



Hormonal correlates of energetic condition in mantled howler monkeys



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ABSTRACT

Hormones have a key role in energy allocation, so their study allows understanding individual metabolic strategies. Because different hormones convey different information on the responses of individuals to energetic demands, a simultaneous analysis of variation in multiple hormones may offer a more reliable picture of metabolic strategies than single hormone assessments. In this study we focused on determining which factors were related to variation in fecal glucocorticoid and thyroid hormone metabolites in wild mantled howler monkeys (*Alouatta palliata*). Over 12 months, we determined fecal glucocorticoid and thyroid hormone metabolite levels of 11 adults belonging to two groups, and examined the relationship between hormone metabolites and a variety of behavioral, physiological, and ecological factors (e.g., food intake, sex/reproductive state, activity, participation in agonistic interactions). We found that glucocorticoids were elevated in gestating and lactating females compared to males and cycling females, and were also higher when individuals were more active and participated in agonistic interactions. Thyroid hormone levels were also related to sex/reproductive state and activity, but were additionally positively related to fruit intake and negatively related to young leaf intake. Our study demonstrates that the non-invasive measurement of glucocorticoid and thyroid hormones of howler monkeys allows assessing different underlying physiological processes. By combining different biomarkers, which has seldom been done with wildlife, we could also parse the influence of psychological vs. metabolic challenges for individual energetic condition, which may be instrumental for deciding which factors should be accounted for when studying different hormone-behavior interactions.

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1. Introduction

Energetic condition affects fitness through its effects on physiology and behavior. Individuals allocate energy to different functions (e.g., growth, reproduction) depending on their energetic condition, and hormones have a key role in energy allocation, as they act as mediators or as products of such allocation (Emery Thompson, 2016). Their study allows therefore understanding the underlying mechanisms and functional consequences of variation in energetic condition.

Recent advances in hormonal measurement in excretions, have made possible the non-invasive assessment of the energetic condition of free-ranging animals (Hodges and Heistermann, 2011; Beehner and Whitten, 2004; Palme, 2005). In this context, glucocorticoids and thyroid hormones are particularly useful as non-invasive biomarkers of energetic condition: on the one hand, both are involved in metabolic

processes, including gluconeogenesis and cellular metabolism (Sapolsky et al., 2000; Kim, 2008); on the other hand, after metabolization these hormones are excreted in the urine and feces, reflecting the biosynthesis occurring over several hours (urine) or days (feces) (Norman and Litwack, 1997). Therefore, the concentrations of excreted glucocorticoid and thyroid hormone metabolites represent an integrative measure of the energetic condition of individuals. Accordingly, fecal metabolite concentrations of both hormones, for example, increase during periods of high energetic demands (molting) in Hawaiian monk seals (*Monachus schauinslandi*; Gobush et al., 2014). However, whereas glucocorticoid secretion occurs in response to environmental (e.g., food availability: Lewanzik et al., 2012; Bourbonnais et al., 2013), physical (e.g., climate: de Bruijn and Romero, 2011; Houser et al., 2011), intrinsic (e.g., reproductive status: Bacci et al., 2014; Viblac et al., 2016), and psychological (e.g., social stress: Corlatti et al., 2014; Stocker et al., 2016) challenges, thyroid hormones seem to be unaffected by psychological stress (Ayres et al., 2012; Gobush et al., 2014; but see Walpita et al., 2007). Additionally, when individuals face nutritional constraints, basal metabolic rate decreases and as a consequence thyroid hormone secretion is downregulated as part of an energy-saving

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strategy (Eales, 1988; Hulbert and Else, 2004; Cristóbal-Azkarate et al., 2016), whereas glucocorticoid levels increase to promote the mobilization of stored energy reserves (Sapolsky et al., 2000). Therefore, the combined measurement of these hormones allows discriminating between 1) psychological and energetic influences on the energetic condition of individuals, and 2) different hormonal coping strategies aimed at maintaining energetic balance (Emery Thompson, 2016). This has been, however, only rarely performed with wildlife (e.g., Ayres et al., 2012; Gobush et al., 2014; Vynne et al., 2014), and to the best of our knowledge, no such information exists for wild primates.

Howler monkeys (*Alouatta* spp.) are frugivore-folivore, arboreal, Neotropical primates with a wide geographic distribution (Mexico to Argentina). The concentrations of fecal glucocorticoid metabolites (for simplicity, glucocorticoid levels hereafter) of howler monkeys have been reported to vary as a function of the exchanging of agonistic interactions, the interaction between female reproductive state and participation in agonistic interactions (i.e., pregnant and lactating females involved in agonistic interactions show higher glucocorticoid levels than males and cycling females: Gómez-Espinosa et al., 2014), male rank (Van Belle et al., 2009), the presence of extragroup males (Cristóbal-Azkarate et al., 2007), time spent traveling (Martínez-Mota et al., 2007; Dunn et al., 2013), protein intake (Martínez-Mota et al., 2016), fruit availability (Behie et al., 2010; Martínez-Mota et al., 2016), habitat size (Martínez-Mota et al., 2007; Rangel-Negrín et al., 2014; but see Rimbach et al., 2013), habitat quality (Aguilar-Melo et al., 2013), anthropogenic disturbance (presence of tourists: Behie et al., 2010; undefined anthropogenic disturbances: Gómez-Espinosa et al., 2014; Rangel-Negrín et al., 2014; but see Rimbach et al., 2013), and climatic seasonality (Gómez-Espinosa et al., 2014; Rangel-Negrín et al., 2014). These factors represent environmental, physical, intrinsic, and psychological challenges to the maintenance of energetic condition, and their impact on glucocorticoid levels has been widely described in primates (Abbott et al., 2003; Beehner and McCann, 2008; Rangel-Negrín et al., 2009; Anestis, 2010; Foerster et al., 2012; Chapman et al., 2015; Schoof et al., 2016).

