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NEWS AND PERSPECTIVES

# Physiological and analytical validations of fecal steroid hormone measures in black howler monkeys

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**Abstract** The measurement of hormones in fecal samples allows for the noninvasive assessment of the endocrine status of free-ranging primates. However, procedures and techniques for hormone analysis in feces must be validated, both analytically and physiologically. Few studies have addressed the endocrinology of black howler monkeys (*Alouatta pigra*). Due to its conservation status, direct handling of individuals from this species and invasive sample collection are highly regulated, and therefore traditional methods for the validation of hormone assays, such as pharmacological challenges, are not allowed. As a consequence, sometimes studies of the fecal hormones of free-ranging black howler monkeys do not report physiological validations and therefore the biological reliability of such measurements cannot be assessed. In order to stimulate future research with this species, the present study aimed at providing methodological bases for fecal endocrine monitoring. Specifically, we compared the validity of two immunoassays (radioimmunoassays, RIA; solid-phase chemiluminescent enzyme immunoassay, SPCEI) performed with commercial kits to measure cortisol, testosterone, estradiol, and progesterone; and demonstrate how the physiological functions of these steroid hormones can be determined through non-pharmacological validations. We found no differences between the analytical validity of RIA and SPCEI assays to measure cortisol and testosterone, whereas for estradiol and progesterone RIA showed better results. Concerning the physiological validation of our assays, we demonstrated that: (1) comparisons between pre- and post-stress situations may be used to assess cortisol response, (2) comparisons between females and males may be used to assess variation in testosterone levels, and (3) comparisons between pregnant and non-pregnant females may be used to determine variation in estradiol and progesterone activity. The analytical and physiological validations that we performed demonstrate that there are currently commercial kits that allow for correct endocrine monitoring of this species, and that there are non-pharmacological alternatives to assess the biological validity of hormone measurements.

**Keywords** Fecal steroids · Cortisol · Testosterone · Estradiol · Progesterone · *Alouatta pigra*

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## Introduction

In mammals, the major groups of steroid hormones are the male and female sex hormones and the hormones produced by the adrenal cortex (Norman and Litwack 1997). These hormones are involved in numerous physiological functions, including the regulation of immune responses or sexual behavior, and have therefore been the major focus of endocrine research on free-ranging non-human primates (Nguyen 2013). Steroid hormones are synthesized from cholesterol, and after transportation and use in target tissues, they are inactivated (mainly in the liver) and excreted via the kidneys into the urine or via the bile into the gut

(Norman and Litwack 1997; Touma and Palme 2005). The measurement of hormones in fecal samples allows for the noninvasive assessment of the endocrine status of free-ranging primates, and in recent years has resulted in a notable growth of field studies on environment-hormone and hormone-behavior interactions (Nguyen 2013).

The development of techniques for the analysis of fecal hormones has been of particular relevance for the study of endangered and/or protected wildlife, which usually cannot be submitted to invasive procedures (Palme 2005). In spite of such developments, and because metabolism and excretion of steroids differ significantly between species (Palme 2005), for many species there is a general lack of information on the feasibility of using fecal sampling for endocrine monitoring. Furthermore, there are now many published studies based on hormone measurement in feces that do not report analytical or physiological validations, preventing its application to further research. Therefore, it is important to stimulate the development of reliable procedures and techniques for hormone analysis in feces (Palme 2005).

