

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 [☆]



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ABSTRACT

Non-invasive methods to assess stress hormone output via fecal glucocorticoid metabolites (FGCMs) have become a powerful tool in behavioral studies and conservation biology because they allow exploring the link between behavior, an animal's socio-ecological environment and its adrenocortical activity. However, FGCM levels are influenced by numerous other factors which often confound their interpretation. Thus, before applying these methods, knowledge on the impact of these factors is important. In this study we investigated the effect of (1) time of day, (2) age, (3) sex and (4) female reproductive state on FGCM levels in brown spider monkeys (*Ateles hybridus*) and red howler monkeys (*Alouatta seniculus*). Initially, we validated a 11 β -hydroxyetiocholanolone enzyme immunoassay for monitoring the physiological stress response via fecal analysis in both species. We determined FGCM levels in fecal samples collected from two and six groups of wild spider monkeys ($n = 461$ samples) and howler monkeys ($n = 166$ samples), respectively. Our analyses revealed a strong effect of time of day on FGCM levels in spider monkeys, but no effect in howler monkeys. Adults of both species had significantly higher FGCM levels than subadults. In neither of the two species we found a sex-effect on FGCM output. Reproductive condition strongly affected FGCM levels in female spider monkeys which showed increasing concentrations with progressing gestation. This was not investigated in female howler monkeys due to an insufficient sample size. Our data indicate that the influence of the tested factors on fecal glucocorticoid metabolite output is species-specific, and that these variables need to be considered when interpreting FGCM levels in the species.

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1. Introduction

In recent years, there has been a substantial increase in the number of studies that investigate the interactions between animal behavior and steroid hormone levels in vertebrates of all major taxa (e.g., Bonier et al., 2009; Cavigelli, 1999; Engh et al., 2006; Ganswindt et al., 2003; Goymann et al., 2003; Kenagy and Place, 2000; Reeder et al., 2004). These studies help to gain insight into the proximate factors underlying and modulating behavioral variation, life history traits, fitness, and survival of animals. Measure-

ment of hormones is also employed in conservation research to assess and monitor the physiology, health and well-being of populations of endangered species in the wild (Chapman et al., 2006; Cyr and Romero, 2008; Franceschini et al., 1997; Hodges and Heistermann, 2003; Tarlow and Blumstein, 2007; Van Meter et al., 2009; Wikelski and Cooke, 2006; Wingfield et al., 1997) as well as to facilitate and ensure the propagation and welfare of animals in captivity (e.g., Dehnhard et al., 2008; Graham et al., 2002; Heistermann et al., 2004; Pirovino et al., 2011).

In the latter contexts, glucocorticoids (cortisol and corticosterone) have received most attention. As front hormones of the vertebrate stress response that reflect physiological stress loads of individuals and populations, they have proven as an important biomarker when assessing the physiological consequences of anthropogenic disturbances and habitat fragmentation for individual and population health (Chapman et al., 2006, 2007; Franceschini et al., 1997; Martínez-Mota et al., 2007; Rangel-Negrín et al., 2009; Thiel et al., 2011; Wasser et al., 1997; Wikelski and Cooke, 2006; Wingfield et al., 1997). Generally, glucocorticoids and their

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metabolites can reliably be measured in blood, urine and feces using enzyme immunoassays (EIAs) (for detailed reviews see (Romano et al., 2010; Sheriff et al., 2011)). When studying stress physiology in wildlife, however, non-invasive methodologies based on the measurement of GC metabolites (GCM) in excreta (urine, feces) is the preferred approach because blood sampling is usually not feasible (and undesirable) in wild animals. Moreover, excreted GCM levels in urine and feces provide a more integrated measure of adrenocortical activity than point serum samples and thus diminish the influence of the pulsatile and episodic patterns of GC secretion (Whitten et al., 1998a). Since excreta can also be collected much more regularly than blood, analyses of urinary and fecal hormone metabolites provide the most suitable way to obtain longitudinal information on endocrine activity.

The metabolism and excretion route of glucocorticoids can differ substantially between species, even closely related ones (Baher et al., 2000; Palme et al., 2005). Thus, prior to applying urinary or fecal analysis, it is crucial to biologically validate the respective method for each new species to assure that the data to be generated will be biologically meaningful (Goymann, 2012; Heistermann et al., 2006; Palme et al., 2005; Schwarzenberger, 2007; Touma and Palme, 2005; Whitten et al., 1998b). In this respect, the validation should not only demonstrate that the GCM measurement reliably detects adrenocortical endocrine activity in response to a stressor, but should also evaluate the specificity of the measurement when immunological detection methods are used (Goymann, 2012; Heistermann et al., 2006). The latter is particularly important given that metabolites of glucocorticoids and other steroids (e.g., testosterone) can be structurally very similar (Ganswindt et al., 2003). Since antibodies used for the quantification of glucocorticoids can potentially cross-react with those metabolites of different origin and function (Ganswindt et al., 2003; Heistermann et al., 2006; Palme et al., 2005), such cross-reactions can have major and distorting effects on the results obtained (see Ganswindt et al., 2003; Goymann, 2012). Further, glucocorticoid metabolism can differ even between sexes within a given species (e.g., Baltic et al., 2005; Touma et al., 2003), making comparisons of GC levels between males and females potentially problematic and meaningless unless the immunological specificity of the assay used is demonstrated (for a detailed review see (Goymann, 2012)).

In many species basal stress hormone levels are affected by a variety of intrinsic factors (for review see (Goymann, 2012; Keay et al., 2006; Millspaugh and Washburn, 2004)) such as age (Sapolsky, 1992; Seraphin et al., 2008), sex (Raminelli et al., 2001; Sapolsky, 1992; Touma et al., 2003), reproductive state (Carnegie et al., 2011; Cavigelli, 1999; Setchell et al., 2008; Weingrill et al., 2004; Ziegler et al., 1995) and body condition (Charbonnel et al., 2008), and they also often show diurnal variation (Bosson et al., 2009; Chapman et al., 2006; Raminelli et al., 2001). All these factors may confound interpretation of GC levels generated in contexts such as behavioral studies or conservation research. Knowledge about whether and in which specific way these variables have an impact on stress hormone output in a given species is therefore of high importance when GC data is collected for such research questions.

