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Correlates of hormonal modulation in mantled howler monkey males, *Alouatta palliata*

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Abstract

Objectives: The study of hormone modulation may offer important insight into the responses of individuals to environmental challenges. Here we studied C-peptide, thyroid hormone (T3), glucocorticoid (GC), and testosterone (T) metabolites of mantled howler males to assess: 1) correlations among hormones; 2) individual and temporal variation in hormone concentrations; and 3) the influence of ecological, climatic, behavioral, social, and reproductive factors on hormone variation.

Methods: We studied 10 adult males at La Flor de Catemaco (Mexico) from January 2012 to December 2016. We collected information on food availability; ambient temperature; time budgets; male involvement in mating, agonistic interactions, and interactions with extragroup males. We analyzed C-peptide concentrations in urine samples and T3, GC, and T in fecal samples.

Results: C-peptide was negatively correlated with other hormones, whereas T3, GC, and T were positively related. Hormonal variation was unrelated to individual or yearly differences. Food availability was positively related to C-peptide and T3, and negatively related to GC. Involvement in mating was positively related to T3 and T, whereas the rate agonistic interactions was positively related to GC and T. The rate of interactions with extragroup males was positively related to T. When males mated, the increase in C-peptide and the decrease in GC with increasing food availability were less notable.

Conclusions: Hormonal variation in mantled howler monkey males is generally stable, but it is influenced by several factors. Our results offer a broad picture of the hormonal modulation of mantled howler monkey males in response to diverse challenges.

KEYWORDS
energy balance, food biomass, glucocorticoids, mating, testosterone, thyroid hormone

1 | INTRODUCTION

The study of the behavioral ecology of primate females has mostly concentrated in understanding how they cope with biotic and abiotic challenges in their environments to maximize their reproductive success (e.g., Lee & Kappeler, 2003; Pusey, 2012). In contrast, studies on male behavioral ecology have heavily focused on intrasexual competition, and particularly on the covariation of dominance rank and reproductive success (Di Fiore, 2003; Majolo et al., 2012). This contrast is related to a basic premise of evolutionary theory: members of the sex

that invest less in offspring (i.e., males in most primate species) are expected to compete among themselves to mate (Trivers, 1972). Still, in the same way that females experience mating competition (Beehner & Lu, 2013; Huchard & Cowlishaw, 2011), the reproductive success of males may be affected by factors other than intrasexual competition. Given their key role in the allocation of resources to different functions, such as metabolism, growth, and reproduction (Nelson & Kriegsfeld, 2016), hormones may be particularly useful to explore the ecological, abiotic, reproductive, and social constraints faced by primate males.

Ecological factors may influence the allocation of time and energy to reproduction and other basic functions. For instance, when food availability is low, long-tailed macaque males (*Macaca fascicularis*) feed less while guarding females (Girard-Buttoz, Heistermann, Rahmi, Agil, et al., 2014). Disruption of foraging by mating activities is frequent among primates (e.g., *Papio cynocephalus*: Alberts et al., 1996; Rasmussen, 1985; *Macaca fascicularis*: Girard-Buttoz, Heistermann, Rahmi, Marzec, et al., 2014; *Macaca mulatta*: Higham et al., 2011; *Macaca fuscata*: Matsubara, 2003; *Saimiri sciureus*: Stone, 2014), and often results in a depletion of energy reserves (chimpanzees, *Pan troglodytes*: Georgiev et al., 2013; white-faced capuchins, *Cebus capucinus*: Schoof et al., 2014). Less is known, however, about time and energy allocation of males in non-reproductive contexts, although barbary macaques (*Macaca sylvanus*) downregulate their energy expenditure when food availability and ambient temperatures are low (Cristóbal-Azkarate et al., 2016). Similarly, yellow baboons (*Papio cynocephalus*) fallback on energy reserves in the dry season, when they reduce food intake and increase energetic expenditure (Gesquiere et al., 2011). Thus, physiological modulation is linked to the interplay of ecological factors and both time and energy allocations, although most evidence comes from studies assessing male behavior and physiology in reproductive contexts.

Gaining access to females is a major component of variation in male reproductive success (Alberts, 2012), and may involve mate guarding, the defense of a territory, ritualized displays, aggressive interactions, alternative mating tactics, and/or the establishment of dominance hierarchies (Emery Thompson & Georgiev, 2014; Muller & Emery Thompson, 2012), all of which may entail costs. In addition to increasing the risk of injury and death (e.g., gray mouse lemurs, *Microcebus murinus*: Kraus et al., 2008; Tibetan macaques, *Macaca thibetana*: Zhao, 1994), male intrasexual competition is linked to high physiological stress (chimpanzees: Muller & Wrangham, 2004a), low energetic balance (chimpanzees: Emery Thompson et al., 2009; *Cebus capucinus*: Schoof et al., 2014), and increased disease risk (yellow baboons: Archie et al., 2012). Even when males that live in the same group share sexual access to females, increases in physiological stress are associated with mating (muriquis, *Brachyteles hypoxanthus*: Strier et al., 1999) and competition with extragroup males may be an important source of male reproductive effort (spider monkeys, *Ateles* spp.: Aureli et al., 2006; Gibson, 2010; muriquis: Strier et al., 2002; Talebi et al., 2009).