Yucatán black howler monkeys (*Alouatta pigra*; hereafter, black howler monkeys) are an endangered tree-dwelling primate species with a geographic distribution restricted to the Yucatan Peninsula in Mexico and Belize, and some parts of northern and central Guatemala (Horwich and Johnson 1986). There are currently few studies on the endocrinology of this species (Martínez-Mota et al. 2007, 2008; Van Belle et al. 2009a, b; Behie et al. 2010; Rangel-Negrín et al. 2011). Research involving direct handling of individuals and invasive sample collection with this species is highly regulated, and the use of traditional methods for the validation of hormone assays, such as pharmacological challenges, is not allowed (e.g., Mexico: Diario Oficial de la Federación 2013). As a consequence, sometimes studies of the fecal steroid hormones of free-ranging black howler monkeys do not report physiological validations (e.g., Martínez-Mota et al. 2007; Van Belle et al. 2009a), and therefore the biological reliability of such measurements cannot be assessed. In order to stimulate future research with this species, the present study aimed at providing methodological bases for fecal endocrine monitoring. We performed our analysis with four steroid hormones, cortisol, testosterone, progesterone, and estradiol, which may be used to study stress physiology, reproductive ecology, and social relationships. Specifically, we first compare the analytical performance of two immunoassays (radioimmunoassays and solid-phase chemiluminescent enzyme immunoassay) performed with commercial kits to measure steroid hormones in feces of black howler monkeys. Second, we demonstrate how the physiological functions of steroid hormones can be determined through non-pharmacological validations, and therefore, biologically validated.

## Materials and methods

Our research complied with the Mexican law and was approved by the corresponding authorities (SEMARNAT SGPA/DGVS/01273/06 & 04949/07).

Fecal samples were collected from adult black howler monkeys living in different locations in Campeche, Mexico. Detailed descriptions of the study area can be found elsewhere (Dias et al. 2011; Rangel-Negrín et al. 2011).

### Fecal sample collection

Samples were collected opportunistically whenever they could be matched with individuals. Fresh fecal samples uncontaminated by urine were collected from the forest floor and deposited in polyethylene bags labeled with the identity of each individual. Fecal samples were kept in a cooler with frozen gel packs while in the field and stored at the end of the day in a freezer at −20 °C in the field station until extraction was performed. Samples were stored at constant temperature for a maximum of 12 months until the extractions were conducted. The freezing procedure used for storing the samples has been reported to have a weak time-storage effect on fecal hormone metabolites in other primate species (Khan et al. 2002). Sample sizes for each validation are given below.

### Steroid extraction

Hormone analyses were conducted at the Departamento de Biología de la Reproducción del Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán, Mexico City. Hormone metabolites were extracted from feces following a modification of the method described in Wasser et al. (2000) for the extraction of steroids. Briefly, 0.6 g of homogenized, lyophilized, and pulverized feces was shaken for 24 h in 4 ml of pure ethanol ($CH_3OH$). Extracts were then centrifuged (460 g for 30 min at −4 °C), and the supernatant containing the steroids was recovered. After complete evaporation of the solvent (at 60 °C), pellets were reconstituted with 3 ml albumin buffer and used for analyses.

### Assays

Hormone radioimmunoassays (RIA) were conducted with cortisol, testosterone, progesterone, and estradiol commercial kits (all from Coat-A-Count, SIEMENS, Los Angeles, CA). We added 25, 50, 100, and 200 µl of the extracts to each tube in the cortisol, testosterone, progesterone, and estradiol kits, respectively, and then 1 ml of radiolabelled ($^{125}I$) hormone. After incubation in a water bath at 37 °C for 45 min, extracts were poured and the tubes were washed with distilled water. Hormone concentrations were determined with a gamma counter (Cobra 5005, Packard, MI).

For hormone measurements with solid-phase chemiluminescent enzyme immunoassay (SPCEI) we used commercial kits (IMMULITE, SIEMENS, Los Angeles, CA for all hormones). We added 25, 50, 100 and 200 µl of the extracts to each tube in the cortisol, testosterone, progesterone, and estradiol kits, respectively, and placed the tubes in an IMMULITE 1000 analyzer (SIEMENS, Munich, Germany).

We selected antibodies based on their commercial availability and on the possibility to compare our data to other studies using the same antibodies (e.g., Cristóbal-Azkarate et al. 2006; Clarke et al. 2007; Martínez-Mota et al. 2007; Rangel-Negrín et al. 2011). All samples were run in duplicate, and mean hormone values are reported as ng/g dry feces.

