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Validation of a Dehydroepiandrosterone-Sulfate Assay in Three Platyrrhine Primates (*Alouatta caraya*, *Aotus azarae infulatus*, and *Sapajus apella*)

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Abstract

The hormone dehydroepiandrosterone (DHEA) and its sulfate ester (DHEAS) are the most abundant circulating steroids in human and some nonhuman primates, and have been implicated in development, aging and in stress modulation. We aimed to validate a commercially available enzyme immunoassay kit to measure DHEAS in feces and serum of three platyrrhine primate species (*Alouatta caraya*, *Aotus azarae infulatus*, and *Sapajus apella*) in captivity. We collected serum samples from one male and one female from each species. To validate the kit for fecal samples, we conducted a physiological validation by administering DHEA orally to one adult female of each species. We also measured fecal DHEAS levels in four female *Alouatta caraya* individuals during the third semester of gestation and in two females following parturition. We obtained a total of 54 fecal samples and 6 serum samples from 10 individuals. We validated the assay analytically by testing parallelism and accuracy tests in both fecal and serum extracts for all species. We observed a peak in DHEAS 24 h following oral DHEA administration in all three species, with *A. caraya* presenting the strongest response and highest baseline concentrations. DHEAS levels were elevated in pregnant *A. caraya* (57,843.86 ± 37,160.31 ng/g) and declined after parturition (1,539.07 ± 2,894.74 ng/g). Our results demonstrated that these platyrrhines secrete measurable concentrations of DHEAS, with *A. caraya* secreting levels comparable to those of catarrhines. The EIA kit is valid for quantification of fecal and serum DHEAS, and it is useful for studies on stress and primate evolution.

Keywords Animal welfare · DHEA/S · Hormonal analysis · Stress monitoring

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Introduction

The hormone dehydroepiandrosterone (DHEA) and its sulfate ester (DHEAS) are produced in large quantities by the adrenal glands in primates and serve as precursors to sex steroids (Leowattana, 2004; Muehlenbein *et al.,* 2003). Both hormones (DHEA/S) decline with aging (Muehlenbein *et al.,* 2003) and play several functions as immunostimulants (Hazeldine *et al.,* 2010), neuroprotective hormones (Maninger *et al.,* 2009), and glucocorticoid antagonists (Prall *et al.,* 2017). These hormones are also important for parturition (Rainey *et al.,* 2004) and pregnancy maintenance, which makes them useful for gestational monitoring (Takeshita *et al.,* 2016). DHEA/S levels increase at late gestation (Takeshita *et al.,* 2016, 2019) owing to the development of a transient layer in the fetal adrenal known as the fetal zone. This layer secretes high levels of DHEA/S, which are transferred to the mother via the placenta and converted to estrogens (Kaludjerovic & Ward, 2012). The fetal zone regresses quickly after birth, so neonates have a steep decline in DHEA/S levels in the first weeks postpartum (Rainey *et al.,* 2004; Walsh *et al.,* 1984).

Following birth, the age-related pattern of DHEA/S secretion varies with species. A postnatal increase in DHEA/S secretion has been reported and defined as adrenarche in *Homo* (Enomoto *et al.,* 2008; Orentreich *et al.,* 1984), *Pan* sp., *Gorilla gorilla* (Bernstein *et al.,* 2012), and *Pongo pygmaeus* (Prall *et al.,* 2015; Takeshita *et al.,* 2019). In contrast, Afro-Eurasian monkeys, such as *Macaca mulatta* (Kemnitz *et al.,* 2000; Muehlenbein *et al.,* 2003), *Macaca nemestrina*, *Papio cynocephalus* (Muehlenbein *et al.,* 2003), and *Macaca fuscata* (Takeshita *et al.,* 2013), have a continuous age decline in DHEA/S levels, although a short increase in DHEA and DHEAS secretion has been reported in the first weeks post-birth in *M. mulatta* (Conley *et al.,* 2012). Few studies have reported the pattern of DHEA/S in platyrrhines. Studies of *Callithrix jacchus* revealed overall lower DHEA/S secretion than in catarrhines and no evidence of adrenarche. Sex differences are also observed in this species: while the male adrenal gland does not produce DHEA/S, ovariectomized females (but not intact females) showed a significant increase in circulating DHEA levels after adrenocorticotropic hormone (ACTH) stimulation, indicating that adrenal secretion of DHEA in females is regulated by ovarian activity (Pattison *et al.,* 2005, 2007). Whether this pattern is unique to *C. jacchus* is unknown. Comparative studies using other platyrrhine species are crucial to elucidate the role of DHEAS in primate evolution.

