High energy or protein concentrations in food as possible offsets for cyanide consumption by specialized bamboo lemurs in Madagascar?

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Short title: Variation in food composition of bamboo lemurs

Abstract

Plants producing toxic plant secondary metabolites (PSM) deter feeding of folivores. Animals that are able to cope with noxious PSMs have a niche with a competitive advantage over other species. However, the ability to cope with toxic PSMs incurs costs for detoxification. In order 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 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 than bamboo lemurs in areas without bamboo and sympatric lemur species that feed on leaves of trees. Concentrations of non-structural 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 sulphur containing amino acids that can be used for detoxification. Due 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 Primates; Strepsirrhine; *Hapalemur* spp.; *Prolemur simus*; Detoxification; Folivory; Plant secondary chemicals; Cyanogenic substances

Introduction

Generalist folivores feeding on toxic plants have to diversify their diet so that different plant secondary metabolites (PSMs) can be detoxified by different metabolic pathways, because single pathways can be limiting by their detoxification rates (Foley and Moore 2005; Nersesian et al. 2012). In contrast, some folivorous specialists focus on only a few plant species (Shipley et al. 2009), such as the giant panda (*Ailuropoda melanoleuca*; Schaller et al. 1985; Nie et al. 2015), 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). Here, plants and folivorous animals are linked in an evolutionary arms race in which plants produce PSM- to deter feeding, and folivores evolve morphological and/or physiological mechanisms for detoxification of these components, or exhibit behavioural selection of higher quality food or reduced energy expenditure to cope with PSMs (Freeland and Janzen 1974;

Guglielmo et al. 1996; Provenza et al. 2003; Iason and Villalba 2006; Moore and DeGabriel 2012). From the plants' perspective, production of PSMs is costly and therefore some PSMs are only produced when plants are threatened by folivores (Dolch and Tscharntke 2000) or after they have actually been fed upon (induced defences; 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 (Glander 1982; Provenza et al. 2003; Dearing et al. 2005; Foley and Moore 2005; Villalba and Provenza 2005; Lee et al. 2006; Shaw et al. 2006). 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 about 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, animals that consume toxic food, possibly in order to avoid competition, should incur higher energy and nutrient requirements.

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 rain forest at Ranomafana, three species of bamboo lemurs (*Hapalemur griseus ranomafanensis*, *H. aureus*, and *Prolemur simus*) spend approximately 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 about 10 - 50 times the lethal dosage for other mammals (Glander et al. 1989; Ballhorn et al. 2009a, 2016). Cyanide is detoxified by conversion to thiocyanate (Huang et al. 2016). This reaction is catalysed by the enzyme rhodanese and requires a sulphur donor, most likely sulphur containing amino acids (Conn 1979; Sousa et al. 2003). While the enzyme is widespread in animal tissues, a 10- to 50-fold increase of cyanogenic substances beyond the lethal dosage ought to require production of supplementary rhodanese and availability of sulphur containing amino acids, making this a protein expensive detoxification mechanism. Detoxification is likely also to be linked to increased energy requirements, which could be provided by non-structural carbohydrates that are easily digestible and metabolized to produce energy.

In contrast to the altitudinal rain forest at Ranomafana, some bamboo lemur species, *Hapalemur alaotrensis* and *H. meridionalis*, occur in reed beds and littoral forests without bamboo. *Hapalemur 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, 2016). Cyanogenesis is frequent in Poaceae and Juncaceae (Conn 1979; Naik 1984; Harborne 1988), but cyanogenic substances are not characteristic for terrestrial grasses (Frohne and Jensen 1973). Neither bamboo nor grasses consumed by mountain gorillas (*Gorilla beringei beringei*) contain cyanogenic substances (Grueter et al., 2016; J. Rothman, pers. comm.). Additionally, a variety of grasses taken from botanical gardens did not contain cyanogenic substances or only in concentrations too low to poison folivorous mammals (Ballhorn, unpubl.).

