

Fluctuations in daily energy intake do not cause physiological stress in a Neotropical primate living in a seasonal forest

Rodolfo Martínez-Mota¹ · Nicoletta Righini^{1,2}  · Rupert Palme³

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Abstract Animals may face periods of nutritional stress due to short-term food shortage and/or low energy consumption associated with seasonal fluctuations in resource availability. We tested the hypothesis that periods of restricted macronutrient and energy intake result in energy deficits and physiological stress in wild black howler monkeys (*Alouatta pigra*) inhabiting seasonal tropical semi-deciduous forests. We conducted full-day follows of focal animals recording feeding rates, time spent feeding, and total amount of food ingested. We carried out nutritional analysis of foods collected from feeding trees and calculated the daily nutrient and energy intake of each focal individual. Fecal glucocorticoid metabolites (fGCM) of focal animals were used as an indicator of physiological stress. We found that fluctuations in daily energy intake across seasons did not have significant effects on fGCM of

individuals. However, protein intake was negatively associated with fGCM, highlighting the interplay among macronutrients, metabolism, and the endocrine system. Fecal glucocorticoid metabolites were also positively related to fruit availability, but this relationship was most likely due to social stress associated with intergroup encounters and resource defense that occurred when preferred trees were fruiting. Behavioral strategies such as dietary shifts and nutrient mixing, and metabolic adaptations such as low energy expenditure allowed individuals to fulfill their minimum energy requirements even during periods of decreased resource availability and intake. The present study suggests that seasonal variations in food, macronutrient, and energy acquisition may have limited physiological costs for animals that exploit different types of plant resources such as howler monkeys.

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✉ Nicoletta Righini
righini2@gmail.com
Rodolfo Martínez-Mota
rmarti39@illinois.edu
Rupert Palme
Rupert.Palme@vetmeduni.ac.at

¹ Department of Anthropology, University of Illinois at Urbana-Champaign, Champaign, USA

² Red de Manejo Biorracional de Plagas y Vectores, Instituto de Ecología, A.C, Xalapa, Veracruz, Mexico

³ Department of Biomedical Sciences, University of Veterinary Medicine, Vienna, Austria

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Introduction

In recent years, there has been increased research on how the ecological characteristics of changing environments affect the physiological response of organisms, and, in particular, how physiology mediates the responses of individuals to the spatial, temporal, and nutritional heterogeneity of the environment (Raubenheimer et al. 2012). Seasonal fluctuations in food availability represent a major challenge to animals that must fulfill their nutritional demands, especially during periods of food scarcity (Wingfield 2013). Failure to consume sufficient nutrients during these periods may result in nutritional stress, defined as a nutrient and energy imbalance (Jeanniard du Dot et al. 2009), and

ultimately in reduced body mass and changes in stress-induced endocrine responses (Kitaysky et al. 1999; Jeanniard du Dot et al. 2009; Stetz et al. 2013).

Malnutrition can affect the health of individuals and have a negative impact on reproductive processes (e.g., ovulation inhibition, impaired embryo implantation, increased inter-birth intervals) and fitness (Schneider 2004; Schoech et al. 2004; Lujan et al. 2005; Bonier et al. 2009). For example, in seasonal environments, a decrease in food abundance and in nutrient and water availability during the dry season (or food shortage and harsh weather in the winter) has been often related to a decline in body condition, nutritional stress, and an increase in glucocorticoid concentrations in mammals such as African elephants (*Loxodonta africana*) (Foley et al. 2001; Woolley et al. 2009), Pyrenean chamois (*Rupicapra pyrenaica pyrenaica*) (Dalmau et al. 2007), red deer (*Cervus elaphus*) (Huber et al. 2003), and short-tailed fruit bats (*Carollia perspicillata*) (Lewanzik et al. 2012). As part of the animal's physiological stress response, the activation of the hypothalamic–pituitary–adrenal (HPA) axis triggered by stressful stimuli results in the release of glucocorticoid hormones, which, if produced in excess and during prolonged periods of time, have deleterious effects such as suppression of the immune and reproductive systems (Sapolsky 2002).

Despite the fact that the tropics are characterized by limited fluctuations in temperature and day length (van Schaik et al. 1993; Moran 2012), seasonal variation in rainfall can be conspicuous, leading to changes in plant phenology, fruiting patterns, and vegetation structure throughout the year (Milton et al. 2005; van Schaik and Brockman 2005). Deciduous tropical forests, in particular, show dramatic seasonal changes in canopy cover (Murphy and Lugo 1986), resulting in periods of lack of foliage (or lack of preferred items such as young leaves) that are likely to affect the behavior and physiology of folivores (Harris et al. 2010). Therefore, tropical forest dwellers including primates may face times of food shortage when resource availability decreases (Hemingway and Bynum 2005; van Schaik and Pfannes 2005). During these periods, dietary shifts are common (van Schaik et al. 1993; Marshall et al. 2009; Lambert and Rothman 2015), but they can lead to changes in the forager's energetic condition (i.e., availability of metabolic fuel in the body) if the diet becomes restricted or nutritionally imbalanced. In this regard, positive relationships between the consumption of energy-rich foods such as mature fruits and levels of energetic biomarkers (i.e., C-peptide of insulin) have been found in free-ranging and captive primates (macaques, Girard-Buttoz et al. 2011; orangutans, Emery Thompson and Knott 2008; and chimpanzees, Emery Thompson et al. 2009).

Several studies have provided valuable insights into the relationship between nutrition and the endocrine system

in free-ranging primates (Table S1). Assessing nutritional condition and energy balance (intake and expenditure) in wild animals is a complex task. Therefore, researchers have used indirect methods to quantify the effects of variation in food intake on physiological stress reactions. For example, out of the 23 primate studies reported in Table S1, 16 used different ecological variables (e.g., fruit availability, fruit intake, percentage of feeding observations on fruits, feeding time, or seasonality) as proxies of energy intake or nutritional status. These studies suggest that fluctuations in food intake and availability have an impact on the nutritional condition of individuals (Muller and Wrangham 2004; Foerster and Monfort 2010). However, more direct measures of nutrient intake would contribute to improve our understanding of primate nutritional ecology and endocrinology. Building on these studies, the present research contributes to the advance of this field by examining the effects of seasonal energy and nutrient intake on the stress response of black howler monkeys (*Alouatta pigra*) living in a seasonal semi-deciduous forest in Mexico. To accomplish this, we estimated daily food ingestion, daily nutrient and energy intake, and assessed noninvasively the physiological stress response in relation to nutrition through measurements of fecal glucocorticoid metabolites (fGCM).

