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Adrenal activity in maned wolves is higher on farmlands and park boundaries than within protected areas

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ABSTRACT

In this study we measured excreted fecal corticoid metabolites (FCM) in maned wolves (*Chrysocyon brachyurus*) living within a protected reserve, on farmlands or in a boundary zone between the two habitats, and determined the impacts of season and reproductive status on adrenal activity. Feces were collected within a national park ($n = 191$ samples), a park boundary zone ($n = 39$) and on nearby farmlands ($n = 27$), processed and analyzed by enzyme immunoassay. FCM amounts from samples collected on farmlands were higher ($P < 0.05$) than in those collected inside the reserve and from the boundary zone. In relation to seasonality, FCM were elevated ($P < 0.05$) in spring (September–November) when wolf pairs were raising young. We then divided the samples collected during breeding season (March–August) into cycling females and male/non-cycling females based on fecal progesterone: fecal testosterone ratio. FCM concentrations of the former collected inside the park were higher than ($P < 0.05$) than the latter group. However, there were no differences in FCM levels between the two groups for samples collected in the boundary zone and on farmlands. Furthermore, FCM concentrations of male/non-cycling females samples collected on farmlands were 2- to 5-fold higher ($P < 0.05$) than in counterparts collected inside the park. The consistently high FCM concentrations in samples collected on farmlands indicate that, in addition to seasonality, gender and reproductive status, anthropogenic pressures also contribute to elevating adrenal steroid for individuals living in altered habitat.

1. Introduction

The maned wolf (*Chrysocyon brachyurus*) is found only in the grasslands of Brazil, Argentina, Bolivia and Paraguay [17,46] and is recognized by the IUCN – World Conservation Union [63] as 'near threatened'. An estimated ~22,000 maned wolves remain in nature, most living in Brazil [41] as solitary individuals. Although breeding pairs are known to share a large home range (averaging 50.9 km^2), individuals are not often seen together [17,46]. The species is facultatively monogamous and seasonally monestrous, that is, mating occurs once from March through June (autumn and part of winter) [17,27,47] when there is decreasing' daylight, rainfall and ambient temperatures. Most maned wolf births occur in winter (June through August; after a 65 day gestation), although some extend into early spring (September) [17,26].

Similar to other rare wildlife species, the most significant threat to the maned wolf is reduction and fragmentation of habitat [17,46]. During the last three decades, 75% of the Brazilian grassland (known as the Cerrado) has been lost to agricultural development, largely to the creation of soybean and coffee plantations and cattle ranches [54]. Although the Brazilian Cerrado is comprised of unique fauna and flora, making it one of the world's 25 biodiversity hotspots [25,36,47], only 2% of this ecosystem is government-protected and managed [21,22], and the remainder is privately-held land. Because the maned wolf requires a large sized home range, any decline in habitat poses a potential threat to the metapopulation [47]. Encroachment associated with habitat conversion

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increases the likelihood of direct or indirect contact with humans and their domesticated animals that, in turn, elevates risk of disease transmission across diverse species [1,16,23,44]. In addition, these environmental changes can represent a potential stress, which can reduce fitness of the free-ranging population [31].

Stressors can be defined as any environmental stimulus that leads to an imbalance of an individual's homeostasis (i.e., normal physiological state) [31,34,45]. The resulting defense reaction of the individual involves the stimulation of the hypothalamic–pituitary–adrenocortical (HPA) axis that, in turn, elicits glucocorticoid secretion from the adrenal cortex [20]. Many situations, including events normally considered beneficial (e.g., courtship, copulation, giving birth, securing prey) or negative (e.g., fighting, capture, transport) can cause a stress response [10,31]. Short-term glucocortiocid elevation generally helps an animal deal with a challenge by rapidly mobilizing energy [51]. However, chronically increased glucocorticoids can reduce fitness through immune suppression, decreased growth, tissue atrophy and compromised reproduction [7,31,35,45]. There have been only a few such studies conducted in free-ranging carnivores. One example is the work of Young and Monfort [61] who have demonstrated that prolonged extra-territorial movement in subordinate meerkats (*Suricata suricatta*) markedly elevates glucocorticoid metabolites that, in turn, compromises fitness and health. An investigation of African wild dogs (*Lycaon pictus*) that were captured from nature and held in enclosures for 22 weeks has revealed an abrupt rise in excreted glucocorticoids followed by a decline of hormone concentration to baseline level within 2 weeks of being in captivity [11]. This indicates that free-ranging wild dogs are able to adapt to a captive environment. Useful insights also have made by studying carnivores managed in *ex situ* collections where the environment can be manipulated and then hormonal activity evaluated. For example, Wielebnowski et al. [60] clearly have demonstrated that excreted corticoid concentrations in clouded leopards (*Neofelis nebulosa*) decrease significantly when animals have vertical climbing opportunities in private enclosures with minimal exposure to humans and nearby species.

These advances in understanding adrenal responsiveness to in *ex situ* [14,43] or *in situ* [9,31,34,47] environmental and seasonal changes have been made possible by the non-invasive assessment of glucocorticoids excreted in urine or feces. For carnivores, most steroid hormones, including glucocorticoids, are excreted in feces [15,32,33,62]. This presents distinct advantages (over urine) for case of collecting specimens. Specifically and depending on species specificities, fecal corticoid concentrations and patterns have reflected changes or differences in carnivore social status (African wild dog [12], gray wolves [Canis lupus, [50]], spotted hyena [*Crocuta crocuta*, [18], meerkat [61]), acclimation to new territory post-translocation (African wild dog [11]) and reaction to increased human activities in native habitat (gray wolf [13], spotted hyena [58]).

