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RESEARCH ARTICLE

# Noninvasive Monitoring of Adrenocortical Function in Captive Jaguars (*Panthera onca*)

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Jaguars are threatened with extinction throughout their range. A sustainable captive population can serve as a hedge against extinction, but only if they are healthy and reproduce. Understanding how jaguars respond to stressors may help improve the captive environment and enhance their wellbeing. Thus, our objectives were to: (1) conduct an adrenocorticotrophic hormone (ACTH) challenge to validate a cortisol radioimmunoassay (RIA) for noninvasive monitoring of adrenocortical function in jaguars; (2) investigate the relationship between fecal corticoid (FCM) and androgen metabolite (FAM) concentrations in males during the ACTH challenge; and (3) establish a range of physiological concentrations of FCMs for the proposed protocol. Seven jaguars (3 M, 4 F) received 500 IU/animal of ACTH. Pre- and post-ACTH fecal samples were assayed for corticoid (M and F) and androgen metabolites (M) by RIA. Concentrations of FCMs increased ($P<0.01$) after ACTH injection (pre-ACTH: $0.90\pm0.12$ μg/g dry feces; post-ACTH: $2.55\pm0.25$ μg/g). Considering pre- and post-ACTH samples, FCM concentrations were higher ($P<0.01$) in males ($2.15\pm0.20$ μg/g) than in females ($1.30\pm0.20$ μg/g), but the magnitude of the response to ACTH was comparable ($P>0.05$) between genders. After ACTH injection, FAMs increased in two (of 3) males; in one male, FCMs and FAMs were positively correlated (0.60; $P<0.01$).

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Excretion of FCMs was assessed in 16 jaguars (7 M, 9 F) and found to be highly variable (range, $<0.11$–$1.56\,\mu g/g$). In conclusion, this study presents a cortisol RIA for monitoring adrenocortical function in jaguars noninvasively. Zoo Biol 30:1–16, 2011. 

**Keywords: endangered cats; ACTH challenge; fecal metabolites; corticoid metabolites; androgen metabolites**

## INTRODUCTION

The jaguar (*Panthera onca*) is the only species of the Genus *Panthera* native to the Americas [Wilson and Reeder, 2005]. Historically, jaguars were found from the southwestern United States through southern Argentina [reviewed by Seymour, 1989]. Currently, the species is listed as threatened with extinction throughout its range [Appendix I, CITES], which is now believed to extend from central Mexico through northern Argentina [Swank and Teer, 1987]. Fragmentation of natural habitats by deforestation and poaching of jaguars are the biggest challenges to the long-term survival of these cats in the wild [Swank and Teer, 1989; Haag et al., 2010; Rosas-Rosas and Valdez, 2010]. Unfortunately, jaguars continue to be poached for reasons that range from sport to retaliation for livestock losses [Swank and Teer, 1989; Rosas-Rosas and Valdez, 2010].

Although preservation of natural habitats and implementation of antipoaching policies are crucial for in situ conservation of jaguars, zoological institutions can play an important role in ex situ conservation by maintaining healthy, sustainable captive populations. Jaguars in captivity can serve both as a genetic resource and as a means of raising public awareness of the species and its ecological importance. However, maintenance of nondomestic species in captivity presents several challenges that might hinder the total expression of the genetic and reproductive potential of individual animals. Several nondomestic species display abnormal behaviors that can be intensified by certain stressors in captivity: lower environmental temperature increases stereotypic behaviors in Asian elephants (*Elephas maximus*) [Rees, 2004], as does ambient noise in giant pandas (*Ailuropoda melanoleuca*) [Owen et al., 2004]. A positive correlation between abnormal behavior and stress, as suggested by increased adrenal activity, has been described in some zoo species, such as clouded leopards (*Neofelis nebulosa*) [Wielebnowski et al., 2002] and giant pandas [Liu et al., 2006]. Chronic stress can negatively impact the reproductive and immune systems, as well as inhibit growth rate, suppress normal exploratory and reproductive behaviors, and increase abnormal behavior [reviewed by Morgan and Tromborg, 2007]. Thus, stress is one of the major concerns in maintaining healthy, self-sustainable captive populations of zoo species.

Previous studies in nondomestic felids have demonstrated that stress due to changes in or inadequacy of housing conditions can result in altered adrenal function as evidenced by fluctuations in glucocorticoid excretion. Moreira et al. [2007] observed an increase in fecal corticoid metabolites (hereafter FCMs) in female tigrinas (*Leopardus tigrinus*) and margays (*Leopardus wiedii*) after transfer from a large, enriched enclosure to a small, barren cage; the tigrinas, but not the margays, had a decrease in FCM concentrations after enrichment of the barren cages. Wielebnowski et al. [2002] observed that clouded leopards housed in enclosures with lower height had higher FCM concentrations compared with those housed in taller enclosures.