Alouatta is traditionally considered as the most folivorous primate genus of the Neotropics; however, these primates include large proportions of fruits in their diet when available, as well as young and mature leaves (Amato and Garber 2014; Righini et al. 2016). The fact that the diet of black howler monkeys is mainly leaf-based during some periods of the year and mainly fruit-based during others (Amato and Garber 2014), makes them a suitable model for testing the effects of nutrient and energy intake on physiological stress in both folivores and frugivores. We first explored the hypothesis that food (fruits and leaves) availability correlates with individual energetic status and physiological stress of black howler monkeys: (1) given that availability of food items in the environment has been used as surrogate of an individual's energy intake, we expected a positive relationship between energy intake and food availability indices. Since fruits are usually characterized by high energy content, we expected energy intake to be lower during periods of low fruit availability. (2) We also tested if decreased food availability predicts the physiological stress of black howler monkeys; thus, we expected a negative association between fGCM and food availability indices. Finally, to address more precisely the hypothesis that periods of lower energy and nutrient intake result in increased physiological stress, (3) we examined the relationship between fGCM and changes in direct measures of individual energy and macronutrient intake (i.e., protein and non-protein energy). We expected fGCM to be lower

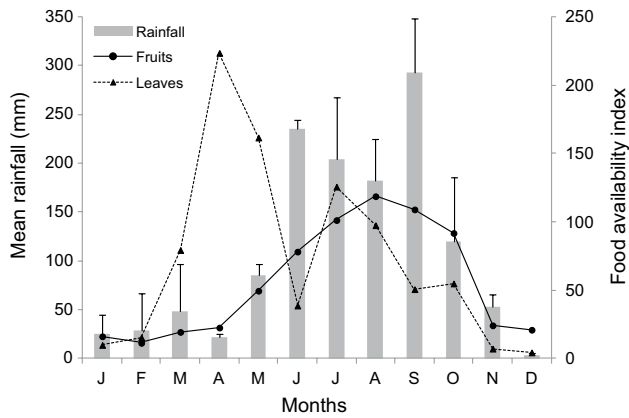


Fig. 1 Monthly rainfall (mean \pm SD) and estimations of fruit (filled circles) and young leaf (filled triangles) availability in a tropical semi-deciduous forest at Escárcega, State of Campeche, Mexico. Bars represent rainfall data recorded from 2010 to 2012

during periods of higher intake of lipids, carbohydrates, and total energy.

Materials and methods

Study site and subjects

This study took place in the Yucatan Peninsula at Escárcega, State of Campeche, Mexico ($18^{\circ}36'44''\text{N}$, $90^{\circ}48'31''\text{W}$). In this area, the dominant vegetation type is tropical semi-deciduous forest. The climate is hot and humid, and is characterized by three seasonal periods that correspond to major changes in food availability: a dry and hot season from February to May (~ 100 mm rain during 4 months, 28.0°C mean daily temperature), a rainy season from June to September (~ 900 mm rain during 4 months, 27.5°C mean daily temperature), and a cooler transitional season (from here on, “nortes”) from October to January (~ 160 mm rain during 4 months, 22.8°C mean daily temperature; Fig. 1).

We studied two groups of black howler monkeys over the course of 14 months (from September 2010 to October 2011, 1300 observation hours). These groups inhabited a 2,100-ha area of semi-deciduous tropical forest owned by Instituto Nacional de Investigaciones Forestales, Agrícolas y Pecuarias (INIFAP). One study group (M) contained 10–12 individuals, including 4–5 adult males and two adult females, and the other (J) contained 6–7 individuals, including 2–3 adult males and two adult females (Table 1). We individually recognized howler monkeys through facial features, scars, and broken digits, and ten adult individuals were darted and marked with color anklets in August 2010, prior to the start of the behavioral data collection. Home

ranges of group M and J were 14.5 and 4.5 ha, respectively, with an overlap of 1.3 ha.

Behavioral data collection

We conducted continuous observations of one adult individual per day, alternating between individuals and obtaining at least two complete focal samples per individual per month. For this study, we included 97 full-day focal samples of nine males ($N = 49$) and four females ($N = 48$) based on the following criteria: (1) days in which we could successfully follow the focal animal for 8–12 consecutive hours without it being out of sight for more than 10 min; (2) days in which we could record all feeding bouts in detail; and (3) days in which we collected fecal samples of the focal animal for fGCM analysis. We used a two-minute instantaneous sampling method on focal animals (Martin and Bateson 2007) to record activities such as feeding, resting, traveling, and social interactions (vocalizations, howling bouts, aggression, play, sexual interactions, affiliative behavior such as grooming). Also, when the focal animal started feeding (i.e., ingesting food items), we temporarily switched to a method of continuous data collection (Martin and Bateson 2007) to record the duration and characteristics of all feeding events. The latter included the species and phenophase (i.e., young/mature leaf, immature/mature fruit, flower/inflorescence) of the items ingested, and feeding rates estimated by counting the number of ingested items, the part(s) of the item, or the bites per minute. Counting the bites per minute and estimating bite weight was mainly necessary for a few species (e.g., *Manilkara zapota* and *Mangifera indica*), since the majority of the fruits eaten were small, and several fruits were ingested at the same time. Once the feeding bout ended, we resumed instantaneous focal animal sampling at 2-minute intervals. For each plant species consumed, we collected ten samples of each food item, weighed (fresh and dry weight) the entire samples or parts of them according to the behavioral observations, and used the average dry weight to calculate the amount of food consumed per feeding bout.

