

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

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24 **Short title:** Variation in food composition of bamboo lemurs

25

26 **Abstract**

27 Plants producing toxic plant secondary metabolites (PSM) deter feeding of folivores. Animals
28 that are able to cope with noxious PSMs have a niche with a competitive advantage over other
29 species. However, the ability to cope with toxic PSMs incurs costs for detoxification. In order
30 to assess possible compensations for the ingestion of toxic PSMs, we compare the chemical
31 quality of plants consumed by bamboo lemurs (genera *Haplemur* and *Prolemur*;
32 strepsirrhine primates of Madagascar) in areas with and without bamboo. Some bamboo
33 lemurs consume bamboo containing concentrations of cyanogenic substances 10 – 50 times
34 above the average lethal dosage for mammals, and we postulate that animals consuming

35 cyanogenic substances need supplementary protein or readily available energy for
36 detoxification. We compared the chemical composition of food consumed by three species of
37 bamboo lemurs that feed mainly (>80% of their time) on bamboo in the evergreen rainforest
38 of Ranomafana (Madagascar) with published data of the diets of bamboo lemurs at two sites
39 without highly cyanogenic plants (reed beds of Lac Alaotra and the evergreen littoral forest of
40 Mandena) and with food of sympatric folivorous lemur species that do not feed on bamboo.
41 Lemurs feeding on bamboo consumed up to twice as much protein than bamboo lemurs in
42 areas without bamboo and sympatric lemur species that feed on leaves of trees.
43 Concentrations of non-structural carbohydrates (a source of energy) showed the opposite
44 trend. This result supports the hypothesis that feeding on cyanogenic plants is linked to high
45 protein intake, either as a source of protein or for sulphur containing amino acids that can be
46 used for detoxification. Due to the high protein concentrations in bamboo, however, we
47 cannot distinguish between the hypothesis that lemurs that eat bamboo target additional food
48 items with higher protein, from the hypothesis that lemurs feeding on bamboo unavoidably
49 obtain higher concentrations of protein than animals feeding on leaves of trees, without an
50 added nutritional benefit.

51

52 **Keywords** Primates; Strepsirrhine; *Hapalemur* spp.; *Prolemur simus*; Detoxification;
53 Folivory; Plant secondary chemicals; Cyanogenic substances

54

55 **Introduction**

56 Generalist folivores feeding on toxic plants have to diversify their diet so that different
57 plant secondary metabolites (PSMs) can be detoxified by different metabolic pathways,
58 because single pathways can be limiting by their detoxification rates (Foley and Moore 2005;
59 Nersesian et al. 2012). In contrast, some folivorous specialists focus on only a few plant
60 species (Shiple et al. 2009), such as the giant panda (*Ailuropoda melanoleuca*; Schaller et al.
61 1985; Nie et al. 2015), red panda (*Ailurus fulgens*; Johnson et al. 1988), pygmy rabbit
62 (*Brachylagus idahoensis*; Shipley et al. 2006), some marsupials (Dearing et al. 2000; Marsh et
63 al. 2003), and the bamboo lemurs of Madagascar (gentle and golden bamboo lemurs:
64 *Hapalemur* spp.; greater bamboo lemur: *Prolemur simus*; Mutschler 1999; Tan 1999, 2006).
65 Here, plants and folivorous animals are linked in an evolutionary arms race in which plants
66 produce PSM- to deter feeding, and folivores evolve morphological and/or physiological
67 mechanisms for detoxification of these components, or exhibit behavioural selection of higher
68 quality food or reduced energy expenditure to cope with PSMs (Freeland and Janzen 1974;

69 Guglielmo et al. 1996; Provenza et al. 2003; Iason and Villalba 2006; Moore and DeGabriel
70 2012). From the plants' perspective, production of PSMs is costly and therefore some PSMs
71 are only produced when plants are threatened by folivores (Dolch and Tschardt 2000) or
72 after they have actually been fed upon (induced defences; Schuman and Baldwin 2012). From
73 the animals' point of view, detoxification also requires additional energy expenditure that
74 sometimes needs to be compensated for by ingestion of more or higher quality food (Glander
75 1982; Provenza et al. 2003; Dearing et al. 2005; Foley and Moore 2005; Villalba and
76 Provenza 2005; Lee et al. 2006; Shaw et al. 2006). A dramatic cost of detoxification of a
77 single PSM (benzoate) has been described for common brushtail possums (*Trichosurus*
78 *vulpecula*) where the costs for detoxification account for about 30% of the dietary nitrogen
79 intake (Au et al. 2013). Common brushtail possums that fed on a low protein diet had a
80 negative protein balance (Au et al. 2013). Thus, animals that consume toxic food, possibly in
81 order to avoid competition, should incur higher energy and nutrient requirements.