The way and extent to which such factors influence adrenocortical activity appears to be species-specific, emphasizing the importance to assess their impact in every previously unstudied species. For example in Columbian ground squirrels (Bosson et al., 2009), common marmosets (Raminelli et al., 2001) and red colobus monkeys (Chapman et al., 2006) a diurnal rhythm of glucocorticoid secretion is reflected in fecal glucocorticoid metabolite (FGCM) levels, while such variation is absent in other species (e.g. white rhinoceros (Turner et al., 2002), baboons (Beehner and Whitten, 2004), western lowland gorillas (Shutt et al., 2012)). Correspondingly, adult male chimpanzees (Seraphin et al., 2008) and Assamese macaques (Ostner et al., 2008) (but only during

the breeding season) have higher GC levels than subadult males and in rats glucocorticoid levels increase with increasing age (Sapolsky, 1992), whereas there is no age-effect in other species (e.g. spiny mice (Nováková et al., 2008)).

In addition to these biological sources of variation, GC levels from feces can also be affected by methodological issues, in particular the way how samples are collected and stored (Khan et al., 2002; Lynch et al., 2003; Shutt et al., 2012). This presents a serious challenge especially for researchers that work in remote areas where there is no access to freezers. One solution to this problem is the immediate extraction of steroids from feces using on-site extraction methodologies (Beehner and Whitten, 2004; Murray et al., 2012; Shutt et al., 2012) in combination with validated methods to store extracts under tropical conditions (Santymire and Armstrong, 2010; Shutt et al., 2012). However, to date it remains unclear how versatile such methods are, i.e. to what extent they can be applied across multiple species.

As part of a larger project that investigates the impact of anthropogenic disturbances and habitat fragmentation on the stress physiology of wild brown spider monkeys (*Ateles hybridus*) and red howler monkeys (*Alouatta seniculus*) in Colombia, we examine here the effect of time of day, age, sex and female reproductive condition on fecal glucocorticoid excretion, information that does not exist for either of the two species. Brown spider monkeys are endemic to Colombia and Venezuela (Defler, 2003). Due to their restricted distribution, their long inter-birth intervals (32–50 months) (Di Fiore and Campbell, 2007), severe habitat loss and high hunting pressure the species is critically endangered (Urbani et al., 2008) and belongs to the 25 most endangered primate species in the world (Mittermeier et al., 2012). As other spider monkey species, they are mainly frugivorous, although young leaves can make up to 50% of their diet (Galvis et al., 2012). In contrast, red howler monkeys have a much wider distribution (Brazil, Ecuador, Peru, Venezuela and Colombia) and are not threatened with extinction (Boubli et al., 2008). They are highly folivorous and can persist even in extremely small forest fragments (Estrada and Coates-Estrada, 1996; Gilbert, 2003; Lovejoy et al., 1986).

For the present study we initially validated an EIA for assessing adrenocortical activity non-invasively from fecal samples of the two species and tested for potential storage effects on FGCM levels in fecal extracts stored for six months at high temperatures. For validation we used 1) the physiological stress response to anesthesia (e.g., Martínez-Mota et al., 2008; Sapolsky, 1982; Whitten et al., 1998b) in zoo-housed and wild animals to test the suitability of four different EIAs in reflecting the stress-related FGCM increase in feces and 2) evaluated the specificity of the most suitable EIA in both sexes of both species by characterizing the pattern of immunoreactive metabolites measured using HPLC analysis.

Thus, with this study, we procure the methodological basis for studying adrenocortical activity non-invasively in *A. hybridus* and *A. seniculus* and provide important comparative baseline information on the influence of several intrinsic variables on FGCM levels in these two endocrinologically unstudied species of New World primates.

2. Material and methods

2.1. Study sites and animals

2.1.1. Field

We collected fecal samples of two wild groups of brown spider monkeys (SJ1, SJ2) and six groups of red howler monkeys (C0, C1, C2, C3, C7, I) in which all individuals were individually recognized and fully habituated (Table 1). All groups ranged in a forest fragment located within the private cattle ranch “Hacienda San Juan

Table 1
Group composition and number of fecal samples collected per study group.

Species	Group	Group composition ^a	No. samples				Total
			AM	AF	SAM	SAF	
<i>Ateles hybridus</i>	SJ1	3–4AM, 5AF, 1–3SAM, 0–2JM, 3–4JF, IF, IM	154	211	40	–	405
	SJ2	AM, 5AF, 2SAM, 3JF, 2IM, IF	11	44	1	–	56
<i>Alouatta seniculus</i>	C0	2AM, 3AF, SAM, JF, 2IF, IM	25	21	1	–	47
	C1	AM, 2AF, SAF, JM, JF	6	5	–	–	11
	C2	2–3AM, 4AF, SAF, SAM, 2JF, JM	17	20	2	1	40
	C3	3AM, 4AF, SAM, SAF, JM	6	15	5	2	28
	C7	AM, 2AF, SAM, JM	6	10	1	–	17
	I	1–2AM, 2AF, SAF, IM	11	10	–	2	23

^a Adult male (AM), adult female (AF), subadult male (SAM), subadult female (SAF), juvenile male (JM), juvenile female (JF), infant male (IM) and infant female (IF).

del Carare" (06° 43' N, 74° 09' W; 150–200 m a.s.l) in Colombia. At the study site both study species have been habituated and studied since 2007 (Link et al., 2010). The fragment comprises 65 ha of seasonally flooded tropical rainforest and is located in the Magdalena River Valley between the eastern and central cordilleras of the Colombian Andes. The area shows two marked rainy seasons, typically one from March to May, and another from October to November. During the rainy seasons the fragment regularly floods for a period of several weeks up to three months. The area receives an annual medium rainfall of 3496.5 mm, shows a medium temperature of 27.9 °C and a medium humidity of 80% (IDEAM, 2008).

2.1.2. Zoo

As part of the validation of a fecal GC assay (see Section 2.4.), we collected fecal samples from a zoo-housed group of brown spider monkeys in the Zoological and Botanical Garden Stuttgart, Germany in 2011 (see Section 2.2.2.). The group consisted of one adult male, four adult females, two juvenile males, one juvenile female and one infant male. The animals had access to an indoor and an outdoor enclosure and they were fed twice a day with fruits and vegetables. Water was available ad libitum.

