



New Route of Investigation for Understanding the Impact of Human Activities on the Physiology of Non-Human Primates

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Abstract

The present study is aimed to assess the impact of human presence on the physiology of the folivorous Golden-mantled Howler Monkey (*Alouatta palliata palliata*) and the frugivorous Ornate Spider Monkey (*Ateles geoffroyi ornatus*) in Tortuguero conservation area in North-eastern Costa Rica. We compared fecal cortisol and fecal testosterone levels between non-habituated monkeys sighted in an area with high human presence (HHP) and in an area with low human presence (LHP). Both sexes of howler monkeys, but not spider monkeys, secreted significantly more cortisol when encountered in the area with HHP. Also, males, but not females, of both species showed higher levels of fecal testosterone when encountered in the area with HHP. In the area with HHP, we found that the proximity to human settlements was the type of human presence inducing the largest rise of cortisol secretion in howler monkeys. On the contrary, the proximity to the canal - which was intensively frequented by motor boats (i.e. ≥80 boats per day) linked to tourism activities – was the type of human presence inducing the largest rise of testosterone secretion in males of both species sighted in area with HHP. Our results suggest that human presence impacts levels of fecal cortisol and fecal testosterone in non-human primates (NHPs), but these impacts could differ across sexes and species. This study opens new investigation perspectives aiming to increase our understanding of the relationship between NHPs' physiology, biology and ecology, as well as mitigating perturbation of human activities on wildlife.

Keywords: Anthropogenic disturbance; Howler monkey; Spider monkey; Cortisol, Testosterone; Conservation

Introduction

In threatening situations, a type of steroid, namely glucocorticoids is released from the hypothalamus-pituitary-adrenal (HPA) axis to prepare the body to cope with crisis and enable it to return to homeostasis [1,2]. The major glucocorticoid, cortisol, promotes tissue degradation and prevents allocation of energy and nutrient uptake for purposes such as digestion, reproduction, growth and immune competence in order to maximize the quantity of energy available to flee [3,4]. This response is an important physiological adaptation for increasing survival rate in animals [5]. Previous studies have correlated the presence of tourists with increased cortisol levels in non-human primates (NHPs; e.g. [6] for the black howler monkey (*Alouatta pigra*); [7] for the Barbary macaques (*Macaca sylvanus*); [8] for the Northeast Bornean Orangutan (*Pongo Pygmaeus morio*); [9] for the Western Lowland Gorilla (*Gorilla gorilla gorilla*)). Other studies found that the level of fecal glucocorticoid metabolites increased in groups of free-ranging Mexican howler monkeys (*Alouatta palliata mexicana*) and black howler monkeys living in small forest fragments in Mexico due to a decrease in food availability [10,11]. One other study in Mexico reported that Geoffroy's spider monkey (*Ateles geoffroyi*) altered their diet in fragmented forests, marking that this species could also suffer from additional feeding cost in fragmented forest [12].

Testosterone is a steroid that promotes tissue construction of muscles and secondary sexual characteristics and permits allocation of all available body energy for optimizing energetic response to (mating) stimuli, thus promoting mating success for males [13,4]. When males are challenged for access to resources, the testis and adrenal glands increase their secretion levels of testosterone [14].

Despite being very important for the maintenance of homeostasis, continuous secretion of steroids at a level beyond a threshold is deleterious for NHPs through the destruction of lymphocytes and

modulation of gene expression of cytokines, inflammatory mediators and lymphocyte receptors [15-17]. Thus, owing to the potential impact of stress hormone levels on many body functions such as reproduction, growth, digestion and immune system efficiency, there is a growing need to understand how human presence may affect physiological alteration in NHPs.