82 The bamboo lemurs of Madagascar are among the very few mammals with a diet
83 dominated by bamboo (Schaller et al. 1985; Tan 1999), a monocotyledonous flowering plant
84 belonging to the grass family Poaceae. In the altitudinal Malagasy rain forest at Ranomafana,
85 three species of bamboo lemurs (*Haplemur griseus ranomafanensis*, *H. aureus*, and
86 *Prolemur simus*) spend approximately 80 - 95% of their feeding time consuming various
87 bamboo species, including cyanogenic parts of the giant bamboo (*Cathariostachys*
88 *madagascariensis*; Tan 1999, 2006). These bamboo lemurs consume cyanogenic items (Tan
89 2000, 2006) and excrete cyanide (or more likely thiocyanate) mainly in urine (Yamashita et
90 al. 2010). The parts consumed contain up to 0.6% cyanide per gram dry weight which is about
91 10 - 50 times the lethal dosage for other mammals (Glander et al. 1989; Ballhorn et al. 2009a,
92 2016). Cyanide is detoxified by conversion to thiocyanate (Huang et al. 2016). This reaction
93 is catalysed by the enzyme rhodanese and requires a sulphur donor, most likely sulphur
94 containing amino acids (Conn 1979; Sousa et al. 2003). While the enzyme is widespread in
95 animal tissues, a 10- to 50-fold increase of cyanogenic substances beyond the lethal dosage
96 ought to require production of supplementary rhodanese and availability of sulphur containing
97 amino acids, making this a protein expensive detoxification mechanism. Detoxification is
98 likely also to be linked to increased energy requirements, which could be provided by non-
99 structural carbohydrates that are easily digestible and metabolized to produce energy.

100 In contrast to the altitudinal rain forest at Ranomafana, some bamboo lemur species,
101 *Haplemur alaotrensis* and *H. meridionalis*, occur in reed beds and littoral forests without
102 bamboo. *Haplemur alaotrensis* feed predominately on Cyperaceae and Poaceae, mainly

103 reeds (*Phragmites*; Mutschler 1999), and *H. meridionalis* on a variety of plants, mainly
104 Poaceae, including terrestrial grass (Eppley et al. 2011, 2016). Cyanogenesis is frequent in
105 Poaceae and Juncaceae (Conn 1979; Naik 1984; Harborne 1988), but cyanogenic substances
106 are not characteristic for terrestrial grasses (Frohne and Jensen 1973). Neither bamboo nor
107 grasses consumed by mountain gorillas (*Gorilla beringei beringei*) contain cyanogenic
108 substances (Grueter et al., 2016; J. Rothman, pers. comm.). Additionally, a variety of grasses
109 taken from botanical gardens did not contain cyanogenic substances or only in concentrations
110 too low to poison folivorous mammals (Ballhorn, unpubl.).

111 Though the notion that protein is a limiting factor for animals feeding on plants is not
112 always supported (Ofstedal 1991; Wasserman and Chapman 2003; Stolter 2008; Rothman et
113 al. 2011), protein is thought to play an important role in primate food selection because food
114 for primate folivores is often characterized by higher concentrations of different measures of
115 protein than items not eaten (protein measured as crude, soluble or digestible protein; e.g.,
116 Milton 1979; Ganzhorn 1988, 1992; Oates et al. 1990; Chapman et al. 2002; DeGabriel et al.
117 2008, 2009, 2014; Wallis et al. 2012). Refined approaches demonstrated that primates may
118 not simply select high protein dietary items, but adjust their food composition to meet their
119 protein requirements by ingesting a certain amount of protein per day (Felton et al. 2009;
120 Johnson et al. 2013). While the emphasis on protein for folivores' food selection has a long
121 tradition, an increasing number of studies indicates that folivores do not need to optimize
122 protein intake because food items contain enough protein to satisfy their needs (Ofstedal 1991;
123 Stolter 2008; Ganzhorn et al. 2017). These studies rather indicate an important role of easily
124 digestible energy for the maintenance of animals (Wasserman and Chapman 2003; Rothman
125 et al. 2011). There are not enough studies available yet to analyse which component is
126 favoured over the other at any given circumstances.

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

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

137 (1) bamboo eating bamboo lemurs consume items with higher protein or non-structural
138 carbohydrate content than bamboo lemurs that do not feed on bamboo,
139 (2) bamboo eating bamboo lemurs consume items with higher protein or non-structural
140 carbohydrate concentrations than sympatric folivorous lemur species that do not feed on
141 bamboo, and
142 (3) items consumed by bamboo lemurs that do not feed on bamboo should not differ in their
143 protein or non-structural carbohydrate concentrations from items consumed by other
144 sympatric folivorous lemur species.