In contrast with the numerous studies on glucocorticoid variation, the concentrations of fecal thyroid hormone metabolites (for simplicity, thyroid hormone levels hereafter) of howler monkeys have been quantified only once. In captive female mantled howler monkeys (*A. palliata*), thyroid hormone levels declined with time post-capture in response to a sharp decrease in feeding (Wasser et al., 2010). This result converges with a positive correlation between thyroid hormone levels and food availability reported in the single study that has measured thyroid hormone levels in a wild primate (Barbary macaques, *Macaca sylvanus*: Cristóbal-Azkarate et al., 2016). Therefore, as in the case of glucocorticoids, thyroid hormone measurements allow assessing the responses of howler monkeys to factors that have the potential to affect their energetic condition.

Habitat size, activity levels, and human presence do not have the same effect on glucocorticoid levels of howler monkeys across studies (e.g., Aguilar-Melo et al., 2013 vs. Behie et al., 2010; Rimbach et al., 2013 vs. Rangel-Negrín et al., 2014). The combined measurement of glucocorticoid and thyroid hormone levels could assist in solving such inconsistencies by parsing different sources of metabolic demands as well as different hormonal coping strategies to deal with such demands (Emery Thompson, 2016). Thus, the aim of the present study was to examine variation in glucocorticoid and thyroid hormone levels in mantled howler monkeys as a function of energy acquisition and energy expenditure in order to better understand which factors affect individual energetic condition. We predicted that glucocorticoid levels should be negatively related to energy acquisition and positively related to energy expenditure, whereas thyroid hormone levels should be positively related to energy acquisition and expenditure. Furthermore, we predicted that energy expenditure factors associated with psychological demands (aggressive behavior) should affect variation in glucocorticoid but not in thyroid hormone levels.

2. Methods

2.1. Ethical note

This study was non-invasive and followed the “Guidelines for the treatment of animals in behavioural research and teaching” (Guidelines for the Use of Animals, 2012). 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 the Mexican law (NOM-059-SEMARNAT-2010).

2.2. Study site and subjects

Our study was conducted from January to December 2014 at La Flor de Catemaco (Veracruz, Mexico; 18°26'39" N, 95°02'57" W), which is part of the Los Tuxtlas Biosphere Reserve. La Flor de Catemaco is a 250-ha ranch dedicated to the sustainable production of ornamental plants, in which ca. 100 ha of the original evergreen forest are preserved.

The mantled howler monkey population living at La Flor de Catemaco has been studied since 2004 (Shedden-González and Rodríguez-Luna, 2010). There are currently 23 individuals in the population divided into three groups, two of which have been followed daily since 2012. Subjects were fully habituated to the presence of researchers (i.e., ignored our presence: Williamson and Feistner, 2011). We identified individuals easily through anatomical and physiognomic characteristics, including body size and proportions, scars, broken fingers, and genital morphology and pigmentation. Several individuals were marked with ankle bracelets. Adult females were classified in one of three reproductive states: lactating (i.e., females with an associated infant of 0 to 20 months: Balcells and Veà, 2009); gestating (i.e., females in the period encompassing the day before parturition to 186 days back from that date, which corresponds to the duration of pregnancy in this species: Glander, 1980); or cycling (neither lactating nor gestating). We based this classification on observations of births and nipple contact during the study, and on observations of newborns during daily visits to the study groups.

2.3. Assessment of ambient temperature

Weekly minimum and maximum ambient temperatures were determined from daily recordings performed at 1-h intervals with a hand-held thermometer (Kestrel 3500 Weather Meter; Nielsen-Kellerman Company, PA, USA). During the study we accumulated a total of 1320 temperature recordings.

2.4. Behavioral sampling

We conducted 1 h continuous focal follows (from sunrise to sunset), during which we recorded the time budgets of 11 adult individuals living in the two main study groups (group 1: 2 adult females and 2 adult males; group 2: 3 adult females and 4 adult males). We categorized behavioral observations into the following: resting (sleep or static without interaction), feeding (inspection of food, bringing food to mouth, chewing and swallowing, moving while feeding within a food patch), traveling (movement to a new area or tree), and other behavior (remaining activities not categorized as resting, feeding or traveling) (Dunn et al., 2009). During feeding we recorded the food item consumed (fruit, flower, mature leaf, young leaf or other item). We also recorded all occurrences of agonistic interactions (Dias and Rangel-Negrín, 2015a). Finally, we numbered and geolocated with GPS each tree used by the howler monkeys.

We organized behavioral recordings in sampling sessions. A sampling session was defined as the period required for all adults to be sampled for their behavior and for collecting at least two fecal samples per individual (see next section). Sampling sessions lasted between one

and two weeks, depending on climatic conditions (i.e., when storms prevented fieldwork, sampling sessions were longer). During the 12 study months, 24 sampling sessions were completed, with a mean \pm SD of 5.2 ± 1.7 observation hours per individual per sampling session. We accumulated a total of 1320 h of behavioral data, with a mean of 122.1 ± 1.2 h per individual.