### Analytical validations of RIA and SPCEI

For each hormone, we performed assay analytical validations and qualitatively compared the results of RIA and SPCEI. Specifically, we compared accuracy, through dose–response curves and parallelism (i.e., running serial dilutions of a fecal extract pool and comparing them with the slope of expected dose versus percent bound to the slope of the standard curve), and recovery (i.e., adding incremental known quantities of steroid to standards and measuring the incremental increase in concentration) to assess the applicability of these kits for black howler monkey fecal extracts. We also ran quality controls that consisted of a pooled fecal sample and three immunoassay controls (high, medium, and low), that allowed us to calculate intra-assay and inter-assay coefficients of variation (CV) for RIA and for SPCEI. For each antibody we report the sensitivity, calibration ranges, and required volumes as provided by the manufacturers.

### Biological validations

All samples used in the biological validations were extracted as described above and analyzed with RIA only.

#### *Cortisol*

Glucocorticoids are the main modulators of physiological stress responses in vertebrates, and its concentrations usually increase in response to a stressor (Sapolsky et al. 2000). Cortisol is the major glucocorticoid in primates (Stratakis and Chrousos 1995). To determine if our assays reflected changes in cortisol metabolites, we determined the effects of capture (an acute stressor) and anesthesia (ketamine; Anesket Vet, Pisa Agropecuaria, Hgo, Mex) on the cortisol excretion profile of three adult howler monkeys (one male and two females), and predicted that cortisol levels should be higher following capture. This capture was part of a concurrent translocation project of black howler monkeys, and our capture and handling procedures (Canales-Espinosa et al. 2011) were approved by Mexican authorities (SEMARNAT, SGPA/DGVS/10637/11).We collected all fecal samples ($N = 42$) from the three individuals from 72 h before to 96 h after capture, and compared pre-capture levels with peak concentrations (i.e., the highest post-stressor values that were ≥2*SD above the mean concentration before capture: Martínez-Mota et al. 2008) with a Wilcoxon signed-rank test.

#### *Testosterone*

Testosterone is the primary androgen, and although it is secreted in the adrenal cortex and ovary of females, it is produced in far greater amounts by the Leydig cells of the testis (Swerdloff et al. 2009). In primates, circulating testosterone levels are higher in males than in females (Dixson 2013). Based on these physiological differences, to determine if our assays reflected changes in testosterone metabolites we compared the testosterone levels of eight adult males and eight adult females ($N = 16$) with Mann–Whitney tests predicting that these should be higher in the former than in the latter.

#### *Estradiol and progesterone*

Progesterone and estrogens are the main reproductive hormones of female primates. Progesterone and estradiol have essential roles in the physiology of pregnancy, including the regulation of the dynamic interaction among mother, placenta, and fetus (Pepe and Albrecht 1995). On average, their concentrations are higher during pregnancy than at other times (Ryan and Hopper 1974; Hodgen and Itskovitz 1988). Therefore, to determine if our assays detected variation in estradiol and progesterone metabolites we compared the fecal concentrations of these hormones between pregnant and non-pregnant adult females and predicted that both estradiol and progesterone should be higher during pregnancy. Females were classified as pregnant by calculating 180 days back from observed parturitions (for a reported gestation length of 184 days: Van Belle et al. 2009b). We compared estradiol and progesterone levels between four pregnant ($N = 21$ fecal samples) and five non-pregnant adult females ($N = 29$) with Mann–Whitney tests.

## Results

### Analytical validation

Dose–response, parallelism and recovery tests suggest that both the RIA and the SPCEI assays that were performed adequately measured cortisol and testosterone in feces of