Our previous study conducted in the Serra da Canastra National Park demonstrates that there are differences in hematology and blood biochemistry between wolves inside the park, around the park boundary and on farmlands [28]. Wolves living on farmlands have higher levels of cholinesterase than those living around park border and inside the park. Furthermore, wolves living on farms and the park border have higher red blood cells, hemoglobin and hematocrit than those living in the park. Because it has been shown that stress induces secretion of acetylcholinesterase, a type of cholinesterase that is modulating hematopoiesis [19], elevation of erythrogram on wolves living on farms may be reflective of stress mediating secretion of cholinesterase (and acetylcholinesterase). Therefore, we were keen to determine if was possible to differentiate level of adrenal activity in a free-ranging canid, the maned wolf, living in the same geographic region (Serra da Canastra region), but under three distinctive environmental conditions. Our specific objective was to determine if fecal corticoid monitoring was sufficiently sensitive to detect differences in excretion concentration among wolves existing solely within the protected park versus those on farmlands versus those living in a marginal zone between the two habitats. A second objective was to determine if wolves living in altered areas with greater human contact excreted higher corticoid metabolite levels that perhaps were indicative of chronic stress. Finally, we evaluated our findings on the basis of seasonality and reproductive status as there is evidence that corticoid production can be influenced by these factors in other species [8,30,49].

2. Methods

2.1. Study area

Serra da Canastra National Park (SCNP) (46°15′ W, 20°00′ S) is in southwestern Minas Gerais State, Brazil and encompasses 2000 km^2 of which 715.2 km^2 are regulated and managed by the Instituto Chico Mendes de Conservação da Biodiversidade (ICMBio), and the remainder is private land. Approximately 50% of this total area is open grassland with 35% being Cerrado vegetation [17]. The ICMBio-regulated lands have been formally defined as undisturbed [28], meaning that there is no or only occasional interaction of wolves with humans (rangers, researchers or tourists). Farm (and ranch) lands surrounding the park mostly are confined to cattle ranching and subsistence plantings, except for large coffee and corn plantations.

Prior to the initiation of the present study, we conducted extensive telemetry surveys that revealed collared maned wolves living exclusively in one of three distinctive habitats [2,28]: (1) inside the park; (2) in boundary areas (i.e., occupying a habitat zone that included both inside and outside the park; and (3) on surrounding farmlands. These areas were identified and mapped as facilitated by ArcView GIS (version 3.2) (Fig. 1). The within the park habitat included all areas inside the reserve boundary that were >500 m from the border. The park border area included a zone ranging 500 m on either side of the boundary. This 1000 m of buffer area is characterized by the apparent transition of typical Cerrado vegetation to exotic grasses on the edge of farmland. All habitats located >500 m beyond the park border where native vegetation had been replaced with crops and exotic grasses were considered farmland.

2.2. Fecal sample collection

The study was facilitated by our access to, and experiences in, studying maned wolves living solely in a protected area (Serra da Canastra National Park), on the boundary and on adjacent farmlands [2,28]. A 6 year long (2004–2009) telemetry study included 18, 16 and 13 maned wolves living exclusively inside the park, on the park border or on farms, respectively (Rocha, Fabiana, personal communication). We collected fresh fecal samples on the basis of the three geographic locations described above: (1) within the park; (2) park boundary zone and (3) farmland. A total of 257 samples was collected from May of Year 1 through August of Year 3 (Fig. 2) by field personnel with extensive experience in identifying canid scats. Of the total, 68 samples were collected from 23 known individuals that were trapped in one of the three areas (within the park, $n = 31$ samples; boundary zone, $n = 25$; farmlands, $n = 12$) for other studies [27]. In these cases, wolves were baited with cooked chicken placed in a box trap, then anesthetized (for biomedical evaluations) and the fresh feces recovered from the floor of the trap, the rectum or immediately after recovered animals defecated upon release. The remaining 189 samples were collected non-invasively from the principal road, on secondary trails or adjacent areas from unseen and