Stress stimulates interactions between the hypothalamus, the pituitary gland, and the adrenal glands, known as hypothalamic–pituitary–adrenal (HPA) axis. The actions of the HPA axis begin with the secretion of corticotrophin-releasing hormone by the hypothalamus, which acts on the pituitary gland causing the release of adrenocorticotrophic hormone (ACTH). Through circulation, ACTH reaches the cortex of the adrenal glands, where it stimulates the production of glucocorticoids [Broom and Johnson, 1993]. Among other actions, glucocorticoids stimulate hyperglycemia and increase blood flow to muscles. In the context of acute stress, such actions prepare the body for the "fight-or-flight" response. Even though the effects of stress are primarily reflected on changes in the HPA axis, the hypothalamic–pituitary–gonadal (HPG) axis may also be affected [Phogat et al., 1997]. In mammals, it was observed that stress can reduce both frequency and amplitude of luteinizing hormone (LH) secretion, which is crucial for reproductive function [Smith and Dobson, 2002]. Therefore, there has been increasing interest in understanding how stress affects the reproductive potential of captive animals.

Adrenal activity, as a parameter for assessing stress levels, can be monitored in carnivores by measuring concentrations of glucocorticoids and/or their metabolites in serum [Brown et al., 1989], saliva [Kobelt et al., 2003], hair [Finkler and Terkel, 2010], urine [McCobb et al., 2005], and feces [Wielebnowski et al., 2002]. The latter can be easily accomplished noninvasively, thus avoiding stress-inducing procedures of animal handling for sample collection. Measuring corticoid metabolites in feces is particularly interesting because fecal concentrations represent a pool of metabolites produced over a period of time; in carnivores, it usually represents the result of hormone production and metabolism ranging from several hours to a couple of days [Goymann et al., 1999; Wielebnowski et al., 2002; Morato et al., 2004]. This is particularly advantageous when assessing endocrine profiles of hormones that undergo circadian fluctuations, such as glucocorticoids, given that levels of hormone metabolites in a fecal sample will reflect the average hormone production over a certain period of time and will be more informative than serum levels of the hormone at one observation time. Moreover, unlike urine, fecal samples can be easily collected from the environment.

In the domestic cat, it has been demonstrated by the infusion of radio-labeled cortisol that the vast majority ($>80\%$) of cortisol metabolites are excreted in feces [Graham and Brown, 1996; Schatz and Palme, 2001]. Because the domestic cat is generally accepted as a research model for nondomestic cat species, and due to the unfeasibility of injecting endangered cats with radio-labeled steroids, it is commonly assumed that felids in general metabolize glucocorticoids in comparable ways. Thus, noninvasive monitoring of adrenal activity in nondomestic cats is usually carried out by assessing concentrations of FCMs [Wasser et al., 2000; Wielebnowski et al., 2002; Morato et al., 2004; Moreira et al., 2007].

Because hormone metabolism may vary across species, fecal assays must be validated for each species. In the case of glucocorticoids, this validation is typically carried out via an ACTH challenge, where fecal samples collected after administration of ACTH are expected to have increased FCM concentrations compared with baseline concentrations [Graham and Brown, 1996; Wielebnowski et al., 2002].

To our knowledge, our study is the first ACTH challenge conducted on jaguars. We hypothesized that concentrations of FCMs would increase in jaguars after administration of ACTH. The main objectives of this study were to: (1) conduct an ACTH challenge in male and female jaguars to validate a cortisol radioimmunoassay (RIA) for monitoring adrenocortical function in the species;

(2) investigate the relationship between FCM and fecal androgen metabolite (FAM) concentrations in males during the ACTH challenge; and (3) establish a reference range of physiological concentrations of FCMs for the proposed protocol in healthy, captive jaguars.

In our study, all wild-born individuals had been in captivity for several years before the study started; thus, we assumed that all subjects had already been acclimated to captivity. However, we decided to compare wild- vs. captive-born individuals to find out whether the supposed acclimation would be reflected on mean baseline concentrations of FCMs in jaguars.

## METHODS

### ACTH Challenge Test

#### *Animals*

Seven jaguars were used in the ACTH challenge: three males (♯1, 2, and 3) and four females (♯5, 6, 7, and 8), all housed in Brazilian zoos (Table 1). Average age ($\pm$SD) was $11 \pm 3$ years for the males (range, 5–13 years) and $8 \pm 3$ years for the females (range, 4–16 years). At RioZoo, females ♯5 and 6 were housed together and alternated use of the enclosure (11.65 m L × 8.37 m W × 5.74 m H) with male ♯1 for public display. When animals were not on public display, they were kept in a smaller

**TABLE 1. Jaguar Individual Data**

| House name | Study ID ♯ | Sex | Age (years) | Reproductive status | Origin | Institution* | Study participation[a] |
|---|---|---|---|---|---|---|---|
| Alexandre | 1 | M | 13 | Intact | Wild | R.Z. | ACTH/Overall |
| Lúcio | 2 | M | 5 | Intact | Wild | I.S. | ACTH/Overall |
| Tonho | 3 | M | 7 | Intact | Wild | I.S. | ACTH/Overall |
| Cabeça | 4 | M | 18 | Vasectomized | Captivity | R.Z. | Overall |
| Preta | 5 | F | 16 | Intact | Wild | R.Z. | ACTH/Overall |
| Gabi | 6 | F | 4 | Intact | Captivity | R.Z. | ACTH/Overall |
| Rebeca | 7 | F | 7 | Intact | Wild | I.S. | ACTH/Overall |
| Bianca | 8 | F | 6 | Spayed | Wild | I.S. | ACTH/Overall |
| Maya | 9 | M | 6 | Intact | Captivity | C.T. | Overall |
| Damien | 10 | M | 20 | Intact | Captivity | T.Z. | Overall |
| Tubarão | 11 | M | 11 | Intact | Wild | Z.P. | Overall |
| Bete | 12 | F | 15 | Intact | Wild | Z.B. | Overall |
| Preta | 13 | F | 18 | Intact | Captivity | Z.B. | Overall |
| Pintada | 14 | F | 11 | Intact | Wild | Z.P. | Overall |
| Cocoy | 15 | F | 9 | Intact | Captivity | F.W. | Overall |
| Taigra | 16 | F | 14 | Intact | Wild | V.R. | Overall |

