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RESEARCH ARTICLE

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Validation of an enzyme immunoassay and comparison of fecal cortisol metabolite levels in black and gold howler monkeys (*Alouatta caraya*) inhabiting fragmented and continuous areas of the humid Chaco region, Argentina

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In the last years, the study of how environmental stimuli influence the physiology and specifically the endocrinology of an organism became increasingly important, relying mainly on the quantification of glucocorticoids to monitor animal welfare. Most studies investigating cortisol levels in primates were focused on the impact of social stressors; however, a major concern for the conservation of howler monkeys is the increased habitat fragmentation led by the advancement of the agricultural frontier. We compared fecal cortisol metabolite levels (FGCM) in howler monkeys (*Alouatta caraya*) living in fragmented and continuous forests of the Argentine humid Chaco region, throughout the warm season (spring-summer). Fecal samples (n = 114) were collected from adult individuals, and steroid extracts analyzed with an enzyme immunoassay also validated in this work. Parallel displacement curves were obtained between dilutions of pooled fecal extracts and the cortisol standard curve (r^2 = 0.99; P = 0.23). Efficiency of the fecal extraction procedure was 79.4% ± 38%; recovery of exogenous hormone added to fecal extracts indicated a low interference of components in the feces with antibody binding. The exogenous administration of ACTH in captive-bred animals demonstrated a "cause-and-effect" relationship between the adrenal gland activation and increased FGCM levels. Contrary to our initial prediction, we were not able to demonstrate a significant difference in FGCM levels of caraya monkeys inhabiting the continuous versus fragmented habitats in our study site (83.2 ± 4.9 ng/g [n = 10 individuals] vs. 71.5 ± 4.9 ng/g [n = 7 individuals]; P = 0.29); this could be the result of low levels of disturbance imposed by a moderate and selective logging, which has proved to be beneficial for this species with high resilience by adjusting their diet to cope with feeding in degraded habitats but with new leaves and buds. Regardless of the habitat, cortisol metabolites were significantly higher in females than in males (86.4 ± 4.2 ng/g [n = 12 individuals] vs. 60.7 ± 5.0 ng/g [n = 5 individuals] respectively; P = 0.007).

KEYWORDS
caraya, cortisol, feces, fragmentation, glucocorticoids

1 | INTRODUCTION

In the last few years, the study of how environmental stimuli influence the physiology and specifically the endocrinology of an organism became increasingly important, relying mainly on the quantification of glucocorticoids (GCs) to understand, monitor and improve animal welfare, health, and reproduction (Touma & Palme, 2005).

Traditionally, the analysis of GC levels has been based on its determination in plasma (Goymann, Möstl, Van't Hof, East, & Hofer, 1999). However, constraints of the blood sampling procedure, the pulsatile secretion pattern and circadian rhythms of GC's (Fulkerson & Tang, 1979) pose some limitations to this approach, particularly for free-ranging animals (Touma & Palme, 2005).

To overcome these problems, efficient alternative techniques have been developed by measuring steroid hormone metabolites in urine, feces, or other non-traditional matrices (i.e., feathers, saliva, hair) (Keay, Singh, Gaunt, & Kaur, 2006; Schwarzenberger, 2007; Sheriff, Dantzer, Delehanty, Palme, & Boonstra, 2011). Using fecal samples offers several advantages, such as avoiding the stress and associated risks of capture, handling, and restraint, the sample collection can be done for long periods of time and finally, the assays used are relatively efficient and easy to adapt from one species to another (Peter, Kapustin, & Critser, 1996). Due to these characteristics, fecal glucocorticoid metabolites (FGCM) monitoring can be an effective tool to determine the prolonged perception of a stressor in free-ranging animals (Washburn & Millspaugh, 2002). However, circulating steroid hormones are metabolized by the liver and excreted as conjugates via the bile into the guts (Touma & Palme, 2005). For a reliable monitoring of adrenocortical activity using FGCM analyses, the technique must be validated for each species and sample material, to ensure proper quantification of the glucocorticoids or their metabolites (Sheriff et al., 2011; Whitten, Brockman, & Stavisky, 1998; Ziegler & Wittwer, 2005).

To date, most of the studies related to cortisol levels using FGCMs in primates were focused on the impact of social stressors (Abbott et al., 2003; Creel, 2001; Engh et al., 2006) and only a few about other potentially stressful factors, such as seasonality (Weingrill, Gray, Barrett, & Henzi, 2004), habitat fragmentation, fruit availability (Pride, 2005), and predation risk (Arlet & Isbell, 2009).