Testosterone, which regulates the male reproductive axis, plays an important role in male intrasexual competition in both mating and non-mating contexts (Oliveira, 2004). For example, testosterone concentrations in male white-faced capuchins can be high when receptive females are present (independent of male rank and age), as well as in alpha vs. subordinate males (independent of the presence of receptive females; Schoof et al., 2014). In black (*Alouatta pigra*) and mantled howler monkeys (*A. palliata*), for instance, variation in testosterone concentrations is mostly linked to competition with extragroup males (Cristóbal-Azkarate et al., 2006; Rangel-Negrín et al., 2011). Thus, the physiological responses of males to mating competition are associated with both challenges coming from within and outside their groups. Given the potential immunosuppressive effects of testosterone (Prall & Muehlenbein, 2014), male responses to intrasexual competition that involve this physiological mechanism may entail health costs.

In the present study, we explore the hormonal modulation of male mantled howler monkeys in relation to ecological, climatic, behavioral, reproductive, and social factors. Mantled howler monkeys have one of the widest geographical distributions among primates (Mexico to Peru) and live under diverse conditions (e.g., 0 to 2000 m a.s.l.: Baumgarten & Williamson, 2007; annual rainfall of 1431 to 4900 mm: Dias & Rangel-Negrín, 2015a). At Los Tuxtlas (Veracruz, Mexico), where we conducted the study, mantled howler monkeys live in tropical evergreen forests where both climate and food availability vary through time. There is a dry season in the months of February to May and a rainy season from June to January. Part of the rainy season (October to January) is a period of storms (locally known as "nortes"), characterized by episodic strong north winds and a decrease in ambient temperature (Soto & Gama, 1997). Food availability is higher in the months of May to September than in the October–April period (Ceccarelli et al., 2020). Mantled howler monkeys respond to this variation with behavioral, physiological, and reproductive flexibility. For instance, in the late "nortes" and early dry season, fruit consumption is significantly reduced (Dunn et al., 2010) and physiological stress levels increase (Dunn et al., 2013). Mantled howler monkeys travel slowly and infrequently when ambient temperature is low (Ceccarelli et al., 2019), and during the dry season, they travel for longer periods than at other times (Gómez-Espinosa et al., 2014). Most conceptions and weaning of offspring occur in the late dry-early wet seasons (Cristóbal-Azkarate et al., 2017), although female energy balance does not seem to be affected by fluctuation in energy intake (Rangel-Negrín et al., 2021). This possibly reflects the fact that variation in fruit availability (the main source of energy in mantled howler monkeys' diet) is relatively low and there are fruiting trees during the entire year (Cano-Huertes et al., 2017).

This species lives in single-male/multi-female or multi-male/multi-female groups (Di Fiore & Campbell, 2007; Treves, 2001). Although intragroup social interactions are infrequent and subtle (e.g., Corewyn & Kelaita, 2014; Wang & Milton, 2003), and extragroup relationships are mainly based on long distance vocal communication (i.e., loud calls: Kitchen et al., 2015), both intra and extragroup male competition is intense, and aggression events (mostly during immigration events) frequently result in physical injury and death (Cristóbal-Azkarate et al., 2004; Dias et al., 2010; Ryan et al., 2008). Aggression directed at extragroup males may serve to avoid infanticidal events

(Crockett, 2003). Dominance relationships among males are generally non-linear, but in some populations, particular males (central males) may show priority of access to resources (females and food), are more active in vocal contests with extragroup individuals, and have a spatially central position within their groups (Dias et al., 2010; Jones, 1980, 1985; Wang & Milton, 2003; but see Corewyn, 2015). Nevertheless, reproductive skew appears to be low in mantled howler monkeys (Corewyn, 2015; Jones, 1995), a probable consequence of the fact that, although central males guard receptive females, alternative reproductive behaviors (e.g., sneaky copulations) have been documented (Corewyn, 2015; Jones, 1995). Males of this species are therefore exposed to a large array of challenges, which should associate with hormonal modulation.

Here, we studied four hormones, C-peptide, the thyroid hormone triiodothyronine, glucocorticoids, and testosterone, all of which may be non-invasively measured in wild mantled howler monkeys (Cano-Huertes et al., 2017; Cristóbal-Azkarate et al., 2006; Dias et al., 2017; Gómez-Espinosa et al., 2014; Thompson et al., 2017). C-peptide is a reliable proxy for insulin production, and thus its excretion is positively related to energy balance (Emery Thompson et al., 2009; Georgiev et al., 2013). Triiodothyronine is involved in the regulation of the basal metabolic rate through the modulation of oxidation rates in tissues and increases in response to increased activity and thermoregulatory demands (Cristóbal-Azkarate et al., 2016; Dias et al., 2017). Glucocorticoids are primary stress hormones, and thus may fluctuate as a function of both psychosocial and metabolic challenges (Gesquiere et al., 2011; Muller & Wrangham, 2004a; Sapolsky, 1986; Schoof et al., 2014; but see Schülke et al., 2014). Finally, testosterone is involved in reproductive physiology, such as spermatogenesis, but also in the expression of male secondary sex characteristics, including those associated with intrasexual competition (Muller & Wrangham, 2004b; Wingfield et al., 1990). Although these hormones are involved in different physiological mechanisms, the first three are directly associated with metabolic regulation, whereas the latter is mainly linked to reproductive function. However, if reproductive events entail the allocation of energetic resources, it is possible that the four hormones could be related.

