# Assessing the influence of biotic, abiotic, and social factors on the physiological stress of a large Neotropical primate in Atlantic forest fragments

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## HIGHLIGHTS

- We assessed fecal glucocorticoid metabolites (fGCM) to determine physiological stress.
- Sex/reproductive state, fruit consumption, and temperature were the main fGCM drivers.
- Group composition, moving effort, and forest cover did not influence fGCM.
- Physiological stress was higher in nursing than in non-nursing females or males.
- Howlers in small fragments showed similar fGCM concentrations than those in large ones.

## GRAPHICAL ABSTRACT
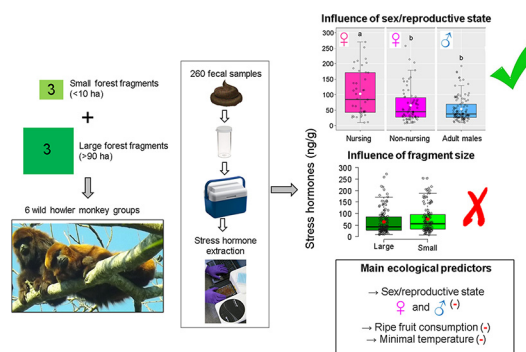


## ARTICLE INFO

## ABSTRACT

Wildlife physiological responses to environmental and human-related stressors provide useful clues on animal welfare. Non-invasive biomarkers, such as fecal glucocorticoid metabolites (fGCM), allow researchers to assess whether variations in habitat quality, behavior, and climate influence the animals' physiological stress. We examined the role of fragment size, ambient temperature, ripe fruit availability and consumption, percentage of records moving, sex, female reproductive state, and group composition as predictors of the level of fGCM in adult brown howler monkeys (*Alouatta guariba clamitans*) inhabiting three small (<10 ha) and three large (>90 ha) Atlantic Forest fragments in southern Brazil. We collected bimonthly behavioral data and fecal samples from adult individuals over three years, and used a multimodel inference framework to identify the main predictors of fGCM. We found that the mean (±SD) fGCM in the study groups ranged from 57 ± 49 ng/g to 93 ± 58 ng/g, which were within the known range for howler monkeys. We found 10 best models including five of the 17 tested variables. Sex and reproductive state were the only variables included in all these models. We found that fGCM was higher in nursing females (mean ± SD = 104 ± 73 ng/g) than in non-nursing females (64 ± 55 ng/g) and males (53 ± 40 ng/g, $P < 0.05$) and that it decreased with increasing ripe fruit consumption and minimum temperature. However, fragment size did not predict fGCM concentration (groups in small fragments = 71 ± 58 ng/g vs. groups in large fragments = 63 ± 54 ng/g, $P > 0.05$). We conclude that factors related to the energetic balance of individuals play major roles in

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modulating the physiological stress of brown howler monkeys. Future studies should investigate the consequences of higher levels of stress hormones on howler monkey health and demography.

## 1. Introduction

Understanding the responses of wildlife to physiological stress caused by environmental, social, and human-related stressors is critical in conservation biology (Gómez-Espinosa et al., 2014; Romero and Wingfield, 2015; Dantzer et al., 2016; Beehner and Bergman, 2017). Overall, the stress response encompasses a suite of behavioral changes and physiological processes presented by an organism to maintain a viable metabolic state when confronted with environmental challenges (Romero and Wingfield, 2015). The complex network of neuronal and hormonal reactions involved in these responses hamper the understanding and interpretation of their positive and negative implications to animal survival at the short- or long-term (Romero et al., 2015; Romero and Wingfield, 2015).

Varying the secretion of glucocorticoid steroid hormones (GC) is one of several strategies employed by animals to deal with stressors. These hormones are widely used stress biomarkers because of two main reasons. Firstly, they have a direct connection with neuroendocrine stress reactions (Romero et al., 2015; Dantzer et al., 2016). Secondly, recent advances in non-invasive methods (e.g., fecal, saliva, and hair sampling) made it easier to measure GCs in wild vertebrates (Dantzer et al., 2016; Beehner and Bergman, 2017). Cortisol is the dominant GC in mammals, whereas the corticosterone is the most common in other vertebrates. The secretion of both hormones is regulated by feedback interactions in the hypothalamic-pituitary-adrenal axis (i.e., HPA axis; Romero and Wingfield, 2015; Beehner and Bergman, 2017). The activation of the HPA axis results in GC secretion in the blood stream in response to internal and external factors (i.e., stressors) that disrupt organismal homeostasis. GC secretion leads to the rapid mobilization of metabolic energy until homeostasis is re-established (Romero et al., 2015). This response contributes to behavioral optimization in animals facing stressors associated with shifts in food availability, variable energetic demands based on female reproductive states, adverse climatic conditions, competition, and predation risk (Romero and Wingfield, 2015; Dantzer et al., 2016; Higham, 2016; Dias et al., 2017).

While acute responses to stressors are necessary to solve immediate challenges, long-term responses to stressors can be deleterious if they compromise an individual's health, growth, and reproduction (i.e., chronic stress: Sapolsky et al., 2000; Beehner and Bergman, 2017). However, evidence of chronic stress in wild vertebrates is weak and controversial (Romero et al., 2015; Dantzer et al., 2016). No clear relationship has been found between chronic stress and GC secretion (reviewed by Romero et al., 2015). Most vertebrates have efficient mechanisms to deal with potential stressors via short-term adaptive actions that maintain homeostasis (i.e., allostasis: Romero and Wingfield, 2015). Therefore, it is likely that the physiological stress reported in many wild vertebrates represents an acute stress response induced by context-specific factors or specific metabolic demands (Romero et al., 2015; Romero and Wingfield, 2015; Beehner and Bergman, 2017).

Researchers have hypothesized that wild animals living in highly disturbed habitats suffer from high levels of physiological stress (e.g., Martínez-Mota et al., 2007; Dantzer et al., 2016; McLennan et al., 2019). For example, habitat loss and fragmentation caused by unsustainable human activities (e.g., deforestation) bring wildlife, humans, and domestic animals closer. In the case of non-human primates, their interaction with humans and domestic animals in anthropogenic landscapes can represent a long-term stressor. Agricultural and urban expansion, mining, and logging are other potential long-lasting stressors afflicting tropical primates (Estrada et al., 2017). These stressors can act via the accumulation of multiple short-term stressors, including

shifts in food availability, particularly the reduction in the availability of highly seasonal, energetic, and preferred foods, such as fleshy fruits (Lambert, 2011; Behie and Pavelka, 2015), intra- and intergroup competition (Castiglione, 2011; Gómez-Espinosa et al., 2014; Markham and Gesquiere, 2017), and extremes of ambient temperature (e.g., McFarland et al., 2014).

Sex-, age-, and reproductive state-based differences in nutritional demands can also represent significant stressors for wild primates (Emery Thompson, 2013). For instance, pregnant, nursing, and infant-carrying females need more energy than adult males (Emery Thompson, 2013; Cantarelli et al., 2017; Dias et al., 2017). These females' potentially higher vulnerability to parasites together with the risk of infanticide can cause higher physiological stress (Martins et al., 2015; Cantarelli et al., 2017; Martínez-Mota et al., 2017).

