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High Energy or Protein Concentrations in Food as Possible Offsets for Cyanide Consumption by Specialized Bamboo Lemurs in Madagascar

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Abstract Plants producing toxic plant secondary metabolites (PSMs) deter folivores from feeding on them. Animals that can cope with noxious PSMs have a niche with a competitive advantage over other species. However, the ability to cope with toxic PSMs incurs the costs of detoxification. To assess possible compensations for the ingestion of toxic PSMs, we compare the chemical quality of plants consumed by bamboo lemurs (genera *Hapalemur* and *Prolemur*; strepsirrhine primates of Madagascar) in areas with and without bamboo. Some bamboo lemurs consume bamboo containing concentrations of cyanogenic substances 10–50 times above the average lethal dosage for mammals, and we postulate that animals consuming cyanogenic substances need supplementary protein or readily available energy for detoxification. We compared the chemical composition of food consumed by three species of bamboo lemurs that feed mainly (>80% of their time) on bamboo in the evergreen rainforest of Ranomafana (Madagascar) with published data of the diets of bamboo lemurs at two sites without highly cyanogenic plants (reed beds of Lac Alaotra and the

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evergreen littoral forest of Mandena) and with food of sympatric folivorous lemur species that do not feed on bamboo. Lemurs feeding on bamboo consumed up to twice as much protein as bamboo lemurs in areas without bamboo and sympatric lemur species that feed on leaves of trees. Concentrations of nonstructural carbohydrates (a source of energy) showed the opposite trend. This result supports the hypothesis that feeding on cyanogenic plants is linked to high protein intake, either as a source of protein or for sulfur-containing amino acids that can be used for detoxification. Owing to the high protein concentrations in bamboo, however, we cannot distinguish between the hypothesis that lemurs that eat bamboo target additional food items with higher protein from the hypothesis that lemurs feeding on bamboo unavoidably obtain higher concentrations of protein than animals feeding on leaves of trees, without an added nutritional benefit.

Keywords Cyanogenic substances · Detoxification · Folivory · *Hapalemur* spp. · Plant secondary chemicals · Primate · *Prolemur Simus* · Strepsirrhine

Introduction

Generalist folivores feeding on toxic plants must diversify their diet so that different plant secondary metabolites (PSMs) can be detoxified by different metabolic pathways, because single pathways can be limited by their detoxification rates (Foley and Moore 2005; Nersesian *et al.* 2012). In contrast, some folivore specialists, such as the giant panda (*Ailuropoda melanoleuca*: Nie *et al.* 2015; Schaller *et al.* 1985), red panda (*Ailurus fulgens*: Johnson *et al.* 1988), pygmy rabbit (*Brachylagus idahoensis*: Shipley *et al.* 2006), some marsupials (Dearing *et al.* 2000; Marsh *et al.* 2003), and the bamboo lemurs of Madagascar (gentle and golden bamboo lemurs: *Hapalemur* spp.; greater bamboo lemur: *Prolemur simus*; Mutschler 1999; Tan 1999, 2006), focus on only a few plant species (Shipley *et al.* 2009). Here, plants and folivorous animals are linked in an evolutionary arms race in which plants produce PSMs to deter feeding, and folivores evolve morphological and/or physiological mechanisms for detoxification of these PSM components, exhibit behavioural selection for higher quality food, show reduced energy expenditure to cope with PSMs, or use excess water to flush out toxins (Freeland and Janzen 1974; Glander 1982; Guglielmo *et al.* 1996; Iason and Villalba 2006; Moore and DeGabriel 2012; Provenza *et al.* 2003). From the plants' perspective, production of PSMs is costly and therefore some PSMs are produced only when plants are threatened by folivores (Dolch and Tscharntke 2000) or after they have actually been fed on (induced defenses; Schuman and Baldwin 2012). From the animals' point of view, detoxification also requires additional energy expenditure that sometimes needs to be compensated for by ingestion of more or higher quality food (Dearing *et al.* 2005; Foley and Moore 2005; Glander 1982; Lee *et al.* 2006; Provenza *et al.* 2003; Shaw *et al.* 2006; Villalba and Provenza 2005). A dramatic cost of detoxification of a single PSM (benzoate) has been described for common brushtail possums (*Trichosurus vulpecula*) where the costs for detoxification account for ca. 30% of the dietary nitrogen intake (Au *et al.* 2013). Common brushtail possums that fed on a low-protein diet had a negative protein balance (Au *et al.* 2013). Thus, for animals that consume toxic food, possibly to avoid competition, the need for detoxification is likely to be associated with costs, either in terms of increased energy or protein requirements (Au *et al.* 2013; Dearing *et al.* 2005; Foley and Moore 2005; Lee *et al.* 2006; Nersesian *et al.* 2012; Shaw *et al.* 2006; Villalba and Provenza 2005).