145

146 **Methods**

147 *Study species and sites*

148 Bamboo lemurs (*Hapalemur* and *Prolemur*) are strepsirrhine primates of Madagascar
149 with body mass ranging from 0.8 – 1.7 kg (*Hapalemur* spp.) and 2.2 – 2.6 kg (*Prolemur*
150 *simus*) (Tan 2006; Mittermeier et al. 2010). All species considered here inhabit the evergreen
151 rainforest or swamps of eastern Madagascar and data are based on observations in the wild
152 (Table 1). Apart from *H. alaotrensis* from the marshes of Lac Alaotra, all other bamboo lemur
153 species occur in sympatry with other folivorous lemur species, such as *Avahi* spp. and
154 *Propithecus* spp., that do not consume bamboo or other grasses but rely on leaves from trees.
155 The species and study sites have been described previously: *Hapalemur alaotrensis* in the
156 reed beds of Lac Alaotra (Mutschler et al. 1998; Mutschler 1999), *H. meridionalis* in the
157 littoral forest of Mandena (Eppley et al. 2011, 2015a), *Avahi meridionalis* from the littoral
158 forest of Ste. Luce (Norscia et al. 2012), a forest fragment close to Mandena, described by
159 Donati et al. (2011), *H. griseus ranomafanensis*, *H. aureus*, *Prolemur simus* and *Propithecus*
160 *edwardsi* in the evergreen rainforest of Ranomafana National Park (Tan 1999, 2006; Arrigo-
161 Nelson 2006). *H. alaotrensis* and *H. meridionalis* occur at sites without bamboo and feed on
162 plants without or with very low cyanogenic potential (HCNp; the maximum amount of
163 cyanide that can be released from the accumulated cyanogenic precursors; Ballhorn et al.
164 2009b). *Hapalemur griseus ranomafanensis*, *H. aureus* and *P. simus* feed mainly on bamboo,
165 some of which are characterized by very high HCNp. The two other lemur species (*A.*
166 *meridionalis* and *P. edwardsi*) do not eat bamboo and were included for comparison of
167 species that ingest other possible food items available at the same site.

168

169

170

171 **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
<u>Sites without cyanogenic bamboo</u>								
<i>Hapalemur alaotrensis</i>	Lac Alaotra	1.2	< 22%	> 60%	13 months: 5 days/month	3 / 2 1 / 1	11	Mutschler 1999
<i>H. meridionalis</i>	Mandena	1.1	42%	44%	12 months: 5 days/month	6 / 8 (3 groups)	72	Eppley et al. 2011, 2016
<i>Avahi meridionalis</i>	Ste. Luce	1.2	15%	67%	7 months: 256 hours	1 / 1 1 / 1	47	Norscia et al. 2012
<u>Sites with cyanogenic bamboo</u>								
<i>H. griseus</i> <i>ranamofanensis</i>	Ranomafana	0.9	48%	41%	12 months: 5 days/month	1 / 2 1 / 2	> 40	Tan 1999, 2006, C. Tan unpubl. data
<i>H. aureus</i>	Ranomafana	1.4	37%	54%	12 months: 5 days/month	1 / 1 1 / 1	> 21	Tan 1999, 2006, C. Tan unpubl. data
<i>Prolemur simus</i>	Ranomafana	2.6	41%	50%	12 months: 5 days/month	3 / 2 1 / 2	7	Tan 1999, 2006
<i>Propithecus edwardsi</i>	Ranomafana	5.8	28%	42%	12 months: 5 days/month	7 / 8 (7 groups)	> 75	Arrigo-Nelson 2006

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

173

174 *Food composition: Comparison between species and sites*

175 In our analyses, we distinguish between food types and food items. A food item
 176 represents the item consumed by an animal in a given feeding event (= one sample). Food
 177 types represent plant parts. The chemical composition of a food type can be represented by the
 178 chemical analysis of a single sample of that food type. In this case, the food type based on just
 179 one or two samples is considered to be representative for the type throughout the study area.
 180 This category of data is available for *Hapalemur alaotrensis* (Mutschler 1999) and *Avahi*
 181 *meridionalis* (Norscia et al. 2012). It ignores temporal, spatial and individual variation of the
 182 chemical composition between plant individuals (Ganzhorn and Wright 1994; Chapman et al.
 183 2003; Rothman et al. 2012). For the other lemur species (*H. meridionalis*, *H. griseus*
 184 *ranamofanensis*, *H. aureus*, *P. simus*, *P. edwardsi*) we collected food items whenever an
 185 animal was seen feeding and sampling was possible. We analysed the chemical composition
 186 of food types for the pooled samples per food type (in case of *H. meridionalis*) or calculated

187 as the mean of the concentration from all items (= samples) collected per food type. We used
188 food consumed by other folivorous lemur species, (*Avahi meridionalis*) in the littoral forest
189 (Norscia et al. 2012), and *Propithecus edwardsi* in Ranomafana (Arrigo-Nelson 2006; Arrigo-
190 Nelson et al. unpublished data) to compare the chemical composition of food consumed by
191 bamboo lemurs with other potential food items available at the same site. Behavioural
192 observations and sample collection for these species are based on systematic observations of
193 habituated animals (Table 1).