Our study subject, the black and gold howler monkey (*Alouatta caraya*), is one of the species found furthest south in the distribution of the *Alouatta* genus (Di Fiore & Campbell, 2007). These howlers can be found in Paraguay, southern Brazil, northern and eastern Bolivia, and northern Argentina (Brown & Zunino, 1994; Di Fiore & Campbell, 2007). Its wide distribution and the marked variability in its habitat, is indicative of their great capability to exploit different resources (Clarke, Collins, & Zucker, 2002; Clarke & Zucker 1994; Fedigan, Rose, & Avila, 1998), a feature that has allow the species to survive in marginal areas and small forest fragments where other species are not capable to live (Crockett, 1998; Jones, 1999).

In Argentina, the caraya monkeys inhabits humid and semi-humid temperate forests in the northeast of the country (Brown & Zunino, 1994; Zunino, Gonzalez, Kowalewski, & Bravo, 2001). In the last 50 years, this forest has been highly modified and fragmented due to deforestation, selective logging, agriculture, and livestock farming. As a result, these primate populations have vastly diminished overtime (Brown & Zunino, 1994; Clarke et al., 2002; Kowalewski & Zunino, 1999; Rumiz, 1990; Zunino, Kowalewski, Oklander, & Gonzalez, 2007; Zunino & Kowalewski, 2008).

In order to predict long-term sustainability of black and gold howler monkey populations, it is important to understand the effects of these threats on animal stress physiology. Understanding how this species responds to anthropogenic disturbance will allow us to develop more effective and comprehensive conservation strategies that could be implemented with certain level of sustainable land use, which is the primary income source for most of the residents in the region.

As part of a larger project that investigates the effects of habitat modification on behavioral and physiological patterns in groups of black and gold howler monkeys (*A. caraya*), the objectives of the present work were to: 1) validate an enzyme-immunoassay for non-invasive monitoring of GC metabolites in caraya feces; 2) evaluate FGCM levels in individuals inhabiting fragmented and non-fragmented areas of the Humid Chaco region of Argentina, during the spring-summer season; and 3) examine sex-related variations in FGCM levels.

2 | METHODS

2.1 | Study site and subjects

We studied four groups of black and gold howler monkeys (*A. caraya*) inhabiting the Humid Chaco ecoregion in the province of Chaco, Argentina; two of these groups inhabited different areas of the Chaco National Park (26°48′29″ S, 59°36′26″ W; hereafter "non-fragmented or continuous forest or habitat"). The other two groups were located in private, surrounding fields undergoing selective logging (hereafter "fragmented forest or habitat") and where animal hunting was allowed. The ecoregion has a humid temperate climate with an average temperature of 22°C and the rainy period is concentrated from October to April corresponding with the warm season (spring-summer).

The studied animals were recognized by their location and group composition, as well as their natural anatomical and physiognomic characteristics (Zunino et al., 2007). In both areas animals are accustomed to human presence; furthermore, the groups inhabiting the Chaco National Park were previously followed and studied. The present study focused only on adult individuals of each group (detailed in Table 1). Animals were studied from December 2011 to March 2012 (summer season). Observations were performed during complete days (06:00–20:00 hr) using focal-animal sampling with continuous observation of the group.

In all cases, adequate measures were taken to minimize animal discomfort or pain, and the study was authorized by the Natural Resources Division, Province of Corrientes, Argentina. The study adhered to the American Society of Primatologists Principles for the Ethical Treatment of Non Human Primates.

2.2 | Fecal sample collection and processing

Fecal samples were collected opportunistically immediately after defecation whenever they could be matched with individuals, directly from the forest floor using latex gloves, and deposited in polyethylene bags with the corresponding ID. These were kept in a cooler box with frozen gel packs while in the field to prevent alteration in hormone

TABLE 1 *Alouatta caraya* groups composition in fragmented and continuous habitats (including all age classes and the number of adults per group)

Habitat	Age class	Group 1	Group 2
Continuous	Adult	MA ♂	LE ♂
		OR ♀	CH ♂
		JU ♀	MA ♀
		FL ♀	FA ♀
		CO ♀	HI ♀
	Juvenile	—	FI ♂
		—	AN ♂
		—	DA ♂
		—	CL ♀
		—	EV ♀
	Infant	—	NE ♀
Total adults in the Continuous habitat		5	5
Total individuals in the Continuous habitat		5	11
Fragmented	Adult	AR ♂	MA ♂
		IS ♀	NEG ♀
		VI ♀	CE ♀
		—	TO ♀
	Juvenile	WI ♂	RA ♂
		CH ♀	JO ♂
		—	KU ♂
		—	CA ♂
		—	NE ♂
	Infant	CA ♂	CR ♂
		—	PE ♂
Total Adults in the Fragmented habitat		3	4
Total individuals in the Fragmented habitat		6	11

The initials preceding sex denotation correspond to the first letters of the animal name.

concentration post-defecation and stored at the end of the sampling day in a freezer at −20°C until extraction was performed.