Though the notion that protein is a limiting factor for animals feeding on plants is not always supported (Oftedal 1991; Wasserman and Chapman 2003; Stolter 2008; Rothman et al. 2011), protein is thought to play an important role in primate food selection because food for primate folivores is often characterized by higher concentrations of different measures of protein than items not eaten (protein measured as crude, soluble or digestible protein; e.g., Milton 1979; Ganzhorn 1988, 1992; Oates et al. 1990; Chapman et al. 2002; DeGabriel et al. 2008, 2009, 2014; 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 protein requirements by ingesting a certain amount of protein per day (Felton et al. 2009; Johnson et al. 2013). While the emphasis on protein for folivores' food selection has a long tradition, an increasing number of studies indicates that folivores do not need to optimize protein intake because food items contain enough protein to satisfy their needs (Oftedal 1991; Stolter 2008; Ganzhorn et al. 2017). These studies rather indicate an important role of easily digestible energy for the maintenance of animals (Wasserman and Chapman 2003; Rothman et al. 2011). There are not enough studies available yet to analyse which component is favoured over the other at any given circumstances.

At Ranomafana and in the littoral forests of eastern Madagascar, bamboo lemurs coexist with other folivorous lemurs that feed on leaves of trees. These sympatric species can be used for comparisons of the chemical properties of food consumed by animals that ingest cyanogenic substances versus species that do not, or ingest cyanogenic components in much lower concentrations. We tested the prediction that the cyanogenic plant parts fed upon by bamboo lemurs contain higher concentrations of protein and/or easily extractable energy than food items consumed by bamboo lemurs or other folivorous lemur species that feed on plants without or with lower cyanide concentrations.

If the consumption of cyanogenic substances requires additional protein or energy, we expect that:

(1) bamboo eating bamboo lemurs consume items with higher protein or non-structural carbohydrate content than bamboo lemurs that do not feed on bamboo,

(2) bamboo eating bamboo lemurs consume items with higher protein or non-structural carbohydrate concentrations than sympatric folivorous lemur species that do not feed on bamboo, and

(3) items consumed by bamboo lemurs that do not feed on bamboo should not differ in their protein or non-structural carbohydrate concentrations from items consumed by other sympatric folivorous lemur species.

Methods

Study species and sites

Bamboo lemurs (*Hapalemur* and *Prolemur*) are strepsirrhine primates of Madagascar with body mass ranging from 0.8 – 1.7 kg (*Hapalemur* spp.) and 2.2 – 2.6 kg (*Prolemur simus*) (Tan 2006; Mittermeier et al. 2010). All species considered here inhabit the evergreen rainforest or swamps of eastern Madagascar and data are based on observations in the wild (Table 1). Apart from *H. alaotrensis* from the marshes of Lac 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: *Hapalemur alaotrensis* in the reed beds of Lac Alaotra (Mutschler et al. 1998; Mutschler 1999), *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), *H. griseus ranomafanensis*, *H. aureus*, *Prolemur simus* and *Propithecus edwardsi* in the evergreen rainforest of Ranomafana National Park (Tan 1999, 2006; Arrigo-Nelson 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). *Hapalemur griseus ranomafanensis, H. aureus* and *P. simus* feed mainly on bamboo, some of which are characterized by very high HCNp. The two other lemur species (*A. meridionalis* and *P. edwardsi*) do not eat bamboo and were included for comparison of species that ingest other possible food items available at the same site.

Table 1 Life history traits of lemur species included in the present study

Species	Site	Body mass	Feeding %	Resting %	Observation time	Group composition M / F	# Food species	Reference
Sites without cyanogenic bamboo								
Hapalemur alaotrensis	Lac Alaotra	1.2	< 22%	> 60%	13 months: 5 days/month	3 / 2 1 / 1	11	Mutschler 1999
H. meridionalis	Mandena	1.1	42%	44%	12 months: 5 days/month	6 / 8 (3 groups)	72	Eppley et al. 2011, 2016
Avahi meridionalis	Ste. Luce	1.2	15%	67%	7 months: 256 hours	1 / 1 1 / 1	47	Norscia et al. 2012
Sites with cyanogenic bamboo								
H. griseus ranamofanensis	Ranomafana	0.9	48%	41%	12 months: 5 days/month	1 / 2 1 / 2	> 40	Tan 1999, 2006, C. Tan unpubl. data
H. aureus	Ranomafana	1.4	37%	54%	12 months: 5 days/month	1 / 1 1 / 1	> 21	Tan 1999, 2006, C. Tan unpubl. data
Prolemur simus	Ranomafana	2.6	41%	50%	12 months: 5 days/month	3 / 2 1 / 2	7	Tan 1999, 2006
Propithecus edwardsi	Ranomafana	5.8	28%	42%	12 months: 5 days/month	7 / 8 (7 groups)	> 75	Arrigo-Nelson 2006