**Table 1** Validation of fecal steroid RIA and SPCEI in black howler monkeys (*Alouatta pigra*)

| Hormone (manufacturer) | Assay | Accuracy | | Recovery ± S.E. | Intra-assay CV (*N*) | Inter-assay CV (*N*) | Sensitivity | Calibration range | Required volume (µl) |
|---|---|---|---|---|---|---|---|---|---|
| | | D-R curves[a] | Parallelism[b] | | | | | | |
| Cortisol (Siemens) | RIA | $y = 4.95 + 0.29*x$; $r^2 = 0.99$, $p < 0.01$ | $t = 1.55$, $p = 0.172$ | 103.4 ± 24.8 % | 7.16 % (58) | 8.35 % (58) | 0.2 µl/dL | 1–50 µg/dL | 25 |
| Cortisol (Siemens) | SPCEI | $y = -0.49 + 0.18*x$; $r^2 = 0.99$, $p < 0.01$ | $t = 0.58$, $p = 0.580$ | 114.4 ± 18.2 % | 5.21 % (60) | 6.28 % (60) | 0.2 µl/dL | 1–50 µg/dL | 100 |
| Testosterone (Siemens) | RIA | $y = -0.02 + 0.47*x$; $r^2 = 0.99$, $p < 0.01$ | $t = 0.68$, $p = 0.519$ | 81.3 ± 21.7 % | 6.45 % (58) | 7.95 % (58) | 4 ng/dL | 20–1,600 ng/dL | 50 |
| Total testosterone (Siemens) | SPCEI | $y = -7.87 + 0.59*x$; $r^2 = 0.97$, $p < 0.02$ | $t = 0.44$, $p = 0.674$ | 60.2 ± 26.6 % | 5.61 % (60) | 6.72 % (60) | 15 ng/dL | 20–1,600 ng/dL | 20 |
| Estradiol ($E_2$, Siemens) | RIA | $y = 490 + 0.89*x$; $r^2 = 0.99$, $p < 0.01$ | $t = 0.64$, $p = 0.543$ | 149.9 ± 33.3 % | 6.52 % (58) | 7.52 % (58) | 8 pg/mL | 20–3,600 pg/mL | 100 |
| Estradiol ($E_2$, Siemens) | SPCEI | $y = 335 + 0.47*x$; $r^2 = 0.82$, $p = 0.09$ | $t = 0.99$, $p = 0.356$ | 26.8 ± 10.0 % | 8.56 % (45) | 9.54 % (45) | 15 pg/mL | 20–2,000 pg/mL | 25 |
| Progesterone (Siemens) | RIA | $y = 3.83 + 0.07*x$; $r^2 = 0.95$, $p = 0.03$ | $t = 1.39$, $p = 0.212$ | 76.1 ± 26.5 % | 7.12 % (58) | 8.15 % (58) | 0.02 ng/mL | 0.1–40 ng/mL | 100 |
| Progesterone (Siemens) | SPCEI | $y = 4.95 + 0.29*x$; $r^2 = 0.86$, $p = 0.07$ | $t = 0.05$, $p = 0.958$ | 42.6 ± 36.2 % | 7.59 % (45) | 8.72 % (45) | 0.2 ng/mL | 0.2–20 ng/mL | 50 |