The Neotropical region harbors the highest primate diversity in the world, with 171 out of 504 commonly recognized species (Estrada et al., 2017). However, few studies have investigated the main predictors of GC secretion in free-ranging Neotropical primates. Research on this topic has focused on a few species of *Alouatta*, *Ateles*, and *Cebus* and often reached contradictory conclusions on the relevance and type of proximal factors driving the secretion of fecal glucocorticoid metabolites (hereafter fGCM, Table 1). In general, the availability and consumption of fruits have an inverse relationship with fGCM secretion (Martínez-Mota et al., 2007; Ordóñez-Gómez et al., 2016; Schoof et al., 2016). Time spent moving (Dunn et al., 2013; Ordóñez-Gómez et al., 2016), exposure to human disturbances (e.g., hunting and logging: Rimbach et al., 2013), social and individual factors related to resource competition or increased nutrient demands (e.g., group size and composition, and inter- and intragroup agonistic interactions, pregnancy and lactation) tend to be positively related to fGCM secretion (Carnegie et al., 2011; Dias et al., 2017).

The brown howler monkey (*Alouatta guariba clamitans*) is an interesting model to study the drivers of physiological stress response. The species is endemic to the Brazilian Atlantic Forest, a highly disturbed and fragmented biome (Scarano and Ceotto, 2015), where most of its remaining populations are confined to <50-ha fragments with varying exposure to humans and domestic animals. Furthermore, its southernmost range in subtropical southern Brazil (~31°S; Culot et al., 2019) is exposed to high seasonal variation in ambient temperature (but not in rainfall); within-day fluctuations of ca. 20 °C are common in this region and between-season differences reach >30 °C (INMET, 2019). Finally, brown howlers may switch their folivorous-frugivorous diet to a predominantly frugivorous one (up to 90% fruit) whenever there is plenty ripe fleshy fruit. In contrast, they exploit a more folivorous diet (>55% of feeding time eating leafy material) during lean periods (Chaves and Bicca-Marques, 2013, 2016).

The goal of this study was to evaluate the role of different biotic and abiotic factors as predictors of fGCM secretion by wild adult brown howlers in Brazilian Atlantic Forest fragments near the species's southernmost range. We accomplished this goal by collecting behavioral data and fecal samples of adult females and males in six wild brown howler groups inhabiting three small and three large fragments. Based on the aforementioned relationships between potential stressors and fGCM secretion in Neotropical primates, we predicted that (1) the availability and consumption of ripe fruits, ambient temperature, sex, and fragment size are major modulators of fGCM as shown for *Alouatta* species (e.g., *A. pigra*: Martínez-Mota et al., 2007; *A. palliata*: Dias et al., 2017) and other primates inhabiting regions with marked fluctuations in ambient temperature (e.g., *Chlorocebus pygerythrus*: McFarland et al., 2014; *Pan troglodytes*: Wessling et al., 2018). We also expect (2) higher fGCM

**Table 1**
Studies assessing the influence of ecological factors on the physiological stress of Neotropical primates via fGCM.

| Species[a] | Prediction[b] | Supported?[c] | Country[d] | #groups[e] | Size[f] | Effort[g] | Ref.[h] |
|---|---|---|---|---|---|---|---|
| *Alouatta pigra* (S, L) | S > L | Yes | ME (1, 2) | 4 (7, 9; **6, 11**) | <2, 1400 | 8 (72) | 1 |
| | ♀ > ♂ | No (♀ = ♂) | | | | | |
| *A. pigra* (L) | Fruit availability (−) | No (+) | ME (4) | 2 (10, 6) | 2100 | 14 (97) | 2 |
| | Energy intake (−) | No (null) | | | | | |
| | Protein intake (−) | Yes | | | | | |
| *A. pigra* (L) | Fruit availability (−) | Yes | BE (3) | 6 (3, 5, 4, 7, 4, 5) | 9600 | 21 (350) | 3 |
| | Tourist presence (+) | Yes | | | | | |
| *A. pigra* (S, L) | S♂ > L♂ | No (S♂ = L♂) | ME (5) | 6 (10, 7, 4; **8, 9, 6**) | 11, 23, 900 | 5 (97) | 4 |
| *A. palliata* (S, L) | S > L | Yes | ME (6) | 2 (10, **8**) | 7, 244 | 9 (202) | 5 |
| | Travel time (+) | Yes | | | | | |
| | Fruit consumption (−) | Yes | | | | | |
| *A. palliata* (L) | Fruit consumption (−) | Yes | ME (6) | 2 (10, 8) | 7, 244 | 9 (233) | 6 |
| | | ♀ > ♂ | | | | | |
| *A. palliata* (S, L) | S > L | Yes | ME (6) | 2 (16; **10**) | 16, 231 | 9 (160) | 7 |
| | Travel time (+) | No (=) | | | | | |
| | Food availability (−) | No (=) | | | | | |
| | Agonistic interactions (+) | Yes | | | | | |
| *A. palliata* (L) | ♀ > ♂ | Yes | ME (6) | 2 (4, 7)* | 100 | 12 (528) | 8 |
| | Fruit consumption (−) | No | | | | | |
| | Agonistic interactions (+) | Yes | | | | | |
| | Activity time (+) | Yes | | | | | |
| *A. seniculus* (S, L) | S > L | No (S = L) | CO (7) | 31 | 4–500 | 24 (373) | 9 |
| | Disturbance level (+) | No (=) | | | | | |
| *A. caraya* (S, L) | S > L | No (S = L) | AR (8) | 4 (6, 11; **5, 11**) | <10, 1400 | 4 (114) | 10 |
| | | ♀ > ♂ | | | | | |
| *A. belzebul* (L) | Sound disturbance (+) | No (=) | BR (9) | 2 (5; **8**) | >190,000 | 8 (85) | 11 |
| *Ateles geoffroyi* (S, L) | Forest cover (−) | No (+) | ME (6, 10) | 6 (22−30) | 28–331,000 | 10 (252) | 12 |
| | Fruit consumption (−) | Yes | | | | | |
| | Fruit availability (−) | Yes | | | | | |
| | Travel time (+) | Yes | | | | | |
| *A. geoffroyi* (S, L) | S > L | Yes | ME (11–13) | 3 | <200, 30,000 | 5 (91) | 13 |
| *A. hybridus* (S, L) | S > L | No (S = L) | CO (7) | 8 | 65–500 | 24 (481) | 9 |
| | Disturbance level (+) | Yes | | | | | |
| *Cebus capucinus* (L) | Dry season > wet season | Yes | CR (14) | 2 (8♀)** | 108,000 | 18 (>1000) | 14 |
| | ♀ pregnant > ♀ nonpregnant | Yes | | | | | |
| | ♀ alpha = ♀ subordinate | Yes | | | | | |
| *C. capucinus* (L) | Intergroup encounters (+) | Yes | CR (14) | 3 (8♂)** | 108,000 | 6 (194) | 15 |
| | ♂ alpha > ♂ subordinate | Yes | | | | | |
| *C. capucinus* (L) | ♂ fGCM: presence ♀f > ♀n | Yes | CR (14) | 3 (14♂)** | 108,000 | 17 (993) | 16 |
| | Photoperiod (−) | Yes | | | | | |
| | Fruit availability (−) | Yes | | | | | |
| | Temperature (+) | No (=) | | | | | |
| | Rainfall (−) | Yes | | | | | |

[a] Habitat type shown in parentheses: small fragment (S) and large fragments or sites within continuous forests (L).

[b] Assessed prediction. Expected correlation: positive (+) and negative (−). The meaning of letters S and L is the same as in the first column. In the final example authors predicted that fGCM in adult males is higher in the presence of fertile females (♀f) than in presence of non-fertile females (♀n).

[c] The result is shown in parentheses when a prediction is not supported by the findings. Findings of studies comparing fGC between sexes are shown when available.

[d] A detailed list of countries and study sites is provided in Appendix A.

[e] Number of study groups. If available, information on group sizes shown in parentheses when the number of study groups was ≤6. The size of groups inhabiting large fragments shown in bold. *Group sizes reported in this study include only adult individuals. **In these studies, authors only mentioned the total number of adult females or adult males studied.

[f] Size of the study habitats in hectares.