Fecal samples were collected from each individual for two consecutive days per month during the 4 months of the study. A total of 114 samples were finally collected, 71 in the continuous habitat, and 43 in the fragmented habitat.

Once in the laboratory, steroid metabolites were extracted following the method described by Palme (2005), with slight modifications: briefly, 5 ml of 80% methanol in water were added to vials containing 0.5 g (wet weight) of each fecal sample, vortexed for 2 min and incubated at 4°C for 12 hr. Once this time elapsed, fecal suspensions were vortexed again for 30 sec and finally centrifuged at ~500*g* for 20 min. The supernatant containing the steroids was recovered and stored at −20°C.

2.3 | Validation of the non-invasive technique for detecting FGCM

Validation of FGCM analysis in caraya monkeys was conducted and reported here by demonstrating: (1) parallelism between dilutions of pooled fecal extracts and the respective standard curves, in order to detect immunological similarities between the standard and sample hormones; (2) recovery of exogenous hormone in the range of the standard curve added to the fecal extracts, in order to examine possible interference of components in feces or the solvent with antibody binding (matrix interference); (3) efficiency of the extraction procedure, evaluated through the recovery of a known amount of exogenous cortisol added to a pool of wet feces before the extraction with the solvent (amount observed/amount expected*100%).

Physiological validity was also established, by demonstrating a cause-and-effect relationship between the activation of the adrenal gland through the administration of exogenous adrenocorticotropic hormone (ACTH), and the corresponding increase in immunoreactive fecal glucocorticoid metabolite levels. Fecal samples were collected from four captive individuals (two males and two females), held at the Corrientes Zoo (Province of Corrientes, Argentina), before and after the exogenous administration of 5 I.U./kg ACTH gel (Acthelea gel, Elea Lab, Buenos Aires, Argentina). Samples were stored at −20°C until further processing.

2.4 | Immunoassay used

The concentration of FGCM was determined by enzyme immunoassay using a polyclonal anti-cortisol antibody, cortisol standard, and the corresponding horseradish peroxidase conjugate (Cortisol R4866, Department of Population Health and Reproduction, C. Munro, UC Davis, CA). Prior to the assay and according to the parallelism results, 200 µl of the fecal extract was dried down at 60°C to evaporate the solvent and reconstituted in 1200 µl of EIA buffer (0.1 mM sodium phosphate buffer, pH 7.0, containing 9 g of NaCl and 1 g of BSA per liter) (final dilution 1:6) and assayed in duplicate.

Flat-bottom microtiter plates (Nunc Maxisorp, VWR, Mississauga, ON, Canada) were first coated with 50 µl of the anti-cortisol antibody diluted in coating buffer (50 mM bicarbonate buffer, pH 9.6; 1:12000), covered with acetate sealers to prevent evaporation and incubated overnight at 4°C. After 16–24 hr, plates were washed to remove any unbound antibody with 0.02% Tween 20 solution using a Bio-Tek ELx 405VR microplate washer (Bio-Tek Instruments, Winooski, VT). Immediately after washing, 50 µl of fecal extracts, standards, and controls diluted in EIA buffer were added in duplicates, followed by 50 µl of horseradish peroxidase conjugate diluted in EIA buffer (1:34000). Plates were then covered and incubated at room temperature for 2 hr. Following incubation, the plates were washed and blotted dry, and 100 µl of substrate solution (50 mM citrate, 1.6 mM hydrogen peroxide, and 0.4 mM 2,20-azino-di-(3-ethylbenzthiazoline sulfonic acid) diammonium salt, pH 4.0) were added to each well (Munro et al., 1991). Absorbance was measured at 405 nm using a microplate reader (MRX microplate reader, Dynex Technologies, Chantilly, VA).

Cross-reactivities reported are: prednisolone (9.9%), prednisone (6.3%), cortisone (5.0%), corticosterone (0.7%), 21-deoxycortisona (0.5%), deoxycorticosterona (0.3%), progesterone (0.2%), 11-desoxycortisol (0.2%), 17a-hydroxyprogesterone (0.2%), and <0.1% with all other steroids tested.

2.5 | Statistical analysis

All hormonal values are expressed as mean ± standard error of mean (SEM), recognizing the fact that hormonal metabolites appear in feces a few hours later than their presence in circulating blood. Additionally, each hormonal data point represents the mean of hormonal values of the two samples obtained for each animal.

