

Reproductive Energetics of Female Mantled Howlers (*Alouatta palliata*)

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Abstract The energetic costs of reproduction have an important influence on the life histories of female primates. At present, however, the interplay of female reproductive state, food availability, and strategies aimed at maintaining energy balance has been described for only a few species, limiting our ability to understand intra- and interspecific variation in female life histories. We assessed how female mantled howlers (*Alouatta palliata*) are affected by reproductive seasonality, and whether they alter their behavior to cope with the energetic demands of reproduction. From August 2013 to July 2015 we measured the reproductive state, behavior (1100 h of focal animal observations), and energetic condition (312 urine samples collected for C-peptide analysis) of 7 adult females, and assessed food availability (weekly phenological sampling of 397 food trees). Female behavior did not vary with reproductive state or reproductive seasonality. There were, however, differences in how females responded to variation in food availability according to reproductive state. Cycling and gestating females spent more time feeding than lactating females, and cycling females less time resting than females in other reproductive states, when food was more available. C-peptide concentrations were unaffected by either individual or overall variation in

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reproductive state, except for cycling females, whose concentrations increased during periods of high food availability. The energetic condition of female mantled howlers is broadly maintained over different stages of reproduction, but is sensitive to variation in food availability.

Keywords *Alouatta* · C-peptide · Foraging · Lactation · Los Tuxtlas · Reproductive seasonality · Time budgets

Introduction

The amount of energy an organism can allocate to reproduction is limited (Kunz and Orrell 2004). Individuals are therefore expected to optimize energetic balance over reproductive cycles to maximize fitness (Beehner and Lu 2013). In mammals, lactation is the costliest state in the female reproductive cycle, with caloric demands (Gittleman and Thompson 1988), vulnerability to disease (Festa-Bianchet 1989; Lloyd 1983), and mortality risk (Altmann and Alberts 2005) all increasing during lactation compared to other reproductive states. Documenting behavioral and physiological strategies to cope with the costs of lactation is therefore important to understanding the energetics of female reproduction, and address life history adaptations, e.g., the duration of gestation and lactation, the timing of reproduction, in mammals, including in primates.

Environmental seasonality is a key factor that can influence primate energetics, and in many species, it results in reproductive seasonality, as females optimize the timing of reproduction to coincide with peak food availability (Janson and Verdolin 2005). There is ample variation among species in terms of which reproductive state is synchronized with food peaks. On the one hand, primates may show condition-dependent conception, such that energy balance must be high for ovarian activity to occur, with the building of energy reserves around the time of conception favoring resulting reproductive success (Brockman and van Schaik 2005; Emery Thompson 2013). On the other hand, females may prioritize high food intake during lactation and weaning (Brockman and van Schaik 2005; Emery Thompson 2013). However, except for small-bodied species (<1 kg), reproductive transitions of female primates span multiple seasons (Lee 1997; van Noordwijk *et al.* 2013). In most anthropoid species, lactation lasts longer than 6 mo (*cf.* Supporting Information in Opie *et al.* 2013), encompassing periods of both food shortage and abundance in seasonal breeders. Reproductive seasonality *per se* may therefore be an insufficient strategy for dealing with the energetic constraints of reproduction for species with lactation that spreads across seasons.

Other strategies, such as behavioral flexibility, may help to maintain energetic balance during lean periods. In support of this prediction, there is evidence for tradeoffs among time budget components across female reproductive cycles in species in which lactation lasts longer than 12 mo, e.g., black howlers (*Alouatta pigra*: Dias *et al.* 2011), chacma baboons (*Papio hamadryas ursinus*: Barrett *et al.* 2006), geladas (*Theropithecus gelada*: Dunbar and Dunbar 1988), and siamangs (*Symphalangus syndactylus*: Lappan 2009), women (Piperata and Dufour 2007); *cf.* Milne-Edwards' sifakas (*Propithecus diadema edwardsi*: Hemingway 1999). Even when there are no changes in time spent feeding according to reproductive state, females may still adjust their food intake. White-faced capuchin (*Cebus capucinus*: McCabe and Fedigan 2007)

and Sanje mangabey (*Cercocebus sanjei*: McCabe *et al.* 2013) females eat faster while lactating than at other times, although only white-faced capuchins increase their consumption of high-quality foods during lactation. It is less clear, however, how behavioral flexibility interacts with food availability in species for which particular reproductive states encompass periods of both food abundance and scarcity.

The energetic cost faced by females during lactation may be so high that behavioral strategies might be insufficient to cope, resulting in a decrease in energetic condition. C-peptide is a byproduct of insulin production and has been recently used as an indirect indicator of energetic condition. The role of insulin in both short-term responses to the ingestion of carbohydrates, and long-term regulation of energy stores, allows for the use of C-peptide measurements not only as a measure of caloric intake, but also as a valid assessment of energy balance (Emery-Thompson 2017). In Sanje mangabeys, lactating and nonlactating females did not differ in C-peptide levels between during periods of high food availability (McCabe *et al.* 2013). This suggests that, although they acquire less energy, lactating females maintain energetic status during lactation via adjustments in feeding rate. Similarly, reproductive state does not correlate with variation in C-peptide in female mountain gorillas (*Gorilla beringei beringei*: Grueter *et al.* 2014), although reproductive females (pregnant or lactating) spend more time feeding than nonreproductive females (Watts 1988). In contrast, female orangutans (*Pongo pygmaeus*) are in their worst energetic condition during lactation, a period in which they reduce feeding and travel time to rest (Morrogh-Bernard *et al.* 2009). Similarly, lactating chimpanzee females that have access to high-quality resources are in better energetic condition than those females who do not (Emery Thompson *et al.* 2012). Variation among studies in study design and sample size could explain why behavioral flexibility across female reproductive states, although widespread among primates, has not always been measured as sufficient to offset the energetic demands of reproduction. Alternatively (and nonmutually exclusively), the interaction between behavioral flexibility, reproductive state, and the energetic demands of reproduction may vary interspecifically.