[g] Sampling effort including the number of study months and the number of fecal samples used for the enzyme immunoassays (in parentheses).

[h] Reference: 1 = Martínez-Mota et al. (2007), 2 = Martínez-Mota et al. (2016), 3 = Behie et al. (2010), 4 = Castiglione (2011), 5 = Dunn et al. (2013), 6 = Dunn et al. (2011), 7 = Gómez-Espinosa et al. (2014), 8 = Dias et al. (2017), 9 = Rimbach et al. (2013), 10 = Cantarelli et al. (2017), 11 = Barros Monteiro et al. (2013), 12 = Ordóñez-Gómez et al. (2016), 13 = Rangel-Negrín et al. (2009), 14 = Carnegie et al. (2011), 15 = Schoof and Jack (2013), 16 = Schoof et al. (2016).

levels in nursing females than in non-nursing females and adult males because of the females' higher energetic demands of pregnancy and lactation (Cantarelli et al., 2017; Dias et al., 2017). Finally, we also expect (3) higher fGCM levels in individuals inhabiting small fragments than in those living in large fragments (e.g., *A. pigra*: Martínez-Mota et al., 2007) given the expected lower food availability for howler monkeys (Arroyo-Rodríguez and Dias, 2010) and the closer contact with humans and domestic animals in the small vs. large study fragments.

## 2. Materials and methods

### 2.1. Study area and climatic conditions

We conducted this study in three large (>90 ha) and three small (<10 ha) fragments of Atlantic Forest in the rural area of the municipalities of Porto Alegre and Viamão, Rio Grande do Sul State, southern Brazil

(Table 2, Fig. 1), from June 2011 to June 2014. The area is composed of a complex mosaic of <1-ha to 40-ha forest remnants with different levels of disturbance and mostly inside private properties, a few larger nature reserves (i.e., Refugio de Vida Silvestre São Pedro and Parque Estadual de Itapuã), natural grasslands, plantations of *Pinus* spp. and *Eucalyptus* spp., agricultural fields, pastures, and rural and suburban human settlements ranging from 2 to ca. 300 homes (IBGE, 2019). The predominant vegetation in this subtropical semi-deciduous Atlantic Forest includes Moraceae, Myrtaceae, and Fabaceae (Sobral et al., 2006). Food species frequently exploited by brown howlers (e.g., *Ficus cestrifolia*, *Erythroxylum argentinum*, and *Enterolobium contortisiliquum*) are common emergent trees (20–30 m in height; Chaves and Bicca-Marques, 2016). Natural grasslands and spiny shrubs (e.g., *Mimosa* spp. and *Sebastiania* spp.) often surround the forest fragments (Setubal et al., 2011).

The region experiences marked seasonal fluctuations in ambient temperature. Summer (22 December–20 March) and spring (23 September–

**Table 2**
Characteristics of the study howler monkey groups and their fecal sample sizes.

| Fragment | FC[a] | GS[b] | Group composition[c] | #groups[d] | Interactions[e] H | Interactions[e] D | Fecal samples (n)[f] ♀ | Fecal samples (n)[f] ♂ | Fecal samples (n)[f] Total |
|---|---|---|---|---|---|---|---|---|---|
| L1 | 93 | 12 | ♂ = 3, ♀N = 2, ♀ = 1, M = 1, F = 1, m = 2, I = 2 | 4 (r) | r (0) | – | 24 | 19 | 43 |
| L2 | 106 | 10 | ♂ = 1, ♀N = 1, ♀ = 2, m = 3, I = 2 | 5 (f) | r (0) | – | 28 | 29 | 57 |
| L3 | 108 | 9 | ♂ = 2, ♀N = 2, ♀ = 1, M = 1, F = 1, m = 1, f = 1 | 7 (r) | o (19) | – | 19 | 13 | 32 |
| S1 | 22 (1.6) | 7 | ♂ = 1, ♀N = 1, ♀ = 1, M = 1, m = 2, I = 1 | 1 (r) | f (42) | f (1–3) | 24 | 14 | 38 |
| S2 | 27 (9.5) | 11 | ♂ = 1, ♀N = 2, ♀ = 1, M = 1, m = 3, f = 1, I = 2 | 2 (f) | f (370) | f (1,3,4) | 29 | 14 | 43 |
| S3 | 4 (3) | 10 | ♂ = 1, ♀N = 3, ♀ = 0, M = 1, m = 3, f = 1, I = 1 | 1 (a) | f (173) | f (1–5) | 30 | 17 | 47 |
| Total | | **59** | ♂ = **10**, ♀N = **10**, ♀ = **7** | **20** | | | **154** | **106** | **260** |

[a] Forest cover (ha) for each fragment. The areas of the small fragments are shown in parentheses. See Methods Section 2.2 for details.

[b] Group size including all sex-age categories.

[c] Average number of adult males (♂) and females (♀N = nursing, ♀ = non-nursing), subadult males (M), subadult females (F), juvenile males (m), juvenile females (f), and infants (I) during the study.

[d] Number of howler groups inhabiting the fragment. Frequency of agonistic interactions with neighbor groups shown in parentheses: a = absent, r = rare, o = occasional, f = frequent.

[e] Interactions with humans (H) and domestic animals (D). The number of houses in a radius of 500 m from the fragment's border or the type of domestic animals interacting with howlers are shown in parentheses: 1 = dogs, 2 = chickens, 3 = horses, 4 = cows, 5 = pigs.

[f] Fecal sample sizes from adult howlers analyzed.

21 December) are the warmest seasons. Their mean temperature during this study was 29 °C (range = 18–39 °C). The lowest mean temperature (17 °C, range = 1–26 °C, considering both day and night air temperatures) was recorded in the winter (21 June–23 September). Mean annual rainfall was ca. 1200 mm during the study, and was evenly distributed throughout the year as expected for Porto Alegre (INMET, 2019).

### 2.2. Study fragment traits

The isolation of the three small fragments from nearby forest patches began in 1985. During the study period these fragments, which have no legal protection, were embedded in a matrix of agricultural fields, pastures, and small human settlements with subsistence orchards. Their resident howlers often interact with people and domestic animals (i.e., dogs, cattle, horses, and chickens) that are common in the matrix and the fragments' edges (Table 2) as observed by Óscar M. Chaves during the data collection and also reported by local inhabitants. These interactions occur mainly when cattle and horses visit the small fragments to drink water in the streams or when the brown howlers descend to the forest floor to access orchards inhabited by dogs and other domestic animals to feed on cultivated fruits (Chaves and Bicca-Marques, 2017). Whereas brown howlers normally ignore cattle and horses, they flee from dogs. The risk of predation by domestic dogs is high at the forest edge and inside orchards as evidenced by many howler casualty reports over the years in the region (Óscar M. Chaves, personal observation). Finally, common human disturbances observed in these habitat patches included selective logging, fire, air and soil pollution (e.g., wastewater, litter, and smoke from grassland fires), and illegal extraction of ornamental plants.

In contrast to the small study fragments, the three large fragments are officially protected and experience low human pressures. They represent patches of Atlantic Forest separated from each other by a matrix of natural grasslands and pastures. Howler interaction with humans can occur when small groups of tourists (2–7 people) occasionally visit two beaches of the Guaíba Lagoon located on the borders of fragments L1 and L2 or during the relatively frequent illegal weekend motocross practicing in the interior and the grasslands surrounding fragment L3 (Fig. 1). Cows, horses, and dogs were occasionally observed along the borders of L3 and on some motocross trails, but not in L1 and L2 (Table 2).