Data analysis were performed using the Infostat statistical software (Di Rienzo et al., 2012). A regression analysis with auxiliary variables (dummy) was used to compare if the curves obtained in the assay of serial dilutions of pooled fecal extracts and the cortisol standard curve were parallel. To assess the effects of type of habitat (continuous/fragmented), sex (male/female) and sampling months (december to march inclusive) (categorical variables) on FGCM levels, a linear mixed effects model was applied. The numerical routines implemented in InfoStat for the estimation of mixed-effects-linear models are based on the nlme package (Pinheiro, Bates, DebRoy, & Sarkar, 2013) of R (R Core Team, 2012). The factors type of habitat, sex, and sampling months were included as fixed effects; meanwhile the group of origin and animal effect and were included as random factors. The animal effect accounts for the intra-class correlation, which is a common correlation structure for repeated measures on the same experimental unit (animal). Samples collected in March were excluded from the analysis because of an inconsistent sampling that month (one of the groups from the fragmented habitat was not found during the field trip in March 2012).

To check the assumptions of normality and variance homogeneity, diagnostic techniques based in the residuals were applied. According to this test, the response variable (FGCM levels) was log transformed. The DGC post hoc test (Di Rienzo, Guzmán, & Casanoves, 2002) was applied to perform the pair-wise comparisons of adjusted means when the overall (F) test was significant. The significance level for all statistical tests was set at 0.05.

3 | RESULTS

3.1 | Validation of the non-invasive technique for detecting FGCM in captive-breed *Alouatta caraya*

To establish the pharmacological validity of the technique, a first trial using diluted fecal extracts (containing methanol) directly into the immunoassay, produced no parallelism between the standard curve