Animals that can feed on plants with specific PSMs have exclusive access to resources not used by competitors. The bamboo lemurs of Madagascar are among the very few mammals with a diet dominated by bamboo (Schaller *et al.* 1985; Tan 1999), a monocotyledonous flowering plant belonging to the grass family Poaceae. In the altitudinal Malagasy rainforest at Ranomafana, three species of bamboo lemurs (*Hapalemur griseus ranomafanensis*, *H. aureus*, and *Prolemur simus*) spend ca. 80–95% of their feeding time consuming various bamboo species, including cyanogenic parts of the giant bamboo (*Cathariostachys madagascariensis*: Tan 1999, 2006). These bamboo lemurs consume cyanogenic items (Tan 2000, 2006) and excrete cyanide (or more likely thiocyanate), mainly in urine (Yamashita *et al.* 2010). The parts consumed contain up to 0.6% cyanide per gram dry weight, which is ca. 10–50 times the lethal dosage of cyanide, which ranges between 74 and 370 mmol/kg for other mammals, such as sheep, mice, cats, or rats (Ballhorn *et al.* 2009a, 2016; Glander *et al.* 1989). Cyanide is detoxified by conversion to thiocyanate (Huang *et al.* 2016). This reaction is catalyed by the enzyme rhodanese and requires a sulfur donor, most likely sulfur-containing amino acids (Conn 1979; Sousa *et al.* 2003). Although the enzyme is widespread in animal tissues, a 10- to 50-fold increase of cyanogenic substances beyond the lethal dosage would require production of supplementary rhodanese and availability of sulfur-containing amino acids, making this a protein-expensive detoxification mechanism. Detoxification is likely also to be linked to increased energy requirements (Ballhorn *et al.* 2007; Berenbaum and Zangerl 1994; Klaassen *et al.* 2013; Torregrossa *et al.* 2012), which could be provided by nonstructural carbohydrates that are easily digestible and metabolized to produce energy (Rothman *et al.* 2012; Van Soest 1994).

In contrast to the altitudinal rainforest at Ranomafana, some bamboo lemur species, *Hapalemur alaotrensis* and *H. meridionalis*, occur in reed beds and littoral forests without bamboo. *H. alaotrensis* feed predominately on Cyperaceae and Poaceae, mainly reeds (*Phragmites*: Mutschler 1999), and *H. meridionalis* on a variety of plants, mainly Poaceae, including terrestrial grass (Eppley *et al.* 2011, 2016a). Cyanogenesis is frequent in Poaceae and Juncaceae (Conn 1979; Harborne 1988; Naik 1984), but cyanogenic substances are not characteristic of terrestrial grasses (Frohne and Jensen 1973). Neither bamboo nor grasses consumed by mountain gorillas (*Gorilla beringei beringei*) contain cyanogenic substances (Grueter *et al.* 2016; Rothman *et al.* 2006). Additionally, a variety of grasses taken from botanical gardens either did not contain cyanogenic substances or only in concentrations too low to poison folivorous mammals (Ballhorn *unpubl. data*).

Though the notion that protein is a limiting factor for animals feeding on plants is not always supported (Oftedal 1991; Rothman *et al.* 2011; Stolter 2008), protein is thought to play an important role in primate food selection because food for primate folivores is often characterized by higher concentrations of protein than items not eaten (protein measured as crude, soluble, or digestible protein; Chapman *et al.* 2002; DeGabriel *et al.* 2008, 2009, 2014; Ganzhorn 1988, 1992; Milton 1979; Oates *et al.* 1990; Wallis *et al.* 2012). Refined approaches demonstrated that primates may not simply select high-protein dietary items, but adjust their food composition to meet their nutritional balance by ingesting a certain amount of protein per day (Felton *et al.* 2009; Johnson *et al.* 2013). Although the emphasis on protein for folivores' food selection has a long tradition, several studies do not support the assumption that folivores prioritize protein. In some cases, researchers assume that items eaten contained enough protein to satisfy the needs of their consumers (Ganzhorn *et al.* 2017; Oftedal 1991; Stolter 2008). Other components such as easily digestible energy could be of greater nutritional importance (Rothman *et al.* 2011; Wasserman and Chapman 2003).

At Ranomafana and in the littoral forests of eastern Madagascar, bamboo lemurs (*Hapalemur* spp. and *Prolemur simus*) coexist with other folivorous lemurs, e.g., *Avahi* spp. and *Propithecus edwardsi*, that feed on leaves of trees. These sympatric species' diets vary such that some species ingest cyanogenic substances whereas others do not, or ingest cyanogenic components in much lower concentrations. This offers the possibility to investigate whether species feeding on cyanogenic bamboo compensate for the assumed costs of detoxification by increased consumption of items that provide easily digestible energy or protein, either to obtain enzymes for detoxification or maintenance, or to obtain sulfur for the detoxification mechanism. To test this hypothesis, we compared the chemical composition of food items consumed by bamboo lemur species living in areas with cyanogenic bamboo with the chemical composition of food in areas where bamboo is absent and where the animals feed on noncyanogenic plants. In addition, we compared the chemical composition of food items consumed by bamboo lemurs with the diet of other folivorous lemurs from the same areas (*Avahi meridionalis* in the littoral forest and *P. edwardsi* in the rainforest of Ranomafana). We predict that

1) bamboo-eating bamboo lemurs (*Hapalemur griseus ranomafanensis*, *H. aureus*, *Prolemur simus*) consume items with higher protein or nonstructural carbohydrate content than bamboo lemurs that do not feed on bamboo (*H. alaotrensis*, *H. meridionalis*);
2) bamboo-eating bamboo lemurs (*H. g. ranomafanensis*, *H. aureus*, *P. simus*) consume items with higher protein or nonstructural carbohydrate concentrations than sympatric folivorous lemur species that do not feed on bamboo (*Avahi meridionalis*, *Propithecus edwardsi*); and
3) items consumed by bamboo lemurs that do not feed on bamboo (*H. alaotrensis*, *H. meridionalis*) should not differ in their protein or nonstructural carbohydrate concentrations from items consumed by other sympatric folivorous lemur species (*A. meridionalis*, *P. edwardsi*).