In order to ascertain whether human presence induced physiological disturbances to howler monkeys and spider monkeys, we compared fecal cortisol and testosterone levels among non-habituated monkeys ranging in an area with high human presence (HHP) and in an area with low human presence (LHP). Spider monkeys were expected to show higher cortisol levels than howler monkeys in the area with LHP, due to the higher intraspecific competition for food [18,19]. In the area with LHP, we also expected the howler monkey to secrete more testosterone than spider monkeys because of the higher level of competition between adjacent groups for membership [20,14]. We expected that human presence would cause a rise of cortisol excretion, especially in the species characterized by a small home range and in which eluding encounters with humans was more difficult, i.e. for the howler monkey [21]. We hypothesized that testosterone levels could rise following human presence due to the increase of inter- or intragroup competition for resources resulting of habitat loss, especially

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in the species living in higher permanent intergroup competition due to higher group density, i.e. howler monkey [21]. In a second step, in order to investigate which human activity was affecting the most the two species of monkeys studied here, we divided the area with HHP into two sites; (i) a site in proximity to the canal and (ii) a site in proximity to human settlements. By comparing results between species, we aimed to assess whether socio-ecological differences between the two species influenced their susceptibility to hormonal dysregulation following human presence.

Materials and Methods

Study site

Caño Palma Biological Station (10° 35' 35.854" N; 83° 31' 39.013" W) is situated along the Caño Palma at the border of Barra del Colorado Wildlife Refuge (92,000 ha) and Tortuguero National Park (18,947 ha) in the conservation district Tortuguero Conservation Area (ACTo) in North-eastern Costa Rica. Caño Palma Biological Station has been owned and managed by the Canadian Organization for Tropical Education and Rainforest Conservation since 1990 [22]. The average daily temperature is 26°C and annual rainfall can exceed 6400 mm (COTERC unpublished data). The NHPs encountered in areas surrounding the station are: Golden-mantled Howler Monkey (Least Concern, [23]), Ornate Spider Monkey (Endangered, [23]) and White-faced Capuchin Monkey (*Cebus capucinus*; Least Concern, [22,24]) Graham et al. found that average number of individuals seen in one location on Caño Palma's ground was between 3 and 6 individuals for howler monkeys and between 2 and 8 individuals for spider monkeys. On Caño Palma's ground, these species showed similar foraging habits to other study sites [25].

Fecal collection and storage

In order to limit any alteration of non-biotic conditions that could lead to variation in hormonal level, sampling was performed during

a short period of five weeks from May 2013 to June 2013. During the sampling period, the minimal temperature and daily rainfall recorded were 22°C and 0 mm; the maximal temperature and daily rainfall were 28°C and 39.3 mm; the cumulative rainfall over the sampling period was 298 mm. In order to minimize circadian alteration in fecal hormone levels, we also collected samples only from 6 am to 10 am [26].

We collected feces exclusively from adult howler monkeys and spider monkeys on Caño Palma's ground and in the Tortuguero National Park (TNP; Figure 1). When sampling, we progressed throughout the forest mainly via an existing trail system while on Caño Palma's ground. In the TNP, we progressed off trail since the tourist trail along the beach is the only existent trail. Caño Palma's ground is in an area characterized by human settlements such as a small village, a resort and the Caño Palma Biological Station lying in the fragmented secondary forest together with an intense boat traffic (i.e. ≥ 80 boats per day) on the canal linked to tourism activities [27]. Therefore, monkeys encountered on Caño Palma's ground were deemed to experience high human presence (HHP). In the TNP, no human activities are allowed except on the short tourist trail along the beach at the Northern of the Park, therefore we deemed that monkeys encountered in the TNP experienced low human presence (LHP). Caño Palma's ground and the TNP are approximately eight kilometers away and the wide Rio Suerte separates them. In virtue of the large distance and the physical barrier between Caño Palma's grounds and the TNP, monkeys encountered in one area were unlikely to be exposed to the human presence of the other area during the 5-week sampling period. Thus, in a first step, we compared hormonal levels in feces samples of monkeys encountered in Caño Palma's ground and in the TNP (Table 1). While sampling a group, we recorded as thoroughly as possible the number of individuals together with the composition of the group so as to diminish the possibility of sampling the same group later. Once we sampled in one location, we took great caution not to sample in the same location later during the sampling period in order to minimize the risk of sampling

