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Analytical and biological validation of a noninvasive measurement of glucocorticoid metabolites in feces of Geoffroy's spider monkeys (*Ateles geoffroyi*)

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Abstract

We report on an analytical and biological validation of a commercial cortisol enzyme immunoassay (EIA) to measure glucocorticoids (GC) in feces of Geoffroy's spider monkeys (*Ateles geoffroyi*). Validation of endocrinological methods for each sample matrix and study species is crucial to establish that the methods produce reliable results. For the analytical validation of the EIA, we assessed parallelism, accuracy, and precision. We carried out a biological validation based on three well-studied GC patterns with the following predictions: (1) increased fecal GC metabolite (fGCM) concentrations after veterinary intervention; (2) increased fGCM concentrations during early morning hours; and (3) higher fGCM concentrations during gestation than in other female reproductive states. For the first prediction, we sampled feces of two zoo-housed females 2 days before, the day of, and 2 days after a veterinary intervention. For the second prediction, we analyzed 284 fecal samples collected from 12 wild males using a linear mixed model (LMM). For the third prediction, we analyzed 269 fecal samples of eight wild females using an LMM. Analytical validation revealed that the EIA showed parallelism, was accurate, and precise within each assay. However, there was elevated variation in between-assay precision. The biological validation supported all predictions: (1) the two zoo-housed females showed a substantial increase in fGCM concentrations 2.5 and 11 h after veterinary intervention; (2) there was a negative effect of sample collection time on fGCM concentrations (i.e., higher concentrations during early morning); (3) gestating females had significantly higher fGCM concentrations than lactating females. Thus, we analytically validated the commercial EIA and, despite between-assay variation, we were able to find three biologically relevant GC signals in captive and wild settings, and in males and females. We are therefore confident that the method can be used to noninvasively address behavioral endocrinology questions in Geoffroy's spider monkeys.

KEYWORDS

capture, circadian rhythm, cortisol, enzyme immunoassay, platyrrhines, reproductive state

Abbreviations: ACTH, adrenocorticotropic hormone; EIA, enzyme immunoassay; fGCM, fecal glucocorticoid metabolites; GC, glucocorticoids; HPA, hypothalamic-pituitary-adrenal; OD, optical density.

1 | INTRODUCTION

Glucocorticoids (GCs) are important metabolic hormones in vertebrates, with corticosterone being the predominant GC in amphibians, reptiles, birds, and rodents, and cortisol in most other mammals including primates (Touma & Palme, 2005). They are crucial for the availability of glucose in the bloodstream which is achieved by decreasing glucose intake in some tissues (MacDougall-Shackleton et al., 2019). Increase in GC blood concentrations is orchestrated by the hypothalamic-pituitary-adrenal axis (HPA axis; Boonstra, 2013; Touma & Palme, 2005). The hypothalamus receives signals to initiate the cascade either from the central nervous system when fast signaling is required (e.g., a threat perceived by the senses) or by hormonal integration when reacting to slower changes in energetic demands within the body (Boonstra, 2013; Stanley et al., 2019). Hence, GCs allow individuals to cope with a wide variety of predictable (e.g., social interaction: Kaisin et al., 2023; seasonal food/water scarcity: Carnegie et al., 2011; Rimbach et al., 2014; seasonal changes in temperature: Beehner & McCann, 2008; reproduction: Strier et al., 2003) and unpredictable challenges (e.g., predation: Wasserman et al., 2013; dominance instability: Carnegie et al., 2011; logging/hunting/fragmentation: Rimbach et al., 2013).

Steroid hormones, like GCs and their metabolites, are relatively stable and can be quantified from a wide range of sample matrices, such as blood, saliva, urine, feces, hair, and feathers. Any sampling that requires capture and/or anesthesia of individuals (usually for blood and saliva) is considered invasive and carries a small but considerable risk of injury and lethality (Caulkett & Arnemo, 2017). These procedures also interfere with the animal's natural behavior and induce HPA activation (Suleman et al., 2004; Wasserman et al., 2013). To investigate the effects of naturally displayed behavior on GC concentrations in highly arboreal species, noninvasive collection of feces has proven to be effective, as feces usually reach the ground where they can be collected (Hodges & Heistermann, 2011; e.g., Carnegie et al., 2011; Kaisin et al., 2023; Rimbach et al., 2013). Fecal samples provide integrative information on HPA activity, as they reflect the GC concentrations of several hours up to several days, depending on the gut-transit time of the study species (Hodges & Heistermann, 2011; Touma & Palme, 2005).

GCs can be analyzed at relatively low cost using enzyme immune assays (EIAs); however, fecal samples pose a unique set of challenges for immunological analysis. One of these challenges is the large variety of substances they contain depending on study species, diet, physiology, gut flora, and health making them a complex sample matrix. These substances can potentially interfere with enzymatic reactions causing a matrix effect (Selby, 1999). The matrix effect is mitigated by subjecting fecal samples to a simple but robust extraction with 60%–100% ethanol or methanol, although it cannot be completely excluded (Touma & Palme, 2005). Most commercial kits are designed for quantification of GC in human blood or saliva; however, some of them have been shown to be suitable for samples of other species and matrices (e.g., Ordóñez-Gómez et al., 2016; Rangel-Negrín et al., 2014; Weingrill et al., 2004). When using commercial kits it is paramount to subject them to analytical validation for the study species and sample matrix including: (1) parallelism between a standard calibration curve and the sample extracts to investigate the dynamics of antibody binding and exclude the presence of a systematic inference by the extract matrix (Selby, 1999): (2) accuracy of analysis (i.e., quantification of a known standard in the presence of sample matrix); and (3) precision of analysis (i.e., within- and between-assay controls; Beehner et al., 2022; Buchanan & Goldsmith, 2004). Another challenge for immunological analysis is the presence of a diverse bacterial community in fecal samples that causes degradation of GC molecules, leading to unknown types and amounts of fecal GC metabolites (fGCM) and low amounts of the original target molecule (Goymann, 2012). If the antibody is highly specific to the original GC molecule it is possible that no biologically relevant signal can be detected by the EIA. The same can occur if the EIA cross-reacts with a large quantity of metabolites derived from structurally similar but functionally different hormones (e.g., testosterone). Both issues can be addressed with a successful physiological or biological validation testing whether expected changes in GC signal specifically related to the HPA physiology can be traced with the employed assay. During physiological validation the secretion of cortisol is either artificially stimulated by injecting synthetic adrenocorticotropic hormone (ACTH) or inhibited by injecting dexamethasone (Touma & Palme, 2005). Alternatively, biological validation takes advantage of external or internal stimuli that are expected to naturally cause an activation of the HPA.

One of the most well-described and reliable natural stimuli to elicit an HPA response is a predation attempt, or "human intervention" (as an umbrella term for capture, medical intervention, anesthesia, translocation, or a combination of those) as its surrogate (e.g., Suleman et al., 2004; Wasserman et al., 2013). These situations are (or are perceived as in case of human intervention) life threatening and require the immediate access to all energy resources, which is reflected in a sharp increase of GC concentrations in the blood and can reliably be traced in fGCM concentrations despite their integrative nature (e.g., Rimbach et al., 2013; Shutt et al., 2012; Wasserman et al., 2013). For that reason, many researchers have used the opportunity of necessary, scheduled "human interventions" to biologically validate protocols for noninvasive measurement of fGCM in multiple mammal species (Touma & Palme, 2005).

The pulsatile cortisol secretion shows a circadian rhythm with highest frequency of GC secretion pulses in the early morning hours (for diurnal animals) and lower pulse frequencies in the afternoon or evening leading to higher GC concentrations in the morning and lower GC concentrations in the afternoon (Tsigos & Chrousos, 2002). The phenomenon is a physiological feature of cortisol secretion that can be found in many vertebrates (mammals: Bohák et al., 2013; Sauropsida: Lance & Lauren, 1984; Teleostei: López-Olmeda et al., 2013). Such circadian rhythm is usually described for serum or salivary GC concentrations, but it might not be readily found in fGCM due to the integrative nature of fecal samples (Heistermann, 2010). Hence, significant effects of time of fecal sample collection have

been found in some primate species (e.g., *Ateles hybridus*: Rimbach et al., 2013; *Leontopithecus chrysopygus*: Kaisin et al., 2023; *Pan troglodytes*: Murray et al., 2013) but not in others (e.g., *Ateles geoffroyi*: Ordóñez-Gómez et al., 2016; Rodrigues et al., 2015; *Procolobus gordonorum*: Barelli et al., 2015; *Gorilla gorilla*: Shutt et al., 2012).