194

195 *Food selection of bamboo lemurs at Ranomafana*

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

201 In order to assess possible selection criteria, we correlated the frequency of
202 consumption of any given food type with the mean concentrations of the various chemical
203 components of the food type. Weighing the consumption of food types by the frequency of
204 consumption seems a suitable procedure since each item was analysed chemically. Repeated
205 consumption of the same item might represent some kind of preference, though we did not
206 consider the availability of the item in question. Other procedures to assess selection criteria,
207 such as measuring the time of consumption or counting bites, calculating intake based on bites
208 or comparing items consumed with items not consumed have other advantages (Zinner 1999;
209 Rothman et al. 2012; DeGabriel et al. 2014; Aristizabal et al. 2017), but are simply not
210 available for comparisons.

211

212 *Chemical analyses*

213 Chemical analyses have been performed and published for food items consumed by
214 *Haplemur alaotrensis* (Pollock 1986; Mutschler 1999), and by *H. meridionalis* (Eppley et al.
215 2011, 2016). We analysed dried samples for all other species in the lab of Hamburg
216 University, following the methods described by Bollen et al. (2004).

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

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

225 Originally, HCNp of the dried food items consumed by the different bamboo lemurs
 226 from Ranomafana had been analysed enzymatically using the Spectroquant® cyanide test
 227 (Merck KGaA, Darmstadt, Germany; Ballhorn et al. 2009a). None of these samples showed
 228 any detectable cyanide. In contrast, plant material from Ranomafana stored in alcohol or
 229 analysed in the field, reliably released up to 0.6% cyanide per dry plant material (Glander et
 230 al. 1989; Ballhorn et al. 2009a) and showed positive reactions for cyanides in semi-
 231 quantitative tests (Tan, 1999, 2006; Yamashita et al. 2010). Therefore, we assume that the
 232 dried samples contained cyanogenic substances but our analyses for HCNp did not produce
 233 viable results likely because potentially specific enzymes (β -glucosidases) necessary to
 234 release cyanide from the bamboo tissue had been destroyed during the drying process or
 235 storage of samples. In order to have at least some qualitative estimates for the cyanogenic
 236 potential of bamboo lemur food items, we performed the analyses of potential food items in
 237 the field as listed below and compiled published information on the cyanide concentrations of
 238 various parts and species of bamboo (Table 2).

239

240 **Table 2** Possible cyanide concentrations found in bamboo and grass from southeastern
 241 Madagascar. All analyzed plant species are known food items of *Hapalemur griseus*
 242 *ranomafanensis* (*H.g.r.*), *H. aureus* (*H.a.*) and/or *Prolemur simus* (*P.s.*) (Tan 2006); however,
 243 only the species/parts consumed within Ranomafana are indicated. Published concentrations
 244 of cyanide (in % per dry weight) are referenced at the end of the table.

Species and Part	Consumed (+)			Cyanide [%]
	<i>H.g.r.</i>	<i>H.a.</i>	<i>P.s.</i>	
<i>Arundinaria</i> 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}
<i>Cathariostachys madagascariensis</i>				
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

Variation in food composition of bamboo lemurs

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}
<i>Sokinochloa chiataniae</i>				
Mature culm				0 ^{6,7}
Growing tip	+	+	+	0.02 ⁶
Leaf blade			+	0 ^{6,7}
<i>Sokinochloa brachyclada</i>				
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}
<i>Nastus elongatus</i>				
Branch shoot	+	+	+	+ ⁷
<i>Nastus sp.</i>				
Stem				0 ^{7,8}
<i>Poecilostachys festucaceus</i>				
Stem	+	+	+	0 ⁹
Leaves			+	0 ⁹
Mature leaf tip				0 ⁹

245 ¹ Eisler (1991), ² assumed to be equivalent to *Cathariostachys madagascariensis* (Eisler
 246 1991), ³ Ballhorn et al. (2009b), ⁴ assumed to be equivalent to “branch shoots”, ⁵ Ballhorn
 247 (unpubl. data), ⁶ Glander et al. (1989), ⁷ Tan (2006),

248 ⁸<http://tech.groups.yahoo.com/group/bamboo-plantations/message/2019>, ⁹ Yamashita et al.
 249 (2010)

250 For statistical analyses, we assigned a concentration of 0.01% HCN to items that showed a
 251 positive response (+) in the Feigl-Anger Test, if only qualitative data are available.

252

253 Total non-structural carbohydrates (TNC) were used as a proxy for easily digestible
 254 carbohydrates (for a discussion see Conklin-Brittain et al. 2006; Rothman et al. 2012). TNC
 255 were calculated as: TNC = 100% - Crude protein – Neutral detergent fibre. Lipids and ash

256 ought to be subtracted from this measure, but these analyses were not available. Lipids are not
257 an important component in leaves. Ash (= mineral) contents can amount to several percent of
258 dry mass in leaves (National Research Council 2003) but has not measured consistently in the
259 samples used for the present analysis. We assume that the error introduced by not subtracting
260 lipids and ash is small compared to the large fibre fraction. Neutral detergent fibre (NDF) was
261 only used to calculate TNC. We did not have any specific hypothesis concerning NDF itself.
262 Therefore, NDF were not discussed specifically.