Our first aim was to explore the correlations among these hormones in mantled howler monkey males. Our second aim was to assess variation in hormone concentrations within and between males as well as through time based on the sampling of 10 males for five continuous years. Our third aim was to assess how the hormone concentrations of males varied in relation to ecological (food availability), climatic (ambient temperature), behavioral (general activity, foraging), social (agonism, interactions with extragroup males), and reproductive (mating) factors. Although in our analysis we follow an exploratory approach to assess the relationships between all factors and hormones (information theory), we outline some general predictions based on the evidence described above. First, energetic challenges, represented here by low food availability, low foraging, high general activity, frequent involvement in agonism and interactions with extragroup males, and participation in mating should relate negatively with C-peptide and thyroid hormone concentrations but positively with

glucocorticoids. Second, due to its involvement in thermoregulation, thyroid hormone concentrations should be negatively related to ambient temperature. Third, testosterone concentrations should be positively related to agonism, interactions with extragroup males, and mating.

2 | METHODS

2.1 | Ethical note

This study was noninvasive, adhered to the ASP Code of Best Practices in Field Primatology, and to the ASP Principles for the Ethical Treatment of Nonhuman Primates. 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

We conducted the study at La Flor de Catemaco, Veracruz, México (18°26′39″ N, 95°02′57″ W). The study site is a 250-ha ranch that includes approximately 100 ha of tropical evergreen forest. The climate is warm and humid, with mean annual ambient temperature of 26°C and 2600 mm of rainfall, and follows the typical seasonal pattern of Los Tuxtlas described above (Figure S1).

Mantled howler monkeys living at La Flor de Catemaco have been monitored since 2004. During the present study, there were 29 individuals in the fragment divided into three groups and four solitary individuals. Two groups have been followed systematically since 2012. Subjects are fully habituated to the presence of researchers (i.e., ignore our presence: Williamson & Feistner, 2003). We identify individuals through anatomical and physical characteristics, including body size and proportions, scars, broken fingers, and genital morphology and pigmentation. We focused on the 10 adult males that resided in the two main study groups from January 2012 to December 2016. Individual adult group membership was relatively stable over the 5-year period in addition to the groups having a similar size and composition: 5 adult males, 5 adult females and a variable number of infants and juveniles. Study subjects were fully grown males, with ages ranging between 10 and 22 years (ages determined through observation of births and inspection of dental condition during capture). No clear patterns of priority of access to receptive females have been observed among males of these groups and all males participate in vocal displays.

2.3 | Assessment of food availability and ambient temperature

To assess food availability, each Monday we sampled 397 trees belonging to 59 plant taxa that mantled howler monkeys use as food

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sources at this site (Cano-Huertes et al., 2017). In each tree, we quantified the presence of young and mature leaves, unripe and ripe fruits, and flowers through a visual estimation of the percentage of stems bearing each phenological phase using the following abundance scores: 0 = none, 0.25 = 1%–25%, 0.5 = 26%–50%, 0.75 = 51%–75%, 1 = 76%–100%. Every week we estimated food biomass by multiplying abundance scores of each food item by allometric formulae (described in Cano-Huertes et al., 2017) and summing the resulting values. We collected a total of 250 weekly phenological samplings.

We determined weekly mean ambient temperatures (temperature, hereafter) from daily recordings performed at 1-h intervals with a handheld thermometer (Kestrel 3500 Weather Meter, Nielsen-Kellerman Company, PA, USA).

2.4 | Assessment of behavioral measures

Each month we visited each group for five consecutive days, during which we collected behavioral data and both fecal and urine samples. During each day, we used focal animal sampling with continuous recording (1-h samples) to study the time budgets of males during complete days of follows (i.e., 06:00–07:00 to 17:00–18:00 h, depending on the time of year). We chose all males in a group randomly as focal animals, and resampled males 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: foraging (inspection of food, bringing food to mouth, chewing, and swallowing, or 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: Dias & Rangel-Negrín, 2015b). We also recorded all occurrences of agonistic interactions (i.e., social interactions that involve threatening, aggression, fighting, or submission) between adult males, sexual interactions (Dias & Rangel-Negrín, 2015b), and involvement in vocal, visual (<50 m), and physical interactions with extragroup individuals (solitary or from other groups). We collected a mean ± SD of 277.3 ± 76.8 h of focal samples per male (55.5 ± 15.4 h per male per year), and 3364 h of all-occurrences sampling.

2.5 | Sample collection and hormonal assays

We collected fecal and urine samples from males immediately after deposition whenever samples were unequivocally matched to subject identity, resulting in the collection of a total of 2671 fecal samples (mean ± SD = 53.4 ± 6.3 samples per male per year) and 2417 urine samples (48.3 ± 2.5 samples per male per year). We collected fecal samples uncontaminated by urine from the forest floor and deposited them in polyethylene bags labeled with the identity of each individual. We collected fresh urine samples uncontaminated by soil, feces, or urine from other individuals by pipetting urine from a plastic sheet positioned underneath males during urination or directly from vegetation. We deposited urine samples in 5 ml snap-cap polypropylene

microtubes. We kept samples in a cooler with frozen gel packs while in the field and stored them at the end of the day in a freezer at −20°C until processing. We freeze-dried (FreeZone 18, Labconco, Kansas City, MO) all fecal samples within a maximum of 6 months after collection.

We extracted fecal triiodothyronine metabolites (T3 hereafter), fecal glucocorticoid metabolites (GC hereafter), and fecal testosterone metabolites (T) following a modification of the method described in Wasser et al. (2000). Briefly, we shook 0.6 g of homogenized, lyophilized and pulverized feces for 20 h in 4.0-ml analytical-grade methanol. We centrifuged extracts (460 g for 30 min) and recovered the supernatant. After complete evaporation of the solvent in a water bath at 60°C for 20 h, we reconstituted pellets with 3-ml albumin buffer which we used for T3, GC, and T assays.