Methods

Study Species and Sites

Bamboo lemurs (*Hapalemur* and *Prolemur*) are strepsirrhine primates of Madagascar with body mass ranging 0.8–1.7 kg (*Hapalemur* spp.) and 2.2–2.6 kg (*Prolemur simus*) (Mittermeier *et al.* 2010; Tan 2006). All species we considered inhabit the evergreen rainforest or swamps of eastern Madagascar and data are based on observations in the wild (Table I). Apart from *H. alaotrensis*, which is confined to the marshes of Lac

Table I Life history traits of lemur species included in the present study

Species	Site	Body mass	Feeding (%)	Resting (%)	Observation time	Group composition M/F	No. of food species	Reference
Sites without cyanogenic bamboo								
Hapalemur alaotrensis	Lac Alaotra	1.2	<22	>60	13 mo: 5 days/mo	3/2 1/1	11	Mutschler (1999)
H. meridionalis	Mandena	1.1	42	44	12 mo: 5 days/mo	6/8 (3 groups)	72	Eppley *et al.* (2011, 2016a)
Avahi meridionalis	Ste. Luce	1.2	15	67	7 mo: 256 h	1/1 1/1	47	Norscia *et al.* (2012)
Sites with cyanogenic bamboo								
H. griseus ranamofanensis	Ranomafana	0.9	48	41	12 mo: 5 days/mo	1/2 1/2	>40	Tan (1999, 2006), C. Tan (*unpubl. data*)
H. aureus	Ranomafana	1.4	37	54	12 mo: 5 days/mo	1/1 1/1	>21	Tan (1999, 2006), C. Tan (*unpubl. data*)
Prolemur simus	Ranomafana	2.6	41	50	12 mo: 5 days/mo	3/2 1/2	7	Tan (1999, 2006)
Propithecus edwardsi	Ranomafana	5.8	28	42	12 mo: 5 days/mo	7/8 (7 groups)	>75	Arrigo-Nelson (2006)

Body mass (kg) from the cited literature or Mittermeier *et al.* (2010)

Alaotra, all other bamboo lemur species occur in sympatry with other folivorous lemur species, such as *Avahi* spp. and *Propithecus* spp., that do not consume bamboo or other grasses but rely on leaves from trees. The species and study sites have been described previously: *H. alaotrensis* in the reed beds of Lac Alaotra (Mutschler 1999; Mutschler *et al.* 1998); *H. meridionalis* in the littoral forest of Mandena (Eppley *et al.* 2011, 2015a); *Avahi meridionalis* from the littoral forest of Ste. Luce (Norscia *et al.* 2012), a forest fragment close to Mandena, described by Donati *et al.* (2011); and *H. griseus ranomafanensis*, *H. aureus*, *P. simus*, and *Propithecus edwardsi* in the evergreen rainforest of Ranomafana National Park (Arrigo-Nelson 2006; Tan 1999, 2006). *H. alaotrensis* and *H. meridionalis* occur at sites without bamboo and feed on plants without or with very low cyanogenic potential (HCNp; the maximum amount of cyanide that can be released from the accumulated cyanogenic precursors; Ballhorn *et al.* 2009b). *H. griseus ranomafanensis, H. aureus*, and *P. simus* feed mainly on bamboo, some of which are characterized by very high HCNp. For comparison, we included two other folivorous lemur species (*A. meridionalis* and *P. edwardsi*) that do not eat bamboo and ingest other possible food items available at the same sites.

Food Composition: Comparison between Species and Sites

We based behavioral observations and sample collection on systematic observations of habituated individuals. Publications cited in Table I give details of the observation procedures for each species. In our analyses, we distinguish between food types and food items. A food item represents the item consumed by an individual in a given feeding event, i.e., one sample. Food types represent plant parts. The chemical composition of a food type can be represented by the chemical analysis of a single sample of that food type. In this case, we considered the food type based on just one or two samples to be representative of the type throughout the study area. This category of data is available for *Hapalemur alaotrensis* (Mutschler 1999) and *Avahi meridionalis* (Norscia *et al.* 2012). It ignores temporal, spatial, and individual variations in chemical composition between plant individuals (Chapman *et al.* 2003; Ganzhorn and Wright 1994; Rothman *et al.* 2012). For the other lemur species (*H. meridionalis, H. griseus ranomafanensis, H. aureus, Prolemur simus, Propithecus edwardsi*) we collected food items whenever we saw an individual feeding and sampling was possible. We analyzed the chemical composition of food types for the pooled samples per food type (in the case of *H. meridionalis*) or calculated it as the mean of the concentration from all items, i.e., samples, collected per food type. We used food consumed by other folivorous lemur species (*A. meridionalis*) in the littoral forest (Norscia *et al.* 2012) and *P. edwardsi* in Ranomafana (Arrigo-Nelson 2006; Arrigo-Nelson *et al. unpubl. data*) to compare the chemical composition of food consumed by bamboo lemurs with other potential food items available at the same site.

Food Selection of Bamboo Lemurs at Ranomafana

In Ranomafana, when possible, we collected items equivalent to the item eaten for chemical analyses at the moment of a feeding event. For this, we collected an item similar to the item consumed from the same plant individual. This procedure allows us to consider the chemical variability within plant species (Chapman *et al.* 2003; Moore and Foley 2005; Rothman *et al.* 2012).