2.5. Fecal sample collection and hormone assays

We collected fecal samples opportunistically whenever they could be matched with individuals: fresh samples uncontaminated by urine were collected from the forest floor and deposited in polyethylene bags labeled with the identity of each individual. The bags were kept in a cooler with frozen gel packs while in the field and stored at the end of the day in a freezer at -20°C until steroid extraction was performed. Samples were stored for 3 to 5 months until the extractions were conducted. The freezing procedure used for storing the samples has been reported to have a weak time-storage effect on fecal hormone metabolites (Khan et al., 2002). We analyzed two fecal samples per individual per sampling session (i.e., 528 samples).

Glucocorticoids and thyroid hormones were extracted following a modification of the method described in Wasser et al. (2000) for the extraction of steroids. Briefly, 0.6 g of homogenized, lyophilized and pulverized feces was shaken for 20 h in 4.0-ml of analytical-grade methanol. Extracts were then centrifuged (460 g for 30 min) and the supernatant was recovered. After complete evaporation of the solvent in a water bath at 60°C for 20 h, pellets were reconstituted with 3-ml albumin buffer and used for the glucocorticoid and thyroid hormone assays.

Assays were conducted at the Instituto de Ciencias Médicas y Nutrición Salvador Zubirán, in Mexico City. Glucocorticoid levels were measured using a commercial radioimmunoassay ^{125}I cortisol kit (Izotop, Institute of Isotopes Ltd., Budapest, HU; sensitivity = 2.9 nmol/L; calibration range = 0–1600 nmol/L) and gamma counter (Cobra Model E5005, Packard, Inc., Downers Grove, IL, USA). Pooled fecal extracts, when added to the standard curve points, exhibited an accuracy of $R^2 = 0.98$ ($N = 5$, $P = 0.001$), and serial dilutions of a fecal pool from howler monkeys yielded results that ran parallel to the kit standards ($R^2 = 0.97$, $N = 5$, $P < 0.001$). Samples were run in the order in which they were collected in a total of six assays, with a new set of quality controls performed in each assay. Glucocorticoid intra-assay variation averaged 6.8% (fecal extract pool, $N = 6$). Inter-assay variation, estimated for the six assays from fecal pools with varying levels of cortisol, averaged 19.3% (low), 15.1% (medium), and 7.2% (high). The protocols that we used to extract and quantify glucocorticoid levels in feces had been previously validated to detect the activation of the HPA axis in response to stressful stimuli in mantled howler monkeys (Gómez-Espinosa et al., 2014).

Thyroid hormone levels were measured using a chemiluminescent enzyme immunoassay commercial kit (Total T3 Immulite, Siemens, CA, USA; sensitivity = 0.59 nmol/L; calibration range = 0.61–9.2 nmol/L) and an Immulite 1000 analyzer (Siemens, CA, USA). Pooled fecal extracts, when added to the standard curve points, exhibited an accuracy of $R^2 = 0.95$ ($N = 5$, $P = 0.01$), and serial dilutions of a fecal pool from howler monkeys yielded results that ran parallel to the kit standards ($R^2 = 0.94$, $N = 5$, $P = 0.001$). Thyroid hormone intra-assay variation averaged 7.2% (fecal extract pool, $N = 6$), and inter-assay variation averaged 5.2%, 7.4%, and 6.1% for low, medium, and high quality controls, respectively.

Traditional thyroid stimulating hormone (TSH) challenge was not permitted for this study due to the endangered status of mantled howler monkeys in Mexico (NOM-059-SEMARNAT-2016). Therefore, we used an opportunistic test for validation of our thyroid hormone measures. Based on multiple evidence that thyroid status is affected by infectious diseases (e.g., Wartofsky and Burman, 1982; Ogwu et al., 1992; Kahl et al., 2002), we predicted that, ceteris paribus, individuals with severe parasite infections should have lower thyroid hormone

levels than non-parasitized individuals. We therefore compared thyroid hormone levels of four adult males, two of which presented severe bot fly (*Cuterebra baeeri*: Cristóbal-Azkarate et al., 2012) infestations. Bot fly parasitism has negative health effects in howler monkeys, and in synergy with other factors (e.g., age) may lead to death (Milton, 1996). As predicted, infected individuals had 54.5% lower (mean \pm SD = 131.6 ± 64.9 ng/g; range = 80.9–300.3) thyroid hormone levels than non-infected individuals (289.1 ± 73.1 ng/g; range = 200.8–387.4; Fig. 1), indicating that our assays reliably measured thyroid function of mantled howlers.

2.6. Data organization and analysis

Young leaves and fruits represented the major food items of mantled howler monkeys during our study (mean \pm SD = $96.8 \pm 2.7\%$ of total feeding time per sampling session; range = 85.7–100%). We therefore based our calculations of energy acquisition on the intake of these items. Time spent eating each food item was multiplied by feeding rates previously calculated for this population (Reynoso-Cruz et al., 2016) to obtain an estimation of the amount of ingested food (in grams) per plant part. From these data, we calculated percentages of ingested food weight per plant part (Table 1).