Recent studies have highlighted the use of DHEA/S in combination with glucocorticoids (GCs) to monitor animal welfare because of their roles in reducing stress, boosting the immune system, and improving mood (Pluchino *et al.,* 2015; Prall *et al.,* 2017). To minimize or avoid stress to the animal, collection of noninvasive biomaterials, such as feces, urine, and saliva, are the preferred methods of sample collection (Andrabi & Maxwell, 2007; Schwarzenberger *et al.,* 1996). Assays using feces must be carefully validated to determine the antibody specificity and suitability for the species, because only metabolites are present in feces (Heistermann *et al.,* 2006; Palme, 2019; Peter *et al.,* 1996; Schwarzenberger *et al.,* 1996). Although several studies have measured DHEA/S levels noninvasively (Behringer *et al.,* 2012; Seraphin *et al.,* 2008; Takeshita *et al.,* 2013, 2014, 2016, 2018a, b, 2019), only two have validated DHEA/S assays physiologically or biologically for use with feces (Takeshita *et al*., 2018a, 2019).

We aimed 1) to validate a commercially available EIA to measure DHEAS in feces of three species of platyrrhine via a pharmacological challenge and 2) to test the effect of pregnancy on fecal DHEAS levels in *Alouatta caraya*. If the assay detects biological differences in DHEAS, we predicted that we would observe higher DHEAS levels in the final third of gestation compared to those in the postpartum or nonpregnant/nonlactating periods. We chose DHEAS because it is more stable than DHEA and present in higher concentrations in the circulation (Kroboth *et al.,* 1999).

Methods

Experimental Subjects

We studied 10 adult animals, based on age classifications established for each species: five female and one male *Alouatta caraya* (7.4 ± SD 3.43 years, range 4–12 years; Rímoli *et al.,* 2012), one female and one male *Aotus azarae infulatus* (14 ± SD 2.82 years, range 12–16 years; Aquino & Encarnación, 1994), and one female and one male *Sapajus apella* (15 ± SD 7.07 years, range 10–20 years; Fragaszy *et al.,* 2004). The animals belong to the breeding colonies of the National Primate Center (Centro Nacional de Primatas, CENP) in the district of Ananindeua, Pará, Brazil, 1°38′26″, 48°38′22″). They were kept in enclosures positioned in a north–south orientation to receive ≤12 h of natural light. The *A. a. infulatus* were housed in individual enclosures (1.5 m *D* × 1.0 m *W* × 2.0 m *H*), and had visual, olfactory, and auditory contact with other members of the colony. The other two species lived in groups of *ca.* 10 individuals. The enclosures measured 3.75 m *D* × 2.2 m *W* × 2.4 m *H* (*A. caraya*) and 3.85 m *D* × 2.6 m *W* × 2.5 m *H* (*S. apella*). Inside the enclosures, there were two wooden walkways, access to food bowls, and external water bottles for drinking. The animals were fed according to CENP's standard management practices. Their diet contained different types of fruits and vegetables, eggs, and commercial primate food with 18% crude protein (Cebidae P18 Megazoo, portion Megazoo, Betim, Minas Gerais, Brazil). We also provided daily supplements of amino acids, vitamins, macro and micro minerals, and 0.5 g of Aminomix Pet® (Vetnil Ind. Veterinary Products Ltda. Louveira, São Paulo, Brazil) per kg of body mass. Water was offered *ad libitum*.

Experimental Procedure

Physiological Validation In compliance with the ethics standards to reduce the use of animals in research, we conducted the pharmacological challenge in only one individual of each species. We selected females for this challenge because, in the only platyrrhine primate studied (*Callithrix jacchus*), males do not secrete DHEAS from the adrenal gland (Pattison *et al.,* 2007).