Howlers (*Alouatta*) show a conservative activity pattern, with long periods of inactivity (Di Fiore *et al.* 2010), and their diet varies along a folivorous–frugivorous continuum, with the degree of folivory and frugivory varying among populations as a function of food availability (Behie and Pavelka 2015; Dias and Rangel-Negrín 2015). Howlers can therefore be a useful model to explore relationships between behavior and the energetics of reproduction, as they allow studies of how variation in diet and degree of activity influence the strategies that females use to cope with the energetic demands of reproduction. The reproductive cycles of female mantled howlers (*Alouatta palliata*) include an ovarian cycle of 16 days (range = 11–24 days: Glander 1980), a mean gestation length of 186 days (range: 180–194: Glander 1980), and a lactation period of up to 20 mo (Balccells and Veà 2009). Females may cycle between 8 and 15 mo before conceiving, so there may be long nonreproductive cycling periods (Glander 1980), suggesting that conception and/or gestation success may be constrained by physiological and/or ecological factors.

It is possible that these constraints are related to variation in female energetic condition resulting from fluctuations in food availability. Although births may occur throughout the year (Di Bitetti and Janson 2000), in some populations more infants are present in some months (the dry season: Fedigan *et al.* 1998; January and June: Milton

et al. 2005), and more infants survive when births are concentrated in a certain climatic season (dry season: Glander 1980). Therefore, although births may occur in any month (Estrada 1982; Glander 1980; Jones 1980), and mantled howlers are often classified as nonseasonal breeders (Di Bitetti and Janson 2000), reproductive seasonality may be dependent on population-specific fluctuations in food availability. Females may face variable energetic demands depending on the scheduling of their reproductive state with respect to food availability, regardless of whether food availability varies seasonally. No published information on variation in female time budgets according to reproductive state is available for this species, but it has been reported that females consume more fats and proteins during gestation and lactation than at other times (Serio-Silva *et al.* 1999). In the absence of strict seasonal breeding, mantled howlers may be coping with seasonal challenges through behavioral flexibility.

We assessed whether female mantled howlers use reproductive seasonality and behavioral flexibility as strategies to cope with the energetic demands of reproduction, and if these strategies influence their energetic condition. We tested three hypotheses.

- 1) Females show reproductive seasonality. Given the uneven distribution of births through time observed in this species, if females offset the energetic demands of reproduction by synchronizing lactation with high food abundance, particularly during early lactation when infants rely exclusively on maternal nursing (Balcells and Veà 2009), we predicted that the probability of finding mantled howler females in different reproductive states (cycling, gestating, early lactation, late lactation) should vary as a function of food availability, and especially of high-quality foods (Dias and Rangel-Negrín 2015).
- 2) Behavior varies among reproductive states. Even if a certain degree of synchronization between energetically demanding reproductive states and high food availability occurs, howlers are not strictly seasonal breeders, and both gestation and lactation may span multiple seasons. Hence, we predicted that gestating and lactating females should eat faster and eat more young leaves and fruits (the main sources of protein and fat for this species: Serio-Silva *et al.* 1999) than cycling females to cope with increased energetic and nutritional demands. As fruit and young leaf availability is spatiotemporally limited (Milton 1980), individuals must devote more foraging time to consume them than to consume mature leaves. Therefore, gestating and lactating females should spend more time active than cycling females, i.e., decrease time spent resting.
- 3) Females preserve their energetic condition across reproductive states. We predicted that if the first or the second hypothesis is supported, the energetic condition of females should not vary among reproductive states, as females would be coping with energetic demands of reproduction through reproductive seasonality or behavioral flexibility, respectively. Given the available evidence that fluctuation in food availability may affect the timing of reproductive events, and perhaps even female energetic condition, we also predicted that food availability, and in particular the availability of high-quality foods, should influence the relationship between female reproductive state and energetic condition. In particular, when food availability is high, the energetic condition of females should not be affected by reproductive state even in the absence of reproductive seasonality and behavioral flexibility.

Methods

Study Site and Subjects

We conducted the study at La Flor de Catemaco, Veracruz, Mexico (Fig. 1), a 250-ha ranch dedicated to the commercial production of ornamental plants, mainly parlor palms (*Chamaedorea elegans*). Although the original understory and forest floor vegetation were replaced by the palm plantations, the canopy and emergent strata (corresponding to evergreen rainforest) are preserved in an area of 100 ha, because palms are grown in the shade of trees. The mean annual rainfall in Catemaco is 1939.4 mm (1981–2010), with most (85.3%) of the rainfall concentrated in the months of June–November, i.e., the rainy season. Mean (\pm SD) monthly temperature is 24 ± 3.2 °C (Comisión Nacional del Agua 2017).

Twenty-three mantled howlers live in this area, in three groups. This population has been studied since 2004 (Shedden-González and Rodríguez-Luna 2010), and we have conducted daily observations of two groups continuously since 2012. We identify individuals easily through anatomical and physiognomic characteristics, including body size and proportions, scars, broken fingers, genital morphology, and pigmentation, as well as blond hairs and skin pigmentation on the feet, hands, and tail.