We used time budget data to calculate an activity index by dividing the sum of time spent feeding, traveling, and in other activities by time spent resting (Cavigelli, 1999; Dunn et al., 2010). This index ranged between 0 (i.e., subjects were resting all the time) and 1 (i.e., subjects were continuously active). We determined ranging distances by calculating the linear distance between trees that were used consecutively by study subjects in each sampling session with the package 'adehabitatLT' (Calenge, 2015) in R 3.3.2 (The R Foundation for Statistical Computing, 2016). Although location recordings were performed at the focal-animal level, we calculated ranging distances per group per sampling session because howler monkey groups are highly spatially cohesive (Hopkins, 2011; Plante et al., 2014). Ranging distances per sampling session were divided by the number of sampling hours per group per session (i.e., converted into a traveling rate) to account for variation in sampling effort between groups.

We explored the relationship between glucocorticoid and thyroid hormone levels with a Pearson correlation on ln-transformed values (function 'cor' in R package 'stats': R Core Team, 2017).

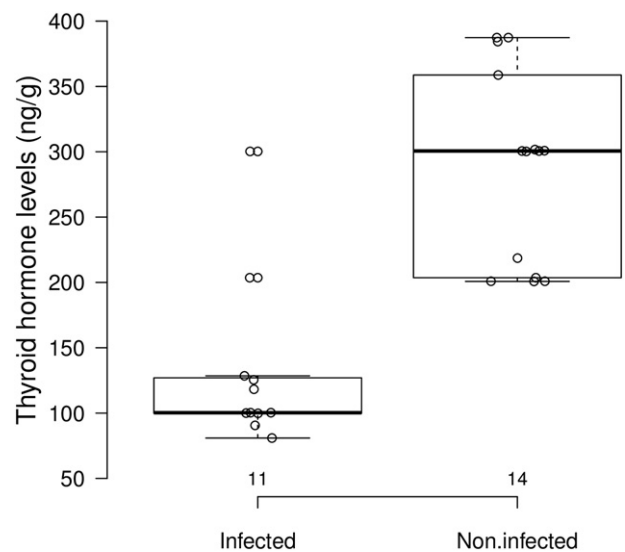


Fig. 1. Thyroid hormone levels of mantled howler monkey males with or without bot fly infections. Center lines show the medians; box limits indicate the 25th and 75th percentiles; whiskers extend 1.5 times the interquartile range from the 25th and 75th percentiles; data points are plotted as open circles.

Table 1
Predictors of energetic condition analyzed in this study.

Variable	Attributes in this study
Energy acquisition	
Fruit intake	Percentage of consumed food weight dedicated to eat fruits per sampling session (min = 1.4%; max = 100%; mean (\pm SD) = 58.2 \pm 25.9%)
Young leaf intake	Percentage of consumed food weight dedicated to eat young leaves per sampling session (min = 0%; max = 95%; mean (\pm SD) = 38.5 \pm 25.1%)
Energy expenditure	
Sex/reproductive state	Male ($N = 6$ individuals), cycling female (3 individuals in 4 to 23 sampling sessions), gestating female (2 individuals in 11 to 17 sampling sessions), lactating female (4 individuals in 2 to 23 sampling sessions)
Activity	Time spent feeding, traveling, and in other activities divided per time spent resting. Calculated per individual per sampling session (min = 0; max = 1.8; mean (\pm SD) = 0.4 \pm 0.3)
Travel rate	Sum of all distances traveled by each group per sampling session divided per the number of sampling hours per session (min = 38.4 m/h; max = 46.7 m/h; mean (\pm SD) = 41.2 \pm 2.7 m/h)
Agonism	Individual involvement (as actor or receiver; recorded as a yes/no dichotomy) in agonistic interactions in each sampling session
Minimum ambient temperature	Minimum ambient temperature recorded in each sampling session (min = 13.8 °C; max = 23.9 °C; mean (\pm SD) = 19.0 \pm 3.0 °C)
Maximum ambient temperature	Maximum ambient temperature recorded in each sampling session (min = 20.1 °C; max = 35.7 °C; mean (\pm SD) = 27.7 \pm 3.4 °C)

To analyze the relationships between each hormone and both energy acquisition (fixed factors: young leaf and fruit intake) and expenditure (fixed factors: sex/reproductive state, activity, travel rate, agonism, minimum and maximum ambient temperatures) we used linear mixed models (LMM: West et al., 2014) to account for repeated measuring of the same individuals in different models (i.e., subject identity was included as a random factor). The interactions between sex/reproductive state and food intake, activity, and participation in aggressive interactions were also included as fixed factors given previous reports of their relevance as predictors of variation in behavior and hormonal responses in howler monkeys (e.g., Cristóbal-Azkarate et al., 2007; Dias et al., 2011; Gómez-Espinosa et al., 2014). We standardized young leaf and fruit intake, activity, travel rate, minimum and maximum ambient temperatures, as they were on very different scales of magnitude (function 'stdize' in R package 'MuMIn': Barton, 2016). We verified the underlying assumptions of the models visually with Q-Q plots of residuals fitted against predicted values, and after ln-transformation, hormone levels had a normal distribution assessed with a Kolmogorov-Smirnov test. We used R package 'lme4' (Bates et al., 2016) with function 'lmer' to obtain LMM parameters, and with function 'confint' to obtain confidence interval for fixed factors.