*Abbreviations of zoological institutions: C.T. (Cat Tales Zoological Park, Mead, Washington); F.W. (Fort Worth Zoo, Fort Worth, Texas); I.S. (Parque Zoológico de Ilha Solteira, Ilha Solteira, São Paulo, Brazil); R.Z. (Fundação RioZoo, Rio de Janeiro, Rio de Janeiro, Brazil); T.Z. (Toronto Zoo, Scarborough, Ontario, Canada); V.R. (Zoológico Municipal de Volta Redonda, Volta Redonda, Rio de Janeiro, Brazil); Z.B. (Zoológico de Bauru, Bauru, São Paulo, Brazil); Z.P. (Bosque-Zoológico Municipal de Pedreira, Pedreira, São Paulo, Brazil).

[a]Study participation in the ACTH challenge test (ACTH) and/or in the overall assessment of mean baseline corticoid metabolite concentrations (Overall).

back enclosure (2.66 m L × 2.00 m W × 2.00 m H). At Zoológico de Ilha Solteira, males ♯2 and 3 and female ♯7 were individually housed in enclosures (9.00 m L × 5.00 m W × 3.00 m H) on public display during visitation hours. Female ♯8 shared an enclosure on public display with an adult male (not included in the study).

### *ACTH preparation and administration*

The ACTH gel was prepared using a sterile technique. Chemical products were purchased from Sigma-Aldrich (St. Louis, MO). Briefly, 0.625 g sodium carboxymethylcellulose (♯C4888, medium viscosity) was slowly added to 40 ml double-distilled water and mixed using a nonheated magnetic stirrer until completely dissolved (~20–30 min). The solution was autoclaved and allowed to cool to room temperature. In a separate beaker, 0.3125 g of liquid phenol (♯P9346, for antimicrobial activity, VSP grade), equivalent to 0.25 g phenol, 2.565 g dextrose (♯D9434, VSP grade), and 10 ml double-distilled water were mixed until the dextrose was completely dissolved. The dextrose/phenol solution was added to a 5,000 IU vial of powdered porcine ACTH (♯A6303-5000IU, amino acids 1–39) and mixed until completely dissolved. ACTH was withdrawn into a syringe and slowly filtered through a 0.22-μm diameter filter (Millex®-GV; Millipore, Carrigtwohill, Ireland) into the swirling solution of carboxymethylcellulose until homogeneous (~5 min). The pH was checked and adjusted with 10% HCl to a pH range of 4.5–6.5. Using a glass pipette, the ACTH gel was transferred to brown glass vials, sealed with rubber stoppers and a crimping tool, and stored at 4°C until use. Approximately 46 ml of ACTH gel at 113 IU/ml were prepared using this protocol.

Jaguars were anesthetized with Zoletil® (10 mg/kg BW, i.m.; Virbac do Brasil, Jurubatuba, SP, Brazil) via a $CO_2$ pistol or a blow dart. Within 20 min of anesthesia induction, animals were injected i.m. with 500 IU ACTH gel. Males ♯2 and 3 were electroejaculated for semen sample collection for another study after chemical restraint and before ACTH injection.

### *Fecal sample collection and hormone metabolite extraction*

Individual fecal samples were collected daily in the morning and the entire bolus was placed in a plastic bag, labeled with the animal's name and date of collection, and stored frozen (−20°C) until processing. Food color dyes (Wilton Industries, Woodridge, IL) mixed in diets were used to differentiate individual fecal samples where jaguars were not housed alone. In the ACTH challenge test, fecal samples were collected daily from all jaguars beginning 10 days before through 10 days after ACTH injection. Some jaguars did not produce samples every day; thus, the post-ACTH samples were considered to be those found on the first 2 days when there were samples available for collection after ACTH injection. Even though sample collection extended over a period of 10 days after ACTH treatment, post-ACTH samples were those found on two of the post-ACTH days because it was expected that FCM concentrations would peak within the 48-hr period after ACTH treatment [Schatz and Palme, 2001; Wielebnowski et al., 2002]. The remaining days of sample collection were included so that we could investigate whether metabolite concentrations had returned to baseline levels within 10 days of ACTH injection.

Extraction of fecal hormone metabolites was performed using a vortexing method adapted from Wasser et al. [2000]. Briefly, 3 ml of 90% ethanol (10% double-distilled water) was added to 16 × 125 mm glass tubes containing approximately 0.2 g

of manually crushed and homogenized freeze-dried feces. Tubes were covered with parafilm® and vortexed for 30 min in a multitube vortexer (VX-2500, VWR™; VWR International, LLC, Radnor, PA). Samples were centrifuged (500 × *g*, 20 min, 4°C), and the supernatants were collected into new set of glass tubes. Pellets were resuspended in 2 ml of 90% ethanol and vortexed briefly for 15 sec. Resuspended pellets were centrifuged (500 × *g*, 20 min, 4°C) and both supernatants were combined. An additional centrifugation step (500 × *g*, 10 min, 4°C) was performed to precipitate any remaining fecal particles. Extracts were transferred to labeled polypropylene tubes, capped, and stored at −20°C until assayed.