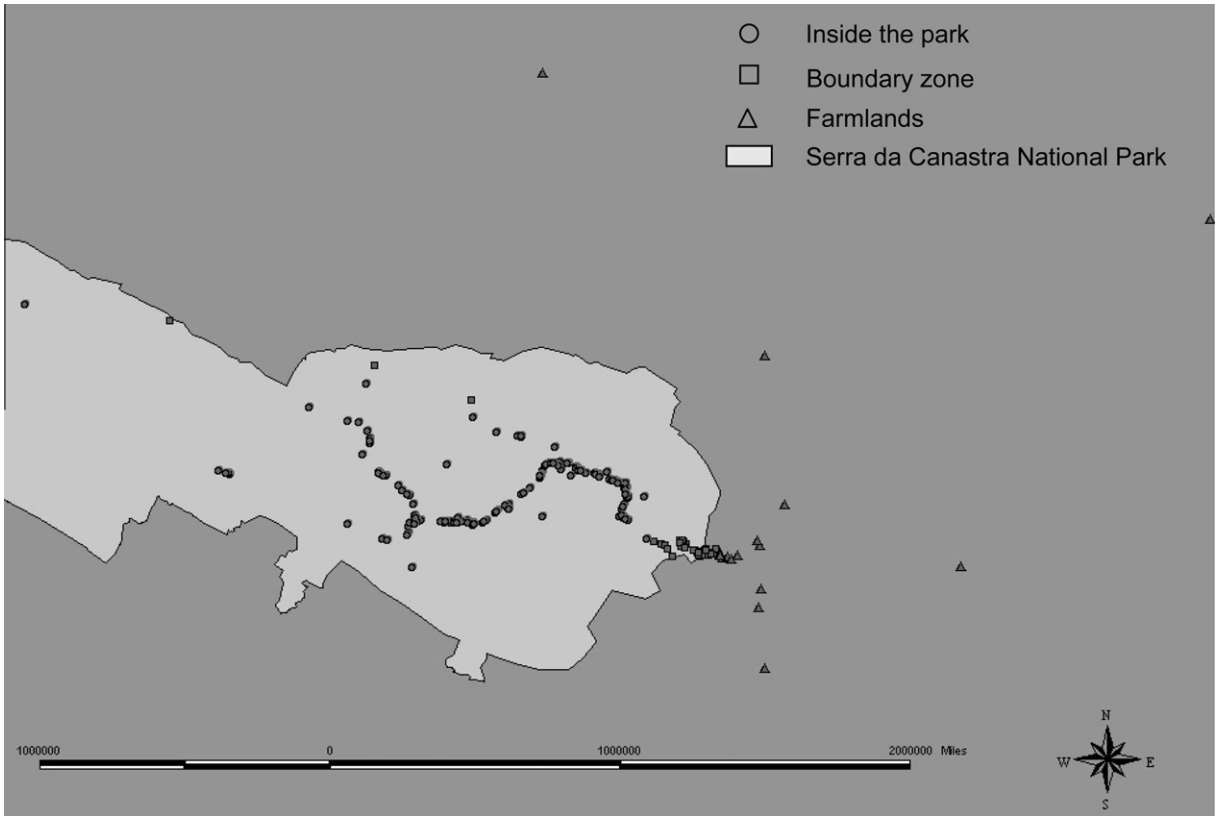

Fig. 1. A map illustrating locations of samples collected inside the Serra da Canastra National Park, Brazil, in boundary zone and on farmlands.

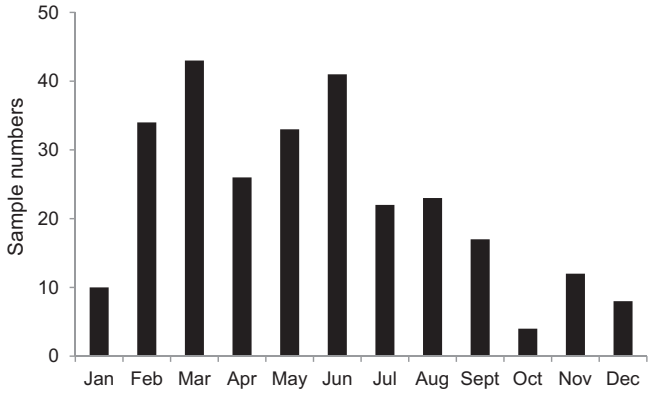

Fig. 2. Distribution of numbers of fecal samples collected throughout the year.

unknown wolves (Fig. 1). Confirmation of species origin was made on the uniqueness of maned wolf fecal shape, size (≥25 mm in diameter), texture, odor and presence of lobeira seeds (*Solanun lycocarpum*) [17]. Only samples that were still moist and with obvious intestinal mucus on the fecal surface were collected (all estimated to be <24 h from defecation). The entire fecal mass was taken for hormone extraction and analysis. A global positioning system (GPS) was used to record the geographical site of each sample, the latter then being placed in a separate plastic bag (labeled with date and GPS location) before being stored on ice for 2–6 h and then at −20 °C before being transported to the laboratory for analysis.

2.3. Fecal extraction and analysis

Fecal extraction and hormone quantification were performed in the Laboratory of Reproductive Physiology, Federal University of Paraná, Curitiba, Paraná State. All reagents (except when specified) were purchased from Sigma–Aldrich (São Paulo, Brazil) and all solutions prepared with Milli-Q water. Fecal extraction was performed by the methods of Brown et al. [6], with slight modifications. Briefly, an aliquot of ~0.5 g of the thawed, well mixed, wet fecal sample was placed in a glass tube containing 5 ml of 90% ethanol:10% distilled water and vigorously shaken for 30 min using a Multi-Pulse vortexer (Glas-Col, Terre Haute, IN). Each sample was centrifuged (1000*g*, 15 min) and supernatant recovered and diluted at a 1:1 ratio with a phosphate buffered solution. The mean (± SEM) of extraction efficiency was 85.5 ± 0.4% with a coefficient of variation (CV) of 8%.

2.3.1. Fecal corticoid analysis

The antibody for cortisol (polyclonal R4866; 1:8500 dilution) was obtained from University of California, Davis, CA, USA. R4866 cross-reacts with 100% cortisol, 9.9% prednisolone, 6.3% prednisone, 5.0% cortisone, 0.7% corticosterone, 0.3% deoxycorticosterone and 0.5% 21-deoxycortisone. Serial dilutions of pooled fecal extracts produced displacement curves parallel to those of the appropriate standard. Recovery of added standard to pooled fecal extract ($y = 0.76x + 17.22$; $r^2 = 0.997$) demonstrated significant recovery ($P < 0.001$). To confirm that FCM assay accurately reflected stress responses in the maned wolf, fecal samples were collected from two adults (one male and one female) housed at the Smithsonian Conservation Biology Institute 2 days before and 5 days after their two pups (3 months old) were separated and restrained for vaccination. The samples were pooled, extracted and assessed for FCM. FCM concentrations initially increased after 2 days and reached the peak value 3 days after pup separation; this was followed by the abrupt declined of steroid concentration (Fig. 3). Based on this result, it appeared that the cortisol assay used in the present study accurately reflected stress responses in the maned wolf. The inter-

and intra-assay CVs were <3% and 15.9%, respectively. Assay sensitivity was 3.9 pg/well.