To validate the DHEAS assay, we administered one dose of DHEA (Youthful You™ DHEA, available in 5-mg capsules) to each female (day zero), by mixing the contents of the capsules with the fruit in the animals' regular diet. Although we did not measure the precise concentrations of the DHEAS powder, we used each capsule as estimates of 5 mg. We calculated the dosages for each species based on a validation in *Macaca fuscata* (Takeshita *et al*., 2018a) and adjusted according to body mass: 35 mg

for *Alouatta caraya* (7.5 kg), 5 mg for *Aotus azarae infulatus* (1.0 kg), and 7.5 mg for *Sapajus apella* (2.7 kg). The animals ingested all the DHEA administered in the fruits.

Sample Collection We transferred the two *Alouatta caraya* and *Sapajus apella* individuals to single cages to facilitate the experimental procedures. We started the experiment 10 days after the transfer to acclimatize the animals and to avoid the confounding effects of stress from the transfer (Takeshita *et al.,* 2014). We did not move the *Aotus azarae infulatus* animals because they were already housed individually. We collected fecal samples for 4 days before the DHEA administration (challenge) to determine DHEAS baseline levels in all species (days −4 to −1). Following DHEA administration, we conducted daily sampling on the challenge day (day 0) and for 5 days post-challenge (days 1–5), totaling 10 samples per animal. All sampling occurred between 08:00 h and 12:00 h to minimize the effect of circadian rhythms on hormonal concentrations. We stored each sample in plastic bags labeled with animal ID, date, and time of collection at −80°C within 1 h of collection. We discarded all samples contaminated with urine or remnants of materials from the enclosures.

After 10 days of fecal sampling, we collected a blood sample from each of the three females to determine their serum DHEAS levels. To mitigate stress from blood collection, we restrained the subjects chemically by administering a combination of 10% ketamine hydrochloride (3–5 mg/kg), dexmedetomidine (0.01 mg/kg), and midazolam (0.2 mg/kg) intramuscularly. We collected 1–4 mL of blood from the femoral vein, using sterile syringes and needles (gauge 14–21). We transferred the samples to a tube and centrifuged them at 4000 rpm for 7 min. We transferred the serum to clear microtubes and stored it at −80°C.

Although the males did not participate in the challenge, we collected three fecal samples and one blood sample from each (totaling nine fecal samples and three serum samples) using the same protocols as above. We combined serum samples from males and females to validate the assay analytically for each species and to provide preliminary data for each species' hormonal levels, given that the literature often reports the average for males and females (Bernstein *et al.,* 2012; Rege *et al.,* 2019).

Effect of Gestation We examined the effect of gestation opportunistically, as there were four pregnancies in the *Alouatta caraya* colony during the study. For the safety of the animals, we collected only fecal samples from these females in their own enclosure. To identify samples, we separated the females from the group temporarily on *x* nonconsecutive mornings. To do this, we moved them to an adjacent area through a sliding door. They rejoined the group as soon as the samples were collected. We collected 1–3 samples per female (total 10), on days ranging from 90 to 1 day before parturition. Following the births, we obtained 5 samples from 2 of these females (2–3 samples/female), 6–16 days postpartum.

Hormonal Analyses

Fecal Extraction We lyophilized fecal samples (L101, Liobras Ltda, São Carlos, Brazil) and extracted them by adding 2 mL of absolute ethanol to 0.2 g of sample. We homogenized the samples in a multivortex (TS - 100 Thermo-Shaker, Biosan SA, Riga, Latvia) at 1000 rpm for 30 min, then centrifuged them at 5000 rpm for 15 min (5427 R Eppendorf, Hamburg, Germany). We stored the supernatant at −80°C until hormonal analyses.

Preparation of Standards, Controls, and Samples We prepared five calibrators by serially diluting the top standard in assay buffer from 60,000 pg/mL to 96 pg/mL, according to the manufacturer's instructions (Arbor Assays EIA Kit, K054-H5; Ann Arbor, MI, USA). To determine the best dilution for each species, we serially diluted a pool of fecal extracts containing six samples (three from the female pre-challenge, and three from the male, for each species) at 1:5, 1:25, 1:125, 1:625, and 1:3125 in the provided assay buffer. For the serum, we serially diluted three pools containing male and female samples from each species at 1:2, 1:4, 1:8, 1:16, 1:32, and 1:64. We prepared the control at a concentration of 600 pg/mL.

Parallelism and Accuracy We performed parallelism tests for feces and serum using the serially diluted pool. We confirmed parallelism between the curves generated by the optical density (OD) of each pool and the standard curve, visually and using an F test. We conducted an accuracy test by adding the pools of fecal extracts and serum to equal parts of known quantities of calibrators (12,000, 2400, 480, 96 ng/mL). We calculated recovery as the observed/expected values based on unspiked samples and expressed as a percentage.