To determine extraction efficiency, 100 µl (~8,000 cpm) of $^{3}$H-cortisol in phosphate-buffered saline gel (PBS-Gel; 2.76 g monobasic phosphate, 11.36 g dibasic phosphate, 1 g sodium azide, 8.76 g NaCl, 1 g gelatin in double-distilled water for a final volume of 1 l of buffer; pH 7.4) were added to lyophilized jaguar fecal samples before extraction [Young et al., 2004]. Two milliliter of scintillation fluid (ScintiVerse®; Fisher Scientific, Pittsburgh, PA) was added to 200 µl of extract from each sample. Vials were vortexed (15 sec) and allowed to incubate in the dark for at least 1 hr before counting in a β counter (LS 6500, Beckman Coulter™; Beckman Coulter, Inc., Brea, CA).

### *Fecal hormone metabolite assessment*

Corticoid metabolite concentrations in fecal samples were measured using a corticosterone double antibody $^{125}$I RIA (♯07-120103; MP Biomedicals, Solon, OH). Fecal extracts were diluted 1:100 (1:500 final dilution) in provided steroid diluent. FAM concentrations were measured using a solid phase $^{125}$I RIA (Coat-A-Count® Total Testosterone, ♯TKTT2; Diagnostic Products Corporation, Los Angeles, CA). Fecal extracts were diluted 1:5 (1:25 final dilution) in PBS-Gel. Fecal metabolite concentrations were calculated on ng/g dry feces basis. The minimum detectable concentration, according to the manufacturer, was 7.7 ng/ml (extract) or 0.11 µg/g dry feces.

Assays were validated by demonstrating parallelism between binding of serial dilutions of jaguar fecal extracts and that of the standard curve, and significant recovery (90–110%) of unlabeled steroid standard added to pooled samples with low endogenous mass. Intra- and inter-assay coefficients of variation for both assays were less than 3 and 8%, respectively.

### *Statistical analysis*

The effects of gender and treatment (ACTH injection), and their interaction, on mean concentrations of FCMs were determined using an Analysis of Variance (ANOVA) general linear models procedure (Proc Glm) (SAS Software, Version 9.1). Nonparametric ANOVA was performed for cases where ANOVA assumptions were violated [Zar, 2009]. Concentrations of FCMs that were below the minimum detectable concentrations were reported at the assay sensitivity, 0.1 µg/g.

Because FCM concentrations vary within and between individuals, the response to ACTH treatment was also analyzed for each jaguar. Corticoid metabolite concentrations in fecal samples collected before ACTH treatment were averaged for each jaguar to obtain individual baseline values; however, metabolite concentrations of two females (♯5 and ♯6) were averaged together because it was not

always possible to discriminate fecal samples between the two individuals, which were a mother and daughter pair that were housed together and received ACTH treatment within 1 hr of one another.

For each individual, an increase in hormone metabolite concentrations was considered to be significant if the hormone concentration in a post-ACTH fecal sample reached a peak. In this study, a peak was defined as a fecal metabolite concentration value that was greater than baseline mean+2SD.

A correlation between concentrations of FCMs and FAMs within each individual male was verified by Regression Analysis using Proc Reg and by Pearson's coefficient of correlation using Proc Corr.

To compare the magnitude of the response with ACTH treatment between male and female jaguars, the FCM concentrations of the first two samples collected after ACTH injection for each individual jaguar were averaged and the resulting mean was divided by the individual baseline mean to obtain a factor of increase. The mean factor of increase was compared between males and females via an unpaired *t*-test with Welch correction; however, due to the small samples size ($n = 3$ per gender), the assumption of a Gaussian distribution could not be tested.

All data are expressed on a dry feces basis. Results are expressed as mean $\pm$ SEM or LSmean $\pm$ SEM. Level of significance was set at $P < 0.05$.

## Mean Baseline Corticoid Metabolite Concentrations

### *Animals*

A total of 16 jaguars (7 M, 9 F) were included in this analysis (Table 1). For females ♯5 and ♯6, only those fecal samples that could be unmistakably identified were considered. The jaguars participating in the ACTH challenge test were included in this calculation by assessing FCM concentrations present in the pre-ACTH samples. The number of jaguars included in each gender/origin group and the respective total number of fecal samples analyzed (plus the range of number of samples per individual) was as follows: 4 wild-born males, 35 samples (8–10 samples/jaguar); 6 wild-born females, 72 samples (3–20 samples/jaguar); 3 captive-born males, 39 samples (5–20 samples/jaguar); and 3 captive-born females, 40 samples (3–19 samples/jaguar).

Fecal sample collection and hormone metabolite extraction and assessment were performed as previously described.