Between August 2013 and July 2015, we studied seven adult females belonging to the two main study groups. At the onset of the study there were five adult females, i.e., sexually active or parous individuals (Balcells and Veà 2009). In November 2013, a subadult individual was observed copulating with a male, and was reclassified as an adult female. In December 2013 one female disappeared and an individual that was classified as subadult gave birth to an infant and was reclassified as an adult female.

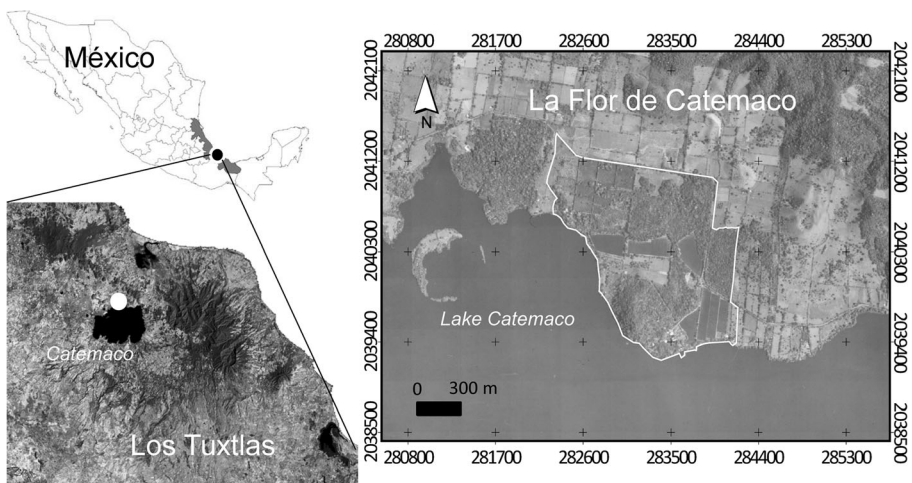


Fig. 1 Map of Mexico (top left) showing Veracruz shaded in gray. Los Tuxtlas region (black dot in top left, bottom left figure) is in southern Veracruz, and includes Lake Catemaco. The study site, La Flor de Catemaco (white polygon in the figure at the right), is on the northern shore of this lake (white dot in bottom left figure)

Assessment of Food Availability

As part of a long-term assessment of tree phenology, we randomly located twenty 50-m transects in the forested areas of La Flor de Catemaco, and identified the four trees with DBH >10 cm nearest to each 10 m of the transect, resulting in 24 sampling trees per transect, and a total of 480 sampling trees (Gómez-Espinosa *et al.* 2014). We sampled all trees every Monday for the abundance of young and mature leaves, unripe and ripe fruits, and flowers using the following abundance scores: 0 = none, 0.25 = 1–25%, 0.5 = 26–50%, 0.75 = 51–75%, 1 = 76–100%. We based scores on a visual estimation of the percentage of stems bearing each phenological phase. To assess food availability, we identified which of the 480 sampling trees were potential food sources for howlers based on previous dietary reports (Dias and Rangel-Negrín 2015), and estimated leaf and fruit biomass per feeding tree using the following allometric formulae that are based on the demonstration that fruit and leaf production are power functions of diameter at breast weight (DBH; Attiwill and Ovington 1968; Clough and Scott 1989; Kira and Shidei 1967; Kittredge 1944; Negi *et al.* 1983; Niklas 1993, 1994; Peters *et al.* 1988; Singh and Yadava 1991; Whittaker and Woodwell 1968):

$$\text{Leaves} = 38.4 \text{ DBH}^{1.65}$$

$$\text{Fruits} = 47 \text{ DBH}^{1.9}$$

Every week we estimated young leaf and unripe and ripe fruit, i.e., the main foods of howlers (Dias and Rangel-Negrín 2015) biomass in 397 potential food trees belonging to 58 species, and one morphospecies (Electronic Supplementary Material [ESM] Table SI), by multiplying the abundance scores of each food item by its biomass formula. We calculated weekly total food biomass as the sum of leaf (young and mature leaves) and fruit biomass (unripe and ripe fruits). Previous studies have demonstrated that fruit and leaf biomass may be accurately predicted for tree assemblages including multiple species (Kira and Shidei 1967; Ogawa and Kira 1977; Peters *et al.* 1988).

Assessment of Reproductive State and Behavior

We organized fieldwork by sampling sessions. We defined a sampling session as the period required for all females to be sampled for their behavior, and for the collection of at least one urine sample per female. Sampling sessions lasted between 2 and 4 weeks, depending on climatic conditions; e.g., when storms prevented fieldwork, sampling sessions were longer.

We classified females as cycling (neither lactating nor gestating); gestating, i.e., females in the period encompassing the day before parturition to 186 days back from that date, which corresponds to the mean duration of pregnancy in this species (Glander 1980); in early lactation, i.e., females with an associated infant of 0–8 mo; or in late lactation, i.e., females with an associated immature of 9–20 mo. We estimated date of

conception as the day from which a female was classified as gestating. Early lactation corresponds to the period in which infants rely on milk as their main food source, whereas late lactation is a period in which immatures complement milk with solid foods (Balcells and Veà 2009). We based this classification on observations of births and the age of the infants, both during the study period and during daily visits to the study groups up to 7 mo after the end of the study, i.e., until December 2015. If we observed females in more than one reproductive state during a single sampling session, we included in our analyses only data on the reproductive state that spanned the most observation days.