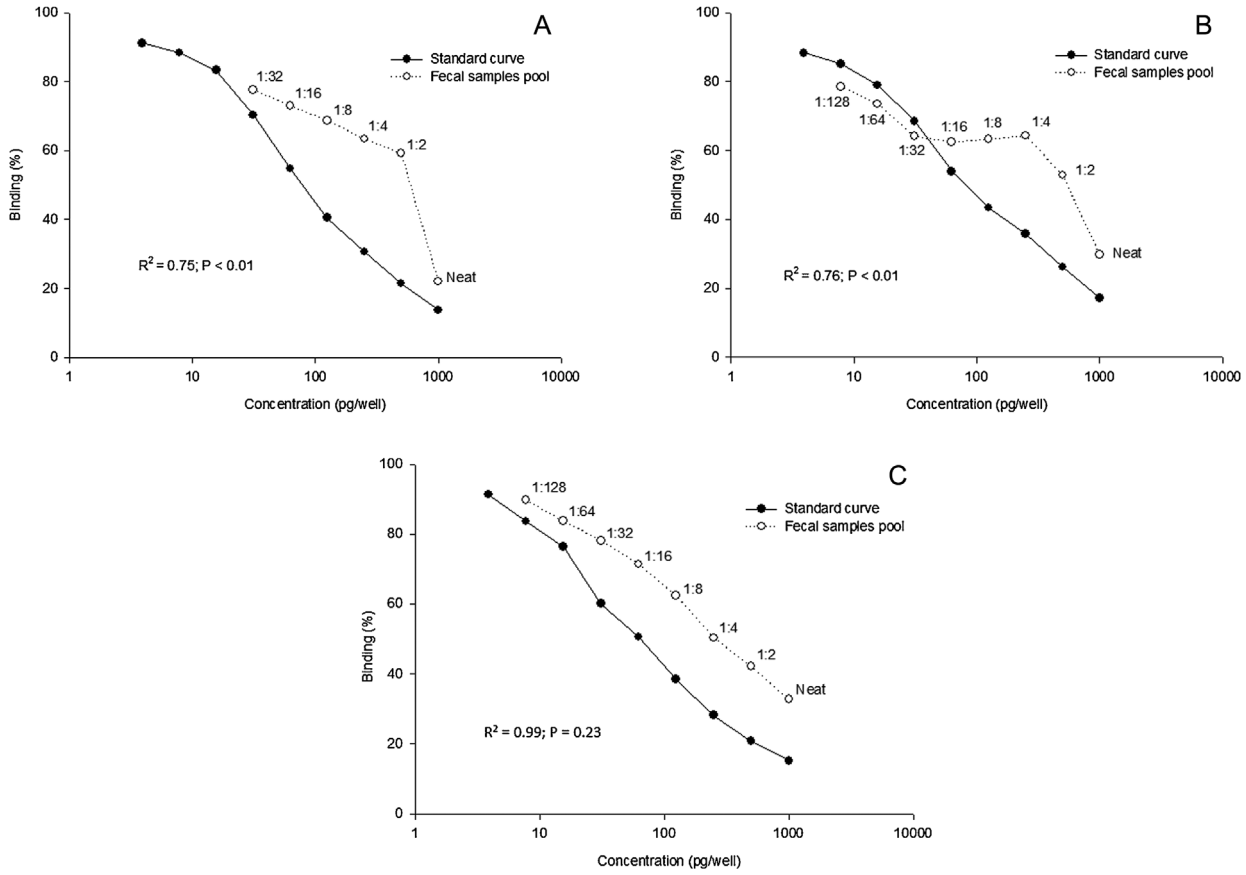

FIGURE 1 Parallelism between a serially diluted methanol extracted pool of fecal samples and the cortisol standard curve. Panel A, 0.5 g of feces extracted with methanol; Panel B, 1 g of feces extracted with methanol; Panel C, 0.5 g of feces extracted with methanol, dried, and reconstituted in enzyme immunoassay buffer

and serial dilutions of the extracts (Figure 1, panel A and B). The same results were obtained when the assay was performed to extracts obtained using a higher amount of wet feces (1 g). Therefore, fecal extracts were dried down to evaporate the solvent that interfered with the hormone-antibody binding and reconstituted in buffer. In that case, the immunoassay performed with a serially diluted pool of fecal extracts showed parallel displacement to the cortisol standard curve ($R^2 = 0.99$; $P = 0.23$) (Figure 1, panel C). According to these results, all fecal extracts in this study were desiccated and reconstituted in phosphate buffer (final dilution 1:6) before immunoassay.

Recovery of exogenous hormone in the range of the standard curve (0.078–20 ng/ml) added to the fecal extracts indicated a low interference of components in the feces with the antibody binding (98.2 ± 17.9%; $y = 0.945x + 0.106$; $R^2 = 0.99$; $P \leq 0.01$). Efficiency of the entire fecal extraction procedure with the solvent was 79.4% ± 38%.

To establish the physiological validity of the technique, FGCM levels in samples collected before and after the exogenous administration of ACTH in captive-bred animals was performed and demonstrated here. A total of 81 samples from two males and two females were collected and analyzed. Exogenous activation of the adrenal gland caused a significant increase in FGCM levels; fecal cortisol metabolite immunoreactivity peaked after 29 ± 8.2 hr post-injection in animals *Florencio*, *Monona*, and *Colincha*. An additional peak of immunoactivity was also detected after ~100 hr in all three animals. The forth challenged animal (*Mogui*) showed a high fluctuation in the FGCM levels in the collected samples and therefore, it was not possible to determine a clear immunoreactivity peak. Interestingly, an additional peak of FGCM excretion was observed in three of the four individuals challenged even before the ACTH administration (Figure 2). A retrospective analysis of the experiment conditions revealed that those peaks occurred right after a Sunday, which is usually the busiest day of the week in terms of public visitors to the zoo. Frequently the howlers are disturbed by people trying to induce the animals to vocalize. These unexpected phenomena could have elicited the additional glucocorticoid peak observed, at the same time providing us with an additional biological validation for the technique.

3.2 | Hormonal determinations in wild *Alouatta caraya*

A total of 114 fecal samples from 17 free-ranging animals (5 males and 12 females) were collected and analyzed using the EIA validated in the present work.

FGCM levels found in howler monkeys did not differ among groups living in continuous and fragmented habitats (forest type, $P = 0.23$)