GC modulation is also linked to female reproductive physiology. The primate placenta produces corticotropin releasing hormone during late gestation to regulate fetus maturation and initiate parturition (McLean et al., 1995). An increase of GC concentrations during gestation or late gestation has been shown in many primate species (e.g., Beehner et al., 2006; Carnegie et al., 2011; Crockford et al., 2008; McLean et al., 1995; Rimbach et al., 2013; but see Rodrigues, 2017; Sousa et al., 2005).

The aim of the present study was to validate a protocol to measure GCs in fecal sample extracts (i.e., fGCM) from Geoffroy's spider monkeys (*Ateles geoffroyi*), a species found throughout Central America and currently listed as Endangered mainly due to habitat loss and fragmentation (Cortés-Ortíz et al., 2021). The species shows a high degree of fission and fusion dynamics which allows for temporal adjustments in subgroup size and composition according to environmental and social challenges making it an interesting study species to investigate variation in GC concentrations as proxy of energetic load and behavioral coping strategies (Asensio et al., 2009; Busia et al., 2016; Chapman et al., 1995). However, little is currently known about GC dynamics in wild Geoffroy's spider monkeys (Ordóñez-Gómez et al., 2016; Rangel-Negrín et al., 2009; Rodrigues, 2017). We first validated the analytical performance of a commercial EIA kit for its use in fecal sample extracts from Geoffroy's spider monkeys by assessing parallelism, accuracy, and precision. We then conducted a biological validation to establish whether we were able to trace biologically relevant and well-documented changes in GC concentrations in fecal samples of Geoffroy's spider monkey. We predicted higher fGCM concentrations after capture and anesthesia for veterinary intervention than before the event (Prediction 1), in the early morning hours than in the rest of the day (Prediction 2), and during gestation than during other female reproductive states (Prediction 3).

2 | METHODS

2.1 | Ethics Statement

The study on captive monkeys was approved by the ethics committee of the zoo "Parque Zoológico del Centenario," Mérida (Yucatan, Mexico). To mitigate the effects of our study on the monkeys, we took advantage of a scheduled routine veterinary examination. The sample and data collection in the wild study group complied with the animal protection and wildlife research laws of Mexico (NOM-126-SEMARNAT-2000, NOM-059-SEMARNAT-2010) and the necessary research permit was granted from the Secretaría de Medio Ambiente y Recursos Naturales (SGPA/DGVS/03005/19). The study followed the Code of Best Practices for Field Primatology provided by the International Primatological Society and the American Society of Primatologists (ASP). Both studies in captivity and in the wild adhered to the ASP Principles for the Ethical Treatment of Non-Human Primates.

2.2 | Study subjects and sites

2.2.1 | Captive study

To address Prediction 1, we carried out the sample collection at the "Parque Zoológico del Centenario," Mérida (Yucatan, Mexico) from October 17, 2020 to October 21, 2020. The study subjects were two female Geoffroy's spider monkeys: Coco and Emilia. Coco was an approximately 4-year-old subadult who lived as a pet before arriving at the zoo in 2018. Emilia was an adult of at least 14 years of age who arrived at the zoo in 2011, probably as a former pet. They lived in a multimale, multifemale group (six females, two males) that had access to a 221 m^2 outdoor enclosure with numerous enrichment elements. The monkeys usually had access to two adjacent indoor enclosures of 4 m^2 each, to shelter from bad weather and public view. These indoor enclosures were used for the purpose of veterinary intervention and the sample collection for biological validation.

2.2.2 | Study in the wild

To assess Predictions 2 and 3 of the biological validation, we collected samples from a study group of wild spider monkeys living at the Otoch Ma'ax yetel Kooh reserve near Punta Laguna (20°38′ N, 87°38′ W) in the center of the Yucatan peninsula, Mexico, during a period of 21 months between April 2019 and December 2020. The study group consisted of 12 sexually mature males (5 subadults, 7 adults), 18 sexually mature females (3 subadults, 15 adults), and 23 infants and juveniles (as of December 2020).

2.3 | Fecal sample collection and storage

We only collected samples from positively identified individuals directly after defecation and samples that had not been contaminated with urine or water. We recorded the identity of the individual who deposited the sample as well as date and time of sample collection. We collected the entire fecal bolus from the ground or leaves into a clean plastic bag and maintained it in a thermos on frozen ice-packs until the end of the collection day (zoo: 7:00–18:00, wild: 5:00–14:00). At that point, we stored the samples in a freezer at approximately −18°C. Every 2–5 months we transported samples on dry ice from the field station to the Instituto de Neuroetología,

Universidad Veracruzana in Xalapa (Veracruz, Mexico), where we stored them at −18°C until further processing.

2.4 | Sample extraction and quantification

We carried out drying and extraction of fecal samples at the Instituto de Neuroetología. To minimize variation in fGCM concentrations between samples caused by differences in water content, we dried the samples for 4.5 h at 70 ± 10°C using a scientific oven. We homogenized the samples with a mortar and pestle after freeing them from any seeds, plant fiber, and debris. We stored the oven-dried and homogenized samples at −18°C until further use.

For extraction, we added 2 mL of pure methanol to 0.2 g of each oven-dried sample. We agitated each sample individually for 30 s using a table-top vortex before agitating the samples an additional 30 min using a multishaker. We then centrifuged samples for 30 min at 15°C and 3000 rpm, and transferred the entire supernatant methanol extract into a labeled glass tube. Because we found in preliminary trials that a substantial amount of fGCM remained in the sediment after the first methanol extraction (Supporting Information: Figure S1), we repeated the extraction using the remaining sample sediment and added the resulting methanol extract in its entirety into the same labeled glass tube containing the methanol extract of the first extraction cycle of the same sample. We transferred 2 mL of the final methanol extract to a glass tube and evaporated it to complete dryness on a water bath at 60°C. We reconstituted the dried methanol extract in 1 mL of albumin buffer (2 g albumin to 1000 mL distilled water) and homogenized it using a table-top vortex and, if necessary, an ultrasound bath. We stored the albumin buffer extracts at −18°C until quantification.

We quantified the sample extracts at the Instituto de Investigaciones Cerebrales, Universidad Veracruzana using a commercial anti cortisol ALPCO® (ALPCO, 2021,11-CRLHU-E01; table of cross-reactivities; Supporting Information: Table S1) enzyme immunoassay (EIA). To run the assay, we prepared a mixture of 20 µL of calibrator, controls, or albumin extracts of sample and 150 µL of cortisol conjugated with horse radish peroxidase within individual microtubes. Then, we transferred the 170 µL from each microtube to the designated positions within the anti-cortisol antibody coated 96-well plate and incubated the assay for 45 min. We washed the plate three times using 340 µL of washing buffer per well in each washing step. Finally, we added 150 µL of tetramethylbenzidine to each well and incubated the plate for 15 min. The colorimetric reaction was terminated using 50 µL of the stop solution provided by the manufacturer. We used a colorimetric plate reader to quantify the optical density (OD) of each well of the 96-well plate at a wavelength of 450 nm and employed a five-parameter curve to fit the calibration points. All equipment items we used are listed in Supporting Information: Table S2 indicating their brand and model.

2.5 | Analytical validation

2.5.1 | Parallelism

To investigate parallelism, we diluted two sample pools in the same five dilution steps (1:1, 1:4, 1:10, 1:40, 1:100, and 0, i.e., reaction buffer only with no cortisol added for the standard and albumin buffer for the sample pools) as the calibration standards provided by the manufacturer.

2.5.2 | Accuracy

To estimate accuracy of the commercial kit in detecting exogenous cortisol in the presence of sample matrix, we spiked a diluted sample pool of known concentrations with four different cortisol standards of known concentrations. We calculated the expected concentration as the sum of the pooled sample and the cortisol standard. We divided the measured concentration by the expected concentration and multiplied the result by 100 as described by Beehner et al. (2022).