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

Food composition: Comparison between species and sites

In our analyses, we distinguish between food types and food items. A food item represents the item consumed by an animal in a given feeding event (= 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, the food type based on just one or two samples is considered to be representative for 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 variation of the chemical composition between plant individuals (Ganzhorn and Wright 1994; Chapman et al. 2003; Rothman et al. 2012). For the other lemur species (*H. meridionalis, H. griseus ranomafanensis, H. aureus, P. simus, P. edwardsi*) we collected food items whenever an animal was seen feeding and sampling was possible. We analysed the chemical composition of food types for the pooled samples per food type (in case of *H. meridionalis*) or calculated as the mean of the concentration from all items (= samples) collected per food type. We used food consumed by other folivorous lemur species, (*Avahi meridionalis*) in the littoral forest (Norscia et al. 2012), and *Propithecus edwardsi* in Ranomafana (Arrigo-Nelson 2006; Arrigo-Nelson et al. unpublished data) to compare the chemical composition of food consumed by bamboo lemurs with other potential food items available at the same site. Behavioural observations and sample collection for these species are based on systematic observations of habituated animals (Table 1).

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 a similar item as 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).

In order 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. Weighing the consumption of food types by the frequency of consumption seems a suitable procedure since each item was analysed chemically. Repeated consumption of the same item might represent some kind of preference, though we did not consider the availability of the item in question. Other procedures 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 (Zinner 1999; Rothman et al. 2012; DeGabriel et al. 2014; Aristizabal et al. 2017), but are simply not available for comparisons.

Chemical analyses

Chemical analyses have been performed and published for food items consumed by *Hapalemur alaotrensis* (Pollock 1986; Mutschler 1999), and by *H. meridionalis* (Eppley et al. 2011, 2016). We analysed dried samples for all other species in the lab of Hamburg University, following the methods described by Bollen et al. (2004).

Samples were dried in the sun or in a drying oven, ground with a Culatti MFC mill to pass a 1 mm sieve, and dried again overnight at 50-60°C prior to analyses. Total nitrogen was determined with a Gerhardt Kjeldatherm using the Kjeldahl procedure. Crude protein was calculated as nitrogen concentration * 6.25. Samples were analysed for neutral detergent fibre

(NDF) (Goering and van Soest 1970; van Soest 1994) modified according to the instructions for use in an ANKOM FIBER ANALYZER. All concentrations are expressed as percentages of dry weight. Biochemical analyses were carried out at the Institute of Zoology, Department of Ecology and Conservation at Hamburg University.

Originally, HCNp of the dried food items consumed by the different bamboo lemurs from Ranomafana had been analysed enzymatically using the Spectroquant® cyanide test (Merck KGaA, Darmstadt, Germany; Ballhorn et al. 2009a). None of these samples showed any detectable cyanide. In contrast, plant material from Ranomafana stored in alcohol or analysed in the field, reliably released up to 0.6% cyanide per dry plant material (Glander et al. 1989; Ballhorn et al. 2009a) and showed positive reactions for cyanides in semi-quantitative 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 viable 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. In order 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 below and compiled published information on the cyanide concentrations of various parts and species of bamboo (Table 2).

Table 2 Possible cyanide concentrations found in bamboo and grass from southeastern Madagascar. All analyzed plant species are known food items of *Hapalemur griseus ranomafanensis* (*H.g.r.*), *H. aureus* (*H.a.*) and/or *Prolemur simus* (*P.s.*) (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.