Phenology and food availability

We conducted bimonthly phenological censuses to monitor the presence and abundance of young/mature leaves, immature/mature fruits, and flowers in the home ranges of the two study groups. We carried out two types of censuses: (1) we walked 10 Gentry’s belt transects (50×2 m each; Gentry 1988) in each home range bimonthly ($N = 196$ trees); (2) we monitored five trees/species for each of the 10 most important tree species in the howler’s monthly diet. In both cases, we estimated the percentage of the crown containing food items for each monitored tree on a scale ranging

Table 1 Composition of the two study groups (J and M), including data on body weight and body mass index of 10 darted and marked adult individuals (*Alouatta pigra*)

Group	ID	Age	Sex	Body weight (kg)	BMI ^a (kg m ⁻²)	<i>N</i>	Entered group	Left group
J	MA	Adult	Male	6.9	28.2	3		Mar 2011
J	MR ^b	Adult	Male	8.7	26.8			Mar 2011
J	CI	Adult	Male			3	Mar 2011	May 2011
J	TO	Adult	Male	7.5	23.1	6	Apr 2011	
J	AM	Adult	Male			3	Apr 2011	
J	CO	Adult	Female			14		
J	GO	Adult	Female			11		
J	MI	Juvenile	Male					Sept 2011
J	JJ	Infant	Male					
M	AZU	Adult	Male	8.4	36.5	10		
M	AMA	Adult	Male	7.9	30.4	2		Mar 2011
M	RBL	Adult	Male	7.7	25.5	8		
M	NAR	Adult	Male	5.7	25.8	11		
M	RAM	Adult	Male	7.9	27.6	3		
M	PAN	Adult	Female	5.8	29.9	11		
M	ROJ	Adult	Female	6.3	24.7	12		
M	AUG	Juvenile	Male					
M	JOR	Infant	Female					
M	IMC	Infant	Male					
M	NAT	Infant	Female					
M	VAL	Infant	Male					

N number of full-day follows of focal animals

^a Body mass index (BMI) was calculated as body weight (kg) divided by the square of crown-rump length (m²)

^b Peripheral male even before leaving the group definitively. Focal samples of this male were not included in the analysis

from 0 to 4 (0; 1 = 1–25 %; 2 = 26–50 %; 3 = 51–75 %; 4 = 76–100 %) (Chapman et al. 2005; Dröscher and Kappeler 2014). To measure tree abundance and calculate basal area of the monitored species, we established six randomly placed 50 × 50 m quadrats and 33 additional Gentry's transects in which we tagged and identified all trees with diameter at breast height (DBH) ≥ 10 cm. This ecological sampling covered 12–25 % of the home ranges of the two study groups. We calculated a food availability index (FAI) for each phenophase of a given species, multiplying the average bimonthly phenology score by its total basal area. We then summed FAIs across items and averaged those values across the two bimonthly surveys to obtain total monthly FAI for young and mature leaves, mature and immature fruits, and flowers (Foerster et al. 2012).

Plant sample collection and nutritional analyses

We marked, measured DBH, and taxonomically identified all feeding trees (*N* = 689) visited by the focal animals. We collected food items from feeding trees either on the same day or within 2 days of the observed feeding bout. We then

air-dried the leaves, flowers, and inflorescences at a constant weight in a dark area in the field lab. We kept fruits in a drying oven (~50 °C) until they reached a stable dry weight.

We conducted nutritional analyses of foods collected from feeding trees (*N* = 127 samples) at Hunter College Nutritional Ecology Laboratory, CUNY. We analyzed all samples in duplicate. We calculated dry matter by drying the field-dried samples in an oven (105 °C) before each analysis to remove atmospheric moisture (Rothman et al. 2012). We determined total nitrogen (N) via combustion using a LECO TruSpec Nitrogen Analyzer (LECO Corporation, St. Joseph, MI, USA). We calculated crude protein (CP) by multiplying N by 6.25 (Rothman et al. 2012). To take into account nitrogen bound to fiber and secondary metabolites, we estimated available protein (AP) as follows: (1) to determine the amount of fiber-bound nitrogen, we subtracted acid detergent insoluble nitrogen (ADIN) from N, multiplied the result by 6.25, and then subtracted it from CP (Rothman et al. 2012); (2) to estimate the effect of tannins on N digestibility, we measured the binding capacity of polyethylene glycol (PEG, a tannin-blocking agent) in

a subset of plant samples [$N = 42$, those that were positive for the qualitative acid-butanol assay for condensed tannins (Waterman and Mole 1994)], followed by in vitro digestion with pepsin and cellulase (DeGabriel et al. 2008). We determined lipids by extraction with petroleum ether with an XT15 Fat Analyzer (ANKOM, Macedon, NY, USA). We determined, sequentially, neutral detergent fiber (NDF), acid detergent fiber (ADF), and acid detergent lignin (ADL) using filter bags in an ANKOM 200/220 Fiber Analyzer. We calculated total nonstructural carbohydrates (TNC) by subtraction: $\%TNC = 100 - \%NDF - \%lipids - \%AP - \%ash$ (Rothman et al. 2012). We calculated the metabolizable energy content of plant samples using standard conversion factors: 16 kJ g⁻¹ for TNC, 17 kJ g⁻¹ for AP, 37 kJ g⁻¹ for lipids (Conklin-Brittain et al. 2006), and 5.7 kJ g⁻¹ for NDF (Righini et al. 2016).

Calculation of nutrient intake

To calculate the daily amount of food ingested (g dry weight) by each focal animal, we multiplied the feeding bout length on each food item (minutes) by the corresponding feeding rate (g dry weight min⁻¹), and then summed all daily feeding events (Felton et al. 2009a; Rothman et al. 2012). To estimate daily nutrient intake, we multiplied the nutrient content of each food item by the estimated amount of items ingested (g dry weight) in each feeding bout. Then, we obtained daily values of AP intake (converted to kilojoule equivalents), non-protein energy (sum of the intake of TNC, lipids, and NDF, converted to kilojoule equivalents), and total energy (sum of AP and non-protein energy) for each focal animal. To take into account the possible effects of different body weights on nutrient and energy intake in male and female howler monkeys, we divided each value of daily nutrient and energy intake by the body mass (kg) of the corresponding focal animal. We obtained body mass values for nine of the 14 focal animals during the darting and marking phase of this project (adult males: 7.6 kg \pm 0.9 SD, adult females: 6.0 kg \pm 0.3 SD). For those individuals whose weights were not available ($N = 4$), we used data on mean body weight of adult male and female *A. pigra* from Kelaita et al. (2011), which were based on a larger sample size (37 males, 32 females).