Repeated consumption of the same item might represent some kind of preference, though we did not consider the availability of the item in question. To assess possible selection criteria, we correlated the frequency of consumption of any given food type with the mean concentrations of the various chemical components of the food type. We weighted the consumption of food types by the frequency of consumption, which is appropriate because we analyzed each item chemically. Other data to assess selection criteria, such as measuring the time of consumption or counting bites, calculating intake based on bites, or comparing items consumed with items not consumed have other advantages (Aristizabal *et al.* 2017; DeGabriel *et al.* 2014; Rothman *et al.* 2012; Zinner 1999), but were not available.

Chemical Analyses

We used the published chemical analyses values of food items consumed by *Hapalemur alaotrensis* (Mutschler 1999; Pollock 1986) and by *H. meridionalis* (Eppley *et al.* 2011, 2016a). We analyzed dried samples for all other species in the lab of Universität Hamburg, following the methods described by Bollen *et al.* (2004).

We dried samples in the sun or in a drying oven, ground with a Culatti MFC mill (IKA® Werke GmbH & Co. KG, Germany) to pass a 1-mm sieve, and dried again overnight at 50–60 °C before analyses. We determined total nitrogen with a Gerhardt Kjeldatherm (C. Gerhardt GmbH & Co. KG, Germany) using the Kjeldahl procedure. We calculated crude protein as nitrogen concentration × 6.25. We analyzed samples for neutral detergent fiber (NDF) (Goering and Van Soest 1970; Van Soest 1994) modified according to the instructions for use in an ANKOM Fiber Analyzer (Ankom Technology, Macedon, NY, USA). All concentrations are expressed as percentages of dry weight. We conducted biochemical analyses at the Institute of Zoology, Department of Animal Ecology and Conservation at Universität Hamburg.

Ballhorn *et al.* (2009a) analyzed the HCNp of the dried food items consumed by the different bamboo lemurs from Ranomafana enzymatically using the Spectroquant® cyanide test (Merck KGaA, Darmstadt, Germany). None of these samples showed any detectable cyanide. In contrast, plant material from Ranomafana, stored in alcohol or analyzed in the field, reliably released up to 0.6% cyanide per dry plant material (Ballhorn *et al.* 2009a; Glander *et al.* 1989) and showed positive reactions for cyanides in semiquantitative tests (Tan 1999, 2006; Yamashita *et al.* 2010). Therefore, we assume that the dried samples contained cyanogenic substances but our analyses for HCNp did not produce results, likely because potentially specific enzymes (β-glucosidases) necessary to release cyanide from the bamboo tissue had been destroyed during the drying process or storage of samples. To have at least some qualitative estimates for the cyanogenic potential of bamboo lemur food items, we performed the analyses of potential food items in the field as listed in the text that follows and compiled published information on the cyanide concentrations of various parts and species of bamboo (Table II).

We used total nonstructural carbohydrates (TNC) as a proxy for easily digestible carbohydrates (for a discussion see Conklin-Brittain *et al.* 2006; Rothman *et al.* 2012). We calculated TNC as: TNC = 100% – Crude protein – Neutral detergent fiber. We

Table II Possible cyanide concentrations found in bamboo and grass from southeastern Madagascar

Species and part	Consumed (+)			Cyanide (%)
	Hapalemur griseus ranomafanensis	*H. aureus*	*Prolemur simus*	
Arundinaria sp.				
Leaf tip				0.40 (mean)–0.81 (max)[1]
Culm pith				0.01 (mean)–0.02 (max)[1,2]
Ground shoot				0.40 (mean)–0.81 (max)[1,2]
Cathariostachys madagascariensis				
Ground shoot	+	+	+	0.40 (mean)–0.81 (max)[3]
Branch shoot	+	+	+	0.61 (mean)–1.08 (max)[3]
Branch complement	+	+	+	0.61 [3,4]
Culm pith			+	0.01 (mean)–0.02 (max)[5]
Leaves			+	0[3,7]
Mature leaf base			+	0[5,7]
Mature leaf tip			+	0[5,7]
Young leaf base	+	+	+	0[5,7]
Young leaf tip			+	0[5,7]
Sokinochloa chiataniae[a]				
Mature culm				0[6,7]
Growing tip	+	+	+	0.02[6]
Leaf blade			+	0[6,7]
Sokinochloa brachyclada[a]				
Branch shoot	+	+	+	0[6,7]
Ground shoot	+	+	+	0[6,7]
Mature leaves			+	0[6,7]
Young leaf base	+	+	+	0[6,7]
Young leaf tip			+	0[6,7]
Nastus elongates				
Branch shoot	+	+	+	+ [7]
Nastus sp.				
Stem				0[7,8]
Poecilostachys festucaceus				
Stem	+	+	+	0[9]
Leaves			+	0[9]
Mature leaf tip				0[9]

All analyzed plant species are known food items of *Hapalemur griseus ranomafanensis*, *H. aureus*, and/or *Prolemur simus* (Tan 2006); however, only the species/parts consumed within Ranomafana are indicated. Published concentrations of cyanide (in % per dry weight) are referenced at the end of the table. For statistical analyses, we assigned a concentration of 0.01% HCN to items that showed a positive response (+) in the Feigl–Anger test, if only qualitative data are available

[a] Revised taxonomy according to Dransfield (2016): *Sokinochloa chiataniae* (formerly *Cephalostachyum* sp.) and *Sokinochloa brachyclada* (formerly *Cephalostachyum perrieri*)