We determined urinary C-peptide concentrations (C-peptide hereafter) using a radioimmunoassay (Human C-Peptide, Merck Millipore, Billerica, MA, USA) and a gamma counter (Cobra Model E5005, Packard, Inc., Downers Grove, IL, USA). We measured T3, GC, and T with chemiluminescent enzyme immunoassay commercial kits (Total T3, Cortisol and Total Testosterone [Immulite, Siemens, CA, USA], respectively) and an Immulite 1000 analyzer (Siemens, CA, USA). We performed analytical validations for all assays as described in Rangel-Negrín et al. (2014) and Table 1. These validations indicate that our assays were accurate (i.e., dose–response curves and serial dilutions of fecal samples ran parallel standards), precise (i.e., low variation in repeated measures of the same samples within and between assays), and that we could recover a significant amount of hormones. The protocols that we used to extract and quantify C-peptide, T3, and GC have been previously biologically validated to detect insulin and thyroid function, and the activation of the hypothalamic–pituitary–adrenal axis, respectively, in mantled howler monkeys (Cañadas-Santiago et al., 2019; Cano-Huertes et al., 2017; Dias et al., 2017). Given that no captive animals were available to perform a gonadotropin releasing hormone (GnRH) challenge test to validate our T assay we performed a noninvasive biological validation. Circulating T levels are higher in male than in female primates (Dixson, 2013). Based on this physiological difference and similarly to previous studies (Rangel-Negrín et al., 2011) we compared the T concentrations of five adult males and five adult females to determine if our assays reflected natural variation in fecal T metabolites. Mean (±SD) T concentrations in male samples (2564.8 ± 1167 ng/g, N = 15) were nearly 370% higher than those in female samples (571.9 ± 258 ng/g, N = 15; Mann–Whitney w = 2, p < 0.001; Figure S2).

We standardized C-peptide measurements by dividing C-peptide concentrations by creatinine concentrations (measured via enzyme immunoassays with an auto analyzer: Gallery, Thermo Fisher Scientific, Waltham, MA, USA). We report C-peptide concentrations as pg/mg of creatinine. We report T3, GC, and T concentrations as ng/g of dry feces. We analyzed a total of 1500 urine and 1500 fecal samples (30 samples per male per year per sample type). Given that the lag between hormone secretion and excretion in feces in this species may be approximately 20 to 28 h (Aguilar-Cucurachi et al., 2010), when selecting fecal samples for analysis we excluded samples

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TABLE 1 Analytical validation of hormone assays performed with urine and fecal samples of mantled howler monkey males studied at La Flor de Catemaco from 2012 to 2016

Hormone	Assay[a]	Accuracy[b]	Parallelism[c]	Recovery ± SE (%)	Intra-assay CV (%)[d]	Inter-assay CV (%)[d]
C-peptide[e]	RIA	$R^2 = 0.99, p < 0.01$	$t = 1.0, p = 0.172$	100.4 ± 20.8	6.0	9.5
T3	EIA	$R^2 = 0.99, p < 0.01$	$t = 0.7, p = 0.623$	114.4 ± 18.2	6.2	8.4
GC	EIA	$R^2 = 0.97, p < 0.01$	$t = 0.7, p = 0.673$	81.3 ± 21.7	9.5	7.7
T	EIA	$R^2 = 0.96, p < 0.02$	$t = 0.3, p = 0.832$	96.0 ± 26.6	4.6	6.3

[a]RIA is radioimmunoassay; EIA is enzyme immunoassay; SE is standard error; CV is coefficient of variation.
[b]Dose-response curves $N = 4$.
[c]$N = 5$. Samples were diluted 1:2, 1:4, 1:8, and 1:16.
[d]$N = 10$.
[e]C-peptide concentrations were analyzed in urine samples, whereas other hormone metabolite concentrations were assayed in fecal samples.

collected during the first day of each monthly sampling session for each group. Variation in hormone concentrations was not influenced by 1) time elapsed from fecal sample collection to sample lyophilization (i.e., no effect of the number of days samples were frozen before drying on hormone metabolite concentrations); 2) time elapsed from sample collection to sample assay (i.e., no effect of the number of days samples were frozen before assay on hormone metabolite concentrations); and 3) time of the day in which samples were collected (i.e., no effect of the sample collection schedule on hormone metabolite concentrations; Table S1).

2.6 | Data analysis

We averaged (mean values) all variables per climatic season (i.e., dry, rainy, and storm seasons) per year. Therefore, each male was represented in the dataset by 15 sampling blocks (i.e., three blocks per five study years). With this data organization, mean hormonal concentrations per male per year/season were based on 10 samples, which we consider that provides a reasonable representation of individual hormone concentrations in each 4-month sampling block. In addition to the hormone concentrations of males, we defined the following predictive variables: 1) food availability; 2) ambient temperature; 3) general activity; 4) foraging; 5) rate of participation in agonistic interactions with other males; 6) the involvement of males in mating with receptive females; and 7) rate of participation in interactions with extragroup males (Table 2).