Samples were diluted 1:2, 1:4, 1:8, and 1:16

[a] $N = 4$

[b] $N = 5$

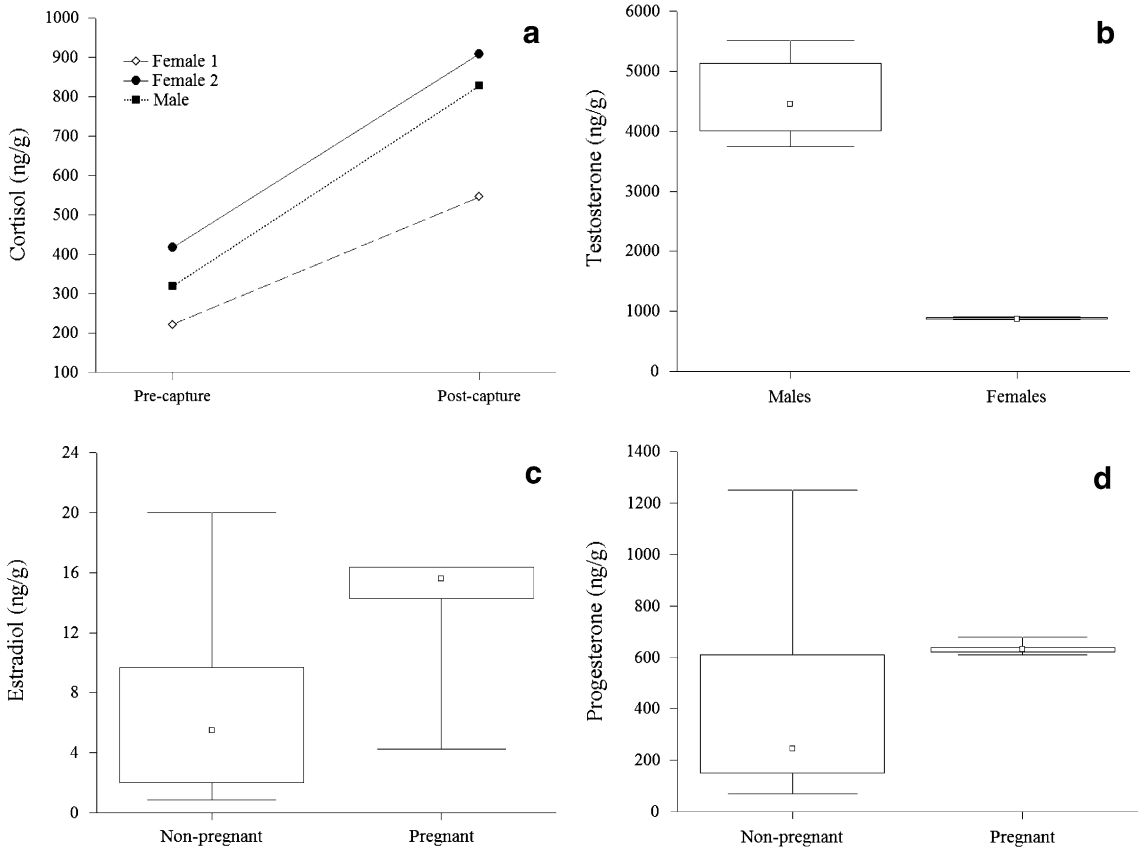


**Fig. 1** Physiological validations of cortisol (**a**; *N* pre-capture = *N* post-capture = three individuals), testosterone (**b**; *N* males = *N* females = eight individuals), estradiol (**c**; *N* non-pregnant females = five, *N* pregnant females = four), and progesterone (*N* non-pregnant females = five, *N* pregnant females = four) measurements in feces of black howler monkeys. In **b**, **c**, and **d**: median ▭ 25 %–75 % ⊤⊥ min–max

black howler monkeys (Table 1). For estradiol and progesterone, the dose–response and recovery results obtained with RIA assays were superior to those of SPCEI.

## Physiological validations

Cortisol levels peaked at a mean (± SD) of 35.3 ± 9.9 h after capture. Peak levels were significantly higher than pre-capture levels for the three individuals (female 1: $Z = 2.27$, $N$ before capture = 6, $N$ after = 7, $p < 0.05$; female 2: $Z = 3.29$, $N$ before = 7, $N$ after = 7, $p < 0.001$; male: $Z = 3.41$, $N$ before = 8, $N$ after = 7, $p < 0.001$; Fig. 1a), indicating that our cortisol assays reliably measured adrenal responses of black howler monkeys to an acute stressor.

The average (± SD) testosterone levels in male samples (4,485.6 ± 234.3 ng/ml) were nearly 580 % higher than those in female samples (659.1 ± 206.7 ng/ml; $Z = 14.03$, $p < 0.001$; Fig. 1b).

Progesterone and estradiol concentrations were significantly higher (progesterone $Z = 2.47$; $N$ pregnant = 21, $N$ non-pregnant = 29, $p = 0.011$; estradiol $Z = 3.17$, $N$ pregnant = 21, $N$ non-pregnant = 29, $p < 0.001$) in pregnant (mean ± SD progesterone = 634.5 ± 21.4 ng/g; estradiol = 14.1 ± 4.4 ng/g) than in non-pregnant (progesterone = 409.1 ± 350.0 ng/g; estradiol = 6.5 ± 4.8 ng/g) females (Fig. 1c and d).

