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RESEARCH ARTICLE

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Validation of an enzyme immunoassay for measurement of fecal dehydroepiandrosterone sulfate in gibbons and siamangs

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Abstract

Monitoring wildlife stress levels is essential to ensure their quality of life in captivity or in the wild. One promising method to assess the stress response is the comeasurement of glucocorticoids (GC) and dehydroepiandrosterone sulfate (DHEAS), adrenal hormones involved in the modulation of the stress response. Although noninvasive methods to measure GCs have been validated in several species, only a few studies have validated DHEAS assays. The aims of this study were (1) to describe an enzyme immunoassay (EIA) to measure DHEAS levels, (2) to validate this assay for fecal samples in gibbons and siamangs, and (3) to test hormonal stability after one freeze-thaw cycle and over time at two freezer temperatures (−20°C and −80°C). Subjects included 32 gibbons and siamangs from U.S. zoological parks. The EIA was validated analytically by parallelism and accuracy tests, and biologically by confirming a DHEAS response 1–2 days after a stressful event (accident, vaccination, or transportation) in three individuals. In addition, fecal DHEAS levels in a pregnant female were above nonpregnant/nonlactating levels and declined progressively the following parturition. The hormonal stability experiments revealed no significant changes in fecal DHEAS levels after one freeze-thaw cycle. Hormonal levels in fecal extracts were stable for 2 months, regardless of the storage temperature, with no significant differences between −20°C and −80°C conditions. The EIA described has high sensitivity and it is suitable for fecal DHEAS measurement in gibbons and siamangs, with a potential to be applied to other species.

KEYWORDS
adrenal steroids, DHEAS, gibbons, noninvasive endocrinology, siamangs

1 | INTRODUCTION

Monitoring wildlife reproduction and stress levels is essential to ensure their quality of life in captivity or in the wild (Behringer & Deschner, 2017; Hodges et al., 2010; Monfort, 2003). Among the methods to monitor animal welfare, glucocorticoids (GC) are one of the most common biomarkers of stress, and have been measured in a wide range of mammals (Keay et al., 2006; von der Ohe & Servheen, 2002; Tilbrook et al., 2000) and birds (Bonier et al., 2011; Messina et al., 2020) due to the development of noninvasive techniques that allow the measurement of these hormones from feces, urine, hair, and saliva (Hodges et al., 2010; Koren et al., 2002; Monfort, 2003). High GC levels are indicative of increased levels of stress that may interfere with reproduction through the inhibition of the hypothalamic–pituitary–gonadal axis (Toufexis et al., 2014). However, recent studies have raised several concerns for using GC as sole indicators of stress due to potential misconceptions of stress as always being detrimental. According to Selye (1956), stress is defined as a series of physiological responses to any disturbance to an organisms' homeostasis. However, these disturbances can include events that are part of an individual's life history, such as changes in season (Takeshita et al., 2014), diet (von der Ohe & Servheen, 2002), reproductive state (Carnegie et al., 2011;

Palme, 2019; Takeshita et al., 2014), and temporary increases in activity or exercise (Chen et al., 2017; Hackney & Walz, 2013; Suarez-Bregua et al., 2018). Under these conditions, the GC response is considered protective, enabling the body to cope with the changes to restore balance. In addition, intrinsic factors can influence GC levels, such as body mass (Edwards et al., 2020), sex, and time of the day (Touma et al., 2003), which may produce confounding results and limit the interpretation of GC levels.

In this context, the term "allostatic load" has been used to refer to unpredictable events that result in the wear and tear on the regulatory systems in the brain and the body (Dowd et al., 2009; Guidi et al., 2021; McEwen, 2005; Seeman et al., 2001). The allostatic load may result in decreased GC levels due to the inability of the organism to cope with the stressful event (Ullmann et al., 2019), which further illustrates the limitation of GCs in studying animal welfare. One promising candidate to assist with the interpretation of GC levels is the comeasurement of dehydroepiandrosterone sulfate (DHEAS), an adrenal hormone involved in the modulation of the stress response (Hechter et al., 1997). During acute stress, DHEAS levels increase and act as a GC-antagonist by binding to GC receptors which assist the body in restoring homeostasis (Prall et al., 2017). Therefore, DHEAS can be interpreted as the ability of the individual to cope with stress. A recent study reported that individuals exposed to stress during early development are permanently affected by a reduction in DHEAS responsiveness to stress. Given the beneficial roles of DHEAS in enhancing the immune response (Bauer et al., 2009; Hechter et al., 1997; Prall et al., 2017) and in neuroprotection (Kimonides et al., 1999; Majewska, 1995; Maninger et al., 2010), a high GC/DHEAS ratio is associated with an inability to cope with stress and maybe detrimental if maintained for a prolonged period (Goncharova et al., 2012; Takeshita et al., 2014). For example, cortisol levels were similar in healthy captive seals and in those suffering from diseases, but the diseased animals had relatively lower DHEA levels and a higher cortisol/DHEA ratio (Gundlach et al., 2018). For this reason, a growing number of studies have reported the use of the cortisol/DHEAS ratio as a biomarker of welfare, with a high GC/DHEAS ratio being commonly associated with depression-like behavior (Goncharova et al., 2010), inflammation (Almeida et al., 2008), disease (Gundlach et al., 2018), or poor environmental conditions in a range of wild and domestic animals (reviewed in Whitham et al., 2020).