2.5.3 | Precision

For each assay plate ($n = 14$) we carried out quality controls to determine the precision of the measurements. We created two sample pools which we used as within- and between-assay controls in three different positions (right after calibration curve, middle, and end) on each of the fourteen 96-well assay plates used for the study. We further added two cortisol standards (low and high concentration) provided by the manufacturer at the beginning and end of each 96-well plate as additional within- and between-assay controls. We ran calibration curves, controls, parallelism, and accuracy experiment in duplicates to continuously assess pipetting accuracy. We calculated the coefficient of variation (CV) for within- and between-assay controls and duplicates.

2.6 | Biological validation

2.6.1 | Data collection for capture and anesthesia at the zoo

On October 17, 2020, the two females were separated from the rest of the group each into one of the two adjacent indoor enclosures where they remained to facilitate the preparation of and the recovery from the veterinary intervention and the fecal sample collection for the present study. The females had the possibility to see and interact with group members through a wire fence, mitigating distress potentially elicited by separation from the group. On the day before the veterinary intervention (October 18th) the two females were fasted in preparation for anesthesia. On the day of the veterinary

TABLE 1 Fecal sample collection for Prediction 1.

	Day 2 10 Oct 2020 Separation (14:00)	Day 1 18 Oct 2020 Fasting	Day 0 19 Oct 2020 Intervention (7:22-9:15)	Day 1 20 Oct 2020	Day 2 21 Oct 2020
Coco	15:23	07:48	18:20	07:23	07:40
		11:15		12:53	12:46
		12:28			
Emilia	15:28	07:34	7:22	06:50	07:27
			10:03		13:03

Note: Displayed are all samples collected from the two Geoffroy's spider monkey females, Coco and Emilia, with the corresponding collection times. Samples were collected at the "Parque Zoologico del Centenario," Merida, Mexico.

intervention (October 19th) they were captured at 7:22 and anesthetized with a ketamine injection, and anesthesia was maintained for the duration of the medical examination until 9:15 using three additional injections of midazolam. The females remained in the indoor enclosure for 2 more days to monitor their recovery from anesthesia and conclude fecal sample collection (October 20 and 21, 2020; Table 1). We expected this timeframe to be sufficient to document a substantial fGCM increase and likely also a decrease of GC concentrations based on previous work in this species (Rangel-Negrín et al., 2009, 2015; Rodrigues et al., 2015).

2.6.2 | Data collection for circadian rhythm and reproductive state in the wild

We used the recorded time of sample collection for each sample as a proxy of circadian patterns of GC secretion. To use sample collection time as a continuous variable, we expressed it in hours with minutes as decimals (i.e., smaller values in early morning).

Reproductive states were assigned retrospectively from birth using a gestation time of 7.5 months (228 days; Eisenberg, 1973). For the 3 months before the estimated conception females were considered to be "likely cycling" (hereafter "cycling"; Campbell et al., 2001; Slater et al., 2008). Females with infants less than 24 months of age were considered "lactating" (Vick, 2008). The lactation period was shorter for four of the eight females as they were estimated to be cycling (according to the above-mentioned criteria) before their infants were 24 months old.

We included control variables in the analyses as GC concentrations can be influenced by a wide variety of environmental, physiological, and methodological factors (see Section 1). Aging has been related to physiological changes in the HPA axis leading to a decreased inhibitory feedback loop and thus prolonged elevations of GC concentrations (Veldhuis, et al., 2013). Evidence from wild primates seem to support a positive relation between GC concentrations and age (e.g., Hämäläinen et al., 2015; Rimbach et al., 2014; Seraphin et al., 2008). Age of all male individuals as well as one adult female that was born in and never left the study group were known. The age of females that immigrated into the group was estimated from the date they were first seen by adding 6 years, which is the approximate age females immigrate into a new group (Shimooka et al., 2008). Three females that were adults with infants at the start of the long-term project in 1997 (Ramos-Fernandez et al., 2018), were considered to be at least 33 years of age at the end of the study in 2020 as they were estimated to be at least 10 years old in 1997. Age of both males and females was represented in years and assigned to the fecal sample according to its collection date.

Climatic seasonality is often correlated with variation in GC concentrations (e.g., Beehner et al., 2022; Carnegie et al., 2011). We collected rain data at the field site using a commercial weather station which was positioned approximately 2.5 m above the ground on a plane surface with no canopy coverage. We categorized the dry season as the period during which the monthly total amount of rain was below 60 mm (Kottek et al., 2006). Using this criterion, we estimated the dry season to last from June to November in 2019 and from May to December in 2020 (Supporting Information: Figure S2). We accordingly assigned fecal samples to the rainy or dry season depending on their collection date.

Fecal GC concentrations can also be influenced by methodological issues, such as the storage time between collection and extraction of the fecal sample. Although freezing fecal samples has been reported to be the most reliable storage method, some studies document an influence of storage time on fGCM concentrations (e.g., Khan et al., 2002), whereas others do not (e.g., Beehner & Whitten, 2004; Kalbitzer & Heistermann, 2013). To account for a possible effect of storage time on fGCM concentrations, we calculated storage time as the number of days between sample collection and sample extraction. The storage time of samples ranged from 1.9 to 3.7 years (Supporting Information: Figure S3C,F).

2.7 | Data analysis

To assess parallelism between a standard dilution curve and the dilution curve of two sample pools, we first calculated the percentage binding as the ratio between the OD measured at a given dilution step and the OD at no cortisol added multiplied by 100. We then plotted the percentage binding of the standard and the two sample pools as well as the mean of the two sample pools as a function of the dilution factor. We transformed the dilution factor using the natural logarithm to obtain linear relation and, then, compared the slope of the standard dilution curve with the slopes of the dilution curve of the sample pools with an ANCOVA (Beehner & McCann, 2008). To do so, we entered percentage binding and dilution factor as variables and sample type as covariate.

To assess the effect of time of sample collection and reproductive state on fGCM concentrations, we implemented two linear mixed models (LMMs) using the lme4 package in R (version 4.3.2, 2023-10-31 ucrt). LMMs allow to account for nonindependence of data, such as the repeated measure of the same individual or group, and are

tolerant of unbalanced samples, a problem frequently encountered in studies in the wild. For both models, fecal GC concentrations were normalized using logarithmic transformation (natural logarithm) and entered the model as the dependent variable.

In the first model, we tested Prediction 2 related to the circadian rhythm of GC secretion in males since the other aspects of the biological validation (capture at the zoo and reproductive state) included only females. We did so to provide evidence that the assay protocol was able to trace a biologically relevant aspects of GC physiology not only in females but also in males. It is crucial for validation to show that the endocrinological method works in both sexes because secretion pathways and metabolism of steroids can vary significantly between females and males (Goymann, 2012; Touma & Palme, 2005). We used the fGCM concentrations of 284 fecal samples collected from 12 male spider monkeys (mean ± SD: 23.7 ± 6.0 samples per male) to fit the LMM. We used time of sample collection as the fixed predictor factor. We further added male age, season, time of sample storage, and plate identity (to control for possible between-assay variation) as fixed control factors (see Supporting Information: Table S3 and Figure S3A–C for sample distribution of each variable). Time of sample collection, age, and storage time were z transformed to avoid issues of scaling within the model. Monkey identity was entered as a random factor to account for resampling of the same individual (see Supporting Information: Table S3 for sample distribution of each individual). We computed generalized variance inflation factors (GVIF) for all fixed factors using the "vif" function of the "car" package in R to evaluate collinearity among them and we checked the assumption normal distribution and homogeneity of variance of the residuals using the "simulateResiduals" function of the "DHARMa" package. We compared the model with the corresponding null model containing only the control fixed factors and random factor via a likelihood ratio test (Dobson & Barnett, 2018) using the "anova" function. Only upon a significant difference between the two models we presented the model results. We finally computed the conditional and marginal effect size of the model using the "r.squaredGLMM" function of the MuMIn package in R.

In the second model, we tested Prediction 3. The model included the fGCM concentrations of 269 samples from eight females that gave birth during the study period (33.6 ± 14.4 samples per female). The fixed predictor factor was reproductive state (cycling, gestating, lactating). Differences in the duration of each reproductive state naturally led to differences in sample size across the three reproductive states (Supporting Information: Table S4). Control variables were time of sample collection, female age, storage time, and plate identity, with monkey identity entered as a random factor (see Supporting Information: Table S4 and Figure S4D–F for sample distribution of each variable). We followed the same procedure as described for the first model.