2.3.2. Fecal testosterone analysis

Fecal testosterone metabolites were quantified by enzyme-immunoassay. The testosterone antibody (polyclonal R156/7; 1:7500 dilution) was obtained from the University of California, Davis. R156/7 cross-reacts with 100% testosterone, 57.37% 5α-dihydrotestosterone and 0.27% androstenedione. Serial dilutions of pooled fecal extracts produced displacement curves parallel to those of the appropriate standard. Recovery of added standard to pooled fecal extract ($y = 0.57x + 4.33$; $r^2 = 0.992$) demonstrated significant recovery ($P < 0.001$). Intra- and inter-CVs were <3% and 15.1%, respectively. Assay sensitivity was 2.3 pg/well.

2.3.3. Fecal progestagen analysis

Fecal progestagen metabolite concentrations were quantified by enzyme immunoassay as described Songsasen et al. [55]. The antibody for progestagen (monoclonal CL425; 1:10,000 dilution) analysis was obtained from the University of California, Davis. Intra- and inter assay CVs were <3% and 14.4%, respectively. Assay sensitivity was 0.78 pg/well.

2.4. Determining sex of unknown fecal donors

It is well-established that the progesterone:testosterone (P/T) metabolite ratio in maned wolf feces is accurate and useful for identifying gender of the individual depositing the sample [59]. Specifically, the, P/T ratio during the breeding season is lower in males/non-cycling females than in cycling females, allowing at least 80% of samples to be correctly identified as to gender (with success particularly high for females in proestrus, estrus or diestrus; i.e., those that are reproductively-active) [59]. As 26% of our samples were from known sex individuals, we first tested the accuracy of the Velloso et al. [59] assertion. We assessed the P/T metabolite ratio in samples of 20 wolves collected in March through August (i.e., breeding and gestation periods) from known male and 12 known female maned wolves, and compared the values using a Mann–Whitney Rank Sum test (Sigma Stat 2.03).

2.5. Corticoid data analysis

All corticoid data were presented as mean (± SEM) concentrations. All data were analyzed using SigmaStat 2.03. Baseline sample concentrations collected from different locations (i.e., within the park versus boundary zone versus farmlands) or seasons were calculated by an iterative process, whereby values that exceeded the mean plus 1.5 standard deviation (SD) were excluded. Each time the mean was recalculated and the elimination process repeated until no value exceeded the mean plus 1.5 SD [5,60].

Thereafter, the FCM values were analyzed to determine: if there were differences in hormone levels between (1) samples collected from known, trapped individuals and those obtained opportunistically and (2) among wolves living in one of three habitat types as well as the influences of (3) seasonality; (4) reproductive status; and (5) reproductive activity on corticoid concentrations. Comparison between samples of known ($n = 68$) and unknown ($n = 189$) donors was performed using a Student's t-test. The influence of locations was evaluated using a Kruskal–Wallis one way ANOVA on ranks followed by a Dunn's multiple comparison procedure.

As a simple assessment of corticoid patterns over time, we examined FCM concentrations across four seasons: (1) spring (September 1 through November 30, $n = 27$); (2) summer (December 1 through February 28, $n = 46$); (3) autumn (March 1 through May 31, $n = 62$); and (4) winter (June 1 through August 31, $n = 57$). Because sampling location affected adrenal function (see below), we evaluated only those data from fecal samples ($n = 191$; known male, 27; known female, 25; unknown individuals, 139) recovered from inside the park. Statistical analysis was by Kruskal–Wallis one way ANOVA on ranks followed by a Dunn's multiple comparison procedure.

To determine the effect of reproductive status on corticoid concentration in samples collected in all habitat types, we separate all samples into two groups based on the time in which the sample was collected, specifically during a reproductive versus non-reproductive phase. The former included samples collected in the months during which breeding and pregnancy normally occur in the wild (March through August, $n = 175$). Non-reproductive phase samples were those collected during the months when wolves raise their young and become reproductively inactive (September through February, $n = 82$). Within each of these time categories, values were further divided into subgroups based on the three site locations and then subjected to a Kruskal–Wallis one way ANOVA followed by a Dunn's multiple comparison procedure. A comparison of FCM content within each area and between the reproductive versus non-reproductive phases was conducted using a Mann–Whitney Rank Sum test.

Finally, comparison in corticoid concentrations between samples predicted to be from reproductively-active females ($n = 53$) and those from males plus inactive females ($n = 123$; determined using P/T ratios, as described above in Section 2.4) were performed in feces obtained during the reproductive phase (March through August). These data were analyzed using a Kruskal–Wallis ANOVA followed by a Dunn's multiple comparison procedure. Then the comparison of these two groups was further assessed on the basis of sample location using a Mann–Whitney Rank Sum test.