Species and Part	Consumed (+)			Cyanide [%]
	H.g.r.	*H.a.*	*P.s.*	
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				
Mature culm				0 [6, 7]
Growing tip	+	+	+	0.02 [6]
Leaf blade			+	0 [6, 7]
Sokinochloa brachyclada				
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 elongatus				
Branch shoot	+	+	+	+ [7]
Nastus sp.				
Stem				0 [7, 8]
Poecilostachys festucaceus				
Stem	+	+	+	0 [9]
Leaves			+	0 [9]
Mature leaf tip				0 [9]

[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)

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.

Total non-structural carbohydrates (TNC) were used as a proxy for easily digestible carbohydrates (for a discussion see Conklin-Brittain et al. 2006; Rothman et al. 2012). TNC were calculated as: TNC = 100% - Crude protein – Neutral detergent fibre. Lipids and ash ought to be subtracted from this measure, but these analyses 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 measured consistently in the samples used for the present analysis. We assume that the error introduced by not subtracting lipids and ash is small compared to the large fibre fraction. Neutral detergent fibre (NDF) was only used to calculate TNC. We did not have any specific hypothesis concerning NDF itself. Therefore, NDF were not discussed specifically.

For analysis of plant HCNp, fresh items were selected from bamboo in Ranomafana several years after the behavioural observation of the bamboo lemurs had been completed (Ballhorn et al. 2009b). These items were used as proxy for cyanogenic components in the original food samples of bamboo lemurs because the analyses of the original food items failed and could not be repeated. For each plant species, we analysed different plant tissues for HCNp to obtain information on the quantitative variability of cyanogenic precursor content as there frequently is substantial within-plant variation with young and reproductive tissues showing the highest amounts of cyanogenic precursors (Ballhorn et al. 2008, 2009b). Fresh plant samples were weighted to the nearest 0.001 g and ground with liquid nitrogen and cooled mortar and pestle (4°C) under addition of the fourfold volume (v fresh weight^{-1}) of ice-cold Na_2HPO_4 solution (67 mmol L^{-1}). Samples were quantitatively analysed 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. Quantification of released HCN was conducted spectrophotometrically at 585 nm using the Spectroquant® cyanide test (Merck KGaA, Darmstadt, Germany; Ballhorn et al. 2009b).

Statistical analysis

Data were tested for deviations from normality with Kolmogorov-Smirnov-one sample tests. If residuals deviated from normality, we used non-parametric 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. All statistical analyses were performed in IBM SPSS Statistics v. 22 (SPSS Inc., Chicago, IL, U.S.A.), with significance set at $p < 0.05$.

Results

Basic information on life history traits and study design of the bamboo lemur species considered are given in Table 1. Analyses were not separated by sex because no published data were available for this comparison. Since sex ratios were balanced in all species, there should not be any bias in the results due to different protein or energy requirements of females and males. The number of food plant species listed in Table 1 represents the total number of species known to be consumed by the different lemur species. Chemical analyses (summarized in Table 3) are only available for subsets of plant species consumed most frequently.

Unweighted samples

The unweighted data in Table 3 are a summation of the nutrients in all of the plant types that have been recorded as consumed by a given species and were analysed for their chemical composition; for example, chemical analyses for *Hapalemur aureus* are available for four different plant types that have been consumed by *H. aureus*. The unweighted data in Table 3 represent the total nutritional value of these plant types as if they were consumed in equivalent amounts.

Hapalemur g. ranomafanensis and *P. simus* consumed plants with much lower cyanogenic potential (HCNp) than *H. aureus*. Due to small sample size of the unweighted samples, this difference is not significant (Table 3). 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 3). According to the unweighted samples, food types consumed by *Hapalemur aureus* have higher concentrations of nitrogen than food consumed all other lemur species, except for the sympatric *H. g. ranomafanensis* (Table 3).

Food of all bamboo lemur species from the sites with non-cyanogenic plants and the non-bamboo-lemur species has lower nitrogen concentrations than the two *Hapalemur* species from Ranomafana (Table 3; Fig. 1a). *Prolemur simus*, with respect to nitrogen in its diet, is closer to the non-bamboo eating *Propithecus edwardsi* than to the other two bamboo lemur species of Ranomafana.