To further investigate how between-assay variation affected LMM fitting, we ran the same two LMMs excluding all samples that were quantified on one of the four assay plates that increased between-assay %CV above the generally accepted 15%. This exclusion resulted in a total of 196 samples for LMM testing Prediction 2 related to the circadian rhythm and 219 samples for the LMM testing Prediction 3 related to female reproductive state. We then carried out a likelihood ratio test comparing each model to a model that included all fixed and random effects but Plate ID. By doing so we assessed whether Plate ID had a significant effect on the model fitting despite variation being below the generally accepted 15% CV.

3 | RESULTS

3.1 | Analytical validation

3.1.1 | Parallelism

The slopes of the standard dilution curve and of the curves of the two sample pool dilution curves showed no significant difference (ANCOVA, Sum Squares = 148.0, $df = 1$, $F = 2.93$, $p = 0.11$; Figure 1).

3.1.2 | Accuracy

A sample pool with a concentration of 1.3 µL/dL that was spiked with 3.3 µL/dL cortisol standard resulted in a measure of 4.5 µL/dL. The same pool spiked with 6.2, 8.3, and 22.4 µL/dL standard resulted in 7.1, 9.7, and 23.7 µL/dL respectively. Based on these results, the accuracy ranged from 94% to 112.7%, with a mean accuracy of 103%.

3.1.3 | Precision

The CVs (expressed as a percentage) of the between-assay control were 21.4% and 15.8% for the two sample pools, whereas CVs of the high and low cortisol standards provided by the manufacturer were 7.1% and 12.7%, respectively ($n = 14$ assay plates). The CVs of within-assay controls were 9.0% and 8.1% for the two sample pools, and 8.3% and 9.7% for the high and low standards. Duplicates had a mean CV of 3.1% ($n = 265$ duplicate pairs).

3.2 | Biological validation

3.2.1 | Capture and anesthesia in the zoo

Subject Coco's preintervention fGCM concentrations ranged from 1747 to 3546 ng/g. Eleven hours after the onset of the intervention (i.e., the capture) fGCM concentration was 8008.8 ng/g. (Figure 2). In the 2 days after the intervention, fGCM concentrations ranged between 2272 and 3511 ng/g comparable with preintervention levels.

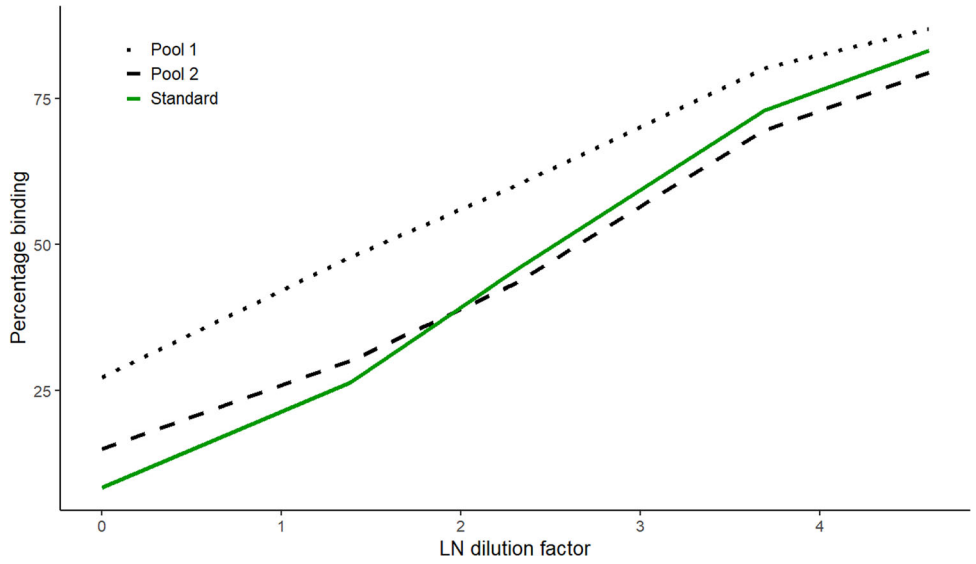

FIGURE 1 Parallelism between standard dilution curve and curves of sample pool dilutions. Curves represent the percentage binding as a function of the dilution factors (LN transformed).

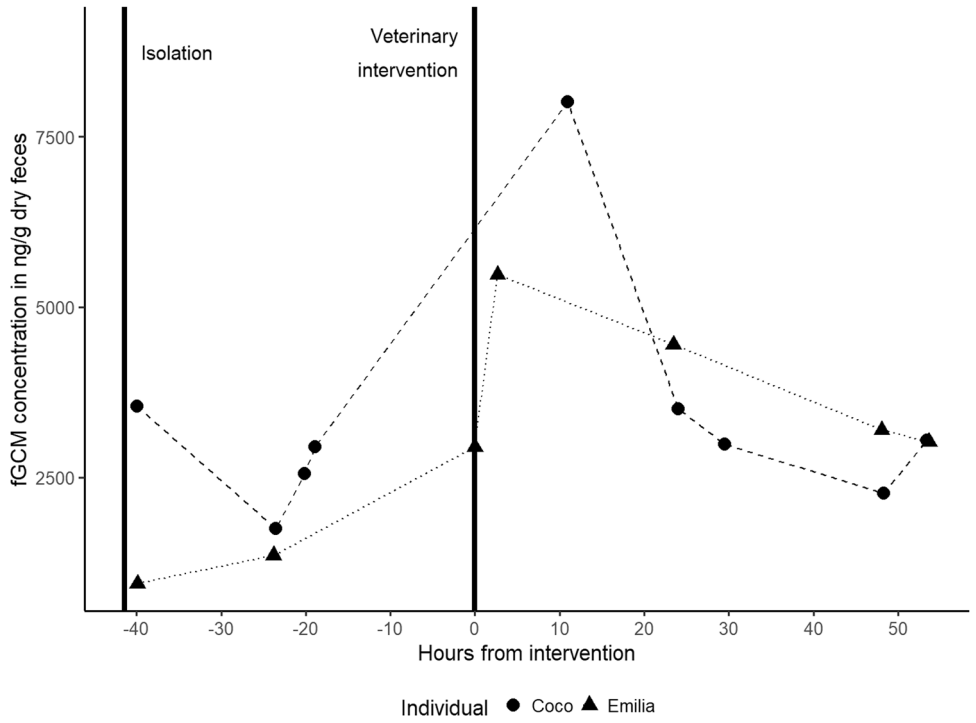

FIGURE 2 Fecal glucocorticoid profiles of the two Geoffroy's spider monkey females, Coco (circles) and Emilia (triangles), in the hours before and after scheduled veterinary intervention. Samples were collected at the Zoo "Parque Zoológico del Centenario," Merida, Mexico.

Subject Emilia's mean pre-intervention fGCM concentrations ranged from 942 to 2950 ng/g. Fecal GCM concentration was 5472.8 ng/g 2.5 h after the onset of the intervention while the individual was still recovering from the anesthesia; this was the only postintervention sample available for the individual on that day (Figure 2). In the 2 days after the intervention, fGCM concentrations ranged from 3020 to 4447 ng/g.

3.2.2 | Circadian rhythm in the wild

The LMM testing the effects of circadian rhythm on fGCM concentrations of males was different from the null model (likelihood ratio test: $\chi^2 = 6.91$, $df = 1$, $p = 0.009$). We found a significant effect of sample collection time on fGCM concentrations (Table 2), indicating higher fGCM concentrations early in the day (Figure 3).

TABLE 2 Results of LMM assessing the effect of time of sample collection on fGCM concentrations in 12 wild Geoffroy's spider monkey males.

	Estimate	SE	5% CI	95% CI	*df*	*t* Value	*p* Value
(Intercept)	6.496	0.122	6.304	6.691	245.456	53.368	<0.001
Collection time	−0.071	0.028	−0.118	−0.027	265.904	−2.524	0.012
Age	0.065	0.033	0.015	0.114	9.754	1.977	0.077
Season	−0.130	0.062	−0.228	−0.029	265.967	−2.086	0.038
Storage time	0.046	0.030	−0.001	0.094	247.699	1.526	0.128
Plate ID[a]							

Note: Samples were collected at the "Otoch Ma'ax yetel Kooh" reserve, Yucatan, Mexico. Effect size of the model: *R2* (conditional) = 0.25, *R2* (marginal) = 0.24.