Assay Procedures We performed hormonal analyses using the DHEA-S Arbor Assays EIA kit (K054-H5) and a plate reader (Thermo Scientific ™ Varioskan ™, Vantaa, Finland), following the manufacturer's instructions. We analyzed the absorbance results in the Microplate Manager v6 software in a four-parameter curve.

The cross-reactivity of the antibody used in the DHEAS kit is 100% DHEAS, 162% for DHEA, 44.5% for epiandrosterone, 28.4% for androsterone, 15.2% for androstenedione, 0.5% for DHT, 0.4% for adrenosterone, 0.4% for testosterone, 0.2% for deoxycorticosterone and progesterone, and <0.1% for the other steroids.

Data Analysis

We carried out all analyses using GraphPad Prism Software (version 7.0, GraphPad Software Inc., San Diego, CA, USA). For the pharmacological challenge, we calculated baseline fecal DHEAS concentrations for each species as the mean of DHEAS concentrations obtained before the challenge. We defined the time lag between hormonal secretion and excretion in metabolites as the time between DHEA administration and the hormonal peak. We tested the effect of gestation by comparing fecal DHEAS levels before and after parturition. We used the fecal DHEAS levels of the female *Alouatta caraya* from the DHEA challenge for DHEAS levels in a female that was not pregnant or lactating. We did not perform statistical analyses because of our small sample size. We assessed analytical validation using accuracy and parallelism tests in a pooled sample of each species to exclude matrix effects in both serum and fecal extracts. Pooled samples are often used to determine the dilution rate for a species because it represents the mean hormonal levels of a given species (Behringer *et al.,* 2012; Bernstein *et al.,* 2012; Rege *et al.,* 2019; Takeshita *et al*. 2018a). We report the value obtained from the pooled (male and female combined) serum of each species at the dilution rate detected at 50% binding for comparison with findings for other species.

We report the mean ± standard deviation (SD) as measures of central tendency and variation. We set α at 0.05.

Ethical Note

The project followed all the guidelines contained in the resolutions of the National Council for the Control of Animal Experimentation – Ministry of Science and Technology (CONCEA-MCT, Brazil), and approved by the Ethics Committee on the Use of Animals (CEUA no. 43/2019) of the Institute Evandro Chagas (IEC), Ananindeua, Pará, Brazil. The authors declare that they have no conflict of interest.

Data Availability The datasets during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Results

Parallelism and Accuracy of the Assay

The ideal dilutions for measuring DHEAS in the three species ranged from 1:50 to 1:200 for fecal extracts and from 1:4 to 1:64 for serum (Table I).

The dilutions performed for the DHEAS assays in the three species were parallel to the standard curve (Fig. 1), and *F* tests showed that the curves were not significantly different (Table II). This indicates no matrix effects in both feces and serum pools. The mean intraassay coefficient of variation for the DHEAS test was 9.22% ($N = 29$) and the interassay coefficient was 14.95% ($N = 3$).

The overall mean recovery for fecal extracts was 114% ± 20 ($N = 4$) for *Alouatta caraya*, 110% ± 2 ($N = 4$) for *Sapajus apella*, and 107% ± 7 ($N = 4$) for *Aotus azarae infulatus*. The mean recovery for serum was 92% ± 21 ($N = 4$) for *A. caraya*, 5% ± 18 ($N = 4$) for *S. apella*, and 120% ± 23 ($N = 4$) for *A. a. infulatus*.

Pharmacological Challenge

The pharmacological challenge (oral administration of DHEA) resulted in an adrenal response in all three individuals. In *Alouatta caraya*, fecal DHEAS concentrations were 13,281.15 ± 4092.38 ng/g ($N = 4$) before the challenge and peaked 24 h after it—an increase of 672% (89,276.27 ng/g) (Fig. 2a). In *Aotus azarae infulatus* fecal DHEAS concentrations were 1217.78 ± 642.03 ng/g ($N = 4$) before the challenge, with two peaks, one at 24 h, with an increase of 308% (3753.84 ng/g) ($N = 4$), and a second peak at 72 h after administration, with an increase of 758% (9227.58 ng/g) (Fig. 2b). In *Sapajus apella* fecal DHEAS