Fecal sample collection

We collected 258 fresh fecal samples from recognized howler monkeys (mean number of samples per individual: 15.5 \pm 5.2 SD; range = 6–22). However, for this study, we selected fecal samples that corresponded to the days of feeding data collection ($N = 97$). Fecal samples were

collected throughout the day (from 0600 to 1800 h). Immediately after fecal deposition, we stored approximately 5 g of thoroughly mixed feces in 20-mL tubes, labeled with the donor's ID, hour and date of collection, and kept in a cooler with frozen gels until transport to the field laboratory. Samples were frozen for 3–5 days until we performed glucocorticoid extractions. Circulating glucocorticoid concentrations show circadian variations, and this effect may be reflected in glucocorticoid metabolites excreted in the feces of certain primates (e.g., *Callithrix jacchus*, Sousa and Ziegler 1998). Thus, before the start of this research, we collected fecal samples ($N = 38$) from 10 individuals both in the morning (0600–1200 h) and in the afternoon (1230–1700 h) to test for the effects of collection time on fGCM. There were no significant differences in fGCM between these two collection periods, indicating that fGCM were not affected by the diurnal rhythm (average morning: 108 ng g⁻¹ \pm 82 SE, average afternoon: 104 ng g⁻¹ \pm 67 SE; likelihood ratio test: $\chi^2 = 0.03$, $df = 1$, $P = 0.86$).

Extraction and analysis of fecal glucocorticoid metabolites

In the field laboratory, we extracted glucocorticoid metabolites from the fecal material following the technique reported in Palme et al. (2013). Briefly, we added 5 ml of 80 % methanol to each fecal sample (0.5 g wet weight). This suspension was vortex-mixed for 10 min, and then centrifuged at 1600g for 20 min. We diluted 1 ml of the supernatant (i.e., hormone extract) in 9 ml of distilled water, and subsequently, we slowly passed this 10 ml diluted extract through a solid-phase extraction (SPE) cartridge (MaxiClean Prevail C18 SPE Cartridges Alltech®). We air-dried SPE cartridges after having been loaded with the steroids extracts and stored in a refrigerator until they were transported for analyses to the Department of Biomedical Sciences/Biochemistry at the University of Veterinary Medicine, Vienna, Austria.

In the laboratory, we eluted steroid extracts from the SPE cartridges using methanol. We measured fGCM with a group-specific 11-oxo-etiocholanolone enzyme immunoassay (EIA) that measured glucocorticoid metabolites with a 5 β -3 α -ol-11-one structure (Möstl et al. 2002). This EIA has been successfully validated to monitor adrenocortical activity in black howler monkeys (Martínez-Mota et al. 2008). Detailed cross-reactivities are reported elsewhere (Möstl et al. 2002). Sensitivity was 5 ng g⁻¹ feces. Intra- and inter-assay coefficients of variation for high and low concentration pool samples were 2.6 and 2.9 % (intra), and 9.7 and 12.5 % (inter), respectively. Concentrations are reported in ng g⁻¹ wet weight.

Table 2 Mean values (\pm SE) of food availability indices (FAI), daily amount of food ingested (grams of dry weight), nutrient and energy intake, and fecal glucocorticoid metabolites (fGCM) of black howler monkeys across three seasons

	Rainy	<i>Nortes</i> ^a	Dry
FAI young leaves	86 \pm 6	50 \pm 3	143 \pm 14
FAI fruits	103 \pm 5	63 \pm 10	25 \pm 3
Young leaves ingested (g)	71 \pm 9	87 \pm 16	89 \pm 9
Fruits ingested (g)	138 \pm 14	84 \pm 13	119 \pm 15
Protein intake (kJ kg ⁻¹)	69 \pm 5	62 \pm 7	61 \pm 5
Non-protein intake (kJ kg ⁻¹)	343 \pm 28	278 \pm 30	340 \pm 25
Daily energy intake (kJ kg ⁻¹)	412 \pm 32	339 \pm 34	401 \pm 29
fGCM (ng g ⁻¹)	930 \pm 121	554 \pm 167	475 \pm 123

^a Refers to a cooler transitional period from October to January, characterized by lower temperature and precipitation compared to the rainy season

Data analysis

We analyzed all data with linear mixed-effects models (LMM's). Before running the models, we inspected the distribution of response variables (i.e., energy intake and fGCM), and after this, energy intake was square root-transformed and fGCM was log-transformed to achieve a normal distribution. We started examining the effects of food availability on energy intake and on the stress response of howler monkeys. First, we created a model (LMM1) that included availability indices of fruits (mature and immature) and young leaves, and seasonality (dry season, rainy season, *nortes* season) as predictors, and square root-transformed energy intake as the response variable. We included seasonality in the model to assess if energy intake changed over time, and included individual reproductive status (nonpregnant, pregnant, lactating, and male) as a covariate to control for this potential effect. Second, we created a model (LMM2) with availability indices of fruits and young leaves as predictor variables, and log-transformed fGCM as the response variable. In this model, reproductive status was also included as covariate since pregnancy and lactation may have effects on excretion of fGCM. Given that mature leaf availability was always higher than young leaf availability across seasons, it was not included in the model.

We then examined whether fluctuations in energy and macronutrient intake directly affected the stress response of black howler monkeys. To accomplish this, we created a model (LMM3) that included energy intake and seasonality as predictor variables and log-transformed fGCM as the response variable. In a separate model (LMM4), we tested the effects of protein and non-protein intake (predictors) on log-transformed fGCM (response variable).

Given that samples were collected from the same individuals over time, in all models the individual ID was set as a random factor (Crawley 2007). We visually inspected Q–Q plots and scatter plots of residuals plotted against predicted values to assess normality and homogeneity of models. For each set of analyses, we ran a likelihood ratio test comparing the fit of the full model (model with predictor variables) with a null model that included only the intercept. We performed a multiple comparison test to detect significant differences among levels of the categorical variable seasonality. We ran all the analyses using the package lmerTest (Kuznetsova et al. 2014) in the R statistical software (version 3.2.4).