2.2. Fecal sample collection

2.2.1. Field

For the validation tests (see Section 2.4.), we collected fecal samples from two wild adult spider monkey males and one wild male adult red howler monkey before and after they were captured and anesthetized to place radio-collars on them as part of a behavioral study.

In order to assess the impact of time of day, age, sex and female reproductive condition on FGCM levels, we collected 461 fecal samples from brown spider monkeys and 166 fecal samples from red howler monkeys between August 2010 and April 2012. To differentiate between adults and subadults we used age-related traits such as body size, coloration and size of primary sexual characteristics (e.g. testes, vulvae) and information on the date of birth (when available). Since in both study species pregnancy cannot reliably be detected by visual inspection, we used the date of parturition in combination with average gestation length (*Ateles* ~7.5 months, *Alouatta* ~6.3 months) (Di Fiore and Campbell, 2007) to identify the samples that were collected during gestation. We categorized females as lactating for the period of time in which they were observed nursing their dependent offspring. Females that did not fall within these two categories (pregnant or lactating) were categorized as cycling (despite their actual cycle status was unknown). We collected fecal samples from all adult and subadult individuals usually on a weekly basis; however, in three less studied groups (C1, C3, C7) samples were collected more sporadically (Table 1).

For sample collection, we homogenized the fecal bolus and removed any obvious undigested matter (e.g. large seeds). We collected only samples uncontaminated with urine. We placed approximately 0.5 g of fresh feces into a 15 ml polypropylene tube pre-filled with 5 ml of 96% ethanol and shook the tube manually until the feces were suspended in the solvent (Shutt et al., 2012). Samples were kept at ambient temperatures until they were extracted after returning to the camp in the evening (see Section 2.3.1.).

2.2.2. Zoo

We collected 70 fecal samples (mainly in the morning) from zoo-housed brown spider monkeys during a capture-translocation event which took place in the Zoological and Botanical Garden Stuttgart, and during which all animals were anesthetized. Samples were collected before the capture commenced to assess pre-treatment baseline FGCM levels and five days thereafter to monitor the FGCM response to the stressor. In addition, samples were collected 55 days following the stressful event, when the animals had completely settled into their new enclosure, to serve as a second control. On Day 3 of the sampling period, the zoo's veterinarian inspected the animals by observing them for a couple of minutes. This caused a high level of distress to all individuals, indicated by high arousal, screaming and defecation of diarrhea samples. During the period of sample collection, the animals could move freely between an indoor and an outdoor enclosure. Because of this and in combination with the large sizes of both enclosures, we could not systematically assign to which individual a certain defecated sample belonged. Thus, we could only assess the stress-related changes in FGCM concentrations on the group level, rather than in individual animals. Samples were collected usually within two hours after defecation and all samples were stored at –20 °C until transportation on ice-packs to the endocrinology laboratory of the German Primate Center for analysis. The samples were processed and stored until analysis as described below (see Section 2.3.2.).

Red howler monkeys are only kept in three European zoos. Unfortunately, during the study period none of these individuals was captured and anesthetized for a medical check-up or transport and stimulating adrenocortical activity using an ACTH challenge was also not possible. Thus, we were unable to collect fecal samples during a stressful event from captive animals as we did for *A. hybridus*.

2.3. Steroid extraction

2.3.1. Field

Prior to extraction we weighted the tube containing the sample to determine fecal wet weight. We then manually shook the fecal suspension firmly for 5 min (Shutt et al., 2012) and, thereafter, centrifuged the samples for 1 min using a manually-operated

centrifuge (from Hettich GmbH & Co. KG Tuttlingen, Germany) to recover the supernatant containing dissolved steroids. We poured off ~2 ml of each fecal extract into 2 ml polypropylene tubes (PPT; SafeSeal Micro Tube; Ref. No. 72.695.200 from Sarstedt AG & Co. Nuernbrecht, Germany), sealed them with parafilm, labeled them with animal ID, date and time of day and stored them at ambient temperatures (~25 °C) in a dark place. Every two months we transported the extracts to the University of Los Andes, Bogotá, where we stored them at –20 °C until shipment to the endocrinology laboratory at the German Primate Center for steroid analysis.

2.3.2. Zoo

We processed and extracted the fecal samples collected from zoo-housed spider monkeys following Heistermann et al. (1995). In summary, we lyophilized and pulverized the feces and extracted an aliquot representing 0.05–0.07 g of fecal powder in 3 ml of 80% methanol by vortexing the suspension for 15 min. Subsequently, we centrifuged the suspension, recovered the supernatant and stored it at –20 °C until analysis.

2.4. Validation of an EIA to measure FGCM

We used the well-documented stress response to anesthesia (e.g., Martínez-Mota et al., 2008; Sapolsky, 1982; Whitten et al., 1998b) to test the ability of four glucocorticoid EIAs, described in detail by Heistermann et al. (2004, 2006), to detect the expected increase in FGCM levels following this stressor. Specifically, we analyzed fecal extracts with EIA systems designed to measure cortisol (CORT) (Palme and Möstl, 1997), corticosterone (CCST) (Heistermann et al., 2006), 11 α -etiocholanolone (Möstl and Palme, 2002) and 11 β -hydroxyetiocholanolone (Ganswindt et al., 2003), the latter two assays representing group-specific assays for the measurement of 5 β -reduced GC metabolites with a 3 α ,11 α - and 3 α ,11 β -dihydroxy structure. All four EIAs have been previously used successfully to monitor adrenocortical activity via FGCM analysis in other primate and non-primate species (Fichtel et al., 2007; Ganswindt et al., 2003; Heistermann et al., 2006; Ostner et al., 2008; Weingrill et al., 2004). In all EIAs antibodies used were raised in rabbit or sheep with steroids coupled to bovine serum albumin (BSA) via a carboxymethylxime bridge. Detailed information on antibody characteristics, standards, and hormone labels as well as on other assay details, e.g., data on assay sensitivities, is given in Heistermann et al. (2006).