We followed an information-theoretic approach (Burnham and Anderson, 2013) to generate a set of models based on information criteria (i.e., AIC_c), and defined the best model for variation in each hormone by averaging parameter estimates of models receiving substantial empirical support (i.e., $\Delta_i < 2$). We also calculated the relative importance for each term included in this model set. Importance is the sum of the Akaike weights w_i of all models which include the term in question. As model weights represent the probability of a model to be the best model in the model set and thus reflect model uncertainty, importance can be understood as the likelihood of a term to be included in the best model. We checked Variance Inflation Factors (VIF) of fixed factors in each of the best models to verify multicollinearity, and found that none had a VIF > 3 (i.e., no collinear effects on other factors: Neter et al., 1990). For these analyses we used R packages 'MuMIn' (Barton, 2016) and 'car' (Fox and Weisberg, 2016).

Significance testing was two-fold: we used a likelihood ratio test to compare a null model including only the random factor with each of the best models (R package 'stats': R Core Team, 2017); we calculated p-values for each fixed factor with the Kenward-Roger approximation for degrees-of-freedom (R package 'afex': Singmann et al., 2016). For effect size assessments, we calculated pseudo-R-squared of each best model (R package 'sjstats': Lüdtke, 2017).

3. Results

Mean (\pm SD) glucocorticoid and thyroid hormone levels of mantled howler monkeys throughout the study were 325.5 \pm 179.3 ng/g (range = 26.0–900.6 ng/g) and 298.9 \pm 127.5 ng/g (range = 80.9–730.0 ng/g),

respectively. Hormone levels were not correlated across all individuals ($r = 0.03$, $N = 253$, $P = 0.592$), in gestating ($r = 0.04$, $N = 28$, $P = 0.838$) and lactating ($r = 0.16$, $N = 56$, $P = 0.251$) females, but were weakly correlated in cycling females ($r = -0.25$, $N = 31$, $P = 0.171$) and males ($r = -0.22$, $N = 138$, $P = 0.009$).

3.1. Correlates of glucocorticoid variation

Variation in glucocorticoid levels of mantled howler monkeys was best explained by an averaged model that included sex/reproductive state, activity, and agonism (Table 2). This model was significantly different from the null model (likelihood ratio test: $\chi^2_5 = 26.8$, $P < 0.001$) and explained 15% ($R^2 = 0.15$) of variation in glucocorticoids. Model 1 included only sex/reproductive state, and was supported 1.7 and 2.7 more than Model 2 (including term activity) and Model 3 (including term agonism), respectively.

Lactating females had the highest mean (\pm SE) glucocorticoid levels (423.7 \pm 29.5 ng/g; range = 103.8–900.6 ng/g), followed by gestating females (390.5 \pm 31.1 ng/g; range = 192.9–752.5 ng/g), males (292.9 \pm 13.2 ng/g), and cycling females (234.2 \pm 19.1 ng/g; range = 73.1–500.0 ng/g) (Fig. 2a). Glucocorticoid levels were also higher when individuals were more active (Fig. 2b), and when they were involved in agonistic interactions (Fig. 2c).

3.2. Correlates of thyroid hormone variation

The averaged model to explain variation in thyroid hormone levels of mantled howler monkeys was significantly different from the null model (likelihood ratio test: $\chi^2_9 = 164.4$, $P < 0.001$). This model

Table 2
Model-averaged LMM of glucocorticoid variation in mantled howler monkeys.

Term	β	SE	95% CI	Model	Importance
Intercept	5.33***	0.12	5.028–5.501	1,2,3	
Sex/reproductive state				1,2,3	1.00
Male	0.16	0.12	−0.068–0.361		
Gestating	0.50**	0.15	0.193–0.758		
Lactating	0.55***	0.14	0.244–0.738		
Activity	0.23*	0.13	0.018–0.483	2	0.29
Agonism	0.13*	0.07	0.006–0.267	3	0.19

Difference in AIC_c values between Model i and the model with the lowest AIC_c (Model 1): $\Delta_1 = 0.00$, $\Delta_2 = 1.18$, $\Delta_3 = 1.98$. Akaike weights: $w_1 = 0.52$, $w_2 = 0.29$, $w_3 = 0.19$. Parameters shown are model-averaged parameter estimates (β), unconditional standard errors which incorporate model uncertainty (SE), 95% confidence intervals, models that included the term, and relative importance.

* $P < 0.05$.