We used focal animal sampling with continuous recording (1-h samples) to study the time budgets and food bite rates of females during complete days of following, i.e., 06:00–07:00 to 17:00–18:00 h, depending on the time of year. We chose all females in a group randomly as focal animals, and resampled females only when we had sampled all of them once. We recorded time budgets using EZrecord for HP iPaq116, and categorized behavioral observations into the following: feeding (inspection of food, bringing food to mouth, chewing and swallowing, moving while feeding within a food patch), resting (sleep or static without interaction), moving (movement to a new area or tree), and socializing (social interactions). During feeding we recorded the time focal individuals dedicated to the consumption of unripe fruits, ripe fruits, young leaves, mature leaves, and flowers, as well as the number of bites, defined as each occasion on which food was put into the mouth during a feeding episode (Reynoso-Cruz *et al.* 2016).

During the 24 study months, we completed 36 sampling sessions, with a mean \pm SD of 5.2 ± 1.7 observation hours per female per sampling session. We accumulated a total of 1100 h of behavioral data, with a mean of 157.1 ± 1.7 h per female (or 176.6 ± 1.6 h if the female that disappeared in December 2013 is excluded).

Assessment of Female Energetic Condition

To determine female energetic condition, we measured C-peptide concentrations in urine samples (Emery Thompson 2017). We collected individual fresh urine samples uncontaminated by soil, feces, or urine from other individuals by pipetting urine from a plastic sheet positioned underneath females during urination or directly from vegetation. We cooled urine samples immediately in a field cooler and froze them at -20 °C within a maximum of 12 h after collection until assayed for C-peptide via radioimmunoassay following the manufacturer's instructions (Human C-Peptide, Merck Millipore, Billerica, MA, USA). We standardized C-peptide measurements by dividing C-peptide concentrations by creatinine concentrations, which we measured in each sample via enzyme immunoassays with an auto analyzer (Gallery, Thermo Fisher Scientific, Waltham, MA, USA). The interassay coefficient of variation (CV) of controls for the C-peptide assay was 9.5% ($N = 10$), and intra-assay CV was 6.3% ($N = 10$). We collected a total of 312 urine samples, with a mean \pm SD number of 44.6 ± 16.3 samples collected per female (or 50.5 ± 4.8 samples if the female that disappeared in December 2013 is excluded), and a mean of 1.6 ± 0.8 samples collected per female per sampling session. Except for three cases (H1 when cycling $N = 1$; H2 when in early lactation $N = 1$; H2 when gestating $N = 2$), we collected more than two urine samples per female per reproductive state. We collected samples during the entire day, some in

the morning after females awoke and some after they started to forage. We found no significant differences in C-peptide concentrations between morning and afternoon samples collected from the same female on a single day (paired t -test on ln-transformed C-peptide values: $t = 0.3$, $N = 16$, $P = 0.761$). Thus, we analyzed C-peptide concentrations independently of sample time of collection.

C-peptide levels covary with food availability (Emery Thompson and Knott 2008; Sherry and Ellison 2007). Therefore, as an indirect biological validation of our assays, we related food availability (food biomass, as described in the next section) with female energetic condition (C-peptide levels). C-peptide levels were positively related to food biomass throughout the study (linear mixed model using female id as a random factor: $F_{1,193} = 9$, $P = 0.003$; Fig. 2), confirming that our assays measured variation in female energetic condition.

Data Analysis

Hypothesis 1: Females Show Reproductive Seasonality To explore the temporal association between food availability measures, we calculated Pearson correlations between total, fruit, and young leaf biomass per month. To determine if the timing of reproductive states was related to monthly variation in food availability, we correlated total fruit and young leaf biomass with the number of births and the proportion of females in each reproductive state using Kendall τ correlations. Owing to differences among variables in measuring scale and distribution, e.g., continuous variable with normal distribution, such as the total food biomass, vs. count variable with Poisson distribution, such as the number of births, we rank-transformed all variables.

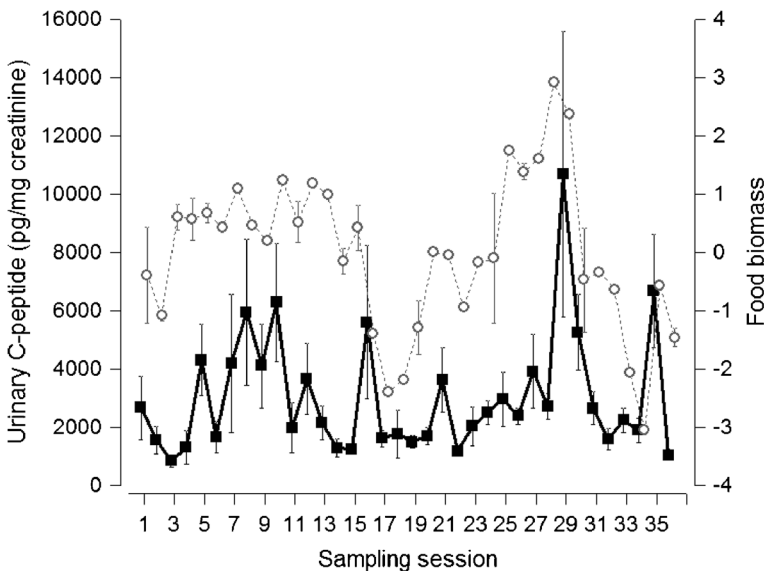


Fig. 2 Variation in mean \pm SE food biomass (PCA of total, fruit and young leaf biomass; open circles) and C-peptide levels (solid squares) per sampling session for seven female mantled howlers studied in La Flor de Catemaco (Mexico) from August 2013 to July 2015