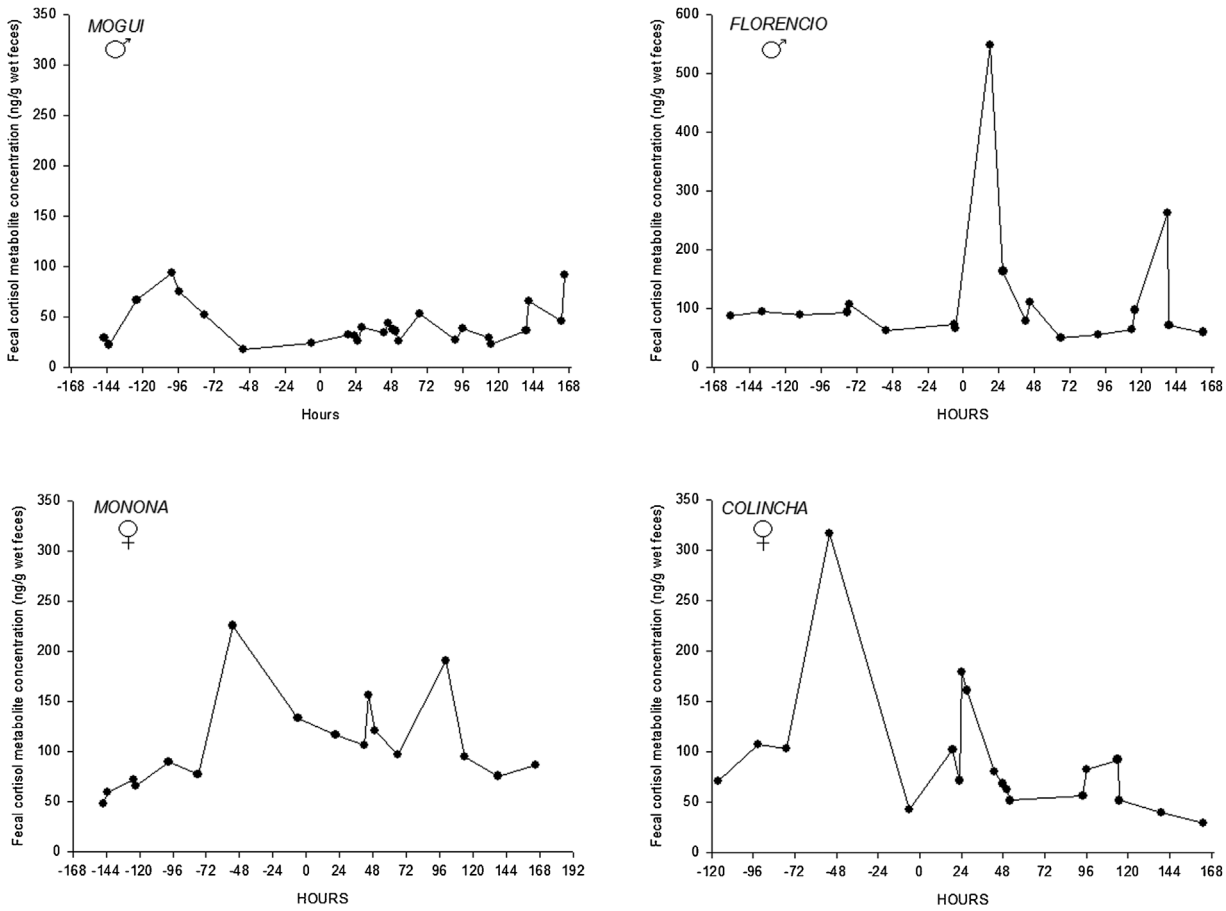

FIGURE 2 Immunoreactivity profiles of cortisol metabolites in fecal samples of *Alouatta caraya* ($n = 4$; two males and two females) before and after the injection of 5 I.U./kg gel ACTH (at time 0)

(Figure 3). In addition, no significant effects were detected when analyzing the effects of the sampling month on FGCM levels, as well as in all the interactions tested in the model (Table 2). However, when the data are analyzed regardless of the habitat, females showed significantly higher FGCM levels than males (86.4 ± 4.2 vs. 60.6 ± 5.0 ng/g wet feces; $P = 0.007$) (Figure 4 and Table 2).

TABLE 2 Results of general linear mixed model application on fecal glucocorticoid metabolite levels data

Effect	d.f.	*F*	*P*-value
Forest type (continuous vs. fragmented)	1	2.92	0.23
Sex (male vs. female)	1	11.14	0.007
Sampling month	2	1.91	0.16
Forest type × Sex	1	1.59	0.23
Forest type × Sampling month	2	1.46	0.24
Forest type × Sex × sampling month	2	0.36	0.69
Sex × sampling month	2	0.41	0.66

Interaction between factors showed.

4 | DISCUSSION

Validation measures for assays determining FGCM concentrations have been described for several other species of non-human primates (e.g., *Pan troglodytes* (Whitten, Stavisky, Aureli, & Russell, 1998), *Lemur catta* (Cavigelli, 1999), *Macaca fascicularis*, and *Papio cynocephalus* (Wasser et al., 2000), among other sp. (Heistermann, Palme, & Ganswindt, 2006)) but not in *A. caraya*. The data reported in this study, clearly demonstrated that the polyclonal antisera and FGCM assay used here can detect some of the GC metabolites excreted via feces in black and gold howler monkeys. Furthermore, these validations demonstrated that the methanol-based fecal extracts should be dissecated and reconstituted in buffer previous to the immunoassay, in order to avoid interference with the antibody that could yield to inconsistent results. Physiological validity was also demonstrated by establishing a "cause-and-effect" relationship between the exogenous activation of the adrenal gland by ACTH administration, and the subsequent excretion of FGCM after an excretion lag-time of approximately ~30 hr.