Both small and large fragments have similar richnesses (range: 34–40 species) and densities (range: 8–35 trunks/ha) of tree species exploited as food sources by howlers (Chaves and Bicca-Marques, 2016), but the total numbers of food trees and palms (particularly *Syagrus romanzoffiana*) in the home ranges of the study groups tend to be higher in the large fragments than in the small ones (Óscar M. Chaves, personal observation). The same pattern is found when we

consider the 'scale of effect' for brown howlers (i.e., the spatial scale that yields the strongest response-landscape relationship for a particular animal species: Fahrig, 2003). We assessed this 'scale of effect' by taking into account howler monkey ability to move in the matrix (i.e., the distance traveled between isolated fragments; maximum: <700 m: Mandujano et al., 2004), home range (i.e., up to 70 ha: Fortes et al., 2015), and ability to use neighbor forest patches and cultivated crops in the matrix (Chaves and Bicca-Marques, 2017). We estimated the forest cover in the landscape (a proxy of habitat amount for arboreal mammals: Fahrig, 2003) for the three small fragments by analyzing high-resolution Landsat 7 images from 2013 (USGS, 2019) using the open software QGIS 3.6.3 (QGIS Development Team, 2018). We calculated the size of each landscape from the centroid of the home range of the respective study group. We summed up the forested areas within each landscape polygon, but we did not estimate the cover of the other landscape elements (e.g., natural grasslands, pastures, and *Eucalyptus* plantations). The estimated forest covers for the small study groups were 22 (S1), 27 (S2), and 4 (S3) ha, while the forest covers for the groups inhabiting the large fragments matched their respective sizes (Table 2).

### 2.3. Study brown howler groups

Brown howlers are the only non-human primates inhabiting the study region. The average size of our groups ranged from 7 to 12 howlers, including 1 to 3 nursing females. Two or more howler groups inhabited each large fragment and one small fragment (S3), while the remaining two small fragments were inhabited by a single group each (Table 2). We did not record any agonistic interaction between howler groups in S3. We classified the frequency of between-group agonistic interactions in the other fragments into rare (i.e., a maximum of a single interaction in early morning and/or afternoon per sampling day) or common (i.e., 3–6 interactions per day, Table 2). Most interactions during group encounters only involved vocalizations and/or one group displacing the other from fruiting trees or other contested resources without physical contact. Interactions with physical contact were extremely rare.

### 2.4. Collection of behavioral data

We conducted a 36-mo study (June 2011 to June 2014) on the behavior of five out of six study groups, while the remaining group (L2) was studied during 33 months (September 2011 to June 2014). We followed each group from dawn to dusk during four to five consecutive days every two months. We recorded the behavior of adult individuals using the instantaneous scan sampling method (Altmann, 1974) with 5-min scan sampling units at 15-min intervals. Using 10 × 42

**Fig. 1.** Location of the three small (S1, S2, and S3) and three large (L1, L2, and L3) study Atlantic Forest fragments in southern Brazil. Fragment perimeters enhanced in yellow. Open-access image published in Chaves and Bicca-Marques (2016).

binoculars, each individual was identifiable based on body traits (e.g., size, pattern of pelage coloration, facial marks, and scars). Adult females were also distinguishable, at least temporarily, if they were carrying an infant. We were successful in including all adult members of a given group (3–6 individuals) in most scan samples (>90%). We did not include some individuals in the remaining samples because they were out of sight or because of poor visibility.

We classified individual behavior into four major states: resting, feeding, moving, and socializing (i.e., play, grooming, agonistic interaction among others). In this study we only analyzed the proportion of records devoted to feeding and moving (mov.effort) because the evidence on the influence of time devoted to resting and socializing on fGCM levels in *Alouatta* spp. is weak (e.g., *A. pigra*: Martínez-Mota et al., 2007; *A. palliata*: Dias et al., 2017). Additionally, we found that the proportion of records devoted to social interactions accounted for <5% of the daily activity budget and were often represented by infant and juvenile play (Óscar M. Chaves, personal observation).

When howlers were feeding, we recorded the following information for each individual participating in the feeding bout: the plant species used as food source and the plant item consumed, i.e., ripe and unripe fruit, young and mature leaves, and flowers. A 'feeding record' is any record of an individual feeding during a given scan sampling unit. We considered two feeding records as independent when they were separated by a minimum of 30 min irrespective of the identity of the food species. We determined that a feeding bout lasted up to 26 min depending on patch size and the exploited plant item. Howlers often traveled 50 to 400 m after each feeding bout to resume feeding in another patch (i.e., large trees used as food sources) or to rest and socialize in a resting site. Sometimes they remained resting in the same patch for 1 to 3 h after feeding had stopped.

Our total sampling effort was ca. 3000 observation hours (=1468 h distributed across 5873 scans in small fragments + 1531 h distributed in 6125 scans in large fragments). The daily investment in each behavior was calculated as the proportion of scan sampling records. Similarly, we estimated the contribution of ripe fruits to the daily diet ($RF_c$) as the number of records feeding on them divided by the total number of feeding records.

### 2.5. Collection of fecal samples

We collected fresh fecal samples from individually recognizable individuals immediately after defecation, selecting portions free of urine, soil, and other detritus. We stored each fecal sample in a $10 \times 20$ cm sterile plastic bag labeled with the following information: individual, group, location, day, and hour. We preserved the samples in the field in dry ice at ca. −4 °C for 5 to 12 h. Following Khan et al. (2002), we stored these samples at the end of the day in a laboratory freezer at −20 °C until fGCM extraction (i.e., 2–8 days after collection). We restricted the analyses to adult howlers to minimize the influence of potential ontogenetic differences in feeding behavior and fGCM. We collected ca. 500 fecal samples, but 48% of them were not included in the analyses because of insufficient amount of fecal material, because they belonged to juveniles, or because of faded bag label. In sum, we measured fGCM in 260 fecal samples (32–57 samples per group) distributed among 17 females and 10 males (Table 2, dataset in Chaves et al., 2019).

While many primate studies have restricted the collection of fecal samples to the morning to control for potential circadian variations in GC secretion (see Beehner and Bergman, 2017), this was not possible in our study because of three main logistical difficulties. First, we did not locate the study groups early in the morning in some observation days, particularly in large fragments. Second, the feces of about ⅓ of the morning defecation events fell in inaccessible places (e.g., streams, pools, or deep rocky crevices), or, third, they were diarrheic, contaminated with detritus and/or urine, or reduced to very small portions after hitting one or more branches and/or leaves before reaching the ground. We believe that these limitations did not influence our results because the observed daily fluctuations did not show a consistent fGCM circadian variation in the study groups (Fig. S1). Furthermore, we performed an LMM analysis using only those samples collected in the morning (8:00–11:30, $N = 99$) to evaluate the potential effect of the timing of collection on fGCM (Table S1). Despite differences between the best supported models when using all samples vs. using only morning samples and their averaged and test values, the results were qualitatively similar (see below). The averaged model based on morning samples was also significant (likelihood ratio test: $X^2 = 22.4$, d.f. = 9, $P < 0.0001$) and included similar variables. Therefore, we used all samples in our subsequent analyses. We also included collection time (hour) as a covariate in the LMM analyses described below and it did not have a significant effect on fGCM.

We classified adult females during sampling into nursing (i.e., those nursing 0- to ca. 12-mo-old infants) and non-nursing (i.e., those not associated with a dependent infant) to control for the potential influence of female reproductive state on fGCM (e.g., Dias et al., 2017). Therefore, we also labeled plastic bags containing a female's fecal sample with information on whether she was carrying a dependent infant or not and, when applicable and possible, the infant's approximate age based on body size. It was not possible to determine whether non-nursing females were cycling or pregnant based on only four to five days of observation every two months.