** $P < 0.001$.

*** $P < 0.0001$.

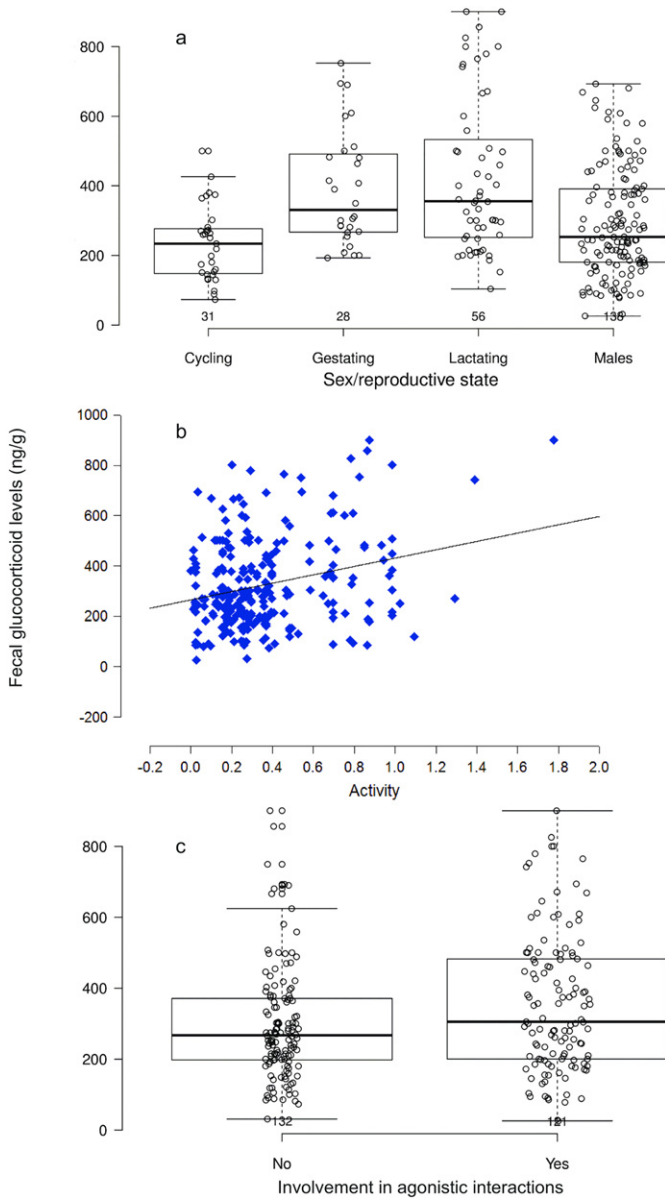


Fig. 2. Variation in fecal glucocorticoid levels of mantled howler monkeys as a function of sex and reproductive state (i.e., males and cycling, gestating or lactating females; a), activity (b), and involvement in agonistic interactions (c). In a and c, center lines show the medians; box limits indicate the 25th and 75th percentiles; whiskers extend 1.5 times the interquartile range from the 25th and 75th percentiles; data points are plotted as open circles. Trend line shown in b only for visualization purposes.

explained 48% of the variation in thyroid hormone levels ($R^2 = 0.48$) and included the factors fruit intake, young leaf intake, sex/reproductive state, activity, and the interaction between sex/reproductive state and activity (Table 3). Sex/reproductive state and activity were the most important factors in the averaged model, although only the former had a significant effect on thyroid hormone levels. Model 1 was supported 1.6, 1.7, and 2.7 times more than Models 2, 3, and 4, respectively.

Thyroid hormone levels were higher in gestating females (mean \pm SE = 359.9 ± 17.0 ng/g; range = 208.7–527.5 ng/g), followed by lactating females (314.1 ± 14.6 ng/g; range = 99.8–540.0 ng/g), males (286.2 ± 11.4 ng/g; range = 80.9–730.0 ng/g), and cycling females (272.6 ± 25.2 ng/g; range = 90.6–705.0 ng/g; Fig. 3). Thyroid hormone levels were also positively related to fruit intake (Fig. 4a) and activity (Fig. 4b), but negatively related to young leaf intake (Fig. 4c). The increase in thyroid hormone at higher activity levels was steeper for males and cycling females than for gestating and lactating females (Fig. 4d).

Table 3
Model-averaged LMM of thyroid hormone variation in mantled howler monkeys.

Term	β	SE	95% CI	Model	Importance
Intercept	5.01***	0.46	4.167–6.149	1,2,3,4	
Fruit intake	0.01***	0.001	0.001–0.012	1,2,4	0.77
Young leaf intake	-0.01***	0.01	-0.012–(-0.009)	3	0.23
Sex/reproductive state				1,2,3,4	1.00
Male	0.09	0.10	-0.114–0.307		
Gestating	0.43**	0.15	0.143–0.751		
Lactating	0.23	0.16	-0.089–0.571		
Activity	0.38		-0.235–1.084	1,2,3	0.86
Activity \times sex/reproductive state				2	0.24
Male	-0.41	0.32	-1.049–0.224		
Gestating	-0.73*	0.35	-1.425–(-0.034)		
Lactating	-0.80*	0.33	-1.449–(-0.157)		

Difference in AICc values between Model *i* and the model with the lowest AICc (Model 1): $\Delta_1 = 0.00$, $\Delta_2 = 0.93$, $\Delta_3 = 1.06$, $\Delta_4 = 1.96$. Akaike weights: $w_1 = 0.39$, $w_2 = 0.24$, $w_3 = 0.23$, $w_4 = 0.14$. Parameters shown are model-averaged parameter estimates (β), unconditional standard errors which incorporate model uncertainty (SE), 95% confidence intervals, models that included the term, and relative importance.

* $P < 0.01$.
** $P < 0.001$.
*** $P < 0.0001$.

4. Discussion

Our results indicate that fecal glucocorticoid and thyroid hormone levels of mantled howler monkeys reflect variation in energetic condition, which is chiefly related to the sex/reproductive state of individuals. However, whereas glucocorticoids were only related to energy expenditure (sex/reproductive state, activity, agonism), thyroid hormone levels were related to both energy acquisition (fruit and young leaf intake) and expenditure (sex/reproductive state, activity). Furthermore, agonism, a source of psychological stress, was related to glucocorticoid but not to thyroid hormone variation. Therefore, we provide evidence that these hormones offer complementary information on the energetic condition of individuals.