### *Statistical analysis*

The GLM procedure of SAS was used to investigate the effects of gender and origin, and their interaction, on FCM concentrations. Because of the uncertainty regarding the age of the wild-born individuals, we did not treat age as an independent variable in the statistical analyses. Normality of data distribution was assessed via a formal test (Anderson–Darling) and by visual analysis using a normal probability plot. If the assumption of normal distribution was violated, nonparametric analysis was conducted [Zar, 2009]. When an interaction between "gender" and "origin" was detected, pairwise comparisons (Unpaired *t*-tests) were performed within "origin" with "gender" being the independent variable and vice-versa.

Concentrations of FCMs were individually averaged and the individual means were used to calculate the mean concentration of FCMs and the confidence interval (CI) per gender/origin.

## RESULTS

### ACTH Challenge Test

Overall, mean concentrations of FCM increased ($P<0.01$) after ACTH treatment (pre-ACTH: $0.90\pm0.12$ μg/g dry feces; post-ACTH: $2.55\pm0.25$ μg/g; LSmean ± SEM). When pre- and post-ACTH samples were combined, FCM concentrations (LSmean ± SEM) were higher ($P<0.01$) in males ($2.15\pm0.20$ μg/g) than in females ($1.30\pm0.20$ μg/g). The difference between mean concentrations of FCMs in male and female jaguars did not change ($P>0.05$) because of ACTH treatment. The intensity of the response to ACTH treatment was comparable ($P>0.05$) between males and females.

Pre- and post-ACTH administration values of FCMs for individual jaguars are found in Table 2. Percentages of mean baseline concentrations of FCMs at each observation time were calculated for individual jaguars throughout the entire period of sample collection for the ACTH challenge (Fig. 1). With the exception of Female ♯ 8, all jaguars exhibited a peak in corticoid metabolite excretion after ACTH injection; the highest numerical values of FCM concentrations were observed in the first or second post-ACTH samples, except male ♯3 (Fig. 1). Female ♯8 did have a relatively elevated concentration of FCMs on day 4 post-ACTH injection but that could not be considered a peak due to a pre-ACTH sample with relatively high amounts of FCMs 2 days before ACTH treatment for unknown reasons.

For males, concentrations of FAMs are also shown in the graphs (Fig. 1). Interestingly, the graphs suggest that males ♯1 and 2, but not male ♯3, experienced an increase in FAMs that seemed temporally associated with the increase in FCMs after ACTH treatment. In fact, after ACTH administration, FAM concentrations

**TABLE 2. Fecal corticoid metabolite (FCM) concentrations (μg/g dry feces) in Male (♯1–3) and Female (♯5–8) Jaguars During an ACTH Challenge**

| | FCMs | | |
|---|---|---|---|
| | Pre-ACTH | Post-ACTH | |
| Jaguar | Mean ± SEM (*n*) | First sample (day) | Second sample (day) |
| 1 | 1.46 ± 0.32 (8) | **3.89** (3) | **3.71** (6) |
| 2 | 1.22 ± 0.35 (9) | **4.29** (3) | 3.14 (4) |
| 3 | 0.66 ± 0.09 (8) | **2.67** (1) | **1.31** (3) |
| 5, 6[a] | 0.82 ± 0.18 (8) | **4.22** (1) | **2.74** (2) |
| 7 | BD (8) | BD (3) | **0.95** (4) |
| 8 | 1.04 ± 0.25 (9) | 1.14 (3) | 2.44 (4) |

Pre-ACTH values are individual baseline means of FCM concentrations (mean ± SEM; *n* = number of samples). Post-ACTH values are FCM concentrations in the first two samples collected after ACTH injection (day = day of sample collection). BD, below detectable.

[a]Pooled samples from Females ♯5 and 6. Bold values are greater than baseline mean plus 2.0SD.