### 2.6. Extraction and quantification of fecal cortisol

We extracted fGCM in the Laboratório de Fisiologia da Conservação, Pontifícia Universidade Católica do Rio Grande do Sul, located between 23 and 47 km from the study sites, following an established lab protocol (Ange-van Heugten et al., 2009). We removed 500 mg of each homogenized fecal sample, stored it in a 15-mL sterile tube, and mixed it with 4.5 mL of methanol (90% purity). We agitated the tubes vigorously for 40 min in a tube shaker and then centrifuged them at 2500*g* for 15 min. We separated and dried the supernatant (i.e., the liquid containing the fecal extract) by evaporation using ultra-pure nitrogen gas (99.9% purity) at room temperature. We used these dried samples to perform the enzyme immunoassay described below. We stored these samples in a freezer at −20 °C during ca. 30 days until obtaining a sample size sufficiently large to use an entire cortisol kit (Ref. 55050, produced by Human GmbH, Max-Planck-Ring 21, D-65205 Wiesbaden, Germany).

During the fGCM assay, we reconstituted each sample with 0.1 mL of the zero calibrator provided by the cortisol kit. Then, we removed two aliquots of this solution for quantifying fGCM using the enzyme immunoassay kit. We used the ELISA plate reader (BIOCHROM EZ READ 400 FLEXI PLUS, Cambridge, UK) set at 405 nm to read the results expressed as ng of fGCM/mL of reconstituted fecal extracts. We calculated the binding percentage and log-transformed the values using a standard curve plotted with known hormone concentrations (i.e., 0, 25, 50, 100, 300, and 500 ng/mL; Fig. S2a). Assay sensitivity ranged from 1.1 to 1.5 ng/mL. We analyzed all fecal samples in duplicate and calculated the mean of their readings. We used these values to determine the amount of fGCM in each fecal sample in ng/mL. We used the fGCM means in the LMM models described below.

We calculated the coefficient of variation (CV) between duplicates obtained with the standard assay. We found intra- and inter-assay CVs, respectively, of 15% and 16%. The kit's manufacturer reports cross-reactivity with other compounds: 60% for prednisone, 29% for corticosterone, 3% for cortisone, and <0.1% for other steroids.

Finally, we transformed fGCM values to nanograms per gram of dry fecal sample (ng/g) to control for the effects of the amount of water in the feces on steroid excretion.

We validated the aforementioned methods to extract and quantify fGCM using the adrenocorticotropic hormone (ACTH) challenge test in captive brown howlers (Madeira Buti et al., 2018). These authors

found a significant increase in fGCM 24 h after the ACTH stressor and did not find differences between males and females. Although this result is reported for a different hormone (corticosterone), it confirms the efficacy of enzyme immunoassays to measure the adrenocortical activity in the study species. Lastly, we also performed a laboratory validation that confirmed data linearity. Alike Madeira Buti et al. (2018), we added known quantities of cortisol in dilutions similar to the standard curve of the kit to fecal extracts with negligible hormone levels. This way we demonstrated the parallelism between the curve representing the serial cortisol dilutions in the fecal material and the standard curve (Pearson's correlation, $R^2 = 0.99$, Fig. S2).

The Scientific Committee of the Faculty of Biosciences of the Pontifical Catholic University of Rio Grande do Sul approved this study (project #3477-SIPESQ). It also meets all Brazilian animal care policies (permits #28578-SISBIO/ICMBio and #372-SEMA).

### 2.7. Ambient temperature data

We recorded the ambient temperature in the shade at a height of ca. 2 m above the ground using a pocket thermo-hygrometer (Yi Chun®, PTH 338) after each scan sampling unit. In most cases the observer and the thermo-hygrometer were within 3 to 10 m from the group's geometric center, near to at least one group member. We assessed the minimum (tmin), maximum (tmax), and mean (tmean) ambient temperature for each analyzed study day.

### 2.8. Fruit availability and intensity of fruit exploitation

We used our tree inventory and food species phenology databases to calculate indexes of spatiotemporal availability of fruits, leaves, and flowers consumed by brown howlers (see Chaves and Bicca-Marques, 2016). We performed bimonthly phenological surveys at each study site from August 2011 to June 2014 one day before the beginning of each group's follow, totaling three sampling periods per fragment in 2011 and 2014 and six in 2012 and 2013. We targeted 16 to 20 trees of the top fruit tree species (i.e., those tree species that together represented >80% of the records devoted to fruit feeding) exploited by brown howlers at the study region (see Chaves and Bicca-Marques, 2013). We determined the presence and abundance of fruits (ripe and unripe), leaves (young and mature), and flowers within the canopy and assigned values ranging from 0 to 4 according to the percentage of the canopy covered by each plant item (0 = 0%, 1 = 1–25%. 2 = >25–50%, 3 = >50–75%, 4 = >75–100%) following Fournier's semi-quantitative method (Fournier, 1974).

We found large monthly variation in ripe fruit production with at least two peaks per year, which were not necessarily synchronized within and between study fragments (Fig. S3). To estimate ripe fruit availability we first averaged the fruit phenological scores of individual trees of each top food species to obtain a ripe fruit phenological index (PIS) for each sampling period. Following Agostini et al. (2010), we multiplied PIS by the basal area of each food species to obtain a fruit species availability index (FAI). We summed all FAIs to estimate ripe fruit availability (RF$_a$) for each sampling period.

We used the Manly Selectivity Ratio $w_i$ (Krebs, 2014) to estimate the intensity with which fruits of a given tree species were ingested by howlers (hereafter fruit exploitation intensity). We calculated fruit exploitation intensity by dividing ripe fruit consumption RF$_c$ (i.e., the proportion of feeding records on ripe fruits of all tree species exploited in each sampling day) by RF$_a$. We used the same procedure to calculate the $w_i$ for the other plant items. Then, we calculated a standardized fruit selection index ($B_i$) by dividing the fruit's $w_i$ by the sum of the $w_i$'s of all food items (fruits + leaves + flowers). $B_i$ ranges from 0, when the item was not eaten (or avoided), to 1, when the diet is made up exclusively of it. Intermediate $B_i$ values (i.e., close to 0.5) indicate that fruit was consumed according to its availability; that is, there was no preference or avoidance. Therefore, we can interpret $B_i$ as the

probability that fruit (or any other food item) was preferred over the other items during a given sampling day. The entire dataset with all variables used in the analyses is available in Chaves et al. (2019).

### 2.9. Statistical analyses

We performed linear mixed-effects models (LMM; Zuur et al., 2009) using the function 'lmer' of the R package lme4 (Bates et al., 2015) to assess the influence of the 17 predictor variables (Table 3) on fGCM; i.e., the averaged fGCM for each individual in each sampling day ($N = 236$ fecal samples). We specified all these variables as fixed factors and individual ID as a random factor to account for measures from the same individual during the study. We simplified the model by including only two second-order, biologically relevant interactions for this study, namely mov.effort/sex and RF$_c$/sex (Table 3), to minimize overparameterization and problems of convergence with the global model (i.e., the model containing all fixed and random factors) due to the inclusion of a large number of variables and their interactions (Grueber et al., 2011). We standardized the variables using the 'stdize' function of the package MuMIn (Barton, 2016) because the predictor variables differed in scale, a characteristic that hampers appropriate comparison of multiple models

**Table 3**
Potential predictors of the physiological stress (fGCM) analyzed in this study and their expected effects according to the data shown in Table 1.