In addition, DHEAS has a direct role in reproduction because it can be converted to sex steroids (Burger, 2002; Labrie, 2010). In marmosets, ovariectomized females had increased DHEAS levels compared to intact females (Pattison et al., 2007), suggesting that DHEAS is an important source of estradiol when their gonadal synthesis is compromised (e.g., during senescence or gonadectomy). The connection between stress and reproduction also makes DHEAS ideal for wildlife monitoring. Moreover, DHEAS levels decline with age, which has prompted its use as a biomarker of physiological aging (Muehlenbein et al., 2003; Orentreich et al., 1984; Takeshita et al., 2013). DHEAS measures can be useful for developing strategies to maximize breeding programs and to improve quality of life. For instance, one study in rhesus monkeys (*Macaca mulatta*) showed that caloric restriction buffered the age-related decline on DHEAS levels (Mattison et al., 2003), which may influence the reproductive rate of the species. Due to its broad utility, an increasing number of studies have incorporated DHEAS in domestic and wildlife monitoring (Poisbleau et al., 2009; Takeshita et al., 2014, 2016; Whitham et al., 2020).

Another advantage of DHEAS is that, like GCs, it can be detected using noninvasive methods because it is measurable in urine, feces, and saliva. While several enzyme immunoassays (EIA) for fecal GC measurement have been validated for a wide range of wild animals (Palme, 2019), only a few studies have validated DHEAS in samples obtained noninvasively, including previous studies in Japanese macaques (*Macaca fuscata*) (Takeshita et al., 2018) and orangutans (*Pongo pygmaeus*) (Takeshita et al., 2019). DHEAS levels have been successfully measured in feces or urine in orangutans (Takeshita et al., 2019), chimpanzees (Seraphin et al., 2008), bonobos (Behringer et al., 2012), and macaques (Takeshita et al., 2018, 2014, 2013, 2016), but no studies have measured DHEAS noninvasively in the lesser apes gibbons and siamangs (family Hylobatidae). Both gibbons and siamangs are characterized by a facultatively monogamous social system (Reichard, 2003), a relatively slow life history that includes sexual maturity at about 6–9 years old (Geissmann, 1991; Reichard & Barelli, 2008; Reichard et al., 2012), and a lifespan of up to 60 years in captivity (Geissmann et al., 2009). Out of the 20 recognized species within the Hylobatidae family, 25% are classified as critically endangered, 70% as endangered, and 5% as vulnerable (IUCN, 2021). Their conservation status and their phylogenetic value as the only lesser apes justify the application of noninvasive methods to monitor their welfare.

The application of EIA techniques to measure steroid hormones from feces requires careful validation to determine the antibody specificity to the target hormone in each species. For instance, cross-reactivity between dehydroepiandrosterone (DHEA) and testosterone has been reported in fecal metabolites of long-tailed macaques (*Macaca fascicularis*) (Möhle et al., 2002). In addition, storage conditions may affect hormonal stability and interfere with the results (Livesey et al., 1980). This is often a concern in field stations where cryopreservation methods are absent or unstable, and fecal samples may undergo freeze-thaw cycles during storage or transportation, particularly during periods of dry ice shortage. Similarly, hormonal changes have been reported in the baboon (*Papio cynocephalus*) fecal extracts stored at different conditions and over time (Khan et al., 2002). Understanding what conditions affect hormonal concentrations is essential to ensure assay reliability and repeatability. In this context, the goals of this study were to (1) describe a new EIA to measure DHEAS concentrations, (2) to validate the EIA for siamang and gibbon feces, and (3) to test hormonal stability after one freeze-thaw cycle and over time in two freezer temperatures.

2 | METHODS

2.1 | Subjects

The subjects were 32 individuals (14 males and 18 females) of the family Hylobatidae (9 *Hylobates* sp., 9 *Nomascus* sp., and 14 *Symphalangus* sp.) from the U.S. Zoological Parks. Their ages ranged from 1 to 54 years old.

They all lived in indoor enclosures with access to outdoor exhibits. All animals were fed according to the zoos' husbandry practices. No changes in the zoo routine were required for this project.