Results

Food availability and food intake

We found that fruit availability was higher during the rainy season (FAI = 103 \pm 5 SE) than in the *nortes* (FAI = 63 \pm 10 SE) and dry (FAI = 25 \pm 3 SE) seasons. In contrast, the availability of young leaves was higher during the dry season (FAI = 143 \pm 14 SE) compared to the rainy (FAI = 86 \pm 6 SE) and *nortes* (FAI = 50 \pm 3 SE) seasons (Fig. 1). Fruit intake was similar during the rainy and dry seasons, but it was reduced by 40 % during the *nortes* season (Table 2). Despite the fact that the availability of young leaves peaked during the dry season, black howler monkeys showed a relatively constant consumption of young leaves across the three different periods (Table 2).

Effects of food availability indices on energy intake and fecal glucocorticoid metabolites

Each full model and the corresponding likelihood ratio test results are shown in Table 3. The first model (LMM1) showed that neither the availability of fruits and young leaves nor seasonality affected the energy intake of black howler monkeys (Table 3). Energy intake showed limited variation between the rainy and dry seasons, and only during the *nortes* season, howler monkeys reduced their energy intake by ~15–18 % compared to the other seasons (412 kJ kg⁻¹ \pm 32 SE in the rainy season, 339 kJ kg⁻¹ \pm 34 SE in the *nortes* season, and 401 kJ kg⁻¹ \pm 29 SE in the dry season). Individual reproductive status did not have significant effects on energy intake (Table 3). The second model (LMM2) revealed that the fruit availability index positively predicted fGCM (Table 4; β = 0.02, SE = 0.00, t = 6.39, P < 0.0001). fGCM increased with an increase in the availability of fruits (Fig. 2). In contrast, the availability of young leaves did not have a significant effect on

Table 3 Hypotheses tested, full models, and results of the likelihood ratio test for each model

Hypothesis tested	Full model	Likelihood ratio test
Food availability correlates with daily energy intake and predicts physiological stress	LMM1: energy intake ~ FAI fruits + FAI young leaves + season + reproductive status	$\chi^2 = 11.2, df = 7, P = 0.12$
	LMM2: fGCM ~ FAI fruits + FAI young leaves + reproductive status	$\chi^2 = 44.1, df = 5, P < 0.0001$
Limitations in daily energy intake and seasonality affect physiological stress	LMM3: fGCM ~ energy intake + season	$\chi^2 = 15.5, df = 3, P < 0.01$
Limitations in nutrient intake affect physiological stress	LMM4: fGCM ~ protein + non-protein	$\chi^2 = 6.9, df = 2, P < 0.05$

Variables in bold were significant predictors

LMM linear mixed-effects model, fGCM fecal glucocorticoid metabolites, FAI food availability index

Table 4 Results of the linear mixed-effects models examining the effects of food availability, seasonality, reproductive status, energy intake, and nutrient intake on fecal glucocorticoid metabolite levels of black howler monkeys at Escárcega, Mexico

Model	Predictor	β	SE	<i>T</i>	<i>P</i>
LMM2	Intercept	4.39	0.46	9.60	<0.0001
	FAI fruits	0.02	0.00	6.39	<0.0001
	FAI young leaves	0.00	0.00	1.40	0.16
	Reproductive status (nonpregnant)	0.11	0.46	0.24	0.80
	Reproductive status (pregnant)	0.16	0.45	0.35	0.72
	Status (males)	0.47	0.45	1.04	0.32
LMM3	Intercept	5.88	0.34	17.35	<0.0001
	Energy intake	-0.00	0.00	-1.45	0.15
	Season <i>nortes</i>	0.04	0.32	0.12	0.90
	Season rainy	0.84	0.24	3.56	<0.001
LMM4	Intercept	7.85	1.48	5.32	<0.0001
	Protein intake	-0.68	0.29	-2.31	<0.05
	Non-protein intake	0.14	0.33	0.43	0.67

Comparisons of reproductive status are done against the lactating stage

Season effects are compared with the dry season. *Nortes*: refers to a cooler transitional period from October to January, characterized by lower temperature and precipitation compared to the rainy season

LMM linear mixed-effects model, FAI food availability index

fGCM (Table 4). Reproductive status did not have significant effects on fGCM (Table 4).

Effects of energy intake and seasonality on fecal glucocorticoid metabolites

The third model (LMM3) indicated that the energy intake of black howler monkeys did not have significant effects on fGCM (Table 4). However, this analysis showed that seasonality predicted fGCM (Table 4). fGCM were higher during the rainy season (930 ng g⁻¹ ± 121 SE) than during

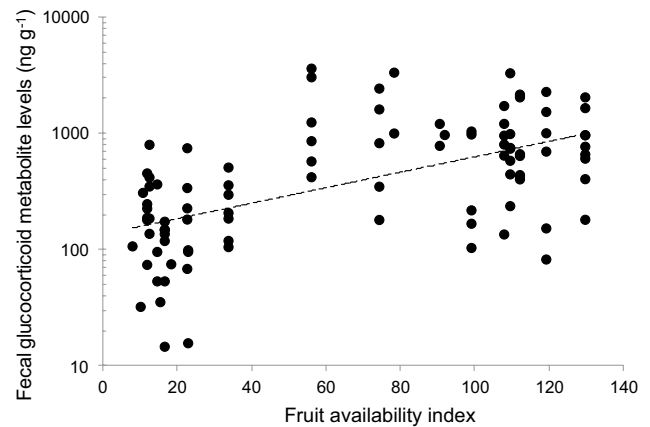


Fig. 2 Relationship between the fruit availability index and fecal glucocorticoid metabolite levels (ng g⁻¹) of black howler monkeys (*Alouatta pigra*). Black howler monkeys showed increased fecal glucocorticoid metabolite levels with an increase in the availability of fruits (*N* = 97, $\beta = 0.02, SE = 0.00, t = 6.39, P < 0.0001$). Trend line is shown only for visual purpose. Note log scale used on y-axis

the *nortes* (554 ng g⁻¹ ± 167 SE, multiple comparison test: $\beta = -0.8, SE = 0.30, t = -2.6, P < 0.01$) and dry seasons (475 ng g⁻¹ ± 123 SE, multiple comparison test: $\beta = -0.8, SE = 0.23, t = -3.6, P < 0.001$), but they did not differ between the dry and *nortes* seasons ($\beta = 0.0, SE = 0.32, t = -0.1, P = 0.90$).