| Variable | Description | Effect[a] |
|---|---|---|
| *Ecological variables* | | |
| 1) % feeding records devoted to ripe fruit (RF$_c$) | Records devoted to ripe fruits/total feeding records | (−) |
| 2) Ripe fruit availability index (RF$_a$) | Spatio-temporal availability of top ripe fruits exploited by brown howlers | (−) |
| 3) Ripe fruit preference index | Manly Selectivity Ratio for top fruit species exploited by brown howlers | (−) |
| 4) % records devoted to moving (mov.effort) | Number of records moving/total number of behavioral records | (+) |
| 5) Forest cover | Proxy of habitat amount for brown howlers taking into account animal ability to move in the matrix, their home range, and their ability to use neighbor fragments | (−) |
| 6) Study group | Groups inhabiting small (S1, S2, S3) and large (L1, L2, L3) fragments | S > L |
| 7) Collection hour (hour) | Time of the day in which each fecal sample was collected | N.A. |
| *Individual variables* | | |
| 8) Sex/reproductive state | Nursing (N) and non-nursing (n) adult females, and adult males | N > n ♀ > ♂ |
| 9) mov.effort/sex | % records devoted to moving by individuals of each sex | N.A. |
| 10) RFc/sex | Fruit consumption per by individuals of each sex | N.A. |
| *Climatic variables* | | |
| 11) Minimum temperature (tmin) | Minimum ambient temperature recorded after each scan sample | (−) |
| 12) Maximum temperature (tmax) | Maximum ambient temperature recorded after each scan sample | (+) |
| 13) Mean temperature (tmean) | Daily mean of all temperature records for each study site | (+) |
| *Group composition* | | |
| 14) Group size | Number of individuals in each study group (including all sex-age categories) during each sampling period | (+) |
| 15) Number of adult males in the group (#males) | Number of adult males in the group | (+) |
| 16) Number of adult females in the group (#females) | Number of adult females in the group | (+) |
| 17) Number of neighbor groups (#groups) | Estimation of the number of groups inhabiting the fragments | (+) |

[a] Predicted effect according to the available evidence: positive (+), negative (−), greater than (>), not-assessed (N.A.).

(Grueber et al., 2011). Furthermore, we controlled for multicollinearity problems between variables prior to the analysis by using the 'VIF' function of the package car. We only included those variables with Variance Inflation Factor (VIF) <3 (Zuur et al., 2009). Based on this criterion, we excluded four variables from the global model: tmean, tmax, #males, and #females. We visually inspected Q-Q plots of residuals plotted against fitted values of the model to check the assumptions of homogeneous and normally distributed residuals. We used an ln-transformation to improve the fit of the fGCM data to the normal distribution (Shapiro-Wilk test, $P > 0.05$).

Following the information-theoretic framework (Burnham and Anderson, 2003), we used the Akaike's Information Criterion (AIC) to select the models that best predict the effects of predictor variables on fGCM. Specifically, we used the AICc as recommended for sample sizes/number predictors <40 (Burnham and Anderson, 2003). According to this criterion, the model with the smallest difference in AICc (ΔAICc) has the strongest empirical support. However, we used the full-model averaging framework to determine which parameters best explained the response variable fGCM because all models with ΔAICc < 2 are considered equally parsimonious (Grueber et al., 2011). This approach is widely recommended to account for model uncertainty and reduce prediction error (Grueber et al., 2011), particularly when the support for the top best model is not strong (i.e., $w_i < 0.9$; Burnham and Anderson, 2003) as is the case in our study.

We used the 'dredge' function of the package MuMIn to generate a full submodel set from the global model, and the 'model.avg' function of the same package to determine the averaged model and the relative importance of each variable or predictor weight ($\sum w_i$). We calculated $\sum w_i$ by summing the Akaike weights ($w_i$) of all models that included that variable. We used a likelihood ratio test over the R function 'anova' to test the significance of the averaged model compared with the model including only the random factor (i.e., null model). We used the 'r.squaredGLMM' function of the MuMIn package to estimate an equivalent of the coefficient of determination or pseudo-$R^2$ for each competing best LMM model (i.e., models with ΔAICc < 2).

To compare fGCM between sexes and between groups in small and large fragments, we used the rarefaction approach over the 'sample' function to control the asymmetry in the number of samples per sex in each fragment (Table 2). Then, we used the rarified data to perform an LMM specifying sex and group as fixed factors and individual ID as random factor. We detected differences between groups using post-hoc contrasts with the function 'glht' of the package multcomp. We performed all statistical analyses in R 3.5.1 (R Core Team, 2018), setting the statistical significance threshold at $P \leq 0.05$.

## 3. Results

We found 10 models with substantial empirical support (i.e., ΔAICc < 2). They included the variables sex/reproductive state, ripe fruit consumption (RF$_c$), minimal temperature (Tmin), group size, and collection hour (Table 4). Sex/reproductive state was the only fGCM predictor found in all these models, while RF$_c$, Tmin, and group size appeared in five models, and hour in only three (Table 4). The model with the highest probability of being the best model included sex/reproductive state and RF$_c$ (ΔAICc = 0.0, $w_i$ = 0.15). The second best model included sex/reproductive state, group size, and Tmin (ΔAICc = 0.29, $w_i$ = 0.13, Table 4). However, the variance explained by all models was similarly low, ranging from 18% to 21% (Table 4).

The averaged and the null models differed significantly (likelihood ratio test: $X^2 = 38.3$, d.f. = 9, $P < 0.0001$). The variance explained by the averaged model was 21%. We found that fGCM was higher in nursing females than in non-nursing females and males and that it was influenced (negatively) by ripe fruit consumption and minimum ambient temperature (Table 4, Fig. 2). Conversely, group size and collection hour did not influence it (β = −0.2, z-value = 2, $P > 0.05$ in both cases; Table 4). The variable with the highest relative importance was

**Table 4**
Best linear mixed models (ΔAICc < 2) and averaged-model that predict the variation of fecal glucocorticoid metabolites (fGCM) in six brown howler groups ($N = 236$).

| Predictor variables[a] | Parameters[b] | | | |
|---|---|---|---|---|
| | AICc | ΔAICc | $w_i$ | $R_c^2$ |
| *Best supported models* | | | | |
| 1) Sex/reproductive state + RF$_c$ | 544.7 | 0.00 | 0.15 | 0.18 |
| 3) Sex/reproductive state + group size + tmin | 545.0 | 0.29 | 0.13 | 0.20 |
| 3) Sex/reproductive state + tmin | 545.1 | 0.4 | 0.12 | 0.20 |
| 4) Sex/reproductive state + group size + RF$_c$ | 545.2 | 0.46 | 0.12 | 0.19 |
| 5) Sex/reproductive state + RF$_c$ + hour | 545.3 | 0.51 | 0.12 | 0.20 |
| 6) Sex/reproductive state + group size + RF$_c$ + hour | 545.7 | 0.97 | 0.09 | 0.20 |
| 7) Sex/reproductive state + RF$_c$ + tmin | 546.2 | 1.51 | 0.07 | 0.20 |
| 8) Sex/reproductive state + group size + tmin + hour | 546.4 | 1.67 | 0.07 | 0.21 |
| 9) Sex/reproductive state + group size | 546.5 | 1.73 | 0.06 | 0.18 |
| 10) Sex/reproductive state + tmin | 546.6 | 1.84 | 0.06 | 0.21 |

| Predictor variables[a] | Parameters[b] | | | | |
|---|---|---|---|---|---|
| | $\beta_i$ | SE | 95% CI | z-value | $\sum w_i$ |
| *Averaged model ($R_c^2 = 0.21$)* | | | | | |
| Intercept | 4.3*** | 0.11 | (3.8, 4.0) | 41.91 | – |
| Sex/reproductive stage | | | | | 1 |
| 　Adult male | −0.61*** | 0.14 | (−0.89, −0.34) | 4.47 | |
| 　Non-nursing adult female | −0.41** | 0.13 | (−0.67, −0.16) | 3.26 | |
| Ripe fruit consumption | −0.25* | 0.10 | (−0.45, −0.06) | 2.50 | 0.55 |
| Minimal ambient temperature | −0.23* | 0.10 | (−0.43, −0.04) | 2.35 | 0.45 |
| Group size | −0.20 | 0.11 | (−0.47, −0.02) | 2.16 | 0.40 |
| Collection hour | −0.19 | 0.10 | (−0.38, −0.01) | 2.02 | 0.34 |