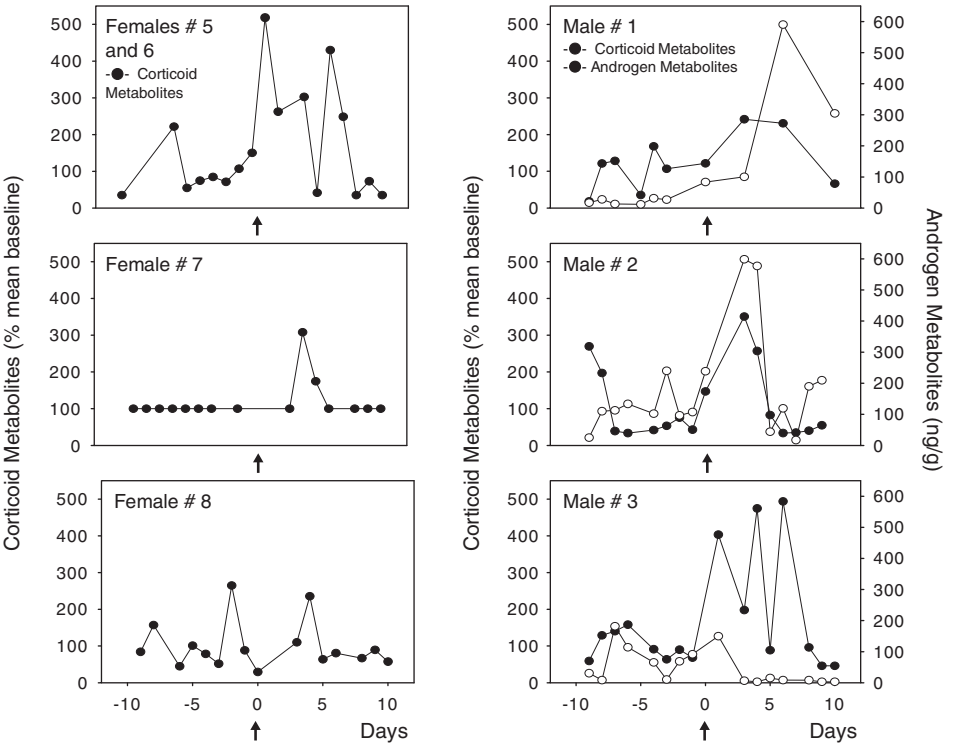


Fig. 1. Percent mean baseline concentrations of FCMs (-●-; left *Y* axis) in males and females, and concentrations of FAMs in ng/g dry feces (-○-; right *Y* axis) in males during the ACTH challenge test. The arrow indicates ACTH injection. FCMs, fecal corticoid metabolites; FAMs, fecal androgen metabolites; ACTH, adrenocorticotrophic hormone.

increased in males ♯1 and 2 only (Table 3). Moreover, in male ♯2, a positive correlation between FCMs and FAMs was found (0.60; $P<0.01$).

### Mean Baseline Corticoid Metabolite Concentrations

The difference between mean baseline concentrations of FCMs in males and females changed ($P<0.05$) according to whether the individuals were born in the wild or in captivity. The only difference detected by pairwise comparisons within "origin" or "gender" was found within the captive-born group where females had lower ($P<0.05$) mean baseline concentrations of FCMs compared with males. The mean concentration of FCMs and the range of physiological values, based on CIs ($\pm$CI) per gender/origin observed in this study were (μg/g dry feces): $1.00\pm0.41$ (wild-born males; range, 0.59–1.41); $0.90\pm0.53$ (wild-born females; range, 0.37–1.42); $1.14\pm0.42$ (captive-born males; range, 0.72–1.56); and $0.37\pm0.14$ (captive-born females; range, 0.23–0.51) (Fig. 2).

## DISCUSSION

In this study, we presented a valid, simplified protocol for noninvasive assessment of adrenal function in the jaguar, and showed its validity using an ACTH challenge. To our knowledge, this is the first report of an ACTH challenge in jaguars.

**TABLE 3. Fecal androgen metabolite (FCM) concentrations (ng/g dry feces) in Male (♯ 1–3) Jaguars During an ACTH Challenge**

| | FAMs | | |
|---|---|---|---|
| | Pre-ACTH | Post-ACTH | |
| Jaguar | Mean ± SEM (*n*) | First sample (day) | Second sample (day) |
| 1 | 27.93 ± 8.46 (8) | **100.48** (3) | **590.16** (6) |
| 2 | 129.66 ± 22.95 (9) | **598.84** (3) | **577.37** (4) |
| 3 | 71.47 ± 20.61 (8) | 149.83 (1) | 6.70 (3) |

Pre-ACTH values are individual baseline means of FAM concentrations (mean ± SEM; *n* = number of samples). Post-ACTH values are FAM concentrations in the first two samples collected after ACTH injection (day = day of sample collection). ACTH, adrenocorticotrophic hormone. Bold values are greater than baseline mean plus 2.0SD.

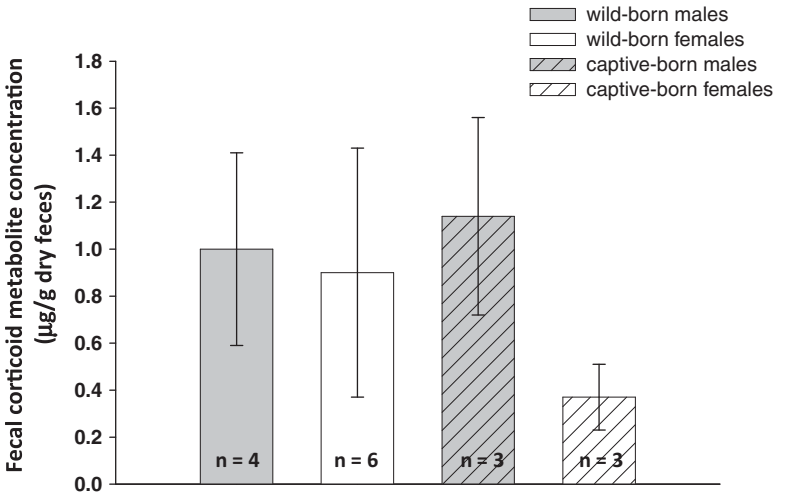


Fig. 2. Mean FCM concentrations ± CI (µg/g dry feces) in adult jaguars according to gender and origin (*n* = numbers of individual jaguars). The total number of samples (and the range of number of samples per individual) in each group is as follows: wild-born males (35; 8–10); wild-born females (72; 3–20); captive-born males (39; 5–20); and captive-born females (40; 3–19). FCM, fecal corticoid metabolites.

