The importance of protein in leaf selection of folivorous primates

JOERG U. GANZHORN¹, SUMMER J. ARRIGO-NELSON², VALENTINA CARRAI³, MUKESH K. CHALISE⁴, GIUSEPPE DONATI⁵, IRIS DROESCHER⁶, TIMOTHY M. EPPLEY¹, MITCHELL T. IRWIN⁷, FLÁVIA KOCH⁶, ANDREAS KOENIG⁸, MARTIN M. KOWALEWSKI⁹, CHRISTOPHER B. MOWRY¹⁰, ERIK R. PATEL¹¹, CLAIRE PICHON¹², JOSE RALISON¹³, CHRISTOPH REISDORFF¹⁴, BRUNO SIMMEN¹², ELEANOR STALENBERG¹⁵, JUANA TERBOVEN¹, PATRICIA C. WRIGHT⁸ AND WILLIAM J. FOLEY ¹⁵

¹ Animal Ecology and Conservation, University of Hamburg, Martin-Luther-King Platz 3, 20146 Hamburg, Germany, ganzhorn@zoologie.uni-hamburg.de
² Department of Biological and Environmental Science, California University of Pennsylvania, California, PA 15419, USA
³ Department of Biology, Zoology and Anthropology Unit, Via A. Volta, 4, I-56126 Pisa, Italy
⁴ Central Department of Zoology, Tribhuvan University, Kirtipur, Nepal
⁵ Nocturnal Primate Research Group, Department of Social Sciences, Oxford Brookes University, Gipsy Lane, OX3 0BP, Oxford, UK
⁶ Behavioral Ecology and Sociobiology, German Primate Center, Kellnerweg 4, 37077 Göttingen, Germany
⁷ Department of Anthropology, Northern Illinois University, DeKalb, IL 60115, USA
⁸ Department of Anthropology, Stony Brook University, and Interdepartmental Doctoral Program in Anthropological Sciences, Stony Brook University, Stony Brook, NY 11794-4364, USA
Estación Biológica Corrientes, Museo Argentino de Ciencias Naturales BR, Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Corrientes, Argentina

Department of Biology, Berry College, Mt. Berry, Georgia 30149, USA

Duke Lemur Center, Durham, N.C., USA

Département Hommes, Natures, Sociétés, CNRS/MNHN, UMR 7206, 4 Avenue du Petit Château, 91800 Brunoy, France

Department of Animal Biology, University of Antananarivo, BP 906, 101 Antananarivo, Madagascar, and Groupe d’Etude et de Recherche sur les Primates de Madagascar (GERP), B.P. 779, 101 Antananarivo, Madagascar

Applied Plant Ecology, University of Hamburg, Ohnhorststr. 18, 22609 Hamburg, Germany

Research School of Biology: Division of Evolution, Ecology and Genetics. The Australian National University, Canberra, ACT 0200, Australia
Abstract
Protein limitation has been considered a key factor in hypotheses on the evolution of life history and animal communities, suggesting that animals should prioritize protein in their food choice. This contrasts with the limited support that food selection studies have provided for such a priority in nonhuman primates, particularly for folivores. Here, we suggest that this discrepancy can be reconciled if folivores only need to select for high protein leaves when average protein concentration in the habitat is low. To test the prediction, we analyzed published and unpublished results of food selection and protein concentrations from 47 studies of folivorous primates. To counter potential methodological flaws, we differentiated between methods analyzing nitrogen and soluble protein concentrations. We found that leaves containing either high concentrations of total nitrogen or high soluble protein were selected more in low protein forests. There was no relationship (either negative or positive) between the concentration of protein and fiber in the food. Overall our study suggests that protein is limiting only in protein-poor environments, explaining the sometimes contradictory results in previous studies on protein selection.