Table I Dilutions of fecal extracts and serum for DHEAS in three species of platyrrhine housed at the National Primate Center, district of Ananindeua, Pará, Brazil, January 2020

Species	Fecal extracts	Serum
Alouatta caraya	1:200	1:64
Aotus azarae infulatus	1:50	1:16
Sapajus apella	1:100	1:4

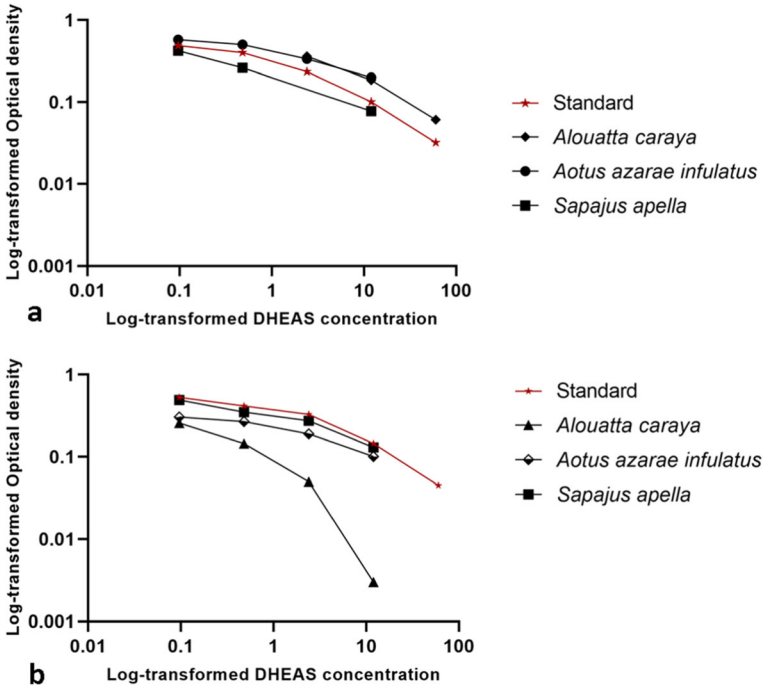

Fig. 1 Log-transformed optical density of DHEAS standard curve and a combined (**a**) fecal extract and (**b**) serum samples in three platyrrhine primate species (housed at the National Primate Center, in the district of Ananindeua, Pará, Brazil, in January 2020), diluted serially in assay buffer.

concentrations were 359.91 ± 255.00 ng/g ($N = 4$) before the challenge and peaked 24 h after administration, with an increase of 341% (1227.92 ng/g) (Fig. 2c).

Effect of Gestation

The mean fecal DHEAS levels in pregnant *Alouatta caraya* (57,843.86 ± 37,160.31 ng/g; $N = 10$) were above those for the female that was not pregnant or lactating (13,281.15 ± 4092.38 ng/g; $N = 4$), and samples from to late gestation had the highest DHEAS levels. After delivery, fecal DHEAS levels were 37-fold lower (1539.07 ± 2,894.74 ng/g; $N = 5$) than mean pregnancy levels (Fig. 3).

Table II Results of F tests for parallelism for DHEAS assays in three species of platyrrhine housed at the National Primate Center, district of Ananindeua, Pará, Brazil, January 2020

Species	Fecal extract			Serum		
	F	df	P	F	df	P
Alouatta caraya	1.64	4, 2	0.826	3.07	4, 3	0.384
Aotus azarae infulatus	1.31	4, 3	0.860	4.78	4, 3	0.229
Sapajus apella	1.29	4, 2	0.974	1.72	4, 3	0.684