Concentrations of non-structural carbohydrates did not differ consistently between Ranomafana and Mandena. According to the unweighted samples, *Prolemur simus* ate the items with the lowest concentrations of total non-structural carbohydrates (TNC) while *Avahi* *meridionalis* and *Propithecus edwardsi* had the highest concentrations of TNC (Fig. 1b). The high concentrations in food of non-bamboo lemur species do not differ between *Avahi meridionalis* and *Propithecus edwardsi* (Table 3).

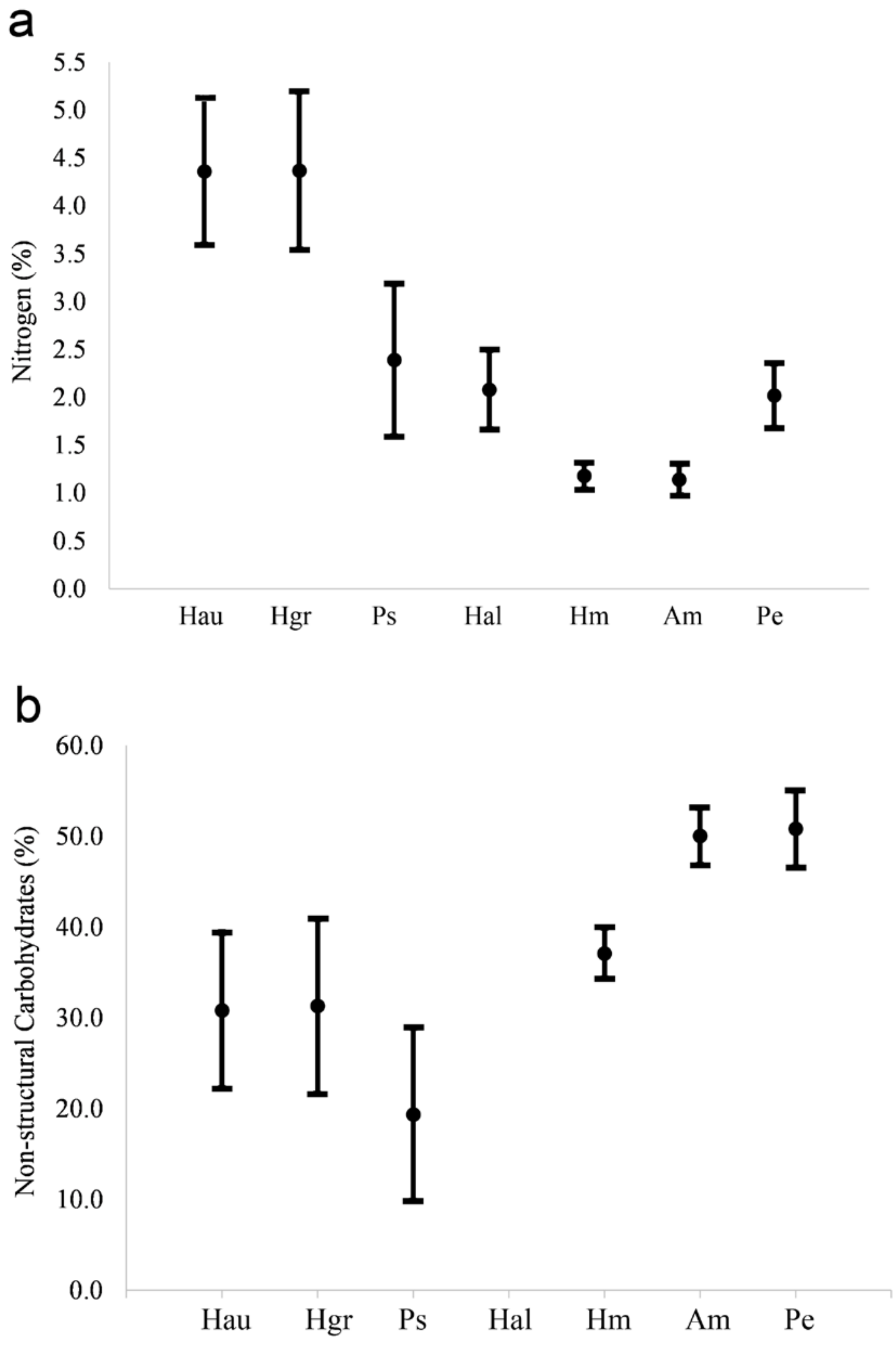

Fig. 1 Concentrations of nitrogen (Fig. 1a) and non-structural carbohydrates as a proxy of energy (Fig. 1b) in food items consumed by bamboo lemurs and sympatric folivorous lemur species at sites with cyanogenic bamboo (rain forest of Ranomafana) and at sites without cyanogenic bamboo (marsh of Lac Alaotra, littoral forests of Mandena and Ste. Luce). Values are means and 95% confidence intervals; items not weighted by frequency of consumption. *Hau*: *Hapalemur aureus*; *Hgr*: *H. griseus ranomafanensis*; *Ps*: *Prolemur simus*; *Hal*: *H. alaotrensis*; *Hm*: *H. meridionalis*; *Am*: *Avahi meridionalis*; *Pe*: *Propithecus edwardsi*.

Weighted samples

Frequency of consumption was considered in the weighted data. Weighted data are only available for *Hapalemur meridionalis* (from a non-bamboo site) and the lemur species that inhabit the sites with cyanogenic bamboo (i.e., Ranomafana). In this case, the diet constituents are weighted by the frequency of consumption, such that the nutritional contents reported in Table 3, are a representation of the lemur's average nutritional intake.

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, $p < 0.01$).

For most species and chemicals, the averages of the weighted samples were remarkably similar to the means of the unweighted samples, but not for the nitrogen concentration in the food of *Hapalemur g. ranomafanensis* (Table 3). While the concentrations of cyanogenic potential in the weighted food item samples were lower for *H. g. ranomafanensis* compared to *P. simus* and *H. aureus*, this difference was not statistically significant (Table 3). 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, food of the three bamboo eating lemur species did not differ in their concentrations of NDF or TNC. All bamboo lemur species from Ranomafana had significantly lower concentrations of NDF and TNC than food items consumed by *H. meridionalis* (Table 3).

Discussion