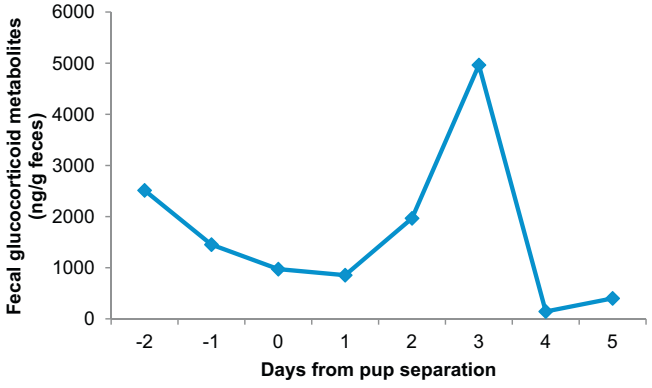

Fig. 3. Fecal corticoid metabolites of two adult maned wolves 2 days before and 5 days after pup separation.

3. Results

3.1. Location

Initially, we evaluated the data to determine if there were differences in corticoid values between samples collected from trapped wolves and those obtained opportunistically. We found that there were no differences ($P > 0.05$) in mean concentrations (88.0 ± 11.7 versus 60.2 ± 7.9, respectively) between the two groups. Therefore, samples collected from known and unknown individuals were combined and analyzed together to assess the impact of location, seasonality, reproductive phase and reproductive activity on corticoid excretion.

Geographical location influenced ($P < 0.05$) FCM concentrations in maned wolves. Excreted corticoid concentration of samples obtained from wolves captured around the park boundary

(116.2 ± 15.9 ng/g feces, $n = 25$) and on farms (136.3 ± 26.8 ng/g feces, $n = 12$) was higher than that from individuals caught inside the park (48.0 ± 5.5 ng/g feces, $n = 31$). When samples collected from captured wolves and those obtained from unknown individuals were analyzed together, the differences in FCM concentrations among geographical location was also apparent (Table 1 and Fig. 4). Specifically, the lowest FCM concentrations were detected in samples collected from wolves living inside the park (26.2 ± 1.1 ng/g feces). Values were more than 2- and 4-fold higher ($P < 0.05$) in the park boundary zone (64.7 ± 7.7 ng/g feces) and on farmlands (127.8 ± 18.2 ng/g feces), respectively.

3.2. Seasonality

When the data were examined for 'seasonality' (only for wolf samples collected in the undisturbed, protected area), there was no variation ($P > 0.05$) in FCM among summer (25.1 ± 2.3 ng/g wet feces), autumn (26.8 ± 1.6) and winter (28.1 ± 2.8, Table 2 and Fig. 5). However, compared to these intervals, corticoid concentrations were 2-fold higher ($P < 0.01$) in spring (54.5 ± 5.8).

3.3. Reproductive phase

There were no differences ($P > 0.05$) in FCM concentrations between reproductive and non-reproductive phases in samples collected exclusively inside the park and in the boundary zone (inside, 26.5 ± 1.4 versus 25.5 ± 2.0 ng/g feces; boundary zone, 68.6 ± 8.9 versus 173.3 ± 89.6, Table 3). However, for wolves living on farmlands, FCM concentration was higher ($P < 0.05$) in the non-reproductive (333.4 ± 94.5) compared to reproductive phase (122.4 ± 20.9, Table 3). Again, when data were compared across locations within each collection time, FCM concentrations were lowest ($P < 0.05$) in samples recovered from wolves living inside the park compared to the other two locations (Table 3 and Fig. 6).

Table 1
Mean (± SEM) baseline and range (ng/g wet feces) of fecal corticoid metabolite (FCM) concentrations in maned wolf samples recovered from within the park, in the boundary zone and on farmlands.

Locale (n)	FCM concentration	Range
Inside the park (191)	26.2 ± 1.1^{A}	9.6–1157.0
Boundary zone (39)	64.7 ± 7.7^{B}	26.3–1533.1
On farmlands (27)	127.8 ± 18.2^{B}	17.8–803.0

^{AB} Different letters indicate significant differences between locations ($P < 0.05$).

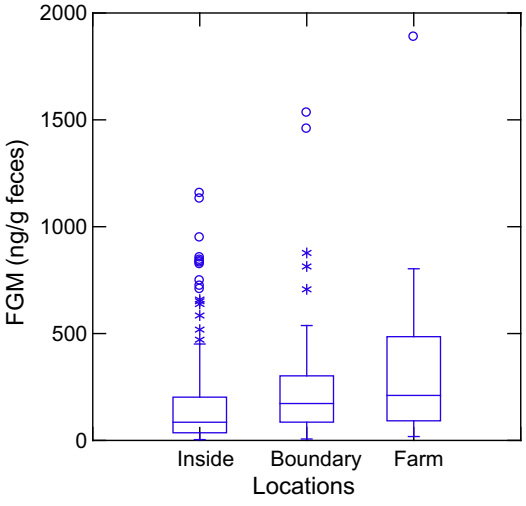

Fig. 4. Box plot of fecal corticoid metabolites concentrations in maned wolf samples recovered from within the park, in the boundary zone and on farmlands.

Table 2
Mean (± SEM) baseline and range (ng/g wet feces) of fecal corticoid metabolite (FCM) concentrations in maned wolf samples recovered from within the Serra da Canastra National Park during various seasons.

Seasons (n)	FCM concentration	Range
Spring (27)	54.5 ± 5.8^{B}	18.6–949.0
Summer (44)	25.1 ± 2.3^{A}	11.8–1157.0
Autumn (65)	26.8 ± 1.6^{A}	11.9–876.9
Winter (55)	28.1 ± 2.8^{A}	9.6–856.1

^{AB} Different letters indicate significant differences between seasons ($P < 0.05$).