[1] Eisler (1991); [2] assumed to be equivalent to *Cathariostachys madagascariensis* (Eisler 1991); [3] Ballhorn *et al.* (2009b); [4] assumed to be equivalent to "branch shoots"; [5] Ballhorn (*unpubl. Data*); [6] Glander *et al.* (1989); [7] Tan (2006); [8] http://tech.groups.yahoo.com/group/bamboo-plantations/message/2019; [9] Yamashita *et al.* (2010)

should have subtracted lipids and ash from this measure, but these data were not available. Lipids are not an important component in leaves. Ash (= mineral) contents can amount to several percent of dry mass in leaves (National Research Council 2003) but has not been measured consistently in the samples used for the present analysis. Ash and wax may not be trivial, especially as many grasses (including bamboo) contain high concentrations of silica (Epstein 1994). Both components are likely to be lower in pith than in leaves. Of the species included in our study, only bamboo lemurs from Ranomafana consume bamboo pith; thus, subtracting ash and wax from the food items consumed by the bamboo-eating lemurs would probably have reinforced the differences between them and other lemurs. We assume that the error introduced by not subtracting lipids and ash is similar for all studies and small compared to the large fiber fraction. While bamboo lemurs employ long gastrointestinal transit times that allow them to efficiently digest dietary fiber, e.g., 18–36 h for *Hapalemur* spp. (Campbell *et al.* 2004a, b; Perrin 2013) and ca. 8 h for *Prolemur simus* (Tan 2000), we used only neutral detergent fiber (NDF) to calculate TNC. We had no specific hypothesis concerning NDF so we do not discuss it specifically.

For analysis of plant HCNp, we selected fresh items from bamboo in Ranomafana several years after the behavioral observation of the bamboo lemurs had been completed (Ballhorn *et al.* 2009b). We used these items as proxy for cyanogenic components in the original food samples of bamboo lemurs because the original food item analyses failed and could not be repeated. For each plant species, we analyzed different plant tissues for HCNp to obtain information on the quantitative variability of cyanogenic precursor content, as there frequently is substantial intraplant variation with young and reproductive tissues showing the highest amounts of cyanogenic precursors (Ballhorn *et al.* 2008, 2009b). We weighed fresh plant samples to the nearest 0.001 g and ground with liquid nitrogen and cooled mortar and pestle (4 °C) under addition of the fourfold volume (vol/fresh wt) of ice-cold Na_2HPO_4 solution (67 mmol/L). We analyzed samples quantitatively for their HCNp by complete enzymatic hydrolysis of cyanogenic precursors under addition of β-glucosidase from almonds (Emulsin®, Merck, NY, USA). We used closed glass vessels (Thunberg vessels) for incubation (20 min at 30 °C in a water bath) of plant extracts together with enzyme solution adjusted to an activity of 20 nkat. We quantified released HCN spectrophotometrically at 585 nm using the Spectroquant® cyanide test (Merck KGaA, Darmstadt, Germany; Ballhorn *et al.* 2009b).

Statistical Analysis

We did not separate analyses by sex because no published data were available for this comparison. Since sex ratios were balanced in all species (Table I), there should not be any bias in the results due to different protein or energy requirements of females and males. We tested data for deviations from normality with Kolmogorov–Smirnov one-sample tests. If residuals deviated from normality, we used nonparametric tests. Kruskal–Wallis analysis of variance was used for comparisons of more than two groups. Post hoc tests for subsequent pairwise comparisons were based on Mann–Whitney *U* tests and significance levels were Bonferroni corrected. For parametric analyses of more than two groups we used analysis of variance with subsequent Scheffé's post hoc test. We ran a chi-square test to determine if the frequency of cyanogenic plant item consumption differed between the three bamboo-eating lemur species. We performed all statistical analyses in IBM SPSS Statistics v. 22 (SPSS Inc., Chicago, IL), with significance set at $P < 0.05$.

Ethical Note

Our research protocols in Ranomafana National Park were approved and permits authorized by the Association Nationale pour la Gestion des Aires Protégées and Direction des Eaux et Forêts de Madagascar, and our research protocols in Mandena were approved and permits authorized by the Commission Tripartite of the Direction des Eaux et Forêts de Madagascar (Autorisation de Recherché n. 240/12/MEF/SG/DGF/DCB.SAP/SCB du 17/09/2012). All research activities reported adhered to the legal requirements of Madagascar.

We declare that we have no conflict of interest.

Results

The number of food plant species listed in Table I represents the total number of species known to be consumed by the different lemur species. Chemical analyses (summarized in Table III) are available only for subsets of plant species consumed most frequently.

Unweighted Samples

The unweighted data in Table III are the sum of the nutrients in all the plant types that each species has been reported to consume, and for which we have chemical composition data; for example, we have chemical analyses for four different plant types (N = 39 samples) consumed by *Hapalemur aureus*. Similarly, we have chemical analyses for five different plant types (N = 38 samples) consumed by *H. griseus ranomafanensis*, and for six different plant types (N = 115 samples) consumed by *Prolemur simus*. The unweighted data in Table III represent the total nutritional value of these plant types as if they were consumed in equivalent amounts.

Hapalemur griseus ranomafanensis and *Prolemur simus* consumed plants with much lower cyanogenic potential (HCNp) than *H. aureus* consumed, but this difference is not significant (Table III). Though the median of plant HCNp is zero or close to zero for *H. g. ranomafanensis* and *P. simus*, respectively, both species include some plant parts with very high cyanide concentration (Table III). According to the unweighted analyses, food types consumed by *H. aureus* have higher concentrations of nitrogen than food consumed all other lemur species, except for the sympatric *H. g. ranomafanensis* (Table III).