Animals that are able to feed on plants with specific plant secondary metabolites have exclusive access to resources not used by competitors. While avoiding competition is advantageous, the need for detoxification is likely to be associated with costs, either in terms of increased energy or protein requirements (Dearing et al. 2005, Foley and Moore 2005, Villalba and Provenza 2005; Lee et al. 2006, Shaw et al. 2006; Nersesian et al. 2012; Au et al. 2013). Bamboo lemurs of Madagascar are of special interest because some of the species ingest cyanogenic bamboo up to about 50 times the lethal dose of cyanide, which range between 74 and 370 mmol kg^{-1} for other mammals, such as sheep, mice, cats or rats (Glander et al. 1989; Ballhorn et al. 2009a). While all bamboo lemur species feed on bamboo where bamboo is available and all of the Ranomafana bamboo lemur species have been found to 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 (Glander et al. 1989; Mutschler et al. 1998; Mutschler 1999; Tan 1999; Eppley et al. 2011, 2016). This offers the possibility to investigate, whether or not 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 for supplying enzymes for detoxification or for maintenance or to supply sulphur for the detoxification mechanism. As tests of this hypothesis, we compared the chemical composition of food items consumed by bamboo lemur species living in areas with cyanogenic bamboo with the food composition in areas where bamboo is absent and where the animals feed on non-cyanogenic 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 *Propithecus edwardsi* in the rainforest of Ranomafana).

The inter-site comparison revealed that all bamboo lemur species feeding on cyanogenic bamboo (*Hapalemur aureus, H. griseus ranomafanensis*, *Prolemur simus*) ingested up to 1.7 times more nitrogen than the two bamboo lemur species (*Hapalemur alaotrensis, H. meridionalis*) studied at sites without cyanogenic bamboo. 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. Food items of *Prolemur simus* have higher nitrogen concentrations than items of the diet of *Propithecus edwardsi*, though this difference is not significant. In contrast to the *Hapalemur* species at Ranomafana, the *Hapalemur* spp. feeding on non-cyanogenic 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 food plants ranges between 2.4% and 4.4% (Table 3). Nitrogen concentrations in food consumed by bamboo lemur species that do not rely on bamboo (in areas where bamboo is absent) ranges between 1.2% and 2.1%. 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 (Moore and Foley 2005; DeGabriel et al. 2009).