[a] Abbreviations of predictor variables: RF$_c$ = ripe fruit consumption, tmin = minimum ambient temperature, and hour = collection hour of fecal sample (see Table 2).
[b] Parameters shown are Akaike's Information Criterion (AICc) for small samples, difference in AICc (ΔAICc), model probability Akaike weights ($w_i$), Pseudo-$R^2$ ($R_c^2$) indicating the percentage of the variance explained by the fixed and random factors, partial regression coefficients of the averaged-model ($\beta_i$), standard errors that incorporate model uncertainty (SE), 95% confidence intervals for the parameter estimates, relative importance of each predictor variable ($\sum w_i$). The degrees of freedom of each model are equal to the number of variables in each model plus four.
* Significance level: $P < 0.05$.
** Significance level: $P < 0.01$.
*** Significance level: $P < 0.001$.

sex/reproductive state ($\sum w_i = 1$), followed by RF$_c$ ($\sum w_i = 0.6$, Table 4).

We observed noticeable variation in fGCM for both sexes regardless of fragment size. In females, fGCM ranged from 7 to 270 ng/g ($N = 154$ samples), while in males it ranged from 9 to 192 ng/g ($N = 106$ samples, Fig. 3a). The LMM model containing sex/reproductive state and group was significantly different from the null model (likelihood ratio test: $X^2 = 30.3$, d.f. = 20, $P = 0.02$). On average, fGCM was ca. 55% higher in nursing females (mean ± SD = 103.6 ± 73.2 ng/g) than in non-nursing females (63.7 ± 54.8 ng/g) and males (53.2 ± 40.5 ng/g, $F_{(2, 149)} = 6.8$, $P = 0.001$; Tukey contrasts, $P < 0.05$ in both cases; Fig. 3a). There were no within- or between-group within-sex/reproductive state fGCM differences (Tukey contrasts, $P > 0.05$ in all cases, Fig. 3a).

Lastly, fGCM (mean ± S.D.) ranged from 57 ± 49 ng/g in group S3 to 93 ± 58 ng/g in S1 (or from 52 ± 45 ng/g to 85 ± 52 ng/g based on rarified data, Fig. 3b). Contrary to our expectation, we found that fGCM was similar between groups in small (70.6 ± 58.1 ng/g) and large fragments (63.3 ± 54.2 ng/g; $F_{(5, 149)} = 1.7$, $P = 0.1$; Fig. 3b). In most cases, we only found slight variation in mean fGCM concentration between groups (Fig. 3b). In addition, forest cover was only included in poorly-supported LMM models (ΔAICc ranging from 4.3 to 10.8).

## 4. Discussion

We found that sex/reproductive state, ripe fruit consumption, and minimum ambient temperature affected the fGCM in adult female and male brown howlers living in forest fragments near the southern limit of the distribution of the species, supporting the influence of a complex
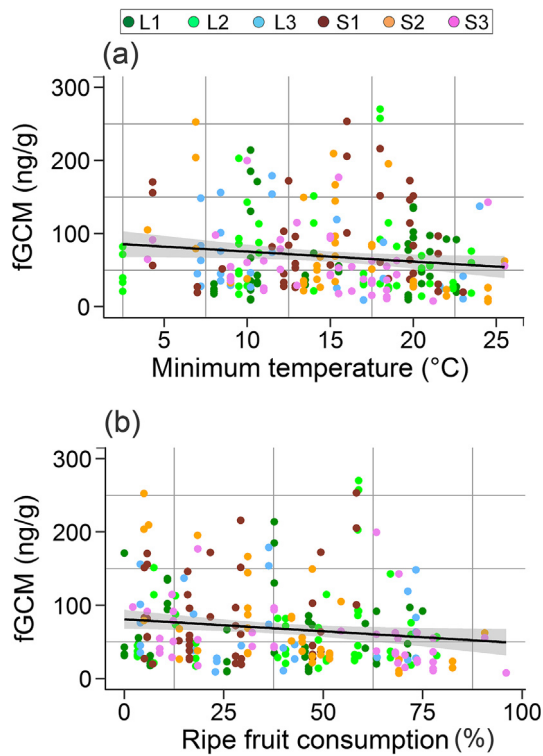
**Fig. 2.** Variation of fecal glucocorticoid metabolites in response to (a) the minimum ambient temperature ($\beta = -0.3$, z-value = 2.5, $P < 0.05$) and (b) the percentage of feeding records devoted to ripe fruits ($\beta = -0.3$, z-value = 2.4, $P < 0.05$). Colored dots represent each study group's fGCM data. The gray area shows the 95% confidence interval.



**Fig. 3.** Fecal glucocorticoid metabolites in six groups of brown howler monkeys in southern Brazil according to (a) sex and female reproductive state in each fragment and (b) the study fragment. Panels at the extreme right represent the overall comparison between sexes or fragment size categories. The line within a box represents the median, the gray square represents the mean, the box represents the first and third interquartiles (IQR), and the whiskers represent the IQR multiplied by 1.5. Dots represent the concentration of cortisol in each sample. The gray number below each bar represents its sample size ($N = 212$ samples). Different lowercase letters above the bars indicate significant differences between the classes (post-hoc Tukey tests, $P < 0.05$). No letters above the bars mean lack of significant differences (Tukey contrasts, $P > 0.05$ in all cases).

set of ecological, individual, social, and climatic variables and their interactions on the physiological stress of wild vertebrates (Crespi et al., 2013; Romero et al., 2015; Romero and Wingfield, 2015). The influence of group size and timing of sample collection was supported in the multimodel analyses, but not in the model averaging. On the other hand, previously reported predictors of stress in howler monkeys, such as group composition (Gómez-Espinosa et al., 2014), habitat size (Martínez-Mota et al., 2007), and moving time (Dunn et al., 2013) did not influence fGCM in the study subjects. Therefore, we found only partial support for our first prediction.

The influence of sex on GC secretion has been widely documented in primates (Behringer and Deschner, 2017), including *Alouatta* species (Table 1). In agreement with these previous studies, we found higher fGCM levels in nursing females than in non-nursing females and males, lending support for our second prediction. It is likely that the higher nutritional demands of pregnancy and lactation promote an increase in GC levels in the blood (Beehner and Bergman, 2017; Dias et al., 2017) that benefit females by quickly mobilizing glucose and restoring homeostasis via enhanced gluconeogenesis (Markham and Gesquiere, 2017). The observed within-group within-sex fGCM variation is probably associated to the high between-individual variability in stress responses resulting from individual differences in age (even between adults), reproductive state, health status, and dominance rank (Crespi et al., 2013; Schoof and Jack, 2013; Romero and Wingfield, 2015). Although we do not have detailed data on the reproductive state of our adult females at all times during the study, all groups contained at least one nursing female that was pregnant during six months. Therefore, these changes in female reproductive state are likely to account for part of the fGCM variation that we recorded.

The inverse relationship between fGCM and fruit consumption may be explained by the higher energy content of fleshy fruits exploited by tropical primates when compared with leaves and other food items (Lambert, 2011; Behie and Pavelka, 2015). This pattern has been reported for other frugivorous taxa (e.g., birds: Jordano, 2000; Blendinger et al., 2016; bats:

Batista et al., 2017). Therefore, when the consumption of wild and/or cultivated fleshy fruits decreases, an increase in GC secretion may compensate for the low 'blood sugar,' as suggested for other primates (e.g., *A. pigra*: Behie et al., 2010, *Pan troglodytes*: McLennan et al., 2019; *Propithecus diadema*: Tecot et al., 2019; *Cercopithecus mitis*: Thompson et al., 2019). This increase in GC secretion decreases the risk of nutritional stress and its long-term consequences on individual health (Behie et al., 2010; but see Martínez-Mota et al., 2016).