Food of all bamboo lemur species from the sites with noncyanogenic plants and the non–bamboo lemur species has lower nitrogen concentrations than the two *Hapalemur* species from Ranomafana (Table III; Fig. 1a). *Prolemur simus* is closer to the non–bamboo eating *Propithecus edwardsi* than to the other two bamboo lemur species of Ranomafana with respect to nitrogen in the diet.

Concentrations of nonstructural carbohydrates did not differ consistently between Ranomafana and Mandena. According to the unweighted samples, *Prolemur simus* ate the items with the lowest concentrations of TNC while *Avahi meridionalis* and *Propithecus edwardsi* had the highest concentrations of TNC (Fig. 1b). The high

Table III Chemical composition of food items frequently eaten by bamboo lemurs (*Hapalemur* spp. and *Prolemur simus*) and sympatric folivorous lemur species (*Avahi meridionalis* and *Propithecus edwardsi*)

		Food types not weighted by frequency of consumption				Food types weighted by frequency of consumption				
Species	Site	HCNp	Nitrogen	NDF	TNC	HCNp	Nitrogen	NDF	TNC	Source
Sites without cyanogenic bamboo										
Hapalemur alaotrensis	Lac Alaotra	nm	$2.08^{b,d} \pm 0.86$ (17)	nm	nm	nm	nm	nm	nm	Mutschler (1999); Pollock (1986)
H. meridionalis	Mandena	nm	$1.18^{b,c} \pm 0.66$ (89)	$55.56^{b,c,d} \pm 13.32$ (89)	$37.09^{b,c,d} \pm 13.48$ (89)	nm	1.41 ± 0.04 (89)	59.44 ± 1.52 (89)	31.73 ± 0.65 (89)	Eppley *et al.* (2016a); this study
Avahi meridionalis	Ste. Luce	nm	$1.14^{b,c} \pm 0.62$ (43)	$43.18^{a,b,c} \pm 10.20$ (43)	$50.03^{a,b,c} \pm 11.07$ (43)	nm	nm	nm	nm	Norscia *et al.* (2012)
Sites with cyanogenic bamboo										
H. griseus ranamofanensis	Ranomafana	0.00 [0.00–0.01] (5)	$4.17^{a} \pm 1.18$ (5)	42.17 ± 16.41 (5)	31.78 ± 11.23 (5)	0.02 [0.00–0.03] (3)	$2.63^{a} \pm 0.84$ (3)	$48.88^{a} \pm 22.68$ (3)	$13.84^{a} \pm 2.92$ (3)	This study
H. aureus	Ranomafana	0.01 [0.00–0.14] (4)	$4.42^{a} \pm 1.64$ (4)	42.04 ± 19.41 (4)	30.33 ± 11.70 (4)	0.31 [0.00–0.46] (3)	$3.51^{a} \pm 1.06$ (3)	$41.17^{a} \pm 9.32$ (3)	$28.79^{a} \pm 11.17$ (3)	This study
Prolemur simus	Ranomafana	0.01 [0.00–0.01] (6)	$2.43^{a,b,c,d} \pm 1.37$ (6)	$62.33^{a,d} \pm 20.61$ (6)	$22.49^{a,c,d} \pm 13.14$ (6)	0.25 [0.00–0.32] (4)	$2.91^{a} \pm 0.87$ (4)	$53.97^{a} \pm 10.15$ (4)	$26.90^{a} \pm 8.84$ (4)	This study
Propithecus edwardsi	Ranomafana	nm	$2.02^{b,d} \pm 1.23$ (51)	$36.52^{a,b,d} \pm 13.32$ (51)	$50.82^{a,b,d} \pm 15.39$ (51)	nm	nm	nm	nm	Arrigo-Nelson *et al.* (*unpubl. data*)
H/F statistics		0.09	22.89	16.55	13.41	0.17	38.73	31.59	25.26	
P-values		0.958	<0.001	<0.001	<0.001	0.920	<0.001	<0.001	<0.001	

Values are means ± standard deviations of percentage dry weight and sample size (in parentheses). Values for HCNp are medians and 25% and 75% quartiles [$Q_{25} - Q_{75}$]. *HCNp* Cyanogenic potential; *NDF* Neutral detergent fiber; *TNC* Total nonstructural carbohydrates

The H-values for cyanogenic substances are based on Kruskal–Wallis analysis of variance. *F*-values at the end of the table are based on one-way ANOVA. Statistical analyses reported in this table were run separately for weighted and unweighted samples. Mean values with the same superscript do not differ but different superscripts indicate differences with $P < 0.05$ according to Scheffé's post hoc test; nm = not measured

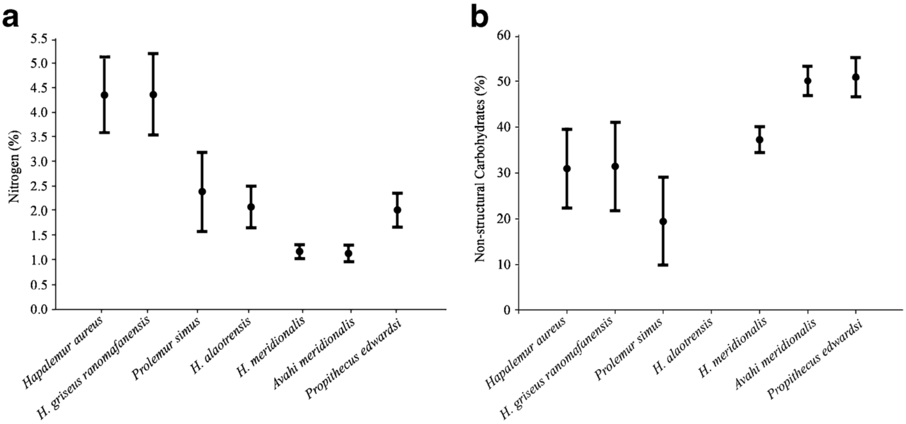

Fig. 1 Concentrations of **(a)** nitrogen and **(b)** nonstructural carbohydrates as a proxy of energy in food items consumed by bamboo lemurs and sympatric folivorous lemur species at sites with cyanogenic bamboo (rainforest of Ranomafana) and at sites without cyanogenic bamboo (marsh of Lac Alaotra, littoral forests of Mandena and Ste. Luce). Values are means and 95% confidence intervals; items are not weighted by frequency of consumption.