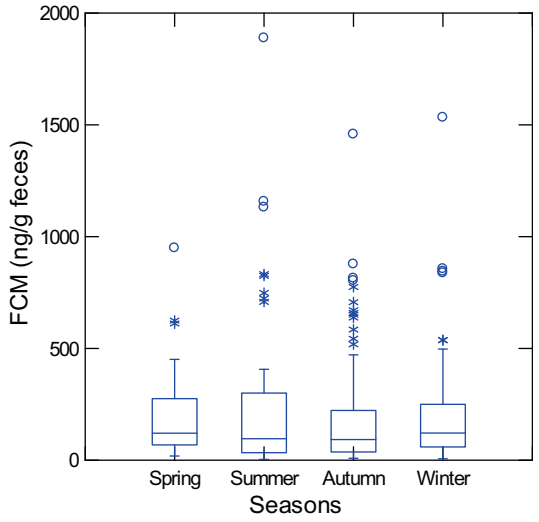

Fig. 5. Box plot of fecal corticoid metabolite concentrations in maned wolf samples recovered from within the Serra da Canastra National Park during various seasons.

3.4. Gender and reproductive status of donor samples

We compared FCM concentrations of captured males versus females and found that there were no differences in FCM concentrations between the two genders (males, 194.6 ± 38.2; females, 272.6 ± 41.7).

There was a difference ($P = 0.037$) in P/T metabolite ratio between males (mean ± SD, 6.9 ± 3.4) and females (19.3 ± 17.7). This affirmation permitted us to predict the gender of the unknown fecal donors in the present study. Every specimen with a ratio >15 was considered a female and likely to be reproductively active, that is, cycling or in a post-ovulation (including pregnancy) phase [56]. Conversely, any ratio <15 was considered a male or a non-cycling female.

Using criteria described above for identifying females that were reproductively active (i.e., cycling), we observed no differences in FCM concentrations ($P > 0.05$) among the three sampling locations (inside, 98.5 ± 12.6 ng/g wet feces; boundary zone, 117.6 ± 28.5; on farms, 123.3 ± 31.5; Table 4 and Fig. 7a). However, when we examined the adrenal activity levels of the 'males plus non-cycling females' group, FCM concentrations of samples collected on farmlands (139.6 ± 31.9 ng/g feces) were 2- to 5-fold higher ($P < 0.05$) than in counterparts collected inside the park (23.4 ± 1.7 ng/g feces, Table 4 and Fig. 7b). When data were compared within the same location, FCM concentrations were higher ($P < 0.05$) in reproductively active females compared to males plus

Table 3
Mean (± SEM) baseline and range (ng/g wet feces) of fecal corticoid metabolite (FCM) concentrations in maned wolf samples recovered from within the park, in the boundary zone and on farmlands during the reproductive phase (i.e., breeding and pregnancy; March through August) versus non-reproductive period (September through February).

Location	Reproductive phase			Non-reproductive phase		
	FCM concentration	Range	*n*	FCM concentration	Range	*n*
Inside the park	26.5 ± 1.4^{Aa}	9.6–876.9	120	25.5 ± 2.0^{Aa}	11.8–1157.0	71
Boundary zone	68.6 ± 8.9^{Ba}	26.3–1,458.3	34	$173.3 \pm 89.6^{B\ a}$	21.6–1533.1	5
On farmlands	122.4 ± 20.9^{Ba}	17.8–803.0	21	333.4 ± 94.5^{Bb}	80.8–624.1	6

[AB] Different letters within the same column indicate significant differences ($P < 0.05$). [a–c] Different letters indicate significant differences ($P < 0.05$) between samples collected during different phases, but within the same location.

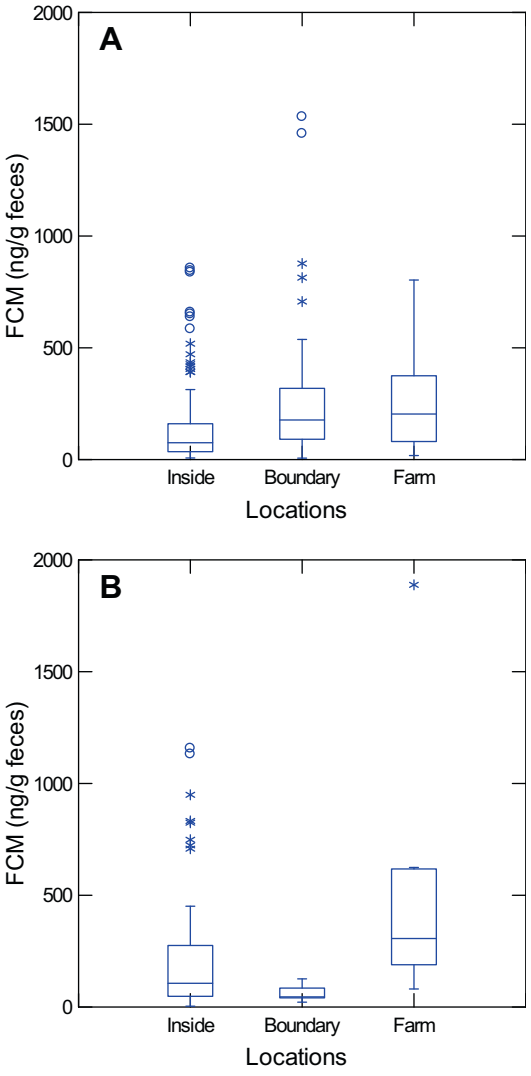

Fig. 6. Box plot of fecal corticoid metabolite concentrations in maned wolf samples recovered from within the park, in the boundary zone and on farmlands during the (A) reproductive phase (i.e., breeding and pregnancy; March through August) and (B) non-reproductive period (September through February).