263 For analysis of plant HCNp, fresh items were selected from bamboo in Ranomafana
264 several years after the behavioural observation of the bamboo lemurs had been completed
265 (Ballhorn et al. 2009b). These items were used as proxy for cyanogenic components in the
266 original food samples of bamboo lemurs because the analyses of the original food items failed
267 and could not be repeated. For each plant species, we analysed different plant tissues for
268 HCNp to obtain information on the quantitative variability of cyanogenic precursor content as
269 there frequently is substantial within-plant variation with young and reproductive tissues
270 showing the highest amounts of cyanogenic precursors (Ballhorn et al. 2008, 2009b). Fresh
271 plant samples were weighted to the nearest 0.001 g and ground with liquid nitrogen and
272 cooled mortar and pestle (4°C) under addition of the fourfold volume (v fresh weight⁻¹) of ice-
273 cold Na₂HPO₄ solution (67 mmol L⁻¹). Samples were quantitatively analysed for their HCNp
274 by complete enzymatic hydrolysis of cyanogenic precursors under addition of β-glucosidase
275 from almonds (Emulsin®, Merck, NY, USA). We used closed glass vessels (Thunberg
276 vessels) for incubation (20 min at 30 °C in a water bath) of plant extracts together with
277 enzyme solution adjusted to an activity of 20 nkat. Quantification of released HCN was
278 conducted spectrophotometrically at 585 nm using the Spectroquant® cyanide test (Merck
279 KGaA, Darmstadt, Germany; Ballhorn et al. 2009b).

280

281 *Statistical analysis*

282 Data were tested for deviations from normality with Kolmogorov-Smirnov-one sample
283 tests. If residuals deviated from normality, we used non-parametric tests. Kruskal-Wallis
284 Analysis of Variance was used for comparisons of more than two groups. Post-hoc tests for
285 subsequent pairwise comparisons were based on Mann-Whitney-U-tests and significance
286 levels were Bonferroni corrected. For parametric analyses of more than two groups we used
287 Analysis of Variance with subsequent Scheffé's post-hoc test. All statistical analyses were
288 performed in IBM SPSS Statistics v. 22 (SPSS Inc., Chicago, IL, U.S.A.), with significance
289 set at $p < 0.05$.

290

291 **Results**

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

300

301 *Unweighted samples*

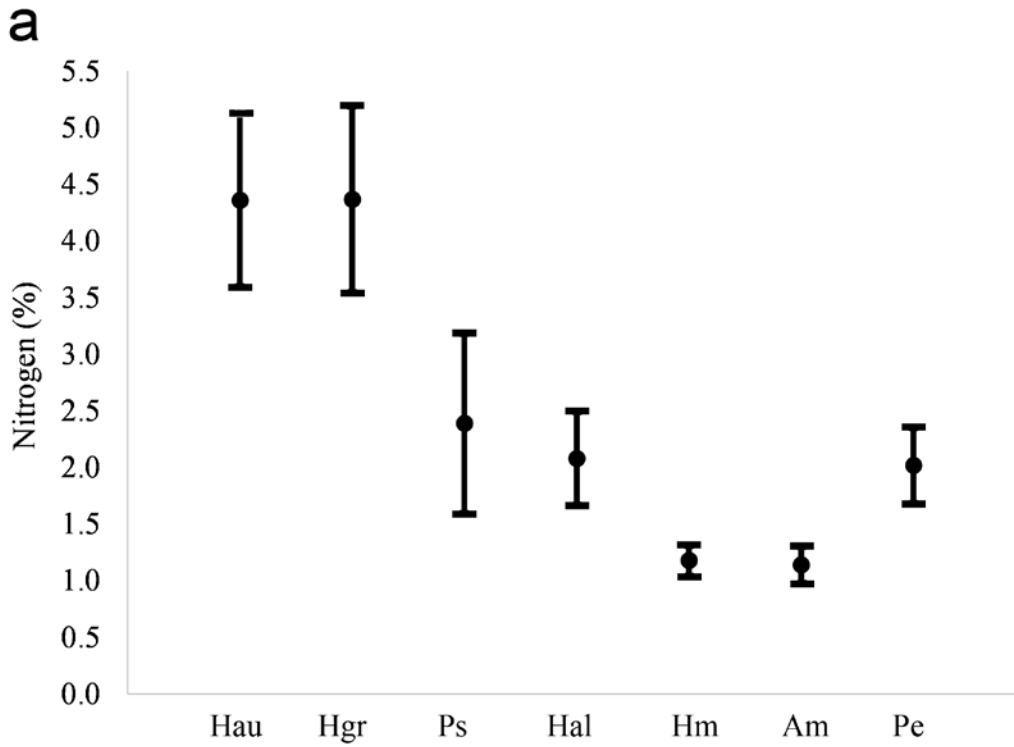
302 The unweighted data in Table 3 are a summation of the nutrients in all of the plant
303 types that have been recorded as consumed by a given species and were analysed for their
304 chemical composition; for example, chemical analyses for *Hapalemur aureus* are available for
305 four different plant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

308 *Hapalemur g. ranomafanensis* and *P. simus* consumed plants with much lower
309 cyanogenic potential (HCNp) than *H. aureus*. Due to small sample size of the unweighted
310 samples, this difference is not significant (Table 3). Though the median of plant HCNp is zero
311 or close to zero for *H. g. ranomafanensis* and *P. simus*, respectively, both species include
312 some plant parts with very high cyanide concentration (Table 3). According to the unweighted
313 samples, food types consumed by *Hapalemur aureus* have higher concentrations of nitrogen
314 than food consumed all other lemur species, except for the sympatric *H. g. ranomafanensis*
315 (Table 3).