concentrations of TNC in food of non-bamboo lemur species do not differ between *Avahi meridionalis* and *Propithecus edwardsi* (Table III).

Weighted Samples

We had weighted data only for *Hapalemur meridionalis* (from a non-bamboo site) and the lemur species that inhabit the sites with cyanogenic bamboo, i.e., Ranomafana. The nutritional contents reported in Table III represent the average nutritional intake for each species.

Based on the frequency of consumption, *Hapalemur aureus* ate the highest proportion of cyanogenic items (65.1% of collected food items), followed by *Prolemur simus* (64.1% of collected food items) and *H. griseus ranomafanensis* (18.2%). The frequency of consumption of cyanogenic plant items differs significantly between the three species ($\chi^2 = 14.11$, df = 2, $P < 0.01$).

While the concentrations of cyanogenic potential in the weighted food item samples were lower for *Hapalemur griseus ranomafanensis* compared to *Prolemur simus* and *H. aureus*, this difference was not statistically significant (Table III). The three bamboo lemur species from Ranomafana have significantly higher concentrations of nitrogen than *H. meridionalis*. Food items of *H. aureus* have somewhat higher concentrations of nitrogen than food consumed by *H. g. ranomafanensis and P. simus*, though this difference is not significant. According to the weighted samples, foods of the three bamboo-eating lemur species did not differ in their concentrations of NDF or TNC. The diets of all bamboo lemur species from Ranomafana had significantly lower concentrations of NDF and TNC than food items consumed by *H. meridionalis* (Table III).

Discussion

Our intersite comparison revealed that all bamboo lemur species feeding on cyanogenic bamboo (*Hapalemur aureus, H. griseus ranomafanensis*, *Prolemur simus*) consumed foods with concentrations that were 1.7 times higher in nitrogen than the two bamboo lemur species (*Hapalemur alaotrensis, H. meridionalis*) studied at sites without cyanogenic bamboo. While all bamboo lemur species feed on bamboo where it is available and all the Ranomafana bamboo lemur species ingest and excrete cyanide (Yamashita *et al.* 2010), the proportion of cyanogenic food ingested by the different species varies and can be negligible at sites without bamboo (Eppley *et al.* 2011, 2016a; Glander *et al.* 1989; Mutschler 1999; Mutschler *et al.* 1998; Tan 1999). The *Hapalemur* spp. feeding on cyanogenic bamboo in Ranomafana also ingest more nitrogen than the sympatric non-bamboo lemur species (*Propithecus edwardsi*) feeding on the leaves of trees. In contrast to the *Hapalemur* species at Ranomafana, the *Hapalemur* spp. feeding on noncyanogenic items do not ingest more nitrogen than the non-bamboo lemur species (*Avahi meridionalis*) that is sympatric to *H. meridionalis*. Cyanogenic glycosides contribute to the nitrogen concentrations in cyanogenic plants but their relative contribution is small (maximum 0.3% nitrogen, corresponding to 0.6% cyanide) compared to the nitrogen contained in protein. The nitrogen concentrations in food plants of the lemur species feeding on cyanogenic plants were considerably higher than those consumed by lemur species that do not rely on bamboo, i.e., in areas where bamboo is absent (Table III). Even when the maximum nitrogen content of 0.3% bound in cyanogenic substances is subtracted from the total nitrogen concentration of cyanogenic food parts, the average nitrogen concentration in food items of bamboo consuming species would be about 1.1% higher than in food plants of species that do not include bamboo in their diet. Nitrogen concentrations of 1.1% are equivalent to about 6.9% more crude protein. According to studies that linked protein in food to reproductive success and growth, this difference can have a major impact for folivorous mammal fitness (DeGabriel *et al.* 2009; Moore and Foley 2005).

The actual difference in the consumption of digestible nitrogen might be more pronounced than indicated by the measure of nitrogen or crude protein. Grasses do not contain condensed tannins and therefore these components do not interfere with protein digestibility as may do tannin-rich leaves of trees (Wallis *et al.* 2012; Waterman and Mole 1994). Thus, although the nitrogen concentrations reported for bamboo are closely linked to the concentrations of digestible nitrogen, the concentrations of digestible nitrogen in leaves of trees are likely to be much lower and do not show a tight relationship between digestible and crude protein (Wallis *et al.* 2012). This would increase the difference in protein between a diet based on bamboo and a diet based on the leaves of trees. This argument could be used to support the finding (in favor of the original hypothesis that additional protein is needed for detoxification) that the nitrogen concentrations in food of *Prolemur simus* do not differ from the nitrogen concentration of the sympatric *Propithecus edwardsi*.