Key words: primates, food chemistry, food selection, leaf-eating, nutrient requirements, protein availability

INTRODUCTION
Protein has been considered a major limiting factor involved in the evolution of animal communities and life history traits [e.g., White, 1993]. The need to satisfy protein requirements plays a central role in hypotheses on the evolution of morphological, physiological and behavioral life history traits (such as gut specialization, reduced metabolism in folivores, social systems linked to the
distribution of different types of food, and community composition; e.g., White, 1993). The essentials of this idea have been developed for primates by Kay [1984] and illustrated by Terborgh [1992]. Specifically, while most primates eat fruit to satisfy their energy requirements, fruits typically do not provide enough available protein for survival and reproduction, though this may not always be the case [reviewed by Klaasen and Nolet 2008; Ganzhorn et al., 2009; Schwitzer et al 2009]. Therefore, smaller-bodied species feed on insects and fruit to support their protein needs. Larger species are unable to obtain enough protein from insects because the capture rate of insects is independent of body mass [Hladik, 1978; Rothman et al., 2014]. Consequently they eat leaves, which usually contain more protein than fruit and can be found in sufficient quantities to satisfy the protein needs of a larger species. According to this scenario, within the broad constraints of body mass, protein represents the ultimate factor that determines whether a species is insectivorous or folivorous. The idea that protein is limiting has received support from the studies of Milton [1979], Oates et al., [1990] and Davies and Oates [1994 and their contributors]. Milton [1979] postulated that the densities and biomass of folivorous howler monkeys are closely related to the average leaf quality of a forest expressed as the ratio of protein to fiber (most commonly measured as acid detergent fiber – ADF) concentrations. Oates and collaborators (1990) tested and found support for this idea through a wide comparison of colobine monkeys. ADF concentrations were included because ADF should represent the refractory fraction of the cell wall (cellulose + lignin) and increasing ADF concentrations are also likely to reflect greater amounts of indigestible protein [Rothman et al., 2008]. The concept of protein to fiber ratios was extended to additional populations of colobines [e.g., Chapman et al., 2002, 2004; Wasserman & Chapman, 2003; Fashing et al., 2007] and supported with independent datasets on lemurs [Ganzhorn, 1992; Simmen et al., 2012] and howler
monkeys [Peres, 1997]. The biological relevance of this ratio has been questioned based on biochemical considerations, statistical issues around the use of ratios [Wallis et al., 2012], and empirical grounds [Gogarten et al., 2012; Chapman et al., 2014] but it seems to retain some predictive capacity.

Restricting the considerations to protein alone, several studies have shown that protein can be limiting with lasting effects on development and lifetime fitness [e.g., Fleagle et al., 1975; Elias & Samonds, 1977; Altmann, 1991, 1998; Degabriel et al., 2009]. However, the evidence that folivorous primates actually select leaves with high protein content is ambiguous. Considering protein alone, some studies found positive selection by primates for high protein leaves [e.g., New World howler monkeys: Milton, 1979, 1998; Glander, 1981; Old World non-colobine monkeys: Beeson, 1989; Barton & Whiten, 1994; Old World colobines: Davies et al., 1988; Waterman et al., 1988; Mowry et al., 1996; Koenig et al., 1998; Yeager et al., 1997; Apes: Calvert, 1985; Lemurs: Ganzhorn, 1988, 1992, 2002; Mutschler, 1999] but others failed to do so [e.g., New World howler monkeys: Gaulin & Gaulin, 1982; Estrada & Coates-Estrada, 1986; Old World colobines: Oates et al., 1980; McKey et al., 1981; Waterman et al., 1988; Kool, 1992; Dasilva, 1994; Chapman et al., 2002; Apes: Conklin-Brittain et al., 1998; Rothman et al., 2011; Lemurs: Ganzhorn, 1988; Ganzhorn et al., 2004; Simmen et al., 2014]. Thus, we are left with the conundrum that protein is hypothesized to be an important component in primate food selection while only about half of the studies on food selection criteria demonstrate that primates actively select high protein leaves. This discrepancy can be due to methodological, ecological, or species-specific reasons, or the hypothesis may simply be wrong.