2.2 | Ethical note

The study complied with the US National Research Council's Guide for the Care and Use of Laboratory Animals, the US Public Health Service's Policy on Humane Care and Use of Laboratory Animals, and the Guide for the Care and Use of Laboratory Animals. Due to the noninvasive and opportunistic nature of sampling, this study was exempted from Kent State University Institutional Animal Care and Use Committee review and oversight (T 115 RT 19-06). The study was approved by the Gibbon Species Survival Plan (SSP) and by all participating zoos.

2.3 | Sample collection

Between 3 and 11 fecal samples/individuals were collected by the zoo staff. Food dye was provided to assist with sample identification. Samples were collected within 2 h from the estimated defecation time. Approximately 5–10 g of feces was collected using wooden sticks and stored in labeled ziplock bags with individual ID, date, defecation and collection time, and feces consistency according to the fecal scoring established by the Gibbon SSP, of which one is the most solid and seven is the most liquid and loose type (B. Richards [personal communication, 2019]). Loose feces (quality 6–7) and samples contaminated with urine were discarded. All samples were stored frozen at −20°C within 1 h after collection and shipped to Kent State University with 2.5 kg of dry ice.

2.4 | Extraction

All fecal samples were freeze-dried (Labconco), pulverized, and filtered to remove nonfecal matter (e.g., leaves, seeds). Approximately 0.1 g of dry feces was added to 5 ml of 80% methanol and vortexed for 30 min. The samples were centrifuged (5000 rpm × 10 min), and the supernatant was transferred to a tube and stored at −20°C until assayed. To evaluate extraction efficiency, one of the samples was extracted in duplicate. The duplicate sample was spiked with 50 µl of DHEAS (0.5 ng/ml, diluted in assay buffer from 1 mg/ml, Miliporeigma; Cat# D-65-1), and the other sample was extracted normally. Extraction efficiency was calculated as the observed/expected concentration of the spiked sample and expressed as a percentage.

2.5 | Parallelism, accuracy, and sensitivity

Parallelism was tested by measuring three pooled samples, each containing fecal extracts from four individuals for each of the three species (*Hylobates* sp., *Nomascus* sp., or *Symphalangus* sp.). The pooled extracts were serially diluted (from 1:2 to 1:1028) with assay buffer (Arbor Assays; Cat#X065). The slope from the curves generated by the optical density (OD) and concentrations of the pooled samples was compared against the standard curve visually and confirmed by an F-test. To test accuracy, the three pooled samples were first diluted to fall within a measurable range in the assay standard curve and then spiked with three known amounts of DHEAS. Sample recovery was calculated as observed/expected values based on the unspiked samples and expressed in percentage. To determine the sensitivity of the assay, the OD of a serial dilution of 14 standards (starting from 50 ng/ml) was measured, along with 32 replicates of assay buffer (B0—maximum binding). The sensitivity was defined as the concentration of the standard with the highest OD value that was lower than two standard deviations (SDs) below the B0 mean.

2.6 | Biological validation

Biological validation was conducted opportunistically by testing hormonal changes under two conditions: response to stress and pregnancy. The response to stress was tested in two female siamangs (*Symphalangus* sp.) and one male gibbon (*Nomascus* sp.). Two females (ages 9 and 27 years old) experienced stressful procedures as part of their zoo husbandry routine. The first female received a tetanus vaccine directly via the enclosure mesh (without capture or anesthesia) followed by positive reinforcement. Before the vaccine, the female had received training for voluntary injection, which may minimize the stress from the procedure. The second female was immobilized and transported to a different exhibit. The male gibbon (21 years old) had fallen from approximately a 10 m height while brachiating on a rope, and although did not sustain any apparent injuries, the staff reported some behavioral changes which included avoidance of heights for a few days following the incident. Given that stress induces an increase in DHEAS levels in nonhuman primates (Goncharova et al., 2012; Takeshita et al., 2018, 2014, 2019), I expected to observe a DHEAS response after the stressful events. Following the sampling protocol described above, daily fecal samples were collected whenever possible, for 3–6 days before the stressful events until 3–7 days following the event (N = 5–11 samples/individual).

For the second validation test (pregnancy), 10 fecal samples were collected from a female siamang (13 years old) from 46 days prepartum to 31 days postpartum. Given that DHEAS levels increase in late gestation as the fetal adrenal glands enlarge (Rainey et al., 2004; Takeshita et al., 2016), I expected that DHEAS levels would be elevated during pregnancy and decline during the postpartum period. For comparative purposes, eight adult females (age range = 14–38 years old), intact, nonpregnant, nonlactating, and noncontracepted were included in this dataset to compare with levels observed during pregnancy. Samples were also collected from four lactating females (age range = 11–29 years) who were between 1 and 4 months postpartum, and these were compared with levels of the recently parturient female to determine the time that takes for DHEAS levels to drop in the mother after parturition.