Reproductive females (i.e., gestating and lactating) had higher glucocorticoid and thyroid hormone levels than cycling females and males. This is in line with studies demonstrating that gestation and lactation are associated with maternal provisioning of thyroid hormone to the fetus, enhanced HPA function due to interaction with estrogens, placental production of corticotropin-releasing hormone, and higher metabolic demands (Blum et al., 1983; Handa et al., 1994; McLean and Smith, 1999; Weingrill et al., 2004; Setchell et al., 2008; Carnegie et al.,

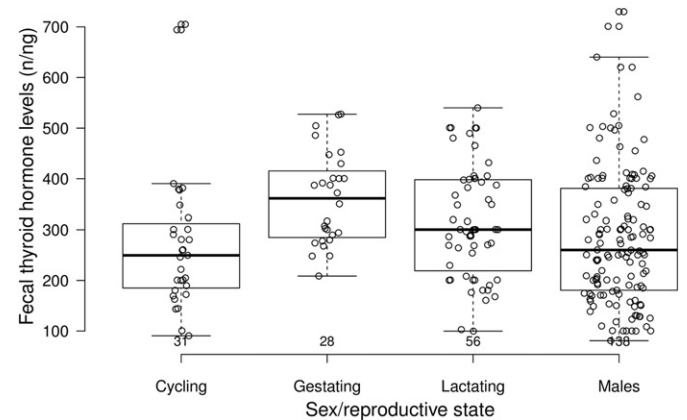


Fig. 3. Variation in fecal thyroid hormone levels of mantled howler monkeys as a function of sex and reproductive state (i.e., males and cycling, gestating or lactating females). Center lines show the medians; box limits indicate the 25th and 75th percentiles; whiskers extend 1.5 times the interquartile range from the 25th and 75th percentiles; data points are plotted as open circles.

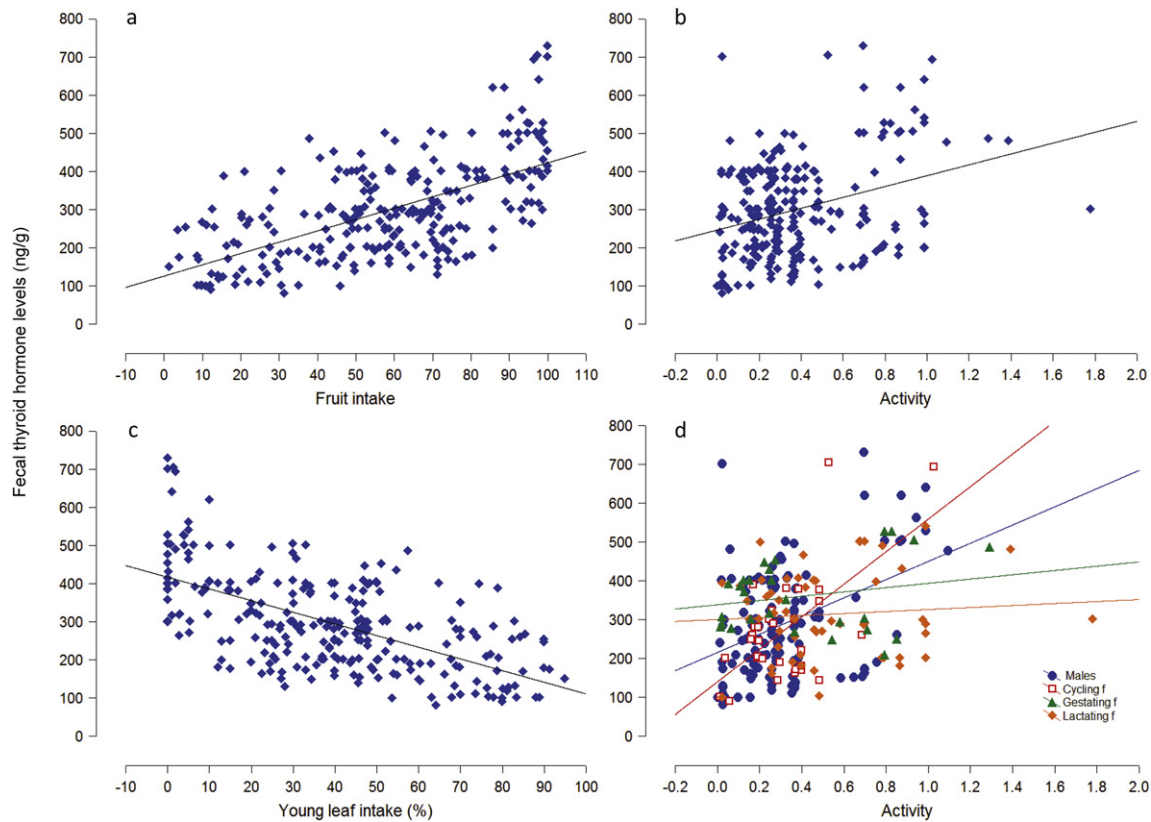


Fig. 4. Variation in fecal thyroid hormone levels of mantled howler monkeys as a function of fruit intake (a), activity (b), young leaf intake (c), and the interaction between activity and sex/reproductive state. Trend lines shown only for visualization purposes.