On the methodological side, different studies have applied different methods to measure “protein”. While the conventional method of measuring crude protein uses...
total nitrogen concentrations multiplied by 6.25 (or a species specific factor [Milton & Dintzis, 1981]) as a surrogate for protein, this measure does not actually distinguish between protein and non-protein nitrogen [e.g. N in cyanogenic glycosides, non-protein amino acids, nitrates or alkaloids], or between available protein and protein bound to other components and thus unavailable for digestion [DeGabriel et al., 2008; Rothman et al., 2008]. To overcome this shortcoming, some studies have analyzed total amino acids [e.g., Glander, 1981; Simmen & Sabatier, 1996; Mutschler, 1999; Curtis, 2004] or soluble protein [e.g., Ganzhorn, 1988; Koenig et al., 1998; Conklin-Brittain et al., 1999; for methodological considerations see Ortmann et al., 2006; Rothman et al., 2012]. Although the selection for high protein items was more consistent in studies that analyzed soluble protein than in studies based on crude protein, none of these methods accounts for differences in protein quality (defined by essential amino acids), or digestibility [Robbins, 1983; NRC, 2003; Wallis et al., 2012; DeGabriel et al., 2014].

From an ecological perspective, the lack of positive selection for high protein items could also be explained by the assumption that primates are able to satisfy their protein requirements with a diet containing about 6.4 – 8% crude protein [NRC, 2003]. The crude protein concentration of leaves and the average concentration of protein in primate foods are around or well above these requirements [e.g., Hladik, 1977; Oftedal, 1991; Conklin-Brittain et al., 1998; Ganzhorn et al., 2009]. Thus, primates might not need to select high protein items but could simply feed according to the average availability of protein in the environment provided that the digestibility of protein from the food was not hindered by other components such as fiber or tannins [Mowry et al., 1996; Yeager et al., 1997; Simmen et al., 2014].

Deviations from selecting high protein leaves may also be caused by species-specific adaptation of gut morphology and digestive physiology [Chivers et al., 1984;
Ganzhorn et al., Protein in leaves

Cork & Foley 1991; Hughes, 1993; Langer & Chivers, 1994; Van Soest, 1994; Lambert, 1998; Milton, 1998, 1999; Campbell et al., 1999, 2004; Edwards & Ullrey, 1999a,b; Godfrey et al., 2004. The effect of gut physiology may be more important than the effect of body mass on dietary characteristics in primates as hindgut-fermenters process food differently than foregut fermenters and both deviate from species with unspecialized digestive tracts, regardless of size. For example, Campbell et al. [2004] found that different adaptations of the digestive tract result in food passage times largely independent of body mass [see also Clauss et al., 2008], such as larger primate species with foregut fermentation (colobines) or hindgut fermentation (gorillas), and small primates with hindgut fermentation and caecotropy (e.g., Lepilemur spp.) [Charles-Dominique & Hladik 1971], or enlargement of the small intestine (Indriidae). This supports the conclusion that body mass is not a useful surrogate to understand primate feeding and digestion, including protein requirements [Lambert, 1998].

Thus, in order to investigate protein selection in folivorous primates, we consider the availability of protein in the environment and test the hypothesis that protein is a limiting component and therefore primates should search for high protein and/or low fiber leaves. According to this hypothesis, selection for high protein items would not be necessary if animals could obtain enough protein from their overall diet. However, if protein concentrations in the environment are low, folivorous primates should seek high protein leaves. Therefore, we predict that selectivity for high protein leaves declines with increasing average protein content in leaves encountered by the animals in their home range. We could expect there to be an inverse relationship between concentrations of protein and fiber in foliage reflecting a maturation of the leaf ontogenetically and temporally. We also tested for this relationship and
separately tested whether fiber in the food selected differed from that of a general sample.

METHODS

Database

The analyses presented here are based on published data from all primate radiations (except for apes; see below), supplemented by new data of folivorous primates from Madagascar, the New World and Nepal (Table 1). Analyses were restricted to forest dwelling species that have been classified as “folivores” because the majority of their food items were from photosynthetic material [Kappeler & Heymann, 1996]. As more studies are conducted, it appears that the classification of species into specific feeding guilds does not reflect the species-specific variability of diet [Hemingway & Bynum, 2005; Garber et al., 2015]. Thus, we call those species “folivores” that are supposed to derive their protein from leaves and not insects according to Kay’s [1984] hypothesis.