Effects of nutrient intake on fecal glucocorticoid metabolites

In general, black howler monkeys maintained a similar protein intake across the three seasons (Table 2). Likewise, the intake of non-protein energy showed limited variation between the rainy and dry seasons, but it was reduced by 19 % during the *nortes* season (Table 2). However, the analysis (LMM4, Table 4) of the effects of macronutrients on physiological stress levels revealed that fGCM decreased with an increase in protein intake (Fig. 3; $\beta = -0.68,$

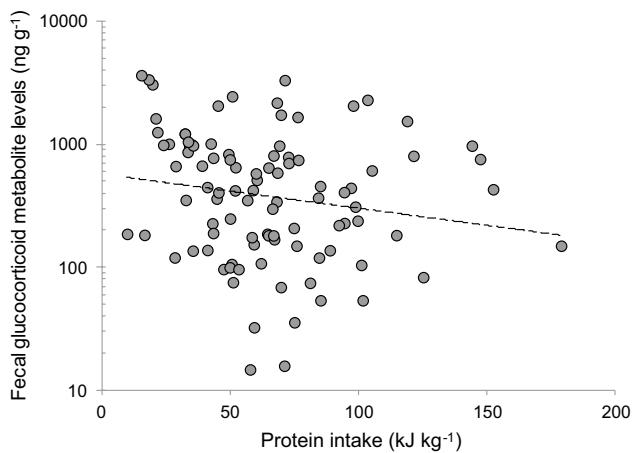


Fig. 3 Relationship between daily intake of protein (kJ kg^{-1}) and fecal glucocorticoid metabolite levels (ng g^{-1}) in black howler monkeys. Levels of fecal glucocorticoid metabolites decrease with an increase in protein intake ($N = 97$, $\beta = -0.68$, $\text{SE} = 0.29$, $t = -2.31$, $P = 0.023$). Trend line is shown only for visual purpose. Note log scale used on y-axis

$\text{SE} = 0.29$, $t = -2.31$, $P < 0.05$), but not in non-protein intake ($\beta = 0.14$, $\text{SE} = 0.33$, $t = 0.43$, $P = 0.67$).

Discussion

Restrictions in energy acquisition associated with periods of starvation and natural fluctuations in food intake may result in significant physiological costs for animals living in seasonal environments (Zhang et al. 2012; Burgess et al. 2013). Here, we examined whether seasonal reductions in food availability and in nutrient, energy, and food intake were associated with physiological stress in black howler monkeys that inhabit a semi-deciduous tropical forest. Although the production of fruits and young leaves fluctuated across the year, we found no statistically significant relationship between changes in the availability of food resources and energy intake. Our data, thus, do not support the hypothesis that food availability measures can be used as surrogate for black howler monkeys' energy intake. We also found that macronutrient and energy intake were relatively similar during the rainy and dry seasons. Only during the *nortes* season, a transitional period characterized by cooler temperatures and increased leaf fall, howler monkeys decreased the daily consumption of fruits (40 % decrease), non-protein intake (19 % decrease), and energy intake (15–18 % decrease). However, there were not significant effects of fluctuating energy intake on stress indicators (i.e., fGCM).

The above could be related to the fact that, despite the fluctuations, energy intake was relatively high throughout the year (averaging $\sim 400 \text{ kJ kg}^{-1}$) and higher than the

estimated energy requirements for howler monkeys (i.e., 355 kJ kg^{-1} , Nagy and Milton 1979). Moreover, according to a nutrient balancing strategy, individuals are expected to ingest foods that enable them to achieve (or approach to) a targeted proportion of nutrients, rather than maximizing the ingestion of any single nutrient or energy (Felton et al. 2009b; Simpson and Raubenheimer 2012). It also has been considered that regulating the ratio of nutrients ingested functions as a strategy for buffering the deficits and surpluses related to an imbalanced diet (Raubenheimer and Simpson 1993). Despite a reduction in energy intake during the *nortes*, the daily ratio of protein and non-protein energy ingested by our study animals across the year fluctuated less ($\text{CV} = 36 \%$) than protein intake ($\text{CV} = 50 \%$) and non-protein energy intake ($\text{CV} = 47 \%$) (Righini 2014), suggesting that howler monkeys selected foods in an attempt to maintain a relatively constant ratio. The latter did not seem to be a mere reflection of the nutrient ratios of the food themselves. In fact, the most consumed items in the annual diet, *Manilkara zapota* immature fruits, *Brosimum alicastrum* immature fruits, and *Acacia usumacintensis* mature leaves (Righini 2014), had protein/non-protein ratios of 0.01:1, 0.75:1, 0.66:1, respectively, which differed from the average intake ratio of 0.19:1. Overall, these data do not support the hypothesis that black howler monkeys inhabiting a seasonal semi-deciduous forest are more physiologically stressed when facing periods of decreased food resources and lower energy intake.

At our study site, the availability of food resources varied seasonally as expected in tropical deciduous forests (Murphy and Lugo 1986). Fruit production peaked during the rainy season and the availability of young leaves increased during the hot dry season, declining during the months of increased rainfall. Contrary to our expectations, individuals showed a significant increase in fGCM with increasing fruit availability, and during the rainy season compared to the drier periods of the year. The fact that the rise in fGCM occurred during the period characterized by increased fruit abundance and availability (i.e., rainy season), and given that energy intake was unrelated to fGCM, suggests that changes in the stress response of black howler monkeys were most likely related to social factors and not to energetic deficits, as has been found in spider monkeys (Rimbach et al. 2014). Despite the fact that black howler monkeys did not display overt within-group aggressions and were tolerant among members of the same group, they engaged in intergroup encounters characterized by short howling bouts (i.e., loud vocalizations displayed by several group members). This agonistic behavior is displayed as a way to expel intruders from ranging areas (Kitchen 2006). In our study, the rate of intergroup encounters per day was higher during the rainy season than in other seasons of the year (11 % increase), supporting the contention that fGCM

exhibited during the period of higher fruit abundance may reflect a type of social stress associated with the defense of preferred resources such as certain fruiting trees.