[a]Too many comparisons to be listed here (see Supporting Information: Table S5 for details). The overall effect of plate ID is: $F_{13,\ 260.9} = 4.49$, $p < 0.001$.

Abbreviations: CI, confidence interval; df, degrees of freedom; SE, standard error.

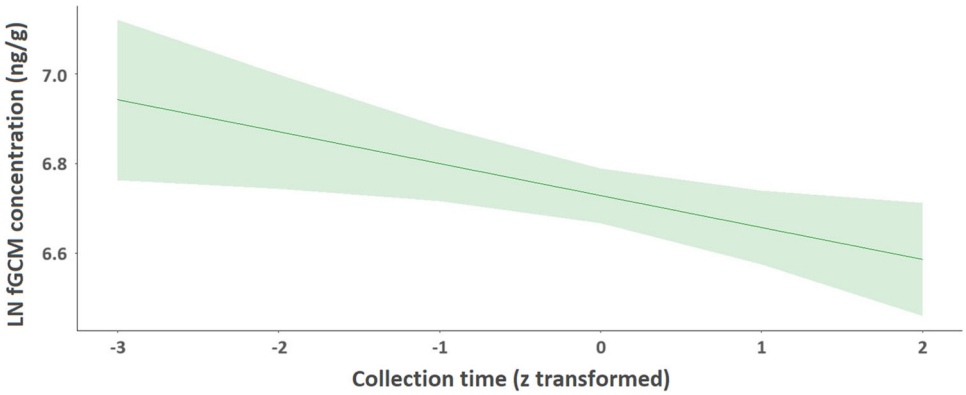

FIGURE 3 Illustration of the association between sample collection time (z transformed) and fGCM concentration (Ln transformed) in 12 wild Geoffroy's spider monkey males, showing the LMM prediction (dark green line) with 95% confidence intervals (in light green). Samples were collected at the "Otoch Ma'ax yetel Kooh" reserve, Yucatan, Mexico.

3.2.3 | Reproductive state in the wild

The LMM testing the effect of female reproductive state on fGCM concentrations (Figure 4) was significantly different from the null model (likelihood ratio test: $\chi^2 = 49.696$, $df = 3$, $p < 0.001$). Gestating females had significantly higher fGCM concentrations than lactating and cycling females (Table 3). In line with the findings from the first LMM, we also found a significant effect of sample collection time (Table 3).

3.2.4 | Between-assay variation

After excluding all samples that were quantified on the four assay plates that increased between-assay %CV above 15%, the between-assay %CV for the reduced data sets ranged from 6.5% to 12.6%. The likelihood ratio test indicated that plate ID still had a statistically significant effect in the LMM testing Prediction 2 related to the circadian rhythm ($\chi^2 = 39.2$, $df = 9$, $p < 0.001$) as well as in the LMM testing Prediction 3 related to female reproductive state ($\chi^2 = 29.3$, $df = 10$, $p = 0.001$).

4 | DISCUSSION

The aim of the present study was to provide an analytical and biological validation for the noninvasive quantification of GCMs in fecal sample extracts of Geoffroy's spider monkeys using a commercial EIA kit. The analytical validation confirmed parallelism between a standard dilution curve and a dilution curve of pooled samples, accuracy of the measurement, within-assay precision as well as between-assay precision for 2 out of 4 controls. During biological validation, we were able to show a clear increase of fGCM concentrations in two zoo Geoffroy's spider monkey females 2.5 and 10 h after veterinary intervention. There was also a significant effect of sample collection time on fGCM concentrations of 12 wild Geoffroy's spider monkey males, with highest concentrations in early morning. Furthermore, we found a significant effect

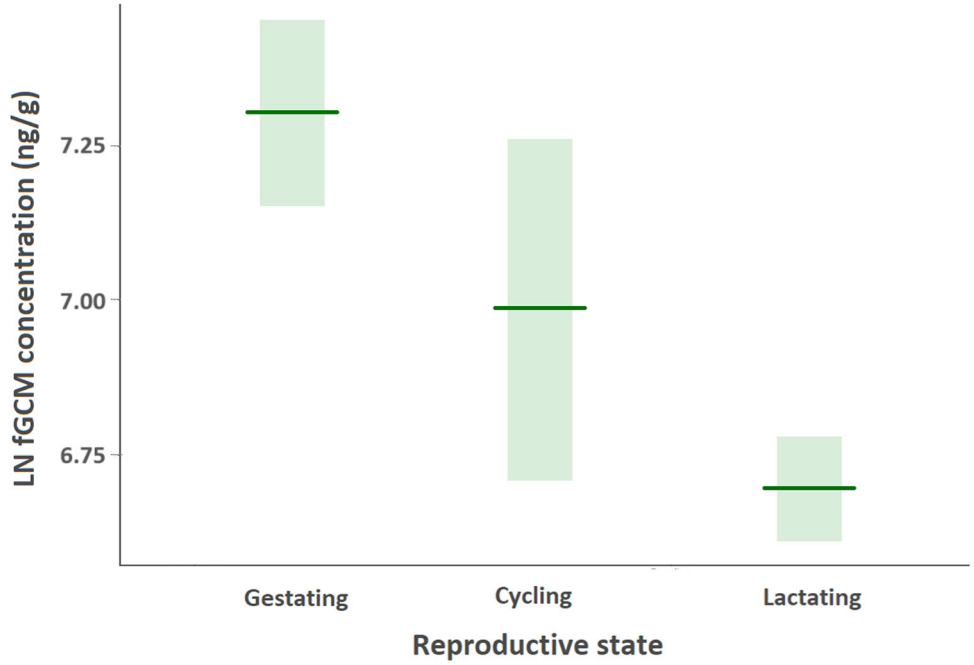

FIGURE 4 LMM estimates of ln transformed fGCM concentrations (dark green line) with 95% confidence intervals (light green bar) in three female reproductive states for eight wild Geoffroy's spider monkey females. Samples were collected at the "Otoch Ma'ax yetel Kooh" reserve, Yucatan, Mexico.

TABLE 3 Results of LMM assessing the effect of female reproductive state on fGCM concentrations in eight wild Geoffroy's spider monkey females.

	Estimate	SE	5% CI	95% CI	*df*	*t* Value	*p* Value
(Intercept)	7.367	0.124	7.161	7.575	197.605	59.386	<0.001
Gestating versus cycling	−0.314	0.142	−0.550	−0.077	264.634	−2.211	0.028
Gestating versus lactating	−0.601	0.083	−0.744	−0.460	233.707	−7.217	<0.001
Age	0.049	0.038	−0.018	0.119	9.055	1.281	0.232
Collection time	−0.082	0.029	−0.130	−0.035	266.353	−2.877	0.004
Season	−0.243	0.062	−0.346	−0.140	268.955	−3.928	<0.001
Storage time	0.044	0.034	−0.014	0.103	230.791	1.301	0.194
Plate ID[a]							

Note: Samples were collected at the "Otoch Ma'ax yetel Kooh" reserve, Yucatan, Mexico. Effect size of the model: *R2* (conditional) = 0.44, *R2* (marginal) = 0.43.

Abbreviations: CI, confidence interval; df, degrees of freedom; SE, standard error.

[a]Too many comparisons to be listed here (see Supporting Information: Table S6 for details). The overall effect of plate ID is: $F_{13,253.2} = 3.16$, $p < 0.001$.

of reproductive state on fGCM concentrations of eight wild Geoffroy's spider monkey females, with highest concentrations during gestation. With the biological validation, we provided evidence that hormonal signals traced in Geoffroy's spider monkey feces using the reported method are sensibly associated with GC physiology. The metabolites detected with the reported method, thus, likely derive from GC and not from other structurally similar but functionally different steroid hormones (Goymann, 2012; Möstl & Palme, 2002).