2.5. Steroid analysis

Based on the outcome of the validation tests (see Section 4.1.), we analyzed all fecal samples collected from wild spider and howler monkeys in the 11 β -hydroxyetiocholanolone EIA. The EIA was performed as described in detail by Heistermann et al. (2004). Prior to steroid measurement, we diluted extracts 1:250–1:2000 (depending on concentration) in assay buffer and took duplicate aliquots to assay. Sensitivity of the assay was 1 pg/well. Serial dilutions of fecal extracts gave displacement curves parallel to those obtained with the 11 β -hydroxyetiocholanolone standard. Intra- and inter-assay coefficients of variation of high- and low-value quality controls were 6.1% (high, $n = 16$) and 7.8% (low, $n = 16$) and 7.4% (high, $n = 37$) and 13.0% (low, $n = 37$), respectively. All steroid concentrations are given as ng/g fecal dry (samples from zoo animals) or wet (samples from wild animals) weight.

2.6. HPLC analysis

We used reverse-phase high pressure liquid chromatography analysis (HPLC) to characterize the immunoreactive metabolites present in the feces of both species and measured by the 11 β -

hydroxyetiocholanolone EIA. HPLC was carried out as described by Möhle et al. (2002) and Heistermann et al. (2006). To evaluate possible sex differences in 11 β -hydroxyetiocholanolone immunoreactivity profiles, we performed HPLC on both a male and a female sample from the wild-living animals of each species. HPLC also allowed us to evaluate whether certain fecal androgens, which could potentially be detected by antibodies raised against cortisol metabolites (Ganswindt et al., 2003; Schatz and Palme, 2001), were co-measured by the 11 β -hydroxyetiocholanolone EIA.

2.7. Storage effect

To investigate whether FGCM levels changed during the two months of storage at ambient temperatures at the field site we conducted a storage experiment. We stored aliquots of eleven fecal extracts (from *A. hybridus*) at an elevated temperature of 30 °C (to simulate storage conditions in the field) at the endocrinology laboratory at DPZ and measured each aliquot in the 11 β -hydroxyetiocholanolone assay immediately (time 0 control) and after one, three and six months of storage to test for a potential change in steroid concentrations as a function of storage duration. For these measurements, inter-assay coefficients of variation of high- and low-value quality controls were 6.1% (high, $n = 4$) and 7.4% (low, $n = 4$), respectively.

3. Statistical analyses

To assess the effects of time of day, sex, age and female reproductive state on FGCM levels we used a generalized linear mixed model (GLMM) (Baayen, 2010) for each study species. We fit all models with the lmer function from the lme4 package (Bates et al., 2010) in R 2.15.1 (R Development Core Team 2012). Individual identity and group were used as random factors in the models. Sex, age (adult or subadult), time of sample collection (morning or afternoon) and female reproductive state (cycling, pregnant or lactating, for definition see Section 2.2.1.) were used as categorical variables. We tested for interactions between sex and age and between time and sex. These were not significant ($P \geq 0.05$) and thus, we did not include any interaction in the final models. We log transformed the response variable (FGCM levels) to achieve normal distribution and we checked that the assumptions of normally distributed and homogeneous residuals were fulfilled in every model by visually inspecting qqplots and the residuals plotted against the fitted values. We checked for model stability by excluding data points one by one from the data and comparing the estimates derived with those obtained for the full model. We derived variance inflation factors (Field, 2005) using the function vif of the R-package car (Fox and Weisberg, 2011) applied to a standard linear model excluding the random effect. To determine the significance of the full model (including all fixed and random effects) we compared it to the corresponding null model (including only the random effects) using a likelihood ratio test (R function 'anova'). To determine more reliable P -values we used the functions pvals.fnc of the package 'language R' (Baayen, 2010). In this function P -values are based on Markov Chain Monte Carlo (MCMC) sampling (Baayen et al., 2011).

Additionally, to examine changes in FGCM levels across the entire gestation period of female spider monkeys in more detail we divided the gestation period into seven monthly intervals and performed a Spearman rank correlation between month of gestation and respective FGCM levels (using Statistica 10). To back up the results of our GLMM we used a T-test (using Statistica 10) to test for potential differences between age categories in howler monkeys (as strongly indicated by Fig. 3f). All statistical tests were two-tailed and the statistical threshold was set at $P \leq 0.05$.

4. Results

4.1. Validation of an EIA to measure FGCM

4.1.1. Zoo-housed *Ateles hybridus*

In absolute terms, the highest levels of FGCMs were measured by the two group-specific assays (mean \pm SE of all samples: 11 β -hydroxyetiocholanolone: 6094 \pm 595 ng/g dry feces; 11oxo-etiocholanolone: 13842 \pm 1123 ng/g dry feces), those measured by the CORT and CCST assay being generally much lower (CORT: 666 \pm 119 ng/g dry feces; CCST: 501 \pm 99 ng/g dry feces). Nevertheless, all four assays showed a clear response to the stress of anesthesia, reflected by a marked rise (200–320%) in FGCM levels within 24 h (Fig. 1). FGCM levels (except CCST) decreased on Days 2 and 3 post-anesthesia, but showed a second clear rise on Day 4 (exception CCST), which probably reflects the stress response to the marked distress caused by the visit of the veterinarian on Day 3 of the sampling period (see Section 2.2.2.). On Day 5, levels of all four FGCM measures started to return to baseline and these low concentrations were also found two months later (Day 55) when the group had completely settled in their new environment. In terms of magnitude of response, the 11 β -hydroxyetiocholanolone, CORT and CCST assays were similar (all ca. 300% increase), while the response of the 11oxo-etiocholanolone assay was slightly less pronounced (ca. 200%).

4.1.2. Wild *Ateles hybridus*

In the few samples collected from the two wild animals before and after the capture event, results were mixed (Table 2). With respect to 11 β -hydroxyetiocholanolone and 11oxo-etiocholanolone

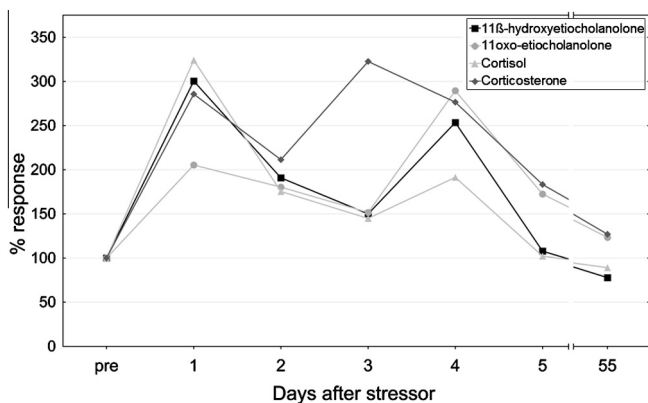


Fig. 1. Percentage response (mean of all samples) in immunoreactive FGCM levels to a capture-translocation event and anesthesia in *A. hybridus*. Pre = samples collected before the anesthesia was applied. Note that on Day 3 the veterinarian checked on the animals which caused a high level of distress (see Methods).