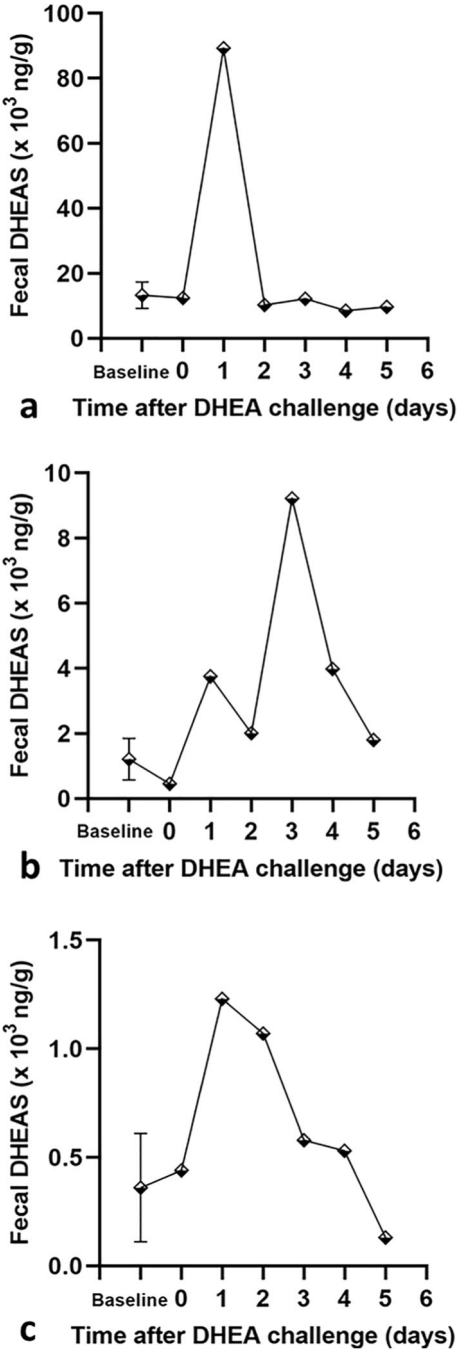

Fig. 2 Changes in fecal concentrations of DHEAS ($\times 10^3$) over time in a female (**a**) *Alouatta caraya*, (**b**) *Aotus azarae infulatus*, and (**c**) *Sapajus apella* (housed at the National Primate Center, in the district of Ananindeua, Pará, Brazil, in January–March 2020) after oral administration of DHEA (day 0). Error bars indicate standard error of three samples collected before the challenge.

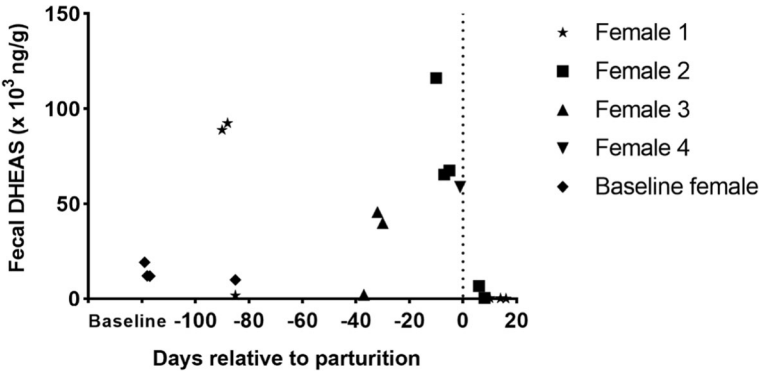

Fig. 3 Comparison of DHEAS concentrations (× 10^3) in *Alouatta caraya* (housed at the National Primate Center, in the district of Ananindeua, Pará, Brazil, in January–April 2020) during pregnancy (N = 4) and lactation (N = 2) with baseline levels (three samples from one nonpregnant/nonlactating female). Day 0 = day of parturition.

Serum DHEAS Concentration

The mean serum DHEAS levels obtained from the combined samples (one male + one female) for each species were 258.93 ng/mL in *Alouatta caraya*, 57.37 ng/mL in *Aotus azarae infulatus*, and 13.30 ng/mL in *Sapajus apella*.

Discussion

The accuracy and parallelism tests demonstrated that neither serum nor fecal samples exhibited matrix effects, indicating that these samples can reliably quantify DHEAS levels. The physiological validation showed that the assay measured DHEAS levels from fecal metabolites. The increase in DHEAS concentrations after oral administration of DHEA is expected due to conversion of DHEA to DHEAS, which occurs through the action of the enzyme DHEA sulfotransferase (SULT2A1) (Rainey *et al.,* 2004).