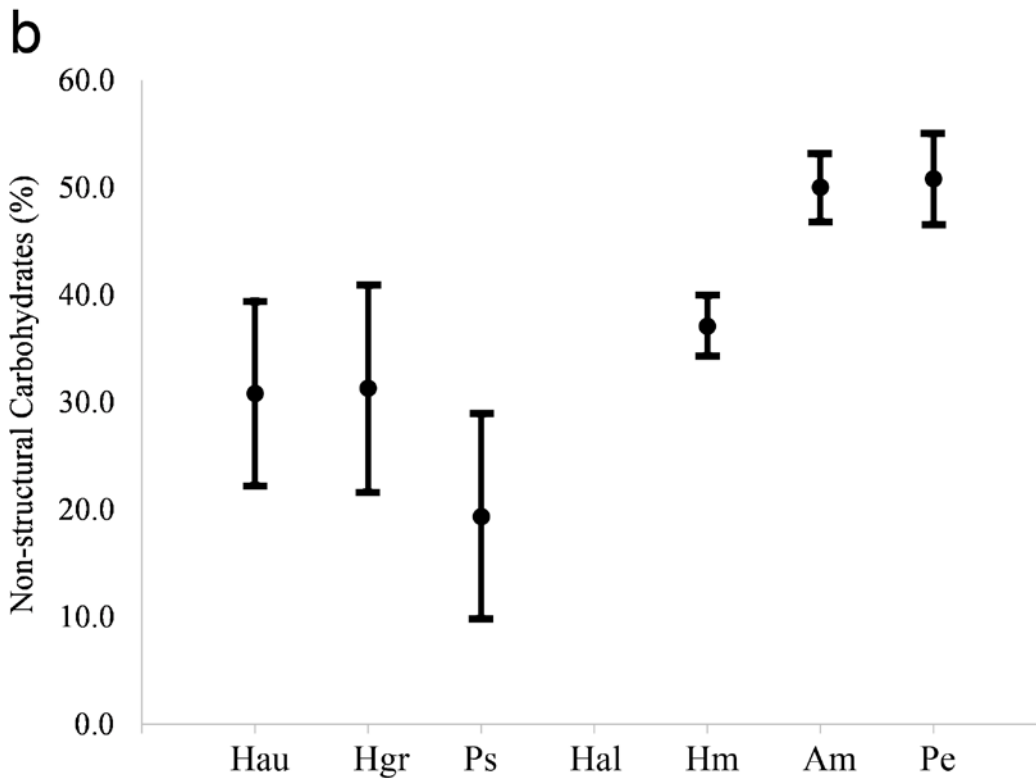
316 Food of all bamboo lemur species from the sites with non-cyanogenic plants and the
317 non-bamboo-lemur species has lower nitrogen concentrations than the two *Hapalemur* species
318 from Ranomafana (Table 3; Fig. 1a). *Prolemur simus*, with respect to nitrogen in its diet, is
319 closer to the non-bamboo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

321 Concentrations of non-structural carbohydrates did not differ consistently between
322 Ranomafana and Mandena. According to the unweighted samples, *Prolemur simus* ate the
323 items with the lowest concentrations of total non-structural carbohydrates (TNC) while *Avahi*

324 *meridionalis* and *Propithecus edwardsi* had the highest concentrations of TNC (Fig. 1b). The
325 high concentrations in food of non-bamboo lemur species do not differ between *Avahi*
326 *meridionalis* and *Propithecus edwardsi* (Table 3).
327



328



329

330 **Fig. 1** Concentrations of nitrogen (Fig. 1a) and non-structural carbohydrates as a proxy of
331 energy (Fig. 1b) in food items consumed by bamboo lemurs and sympatric folivorous lemur
332 species at sites with cyanogenic bamboo (rain forest of Ranomafana) and at sites without
333 cyanogenic bamboo (marsh of Lac Alaotra, littoral forests of Mandena and Ste. Luce). Values
334 are means and 95% confidence intervals; items not weighted by frequency of consumption.
335 *Hau: Hapalemur aureus; Hgr: H. griseus ranomafanensis; Ps: Prolemur simus; Hal: H.*
336 *alaotrensis; Hm: H. meridionalis; Am: Avahi meridionalis; Pe: Propithecus edwardsi.*
337

338 *Weighted samples*

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

344 Based on the frequency of consumption, *Hapalemur aureus* ate the highest proportion
345 of cyanogenic items (65.1% of collected food items), followed by *Prolemur simus* (64.1% of
346 collected food items) and *H. griseus ranomafanensis* (18.2%). The frequency of consumption
347 of cyanogenic plant items differs significantly between the three species ($\chi^2 = 14.11$, $df = 2$, p
348 < 0.01).