It should be considered that the route, time and form of metabolic glucocorticoid excretion differ in each species (Monfort, 2003). Furthermore, the same metabolite could sometimes present a different lag-time of excretion in different individuals due to inherent metabolic rates of each individual or even to constipation, indicating an "animal effect." Although in human and non-human primates the average excretion time is calculated in approximately 24 hr (e.g., *P. troglodytes*, *M. fascicularis*, and *Callithrix jacchus* (Bahr, Palme, Mohle, Hodges, & Heistermann, 2000)), the time delay differences in the present study could likely be attributed to this animal effect (i.e., 1–3 fecal samples post-injection to reach the excretion peak in the three responding animals).

In general, anthropogenic changes to primate habitats impact primate populations by changing the availability, density, abundance, and distribution of plant species (Milich, Stumpf, Chambers, & Chapman, 2014) which in turn, can trigger a physiological stress response reflected in higher cortisol levels (e.g., *Alouatta pigra* (Arroyo-Rodriguez & Dias, 2010; Martínez-Mota, Valdespino, Sánchez-Ramos, & Serio-Silva, 2007), *Piliocolobus tephrosceles* (Chapman, Saj, & Snaith, 2007), *L. catta* (Cavigelli, 1999), *P. troglodytes* (Muller & Wrangham, 2004), *Papio anubis* (Sapolsky, 1986)). For arboreal primates like howler monkeys, the potential stressors include terrestrial locomotion (an atypical situation that may increase susceptibility to predation) or increased human presence in the fragment (Rangel-Negrín, Coyohua-Fuentes, Chavira, Canales-Espinosa, & Dias, 2014).

Although howlers have been cited as a relatively tolerant taxon to habitat disturbance (Arroyo-Rodriguez & Dias, 2010), a number of

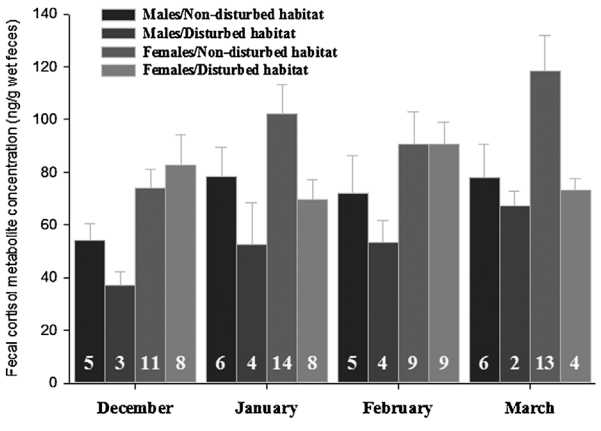

FIGURE 3 Fecal glucocorticoid metabolite levels (ng/g wet feces) in *Alouatta caraya* males and females inhabiting disturbed and non-disturbed habitats of the humid Chaco ecoregion. Data are expressed as mean ± S.E.; number of samples indicated at the bottom of the bars

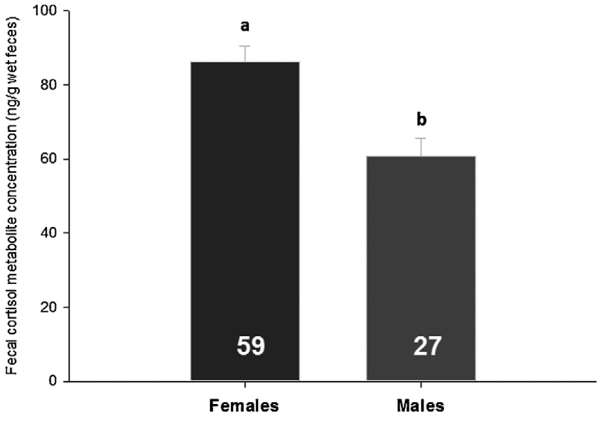

FIGURE 4 Fecal glucocorticoid metabolite levels (ng/g wet feces) in *Alouatta caraya* males and females inhabiting disturbed and non-disturbed habitats of the humid Chaco ecoregion. Data are expressed independently of the sampling month and habitat, as mean ± S.E.; number of samples indicated at the bottom of the bars. *$P = 0.007$