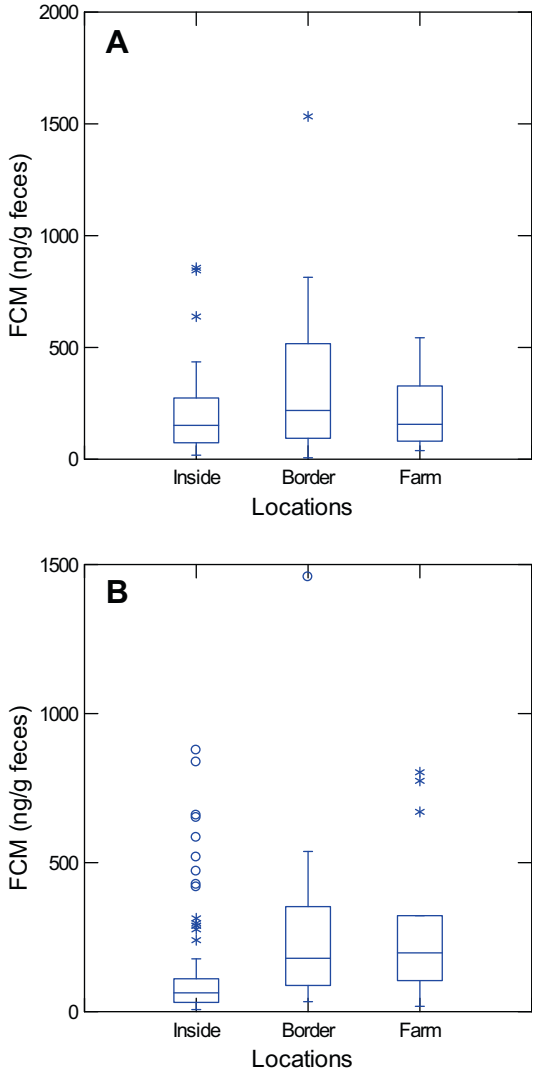

Fig. 7. Box plot of fecal corticoid metabolite concentrations in samples of (A) cycling females and (B) males plus non-cycling females collected inside the park, in the boundary zone and on farmlands in the breeding season (March through August).

Table 4
Mean (± SEM) of baseline and range (ng/g wet feces) of fecal corticoid metabolite concentrations in samples of cycling females versus males plus non-cycling females collected inside the park, in the boundary zone and on farmlands in the breeding season (March through August).

Location	Cycling females			Males plus non-cycling females		
	FCM concentration	Range	*n*	FCM concentration	Range	*n*
Inside the park	98.5 ± 12.6^{Aa}	17.8–856.1	29	23.4 ± 1.7^{Ab}	7.0–876.9	90
Boundary zone	117.6 ± 28.5^{Aa}	6.4–1,533.3	16	102.5 ± 16.2^{Ba}	33.4–1458.3	20
On farmlands	123.3 ± 31.5^{Aa}	38.3–543.3	8	139.6 ± 31.9^{Ba}	17.8–803.0	13

[AB] Different letters within the same column indicate significant differences ($P < 0.05$). [a–d] Different letters indicate differences ($P < 0.05$) between samples of cycling female versus males plus non-cycling females within the same location.

inactive females, but only for wolves living solely within the park (98.5 ± 12.6 versus 23.4 ± 1.7 ng/g feces, Table 4).

4. Discussion

Our findings affirmed the ability to measure corticoid metabolites in the feces of a free-ranging carnivore, the maned wolf, as already identified in the gray wolf [12], African wild dog [12] and spotted hyena [58]. Our study demonstrated the impact of seasons, sex, reproductive activity and status as well as location on corticoid excretion in maned wolves. Irrespectively to sex, reproductive activity and status, corticoid values were at least 2-fold higher in fecal samples of maned wolves living in the boundary of protected areas and 4-fold higher on farmlands compared to within the park. Because sustained elevations in this hormone generally indicate increased adrenal function [30,31], it could be speculated that the higher FCM values measured in the boundary zone and on farmlands were related to greater 'stress' associated with living in these areas where natural habitat is altered and encounters between wolves and humans and their domestic animals are common. Our results have two implications, the first being that non-invasive FCM monitoring has value in reflecting physiological function in this carnivore that is living in rapidly degraded and converted habitat. Secondly, if chronically elevated corticoids are eventually demonstrated to lead to other negative consequences, such as reduced reproduction or survivorship, then monitoring adrenal corticoids from opportunistically collected scat may be a valuable tool for tracking the well being of this and other threatened wild carnivores.

Our discovery that non-invasive hormone monitoring has utility for free-ranging maned wolves compliments earlier studies demonstrating the benefits of this approach for this species managed *ex situ* [11,14,59]. For example, monitoring ovarian reproductive steroids has suggested that females failing to reproduce and/or rear pups produce less excreted progestagens [55]. Interestingly, females with poorer reproduction while living in captivity also simultaneously excrete higher FCM concentrations [11]. Such reports reinforce the significance of the present findings that fecal corticoid monitoring can identify populations with different physiological status, at least in the context of adrenal activity.