Before data analysis, we standardized all continuous variables by centering and scaling. Our analysis was three-fold. First, we explored the relationships between hormone concentrations with Pearson correlation tests on mean hormone concentrations per male per sampling block (please note that animal identity and sampling period had no consistent effects on hormone concentrations: Tables S3–S6). Second, we examined the influence of inter-individual and annual variation on hormonal concentrations with generalized linear models (GLMs) fitted with a Gaussian distribution and an identity link function. We followed an information-

TABLE 2 Variables assessed in this study pertaining to mantled howler monkey males studied at La Flor de Catemaco from 2012 to 2016

Variable	Attributes in this study
Total food availability[a]	The sum of leaf (young and mature leaves) and fruit (unripe and ripe fruits) biomass (min = 12,156; max = 20,803; mean (±SD) = 15,888 ± 2069 t/ha)
Ambient temperature[a]	Mean ambient temperature (min = 17; max = 30; mean (±SD) = 25 ± 3°C)
General activity[b]	The ratio of time spent foraging, moving, and socializing to time resting per focal sample (min = 0.02; max = 0.99; mean (±SD) = 0.29 ± 0.23)
Foraging[b]	Percentage of time spent foraging per focal sample (min = 5.6; max = 53.4; mean (±SD) = 15.6 ± 26.8%)
Agonism[b]	Frequency of agonistic interactions exchanged with other males divided by observation time (min = 0; max = 1.4; mean (±SD) = 0.24 ± 0.27 interactions/h)
Mating[b]	Individual involvement (recorded as a yes/no dichotomy) in sexual interactions (Table S2)
Interactions with extragroup males[b]	Frequency of physical, visual, and vocal encounters with other males (solitary or from other groups) divided by observation time (min = 0; max = 1; mean (±SD) = 0.1 ± 0.1 encounters/h)
C-peptide[b]	Urinary C-peptide concentrations (min = 54.7; max = 16,366.3; mean (±SD) = 2960.6 ± 3314.3 pg/mg creatinine)
T3[b]	Fecal triiodothyronine metabolite concentrations (min = 80.9; max = 702.5; mean (±SD) = 280.0 ± 141.7 ng/g)
GC[b]	Fecal glucocorticoid metabolite concentrations (min = 26.0; max = 694.4; mean (±SD) = 319.7 ± 166.3 ng/g)
T[b]	Fecal testosterone metabolite concentrations (min = 898.8; max = 8001.5; mean (±SD) = 2878.1 ± 1269.0 ng/g)

[a]Variables calculated per season per year.
[b]Variables calculated per male per season per year.

theoretic approach and multimodel inference to generate a set of models based on all combinations of predictors and averaged parameters across all resulting models (Burnham & Anderson, 2013). We calculated the relative importance of each predictor based on its Akaike weight in all models (Σw_i). As model weights represent the probability of a model to be the best in the model set and thus reflect model uncertainty, importance can be understood as the likelihood of a predictor to be included in the best model (Burnham & Anderson, 2013).

Third, we built four linear mixed models (LMMs) to analyze variation in each hormone in which the fixed predictors were total food availability, ambient temperature, general activity, foraging, rate of participation in agonistic interactions, the involvement of males in mating, and rate of participation in interactions with extragroup males. Male identity and year were random factors to account for the repeated sampling of males through time. We diagnosed collinearity among predictors via variance inflation factors (VIF) and found that food availability (VIF = 22.5), ambient temperature (VIF = 15.1), general activity (VIF = 7.7), and foraging (VIF = 15.6) had high VIF (Figure S3). We simplified models by excluding one variable at a time, then two at a time, and so on until all predictors had VIF < 3. Following this process, we retained four predictors: food availability (VIF = 2.9), occurrence of mating (VIF = 1.9), rate of agonistic interactions (VIF = 2.1), and rate of extragroup interactions (VIF = 1.5). We also added to models the interactions between continuous predictors and involvement in mating. In these LMMs we followed the information-theoretical approach described above. We compared complete models (i.e., with fixed and random predictors) with a null model including only the random factors with likelihood ratio tests to determine whether the fixed factors explained more variation in
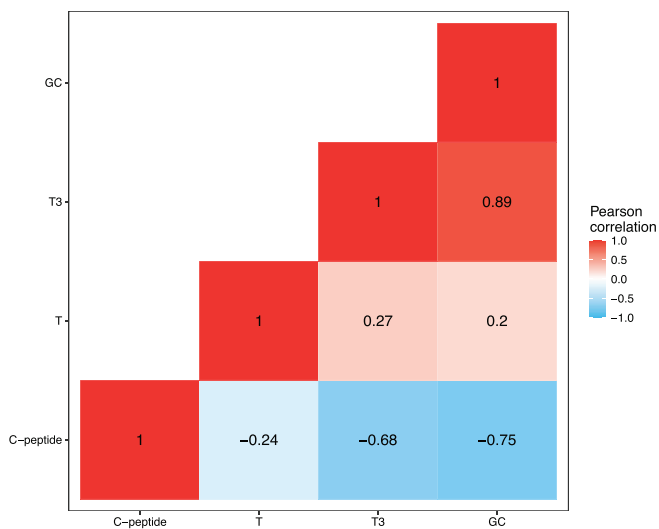

FIGURE 1 Correlogram depicting the relationships among hormone metabolite concentrations of male mantled howler monkeys studied at La Flor de Catemaco (Mexico) from 2012 to 2016. T3 are fecal triiodothyronine metabolites, GC are fecal glucocorticoid metabolites, and T are fecal testosterone metabolites. Color scale (defined on the right) corresponds to variation in Pearson correlation coefficients

TABLE 3 Information-theoretic based linear mixed models of the factors influencing hormone concentrations in mantled howler monkey males studied at La Flor de Catemaco from 2012 to 2016