A previous study in male jaguars measured changes in FCMs after chemical restraint and electroejaculation [Morato et al., 2004]. Our ACTH challenge test showed that both male and female jaguars responded to stimulation of the adrenal glands in a way that was detectable in fecal samples as a significant increase in corticoid metabolites concentrations. When pre- and post-ACTH samples were considered, male jaguars had overall higher concentrations of FCMs compared with females. However, there was no significant difference in the magnitude of the response to ACTH between males and females.

We could not investigate the effect of origin (wild- vs. captive-born) on FCM concentrations after acute stimulation of the adrenal glands via ACTH injection because, with the exception of female ♯6, all jaguars participating in the ACTH

challenge test were wild-born. In the assessment of mean baseline corticoid metabolite concentrations, where more jaguars were included, mean baseline concentrations of FCMs in captive-born female jaguars were numerically lower compared with those of other gender/origin groups. A significant interaction between gender and origin was detected; therefore, the effect of gender on mean baseline concentrations of FCMs could not be considered separately from the origin of the jaguars. Within the captive-born group, females had lower mean baseline concentrations of FCMs than males.

A tempting extrapolation would be to conclude that once acclimated, wild-born male jaguars seem to adapt well to the captive environment because their mean baseline FCM concentrations did not seem to differ from those of their captive-born counterparts. However, baseline FCM concentrations may not differ between animals that are or are not under chronic stress due to adaptation to a frequent stressor. Sakellaris and Vernikos-Danellis [1975] observed that plasma corticosterone concentrations in rats exposed to chronic stressors returned to pre-stress levels regardless of the continuation of the stimuli indicating some adaptation to chronic stress. However, the response of the pituitary–adrenal system to new stimuli was faster in those rats compared with control rats, as evidenced by a faster increase in plasma corticosterone concentrations in the chronically stressed rats upon additional stimuli. Moreover, stress is a multifaceted condition; thus, a comprehensive approach on how stress affects an organism, including other parameters (e.g., immunologic health, behavior patterns) would be necessary in order to draw more meaningful conclusions.

Differences in the way that wild- and captive-born individuals cope with stressors may be reflected in parameters other than FCM concentrations. A study in bighorn sheep (*Ovis canadensis canadensis*) concluded that once the wild-born group had acclimated to the captive environment, baseline concentrations of FCMs were similar to those of the captive-raised group and both groups had comparable responses to an acute stress event in terms of FCM concentrations [Coburn et al., 2010]. However, the autonomic nervous system response to the same acute stressor (a drop-net restraint) was stronger in the captive-raised group compared with the wild-born group, suggesting that the two groups perceived the stressor in distinct ways.

Another study compared the response with changes in housing situation, such as transfer to a new room or a change in cage size in the pigtailed macaque (*Macaca nemestrina*) and observed that the subject's origin did not influence any of the variables tested (e.g. urinary cortisol, abnormal behavior) except for appetite suppression after room change, which was greater in wild-caught females than in their captive-born counterparts [Crockett et al., 2000].

Those studies indicate that at least in some species, after acclimation to captivity, wild-born individuals have similar baseline levels of glucocorticoids as those born in captivity, as well as similar HPA responses to acute stress. However, other measurements of response to acute stress might differ between wild- and captive-born individuals in ways that are not fully understood at present.

One aspect of the HPA axis that is still poorly understood is the nature of its interactions with the HPG axis. In nondomestic felids, some progress toward a better understanding of the HPA–HPG interactions was made in studies investigating how changes in glucocorticoid concentrations would affect reproductive hormones [Wildt et al., 1988; Brown et al., 1989]. Increased serum cortisol concentrations following anesthesia and electroejaculation or ACTH treatment did not affect LH and

testosterone profiles in cheetahs (*Acinonyx jubatus*) and pumas (*Puma concolor*), but caused testosterone concentrations to decrease in leopards (*Panthera pardus*), whereas results in tigers (*Panthera tigris*) were unclear [Wildt et al., 1988].

Another study also detected increased cortisol and decreased testosterone concentrations in serum of male leopards (*P. p. kotyia*) following an anesthesia/electroejaculation procedure; the same study showed that injection of ACTH inhibited a GnRH-induced increase in testosterone patterns—in both experiments, pituitary LH and FSH patterns were unaffected [Brown et al., 1989]. In jaguars, a previous study reported an increase in FCM concentrations after anesthesia/electroejaculation, without a concomitant increase in FAMs [Morato et al., 2004]. In our study, all three males participating in the ACTH challenge had increased concentrations of FCMs, and two of them experienced an increase in FAMs, whereas the third jaguar did not. In fact, the latter showed consistently low concentrations of FAMs on the days following the procedure (Fig. 1). Together, these findings suggest that not only do the effects of adrenal stimulation on androgen concentrations vary across cat species, as previously suggested [Wildt et al., 1988], but also between individuals of the same species.

One could speculate that electroejaculation itself could have an effect on androgen concentrations. In this study, two (of 3) male jaguars participating in the ACTH challenge were electroejaculated while under anesthesia for ACTH gel administration (males ♯2 and 3). Our study was not designed to investigate the effects of electroejaculation on FAM concentrations; thus, we cannot draw any conclusions on the subject. However, we observed that of the two males that were electroejaculated, one had an increase in FAMs (male ♯ 2) and the other did not (male ♯ 3). Thus, it is possible that electroejaculation itself may not be a factor in the changes observed in FAMs profiles. In fact, Wildt et al. [1988] reported that, within the species studied (tigers, leopards, pumas, and cheetahs), anesthesia alone or anesthesia followed by electroejaculation resulted in similar serum testosterone profiles.