Our results on parallelism and accuracy indicate that the extraction procedure reduced the amount and variability of molecules other than the target molecules to a point where they did not significantly interfere with the reaction dynamics of the EIA system (Buchanan & Goldsmith, 2004; Selby, 1999). Running within- and between-assay controls we found that the EIA was sufficiently precise within the same assay plates; however, there was elevated variation for the between-assay controls that consisted of sample pools but not in the standard controls provided by the manufacturer.

This pattern of results highlights the importance to include sample pool controls rather than exclusively relying on standard controls as those could potentially underestimate within- and between-assay variation (Beehner et al., 2022). Variation between EIA plates can be influenced by multiple factors such as changes in temperature, variation in performance while using the plate, and plate batch. These factors were maintained as stable as possible in the study so that we cannot pinpoint an obvious source of variation. The usual approach for addressing high variation between assay plates is to rerun plates that have the most impact on between-assay CV until the generally accepted threshold of <15% CV is reached. However, we showed that between-assay variation even lower than 15% CV still has significant effects in LMMs. We thus strongly encourage to statistically control for between-assay variation. Intensity normalization (randomized studies) or reference sample normalization (non-randomized studies; Olink, 2021) could also effectively address between-assay variation. Comparing applicability and strength of different methods to tackle between-assay variation could be an endeavor for future research as projects working with limited resources would greatly benefit from the knowledge obtained from such a comparison.

As expected, we found a substantial increase in fGCM concentration following veterinary intervention. Increases in fGCM concentration occurred as early as 2.5 and 11 h after the event. In previous studies in *Ateles* spp. with similar validation protocols, fGCM peaks were reported earliest at 9 h up to 1 day after capture (*A. geoffroyi*: Rangel-Negrín et al., 2009; Rodrigues et al., 2015; *A. hybridus*: Rimbach et al., 2013). It could be argued that the detected earlier increase was due to the separation of the monkeys approximately 1 day and 16 h before the veterinary intervention. However, that would mean that the individuals showed lag time comparable to or exceeding the ones found in animals with substantially longer gut transit times (Palme et al., 2000; Murray et al., 2013; Rangel-Negrín et al., 2014; Shutt et al., 2012). Even if absolute fGCM peaks were missed because we could not collect samples during closing hours of the zoo, the detected increases are likely associated to capture and anesthesia for two reasons. First, as ketamine injections are known to elicit GC increase (Khalili-Mahani et al., 2015), it is likely that the highest measured fGCM concentrations were associated with the injection. Second, the females were trained to be separated into the adjacent indoor enclosure and showed no behavioral signs of distress during separation, whereas they showed such signs (e.g., trying to flee, scream) during capture. Our findings also align well with reports of relatively short gut-transit times of 4.4 ± 1.5 h in Geoffroy's spider monkeys (Milton, 1981). It is important to stress that the primary aim of a biological validation protocol like ours is not to detect the time lag between a challenging event and the associated increase in fGCM concentrations but rather to examine whether a substantial and biologically relevant fGCM increase can be detected. Hence, regardless of whether peaks were associated with the separation event or the capture event, the validation of the method was successful. To establish a time lag of GC increase, other types of protocols are needed such as the ACTH challenge where synthetic ACTH is injected causing severe adrenal activation (Möstl & Palme, 2002; Touma & Palme, 2005). This allows to estimate how long it takes an individual to metabolize GC in the liver, and excrete GCM via feces without the need to fast the animal, which in itself can potentially influence gut transit time.

As predicted, we were able to detect higher fGCM conentrations in early morning than in late morning and early afternoon. A major challenge in detecting effects of circadian rhythm in fecal samples is that fGCMs are integrative measurements because the fecal bolus contains all GC that are excreted via the liver and bile (or more precisely any metabolites deriving from those GCs) into the intestines throughout the time the feces are in the digestive tract (i.e., the gut-transit time) (Goymann, 2012; Möstl & Palme, 2002). Fecal GCM concentrations therefore reflect excretion of GC throughout that time period (i.e., are integrated), resulting in a less defined temporal representation of hormone dynamics in feces than in saliva, blood, or even urine (Heistermann, 2010). The integration of fGCM concentrations is much more pronounced when the gut transit time is long than when gut transit time is short. For example, frugivore spider monkeys (*Ateles paniscus*) have a shorter gut transit time (4.4 h) than the more folivore howler monkeys (*Alouatta fusca*), which have a longer gut transit time (20.3 h), despite having approximately the same body size (Milton, 1981). In sympatric species of the same two genera, effects of sample collection time were found in *Ateles hybridus* but not in *Alouatta seniculus* despite the use of the same endocrinological methodology (Rimbach et al., 2013). We were likely able to track a significant effect of sample collection time due to relatively fast metabolism of GC and a short gut transit time in Geoffroy's spider monkeys.

Evidence for the circadian rhythm in fGCM concentrations in males was confirmed in our analysis focusing on reproductive state in females. Males and females can show substantial differences in quantities of GC excreted via urine and feces (Touma & Palme, 2005). Further, steroid metabolites derived from androgens seem to be more likely to cross-react with cortisol specific antibodies due to the structural similarities of the metabolites increasing chances for overestimating fGCM in males (Touma & Palme, 2005). Showing the same characteristics of GC physiology in both males and females, is, thus, a crucial part of our biological validation.

We supported our prediction that fGCM concentrations are higher during gestation than during other reproductive states. Because of the variation of GC concentrations throughout reproductive states, many studies include reproductive state as a control variable (e.g., Carnegie et al., 2011; Rimbach et al., 2014; Rodrigues, 2017), but only few studies focused on GC concentrations during gestation. Beehner et al. (2006) found a steady increase in GC concentrations during successful gestation in wild yellow baboons (*Papio cynocephalus*) similar to the one described in humans (McLean et al., 1995). Likewise, a validation study in brown spider monkeys (*Ateles hybridus*) observed a steady increase of 11b-hydroxyetiocholanolone concentrations during gestation and, when comparing different reproductive states, gestating females had the highest fGCM concentrations

(Rimbach et al., 2013). Some studies, however, suggest that social factors can modulate or mask those general dynamics. In chimpanzees (*Pan troglodytes*), for example, the prepartum increase in GC concentrations depended on dominance rank with only lower ranking females showing differences between pre- and postpartum GC concentrations (Murray et al., 2018). Along similar lines, the highest GC concentrations were found in Geoffroy's spider monkey females when they were cycling (Rodrigues, 2017), which is the reproductive state with the highest rates of male-to-female aggression (i.e., prolonged chases), which is possibly linked to courtship display (Slater et al., 2008). Such dynamics need to be considered when interpreting results, as they do not per se contradict the prediction of elevated GC concentrations during gestation but rather indicate a necessity to control for additional variables, such as dominance rank and aggression.

In conclusion, the present study shows that a commercially available EIA designed for human serum can be used to noninvasively measure fGCM concentrations in Geoffroy's spider monkeys. Our study supports previous work demonstrating the suitability of using two other commercial EIA systems to measure GCM concentrations in howler monkeys and spider monkeys' fecal samples (Rangel-Negrín et al., 2009, 2014). Like those studies, we point out the importance of validating EIA systems that are accessible in primate-range countries. Despite a high between-assay variation, we were able to detect the expected variation in HPA activation in three biologically relevant contexts of varying severity. The method is thus likely suitable for research questions focusing on factors that influence wild Geoffroy's spider monkeys' HPA activation in a similar magnitude as the physiological phenomena demonstrated here. However, it remains to be explored whether the methodology also allows to capture more subtle or more acute changes in HPA activation in an uncontrolled wild setting, such as those linked to involvement in affiliative or aggressive interactions (e.g., intergroup aggression: Kaisin et al., 2023; Schoof & Jack, 2013, affiliative interactions: Crockford et al., 2008).

AUTHOR CONTRIBUTIONS

Juliane Damm: Conceptualization (equal); data curation (lead); formal analysis (equal); funding acquisition (supporting); investigation (lead); methodology (equal); resources (supporting); validation (equal); visualization (equal); writing—original draft (equal). **Filippo Aureli**: Conceptualization (equal); funding acquisition (equal); methodology (equal); resources (equal); writing—review and editing (equal). **Ariadna Rangel-Negrín**: Investigation (supporting); methodology (equal); resources (equal); validation (equal); writing—review and editing (equal). **Miriam Barradas-Moctezuma**: Investigation (supporting); methodology (equal); validation (equal); writing—review and editing (equal). **Pedro A. D. Dias**: Formal analysis (equal); funding acquisition (equal); resources (equal); visualization (equal); writing—review and editing (equal).