Predictor	C-peptide 94.5%[a] β	SE	95% CI	Σwi	T3 80.9% β	SE	95% CI	Σwi	GC 88.4% β	SE	95% CI	Σwi	T 68.9% β	SE	95% CI	Σwi
Food availability	**1.03**	**0.04**	**1.01 to 1.58**	**1.00**	**0.90**	**0.07**	**0.76 to 1.04**	**1.00**	**−1.15**	**0.10**	**−1.35 to −0.95**	**−1.00**	0.04	0.18	−0.32 to 0.41	0.13
Food availability × mating	**0.99**	**0.19**	**0.62 to 1.36**	**1.00**	0.04	0.10	−0.16 to 0.23	1.00	**−0.71**	**0.11**	**−0.94 to −0.48**	**−1.00**	−0.25	0.24	−0.75 to 0.25	−0.04
Mating	0.15	0.09	−0.56 to 0.25	0.76	**0.14**	**0.06**	**0.02 to 0.26**	0.76	−0.04	0.14	−0.32 to 0.24	−0.75	**0.72**	**0.30**	**0.14 to 1.31**	**0.80**
Agonism	0.12	0.04	−0.04 to 0.59	0.64	−0.01	0.11	−0.23 to 0.22	0.64	**0.21**	**0.08**	**0.05 to 0.37**	**0.98**	**0.27**	**0.16**	**0.03 to 0.58**	**0.75**
Agonism × mating	−0.34	0.23	−0.79 to 0.11	0.40	0.09	0.09	−0.10 to 0.28	0.40	−0.13	0.15	−0.43 to 0.17	0.17	−0.31	0.22	−0.76 to 0.13	0.20
Intergroup encounters	0.03	0.04	−0.23 to 0.13	0.06	0.07	0.04	−0.19 to 0.18	0.06	0.11	0.06	−0.00 to 0.22	0.30	**0.66**	**0.14**	**0.37 to 0.95**	**1.00**
Intergroup encounters × mating	0.02	0.18	−0.33 to 0.37	<0.01	−0.01	0.09	−0.02 to 0.15	<0.01	−0.05	0.11	−0.27 to 0.17	0.05	−0.26	0.22	−0.71 to 0.18	0.18

Note: Model-averaged parameter estimates (β), unconditional standard errors which incorporate model uncertainty (SE), 95% confidence intervals (95% CI), and importance values (Σw_i). Numbers in bold represent the cases in which predictor beta values did not include zero.
[a]Pseudo R^2 values.

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hormone concentrations than the random factors alone (Pinheiro & Bates, 2000) and in all cases found significant differences ($p < 0.001$ in all tests).

In both GLMs and LMMs we checked that the assumptions of normally distributed and homogeneous residuals were fulfilled by visually inspecting Q–Q plots and the residuals plotted against the fitted values for each model. Model residuals did not deviate significantly from normality (all Kolmogorov–Smirnov tests $p > 0.05$). We calculated marginal pseudo-coefficients of determination for each model to assess the variance (deviance) in hormonal concentrations explained by each model (i.e., goodness-of-fit). We conducted all analysis in R 4.0.4 (R Core Team, 2021).

3 | RESULTS

3.1 | Correlations among hormones

T3 was strongly and positively related with GC concentrations (95% CI $= 0.845$ to 0.916), whereas C-peptide was negatively related to both T3 (95% CI $= -0.756$ to -0.581) and GC (95% CI $= -0.814$ to -0.673) concentrations (Figure 1). T concentrations were moderately related with other hormones: positively related with T3 (95% CI $= 0.116$ to 0.413) and GC (95% CI $= 0.045$ to 0.353); negatively related with C-peptide concentrations (95% CI $= -0.388$ to -0.086).

3.2 | Interindividual and temporal variation in hormone concentrations

Variation in hormone concentrations was not related to interindividual (C-peptide $\Sigma w_i = 0.01$; T3 $\Sigma w_i < 0.01$; GC $\Sigma w_i < 0.01$; T $\Sigma w_i = 0.09$) or annual differences (C-peptide $\Sigma w_i = 0.18$; T3 $\Sigma w_i = 0.38$; GC $\Sigma w_i = 0.03$; T $\Sigma w_i = 0.01$), as well as to interindividual variation per study year (C-peptide $\Sigma w_i < 0.01$; T3 $\Sigma w_i < 0.01$; GC $\Sigma w_i < 0.01$; T $\Sigma w_i = 0.01$; Tables S3–S7).

3.3 | Factors influencing variation in hormone concentrations

All LMMs pertaining to the influence of ecological and social factors on male hormone concentrations had relatively high explanatory power (>80% of deviance explained) except for the T model which accounted for 68.9% of deviance. Food availability was positively related to both C-peptide and T3 concentrations, and negatively related to GC concentrations (Table 3). The occurrence of mating was positively related to T3 and T concentrations, whereas the rate of participation in agonistic interactions was positively related to GC and T concentrations. T concentrations were also positively related to the rate of extragroup interactions. The interaction between food availability and mating was also an important factor explaining variation in C-peptide and GC concentrations. Specifically, the increase in C-