349 For most species and chemicals, the averages of the weighted samples were
350 remarkably similar to the means of the unweighted samples, but not for the nitrogen
351 concentration in the food of *Hapalemur g. ranomafanensis* (Table 3). While the
352 concentrations of cyanogenic potential in the weighted food item samples were lower for *H. g.*
353 *ranomafanensis* compared to *P. simus* and *H. aureus*, this difference was not statistically
354 significant (Table 3). The three bamboo lemur species from Ranomafana have significantly
355 higher concentrations of nitrogen than *H. meridionalis*. Food items of *H. aureus* have
356 somewhat higher concentrations of nitrogen than food consumed by *H. g. ranomafanensis* and
357 *P. simus*, though this difference is not significant. According to the weighted samples, food of
358 the three bamboo eating lemur species did not differ in their concentrations of NDF or TNC.
359 All bamboo lemur species from Ranomafana had significantly lower concentrations of NDF
360 and TNC than food items consumed by *H. meridionalis* (Table 3).

362 **Discussion**

363 Animals that are able to feed on plants with specific plant secondary metabolites have
364 exclusive access to resources not used by competitors. While avoiding competition is
365 advantageous, the need for detoxification is likely to be associated with costs, either in terms
366 of increased energy or protein requirements (Dearing et al. 2005, Foley and Moore 2005,

367 Villalba and Provenza 2005; Lee et al. 2006, Shaw et al. 2006; Nersesian et al. 2012; Au et al.
368 2013). Bamboo lemurs of Madagascar are of special interest because some of the species
369 ingest cyanogenic bamboo up to about 50 times the lethal dose of cyanide, which range
370 between 74 and 370 mmol kg⁻¹ for other mammals, such as sheep, mice, cats or rats (Glander
371 et al. 1989; Ballhorn et al. 2009a). While all bamboo lemur species feed on bamboo where
372 bamboo is available and all of the Ranomafana bamboo lemur species have been found to
373 ingest and excrete cyanide (Yamashita et al. 2010), the proportion of cyanogenic food
374 ingested by the different species varies and can be negligible at sites without bamboo
375 (Glander et al. 1989; Mutschler et al. 1998; Mutschler 1999; Tan 1999; Eppley et al. 2011,
376 2016). This offers the possibility to investigate, whether or not species feeding on cyanogenic
377 bamboo compensate for the assumed costs of detoxification by increased consumption of
378 items that provide easily digestible energy or protein, either for supplying enzymes for
379 detoxification or for maintenance or to supply sulphur for the detoxification mechanism. As
380 tests of this hypothesis, we compared the chemical composition of food items consumed by
381 bamboo lemur species living in areas with cyanogenic bamboo with the food composition in
382 areas where bamboo is absent and where the animals feed on non-cyanogenic plants. In
383 addition, we compared the chemical composition of food items consumed by bamboo lemurs
384 with the diet of other folivorous lemurs from the same areas (*Avahi meridionalis* in the littoral
385 forest and *Propithecus edwardsi* in the rainforest of Ranomafana).

386 The inter-site comparison revealed that all bamboo lemur species feeding on
387 cyanogenic bamboo (*Hapalemur aureus*, *H. griseus ranomafanensis*, *Prolemur simus*)
388 ingested up to 1.7 times more nitrogen than the two bamboo lemur species (*Hapalemur*
389 *alaotrensis*, *H. meridionalis*) studied at sites without cyanogenic bamboo. The *Hapalemur*
390 spp. feeding on cyanogenic bamboo in Ranomafana also ingest more nitrogen than the
391 sympatric non-bamboo lemur species (*Propithecus edwardsi*) feeding on the leaves of trees.
392 Food items of *Prolemur simus* have higher nitrogen concentrations than items of the diet of
393 *Propithecus edwardsi*, though this difference is not significant. In contrast to the *Hapalemur*
394 species at Ranomafana, the *Hapalemur* spp. feeding on non-cyanogenic items do not ingest
395 more nitrogen than the non-bamboo lemur species (*Avahi meridionalis*) that is sympatric to *H.*
396 *meridionalis*. Cyanogenic glycosides contribute to the nitrogen concentrations in cyanogenic
397 plants. But their relative contribution is small (maximum 0.3% nitrogen, corresponding to
398 0.6% cyanide) compared to the nitrogen contained in protein. The nitrogen concentrations in
399 food plants of the lemur species feeding on cyanogenic food plants ranges between 2.4% and
400 4.4% (Table 3). Nitrogen concentrations in food consumed by bamboo lemur species that do

401 not rely on bamboo (in areas where bamboo is absent) ranges between 1.2% and 2.1%. Even
402 when the maximum nitrogen content of 0.3% bound in cyanogenic substances is subtracted
403 from the total nitrogen concentration of cyanogenic food parts, the average nitrogen
404 concentration in food items of bamboo consuming species would be about 1.1% higher than
405 in food plants of species that do not include bamboo in their diet. Nitrogen concentrations of
406 1.1% are equivalent to about 6.9% more crude protein. According to studies that linked
407 protein in food to reproductive success and growth, this difference can have a major impact
408 for folivorous mammal fitness (Moore and Foley 2005; DeGabriel et al. 2009).