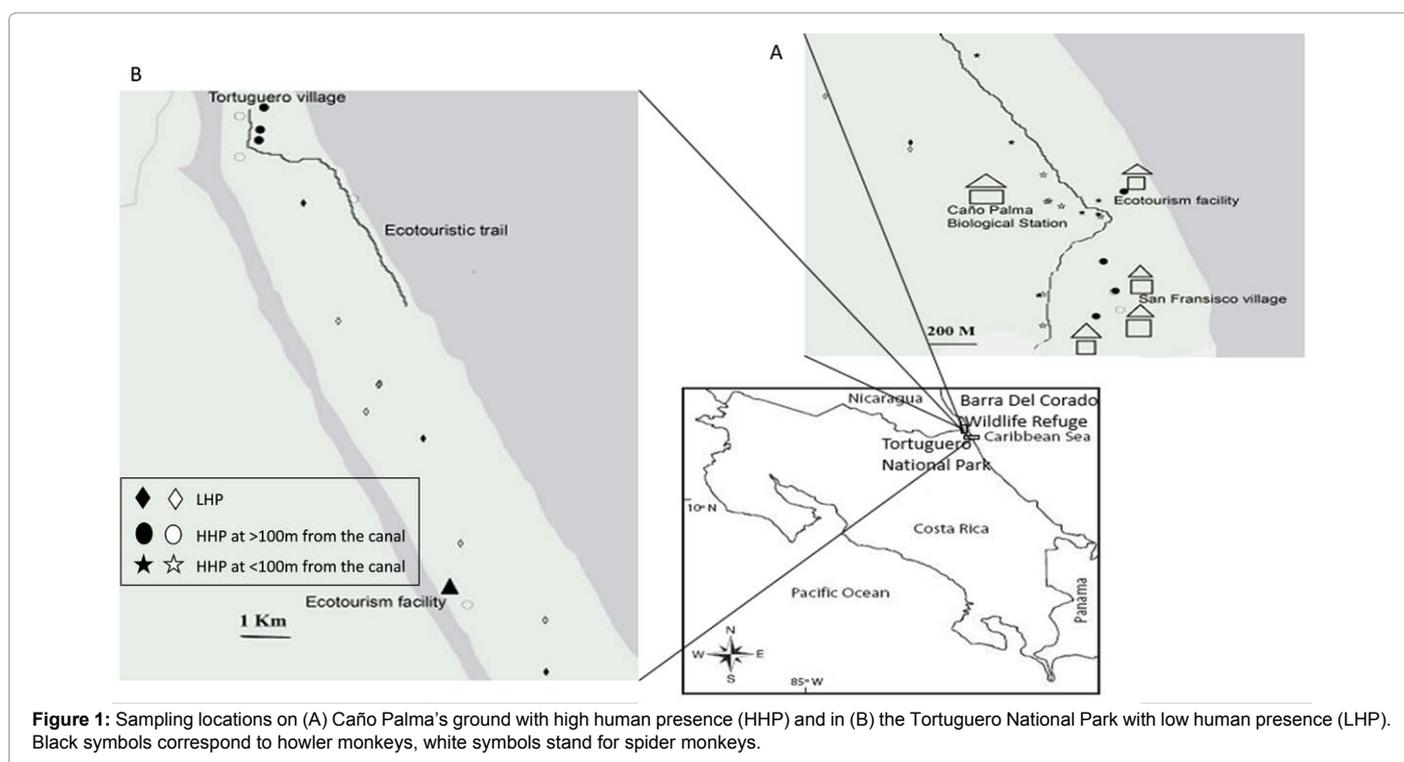


Figure 1: Sampling locations on (A) Caño Palma's ground with high human presence (HHP) and in (B) the Tortuguero National Park with low human presence (LHP). Black symbols correspond to howler monkeys, white symbols stand for spider monkeys.

	No. of groups	No. of males	No. of females
Howler Monkeys			
Area with HHP	8	7	11
Area with LHP	5	6	5
Spider Monkeys			
Area with HHP	8	9	11
Area with LHP	5	5	4

Table 1: Numbers of groups, males and females sampled for both species on sites in proximity to the canal and to human settlements in the area with high human presence (HHP) and in the area with low human pressure (LHP).

twice the same group or party. Also, within a group, individuals could not have been sampled twice, since we never were focused on a same group collecting feces for more than two hours. In a previous study in Panama, Milton [28] estimated that the time of gut passage was 20.4 h in howler monkey and 4.4 h in spider monkey. If we sampled unintentionally twice a group, it was very unlikely that it happened within a two day-time frame. Two days is the approximate lag time necessary to find peak of cortisol concentration in feces after a stressful event in howler monkeys [29]. In spider monkeys, peak of cortisol in feces appears only 20 hours after the stressful event [30].