Among the most visited fruiting trees during the rainy season, there were *Brosimum alicastrum*, *Vitex gaumeri*, and *Metopium brownei* (Righini et al. 2016). Mature fruits found on these trees accounted for ~40 % of the seasonal diet of the two study groups (Righini et al. 2016). They were relatively large trees [mean DBH ranging from 29.5 (± 1.3 SE) cm to 59.7 (± 5.5 SE) cm] with an average density of 3.2 trees/ha per home range. However, their fruiting patterns were not completely synchronous, and the peaks of mature fruit availability on each tree occurred a few days or weeks apart. For this reason, several howler groups were at times gathering at the same trees foraging on mature fruits. All the above is suggesting that intergroup competition could have been present at least during the peak periods of mature fruit availability. These data agree with studies in primates such as ring-tailed lemurs (*Lemur catta*, Pride 2005) and spider monkeys (*Ateles hybridus*, Rimbach et al. 2014), and other animals including spotted hyenas (*Crocuta crocuta*, Benhaiem et al. 2013) and blue-footed boobies (*Sula nebouxii*, Nuñez-de la Mora et al. 1996), which show that agonistic interactions related to competition and defense over food resources mediate the stress response and glucocorticoid output among conspecifics.

Although daily protein intake was negatively related to fGCM, this relationship was relatively weak (Fig. 3). Other New World primate species such as spider monkeys are known to strictly regulate and prioritize daily protein intake (Felton et al. 2009a, c); thus, a reduction in its ingestion could possibly lead to physiological stress. However, in our study, available protein intake, on average, was higher ($3.8 \text{ g kg}^{-1} \pm 0.2 \text{ SE}$) (Righini 2014) than the estimated requirements calculated for howler monkeys (3.2 g kg^{-1} , Milton 1979), suggesting that the inverse relationship with fGCM should be taken cautiously. Alternatively, we could infer that protein intake may be playing a role in hormone regulation, possibly contributing to increase fGCM during periods of lower than average protein ingestion. Changes in diet nutritional content, and not only in caloric intake, have the potential to affect metabolism, amino acid homeostasis, body condition, and, consequently, to result in the activation of the HPA axis, affecting steroid concentrations (Knepper et al. 1975; Jeanniard du Dot et al. 2009).

Another factor that must be taken into account is the effect of food intake and fecal output on fGCM production (Goymann 2005; Lepschy et al. 2010). Since the daily collection of all voided feces in free-ranging animals is practically impossible, we could not exactly control for this factor. However, we could estimate total daily food ingestion in our study animals, and we did not find a high variability within and across seasons (Rainy: $215 \text{ g dry weight} \pm 17$

SE, CV = 52 %; Nortes: $191 \text{ g dry weight} \pm 18 \text{ SE}$, CV = 37 %; dry: $224 \text{ g dry weight} \pm 15 \text{ SE}$, CV = 40 %). The latter confirms that the differences found in levels of fGCM were not likely a reflection of different amounts of food ingested.

The present study provides important information for understanding the interplay between nutritional ecology and stress physiology in animals that inhabit seasonal tropical habitats. Through an assessment of the relationship between measures of energy and macronutrient consumption and fGCM, we showed that individuals that exploit seasonally different food resources are able to fulfill their daily energetic demands and avoid energy deficits regardless of the availability of specific food items (e.g., fruit availability). This is most likely related to the fact that black howler monkeys, as many other animals (e.g., South American foxes: *Pseudalopex culpaeus*; spider monkeys: *Ateles chamek*), switch and mix food items to regulate their nutrient intake according to their nutritional needs (Silva et al. 2005; Felton et al. 2009c; Righini 2014), which generally results in decreased physiological stress (Lodge et al. 2013). Moreover, feeding trials with captive animals indicate that foragers tend to choose “mixed diets” even when foods that are considered preferred are offered ad libitum (e.g., tortoises (*Kinixys spekii*): Hailey et al. 1998; brown bears (*Ursus arctos*): Erlenbach et al. 2014). The black howler monkeys of our study consumed food items from 44 plant species (Righini et al. 2016). Their diet varied seasonally, and they fed on leaves (49.5 %), fruits (40.8 %), and flowers (9.7 %) during the nortes. During the rainy and dry seasons, they instead consumed more fruits (69.2 and 54.1 %, respectively) than leaves (30.2 and 38.4 %) and flowers (0.6 and 7.1 %) (Righini et al. 2016). Also, howlers frequently left an undepleted feeding patch (i.e., a tree) of a particular food type to move to another patch containing a nutritionally complementary food type (~50 % of all patch-leaving events). In particular, individuals frequently switched between young leaves and immature fruits, mature fruits and young leaves, and immature fruits and young leaves during successive feeding bouts (Righini 2014). These types of resource mixing probably allowed howler monkeys to fulfill their nutritional and energetic needs also during fluctuations in food availability.

Our results contrast with previous primate studies (e.g., Muller and Wrangham 2004; Pride 2005) which found that a decrease in the consumption of certain resources, particularly fruits characterized by a high content of carbohydrates and lipids used as energy reserve, results in energetic deficits and physiological stress. Drastic limitations in energy intake occur most likely in animals that exploit inclement environments (e.g., north-temperate ungulates: Parker et al. 2009; pinnipeds: Champagne et al. 2012), where the amount and quality of food during certain

seasons are scarce and poor. However, our findings indicate that black howler monkeys, at least for the duration of our 14-month study, did not face periods of extreme food scarcity regardless of seasonal fluctuations in food abundance and availability. In this sense, long-term studies are needed to ascertain the effects of interannual variation in climate and phenology on tropical forest foragers. Moreover, a caveat of our study was that we could not provide more precise measures such as adipose tissue gain/loss, ketosis, or energy balance to reflect the individuals' nutritional status. Yet, the fact that total energy expenditure in many primates, and in black howler monkeys in particular, is generally lower than in other mammals of similar size (Pontzer et al. 2014), could also help them endure periods of reduced energy intake without negative physiological consequences (e.g., energy deficiency). The present study suggests that seasonal variations in food, macronutrient, and energy acquisition do not necessarily have high physiological costs for primates that exploit different types of plant resources such as howler monkeys.