The DHEAS excretion time lag varied from 24 h to 72 h across species. The three species also differed in the time taken to return to baseline levels, with *Alouatta caraya* doing so faster than the other species. This is surprising, given that *A. caraya* has the slowest metabolism and highest degree of folivory of the three species (Glander, 1980; Milton, 1980). *Sapajus apella* have relatively higher metabolism and feed on a variety of foods (Fragaszy *et al.,* 2004). The gastrointestinal transit time of *Alouattas* sp. is approximately eight times greater than that of *Sapajus* sp. (Milton, 1984, 1993), which is related to the higher amount of fiber in the diet of folivores. However, the animals described in this study live in captivity, and their diets contain fruits and vegetables, which may have decreased the species differences in gut retention time and fecal frequency (Palme, 2019). Other factors that could have contributed to our findings are species-specific differences in the metabolic route, enzymatic activity of gastrointestinal bacteria, and differences in body mass (Bahr *et al.,* 2000; Goymann, 2012; Möstl & Palme, 2002).

The DHEAS fecal excretion time observed in *Alouatta caraya* is similar to the excretion of fecal GC metabolites in *A. caraya* challenged with ACTH (Buti *et al.,*

2018). This suggests that 24 h may be the standard time of secretion of adrenal steroids in this genus. Although *Sapajus apella* and *Aotus azarae infulatus* had similar peaks at 24 h, they had a slower decrease in DHEAS concentrations, taking 96 h to return to baseline levels.

The results of the DHEA challenge in *Sapajus apella* and *Aotus azarae infulatus* were similar to those in female *Macaca fuscata*, in which fecal DHEAS concentrations increased at 24 h and returned to baseline at 72 h after oral DHEA administration (Takeshita *et al*., 2018a). In addition, there were two peaks in *A. a. infulatus*, with the second peak being higher than the first. The explanation for the second peak is unclear, but it might be related to 1) gut reabsorption (Palme, 2019) or 2) further conversion of the DHEA to DHEAS in other tissues (Klinge *et al.,* 2018). It is also possible that the individual experienced acute stress during the experimental procedure (e.g., during feeding procedures or from visual communication with conspecifics), which could have elicited a DHEAS response (Takeshita *et al*. 2018, 2019). Furthermore, considering that *Callithrix* has a peculiar adrenal mechanism not observed in other species (Pattison *et al.,* 2005; 2007), the owl monkey adrenal may also have a different mechanism. Further studies are needed to test these hypotheses.

We observed an increase in DHEAS concentrations in pregnant females in relation to a female that was not pregnant or lactation, followed by a decrease after parturition. These findings are consistent with those for *Macaca fuscata* and *Pongo pygmaeus*, which detected high fecal DHEAS levels at the end of pregnancy (Takeshita *et al.,* 2016, 2019). Gestation lasts a mean of 180 days in *Alouatta caraya* (Calegaro-Marques & Bicca-Marques, 1993; Kowalewsky & Zunino, 2004), and we found an increase in DHEAS concentrations in the final third of pregnancy, *ca.* 30 days before parturition and at 83% of the gestational period, with the highest concentrations close to parturition, at about 95% of the gestational period. The increase observed at the end of pregnancy and decline after parturition may be associated with the development of the fetal adrenal gland. In *Sapajus* sp., which have a 155-day gestation period (Rylands & Mittermeier, 2013), the adrenal gland of the fetus increases in size significantly between 90 and 141 days (from 58% to 90% of the gestational period) (Torres-Farfan *et al.,* 2003). The development of the fetal zone at the end of pregnancy contributes to the high concentrations of DHEAS as a source of estrogens necessary for parturition, which plummet to lower levels after birth due to the regressing fetal zone (Klinge *et al.,* 2018; Rainey *et al.,* 2004; Walsh *et al.,* 1984). A study in *M. fuscata* suggested that low DHEAS levels at this stage may indicate fetal death, which makes this method useful for monitoring reproductive success in free-ranging primates noninvasively (Takeshita *et al.,* 2016).

Alouatta caraya showed higher baseline concentrations of DHEAS in feces and serum, followed by *Aotus azarae infulatus*, with lower concentrations in *Sapajus apella*. The concentration of DHEAS is highly variable among primates and is related to differences in the expression of adrenal enzymes involved in the conversion of DHEA to DHEAS (Rege *et al.,* 2019). In addition, circadian rhythm may have influenced these differences (Hucklebridge *et al.,* 2005). We collected samples in the morning, but the circadian rhythm of the nocturnal *A. a. infulatus* is likely to differ to that for the two diurnal species and could have influenced comparisons (Pieper & Lobocki, 2000). In addition, *A. a. infulatus* were housed singly, which increases DHEAS levels in *Macaca fuscata*, in comparison to socially living individuals

(Takeshita *et al.,* 2014). Future studies controlling for these two variables are needed to compare DHEAS levels across these species.