Table 2

Fecal glucocorticoid metabolite concentrations (as detected by four different assays) in response to anesthesia in individual spider monkeys and a howler monkey.

Animal ^a	11 β -hydroxyetiocholanolone				11oxo-etiocholanolone				CORT				CCST			
	Pre ^b	Peak ^c	Post ^d	Lag ^e	Pre	Peak	Post	Lag	Pre	Peak	Post	Lag	Pre	Peak	Post	Lag
Wampi (A.h.)	140	466	83	24.5	378	1142	215	24.5	29	554	394	24.5	11	49	27	24.5
Roko (A.h.)	49	175	14	24.5	86	525	27	24.5	62	104	15	28	27	33	5	28
Cirilo (A.s.)	112	473	266	46	598	2083	1515	46	72	126	50	50.3	21	57	38	24

^a A.h. = *Ateles hybridus*; A.s. = *Alouatta seniculus*.

^b Pre-treatment levels in ng/g wet feces (see Methods).

^c Peak levels in response to stressor in ng/g wet feces.

^d Wampi: levels 50 h after stressor; Roko: levels one week after stressor; Cirilo: 72 h after stressor.

^e Lag time in hours between occurrence of the stressor and peak FGCM level.

measurements, findings were similar to those obtained in the zoo-housed animals. Specifically, both individuals showed a 3- to 4-fold increase in 11 β -hydroxyetiocholanolone and 11oxo-etiocholanolone levels in the sample collected 24.5 h after the capture and levels declined to pre-capture baseline concentrations in the samples collected following the stressful event (Table 2). The patterns for the CORT and CCST measures were inconsistent between individuals, i.e. a response to the stressor in terms of a clear rise in the respective FGCM levels was only seen in one of the two animals

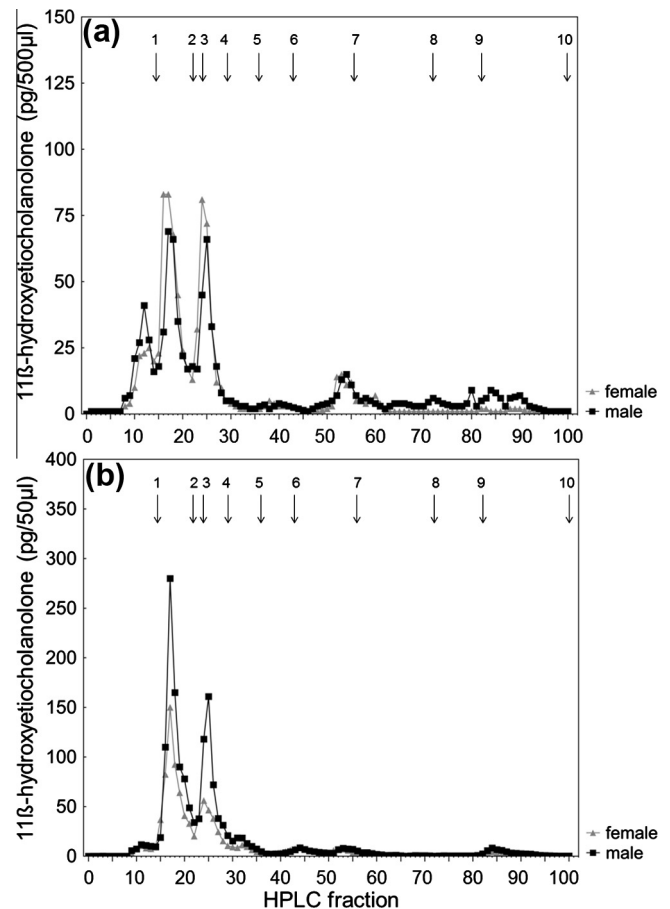


Fig. 2. HPLC profiles of immunoreactivity detected with the 11 β -hydroxyetiocholanolone EIA in samples of a wild female and a male (a) spider monkey and (b) howler monkey. Arrows indicate elution positions of reference standards: [1] cortisol (fractions 14–15), [2] corticosterone (22), [3] 11 β -hydroxyetiocholanolone (24–25), [4] 11-oxoetiocholanolone (29–30), [5] 5 β -androstane-3,11,17-trione (36), [6] testosterone (43), [7] androstendione, dehydroepiandrosterone (55–56), [8] epiandrosterone, 5 β -DHT, 5 β -androstane-3 β -ol-17-one (72), [9] 5 β -androstane-3 α -ol-17-one (82), and [10] androsterone (100).

(Table 2). Based on the combined validation results (zoo and wild), we chose the 11 β -hydroxyetiocholanolone assay for all further analyses.

4.1.3. *Alouatta seniculus*

FGCM levels in the individual male howler monkey showed a similar response to capture as in spider monkeys (Table 2). Specifically, 11 β -hydroxyetiocholanolone, 11oxo-etiocholanolone and CCST levels showed a clear rise (2.5–4.2-fold) within 24 h after capture, while CORT levels remained largely unchanged (Table 2). In contrast to spider monkeys, peak response in the two group-specific assays was, however, seen later, i.e. on Day 2 after capture. Levels of all FGCM measures showed a decline towards baseline in the sample collected on Day 3. Based on these findings we selected the 11 β -hydroxyetiocholanolone EIA for all further analyses (as for spider monkeys).

4.2. HPLC analysis

HPLC analysis indicated that the vast majority of immunoreactivity (>80% for *A. hybridus* and >90% for *A. seniculus*) was detected as distinct peaks between fractions 9 and 31 – positions where cortisol metabolites in our HPLC system elute (Fig. 2) (Heistermann et al., 2006). In both species the highest amounts of immunoreactivity were detected around fractions 16 and 25, the latter being the elution position of 11 β -hydroxyetiocholanolone, indicating a high abundance of this metabolite of cortisol in the feces of both

species. The presence of only small amounts of immunoreactivity measured after fraction 40 (positions where certain potentially cross-reacting androgen metabolites elute (Ganswindt et al., 2003; Heistermann et al., 2006)), suggests a low degree of co-measurement of these androgens in our assay (Fig. 2). In both species, HPLC profiles were very similar between males and females in terms of both number and elution position (i.e. characteristic) of metabolites measured.