## Discussion

The present study had two objectives: to compare the validity of two immunoassays performed with commercial kits to measure steroid hormones in feces of black howler monkeys, and to demonstrate how the physiological functions of the steroid hormones that were measured in feces of this species can be determined through non-pharmacological validations. We found no differences between the validity of RIA and SPCEI assays to measure cortisol and testosterone, whereas for estradiol and progesterone RIA showed better results. Concerning the physiological validation of our assays, we demonstrated that: (1) comparisons between pre- and post-stress situations may be used to assess cortisol response, (2) comparisons between females

and males may be used to assess variation in testosterone levels, and (3) comparisons between pregnant and non-pregnant females may be used to determine variation in estradiol and progesterone activity. It is important to note that comparative evidence supports the biological relevance of our hormone measurements: fecal cortisol levels that we found are comparable to those reported by Martínez-Mota et al. (2007) despite several differences in assay methodology between studies (e.g., immunoassay method). In addition, fecal cortisol levels of black howler monkeys measured in the present study were lower than those of spider monkeys measured through a similar method (Rangel-Negrín et al. 2009), as expected by previous interspecific comparisons performed under controlled conditions (Coe et al. 1992).

Our results indicate that the analytical performance of the cortisol and testosterone kits that are available in Mexico to measure these hormones in feces of black howler monkeys does not vary between RIA and SPCEI. Because RIA is highly regulated due to security requirements associated with the use and disposal of radioactive material and requires more expensive instrumentation (Hodges et al. 2010), commercial kits based on SPCEI analysis may be the best option for studying these hormones in black howler monkeys. For female sexual hormones, SPCEI had a lower performance, probably because both kits had higher cross-reactivity with other compounds than RIA kits (Siemens 2014), suggesting that RIA should be used and that alternative kits must be tested.

Because hormone metabolism and excretion routes may differ between species (Palme et al. 2005), it is crucial to biologically validate assays to ensure the biological meaningfulness of analyses (Heistermann et al. 2006; Goymann 2012). This is particularly important when using commercial kits developed for hormone measurement in blood with feces or urine samples, as in the case of the present study (Hodges et al. 2010). Commercial kits are often based on antibodies highly specific to the native hormone but with unknown cross-reactivities with hormone metabolites, which usually represent the majority of excreted hormones. Native steroids are degraded into a number of metabolites through liver metabolism, microbial activity during intestinal passage, and re-absorption into the enterohepatic circulation (Palme et al. 1996). In addition, metabolism of stress hormones such as cortisol, and gonadal hormones such as testosterone may result in fecal metabolites with similar structures because the native hormones have similar structures (Norman and Litwack 1997). Thus, immunoassays designed to assess stress by measuring the concentration of fecal glucocorticoid metabolites may cross-react with other steroid metabolites, distorting the results. To ensure that an immunoassay reliably measures an animal's endocrine status, it should be experimentally demonstrated that it is able to quantify variation in hormone concentrations as a function of its known physiological functions. In this study we demonstrated that such validations may be performed noninvasively. To further improve the reliability of noninvasive hormonal analysis, future studies could use high-pressure liquid chromatography analysis to obtain information on the hormones and hormone metabolites excreted in feces of black howler monkeys, and assess the specificity of the performed assays (Bahr et al. 2000; Rangel-Negrín et al. 2011; but see Hodges et al. 2010).

In conclusion, our results provide methodological bases for the noninvasive study of steroid hormones in feces of the endangered black howler monkey. The analytical and physiological validations that we performed demonstrate that currently available commercial kits allow for a correct endocrine monitoring of this and other primate species, and that there are non-pharmacological alternatives to assess the biological validity of hormone measurements. It remains for future research to analyze other basic topics on the non-invasive study of the endocrinology of black howler monkeys. Among these, assessing the effects of sample storage on hormone concentrations (e.g., Khan et al. 2002) and comparing the efficiency of "field-friendly" extractions (e.g., Van Belle et al. 2009a) with conventional laboratory extractions (e.g., Rangel-Negrín et al. 2011) will be of paramount importance.

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