We collected feces mostly found on leaves in 15 ml test tubes when the monkey was seen defecating or still in sight. By this method, the investigator ensured identification of the species, as well as the freshness of the feces. We desiccated the feces collected directly when coming back from the field, i.e. within 1 to 6 hours after collection. After desiccation at 80°C for 2 h 30 m in an oven, feces were stored in a Ziplock plastic bag with two grams of silica gel, until hormone extraction at the laboratory of the School of Biology of the University of Costa Rica in San José. For each fecal sample, depending on the date of collection, hormone extraction occurred between 3 and 12 weeks after desiccation.

Then, we compared hormonal level of i) monkeys from site in proximity to the canal in the area with the HHP on Caño Palma's ground; ii) monkeys from site in proximity to human settlements in areas with HHP encountered on Caño Palma's ground (N=14) and in the TNP (N=10); iii) monkeys from the area with LHP encountered in the TNP (Figure 1 and Table 2). On Caño Palma's ground, we assumed that a group of monkeys experienced more pressure from the human presence from which it was close at sampling time than the other for the two day-period before sampling. Nevertheless, since there was no physical, nor geographical separation between sites, we could not ensure that monkeys encountered on one site did not range in the other site during the two day-period before sampling.

Hormone quantification

We used a Steroid Solid Extraction protocol developed by Arbor Assay for their DetectX™ Steroid Immunoassays before conducting the Immunoassays. We used kits of NCal™ NIST-Calibrated DetectX® Cortisol Enzyme Immunoassay Kit K003-H to quantify total cortisol concentration in feces. This assay uses a monoclonal antibody to cortisol specially manufactured for detecting cortisol in animal feces. In previous studies, NCal™ NIST-Calibrated DetectX® Cortisol Enzyme Immunoassay Kit K003-H was validated and used for assessing cortisol level in numerous animal species of different families (e.g: [31] in *Homo sapiens*; [32] in *Balaena mysticetus*). We also used kits of DetectX® Testosterone Enzyme Immunoassay Kit K032-H to quantify testosterone. This assay is a mix of two polyclonal antibodies (R156/R157) developed by C. Munro at UC Davis. This combination of antibodies has also been validated and used to quantify testosterone and its metabolites in various animal species (e.g: [33] in *Gorilla gorilla*

gorilla; [34] in *Mus musculus*). Additional information on the protocols, previous uses, sensitivity, accuracy, precision and cross-reactivity of the kits are available online at: <http://www.arborassays.com/product/k003-h-cortisol-eia-kit/> and <http://www.arborassays.com/product/k032-h-testosterone-eia-kit/>.

The two kits were utilized according to the manufacturer's instructions in the Center of Investigation of Tropical Diseases (CIET) of the University of Costa Rica in San José. We calculated hormonal concentration per gram of dry feces. Some feces provided insufficient material to assess both hormones, thus 8 samples (4 for howler monkeys and 4 for spider monkeys) of the 72 samples collected were not used when calculating levels of testosterone.

Because sex determination in the field was sometimes challenging due to the height at which monkeys were, sex of the defecating individual was verified based on the individual testosterone level. Based on the testosterone concentration in dry feces, we were able to separate samples into two groups representing the sexes (males: 156.5 - 355 ng/g, and females: 36 - 89.5 ng/g for howler monkeys; males: 62 - 159 ng/g, and females 16 - 29 ng/g for spider monkeys). Sex determination using this method was similar to what we had seen in the field except for seven individuals (4 spider monkeys and 3 howler monkeys). Although the collector determined those seven individuals as males while in the field, their fecal testosterone level were low and could be classified as females. We ascribed this variation to the fact that young males with testosterone levels similar to females, could be misclassified as females using the method of testosterone level to determine sex. For this reason, in this article, mentions of "males" signify more accurately "older males" and mentions of "females" encompass "females and, potentially, younger males."