4.3. Storage experiment

Our storage experiment revealed that 11 β -hydroxyetiocholanolone concentrations remained stable over the 6 months of storage at 30 °C with levels deviating from time 0 control measurements by maximally 5.1% at any time point tested (1 month: –5.1%, 3 months: –2.4%, 6 months: +4.9%). The mean CV value calculated across the 11 individual CVs for each sample measurement over the 6 months of analysis was 6.6% (range 3.8–10.7%) and was thus exactly in the range of our inter-assay variation (6.1% and 7.4%, see Section 2.7).

4.4. Effect of time of day, age, sex and female reproductive state on FGCM levels

Fecal samples of *A. hybridus* collected in the morning (mean \pm SE: 203 \pm 10 ng/g) had significantly higher FGCM levels than samples collected in the afternoon (83 \pm 13 ng/g; GLMM:

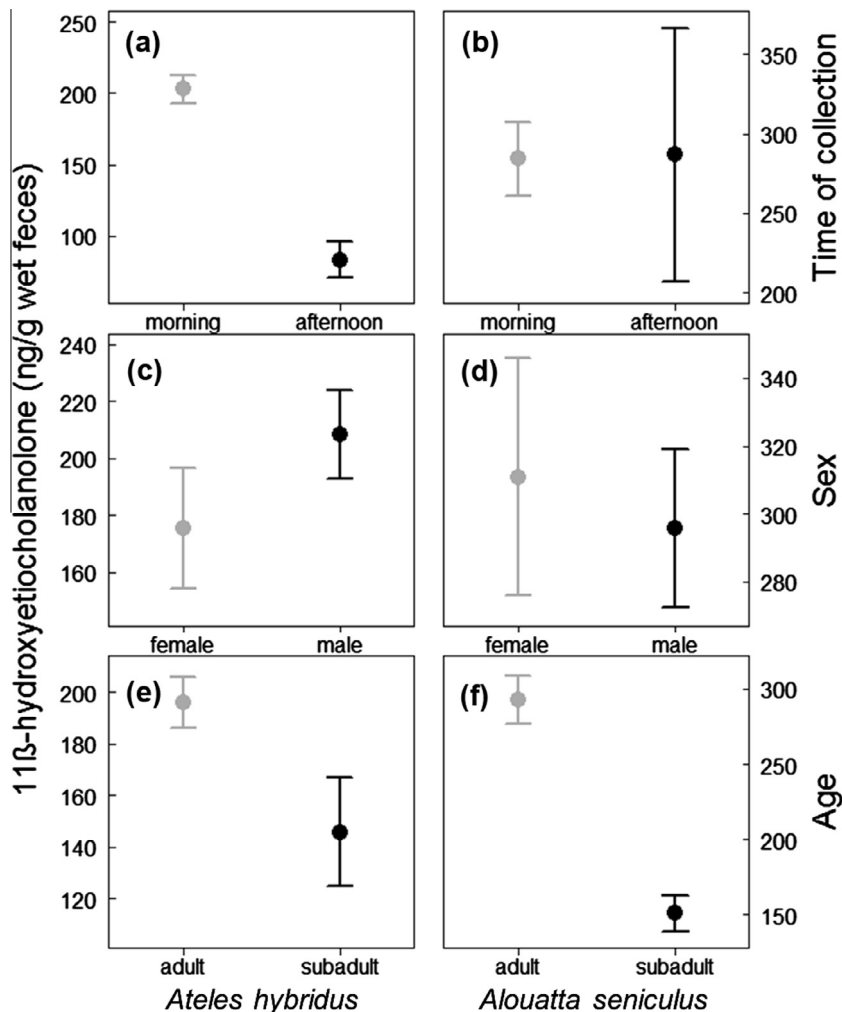


Fig. 3. Mean \pm SE FGCM levels of *A. hybridus* (left graphs) and *A. seniculus* (right graphs) in relation to time of day (a and b), sex (c and d) and age category (e and f).

$P_{\text{MCMC}} = 0.0001$, Fig. 3a). Adult individuals had significantly higher FGCM levels than subadults (adults: 196 ± 10 ng/g; subadults: 146 ± 21 ng/g; $P_{\text{MCMC}} = 0.027$, Fig. 3e) but there was no difference in FGCM concentrations between the sexes (males: 208 ± 15 ng/g; cycling females: 175 ± 21 ng/g; $P_{\text{MCMC}} = 0.17$, Fig. 3c). Within the females, however, reproductive state significantly influenced FGCM levels ($X^2 = 15.32$, $P = 0.001$), with concentrations being significantly elevated during pregnancy compared to the cycling ($P_{\text{MCMC}} = 0.0004$) and lactating ($P_{\text{MCMC}} = 0.003$) condition (Fig. 4). Cycling and lactating females did not differ in FGCM levels ($P_{\text{MCMC}} = 0.58$). Furthermore, in pregnant females, FGCM levels were significantly and positively correlated with the duration of gestation (Spearman: $R = 0.54$, $T = -3.59$, $P = 0.004$; Fig. 5).

In *A. seniculus* neither time of day (morning: 284 ± 23 ng/g; afternoon: 287 ± 80 ng/g), nor sex (males: 296 ± 23 ng/g; cycling females: 311 ± 35 ng/g) significantly influenced FGCM levels ($N = 166$ samples, full versus null model $X^2 = 3.46$, $df = 9$, $P = 0.62$; Fig. 3b and d). However, in accordance with spider monkeys, adult howler monkeys (286 ± 16 ng/g) had significantly higher FGCM levels than subadult individuals (151 ± 12 ng/g; $T = 2.74$, $df = 33$, $P = 0.009$; Fig. 3f). Due to an insufficient sample size, the influence of female reproductive state on FGCM levels could not be tested in this species.

