 0.05$) from the hay standard are indicated with a *.

Chapter 4: **Conclusions**

Rhinopithcus bieti, a colobine monkey, is uniquely able to survive in its harsh, high-elevation habitat as a result of several key adaptations. The first is its colobine lineage with its foregut fermentation system that likely allows it greater flexibility to consume a low-protein diet such as lichen. Further investigation is still needed to examine what other sources of protein may be available to the monkeys in winter, or the mechanisms by which they cope with seasonal protein deficits. The second adaptation is a mechanism, still unknown, that allows *R. bieti* to consume large quantities of lichen despite the presence of antimicrobial compounds. One possibility is that *R. bieti* possess a unique microbial community that is able to break down the antimicrobial compounds, such has been seen in sheep able to consume *Leucaena* or reindeer able to consume usnic acid. Anecdotally, there seemed to be some slight evidence for this during the digestibility trials when the animals initially consumed lichen eagerly, then stopped, then slowly began eating increasing amounts of lichen. This would be consistent with lichen being palatable, but causing mild discomfort, followed by a slowly increasing tolerance for it. Another possibility is careful selection by the monkeys for lichens with lower antimicrobial activity. This study tested whether semi-wild *R. bieti* were selective when consuming lichen and found that the monkeys could be very selective between at least two chemotypes of lichen, although what, exactly, they were selecting for or against is still unclear. Lichen is highly digestible (~81%) and likely provides as much or more energy to the monkeys than leaves do. Because of its ubiquity in the region, lichen clearly serves as a fallback food, being one of the only foods available throughout the coldest months. That *R. bieti* continues to consume lichen during warmer months indicates its continuing value as an energy source.

This study also examined how *R. bieti*'s digestive performance compared with that of other colobine monkeys. Like other colobines, protein digestibility increased as the protein content of the diet increased. Overall diet digestibility was similar to that of other colobine monkeys. Like other colobine monkeys, *R. bieti* tended to select diets (from what they were offered) with lower protein-to-fiber ratios, although this did not hold true when they were fed diets high in lichen. Because habitats with elevated protein-to-fiber ratios are associated with a larger colobine biomass in other colobines, it would be interesting to compare the biomass of *R. bieti* in the groups who rely largely or minimally on lichen as a food source in winter. In addition, because some anti-nutritive compounds can influence fitness, it would be interesting to explore whether monkey groups that have a higher reliance on lichen would have any loss in fitness as a result.

There are no known food sources available in winter that could sustain large groups of large primates in the central and northern parts of the monkeys' range other than lichen. Given the speed of climate change in the area, and lichens' sensitivity to environmental changes, a rapid change in lichen abundance could have severe consequences for *R. bieti*. Although several climate models for the area actually predict increasing forest area, it remains to be seen whether the new forest area will support sufficient winter food supply for many *R. bieti* groups. Assessment of lichen abundance and the impact of climate change on the main winter fallback food of *R. bieti* should be priorities for future research and conservation of this valuable species.

Appendix A: Use of high-pressure liquid chromatography to separate lichen compounds and assess them for anti-fermentative activity

INTRODUCTION

To investigate whether there were individual components of lichen that could influence selection or fermentation, five lichen samples (Table 3-1) were separated using high-pressure liquid chromatography (HPLC), and then each of these separated extracts was assessed in an in vitro fermentation system to examine whether there was a reduction in fermentative activity. The use of HPLC enabled separation of compounds (or combinations of compounds that eluted at the same time) so that they could be used for the *in vitro* fermentation analysis, but did not allow identification of the compounds. For that, thin-layer chromatography or mass spectroscopy would be needed.

METHODS

Compounds from five samples of *Usnea* (Table 3-1) were separated using high-pressure liquid chromatography (HPLC). The extraction and HPLC separation followed the methods of Feige et al. (1993).

To extract the lichen compounds, subsamples weighing 0.5 g of each ground lichen sample were mixed with acetone for 60 minutes on a laboratory shaker. The acetone supernatant was transferred to a clean vial, and then evaporated in a fume hood, leaving behind a residue of the extracted compounds. The resulting residue was resuspended in a mixture of 30% methanol and 70% of a 1% ortho-phosphoric acid solution and placed in an ultrasonic vibrator for 5 minutes. The samples were then filtered through a 0.45-μm acrodisk with low protein binding (Gelman polysulfone #4497) and stored in a -80 °C freezer.