Ethical notes

This research was carried out on free ranging animals with non-invasive protocols concurring with the American Society of Primatologists (ASP) Principles for the Ethical Treatment of non-human primates. This study was also approved by the ethical committee of the School of Anthropology and Conservation at the University of Kent, UK. The Costa Rican committee SINAC (National System of Conservation Areas) reviewed all methods used in this study and granted this research with the permit number 003-2014. The Ministry of Environment and Energy of Costa Rica (MINAE) also approved this study (scientific passport number 03094).

	No. of groups	No. of males	No. of females
Howler Monkeys			
Area with HHP in proximity to the canal	5-0	5-0	7-0
Area with HHP in proximity to human settlements	3-3	2-3	4-3
Area with LHP	1-5	1-6	1-4
Spider Monkeys			
Area with HHP in proximity to the canal	6-0	6-0	6-0
Area with HHP in proximity to human settlements	2-3	3-2	5-2
Area with LHP	1-5	2-5	1-4

Table 2: Numbers of groups, males and females sampled for both species in the area with high human presence (HHP) and in the area with low human pressure (LHP). The first number is the number of samples collected on Caño Palma's ground. The second number is the number of samples collected in the Tortuguero National Park.

For fecal collection, when one group of monkey was in sight, the investigator waited nearby until the monkeys defecated. The investigator would leave the zone if the monkeys showed any signs of disturbance, such as displaying or alarm calling.

Statistics

The software IBM SPSS Statistic 20 was used for all our statistical tests. We transformed data into logarithmic base 10 of the value in order to obtain a dataset not deviating significantly from the normal distribution assessed using a Kolmogorov-Smirnov test. These transformations allowed us to use parametric statistics to analysis all our data. We compared levels of cortisol and testosterone between both species in areas with LHP using T-tests. We also used T-tests to compare hormonal levels between feces of monkeys encountered in areas with HHP and LHP for both species. We performed one-way ANOVAs and post-hoc Tukey tests to investigate which kind of the human presences had the highest impact on monkeys' hormonal levels in area with HHP. For all tests, where appropriate, the equality of variance was checked and statistical significance was set at $p < 0.05$.

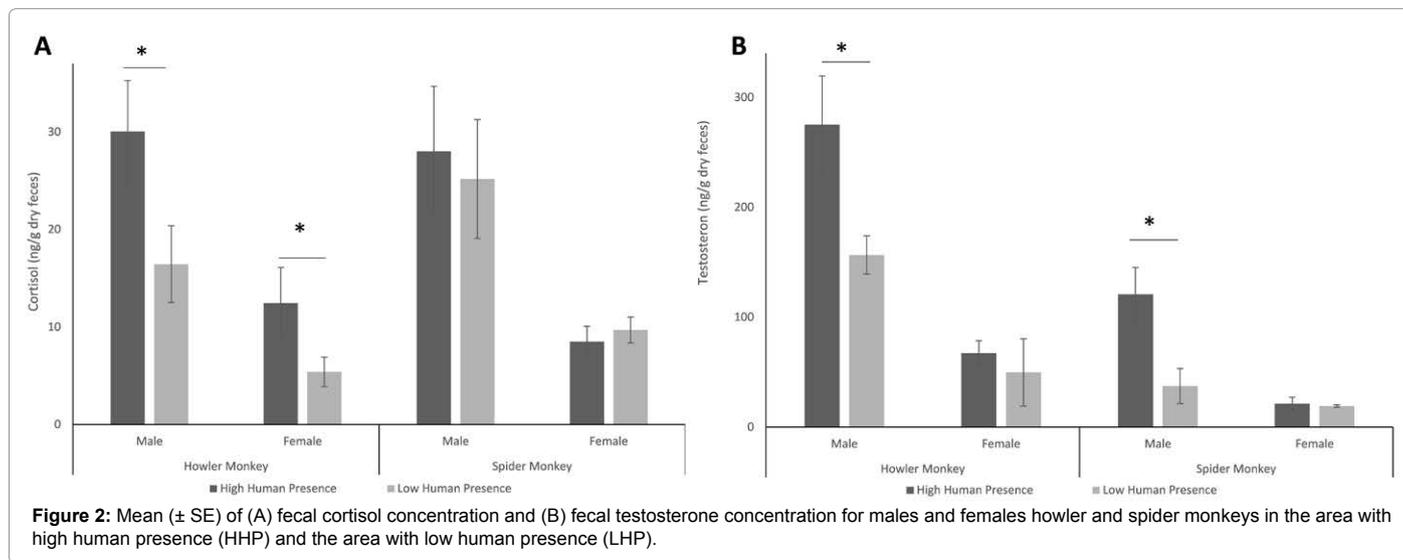
Results

As expected, in the area with low human presence (LHP), levels of cortisol were higher in spider monkeys than in howler monkeys. These

differences were significant only in males ($t(10)=4.06$, $P < 0.005$; Figure 2a).