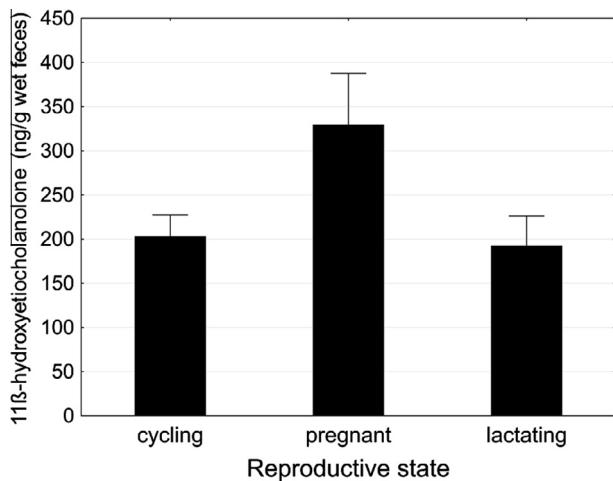


Fig. 4. Mean \pm SE FGCM levels in relation to female reproductive condition.

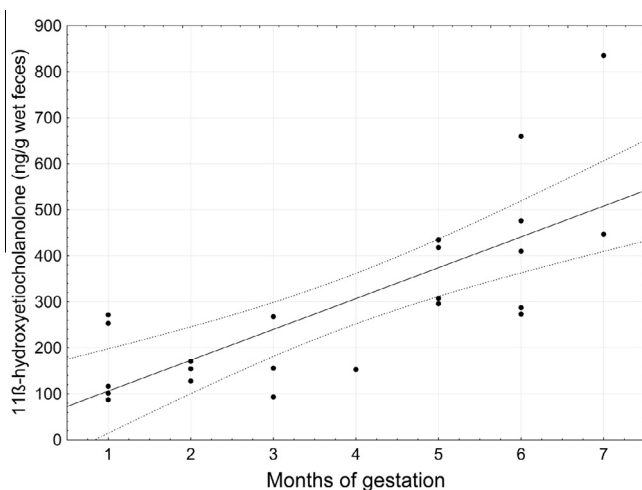


Fig. 5. Mean \pm SE FGCM levels in relation to months of gestation in female spider monkeys. Dashed lines indicate the 95% confidence intervals.

5. Discussion

This is the first study determining adrenocortical activity in brown spider and red howler monkeys and evaluating the potential impact of various intrinsic factors on FGCM output in these two species of New World monkeys. Our results demonstrate the validity of a group-specific EIA (11 β -hydroxyetiocholanolone) for monitoring the physiological stress response in both species by measuring 5-reduced GC metabolites with a 3 α ,11 β -dihydroxy structure in the feces. They also indicate that time of day, age and female reproductive state significantly influence FGCM output in *A. hybridus*, whereas age but not time of day affects FGCM levels in *A. seniculus*, and that sex did not affect FGCM concentrations in either species. Besides providing important information on the suitability of FGCM assays for tracking HPA axis activity in brown spider and red howler monkeys our study emphasizes species differences in terms of basic factors influencing adrenocortical activity.

5.1. Validation of an EIA to measure FGCM

Due to logistical constraints we were not able to determine the physiological stress responses individually in our zoo-housed spider monkeys and therefore can only present changes in FGCM concentrations on a group mean level. While this prevented us from obtaining information on individual variability in terms of the adrenocortical response to capture and anesthesia, it did not restrict our evaluation of the suitability of different GC assays for detecting the stressor associated changes in FGCM levels. Of the four assays tested, the two group-specific EIAs were the ones that responded consistently to the stressor(s) in both the captive and wild animals, indicating their higher biological sensitivity for tracking fluctuations in HPA axis activity compared to the cortisol and corticosterone EIAs tested. In terms of magnitude and time course of response, the two group-specific measures were within the range of those reported in other studies on primate (Heistermann et al., 2006; Martínez-Mota et al., 2008; Whitten et al., 1998b) and non-primate species (Wasser et al., 2000; Young et al., 2004). Although both assays thus appear to be of similar value, for practical reasons we selected the 11 β -hydroxyetiocholanolone assay for all subsequent analyses. Our HPLC data indicated that the 11 β -hydroxyetiocholanolone measure was largely specific for glucocorticoid metabolites and not affected by a substantial co-measurement of androgens which potentially can have distorting effects on FGCM results (Ganswindt et al., 2003; Goymann, 2012; Schatz and Palme, 2001). We found similar results for the red howler monkey used for biological validation, although the findings of this case study should be confirmed in a larger number of animals. The 11 β -hydroxyetiocholanolone assay has, however, been applied successfully to monitor stress physiology in many other primates, (lemurs (Fichtel et al., 2007); macaques (Girard-Buttoz et al., 2009; Ostner et al., 2008); gibbons (Pirovino et al., 2011); apes (Shutt et al., 2012; Weingrill et al., 2011)) indicating its versatility for non-invasively monitoring adrenocortical activity in primates of all major taxa. Based on these and our present findings, we are therefore confident that the 11 β -hydroxyetiocholanolone assay is valid for monitoring adrenocortical activity in the two study species.

5.2. Effect of time of day, age, sex and female reproductive state on FGCM levels

We extracted all fecal samples on-site in our field camp and stored fecal extracts at temperatures of about 25 °C for up to two months before we were able to freeze them, the gold standard

for storing hormone samples long-term (Herring and Gawlik, 2009; Hunt and Wasser, 2003; Shutt et al., 2012). Our storage experiment showed that FGCM levels were not largely affected by storing the alcoholic extracts at elevated temperatures for up to 6 months as indicated by changes that were within 5.1% of the controls. Our data thus confirm recent findings from a study on gorillas (Shutt et al., 2012) and baboons (Kalbitzer and Heistermann, 2013), showing that FGCMs in alcoholic extracts remained stable for up to one year when stored unfrozen. As the variation in repeated sample measurements across the 6 months was almost identical to our measure of inter-assay variability, the small differences observed are very likely due to assay variation and thus do not reflect true changes in FGCM concentrations (see also (Shutt et al., 2012; Kalbitzer and Heistermann, 2013)). Therefore, our method of storing fecal extracts for two months at elevated temperatures at the field site did not affect the FGCM levels obtained and thus is unlikely to have biased our data.