Hypothesis 2: Behavior Varies Among Reproductive States To test our second hypothesis, we ran seven mixed models (Baayen 2010) that assessed the influence of female reproductive state (cycling, gestation, early lactation, and late lactation) on each behavioral variable (time spent feeding, resting, moving, socializing, consuming fruits, young leaves, and bite rate) measured per sampling session per female. We included the interaction between food availability and reproductive state as a fixed factor in these models to account for the putative influence of variation in food availability in each reproductive state on female behavior, e.g., lactation spanning through sampling sessions with variation in food availability. Mixed models circumvent some of the limitations of statistical approaches for data that are not independent (repeated measures), such as the sampling of females in several sampling sessions in our study, avoiding the problems of pseudoreplication (Baayen 2010). Accordingly, we included female identity as a random factor in all models.

As food availability variables were correlated ($r > 0.50$), we used principal components analysis (PCA) to reduce them to a subset of orthogonal factors (Quinn and Keough 2002). This analysis resulted in a single component with an eigenvalue of 2.3 that explained 75.5% of the variance in the original variables, and was positively correlated with total food biomass ($r = 0.96$), fruit biomass ($r = 0.92$), and young leaf biomass ($r = 0.70$). This component, named food biomass, was subsequently used in models of behavior and C-peptide.

We analyzed time spent feeding, resting, moving, consuming fruits, and consuming young leaves (in seconds) with generalized mixed models adjusted for a Poisson distribution and a log link function. We entered observation time per female per sampling session as the offset variable. We corrected these models for overdispersion as described by Harrison (2014). Because females did not socialize in several sampling sessions, we analyzed time spent socializing as a binary variable, i.e., involved/not involved in social interactions, with a generalized mixed model with a binomial distribution and a logit link function. We transformed bite rates to their natural logarithms and analyzed it with a linear mixed model adjusted for a normal distribution and an identity link function.

Hypothesis 3: Females Preserve Their Energetic Condition Across Reproductive States To test our third hypothesis, we ran one linear mixed model to analyze variation in female energetic condition via transformed C-peptide levels as a function of female reproductive state. We included the interaction between reproductive state and food biomass as a predictor, to account for the putative effects of variation in food availability within each reproductive state on female energetic condition, and female identity as a random factor.

We diagnosed model validity by inspection of the distribution of residuals and quantile–quantile plots to verify normality, and of residuals plotted against predicted values to assess homogeneity. Bite rates and C-peptide levels had normal distributions after transformation, as assessed by Kolmogorov–Smirnov tests. We present coefficients and confidence intervals of all terms included in mixed models to assess effect sizes. We calculated statistical significance in tests of the second hypothesis controlling for the False Discovery Rate (Benjamini and Hochberg 1995). We performed all tests in R 3.3.3. (R Core Team 2017) using packages *car* (Fox and Weisberg 2016), *lmer4* (Bates *et al.* 2016), and *MuMIn* (Bartoń 2016).

Ethical Note

Our study was noninvasive and followed the Guidelines of Best Practices for Field Primatology of the International Primatological Society. Research protocols were approved by the Secretaria de Medio Ambiente y Recursos Naturales (permits SGPA/DGVS/10637/11 and SGPA/DGVS/04999/14) and adhered to the legal requirements of Mexican law (NOM-059-SEMARNAT-2010). We have no conflicts of interest to declare.

Results

Hypothesis 1: Females Show Reproductive Seasonality

Total food, fruit, and young leaf biomass were positively correlated during the study (total and fruit: $r = 0.97$, $P < 0.001$; total and leaf: $r = 0.68$, $P < 0.001$; fruit and leaf: $r = 0.64$, $P = 0.001$; $N = 24$ mo in all tests). Food biomass was higher at the beginning of the wet season, i.e., June and July, and lower in the dry season (Fig. 3). Of the five adult females that were present at the beginning of the study, two were lactating, two were pregnant, and one was cycling, suggesting a low degree of synchronization in the timing of reproductive states, which was maintained throughout the study. The number of births and the proportion of females in each reproductive state were not correlated with monthly total, fruit, or young leaf biomass ($P > 0.05$ all Kendall τ tests; Table I).

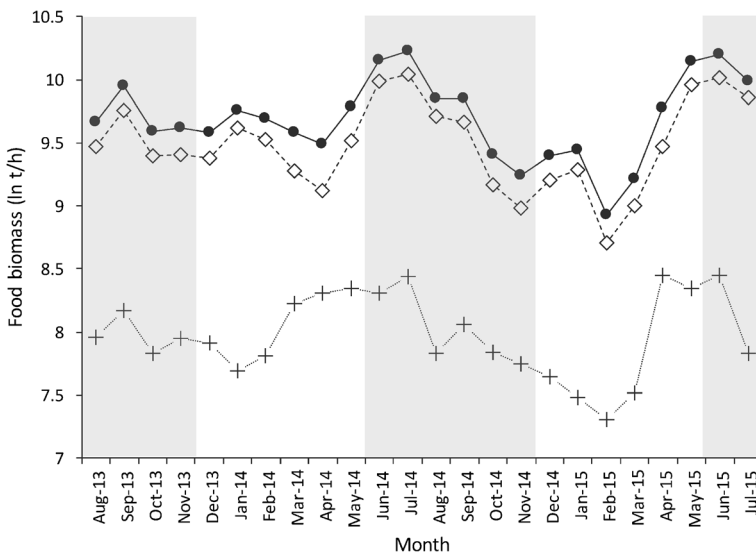


Fig. 3 Total (●), fruit (◊), and young leaf (⊕) biomass (ln-transformed t/ha) in 397 food trees of mantled howlers studied between August 2013 and July 2015 in La Flor de Catemaco, Mexico. Rainy seasons are shaded in gray