Levels of cortisol were higher in the area with high human presence (HHP) compared with the area with LHP only in howler monkeys, for both sexes ($t(18)=2.51$, $P < 0.05$ and $t(15)=2.92$, $P < 0.02$, respectively for males and females; Figure 2a). We found significant differences of cortisol levels in both sexes of howler monkeys according to the site of sampling ($F=5.31$, $df=16$, $P=0.019$ for males and $F=9.42$, $df=15$, $P=0.03$ for females; Table 3). Both sexes of howler monkeys showed significantly higher levels of cortisol when encountered on site in proximity to human settlements in the area of HHP as compared with howler monkeys encountered in the area with LHP (Tukey post hoc test: males: $p=0.015$; females: $p=0.002$; Table 3).

Also, as we predicted, in the area with LHP, testosterone levels were higher for howler monkeys than for spider monkeys. These differences were significant in males ($t(9)=4.15$, $P < 0.005$), but not in females (Figure 2b). Levels of testosterone were higher in the area with HHP as compared with the area with LHP only in males, for both species ($t(15)=2.45$, $P < 0.05$, $t(16)=3.59$, $P < 0.005$; respectively for male howler monkeys and male spider monkeys; Figure 2b). Among sites, testosterone levels of males of both species were significantly different ($F=4.78$, $df=13$, $P=0.032$ for male spider monkeys and $F=8.34$, $df=14$, $P=0.011$ for male howler monkeys). Males of both



	N	ANOVA	p-value of post-hoc test between area with HHP in proximity to the canal and area with HHP in proximity to human settlements	p-value of post-hoc test between area with HHP in proximity to the canal and area with LHP	p-value of post-hoc test between area with HHP in proximity to human settlements and area with LHP
Cortisol					
Howler male	17	F=5.31; P=0.019	0.38	0.23	0.015
Howler female	16	F=9.42; P=0.03	0.056	0.1	0.002
Spider male	14	F=0.12; P=0.88	NA	NA	NA
Spider female	18	F=1.56; P=0.24	NA	NA	NA
Testosterone					
Howler male	14	F=8.34; P=0.011	0.085	0.009	0.16
Howler female	15	F=3.35; P=0.11	NA	NA	NA
Spider male	14	F=4.78; P=0.032	0.18	0.028	0.45
Spider female	14	F=2.59; P=0.12	NA	NA	NA

Table 3: Results of one-way ANOVA test and P-value of Tukey post-hoc test comparing hormonal concentrations of sites in proximity to the canal or in proximity to human settlements in the area with high human presence (HHP) to the area with low human presence (LHP).

species found on site in proximity to the canal in the area with HHP showed significantly higher testosterone levels than males encountered in the area with LHP (Tukey post hoc test: howler monkeys: $p=0.009$; spider monkeys: $p=0.028$; Table 3).

Discussion

Our results corroborated our prediction that spider monkeys should secrete a higher baseline level of cortisol than howler monkeys owing to the higher level of intraspecific competition for food. If the cortisol level in the area with LHP of spider monkeys compared to howler monkeys was indeed higher due to the intraspecific competition for food, our results show that female spider monkeys were the ones most affected by the higher level of competition for food. The fact that males are dominant over females in spider monkeys may substantiate this hypothesis [35]. The fact that only howler monkeys showed higher cortisol levels in the area with HHP compared to the area with LHP could confirm our prediction that howler monkeys are more susceptible to be disturbed by the type of human presence studied here, perhaps due to their smaller home range and social habits [21]. The smaller territory of howler monkey could i) limit their ability to evade or elude encounters with humans or ii) hamper them from meeting their food requirements due to decrease of food availability which might result from proximity to human settlements. This latter possibility mirrors results of Dunn et al. [11] showing an increase of feeding efforts leading to an increase of fecal cortisol level among howler monkeys living in fragmented forests. Further studies across various species with differing social habits and ecologies are needed to challenge these primary results. Results of the analysis aiming to discriminate between the proximity to human settlements and the proximity to the canal in regard to the induction of hormonal dysregulation in areas with HHP can only suggest routes for investigation for further studies. Indeed, it is not unlikely that monkeys sampled on one site experienced the human presence of the other site for at least a few moments during the time necessary for excreted steroids to be found in feces (i.e. ± 2 days for howler monkeys and ± 20 hours for spider monkeys). This analysis shows that the proximity to human settlements was the factor inducing the largest rise of cortisol level in howler monkeys when they were in area with HHP.