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

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

428 Morphological traits could provide hints for special adaptations to deal with food
429 peculiarities, such as a complex forestomach that could facilitate detoxification (Chivers and
430 Hladik 1980; Alexander 1993; Waterman and Kool 1994). However, at least *Hapalemur*
431 *griseus* does not have any morphological specialization that indicates any specific adaptation
432 of the digestive tract. The only difference between *H. griseus* and a dietary generalist lemurid
433 species such as *Lemur catta* is a shorter cecum and a shortened and sacculated colon of *H.*
434 *griseus* (Campbell et al. 2004; Perrin 2013). However, these are unlikely candidates for

435 detoxification adaptations, as detoxification should occur at the beginning of the digestive
436 tract rather than at the end. However, *H. griseus* achieves higher digestion of fibre and protein
437 than other lemurs studied so far, indicating some kind of specialization that cannot be linked
438 to morphological characteristics as both features of the digestive tract hint towards lower
439 rather than higher digestion rates, just the opposite of what has been found in digestion studies
440 (Campbell et al. 2000). Thus, it remains unclear what the surplus protein in bamboo is used
441 for.

442 On the basis of the comparison between lemur species occurring at different sites, the
443 utilization of protein for detoxification is consistent with the original hypothesis. It is also
444 consistent with studies on sheep and brushtail possums, which describe that animals can
445 ingest more PSM if supplemented with protein (Villalba and Provenza 2005; Nersesian et al.
446 2012; Au et al. 2013). Our comparison of the three sympatric bamboo lemur species at
447 Ranomafana matches this scenario, providing evidence that cyanide tolerance may be related
448 to protein ingestion. In Ranomafana, *Haplemur g. ranomafanensis* is the species that
449 consumes the lowest amounts of cyanogenic substances (Table 3; Yamashita et al. 2010),
450 while it is also the species ingesting the lowest portion of nitrogen in its diet.

451 Despite our results supporting the hypotheses, our sample size is small and we are
452 aware that the study design is not optimal as it is non-experimental and lacks direct
453 physiological measurements of energy and protein expenditure, but all the lemur species
454 involved are threatened, with most being classified as “Endangered” or “Critically
455 Endangered” according to the most recent IUCN classification (July 2012; Schwitzer et al.
456 2013). Therefore, it is impossible to use these animals in standard physiological experiments
457 and, in particular, it is out of the question that the animals could be fed cyanogenic food for an
458 experimental study. Thus, we had to rely on descriptive field studies. The results of our
459 comparisons are consistent with the hypothesis that lemurs consuming cyanogenic bamboo
460 can compensate for the toxins by consuming more protein because (1) bamboo eating bamboo
461 lemurs consume items with higher protein content than bamboo lemurs that do not feed on
462 bamboo, (2) two out of three bamboo eating lemur species consume items with higher protein
463 than sympatric folivorous lemur species that do not feed on bamboo, and (3) items consumed
464 by bamboo lemurs that do not feed on bamboo do not differ in their protein concentrations
465 from items consumed by other sympatric folivorous lemur species. The results for total non-
466 structural carbohydrates show the reverse trend. Therefore, we conclude that protein serves a
467 greater function than simply providing a source of energy for these animals.

468 Yet, bamboo is rich in protein but low in total non-structural carbohydrates. Therefore,
469 it could well be that if lemur species feed on bamboo they unavoidably ingest high
470 concentrations of protein. Also, it would have to be expected that lemurs feeding on
471 cyanogenic substances would show some indication of protein selection. This was not found
472 in our analyses and it remains enigmatic what the bamboo lemurs do with the high
473 concentrations of protein in their diet. These issues cannot be further resolved without
474 experimental approaches or at least by applying new conceptual frameworks on how to study
475 food selection (Felton et al. 2009; Rothman et al. 2011; Johnson et al. 2013; DeGabriel et al.
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477

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497

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814 **Table captions**

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

816

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

822

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

825

826 **Figure legend**

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

832 *Hau*: *Hapalemur aureus*; *Hgr*: *H. griseus ranomafanensis*; *Ps*: *Prolemur simus*; *Hal*: *H.*

833 *alaotrensis*; *Hm*: *H. meridionalis*; *Am*: *Avahi meridionalis*; *Pe*: *Propithecus edwardsi*.