studies have in fact indicated that individuals who live in small and very disturbed patches consume less fruits, have a higher energy cost directed to feeding and present higher levels of cortisol, than those inhabiting larger and preserved patches (*Alouatta pigra* (Dunn, Cristobal-Azkarate, & Veà, 2009; Dunn, Cristóbal-Azkarate, & Veà, 2010; Martínez-Mota et al., 2007)). Also, according to Arroyo-Rodriguez and Dias (2010), the patch size is positively related to food availability and negatively related to anthropogenic and physiological stress. Similarly, Dunn et al. (2009) reports that fecal glucocorticoid metabolite concentrations are higher among an *A. palliata* group inhabiting a small forest patch, than those individuals inhabiting a larger patch (Arroyo-Rodriguez & Dias, 2010). On the other hand, a study in free ranging spider monkeys showed that, at least in the short term, animals inhabiting landscapes with low forest cover do not necessarily show higher FGCM levels, and highlighted the importance of preserving fruit sources and controlling hunting activities for reducing FGCM levels (Ordóñez-Gómez et al., 2016). Similarly, Dunn, Cristóbal-Azkarate, Schulte-Herbrüggen, Chavira, and Veà (2013) found that travel time related to fruit consumption is the main factor predicting elevations in FGCM levels.

Likewise, and contrary to our initial prediction, we were not able to demonstrate a significant difference in the FGCM levels of *A. caraya* monkeys inhabiting the fragmented versus continuous forest fragments in our study site.

Some studies and in accordance to ours, found that individuals in degraded environments could show a behavioral plasticity in feeding behaviors and foraging strategies to expand their diet to include a greater variety of foods than seen in individuals living in the old-growth forest, which would effectively increase their resource availability (Milich et al., 2014). In fact, the *Alouatta* genus is known by its high resilience and pioneer capacity (Bicca-Marques, 2003; Garber, Estrada, & Pavelka, 2006; Lovejoy et al., 1986; Schwarzkopf & Rylands, 1989; Van Belle & Estrada, 2006). This characteristic allows them to adapt and survive in modified woods in the first stages of disturbance where other primate species could not survive. According to Arroyo-Rodriguez and Dias (2010) in their detailed review of the genus, the success of howlers in coping with habitat disturbance has been related to their high feeding plasticity reflected in their capacity to consume a high amount of new and exotic leaves (frequent in disturbed habitats) in absence of fruits, use small home ranges and utilize energy-saving activity budgets. They also proposed from evidences recorded in *A. caraya*, *A. guariba*, *A. palliata*, *A. pigra*, and *A. seniculus*, that patch size is an important factor negatively affecting the presence, abundance and persistence of howler populations in fragmented habitats, but that habitat loss probably has larger consistent negative effects on howler populations than habitat fragmentation per se (Arroyo-Rodriguez & Dias, 2010).

Furthermore, the selective logging activities in our study site seemed to be insufficient to impact the park caraya populations. We should also keep in mind that, large predators in this region (i.e., *Puma concolor*) are the first species to disappear from degraded fields (Terborgh et al., 2001) adding a possible explanation to the similar fecal cortisol metabolite levels detected in the groups from both areas.

In this sense, a major focus of future studies should be the identification of threshold values for habitat disturbance under which the glucocorticoid secretion balance of howler populations could be compromised (Arroyo-Rodriguez & Dias, 2010).

Finally, the present work also found that regardless of the habitat, females showed significantly higher FGCM levels than males (~1.5 times higher). Previous research on howler monkeys has reported that glucocorticoid levels are higher in females than males (Aguilar-Cucurachi et al., 2010) and in reproductive than non-reproductive females (Dunn, Cristóbal-Azkarate, Veà, & Chavira, 2011), independently of habitat characteristics. Clearly, glucocorticoids are expected to be elevated during moments of increased metabolic demands (Sapolsky, Romero, & Munck, 2000), such as those associated with gestation, lactation (Gittleman & Thompson, 1988), and even oestrus. Additionally, some forms of female–female competition have also been observed in *A. caraya* (Calegaro-Marques & Bicca-Marques, 1996) in example, female howler monkeys may compete to avoid infanticide risk (Crockett & Janson, 2000), aggressively prevent extragroup females from immigrating thereby maintaining limited group size (Crockett, 1984; Crockett & Pope, 1993) or compete to recruit their own daughters as additional breeding females and other females' daughters are forcefully evicted as juveniles. These social interactions could have an impact in the glucocorticoids levels in females.

However, we should also recognize that in general, sex-specific variations in GC metabolism (i.e., route of excretion, lag-time and even type of metabolites excreted) has been described in several species (Touma, Sachser, Möstl, & Palme, 2003). Although in caraya monkeys we do not really know if there are sex differences in how GC are metabolized, if this is the case, then we should consider that it is possible that females have shifted their output in metabolites and that we are actually detecting more of a specific kind of metabolite that cross-reacts with the antibody, but not an actual difference in hormone levels between the sexes.

Finally, by building on previous and ongoing studies examining habitat quality, food availability, travel time/distance between food patches, predation danger, parasite presence and behavior strategies within and around the park, our results can help to shed light on conservation policy and management practices for *A. caraya* populations and their ecosystem.

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