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 (Waterman and Mole 1994; Wallis et al. 2012). Thus, while 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 favour 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*.

Based on our limited knowledge of the population dynamics of these species, the bamboo lemur species at the different sites do not seem to differ in their reproductive output or population dynamics (Nievergelt et al. 2002; Tan 2006; Eppley et al. 2015b, 2016b). 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 non-structural 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 (Chivers and Hladik 1980; Alexander 1993; Waterman and Kool 1994). However, at least *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. 2004; Perrin 2013). However, these are unlikely candidates for detoxification adaptations, as detoxification should occur at the beginning of the digestive tract rather than at the end. However, *H. griseus* achieves higher digestion of fibre and protein than other lemurs studied so far, indicating some kind of specialization that cannot be linked to morphological characteristics as both features of the digestive tract hint towards lower rather than higher digestion rates, just the opposite of what has been found in digestion studies (Campbell et al. 2000). Thus, it remains unclear what the surplus protein in bamboo is used for.

On the basis of the comparison between lemur species occurring at different sites, the utilization of protein for detoxification is consistent with the original hypothesis. It is also consistent with studies on sheep and brushtail possums, which describe that animals can ingest more PSM if supplemented with protein (Villalba and Provenza 2005; Nersesian et al. 2012; Au et al. 2013). 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 g. ranomafanensis* is the species that consumes the lowest amounts of cyanogenic substances (Table 3; Yamashita et al. 2010), while it is also the species ingesting the lowest portion of nitrogen in its diet.

Despite our results supporting the hypotheses, our sample size is small and we are aware that the study design is not optimal as it is non-experimental and lacks direct physiological measurements of energy and protein expenditure, but all the lemur species involved are threatened, with most being classified as “Endangered” or “Critically Endangered” according to the most recent IUCN classification (July 2012; Schwitzer et al. 2013). Therefore, it is impossible to use these animals in standard physiological experiments and, in particular, it is out of the question that the animals could be fed cyanogenic food for an experimental study. 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 non-structural carbohydrates show the reverse trend. Therefore, we conclude that protein serves a greater function than simply providing a source of energy for these animals.

Yet, bamboo is rich in protein but low in total non-structural carbohydrates. Therefore, it could well be that if lemur species feed on bamboo they unavoidably ingest high concentrations of protein. Also, it would have to be expected that lemurs feeding on cyanogenic substances would show some indication of protein selection. 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. These issues cannot be further resolved without experimental approaches or at least by applying new conceptual frameworks on how to study food selection (Felton et al. 2009; Rothman et al. 2011; Johnson et al. 2013; DeGabriel et al. 2014).

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Table captions

Table 1 Life history traits of lemur species included in the present study.

Table 2 Possible cyanide concentrations found in bamboo and grass from southeastern Madagascar. Plant species are known food items of *Hapalemur griseus ranomafanensis* (*H.g.r.*), *H. aureus* (*H.a.*) and/or *Prolemur simus* (*P.s.*), with those parts consumed within Ranomafana indicated. Published concentrations of cyanide are referenced at the end of the table.

Table 3 Chemical composition of food items eaten by bamboo lemurs *Hapalemur* spp., *Prolemur simus*; and sympatric folivorous lemur species.

Figure legend

Fig. 1 Concentrations of nitrogen (Fig. 1a) and non-structural carbohydrates as a proxy of energy (Fig. 1b) in food items consumed by bamboo lemurs and sympatric folivorous lemur species at sites with cyanogenic bamboo (rain forest 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 not weighted by frequency of consumption. *Hau*: *Hapalemur aureus*; *Hgr*: *H. griseus ranomafanensis*; *Ps*: *Prolemur simus*; *Hal*: *H. alaotrensis*; *Hm*: *H. meridionalis*; *Am*: *Avahi meridionalis*; *Pe*: *Propithecus edwardsi*.