Using the newly established FGCM assay methodology we were able to examine the potential impact of time of day, age, sex and female reproductive state on adrenocortical activity in wild brown spider and red howler monkeys. Our results revealed species differences in this respect. For example, brown spider monkeys showed a marked diurnal variation in FGCM levels with morning samples showing, on average, 2.5-fold higher concentrations than afternoon samples. Similar results of the same magnitude have been found by Davis et al. (2005) for urinary cortisol excretion in closely related Colombian spider monkeys (*Ateles geoffroyi* [sic] *rufiventris*). By contrast, we did not find evidence for diurnal changes in FGCM levels in red howler monkeys, a finding that concurs with that reported for males of black howler monkey (*Alouatta pigra* (Van Belle et al., 2009); but see (Rangel-Negrín, 2010)). These interspecific differences can presumably be ascribed to the distinct foraging strategies and differences in gut passage rates of the two species. Spider monkeys are mainly frugivorous and defecate very frequently (average of 13.7 times/12 h) (Link and Di Fiori, 2006) as the gut passage time is very short, i.e. only 4.4 h on average (Milton, 1981). This should result in a high temporal resolution of endocrine information derived from feces which may explain why the diurnal variation in HPA axis activity is well reflected in FGCM levels in spider monkeys. In contrast, howler monkeys are mainly folivorous and have a bimodal defecation pattern (Andersen, 2002) and an average gut passage rate of 20.4 h (Milton, 1981). Consequently, in howler monkeys information on endocrine state from feces is more dampened, and, therefore, it is likely that short-term temporal changes in FGCM output cannot be tracked from feces in howler monkeys. Overall, our data support the prediction made by Touma and Palme (2005), stating that it might be impossible to detect diurnal changes in FGCM levels in species with a long gut passage rate and/or animals that defecate rather infrequently. More importantly, our results emphasize that diurnal variation in HPA axis activity is an important factor to consider when interpreting FGCM data in brown spider monkeys and presumably other species of the genus.

In both species we found age-related differences where adult individuals showed significantly higher FGCM levels than subadult individuals. Our results are in accordance with findings from studies on wild chimpanzees (Seraphin et al., 2008) and wild Assamese macaques (Ostner et al., 2008) which both showed that fecal glucocorticoid levels were higher in aged individuals. Cortisol has also been reported to increase with age in humans (Touitou et al., 1983). Besides possible maturation-related changes in adrenocortical function, elevated FGCM levels in older individuals compared to younger ones may reflect the higher engagement of adult animals in physiologically demanding and potentially stressful activities such as territorial defense (Peres, 1989; Pride, 2005) and reproductive competition (Bercovitch and Ziegler, 2002; Ostner et al., 2008).

Consequently, for both species age needs to be recognized as a significant individual characteristic that interacts with social and ecological variables when shaping glucocorticoid production.

We did not find differences in FGCM levels between males and cycling females in either of the two primate species studied. We believe that this is a valid result because our HPLC data show that the 11β -hydroxyetiocholanolone assay measures the same immunoreactive compounds in both males and females of both species. Thus, our quantitative comparison of FGCM concentrations between the sexes is not significantly biased by cross-reactivity of the antibody with particular sex-specific hormone metabolites, the latter being a major concern with respect to data interpretation when comparing absolute hormone levels between sexes (Goymann, 2012; Preis et al., 2011). Absence of a sex-specific effect on FGCM levels as found in our two New World primate species has also been reported in other mammal species such as California mice (Harris et al., 2012) and black howler monkeys (*A. pigra* (Behie et al., 2010; Martínez-Mota et al., 2007); but see (Rangel-Negrín, 2010)). However, our results for the brown spider monkey contrast with those reported for a closely related species, the Yucatan spider monkey (*Ateles geoffroyi yucatanensis*) where captive-housed males show significantly higher fecal cortisol levels than females (Rangel-Negrín et al., 2009). Whether the latter finding may indicate a species-specific physiological difference in baseline adrenocortical activity within the genus *Ateles* or whether it merely reflects sex differences in the response to the potentially more stressful captive conditions remains unclear. However, a comparison of our results with those of other studies may be generally of limited value since the extent to which FGCM levels in males and females reflect measurements of the same or different metabolites (see (Goymann, 2012)) is usually not reported.

Reproductive condition had a significant effect on FGCM concentrations in female spider monkeys, with levels increasing with progressive gestation. Unfortunately, due to small sample size we were not able to examine the effect of female reproductive state on FGCM output in howler monkeys. We envisage, however, that pregnancy-related increases in FGCM output are also present in howler monkeys given that this pattern is commonly found in female mammals, incl. primate species of all major radiations (e.g.: *Rattus norvegicus* (Atkinson and Waddell, 1995); *Myotis lucifugus* (Reeder et al., 2004); *Lemur catta* (Cavigelli, 1999); *Cebus capucinus* (Carnegie et al., 2011); *Saguinus oedipus* (Ziegler et al., 1995); *Papio ursinus* (Engh et al., 2006; Weingrill et al., 2004); *Mandrillus sphinx* (Setchell et al., 2008); *Macaca mulatta* (Hoffman et al., 2011)). Elevated glucocorticoid levels during gestation are likely caused by interactions of increased metabolic demands on the female and stimulation of the HPA axis by pregnancy-related increases in levels of estrogens (Jones et al., 1989; McLean and Smith, 1999). In addition, elevated glucocorticoid levels during gestation may also arise from placental production of cortisol (Clapp et al., 1982). Our data indicate that female reproductive condition, specifically whether an individual is pregnant or not, strongly influences adrenocortical function and FGCM concentrations (at least in spider monkeys).

Collectively, the present study demonstrates that time of day, age and female reproductive state need to be considered in spider monkeys and to a lesser extent in howler monkeys when FGCM data generated as part of behavioral, ecological or conservation-related studies are interpreted. Specifically, by raising awareness to intrinsic factors confounding FGCM levels our findings should help to better interpret future data on the effects of anthropogenic disturbances and habitat fragmentation on stress hormone metabolite output in these two New World primate species. Using FGCM analysis as part of such conservation management studies might be especially important for the future survival of the critically endangered brown spider monkey. Subsequent studies may help

to detect the most susceptible populations of *A. hybridus* and support the implementation of conservation plans for the species in Colombia and Venezuela.

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