Species that feed primarily on the leaves of grasses, bamboo (Hapalemur spp., Prolemur simus) and herbs (Gorilla spp.) were not included, as grass and herbs have different physico-chemical properties than leaves from trees, such as different lignin, a general lack of tannins and incorporation of silica in grasses [Robbins, 1983]. However, Hapalemur meridionalis from Mandena (south-eastern Madagascar) was included as these animals live in an area without bamboo and feed on grass and other leaves [Epplley et al., 2011]. For the current analysis we removed all grasses that were used as food and restricted the analysis to the proportion of their diet that consists of leaves from trees. We also included body mass in the database provided in Table I. Data for primate body mass were taken from Smith and Junger [1997] and Mittermeier et al. [2010] and averaged between sexes.
Food Types and Nutritional Analyses

Foods included in the present analysis were leaves or flower buds from trees, shrubs or vines. We further restricted the analysis to concentrations of nitrogen (measured by the Kjeldahl method), or by a combustion procedure with subsequent analysis of elementary nitrogen (the Dumas method), or based on near infrared reflectance spectroscopy (NIRS) (calibrated against the Kjeldahl or Dumas method), soluble protein and acid detergent fiber (ADF). Data presented as “crude protein” (i.e. total nitrogen multiplied by 6.25) was re-transformed to total nitrogen concentration as the biological significance of the conversion factor is presently debated and its biological meaning is unclear (Milton & Dintzis, 1981; NRC, 2003; for methodological reviews see Ortmann et al., 2006; Rothman et al., 2012). The Kjeldahl and Dumas methods yield almost identical results (regression between nitrogen measured by Kjeldahl [y] and by the Dumas method [x] forced through the origin: $y = 0.94x; R^2 = 0.99; n = 90$; Supplementary Material [Terboven, 2014]). Near infrared reflectance spectroscopy also provides accurate estimates for nitrogen concentrations when models were tested with truly independent data (Kjeldahl: $y = 1.06x, R^2 = 0.97, N = 18$; combustion: $y = 0.97x, R^2 = 0.97; N = 18$; Supplementary Material).

Studies that published soluble protein concentrations (measured by the method outlined by Bradford, [1976]) but without estimates of crude protein were included in the analysis, when available. However, these two datasets were analyzed separately. “Available protein” would be a more biologically appropriate measure of protein than crude protein [DeGabriel et al., 2008, 2014; Wallis et al., 2012] and probably also than soluble protein as soluble protein concentrations are correlated with available protein in some studies but not in others [Ganzhorn, unpubl.]. To date, too few data exist for available protein to allow for comparative analyses.
In primate studies, fiber concentrations are most commonly reported as acid detergent fiber (ADF). However, not all studies report exact details of the procedures (e.g. whether ADF is analyzed sequentially following isolation of neutral detergent fiber (NDF)). In addition, most studies do not specify whether ADF is reported on an ash-free basis or corrections are made for residual dry matter. Furthermore, there is little appreciation in primate literature that fiber residues can be contaminated with tannin-protein complexes [Wallis et al., 2012]. All these factors can contribute to unknown errors in the reported ADF concentrations, but how significant they are in different studies is hard to gauge and it is not possible to apply a consistent correction factor to compensate for methodological differences. We emphasize the need for rigorous analysis to avoid these uncertainties [Rothman et al., 2012]. As a result, the accuracy of the “ADF” data is likely to be low and conclusions derived from fiber concentrations should be considered with these limitations in mind.

All as yet unpublished chemical analyses were carried out in the laboratory of the University of Hamburg [Donati et al., 2007] (Table I). All results are expressed as % of dry matter.