Our overall findings also supported a growing abundance of data that adrenal activity is more attenuated in wildlife that exists in the 'wild' – in areas that are devoid and/or unaffected by people. For example, in this same country (Brazil), pampas deer (*Ozotoceros bezoarticus*) living outside of the Emas National Park have a 1-fold higher fecal corticoid content than counterparts located within the reserve [42]. Similar observations also have been made recently in spotted hyenas existing on the edge of the Masai Mara National Reserve and Amboseli National Park in East Africa [58]. Longitudinal data collected over 12 years have revealed that increasing human density along the edge of this population's home range has increased amounts of excreted corticoids. This increased adrenal (2-fold higher) activity is especially prevalent under conditions of intensive cattle ranching compared to areas void of pastoralists. Recent publication from our group has shown that maned wolves living on farms surrounding the Serra da Canastra National Park and in the park boundary zone have greater tick infestations, experience microcytic anemia and have higher cholinesterase concentrations and red blood cell counts (the latter two metrics being physiological traits associated with stress) [19] than counterpart wolves living inside the park [28]. It has been shown that excreted corticoids can be influenced by altered food availability, exposure to environmental contaminants and parasitic infestation [8,38,39]. Thus, our data and those of previous research [58] indicate that altered environment can potentially impact overall health and physiology of carnivores.

The literature contains significant numbers of papers describing seasonal corticoid patterns in diverse taxa [45,49], including in mammals [37,49,52], reptiles [53], amphibians [24,40] and birds [48]. Most of these articles support the notion that increased and sustained adrenal activity is associated with seasonality and reproductive activities [49]. Although the mechanisms mediating this phenomenon are not well understood [49], there is general consensus that increased glucocorticoids are necessary to help mobilize energy for crucial events and, in the case of carnivores, preparing for a competitive breeding season (weddell seals [3]; gray wolf, African wild dog and dwarf mongoose [12]. When we examined the maned wolf population living inside the park, the FCM concentrations during summer (December through February), autumn (March through May) and winter (June through August) were invariant. However, there was a seasonal corticoid rise during the spring (September through November), the period when wolves are lactating and raising young [17]. This discovery in the wild population was in agreement with a zoo-based study that showed a rise in FCM concentrations in both female and male maned wolf parents post-parturition, which then remained high for at least 2 months [56]. Therefore, it appeared that the elevated FCM content observed in the spring in our field study had the potential of being related to energy demands during lactation and early pup-rearing.

It has been suggested that seasonal differences in FCM may be due to diet variations [29]. In the wild, maned wolf' diet consists of 50% plant materials and 50% animal items [17]. However, it has been shown that fruits consumed by maned wolves are abundant during the wet season (October–February, i.e., spring and summer) while prey species are readily available during the dry period (March–September, i.e., fall and winter). Thus, differences in FCM concentrations among seasons may also be due to the variations in food availability.

When compared between reproductive versus non-reproductive periods, differences were observed only in samples obtained on farmlands. It has been shown that size of home range varies between the two reproductive periods; smaller home ranges were observed during the reproductive period when females become pregnant and provide care to their offspring [2]. Thus, increased home range of wolves living on farmlands during the non-reproductive phase may subject individuals to contact with human or domestic animals more frequently than during reproductive period, and cause a rise in corticoid excretion. However, due to the small sample size ($n = 6$), future studies with a larger sample size are warranted.

Within the reproductive phase (March through August), there also appeared to be a relationship between sexual activity (and gender) and quantity of corticoid produced. High FCM concentrations in 'cycling and/or pregnant' females for samples recovered from inside the park were probably due to two reasons. The first is a higher degree of overlapping home-ranges among females than among males. This phenomenon is well known for maned wolves living inside the Serra da Canastra National Park and monitored by radiotelemetry [2]. Aggression among individuals, especially within the same gender and over territorial disputes, also has been observed [47]. The greater maned wolf density from within the park (compared to the boundary zone and farmlands) increased the likelihood of confrontation and aggression among female conspecifics, thereby perhaps explaining their higher FCM concentrations. A second explanation probably was related to these cycling/pregnant females having overall high metabolic demands, especially during the second half of gestation, at parturition and during a subsequent lactation as observed in other species, including the arctic ground squirrel [4], ring-tailed lemur [57] and harbor seal [18]. The lack of elevated corticoids in reproductively-active females in the boundary zone and on farmlands probably was related to these

wolves already having an overall high FCM concentration (as well as a smaller sample size due to lower animal density).

In conclusion, this study has reaffirmed the ability to monitor adrenal activity in a free-living carnivore and, most importantly, distinguish adrenal activities among three distinctive habitats with different levels of human disturbance. The highest FCM concentrations were observed on farmlands where habitat conversion is the most apparent. Future studies should focus on clarifying the specific consequences and mechanisms whereby chronically elevated corticoids eventually compromise reproduction, health and survivorship. It would be interesting to examine adrenal functional among (and within) wolves in different (or the same) habitats over time. Such studies would be possible through a combination of telemetry and serial fecal sampling for endocrine (and DNA) evaluations, with findings related to offspring production, survival and dispersal. Regardless, our current findings and a growing number of publications by others suggest that FCM monitoring is likely to become increasingly important for not only for demonstrating the consequences of habitat contractions/degradations, but also for predicting the necessary size of protected areas to minimize chronic stress.

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