2.7 | Hormonal stability tests

To test the effect of one freeze-thaw cycle on DHEAS concentrations, 23 frozen samples from 10 individuals were broken down with a mallet to homogenize the samples. Part of each sample was transferred to a new ziplock bag and stored in a cooler box placed at room temperature but containing one ice pack. This method aimed to mimic conditions where sample transportation would be done without dry ice. The remainder of the sample was immediately stored at −20°C without thawing. After 24 h, the samples that were stored in the cooler box were completely thawed and were then transferred to the freezer (−20°C) containing the control (non-thawed) samples until extraction.

To test the effect of freezer temperature on fecal extract storage over time, the supernatants of 17 samples from 10 individuals collected at the end of extraction were aliquoted into two tubes, one tube was stored at −20°C and the other at −80°C. Samples from both freezers were measured in the same plate at 1 day, 1 week, 2 weeks, 1 month, and 2 months following extraction to determine whether freezer temperature can influence sample stability over time. All other samples were stored at −20°C and assayed within 1 week.

2.8 | Cross-reactivity

To determine cross-reactivity of other steroids with this assay, serial dilutions of DHEA, epiandrosterone, progesterone, testosterone, dihydrotestosterone (DHT), β-estradiol, and prednisolone were measured in concentrations ranging from 1000 to 1.9 ng/ml. The cross-reactivity of each steroid was calculated by the DHEAS/steroid concentration at 50% binding.

2.9 | Standards, controls, and sample preparation

The standards were prepared from a DHEAS sodium salt solution (1 mg/ml, MiliporeSigma; Cat# D-65-1). For each assay, the top standard was prepared by diluting the DHEAS solution in assay buffer to 12.5 ng/ml. Eight additional standards were prepared by serially diluting the top standard at 1:2, with the lowest standard at 0.048 ng/ml. The control was set at 0.5 ng/ml. The optimal dilution for siamang and gibbon fecal samples was determined using the parallelism test, and it was set as the dilution detected at 50% binding of the labeled hormone to the primary antibody.

2.10 | Assay procedures

To precoat the plates, goat anti-mouse immunoglobulin G (Cat. No. A008; Arbor Assays) was diluted in coating buffer (X108; Arbor Assays) at a concentration of 10 μg/ml. Using a repeater pipette, 150 μl of the solution was added to each well of the 96-well microplates. Following incubation for 15-24 h at room temperature, the wells were aspirated using a plate washer (50 TS; BioTek), and 250 μl of blocking buffer (X109; Arbor Assays) was added to each well. Following another incubation for 4–24 h at room temperature, the plates were double aspirated, blotted dry, and placed in a vacuum desiccator chamber containing silica gel. When humidity was <20%, plates were removed from the chamber and stored in sealed aluminum foil pouches at 4°C. At the time of the assay, the microplate and assay buffer were brought to room temperature. Fifty microliters each of standards, control, and samples were added to the designated wells of the precoated plate in duplicate. Assay buffer was added to two nonspecific binding (NSB) wells (75 μl) and two B0 wells (50 μl). Then, 25 μl of DHEAS-horseradish peroxidase (Cat. No. C175; CalBioreagents) diluted in assay buffer (1:13,000) was added to each well, and 25 μl of antibody mouse monoclonal anti-DHEA 3-sulfate (Cat. No. M674; CalBioreagents) diluted in assay buffer (1:13,000) was added to each well, except NSB wells. The microplate was sealed and incubated on a plate shaker (SBT1500-H; Southwest Science) at 300 rpm at room temperature for 2 h. After washing the plates four times with 1:10 diluted wash buffer (0.5% Tween-20, 1.5 M sodium chloride), the assay was developed with 100 μl/well of 60% High Kinetic 3,3′,5,5′-tetramethylbenzidine (Moss, Inc.) and incubated in the dark at room temperature for 30 min. Finally, the reaction was stopped with 50 μl/well of stop solution (1 N HCl) and the microplate was read at 450 nm (800 TSI; BioTek).

2.11 | Data analyses

The data were analyzed using Genie5™ software (license obtained from BioTek upon equipment purchase). All OD values were subtracted from the NSB OD mean. Hormonal concentrations were calculated based on the standard curve generated by the software. Binding for each sample or standard was calculated by dividing their OD by the mean maximum binding OD and expressed as percentage. The intra-assay coefficient of variation (CV) was calculated by the average variance of all duplicate samples ($N = 278$), and the interassay CV was calculated by the variance of controls across different assay plates ($N = 15$).

To assess hormonal changes due to stressful events, I used iterative processes previously described (Brown et al., 1999, 1994; Heintz et al., 2011; Leeds et al., 2018; Moreira et al., 2001; Wark et al., 2016). Briefly, the mean and SD of DHEAS were initially calculated per individual using all their samples. All samples that had DHEAS levels above the mean + 1.5 SD were considered outliers and were removed. The mean and SD were recalculated, and the process was repeated until all samples fell within the mean + 1.5 SD. The new mean defined the baseline level of each individual, and samples that had DHEAS levels above the new mean + 1.5 SD were considered elevated.