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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REFERENCES

ALPCO. (2021). 11-CRLHU-E01 instructions for use (IFU), version 8.0.

Asensio, N., Korstjens, A. H., & Aureli, F. (2009). Fissioning minimizes ranging costs in spider monkeys: A multiple-level approach. *Behavioral Ecology and Sociobiology*, *63*(5), 649–659.

Barelli, C., Rovero, F., Hodges, K., Araldi, A., & Heistermann, M. (2015). Physiological stress levels in the endemic and endangered Udzungwa red colobus vary with elevation. *African Zoology*, *50*(1), 23–30.

Beehner, J., & Whitten, P. (2004). Modifications of a field method for fecal steroid analysis in baboons. *Physiology & Behavior*, *82*(2–3), 269–277.

Beehner, J. C., Alfaro, J., Allen, C., Benítez, M. E., Bergman, T. J., Buehler, M. S., Carrera, S. C., Chester, E. M., Deschner, T., Fuentes, A., Gault, C. M., Godoy, I., Jack, K. M., Kim, J. D., Kolinski, L., Kulick, N. K., Losch, T., Ordoñez, J. C., Perry, S. E., … Wasserman, M. D. (2022). Using an on-site laboratory for fecal steroid analysis in wild white-faced capuchins. *General and Comparative Endocrinology*, *329*, 114109.

Beehner, J. C., & McCann, C. (2008). Seasonal and altitudinal effects on glucocorticoid metabolites in a wild primate (*Theropithecus gelada*). *Physiology & Behavior*, *95*(3), 508–514.

Beehner, J. C., Nguyen, N., Wango, E. O., Alberts, S. C., & Altmann, J. (2006). The endocrinology of pregnancy and fetal loss in wild baboons. *Hormones and Behavior*, *49*(5), 688–699.

Bohák, Z., Szabó, F., Beckers, J. F., Melo de Sousa, N., Kutasi, O., Nagy, K., & Szenci, O. (2013). Monitoring the circadian rhythm of serum and salivary cortisol concentrations in the horse. *Domestic Animal Endocrinology*, *45*(1), 38–42.

Boonstra, R. (2013). Reality as the leading cause of stress: Rethinking the impact of chronic stress in nature. *Functional Ecology*, *27*(1), 11–23.

Buchanan, K. L., & Goldsmith, A. R. (2004). Noninvasive endocrine data for behavioural studies: The importance of validation. *Animal Behaviour*, *67*(1), 183–185.

Busia, L., Schaffner, C. M., Rothman, J. M., & Aureli, F. (2016). Do fruit nutrients affect subgrouping patterns in wild spider monkeys (*Ateles geoffroyi*)? *International Journal of Primatology*, *37*(6), 738–751.

Campbell, C. J., Shideler, S. E., Todd, H. E., & Lasley, B. L. (2001). Fecal analysis of ovarian cycles in female black-handed spider monkeys (*Ateles geoffroyi*). *American Journal of Primatology*, *54*(2), 79–89.

Carnegie, S. D., Fedigan, L. M., & Ziegler, T. E. (2011). Social and environmental factors affecting fecal glucocorticoids in wild, female white-faced capuchins (*Cebus capucinus*). *American Journal of Primatology*, *73*(9), 861–869.

Caulkett, N. A., & Arnemo, J. M. (2017). Comparative anesthesia and analgesia of zoo animals and wildlife. In K. A. Grimm, L. A. Lamont, W. J. Tranquilli, S. A. Greene, & S. A. Robertson (Eds.), *Veterinary anesthesia and analgesia* (pp. 764–776). John Wiley & Sons, Ltd.

Chapman, C. A., Chapman, L. J., & Wrangham, R. W. (1995). Ecological constraints on group size: An analysis of spider monkey and chimpanzee subgroups. *Behavioral Ecology and Sociobiology*, *36*, 59–70.

Cortés-Ortíz, L., Solano-Rojas, D., Rosales-Meda, M., Williams-Guillén, K., Méndez-Carvajal, P. G., Marsh, L. K., Canales-Espinosa, D., & Mittermeier, R. A. (2021). Ateles geoffroyi (amended version of 2020 assessment). *The IUCN Red List of Threatened Species*.

Crockford, C., Wittig, R. M., Whitten, P. L., Seyfarth, R. M., & Cheney, D. L. (2008). Social stressors and coping mechanisms in wild female baboons (*Papio hamadryas ursinus*). *Hormones and Behavior*, *53*(1), 254–265.

Dobson, A. J., & Barnett, A. G. (2018). *An introduction to generalized linear models*. CRC Press.

Eisenberg, J. F. (1973). Reproduction in two species of spider monkeys, *Ateles fusciceps* and *Ateles geoffroyi*. *Journal of Mammalogy*, *54*(4), 955–957.

Goymann, W. (2012). On the use of non-invasive hormone research in uncontrolled, natural environments: The problem with sex, diet, metabolic rate and the individual. *Methods in Ecology and Evolution*, *3*(4), 757–765.

Hämäläinen, A., Heistermann, M., & Kraus, C. (2015). The stress of growing old: Sex-and season-specific effects of age on allostatic load in wild grey mouse lemurs. *Oecologia*, *178*, 1063–1075.

Heistermann, M. (2010). Non-invasive monitoring of endocrine status in laboratory primates: Methods, guidelines and applications. *Advances in Science and Research*, *5*(1), 1–9.

Hodges, J. K., & Heistermann, M. (2011). Field endocrinology: Monitoring hormonal changes in free–ranging primates. In J. M. Setchell, & D. J. Curtis (Eds.), *Field and Laboratory Methods in Primatology: A Practical Guide* (2nd ed., pp. 353–370). Cambridge University Press.

Kaisin, O., Bufalo, F., Amaral, R., Palme, R., Poncin, P., Brotcorne, F., & Culot, L. (2023). Linking glucocorticoid variations to monthly and daily behavior in a wild endangered neotropical primate. *American Journal of Primatology*, *85*, e23503.

Kalbitzer, U., & Heistermann, M. (2013). Long-term storage effects in steroid metabolite extracts from baboon (*Papio sp.*) faeces—A comparison of three commonly applied storage methods. *Methods in Ecology and Evolution*, *4*(5), 493–500.

Khalili-Mahani, N., Martini, C. H., Olofsen, E., Dahan, A., & Niesters, M. (2015). Effect of subanaesthetic ketamine on plasma and saliva cortisol secretion. *British Journal of Anaesthesia*, *115*(1), 68–75.

Khan, M. Z., Altmann, J., Isani, S. S., & Yu, J. (2002). A matter of time: Evaluating the storage of fecal samples for steroid analysis. *General and Comparative Endocrinology*, *128*(1), 57–64.

Kottek, M., Grieser, J., Beck, C., Rudolf, B., & Rubel, F. (2006). World Map of the Köppen-Geiger climate classification updated. *Meteorologische Zeitschrift*, *15*(3), 259–263.

Lance, V. A., & Lauren, D. (1984). Circadian variation in plasma corticosterone in the American alligator, *Alligator mississippiensis*, and the effects of ACTH injections. *General and Comparative Endocrinology*, *54*(1), 1–7.

López-Olmeda, J. F., Blanco-Vives, B., Pujante, I. M., Wunderink, Y. S., Mancera, J. M., & Sánchez-Vázquez, F. J. (2013). Daily rhythms in the hypothalamus-pituitary-interrenal axis and acute stress responses in a teleost flatfish, *Solea senegalensis*. *Chronobiology International*, *30*(4), 530–539.

MacDougall-Shackleton, S. A., Bonier, F., Romero, L. M., & Moore, I. T. (2019). Glucocorticoids and "stress" are not synonymous. *Integrative Organismal Biology*, *1*(1):obz017.

McLean, M., Bisits, A., Davies, J., Woods, R., Lowry, P., & Smith, R. (1995). A placental clock controlling the length of human pregnancy. *Nature Medicine*, *1*(5), 460–463.