We worked with averages for comparisons because our data lacked temporal and spatial resolution across all species. According to our subjective impression, *Prolemur simus* might consume higher amounts of unweighted cyanogenic substances than *Hapalemur griseus ranomafanensis*, though this is not reflected in the quantitative data, possibly due to small sample size. The amount of cyanogenic bamboo parts consumed by *P. simus* differs seasonally between the warm–wet and cool–dry season, with this species consuming more shoots of *Cathariostachys madagascariensis* (containing high concentrations of cyanogenic substances) during the wet season and then shifting to its culm (containing no cyanogenic substances) during the dry season (Tan

1999). The developing shoots of *C. madagascariensis* have concentrations of >200 μmol cyanide/g dry wt (Ballhorn *et al.* 2009b), which *P. simus* feeds almost exclusively during the wet season (Tan 1999). Thus, it is likely that *P. simus* ingests the most HCNp seasonally, whereas *H. aureus* is more constant in HCNp consumption throughout the year.

Based on our limited knowledge of their population dynamics, the bamboo lemur species at the different sites do not seem to differ in their reproductive output or population dynamics (Eppley *et al.* 2015b, 2016b; Nievergelt *et al.* 2002; Tan 2006). Therefore, the surplus of protein ingested from bamboo in Ranomafana is not reflected in higher reproductive rates. In contrast to the nitrogen content, there is no indication that energy (measured as total nonstructural carbohydrates, though proteins can certainly also be converted to energy) plays a role in detoxification or food selection.

Morphological traits could provide hints for special adaptations to deal with food peculiarities, such as a complex forestomach that could facilitate detoxification (Alexander 1993; Chivers and Hladik 1980; Waterman and Kool 1994). However, *Hapalemur griseus* does not have any morphological specialization that indicates any specific adaptation of the digestive tract. The only difference between *H. griseus* and a dietary generalist lemurid species such as *Lemur catta* is a shorter cecum and a shortened and sacculated colon of *H. griseus* (Campbell *et al.* 2000; Perrin 2013). However, compared to other lemurs studied so far, *H. griseus* achieves higher digestion of fiber and protein, indicating some kind of specialization that cannot be linked to morphological characteristics. Both features of the *H. griseus* digestive tract hint toward lower rather than higher digestion rates (Campbell *et al.* 2000), just the opposite of what has been found in digestion studies (Campbell *et al.* 2004a, b). Thus, it remains unclear what the surplus protein in bamboo is used for by *Hapalemur* spp. and *Prolemur simus*.

On the basis of the comparison between lemur species occurring at different sites, the use of protein for detoxification is consistent with the original hypothesis. It is also consistent with studies on sheep and brushtail possums, which describe the increased consumption of PSMs, e.g., terpenes (Villalba and Provenza 2005), cineole (Nersesian *et al.* 2012), and benzoate (Au *et al.* 2013), as being associated with the increased ingestion of protein. Our comparison of the three sympatric bamboo lemur species at Ranomafana matches this scenario, providing evidence that cyanide tolerance may be related to protein ingestion. In Ranomafana, *Hapalemur griseus ranomafanensis* is the species that consumes the lowest amounts of cyanogenic substances (Table III; Yamashita *et al.* 2010), while it is also the species ingesting the lowest (weighted) portion of nitrogen in its diet.

Although the results support our hypothesis, our sample size is small and the study design is not optimal as it is nonexperimental and lacks direct physiological measurements of energy and protein expenditure. Furthermore, our analyses are hampered by the lack of information on the actual amount of food and chemical components consumed. The geometric framework approach could theoretically help to resolve this problem. While most studies still assume that time spent feeding reflects food intake, there may be as much support for this as there are exceptions (Schülke *et al.* 2006). Also, some studies produced consistent results using weighted or unweighted samples (the two lemur examples in Ganzhorn *et al.* 2017), whereas others (such as the present study) indicated different outcomes from the two approaches. Without controlled experiments, it will be impossible to assess the value of the two methods. However, the threatened status of the study species—i.e., most bamboo lemurs are classified as Endangered or Critically Endangered according to the most recent IUCN classification (Schwitzer *et al.* 2013)—precludes standard physiological experiments and it would be unethical to feed animals cyanogenic food. Thus, we had to rely on descriptive field studies. The results of our comparisons are consistent with the hypothesis that lemurs consuming cyanogenic bamboo can compensate for the toxins by consuming more protein because 1) bamboo-eating bamboo lemurs consume items with higher protein content than bamboo lemurs that do not feed on bamboo, 2) two out of three bamboo-eating lemur species consume items with higher protein than sympatric folivorous lemur species that do not feed on bamboo, and 3) items consumed by bamboo lemurs that do not feed on bamboo do not differ in their protein concentrations from items consumed by other sympatric folivorous lemur species. The results for total nonstructural carbohydrates show the reverse trend. Therefore, we conclude that protein serves a greater function than simply providing a source of energy for these animals.

Bamboo is rich in protein but low in total nonstructural carbohydrates. Therefore, it could well be that if lemur species feed on bamboo they unavoidably ingest high concentrations of protein. Thus, it would be expected that lemurs feeding on cyanogenic substances would show some indication of protein selection; however, this was not found in our analyses and it remains enigmatic what the bamboo lemurs do with the high concentrations of protein in their diet. Until we are able to apply new experimental approaches or at least new conceptual frameworks on how to study food selection (DeGabriel *et al.* 2014; Felton *et al.* 2009; Johnson *et al.* 2013; Rothman *et al.* 2011), these issues cannot be further resolved.

Data Availability

The datasets analyzed in this study are available from the corresponding author on reasonable request.

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