FIGURE 2 The influence of male involvement in mating on the relationship between food availability and hormone metabolite concentrations of male mantled howler monkeys studied at La Flor de Catemaco (Mexico) from 2012 to 2016: (a) C-peptide; (b) fecal glucocorticoid metabolites (GC). Solid lines and circles (blue, in color version of the figure) are males that did not mate, and dashed lines and triangles (red, in color version of the figure) are males that mated. Areas shaded in gray represent 95% confidence intervals. In panel (a), we fitted the trend line with a gam function
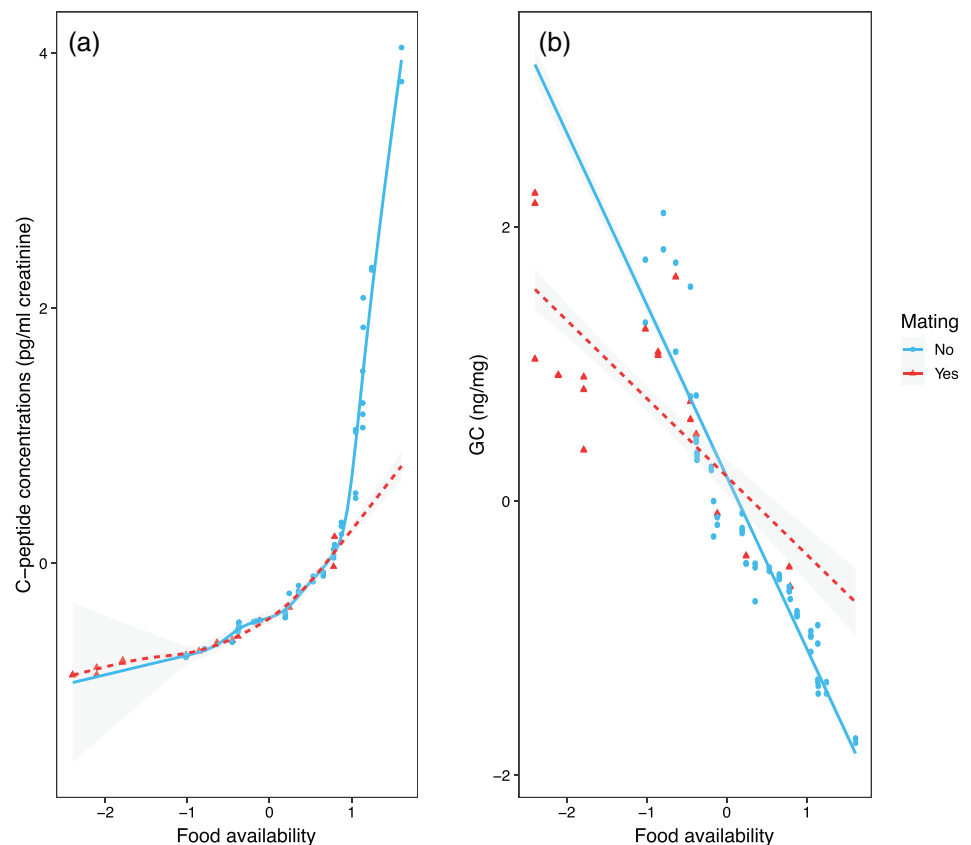
peptide and the decrease in GC concentrations with increasing food availability were less steep in periods when males mated (Figure 2).

4 | DISCUSSION

In this study we examined variation in the concentrations of four hormones, C-peptide, T3, GC, and T in mantled howler monkey males that were sampled for 5 years. C-peptide concentrations were negatively correlated with all other hormones, whereas correlations among the remaining hormones were positive. Food availability was positively related to C-peptide and T3 concentrations and negatively related to GC. Involvement in mating was positively related to T3 and T, whereas the rate of agonistic interactions was positively related to GC and T. The rate of interactions with extragroup males was positively related to T. In periods in which males mated the increase in C-peptide and the decrease in GC concentrations with increasing food availability were less notable. The sample collection regime that we followed (and resulting small sample size) did not allow matching behavioral events (i.e., mating, agonism, intergroup interactions) with urine and fecal samples, and thus, with hormone concentrations. Therefore, the associations between predictors and hormone concentrations that we report pertain to the complete 4-month sampling blocks in which data were organized and do not allow discussing directionality in hormone-behavior relationships. Nevertheless, we believe that the long duration of the study and the detailed sampling regime resulted in a dataset that provides a broad picture of the hormonal modulation underlying male responses to ecological, reproductive, and social factors.

Food availability correlated with variation in hormone concentrations, except for T. Given its positive relationships with ambient temperature, general activity, and foraging, our measure of food availability proxies variation in ecological and climatic factors, as well as the investment howler monkeys make to obtain resources from the environment (general activity and foraging). Although variation in abiotic factors (temperature, photoperiod) and in plant biomass are lower in the tropics than in other regions (van Schaik et al., 1993), it has been demonstrated that they still affect animal behavior and reproductive patterns (Brockman & van Schaik, 2005). In this same population T3 concentrations are positively related to fruit intake (Dias et al., 2017), which in turn is higher when more fruit is available (Gómez-Espinosa et al., 2014). It is possible that, as observed in other primates (Cristóbal-Azkarate et al., 2016; Gesquiere et al., 2011), low C-peptide and T3 and high GC concentrations of males during periods of low food availability and low ambient temperatures are associated with increased difficulty to find food (Dunn et al., 2010; Dunn et al., 2013), energy saving (Dias et al., 2017), and thermoregulatory demands (Thompson et al., 2017). Therefore, ecological and climatic factors have a strong impact on the fluctuation of hormones linked to energy allocation in male mantled howler monkeys.