Milton, K. (1981). Food choice and digestive strategies of two sympatric primate species. *The American Naturalist*, *117*(4), 496–505.

Möstl, E., & Palme, R. (2002). Hormones as indicators of stress. *Domestic Animal Endocrinology*, *23*(1–2), 67–74.

Murray, C. M., Heintz, M. R., Lonsdorf, E. V., Parr, L. A., & Santymire, R. M. (2013). Validation of a field technique and characterization of fecal glucocorticoid metabolite analysis in wild Chimpanzees (*Pan troglodytes*). *American Journal of Primatology*, *75*(1), 57–64.

Murray, C. M., Stanton, M. A., Wellens, K. R., Santymire, R. M., Heintz, M. R., & Lonsdorf, E. V. (2018). Maternal effects on offspring stress physiology in wild chimpanzees. *American Journal of Primatology*, *80*(1):e22525.

Olink. (2021). White paper – Data normalization and standardization. https://olink.com/application/data-normalization-and-standardization/

Ordóñez-Gómez, J. D., Cristóbal-Azkarate, J., Arroyo-Rodríguez, V., Santillán-Doherty, A. M., Valdez, R. A., & Romano, M. C. (2016). Proximal and distal predictors of the spider monkey's stress levels in fragmented landscapes. *PLoS ONE*, *11*(2), e0149671.

Palme, R., Robia, C., Baumgartner, W., & Möstl, E. (2000). Transport stress in cattle as reflected by an increase in faecal cortisol metabolite concentrations. *The Veterinary Record*, *146*(4), 108–109.

Ramos-Fernandez, G., Aureli, F., Schaffner, C. M., & Vick, L. G. (2018). Ecología, comportamiento y conservación de los monos araña (*Ateles geoffroyi*): 20 años de estudio en Punta Laguna, México. *La primatología en Latinoamérica*, *2*, 531–544.

Rangel-Negrín, A., Flores-Escobar, E., Chavira, R., Canales-Espinosa, D., & Dias, P. A. D. (2014). Physiological and analytical validations of fecal steroid hormone measures in black howler monkeys. *Primates*, *55*(4), 459–465.

Rangel-Negrín, A., Flores-Escobar, E., Coyohua-Fuentes, A., Chavira-Ramírez, D. R., Canales-Espinosa, D., & Dias, P. A. D. (2015). Behavioural and glucocorticoid responses of a captive group of Spider monkeys to short-term variation in food presentation. *Folia Primatologica*, *86*(5), 433–445.

Rangel-Negrín, A., Alfaro, J. L., Valdez, R. A., Romano, M. C., & Serio-Silva, J. C. (2009). Stress in Yucatan spider monkeys: Effects of environmental conditions on fecal cortisol levels in wild and captive populations. *Animal Conservation*, *12*(5), 496–502.

Rimbach, R., Heymann, E. W., Link, A., & Heistermann, M. (2013). Validation of an enzyme immunoassay for assessing adrenocortical activity and evaluation of factors that affect levels of fecal glucocorticoid metabolites in two New World primates. *General and Comparative Endocrinology*, *191*, 13–23.

Rimbach, R., Link, A., Heistermann, M., Gomez-Posada, C., Galvis, N., & Heymann, E. W. (2013). Effects of logging, hunting, and forest fragment size on physiological stress levels of two sympatric ateline primates in Colombia. *Conservation Physiology*, *1*(1), cot031.

Rimbach, R., Link, A., Montes-Rojas, A., Di Fiore, A., Heistermann, M., & Heymann, E. W. (2014). Behavioral and physiological responses to fruit availability of spider monkeys ranging in a small forest fragment. *American Journal of Primatology*, *76*(11), 1049–1061.

Rodrigues, M. A. (2017). Female spider monkeys (*Ateles geoffroyi*) cope with anthropogenic disturbance through fission–fusion dynamics. *International Journal of Primatology*, *38*(5), 838–855.

Rodrigues, M. A., Wittwer, D., & Kitchen, D. M. (2015). Measuring stress responses in female Geoffroy's spider monkeys: Validation and the influence of reproductive state. *American Journal of Primatology*, *77*(9), 925–935.

Schoof, V. A. M., & Jack, K. M. (2013). The association of intergroup encounters, dominance status, and fecal androgen and glucocorticoid profiles in wild male white-faced capuchins (*Cebus capucinus*). *American Journal of Primatology*, *75*(2), 107–115.

Selby, C. (1999). Interference in immunoassay. *Annals of Clinical Biochemistry: International Journal of Laboratory Medicine*, *36*(6), 704–721.

Seraphin, S. B., Whitten, P. L., & Reynolds, V. (2008). The influence of age on fecal steroid hormone levels in male Budongo Forest chimpanzees (*Pan troglodytes schweinfurthii*). *American Journal of Primatology*, *70*(7), 661–669.

Shimooka, Y., Campbell, C. J., Di Fiore, A., Felton, A. M., & Izawa, K., et al (2008). Demography and group composition of spider monkeys. In C. Campbell (Ed.), *Spider monkeys: Behavior, ecology & evolution of the genus Ateles* (pp. 329–348). Cambridge University Press.

Shutt, K., Setchell, J. M., & Heistermann, M. (2012). Non-invasive monitoring of physiological stress in the western lowland gorilla (*Gorilla gorilla gorilla*): Validation of a fecal glucocorticoid assay and methods for practical application in the field. *General and Comparative Endocrinology*, *179*(2), 167–177.

Slater, K. Y., Schaffner, C. M., & Aureli, F. (2008). Female-directed male aggression in wild *Ateles geoffroyi* yucatanensis. *International Journal of Primatology*, *29*(6), 1657–1669.

Sousa, M. B. C., Albuquerque, A. C. S. R., Albuquerque, F. S., Araujo, A., Yamamoto, M. E., & Arruda, M. F. (2005). Behavioral strategies and hormonal profiles of dominant and subordinate common marmoset (*Callithrix jacchus*) females in wild monogamous groups. *American Journal of Primatology*, *67*(1), 37–50.

Stanley, S., Moheet, A., & Seaquist, E. R. (2019). Central mechanisms of glucose sensing and counterregulation in defense of hypoglycemia. *Endocrine Reviews*, *40*(3), 768–788.

Strier, K. B., Lynch, J. W., & Ziegler, T. E. (2003). Hormonal changes during the mating and conception seasons of wild northern muriquis (*Brachyteles arachnoides hypoxanthus*). *American Journal of Primatology*, *61*(2), 85–99.

Suleman, M. A., Wango, E., Sapolsky, R. M., Odongo, H., & Hau, J. (2004). Physiologic manifestations of stress from capture and restraint of free-ranging male African green monkeys (*Cercopithecus aethiops*). *Journal of Zoo and Wildlife Medicine*, *35*(1), 20–24.

Touma, C., & Palme, R. (2005). Measuring fecal glucocorticoid metabolites in mammals and birds: The importance of validation. *Annals of the New York Academy of Sciences*, *1046*, 54–74.

Tsigos, C., & Chrousos, G. P. (2002). Hypothalamic-pituitary-adrenal axis, neuroendocrine factors and stress. *Journal of Psychosomatic Research*, *53*(4), 865–871.

Veldhuis, J. D., Sharma, A., & Roelfsema, F. (2013). Age-dependent and gender-dependent regulation of hypothalamic-adrenocorticotropic-adrenal axis. *Endocrinology and Metabolism Clinics of North America*, *42*(2), 201–225.

Vick, L. G. (2008). Immaturity in spider monkeys: A risky business. In C. Campbell (Ed.), *Spider monkeys: Behavior, ecology & evolution of the genus Ateles* (pp. 329–348). Cambridge University Press.

Wasserman, M. D., Chapman, C. A., Milton, K., Goldberg, T. L., & Ziegler, T. E. (2013). Physiological and behavioral effects of capture darting on red colobus monkeys (*Procolobus rufomitratus*) with a comparison to chimpanzee (*Pan troglodytes*) predation. *International Journal of Primatology*, *34*(5), 1020–1031.

Weingrill, T., Gray, D. A., Barrett, L., & Henzi, S. P. (2004). Fecal cortisol levels in free-ranging female chacma baboons: Relationship to dominance, reproductive state and environmental factors. *Hormones and Behavior*, *45*(4), 259–269.

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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