The peaks were characterized by HPLC using a two-solvent system (1% ortho-phosphoric acid and methanol) on a Beckman Coulter System Gold System 126 machine . Absorbance was measured at λ = 245 nm. Peaks with amplitudes over 100 mAU were targeted for collection (Figure 4-1). The compounds eluted at the selected peaks were collected by running multiple aliquots of the lichen extracts through the HPLC and collecting the output during each peak in absorbance into individual vials.

Because the amount of fluid obtained from each HPLC peak varied depending upon the width of the peak, each peak was brought to volume with additional amounts of the HPLC solvent so that the same amount of fluid extracts could be added to each fermentation. The extracts were identified by sample letter and peak number (e.g. H2 was the second peak from lichen sample H).

Fermentations were conducted in 20-ml serum vials that had known weights and volumes. The fermentations including the preparation of the rumen fluid inoculum followed the methods of Weimer et al. (2004), except that amounts were adjusted to use 20-ml serum vials. Specifically, each vial received 0.10 g of substrate, 6.7 ml of buffer, 300 µl of reducing agent (Goering and Van Soest 1970) and 3 ml of rumen fluid inoculum . Gas pressure readings were recorded at 48 h. The final gas volume produced was calculated by converting from PSI recorded using the known volume of each vial and subtracting the volume of air lost in the syringe at each measurement point.

Colobine monkeys can have low ruminal pH s (Bauchop and Martucci 1968; Ohwaki et al. 1974), and the mean transit time has been reported as 27 h for *R. bieti* (Kirkpatrick et al. 2001), and 13.5-43.5 h for other colobine species (Edwards and Ullrey 1999), depending on methodology. Therefore, fermentations were tested using buffers with pH 5.6, 6.3, and 6.8 and with gas pressure readings at 0, 12, 34, and 48 h. However, pH 6.8 and 48 h, as used in standard bovine in vitro fermentation setups, produced the closest values to *in vivo* digestibility values, and so these were used here.

Samples were run in triplicate across at least two different runs (n=6). Differences were analyzed using an ANOVA followed by a one-sided Dunnet's test to determine whether the mean gas production in samples with lichen extract was less than the mean of those without lichen extract (solvent blanks). All analyses were conducted using the R statistical package (R Core Team 2012) with the *multcomp* (Hothorn, Bretz, and Westfall 2008) and *car* (Fox and Weisberg 2011) libraries.

RESULTS

A total of 32 peaks were selected from the five lichen sample extracts (Table 4-1). Each sample differed from the others in terms of the number of peaks and their elution times within the run. Hay incubated with extracts H1, H2, M1, M2, and M3 produced significantly less gas at 96 h than the control samples with only the solvent added ($p < 0.05$), and two other peaks (K1 and K3) tended to produce less gas ($p < 0.1$;Figure 4-2). Three out of the five lichen samples (H, M and K) contained compounds that inhibited or tended to inhibit fermentation. The two lichen samples that were used in the selection trial (G and W) did not.

DISCUSSION

Two extracts, K1 and K3, from the lichen used for the digestibility trials in Chapter 2, inhibited gas production in an *in vitro* system. If sample K contained compounds that negatively influenced monkey digestion or palatability, it could explain why the monkeys were not more willing to consume diets high in lichen during the lichen-feeding trials (Chapter 3). None of the extracts from the two lichen samples (G and W) used in the selection trials described in Chapter 3 reduced *in vitro* gas production, although the whole lichen samples G and W both did. It is possible that the antimicrobial substance(s) that reduced gas production when whole were not separated out or were not separated out in sufficient quantity to alter the *in vitro* fermentation, or that it is an interaction of substances that inhibits fermentation.