Serum DHEAS levels in our *Alouatta caraya* sample were higher than those reported in most catarrhine primates, such as *Cercopithecus* (134.87 ng/mL), *Macaca* (186.03 ng/mL), *Symphalangus* (116.19 ng/mL), and *Pongo* (109.06 ng/mL), comparable to that described in *Gorilla* (227.55–355 ng/mL) (Bernstein *et al.,* 2012; Edes, 2017) and lower than that described in the genera *Pan* (669.46 ng/mL) and *Homo* (1206.7 ng/mL) (Bernstein *et al.,* 2012). Previous studies did not consider the ability of platyrrhine primates to secrete DHEAS from the adrenal gland based on studies on *Callithrix*¸ which secretes low or nondetectable levels of DHEAS (Pattison *et al.,* 2005; Pattison *et al.,* 2007). However, due to the great diversity of platyrrhine primates, comparative studies that include other platyrrhine species with different ecology, behavior, and life histories are needed to understand the possible role of DHEAS in their evolution. This is supported by our data on the interspecies variation in DHEAS levels, and by the fact that *A. caraya* had levels comparable to those of catarrhine primates.

Serum DHEAS concentrations in *Aotus azarae infulatus* were similar to those described in *Colobus* (35.54 ng/mL) (Bernstein *et al.,* 2012). Only one study has measured DHEA in *Aotus* sp., noninvasively (from urine samples), to investigate the effect of reproductive experience on the DHEA/cortisol ratio in mated pairs (Bardi *et al.,* 2014). The authors found that mated pairs with reproductive experience showed more efficient foraging responses than those without reproductive experience, and a higher DHEA/cortisol ratio, suggesting that behavioral and social factors influence DHEA, and consequently, DHEAS levels in this species. Further studies and a larger sample size that controls for social factors are needed for interspecies comparison in DHEAS secretion.

Sapajus apella had the lowest DHEAS concentration among the three platyrrhine primates we evaluated. Its concentration was also low relative to those in catarrhines. However, the number of samples presented in this study is limited in comparison to studies of catarrhines. We used only adult animals and combined the sexes in our sample, and the lack of infants and juveniles in our dataset and potential sex differences may have influenced the overall DHEAS levels of this species. One study reports serum DHEAS concentrations in fetuses of *S. apella* (Torres-Farfan *et al.,* 2003) but we cannot compare our data with the data in that study because given that fetuses have significantly higher DHEAS levels due to the fetal adrenal (Raineyet *al.,* 2004; Takeshita *et al.,* 2013, 2016).

In addition to the enzymes involved in DHEAS biosynthesis, the percentage of free and bound hormone in the serum can influence the results (Barsano & Baumann, 1989). Other factors that need to be considered are related to the metabolism of DHEA and DHEAS, which varies according to sex and age, as well as species-specific metabolic clearance rates (Whitham *et al.,* 2020). Furthermore, DHEA and DHEAS can be metabolized in several tissues, such as the placenta, ovary, testicles, prostate, adipose tissue, liver, and brain, which are tissues associated with processes directed by a nuclear sex hormone receptor (Klinge *et al.,* 2018). All these factors may have influenced the interspecies variation in DHEAS levels found in this study.

In summary, we validated a noninvasive method to measure DHEAS in three platyrrhine species. Differences in DHEAS levels among these species suggest that

some platyrrhine species secrete DHEAS at levels comparable to catarrhines. To better understand the functions and age-related patterns of DHEAS secretion and excretion in primate evolution, comparative research involving platyrrhines is needed. These studies may help us to develop platyrrhine models for studying the effects of DHEAS on cognition and neonatal development, age-related disorders, and on the evolution of the adrenal gland among primate species (Campbell, 2020; Takeshita *et al*., 2018b). Furthermore, the GC/DHEAS index can be used as part of a multifaceted and more accurate assessment of stress (Takeshita *et al.,* 2019; Whitham *et al.,* 2020) and can be applied to monitor primate welfare and reproduction.

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Author Contributions RSCT and FOBM conceived and designed the study. GPS, JTM, and ANPF performed the experiments and collected data. GPS and RSCT analyzed the data. GPS, RSCT, and FOBM wrote the manuscript; the other authors provided editorial advice.

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