The negative influence of minimum ambient temperature on fGCM is also probably related to the energy balance of howler monkeys. The thermal environment of the study groups shows high seasonal fluctuations, whose absolute minimum near 1 °C (INMET, 2019) occurs exactly during the months with lower fruit availability and consumption (Chaves and Bicca-Marques, 2016). Such colder periods might increase GC secretion by causing 'cold' stress that increases the energetic demands of thermoregulation, as reported for temperate primates (e.g., *Chlorocebus pygerythrus*: McFarland et al., 2014; *Rhinopithecus roxellana*: Guo et al., 2018). This hypothesis is compatible with the adoption of positional adjustments by brown (Bicca-Marques and Azevedo, 2004) and black-and-gold (Bicca-Marques and Calegaro-Marques, 1998) howlers in response to temperature extremes. In this respect, the influence of huddling, a positional behavior frequently observed in brown howlers during cold periods (Óscar M. Chaves, personal observation), on physiological stress remains to be investigated.

The finding that the averaged model explained only 21% of the variance in fGCM is consistent with previous studies showing that the stress response of vertebrates is site-specific and context-dependent (Crespi et al., 2013; Romero and Wingfield, 2015). In the case of

brown howlers, future multi-year studies should assess whether and how the proximity to and the frequency of contact with humans, the level of human-related noise, dominance rank, and intra- and inter-group agonistic interactions (Table 1) influence the physiological stress of individuals. The unexplained fGCM variance can also be influenced by a variety of factors affecting non-invasive hormone biomarkers, such as sex differences in hormone metabolism and secretion, the effect of gut bacterial composition on hormone decomposition, and the effect of dietary fiber on the amount of excreted hormone metabolites (Goymann, 2012). In this respect, the members of the various sex-age classes within groups ingested similar amounts of dietary fiber (Flavia M. Lisboa and Júlio César Bicca-Marques, personal communication), suggesting that this factor probably does not contribute to the variation in fGCM observed in our study. However, adult female *A. pigra* host gut microbiota communities that are partially distinct from those of adult males (Amato et al., 2014), which can influence both nutrient extraction efficiency and hormone metabolism.

We did not find support for the prediction that howler monkeys living in small forest fragments are more stressed than those living in large well-protected forests, as reported for African (e.g., *Procolobus* (*Piliocolobus*) *rufomitratus*: Chapman et al., 2006, 2015; *Pan troglodytes*: McLennan et al., 2019) and Neotropical (*Ateles geoffroyi*: Rangel-Negrín et al., 2009; *Alouatta pigra*: Martínez-Mota et al., 2007; *A. palliata*: Dunn et al., 2013) primate species. This expectation is based on the assumption that primates inhabiting small habitat patches face adverse conditions that are absent or uncommon in larger patches. These adversities can include longer periods of food scarcity, frequent human presence, logging, hunting, and predation by domestic dogs (Arroyo-Rodríguez and Dias, 2010; Chaves and Bicca-Marques, 2017; Estrada et al., 2017). In our study, the small fragments can be considered of lower quality for brown howlers than the large ones because (1) the home ranges of the study groups in the former habitats are constrained spatially to about half the size of those used by groups in the latter habitats (mean ± SD = 4 ± 2 ha vs. 9 ± 2 ha, Óscar M. Chaves, personal observation), (2) their absolute food availability is lower (Chaves and Bicca-Marques, 2016), and (3) the howler monkeys within them interact more frequently with humans and domestic animals (Table 2). Despite this potential stress, we did not observe higher fGCM levels in the howlers living in the small fragments compared with those living in the large fragments.

It is possible that the degree of habitat deterioration of the small study fragments is below the threshold that triggers a consistent increase in cortisol level, as suggested for other South American atelid monkeys (e.g., *A. seniculus* and *Ateles hybridus*: Rimbach et al., 2013; *A. caraya*: Cantarelli et al., 2017; Table 1). The potential nutritional stress experienced by other animals inhabiting small fragments may be avoided by *Alouatta* spp. owing to their high resilience to human disturbances, particularly their ability to deal with food reduction via their flexible and opportunistic diets that can include cultivated fruits, climbers, and even animal protein (Bicca-Marques, 2003; Bicca-Marques et al., 2009, 2014; Dias and Rangel-Negrín, 2015). Additionally, howlers can exploit resources available in landscape elements such as neighboring forest patches, riparian corridors, isolated trees, live fences (reviewed by Arroyo-Rodríguez and Mandujano, 2009), backyards and orchards (Chaves and Bicca-Marques, 2017). Wild chimpanzees also exploit nutritious fruits cultivated in human-modified habitats to supposedly mitigate their energetic stress (McLennan et al., 2019).

Finally, whereas only one study group inhabiting a small forest fragment shared resources with a neighboring group, all groups inhabiting large fragments had several neighbors (Table 2). Group encounters at feeding patches in the large fragments can represent an additional source of stress that is absent in two of the small ones. Therefore, this difference in their social environments cannot be discarded as a potential factor reducing the discrepancy in the level of stress hormone between adult howlers inhabiting the small and large study fragments.

Although we did not collect data on individual health and the reproductive rate of the study groups, we found no evidence that these parameters differ between groups in small and large fragments (Óscar M. Chaves, personal observation). However, it is likely that habitat shrinking and other disturbances in these landscapes shaped by human activities, such as predation by domestic dogs, electrocutions, and roadkills (Lindshield, 2016; Villatoro et al., 2019), have synergistic negative long-term effects on the persistence of brown howler populations (Bicca-Marques, 2003; Chaves and Bicca-Marques, 2016), as reported for African primates (e.g., Chapman et al., 2006, 2013). Therefore, small isolated populations can play an important role in brown howler conservation if they are connected in a functional metapopulation. This role highlights the value of conserving even the smallest habitat patches as their temporary or permanent provisioning of essential resources for howler monkeys (e.g., food, water, sleeping sites) contributes to the long-term survival of isolated groups (Asensio et al., 2009). Similarly, this may influence the genetic diversity of the species as the likelihood of harboring individuals with rare alleles increases with increasing metapopulation size.

Therefore, management strategies should aim to promote the protection of these forest fragments with the active participation of local inhabitants, governments and researchers, and to integrate them in the establishment of corridors to facilitate dispersal between habitat patches. The planting of fast-growing native fruit species or even alien non-invasive cultivated species in the fragments' borders and the matrix (Bicca-Marques and Calegaro-Marques, 1994) can also be effective ways to fulfill these goals. Finally, outreach activities informing the laypeople and the educational institutions of all instruction levels on the importance of conserving the remaining small habitat patches immersed in landscapes highly fragmented by economic activities are necessary to develop an ecofriendly culture that promotes the long-term safeguarding of biodiversity and ecosystem services.

## 5. Conclusions

In conclusion, we found that fruit consumption, ambient temperature and, for adult females, nursing modulate the physiological stress of brown howlers, whereas group composition, moving effort, and the forest cover in the neighboring landscape did not. Our finding that brown howlers cope with spatial habitat restrictions at the individual level without evident signs of increased stress highlights the potential importance of groups inhabiting small habitat patches for the conservation of the species in fragmented landscapes. Therefore, management strategies in these landscapes should aim to integrate them into metapopulations while reducing the risks of dispersal between discrete populations.

**Declaration of Competing Interest**

The authors declare no conflict of interest.

Felipe Vianna (Instituto Econsciência) for granting us permission to conduct this research in their properties.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2019.07.033.

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