Table I Mean monthly food biomass and reproductive data for seven female mantled howlers in La Flor de Catemaco (Mexico) studied between August 2013 and July 2015

Month ^a	Food biomass ^b			No. of births	Proportion of females in each reproductive state			
	Total	Fruit	Young leaves		Cycling	Gestation	Early lactation	Late lactation
January	15,125	12,916	1981	1	0.3	0.1	0.3	0.3
February	13,051	9893	1981	0	0.4	0.1	0.3	0.4
March	12,892	9425	2798	0	0.3	0.2	0.3	0.3
April	15,123	11,034	4356	1	0.3	0.1	0.2	0.3
May	19,005	17,378	4223	0	0.4	0.1	0.4	0.4
June	21,931	21,964	4356	0	0.3	0.1	0.4	0.3
July	21,673	21,104	3563	0	0.4	0.0	0.4	0.4
August	17,355	14,706	2687	3	0.3	0.1	0.4	0.3
September	19,018	16,503	3355	0	0.4	0.1	0.4	0.4
October	15,373	10,832	2521	1	0.2	0.2	0.4	0.2
November	14,890	10,069	2578	0	0.4	0.1	0.4	0.4
December	14,970	10,840	2409	2	0.4	0.2	0.3	0.4
Mean	16,700.5	13,888.6	3067.3	0.67	0.34	0.11	0.33	0.33
SE	887.8	1276.7	254.2	0.28	0.01	0.02	0.02	0.01

^a With the exception of the number of births, all data are presented as means per study month

^b Biomass calculated as t/ha

Hypothesis 2: Behavior Varies Among Reproductive States

Females spent most their time resting (>70% of observation time in all reproductive states), followed by feeding, moving, and socializing (Table II). Females in all reproductive states consumed more fruits than young leaves, and performed approximately seven bites per minute during feeding episodes.

Only socialization was related to female reproductive state (Table II), such that females in early lactation socialized less than females in all other reproductive states (Fig. 4). Feeding and resting were affected by the interaction between reproductive state and food biomass. Specifically, cycling and gestating females spent more time feeding than lactating females when food availability was higher, and cycling females spent less time resting than gestating and lactating females when food was more available.

Hypothesis 3: Females Preserve Their Energetic Condition Across Reproductive States

Gestating females had the highest C-peptide levels, followed by females in late lactation, females in early lactation, and cycling females (Table II). C-peptide levels did not vary according to reproductive state but were affected by the interaction between reproductive state and food biomass (Table II; Fig. 4), such that cycling females had higher C-peptide levels when food biomass increased.

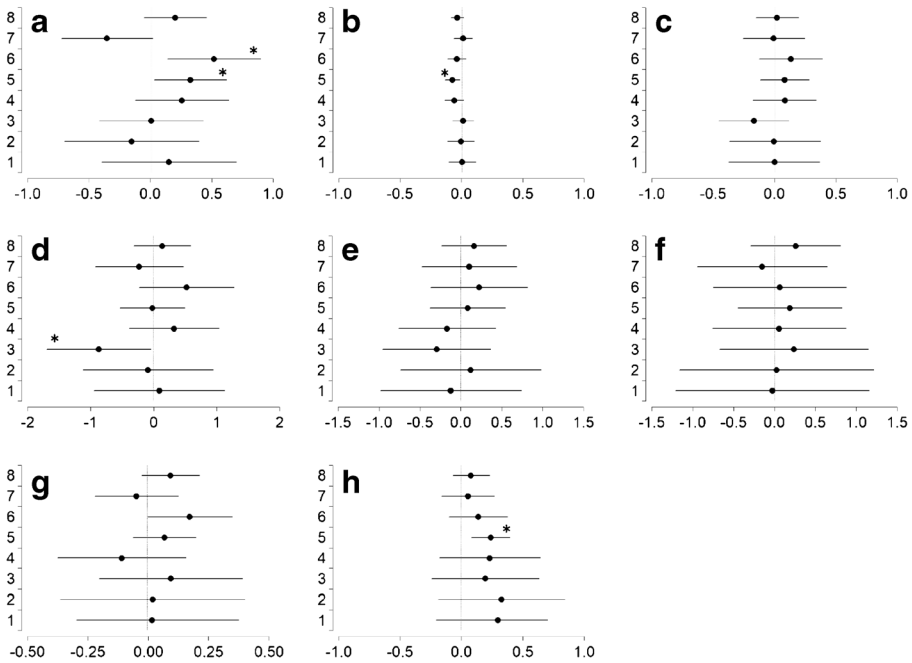


Fig. 4 Coefficients and 95% confidence intervals for the effects of female reproductive state and the interaction between female reproductive state and food biomass on **(a)** feeding time; **(b)** resting time; **(c)** moving time; **(d)** socializing time; **(e)** time spent eating fruits; **(f)** time spent eating young leaves; **(g)** bite rate; and **(h)** C-peptide levels. Asterisks represent significant effects adjusted for a False Discovery Rate of 0.5. Model terms: 1) cycling; 2) gestating; 3) early lactation; 4) late lactation; 5) cycling × food biomass; 6) gestating × food biomass; 7) early lactation × food biomass; 8) late lactation × food biomass