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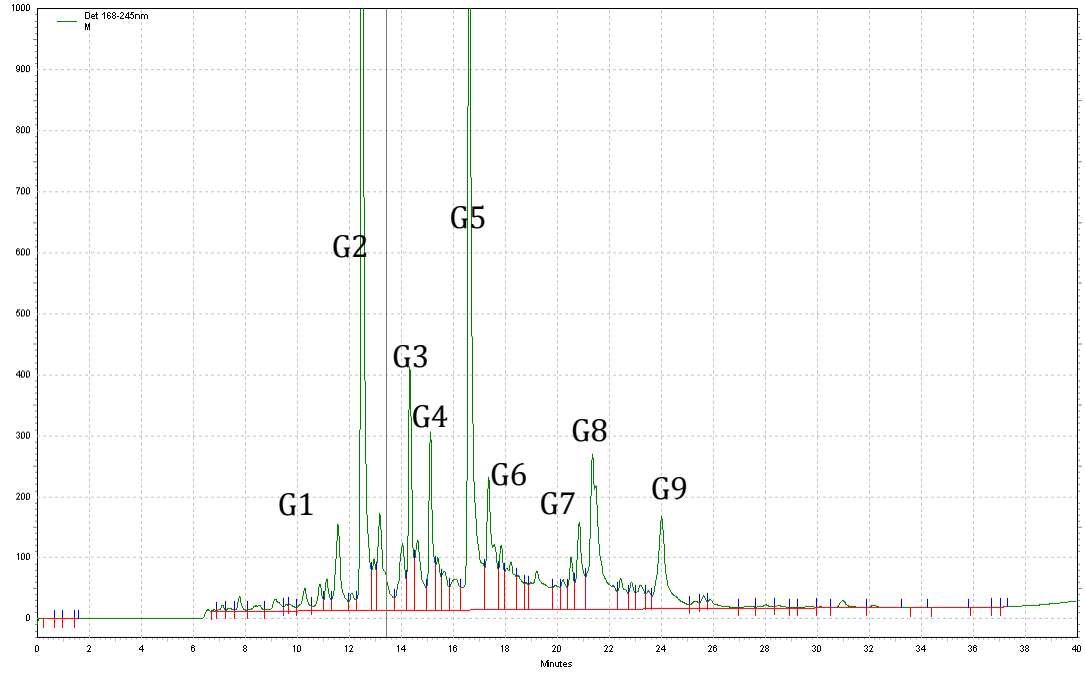

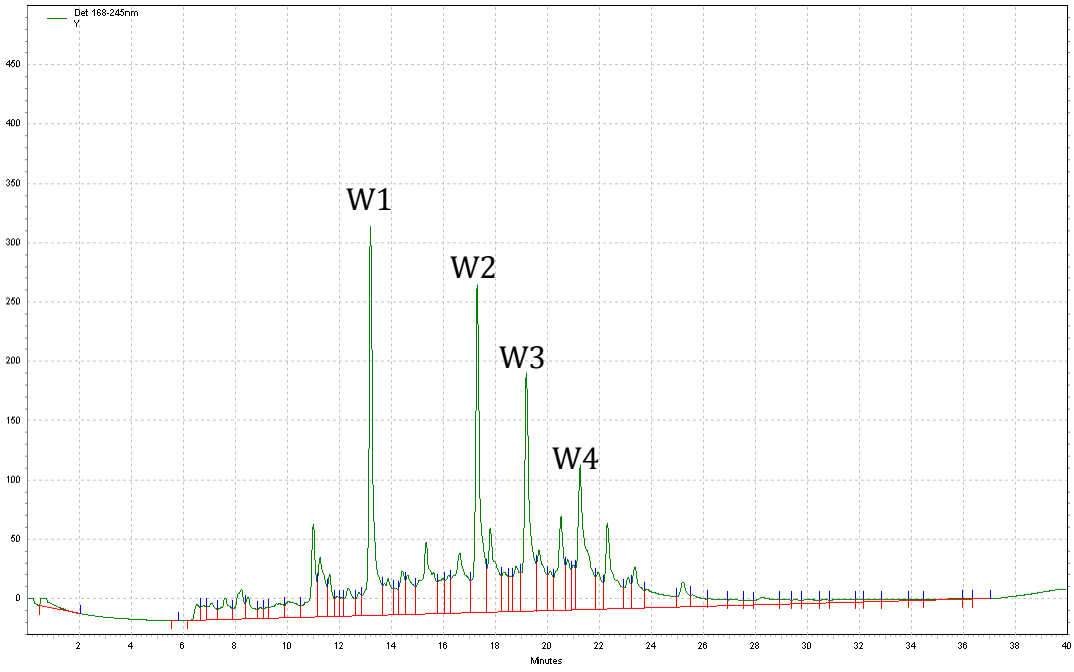

Figure 4-1**.** Peaks obtained via high-pressure liquid chromatography for samples G and W.