All statistical analyses were conducted in R Core Team (2017) and figures were produced using the package ggplot2 (Wickham, 2009). To confirm parallelism, I used an *F*-test to compare the curves generated by the serial dilutions of the standards and pooled sample. To test hormonal stability, I used repeated-measures analysis of variance (ANOVA) one-way (freeze-thaw cycle) or two-way (temperature and storage time). Due to non-normality, the data were transformed using a log transformation. Normality and homogeneity of variances were confirmed by diagnostic

plots (histogram of frequency, quantile–quantile plot, distribution of residuals, and Levene's test). α was set at .05.

3 | RESULTS

3.1 | Parallelism, accuracy test, and sensitivity

The curve generated by the OD of the serially diluted fecal pool was parallel to the standard curve with no difference between slopes (Figure 1; $F_{7,\ 7} = 1.21$, $p = .8$), indicating that the fecal extract is suitable for the EIA. The sample dilution was set at 1:60, as this dilution resulted in approximately 50% binding.

The three spiked pools yielded a mean recovery of 111% ± 8% (Table 1), which is within the acceptable range in the accuracy test. This result indicates no matrix effects in the sample and confirms that the fecal extract is appropriate for the EIA. The sensitivity was 0.1 ng/ml. The mean extraction efficiency was 89%. The intra-assay CV was 5.2% ($N = 278$) and the interassay CV was 8.4% ($N = 15$).

3.2 | Cross-reactivity

The monoclonal DHEAS antibody cross-reacted 100% with DHEAS, 40% with DHEA, 40% with epiandrosterone, 9.6% with progesterone, 0.2% with testosterone, and 0.1% with prednisolone and DHT.

3.3 | Biological validation

The stressful event resulted in an increase in DHEAS levels in the male gibbon 1 day after the stressor (Figure 2—left), and in the female siamang who was transported 1 and 2 days after the stressor (Figure 2—center). In both individuals, DHEAS fell within baseline levels within 2 days after the peak. In the individual that was vaccinated, the peak was not above baseline levels, though it is worth noting that the baseline levels in this individual were higher than in the other two cases, with one sample falling below baseline levels (Figure 2—right). In addition, as noted previously, this individual had been trained for injections.

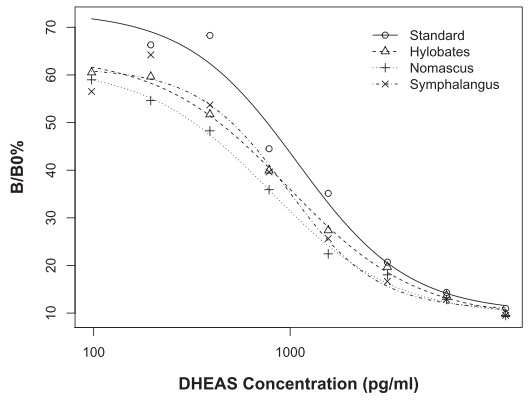

FIGURE 1 Parallelism test showing the percentage binding of the standard curve (solid line) and three serially diluted pooled fecal extracts from *Hylobates* sp. (dashed line), *Nomascus* sp. (dotted line), and *Symphalangus* sp. (dashed and dotted line). DHEAS, dehydroepiandrosterone sulfate

TABLE 1 Recovery from three pooled fecal extracts unspiked and spiked with DHEAS standards

Pool	Amount spiked (pg/ml)	Observed (pg/ml)	Expected (pg/ml)	Recovery (%)
Pool 1	Unspiked	1018.4	–	
	781.25	910.016	899.825	101.132554
	390.63	744.814	704.515	105.720105
	195.31	619.83	606.855	102.138073
Pool 2	Unspiked	1744.779	–	
	781.25	1498.345	1263.0145	118.632446
	390.63	1256.666	1067.7045	117.697921
	195.31	1189.39	970.0445	122.611901
Pool 3	Unspiked	1571.02	–	
	781.25	1112.283	1176.135	94.5710314
	390.63	1195.572	980.825	121.894528
	195.31	1023.627	883.165	115.904389
				111 ± 8

Abbreviation: DHEAS, dehydroepiandrosterone sulfate.

Samples from the pregnant female revealed that DHEAS levels in all pregnant samples were above control levels. The postpartum samples were still elevated and above control lactating levels (>1 month postpartum), but there was a pattern indicating lower DHEAS levels with advancing postparturition time (Figure 3). Although no statistical tests were conducted due to the sample size of one female, mean DHEAS levels were 7.3 times higher at late gestation (15626 ± 7867 ng/g) than nonlactating controls (2134 ± 966 ng/g), and 2.6 times higher than mean DHEAS levels in the first month postpartum (5949 ± 1847 ng/g).