Discussion

Our results suggest that female mantled howlers do not synchronize reproductive states with periods of food availability as a strategy to maintain energetic balance (hypothesis 1). Additionally, behavioral flexibility (hypothesis 2) was not associated with reproductive state, but rather with the interaction between reproductive state and food availability. Cycling females traded-off feeding for resting time when food availability was low, and variation in feeding time of gestating females was positively correlated with food availability. Lactating females did not show variation in resting or feeding time according to food availability. Consistent with the absence of reproductive seasonality and variation in behavioral flexibility among reproductive states, energetic condition, i.e., urinary C-peptide levels, did not differ among reproductive states (hypothesis 3), but varied for cycling females as a function of food availability. Thus, the energetic condition of female mantled howlers is maintained throughout the reproductive cycle, but is sensitive at some stages to variation in food availability. Owing to the difficulties involved in collecting urine samples from an arboreal primate, we studied a small number of females, reducing the power of our conclusions. Nonetheless, our results are based on 2 years of fieldwork.

Table II Mean \pm SE behavior and energetic condition (measured using C-peptides) of female mantled howlers ($N = 7$) by reproductive state in La Flor de Catemaco (Mexico) between August 2013 and July 2015, and their variation as a function of reproductive state and the interaction between reproductive state and food biomass

Behavior	Reproductive Cycling (72) ^d	Gestation (23)	Early lactation (48)	Late Lactation (70)	Predictors	χ^2	P ^d
Feed ^a	15 \pm 1.2	16 \pm 2.0	16 \pm 1.1	16 \pm 1.0	Intercept	280	<0.001*
					Reproductive state	3	0.379
Rest ^a	74 \pm 1.7	73 \pm 2.8	75 \pm 1.2	73 \pm 1.2	Food biomass \times reproductive state	18	0.001*
					Intercept	133	<0.001*
Move ^a	10 \pm 0.7	11 \pm 1.5	8 \pm 0.5	11 \pm 0.7	Reproductive state	4	0.327
					Food biomass \times reproductive state	9	0.066*
Socialize ^a	1 \pm 0.2	0 \pm 0.1	0 \pm 0.1	1 \pm 0.1	Intercept	742	<0.001*
					Reproductive state	3	0.393
Fruits ^b	57 \pm 3.8	62 \pm 6.3	56 \pm 3.2	60 \pm 3.0	Food biomass \times reproductive state	2	0.779
					Intercept	0.04	0.843
Young leaves ^b	35 \pm 3.6	30 \pm 6.2	39 \pm 3.0	35 \pm 2.9	Reproductive state	9	0.032*
					Food biomass \times reproductive state	3	0.606
Bite rate ^b	7 \pm 0.3	7 \pm 0.4	7 \pm 0.3	7 \pm 0.2	Intercept	19	<0.001*
					Reproductive state	1	0.746
				Food biomass \times reproductive state	1	0.843	
				Intercept	57	<0.001*	
				Reproductive state	0.3	0.963	
				Food biomass \times reproductive state	1	0.853	
				Intercept	356	<0.001*	
				Reproductive state	0.3	0.963	
				Food biomass \times reproductive state	3.5	0.485	

Table II (continued)

Behavior	Reproductive Cycling (72) ^d	Gestation (23)	Early lactation (48)	Late Lactation (70)	Predictors	χ^2	<i>P</i> ^d
C-peptide ^e	2621 ± 399	3944 ± 947	2741 ± 220	2801 ± 221	Intercept Reproductive state Food biomass × reproductive state	2 0.6 3	0.051* 0.586 0.023*

^a Mean percentage of time dedicated to each activity during 1-h continuous focal samples

^b Mean percentage of feeding time dedicated to each food item during 1-h continuous focal samples, with the exception of bite rate, which represents the mean number bites per minute

^c Urinary C-peptide levels in pg/ug of creatinine

^d Numbers in parentheses represent the total number of sampling sessions analyzed for each reproductive state

^e Asterisks indicate significant effects adjusted for a False Discovery Rate of 0.5 (Benjamini and Hochberg 1995)

Although howlers have faster life histories than other primates of similar body size (Di Fiore *et al.* 2010), there is evidence suggesting that female mantled howlers may face high reproductive constraints compared with other anthropoids (Mitani *et al.* 1996), as they experience a very high number of nonconceptive cycles (Glander 1980). Furthermore, infant survival is influenced by the timing of births (black howlers: Behie and Pavelka 2015; Dias *et al.* 2016; mantled howlers: Glander 1980). Given the positive correlations observed in other primates between food availability, energetic condition, and ovarian function (Ellison and Valeggia 2003; Emery Thompson *et al.* 2012), we predicted that some degree of reproductive seasonality resulting from variation in food availability should occur in mantled howlers. At the onset of our study, however, only two of the five females were in a similar reproductive state, and food availability *per se* was a poor predictor of reproductive state, corroborating previous classifications of mantled howlers as nonseasonal breeders (Di Bitetti and Janson 2000). Interestingly, there was a coincidence in the timing of conception of three females (H1 in session 24, HCC in session 28, and HPM in session 29) during the dry season, suggesting that the onset of lactation would occur in a period of high food availability. Thus, although no strict breeding seasonality likely exists in our study population, it is possible that female howlers follow an income-II breeding strategy (Janson and Verdolin 2005), a term coined to refer to species that correlate peak lactation with peak food abundance, e.g., black howlers (Dias *et al.* 2016) and white-faced capuchins (Carnegie *et al.* 2011). The small sample of females in our study makes further analysis necessary to confirm this interpretation.