2011; Lazarus, 2011). The high energetic costs of female reproduction are confirmed by the comparatively lower thyroid hormone levels of males. Basal metabolism is an allometric function of body size (White and Seymour, 2003), and thyroid hormones are deeply involved in the regulation of basal metabolism, so males could be expected to have higher thyroid hormone levels than females when sexual dimorphism in body size exists (Boucai et al., 2011). Such expectation was confirmed when comparing males (which in this species are 32.1% larger than females: Kelaita et al., 2011) with cycling females, but not when comparing them with gestating and lactating females. This result, the observed variable influence of the interaction between activity and sex/reproductive state on thyroid hormone levels, and the varying correlation between both hormones depending on subject sex/reproductive state, further highlight the metabolic constraints faced by females during gestation and lactation.

Whereas glucocorticoids were higher in lactating females, thyroid hormone levels were higher in gestating females, suggesting that different mechanisms for metabolic regulation may act in each reproductive state. Previous research suggests that, as in other primates and carnivores (e.g., Abbott et al., 2003; Sands and Creel, 2004), variation in glucocorticoid levels of howler monkeys signals social challenges (e.g., Cristóbal-Azkarate et al., 2007; Rangel-Negrín et al., 2014; Martínez-Mota et al., 2016), and that the glucocorticoid levels of reproductive females increase when they participate in agonistic interactions (Gómez-Espinosa et al., 2014). Lactating females should be particularly sensitive to psychological stressors, as they are more vulnerable to ecological (e.g., predation) and social (e.g., infanticide) risks than other individuals (Caro, 1987; Cowlshaw, 1999; Wolff and van Horn, 2003) and, as a consequence, invest more energy in vigilance (Barrett et al., 2006). Our results converge with that evidence, and highlight the importance of accounting for both reproductive status and individual involvement in agonistic interactions, as well as other sources of psychological stress (e.g., inter-group encounters: Schoof and Jack,

2013), when studying glucocorticoids. Regarding thyroid hormone, the most parsimonious explanation for the higher levels observed among gestating females, is excess production to be delivered to the fetus (Lazarus, 2011), as preliminary evidence indicates that in this species C-peptide levels (another marker of energetic condition: Emery Thompson, 2017) do not vary between gestating and lactating females (Cano-Huertes et al., under review).

Thyroid hormones regulate energy expenditure/saving strategies, and are positively related to food intake (Fisher, 1996; Moon et al., 1999; Flier et al., 2000; Wasser et al., 2010). In our study, however, thyroid hormone levels were positively related to fruit intake but negatively related to young leaf intake. Yet, because fruits were the main foods of mantled howler monkeys, it is possible that the consumption of young leaves was associated with a decrease in the availability of fruits, in which case the decrease in thyroid hormone levels could be associated with an energy saving strategy. Such possibility is further supported by the positive relationships between activity and both thyroid hormone levels (Table 3) and young leaf consumption ($r = -0.20$, $P = 0.001$). Our results confirm therefore the role of thyroid hormones in the regulation of basal metabolic rate associated with varying activity levels (Pakarinen et al., 1988; Simsch et al., 2002) and nutritional stress (Eales, 1988; Cristóbal-Azkarate et al., 2016). Interestingly, glucocorticoid levels were not affected by food intake. Covariation of glucocorticoids and food intake is usually interpreted as either a consequence of fluctuations in nutrient acquisition that impact the use of energy reserves (Sapolsky et al., 2000; Muller and Wrangham, 2004; Pride, 2005), or as a byproduct of the energetic investment in food acquisition (e.g., Cavigelli, 1999; Dunn et al., 2013; but see Pontzer et al., 2014; Pontzer, 2015). The latter explanation is supported in our study by the inclusion of activity levels in the averaged glucocorticoid model, and is in line with evidence of a positive correlation between hormone secretion and physical activity (e.g., Tharp, 1975; Girard and Garland, 2002). Still, interpretation of these results must be made with caution in light

of the fact that we did not measure actual nutrient intake (Martínez-Mota et al., 2016), and that travel rate was not an influential factor in our models.

Finally, ambient temperatures were not related to variation in both hormones. There is evidence that variation in temperature is positively correlated with basal metabolic rates, influencing therefore thyroid function (Jenni-Eiermann et al., 2002; Silva, 2006; Bianco et al., 2005). There is also evidence that glucocorticoid levels increase under exposure to extreme temperatures (Wingfield et al., 1992; Beehner and McCann, 2008; Rogovin et al., 2008; Houser et al., 2011). Because hormonal responses of individuals to ambient temperature have been usually found in species that live in environments with extreme temperatures (e.g., deserts: Wingfield et al., 1992; Rogovin et al., 2008; high altitude: Beehner and McCann, 2008; Cristóbal-Azkarate et al., 2016), it is possible that the tropical climate (Afin Köppen climate classification) of our study area does not elicit such responses.

The present study demonstrates that the metabolic function of howler monkeys may be assessed through the non-invasive measurement of glucocorticoid and thyroid hormones. By combining glucocorticoid and thyroid hormone measures, we could also parse the influence of psychological vs. metabolic challenges for individual energetic condition, which may be instrumental for deciding which factors should be accounted for when studying different hormone-behavior interactions. These contributions will most certainly help in understanding the physiological basis for the notable degree of behavioral flexibility exhibited by howler monkeys (e.g., feeding habits: Dias and Rangel-Negrín, 2015b; social organization: Di Fiore and Campbell, 2007; mating systems: Van Belle and Bicca-Marques, 2015), but most importantly, open the window into the study of the adaptive significance of glucocorticoid and thyroid hormone variation in wild primates (Beehner and Bergman, 2017).

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