3.4 | Sample stability

The sample stability experiments revealed no significant effect of a freeze-thaw cycle in preprocessed fecal samples (ANOVA: $F_{1,\ 22} = 0.4$, $N = 23$, $p = .53$, Figure 4a). In addition, DHEAS concentrations in fecal extracts (post-processing) were not affected by freezer temperature, storage time or their interaction (ANOVA: $F_{1,\ 20} = 0.34$, $N = 17$, $p = .57$). This indicates that in both −20°C and −80°C conditions, DHEAS levels in the fecal extracts were stable for 2 months (Figure 4b).

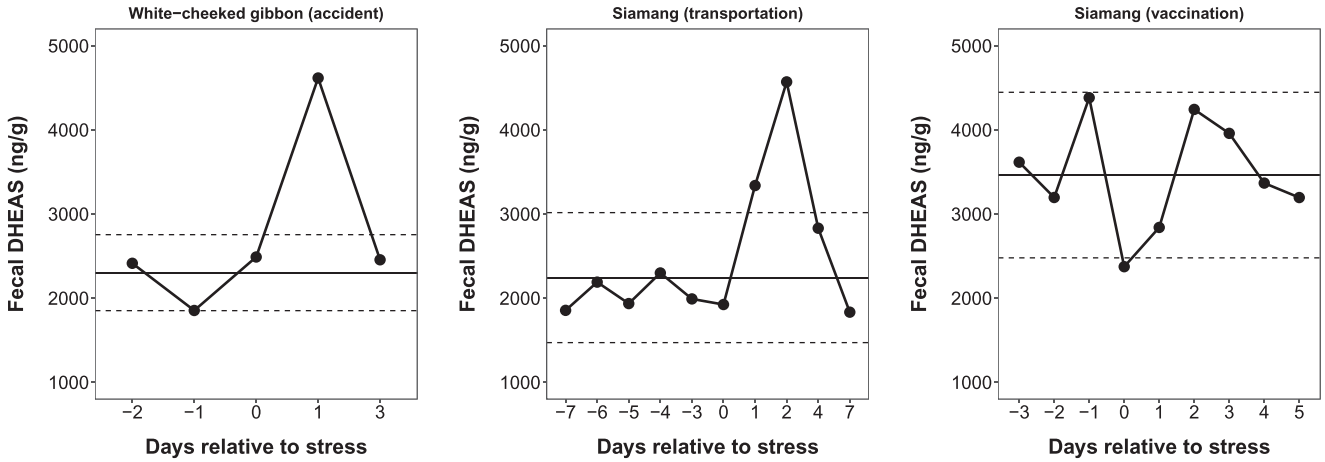

FIGURE 2 Daily changes in fecal DHEAS levels in one male gibbon (left) and two female siamangs (middle and right) in relation to a stressful event (Day 0), described in parenthesis. The horizontal lines indicate baseline levels, with a mean (solid line) and mean ± 1.5 SD (dashed lines) of all samples per individual, excluding outliers. Samples above baseline levels were considered elevated. DHEAS, dehydroepiandrosterone sulfate

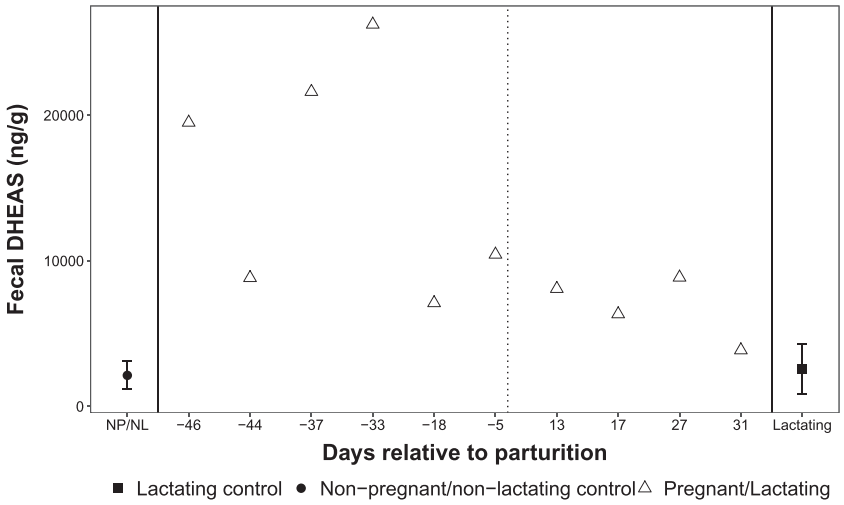

FIGURE 3 Changes in fecal DHEAS levels in a female siamang during pregnancy and postpartum (open triangles) with eight nonpregnant/nonlactating (NP/NL) controls (closed circle) and four lactating controls (>1 month postpartum, closed square). The dotted line indicates the day of parturition (Day 0). Error bars indicate standard error. DHEAS, dehydroepiandrosterone sulfate

4 | DISCUSSION

This study described and validated a newly developed EIA to measure DHEAS levels. The assay sensitivity of 0.1 ng/ml was higher than my previously described methods (Takeshita et al., 2018, 2013), and similar to the Arbor Assays DHEAS Kit (K054-H; Arbor Assays), although other commercial kits (Abcam, Eagle Biosciences, and Abnova) have reported a sensitivity of 0.05 ng/ml. The antibody cross-reacted mainly with DHEA and epiandrosterone, but less than 1% cross-reactivity with other steroids.