Contribution of androgens from adrenal origin should be considered when adrenal stimulation results in an increase in fecal androgens. We did not assess FAM fluctuations in female jaguars participating in the ACTH challenge test, because that was out of the scope of this study. If observed in females, an increase in FAMs in response to exogenous ACTH would suggest that at least part of the increased androgen metabolites observed in males would likely have adrenal origin.

The effects of reproductive status (i.e. intact vs. castrate) on FCM concentrations during an ACTH challenge were not assessed in this study given that all individuals were intact with the exception of one jaguar (Female ♯8), which, interestingly, was the only jaguar whose increase in FCMs after ACTH injection could not be considered a peak. Assessing the effects of reproductive sterilization on baseline concentrations of fecal corticoids would be another interesting future study since it could provide some insight on how castration may affect the responsiveness of jaguars to the captive environment.

In felids, protocols for extracting steroid metabolites from feces typically include boiling of pulverized fecal samples in 90% ethanol for 20 min [Brown et al., 1994, 1995; Wielebnowski et al., 2002; Morato et al., 2004]. Alternatively, some laboratories have developed protocols for fecal steroid extraction that, instead of boiling the samples agitate them for 30 min in a multi-tube vortexer [Schwarzenberger et al., 1991; Wasser et al., 2000]. As a whole, the vortexing (nonboiling)

method is simpler and less time-consuming than its counterpart, and therefore, it has become the method of choice for some authors [Wasser et al., 2000]. For the same reason, we wanted to use a vortexing method for fecal steroid extraction in our study, so we conducted preliminary tests using jaguar fecal samples to compare extraction efficiency between the boiling and the vortexing methods (data not shown). In agreement with Wasser et al. [2000], our tests indicated that extraction efficiency was comparable between the two methods. We believe this study is the first report of fecal steroid extraction using a vortexing method in jaguars.

The content of steroid metabolites in fecal samples can be altered by several factors, such as bacterial enzyme activity, and environmental humidity and temperature [Winter et al., 1979; Cerone-McLernon et al., 1981; Bokkenheuser et al., 1986; Abáigar et al., 2010]. We did not assess stability of the immunoreactive compounds in jaguar fecal samples; however, all samples in our study were frozen while still relatively "fresh" (i.e. less than 30 h between defecation and freezing of the samples). A previous study investigated the stability of steroid metabolites in Iberian lynx (*Lynx pardinus*) fecal samples concluding that those compounds remained stable in feces for at least 1 week under field conditions [Abáigar et al., 2010]. Even though we did not perform the same assay as the latter study, we extrapolate from those findings to our study and believe there was enough stability of steroid metabolites in the jaguar fecal samples.

We did not investigate the chemical structure of the glucocorticoid metabolites found in jaguar feces so we cannot affirm that adding $^{3}$H-cortisol to jaguar fecal samples before extraction and measuring it afterwards was an effective way of assessing extraction efficiency of FCMs in jaguars; however, this method has been accepted as an estimate of recovery in other carnivore species [Young et al., 2004]. It is worth noting, nevertheless, that among the most prevalent immunoreactive cortisol metabolites excreted in feces of domestic cats are 11,17 dioxoandrostanes [Schatz and Palme, 2001].

In our study, we assumed that the amount of variation in baseline measurements of FCMs within and between individuals was "normal," as opposed to reflections of acute stress events, because during the period of time where fecal samples were collected, the jaguars did not experience any change in their routines, according to the keepers. The amount of variation between individuals in FCMs indicates that to conduct meaningful monitoring of individual adrenal responses to acute stress it is important to be based on individual FCM baseline levels. Thus, long-term longitudinal monitoring of individuals is recommended if the impact of potential stressors is to be understood. Other measurements, such as assessment of immune health and behavioral patterns, should also be incorporated into a comprehensive investigation of how stressors may impact an animal's health.

The protocol described in this study is a valid method for monitoring adrenocortical function in response to acute stressors in captive jaguars. Our study also provides a physiological range of FCM concentrations for healthy, captive adult jaguars that can be used as a reference for the assay system proposed here. Thus, the information generated in this study can be useful in helping researchers and zoo professionals monitor how changes in the captive environment and in husbandry practices affect their animals. We hope that an increasing number of institutions adopt FCM monitoring to help them make more meaningful changes in the captive environment so that the wellbeing of their jaguars can be enhanced.

## CONCLUSIONS

(1) An ACTH challenge was performed in male and female jaguars to validate the assay system proposed in this study for monitoring adrenal function in the species.
(2) An increase in FCM concentrations was observed in males and females after ACTH injection. No difference was observed in the magnitude of the response to ACTH treatment between male and female jaguars.
(3) When pre- and post-ACTH injection samples were considered, overall, males had higher concentrations of FCMs than females.
(4) The existence of a positive correlation between concentrations of FCMs and FAMs in response to acute adrenal stimulation seemed to depend on the individual male jaguar.
(5) The difference between mean baseline concentrations of FCMs in wild-born vs. captive-born changed according to whether the individuals were males or females. Within the captive-born group, females had lower mean baseline concentrations of FCMs compared with males.

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