We predicted that female mantled howlers would use behavioral flexibility to cope with the energetic demands of reproduction. Females of other primate species trade off components of their time budgets to optimize energy balance throughout their reproductive cycles, e.g., black howlers (Dias *et al.* 2011), chacma baboons (Barrett *et al.* 2006), geladas (Dunbar and Dunbar 1988), and mountain gorillas (Watts 1988) and adjust their foraging behavior, either by eating faster or eating smarter, i.e., consuming higher proportions of key nutrients or energy, e.g., Sanje mangabeys (McCabe *et al.* 2013) and white-faced capuchins (McCabe and Fedigan 2007). In our study female time budgets were strikingly constant across reproductive states, supporting previous contentions that howler behavior is highly conserved, perhaps because of phylogenetic constraints (Bicca-Marques 2003; Dias *et al.* 2011), or of an energetically conservative life style (Nagy and Milton 1979). Additionally, recent evidence suggests that the gut microbiota of howlers provides energy and nutrients that compensate for changes in the diet, such as those associated with seasonal variation in food availability (Amato *et al.* 2015). Such microbial activity could downplay the importance of behavioral flexibility for the maintenance of energetic balance. Nonetheless, cycling and gestating females showed variation in resting and feeding time related to fluctuations in food availability, highlighting the importance of considering the interplay of phenology and behavior as a determinant of female energetic condition in species with multiseasonal reproductive states. It is less clear, however, why such interplay was not observed among lactating females, as documented in other primates, e.g., chimpanzees (Emery Thompson *et al.* 2012), Sanje mangabeys (McCabe *et al.* 2013), and siamangs (Lappan 2009), especially because a lower involvement in social interactions during early lactation could allow females to use more time to feed (Altmann 1980; Barrett *et al.* 2006). Considering that lactation may last 20 mo in mantled howlers (Balcells and Veà 2009),

encompassing several periods of food abundance and scarcity, a future analysis of variation in behavior and energetic condition within lactation states based on a larger sample of females could provide more clarity to our results.

Cycling females displayed the strongest behavioral and C-peptide responses to variation in food availability, and were in the worst energetic condition. Although C-peptide levels for some females could sometimes be measured only by a single sample in some reproductive states, C-peptide levels consistently corresponded to food availability during our study, and the observed patterns of energetic state appear consistent with the known reproductive biology of this species. The long nonreproductive cycling periods reported for this species (Glander 1980) indicate that the ovarian function of females is highly constrained, and our results suggest that variation in energetic condition in response to resource availability could be a mechanism underlying such constraints. In other species, energetic condition is related to ovarian hormone production, e.g., chimpanzees (Emery Thompson *et al.* 2012), humans (Ellison and Valeggia 2003), orangutans (Knott 1999), and Sanje mangabeys (McCabe *et al.* 2013), and therefore to conception probabilities. In our study, two females cycled for long periods. Subadult HB was observed copulating with a male in November 2013 and was classified from that moment as an adult female. She copulated at irregular intervals during the study but was never observed with an associated infant. It is possible that this young adult female was still investing a large portion of her energy budget in her own growth, which could preclude investment in ovarian activity. Female HCC had weaned her 18-mo juvenile in January 2013, and remained cycling for the first 19 mo of this study, resulting in an interbirth interval of 42 mo, a new maximum for howlers (Van Belle and Bicca-Marques 2015). She was also observed copulating several times during those 19 cycling months, so she either did not conceive or lost a fetus during this period. Her C-peptide levels were similar between cycling and gestation states, suggesting that other factors besides energetic condition may have affected her reproductive performance. Regardless of interindividual variation in causes of long cycling periods, cycling females traded off resting time for feeding when food availability was high, reinforcing the possibility that this may be a critical period in the reproductive cycle of female mantled howlers.

Finally, variation in total and fruit biomass was small during the study (CV <4%; Fig. 2), we detected fruiting trees in all months (Fig. 3), and DBH of food trees (ESM Table SI) is higher at La Flor de Catemaco than in other mantled howler habitats (Gómez-Espinosa *et al.* 2014). Additionally, mean group size (7.7 individuals) and population density (0.23 ind/ha) are smaller at La Flor de Catemaco than at other sites (Clarke *et al.* 1986; Estrada 1982; Fedigan *et al.* 1998). Consequently, it is unlikely that female behavior and energetic condition were affected by food constraints associated with food scarcity or by intra- and intergroup food competition, as observed in some other primate species and populations, e.g., chimpanzees (Emery Thompson *et al.* 2012) and primates (Koenig 2000). In this sense, our data may be particularly informative for understanding the interplay of female reproductive strategies and energetics under high food availability conditions.

In conclusion, in this study female mantled howlers showed neither reproductive seasonality nor behavioral flexibility in relation to reproductive state. Food availability influenced the energetic condition of cycling females, suggesting that conception may be energetically constrained in this species. Our study population has a low population

density and high food availability throughout the year, so it is possible that our results reflect the behavioral and physiological responses of females living under non-food-restricted conditions.

Data Availability

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

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