Quality of Leaves Available in Different Forests (“representative samples”)

Most measures of the availability of protein and leaf quality in different forests (here termed “representative samples”) are based on mature tree leaves. Leaves were collected opportunistically or from the most abundant tree species and were assumed to represent a proxy for year-round leaf quality [e.g., Oates et al., 1990; Ganzhorn, 1992; Chapman et al., 2002, 2004; Wasserman & Chapman, 2003; Simmen et al., 2014]. The representative samples for *Semnopithecus schistaceus* in
Ganzhorn et al.,: Protein in leaves

249 Ramnagar (Nepal) are based on mature leaves of the 25 most abundant tree species [Chalise 1995; Chalise & Koenig, unpubl.] and for Propithecus edwardsi in
250 Ranomafana (Madagascar) on 14 tree species sampled haphazardly [Wright &
251 Daniels, unpubl.].
252
253 Some studies collected separate representative samples for young and
254 mature leaves [Mowry et al., 1996; Liu et al., 2013] or separate samples for the wet
255 and the dry season [Ganzhorn, 2002]. These samples were considered as
256 independent data points and were entered in the analyses as independent units. Our
257 rationale is that we wanted to have some measure of leaf nutritional quality in
258 samples of leaves that we could use for the analyses of selection of leaves
259 consumed as food against this representative sample (see “Selection Criteria for
260 Consumed Leaves” below).
261
262 Selection Criteria for Consumed Leaves
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264 Determination of the significance of selection for specific chemical
265 components was restricted to photosynthetic parts (leaves, sometimes differentiated
266 in different parts of leaves). Analyses of selection were always restricted to the same
267 types of plant parts because we wanted to know when selection occurs with respect
268 to the representative sample. For example; if the representative sample consisted of
269 mature leaves, then only food items consisting of mature leaves were considered. If
270 the representative sample consisted of young leaves, then only young leaf food items
271 were considered. If the representative sample consisted of mature leaves and the
272 animals were feeding only on young leaves, no comparison was calculated.
273
274 The data for Propithecus coronatus are based on the early dry season.
275 During this time of the year, the diet consisted of 85-90% leaves. The chemical
analyses were based on a reconstructed diet, made by mixing aliquot proportions of each food species consumed according to its dietary proportion [Pichon, 2012].

Selection criteria were taken from the original paper, or leaves that were consumed were compared with representative samples from the forest, or concentrations of chemical components were correlated with the frequency of consumption (assumed to represent the amount of leaf material ingested). Thus, p-values listed in Table I and Figure 1 are based on t-tests between samples of material consumed versus the representative sample or on correlations between the frequency of consumption and the concentration of the chemical component in question.

Statistical Analyses

Published data are based on the analysis of a single individual per plant species or averages based on several different individuals of the same plant species or on averages weighted by the frequency of abundance or the frequency of consumption. When possible, we base our analyses on unweighted means of plant species. Surprisingly, and despite the known temporal and inter-individual variation within plant species [Ganzhorn & Wright 1994; Chapman et al., 2003], the variation between weighted and unweighted samples seems to average out in large samples (Table II). Statistical tests were made with SPSS 21.0.

RESULTS

Selection of Leaves in Relation to the Average Concentrations of Nitrogen, Soluble Protein or ADF in a Given Forest
Measures of nitrogen, soluble protein and ADF in representative samples of plant leaves were found for 19, 18 and 33 studies, respectively (Table I). Concentrations of the same components in food plants were found for 35, 22 and 41 studies. The data for soluble protein were unevenly distributed in the dataset, and were mainly available for foods of lemurs. Studies of the same species in different areas or during different times of the year were treated as independent units since the concentrations of chemical components vary significantly between sites and seasons.

Selectivity for leaves containing high concentrations of nitrogen increased significantly with declining nitrogen concentrations in forests ($r_s = 0.62$, $P = 0.008$, $N = 17$; Figure 1; Table I). Restricting the correlation to the Colobinae does not alter the principal result but removes significance ($r_s = 0.51$, $P = 0.075$, $N = 13$).

For soluble protein data, selection of high protein leaves was stronger in forests with low concentrations of soluble protein in representative samples of leaves than in forests with high concentrations ($r_s = 0.66$, $P = 0.004$, $N = 17$). Removing *Semnopithecus schistaceus* from the correlation (the only species for which soluble protein data are available for representative samples of leaves outside Madagascar; thus restricting it to lemurs) does not change the result ($r_s = 0.66$, $P = 0.005$, $N = 16$).

Combining the data for the two measures of protein and including the type of protein analysis as a random categorical variable in a GLMM results in a highly significant effect of the concentrations of protein in representative samples of leaves on the strength (significance) of selection ($F = 21.58$; $P < 0.001$).