As we predicted based on the territorial behavior and the intensity of the intra- and inter-group competition for access to mates [18,19], male howler monkeys presented higher levels of testosterone than male spider monkeys in the area with LHP. Despite the small sampling size, we think that our results are representative of the groups sampled. Using the group size of primates reported by Graham et al. [25] together with long-term monitoring on Caño Palma's ground, we estimated that groups were composed of no more than 2 males for groups of howler monkeys and no more than 3 males for groups or party of spider monkeys (COTERC, unpublished data). Thus, we estimated that sampling two or three individuals per group allowed us to assess hormonal levels of almost all males of a given group. In both species, only males showed higher level of testosterone in the area with HHP compared to males in the area with LHP. When comparing testosterone levels within species, we assumed that competition for resources, whether food or mates in the area with LHP and the area with HHP were similar. However, we were unable to validate these latter assumptions and further research will be required to ascertain the underlying causes of this noted rise in testosterone levels among males of both species on site in proximity to the canal in the area with HHP. Nevertheless, here, we argue that in the area with HHP, males of both species would aggressively display in the presence of humans, as they would in the presence of any predator. The hormonal response

to stimuli leading to aggressive displays is an increase of testosterone levels [13]. Our data suggests that the proximity to the canal was the factor impacting the most secretion of testosterone in males of both species when in areas with HHP. Thus, our study suggests that noise from motor boats was a stimuli that might amplify the aggressive behavior and therefore led to a higher level of testosterone in males. It is noteworthy that, throughout this study, male howler monkeys almost systematically began howling when boats with motor on passed within proximity. In contrast, they rarely howled when motors were off. Male howler monkeys typically howl to express or to assess fighting ability in the context of territorial protection or expansion [36,37]. Such observations provide some credence to the possible effect of noise disturbance from motor boat passage along the canal on male testosterone levels. A previous study in a logging concession argued that noise disturbance from machinery and vehicles affects the behavior of NHPs [38]. In a study carried out in Brazil, Monteiro et al. [39] also reported that free ranging male howler monkeys (*Alouatta belzebuch*) exposed to higher noise intensity from mining activity excreted more androgens, including testosterone, than monkeys ranging in an area without mining activity.

Because of the assumptions that we had to make, we only suggest that human presence impacts the physiology of howler monkeys and spider monkeys. We think that our results show patterns justifying further long term studies. Further studies should be undertaken in areas where noise from human activities such as boat traffic and human settlement are physically separated. Larger sample sizes and repetitive sampling could help promote our understanding of the impact of noise disturbances and human activities on the physiology of NHPs and the underlying endocrine response.

In conclusion, although further studies are needed to confirm the actual impact of human activities on NHPs and wildlife physiology, this study strongly suggests that human presence and potentially noise have an impact on the physiology of NHPs. Our study upheld the hypotheses that human presence can have differing disruptive effects on different NHP species that reflect differences in their socio-ecology and their ability to buffer environmental changes. Should further studies confirm a relationship between human-related noise disturbance and hormonal levels among NHPs, strong policy guidelines concerning tourism management and/or noisy human activities should be developed and implemented in order to prevent or mitigate impacts on NHPs and possibly other wildlife. Also, given the roles of hormones such as cortisol and testosterone in the body, we suggest that primatologists together with conservationists should undertake long-term studies on the impact of hormonal dysregulations caused by human activities on NHPs' behaviors, body functions and physical condition.

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