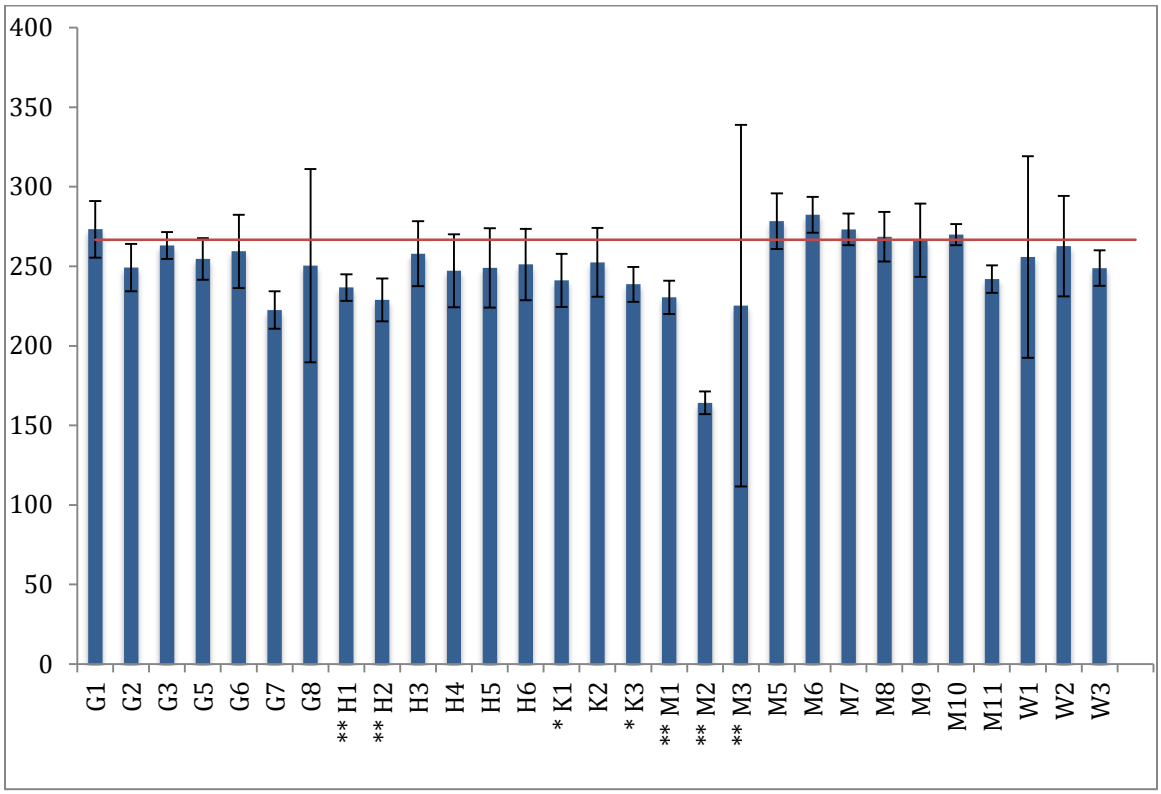

Figure 4-2. 96-hour gas production from fermentation of individual lichen extracts compared with the standard sample (red, horizontal line). Samples that are different from the control with a p-value of < 0.05 are indicated with **, samples that differ with a p-value < 0.1 are indicated with a *.

Table 4-1. Individual lichen extracts separated by HPLC with elution times. The letter in the peak name corresponds to the plant samples listed in Table 1. The number in the peak name corresponds to the order the peak emerged off the column.

Peak	Start (min)	Stop (min)	Different from control (p-value)
G1	11	12	
G2	12.5	14	
G3	14	15	
G5	16.5	17.5	
G6	17.5	18	
G7	21	21.5	
G8	21.5	22.5	
H1	12	12.5	0.029
H2	12.5	13.5	0.002
H3	13.5	14.5	
H4	16	16.5	
H5	16.5	18	
H6	19	20	
K1	13	14	0.097
K2	17	18	
K3	18.5	19.5	0.051
M1	11	11.5	0.004
M2	12	13	<0.001
M3	13	14	<0.001
M5	15	15.5	
M6	16	17	
M7	17	18	
M8	18	19	
M9	19	19.5	
M10	20.5	21	
M11	21	22	
W1	13	14	
W2	17	18	
W3	19	20	