There was no relationship between concentrations of nitrogen or soluble protein and fiber in the data set. There were no significant correlations between the selection (or rather discrimination) against ADF and the ADF in representative
samples, either over all the data ($r_s = 0.12, P = 0.534, N = 31$), or when considering various primate radiations separately.

DISCUSSION

The present analysis sought to better understand the discrepancy between the findings of some studies that identify protein as a limiting resource, including those that focus on non-human primates [Kay 1984] and others that find no evidence for this phenomenon. Primates (and animals in general) need to satisfy their protein needs by selecting protein-rich food, but we found that many primatological studies failed to demonstrate such a selection for high protein food (Table I). A number of studies have pointed out that selection of high protein food would only be required if the food items in the environment have average protein concentrations below the required needs [e.g., Mowry et al., 1996; Yeager et al., 1997; Ganzhorn et al., 2009; Simmen et al., 2014] and that, once protein concentrations are above requirements, selection could be based on other components and criteria, such as the availability within the environment [e.g., Oftedal, 1991; Fashing et al., 2007] or secondary plant chemicals [Moore & Foley 2005] or minerals such as sodium [Rothman et al 2006]. While this idea has been around for some time, it has rarely been tested [Marsh et al., 2014; Jensen et al., 2015]. Studies started to focus instead on long-term nutrient budgets and nutrient balancing using the conceptual approach of geometric frameworks [e.g., Felton et al., 2009; Rothman et al., 2011; Johnson et al., 2013; DeGabriel et al., 2014; Irwin et al., 2014], on new methods on how to measure protein that is actually available [DeGabriel et al., 2008], or on an understanding of other confounding variables [Wallis et al., 2012]. Our results illustrate that primates
select for high protein leaves especially in situations where the average protein content of leaves in a forest is low. No such correlation was found with respect to fiber concentrations. Thus, it appears that protein is limiting for folivorous primates under certain conditions, but clearly not in the majority of tropical forests studied. In contrast, we found no evidence of either an expected inverse relationship between protein and fiber concentrations in food or indeed any evidence that animals were selecting against fiber. We cannot judge whether there is a significant effect of methodology on this result but it is clear that fiber is analyzed inconsistently in primatological studies with little regard to the effects of ash, tannins or other interfering substances [Makkar & Singh 1995; Wallis et al., 2012].

Our comparative study also indicates a fundamental problem of field studies on food selection. Animals are most frequently studied where they occur in high densities. These are probably the best areas for survival and reproduction with high quality food availability. Under these conditions, it is probably hard, if not impossible, to identify factors that are actually limiting. Having enjoyed considerable time in forests with plentiful animals, it may be an unfortunate conclusion, but in order to find out what limits primates, researchers will likely need to turn their attention to regions where animals are naturally scarce (e.g. Stalenberg 2015).

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Ganzhorn et al.,: Protein in leaves

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We confirm that the research adhered to the legal requirements of the country in which the research was conducted and that this research adhered to the American Society of Primatologists (ASP) Principles for the Ethical Treatment of Non-Human Primates.

References


Ganzhorn et al.: Protein in leaves


Tables

**TABLE I.** Nitrogen, soluble protein and acid detergent fiber (ADF) concentrations in leaves eaten by folivorous primates and in “representative samples” of leaves (RS) in a given forest. “P” indicates significance of selection for high protein or low ADF concentrations.

**TABLE II.** Comparison of the concentration of chemical components in leaves based on measures of several individuals of the same plant species and on the mean per plant species. Values are means ± standard deviations; N = sample size. Data on *Propithecus edwardsi* from Arrigo-Nelson (2006; unpubl.) based on mature leaves; data on *P. candidus* from Patel [2012; unpubl.], restricted to leaves of species identified unambiguously.
Fig. 1. Significance values for the selection of leaves in relation to the average concentrations of nitrogen (upper graph), soluble protein (middle graph) and ADF (lower graph) in leaves available in different forests. Dots are lemurs, squares are Old World Monkeys (Colobinae) and triangles are New World monkeys (Alouatta spp.).