The critical role of testosterone in male reproductive function through the modulation of physiological processes that sustain reproduction (spermatogenesis: Dixson, 2013) may explain the positive relationship that we found between testosterone concentrations and the participation of males in mating, whereas the positive relationship between mating and T3 suggests covariation between reproductive and male metabolism. In mantled howler monkeys, some males may have priority of access to receptive females (Jones, 1985), but such priority usually involves mate guarding (Corewyn, 2015; Jones, 1995), a behavior that entails time and energy costs resulting from monitoring females and other males (Alberts et al., 1996; Matsubara, 2003). Males that do not have priority of access to females, in turn, display alternative reproductive behaviors, such as sneaking copulations. As all males attain a share of reproduction (Corewyn, 2015; Jones, 1995) reproductive success is not highly skewed in this species (Ellsworth, 2000; Milton et al., 2009). Perhaps due to this unbiased reproductive system, all males may increase general activity when receptive females are present (e.g., Chastel et al., 2003), which could impact their basal metabolic rate (proxied here by T3). Additionally, at high food availability periods, males that were involved in mating had lower C-peptide and higher GC concentrations than males that did not mate. Thus, as observed in other primate species (e.g., Higham et al., 2011; Ostner et al., 2008; Rudolph et al., 2020; Strier et al., 1999), reproduction and energetics are strongly associated in male mantled howler monkeys.

Involvement in agonistic interactions was related to variation in GC and T concentrations. These results are in line with studies demonstrating that intrasexual competition represents a strong challenge for primate males and is linked to hormonal mechanisms (e.g., Bergman et al., 2005; Cristóbal-Azkarate et al., 2006; Jaeggi et al., 2018; Muller & Wrangham, 2004a; Wittig et al., 2015). The positive relationship between rates of agonism and GC also supports previous findings from this population (Dias et al., 2017; Gómez-Espinosa et al., 2014). Mantled howler monkey males do not display aggressive behavior frequently, and most agonistic interactions are of low intensity, usually involving supplants, avoidance, and threats (Corewyn, 2015; Dias et al., 2010; Wang & Milton, 2003). Given that agonism was unrelated to C-peptide and T3 variation, which are primarily energetic markers, our results suggest that these events, even if infrequent, represent sources of psychological stress and may activate the physiological stress response of males. The increase in T in sampling bocks with higher agonism rates is probably linked to the involvement of testosterone in mechanisms that underly the expression of competitive behaviors (Oliveira, 2004; Wingfield et al., 1990).

Increasing T concentrations when challenged by extragroup males may represent a preparative mechanism for subsequent interactions (Booth et al., 2006), as noted by previous research on mantled howler monkeys (Cristóbal-Azkarate et al., 2006). Extragroup males, either living solitarily or in other groups, represent competitors for resident males for the access to females and for the limited number of reproductive positions available in groups (Van Belle & Bicca-Marques, 2015). Additionally, extragroup males may commit infanticide, which has a negative impact on the reproductive success of residents (Crockett, 2003). Extragroup males are therefore usually chased and attacked by resident males (Glander, 1992). Visual and physical

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interactions with extragroup males are infrequent, but involve high risks, as they may escalate into direct fights and result in physical injury or death (Cristóbal-Azkarate et al., 2004; Dias et al., 2010). Vocal interactions, which are the most frequent type of interaction with extragroup males, are less risky but still involve an investment of time and energy (Da Cunha & Byrne, 2006; Kitchen, 2004). However, interactions with extragroup males were not associated with variation in C-peptide, T3, and GC, suggesting that they do not represent strong energetic and psychosocial challenges to resident males. Still, as the upregulation of T may entail costs unrelated to the metabolism, such as the inhibition of the immune system (Klein, 2000; Prall & Muehlenbein, 2014; Schmid-Hempel, 2003) or increased exposure to infectious diseases (Nunn et al., 2014), it remains for future research to determine whether interactions with extragroup influence the health status of mantled howler monkey males.

Given previous evidence that individual attributes, such as age, ontogenetic trajectory, and rank influence the reproductive strategies of males (e.g., Fischer et al., 2004), we assessed whether hormone concentrations varied among males through time, and among males per study year. It is possible that we could not detect those effects due the small sample of males that we analyzed, but it is also plausible that the observed trends correspond to normal fluctuations in behavioral and physiological mechanisms that allow males to cope with challenges in their environment. A demonstration of this proposal, besides requiring a larger sample of males and a more frequent endocrine sampling regimen to ensure that distinctive behaviors are accounted for, should include the study of individuals living under more diverse environmental conditions.

CONFLICT OF INTEREST

The authors declare no potential conflict of interest.

AUTHOR CONTRIBUTIONS

Pedro Américo Duarte Dias: Conceptualization (lead); data curation (lead); formal analysis (equal); funding acquisition (equal); investigation (equal); methodology (equal); project administration (equal); resources (equal); software (equal); supervision (equal); validation (equal); visualization (equal); writing – original draft (equal); writing – review and editing (equal). **Alejandro Coyohua-Fuentes:** Data curation (equal); investigation (equal); writing – original draft (equal); writing – review and editing (equal). **David Roberto Chavira Ramírez:** Formal analysis (equal); investigation (equal); methodology (equal); resources (equal); writing – original draft (equal). **Domingo Canales Espinosa:** Funding acquisition (equal); project administration (equal); resources (equal); writing – original draft (equal); writing – review and editing (equal). **Ariadna Rangel Negrín:** Conceptualization (lead); data curation (equal); formal analysis (lead); funding acquisition (equal); investigation (equal); methodology (equal); project administration (equal); resources (equal); software (equal); supervision (lead); validation (equal); visualization (equal); writing – original draft (equal); writing – review and editing (equal).

DATA AVAILABILITY STATEMENT

Data that support the findings of this study are available from the corresponding author at a reasonable request.

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