One of the main concerns of DHEAS assays is the potential cross-reactivity with testosterone. One study reported that DHEAS cross-reacts with the Abbot Architect direct assay for testosterone (Warner et al., 2006). In the present assay, testosterone and DHT cross-reactivities were 0.2% and 0.1%, respectively, which is similar to those reported for most commercially available kits (Arbor Assays, Abcam, Eagle Biosciences, Abnova, and ALPCO). Nevertheless, cross-reactivity with testosterone may occur in fecal samples because they are excreted as metabolites and not in their pure form (Möhle et al., 2002). For this reason, analytical and biological or physiological validations are necessary to confirm whether the desired metabolites are being detected by the EIA.

The analytical validation performed by parallelism and accuracy tests demonstrated that the fecal extracts do not interfere with the assay, and the extraction efficiency of 89% indicates good hormone recovery. These findings confirmed that the assay protocol and the extraction method are suitable for the measurement of DHEAS levels from gibbon and siamang fecal samples. The biological validation with the three individuals who experienced a stressful event showed that DHEAS levels peaked within 2 days, which further confirms that the assay is valid for gibbon and siamang feces. Although the peak in the gibbon was seemingly earlier than in the siamang, it is worth noting that a sample for Day 2 from the gibbon was missing and that the siamang had increased DHEAS levels in Days 1 and 2. In contrast, there was no clear response in the siamang that was vaccinated. This contrast illustrates how different events might elicit differences in DHEAS response. Although vaccination can be often considered stressful, no animal manipulation was needed in the present

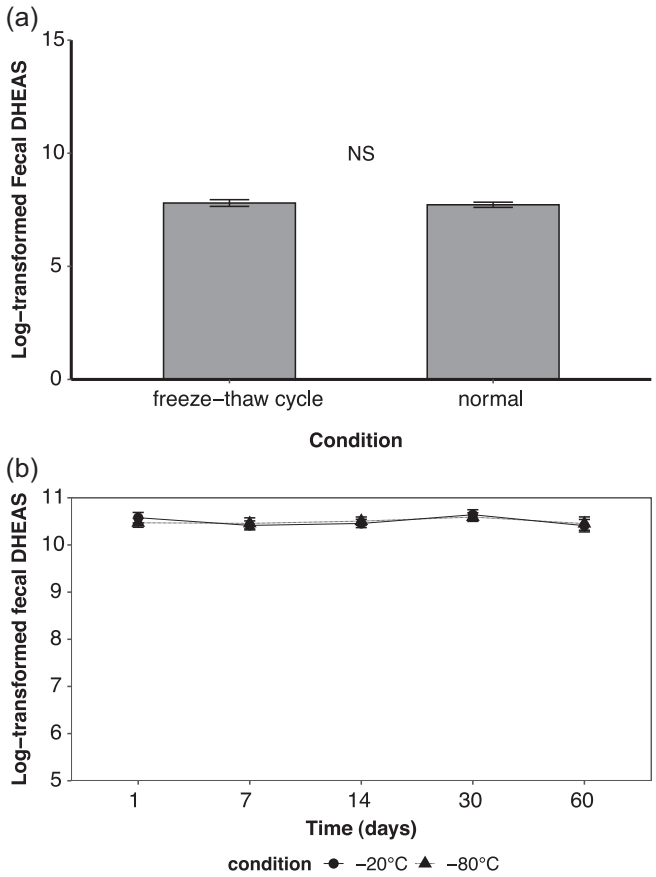

FIGURE 4 Comparison of fecal DHEAS levels (a) after fecal samples undergo one freeze-thaw cycle ($N = 23$) and (b) between fecal extracts ($N = 17$) stored at −20°C and −80°C, assayed at 1, 7, 14, 30, and 60 days from extraction day. Data are represented by log transformations. (b) The *y*-axis has been adjusted to facilitate data visualization. DHEAS, dehydroepiandrosterone sulfate; NS, not significant