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Author contribution statement RMM and NR formulated the idea, collected and analyzed the data, and wrote the manuscript; RP provided funding and technical support for hormone analyses, and wrote the manuscript.

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Supplementary Table 1. Relationships between physiological measures and proxies of nutritional status across primate taxa

Primates	Species	Setting	Nutritional status proxy	Physiological measure	Sex of subjects	Findings	Reference
Prosimians	<i>Lemur catta</i>	wild	Fruit intake	fGCM	Females	Negative relationship between fruit intake and fGCM levels	Pride 2005
	<i>Eulemur rubriventer</i>	wild	Fruit availability	fGCM	Males and females	Increased fGCM levels during low fruit availability	Tecot 2013
	<i>E. collaris</i>	wild	Seasonality	fGCM	Males and females	Increased fGCM levels during the season of low food availability	Balestri et al. 2014
New World	<i>Callithrix jacchus</i>	captive	Food intake	uCortisol	Females	Decreased uCortisol levels during periods of energy restriction	Tardif et al. 2005
	<i>Ateles geoffroyi</i>	wild	Time spent feeding and basal area of fruiting trees	fGCM	Males and females	No relationship between time spent feeding on fruits and fGCM levels. Negative relationship with basal area of fruiting trees.	Ordóñez-Gómez et al. 2016
	<i>A. hybridus</i>	wild	Fruit availability	fGCM	Males and females	Positive relationship between fruit availability and fGCM levels	Rimbach et al. 2014
	<i>Alouatta pigra</i>	wild	Energy intake, protein and non-protein intake, fruit and young leaf availability	fGCM	Males and females	No relationship between fGCM levels and energy intake, non-protein intake, and young leaf availability. Negative relationship between fGCM levels and protein intake. Positive relationship between fGCM levels and fruit availability	This study

Supplementary Table 1 continued

Primates	Species	Setting	Energy/Nutrient intake proxy	Physiological measure	Sex of subjects	Findings	Reference
New World	<i>A. pigra</i>	wild	Fruit availability	fGCM	Males and females	Negative relationship between fruit availability and fGCM levels	Behie et al. 2010
	<i>A. palliata</i>	wild	Fruit, leaf, and flower availability	fGCM	Males and females	No relationship between food availability and fGCM levels	Gómez-Espinosa et al. 2014
	<i>A. palliata</i>	wild	Time spent feeding on fruits, time spent traveling	fGCM	Males and females	Weak effect of time spent feeding on fruits on fGCM levels. Positive relationship between travel time and fGCM levels	Dunn et al. 2013
Old World	<i>Procolobus rufomitratu</i> s	wild	Protein/fiber intake	fGCM	Males and females	Negative relationship between protein/fiber intake and fGCM levels	Chapman et al. 2007
	<i>Cercopithecus mitis albogularis</i>	wild	Fruit availability and feeding time	fGCM	Females	Positive relationship between neem fruit availability and fGCM levels. No relationship between total fruit availability and fGCM levels	Foerster & Monfort 2010
	<i>Papio cynocephalus</i>	wild	Seasonality	fGCM	Males	Higher fGCM levels in the dry season than in the wet season	Gesquiere et al. 2011
	<i>Macaca mulatta</i> and <i>M. fascicularis</i>	captive	Food intake	uC-peptide	Males and females	uC-peptide levels decreased during the period of food restriction	Girard-Buttoz et al. 2011

Supplementary Table 1 continued

Primates	Species	Setting	Energy/Nutrient intake proxy	Physiological measure	Sex of subjects	Findings	Reference
Old World	<i>Pan troglodytes schweinfurthii</i>	wild	Percentage of feeding observations on fruits	uCortisol	Males	Negative relationship between percentage of feeding observations and uCortisol levels	Muller & Wrangham 2004
	<i>P. t. schweinfurthii</i>	wild	Percentage of feeding observations on fruits	uCortisol	Females	Negative relationship between percentage of feeding observations and uCortisol in lactating females	Emery Thompson et al. 2010
	<i>P. t. schweinfurthii</i>	wild	Percentage of feeding observations on fruits	uC-peptide	Males	Positive relationship between percentage of feeding observations and uC-peptide levels	Emery Thompson et al. 2009
	<i>P. paniscus</i>	captive	Dietary energy content	uC-peptide	Males and females	Low-energy food intake decreased uC-peptide levels	Deschner et al. 2008
	<i>Gorilla gorilla beringei</i>	wild	Percentage of bamboo shoots in the diet	uC-peptide	Females	Positive relationship between uC-peptide levels and bamboo availability	Grueter et al. 2014
	<i>Pongo pygmaeus</i>	wild	Energy intake, fruit availability	uC-peptide	Males and females	Positive relationship between fruit availability, energy intake, and C-peptide levels	Emery Thompson & Knott 2008
	<i>P. pygmaeus</i>	wild	Energy intake, percentage of trees bearing fruit	uKetones	Males and females	No relationship between ketones and fruiting trees	Harrison et al. 2010

Supplementary Table 1 continued

Primates	Species	Setting	Energy/Nutrient intake proxy	Physiological measure	Sex of subjects	Findings	Reference
Old World	<i>P. pygmaeus</i>	wild	Energy intake, fruit availability	uKetones	Males and females	Positive relationship between fruit availability and energy intake. Negative relationship between fruit availability and ketones	Knott 1998
	<i>P. p. wurmbii</i>	wild	Protein intake, fruit availability	Urea concentration	Males and females	Positive relationship between fruit availability and urea concentration. No relationship between daily protein intake and urea concentration	Vogel et al. 2012

fGCM: fecal glucocorticoid metabolites; uC-peptide: urinary C-peptide; uCortisol: urinary cortisol; uKetones: urinary ketones

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