study. Vaccination is part of regular husbandry procedures in zoos and research facilities, and the results show that preinjection training with positive reinforcement was efficient in reducing or eliminating a robust stress response to this procedure. In contrast, transportation to a new enclosure and accidents are unexpected events. Takeshita et al. (2014) reported an increase in fecal DHEAS and fecal GC levels up to 10 days after a Japanese macaque (*Macaca fuscata*) was transferred from a social group to a single cage. In addition to the stress to the body, the novelty of the situation is a contributing factor that may accentuate the stress response. Alternatively, an unknown event could have elicited a stress response before the vaccination procedure (e.g., fight with conspecifics, aggression towards tourists). This is a possible hypothesis because one sample collected on the day before the vaccination had an elevated DHEAS level, which resulted in high variation and an increased baseline level for that individual. Nevertheless, the DHEAS increase observed 2 days following the day of vaccination was still weaker compared to the transportation and accident events, which supports the hypothesis that the magnitude of the physiological response varies according to stressful events, and can be reduced with previous training or exposure to these procedures (Palme, 2019).

The present data also revealed that the time lag between DHEAS response and excretion in feces varies between 24 and 48 h, with a slightly faster response in the gibbon than the siamangs in this sample. This difference could have been due to sex differences in gastrointestinal transit time, given that the siamang was female and the gibbon was male. Variations in steroid hormone excretion time lag within and between closely related species have been reported in mammals and birds due to sex, diet, and body size (Hodges et al., 2010; Keay et al., 2006; Palme, 2005). Another possible explanation is that adaptation to a new environment in the transportation event resulted in a prolonged response (Takeshita et al., 2014), whereas the gibbon male could choose to avoid the items (rope) associated with the accident. The time lag observed in the present study is similar to the time lag of fecal DHEAS in orangutans (Takeshita et al., 2019), which seems to be associated with the role of DHEAS as GC antagonist. During a stress response, GC levels rise via activation of the hypothalamic–pituitary–adrenal axis in the so-called "fight-or-flight" response. DHEAS is then secreted from the adrenal gland and acts as a competitor for GC receptors to help restore the body to homeostasis. In orangutans, there is a 24 h time lag between fecal GC and fecal DHEAS response during a stressful event (Mendonça et al., 2016; Takeshita et al., 2019). One study in pileated gibbons (*Hylobates pileatus*) reported a 48 h time lag of fecal GCs (Pirovino et al., 2011). However, no studies have determined the GC time lag in siamang or white-cheeked gibbon feces. Further comparative studies are needed to determine whether there is a delay between fecal GC and fecal DHEAS excretion in these species.

The finding of high DHEAS levels during pregnancy in the siamang is similar to what was reported in orangutans (Takeshita et al., 2019) and Japanese macaques (Takeshita et al., 2016). The latter study reported that high DHEAS levels at late gestation may originate from the fetus, and low DHEAS levels at that stage may be indicative of fetal death. This is because the fetal adrenal develops a transient layer (fetal zone), which secretes high levels of DHEAS at the late stages of gestation. Previous studies have shown that fetal DHEAS is transferred to the mother via the placenta, and it is an essential source of estrogens necessary to maintain gestation and to regulate the mechanism of parturition (Makieva et al., 2014; Rainey et al., 2004; Walsh et al., 1984). In this study, all samples obtained during pregnancy had DHEAS levels that were above control levels, with a mean 7.3-fold higher than non-pregnant samples, which confirms the general trend observed in non-human primates. The mean postpartum DHEAS levels were also above the baseline. This finding is in contrast with previous studies that reported lower fecal DHEAS levels in lactating females in comparison to pregnant females (Takeshita et al., 2016, 2019). The contrasting result is probably related to the timing of fecal sampling. The present study reported fecal DHEAS changes in postpartum over time within the same individual and within 1 month postpartum, revealing that DHEAS levels decline progressively during the postpartum period. These data show that DHEAS levels were close to those of other lactating females at approximately one month postpartum.

With regard to sample stability, neither fecal freeze-thaw cycle nor fecal extract storage temperature affect DHEAS concentrations. Most studies have reported no changes in steroid hormone concentrations of

serum samples after a few freeze-thaw cycles for humans (Hillebrand et al., 2017; Livesey et al., 1980; Nwankpa et al., 2018) and nonhuman mammals (Gholib et al., 2019). One study using a C18 cartridge to extract fecal steroids from geladas (*Theropithecus gelada*) reported that two and six freeze-thaw cycles were necessary to affect fecal GC and fecal testosterone levels, respectively (Pappano et al., 2010). With regard to storage time, one study in crested macaques (*Macaca nigra*) reported that fecal extracts are stable at room temperature for 8 days (Gholib et al., 2017). Another study using baboon (*Papio cynocephalus*) fecal extracts found that hormonal steroids change with storage time, at approximately 30 days if stored at room temperature and at 90–120 days if stored at −20°C (Khan et al., 2002). In the present study, there were no effects of time or storage condition in DHEAS concentrations, but the tests were limited to only one freeze-thaw cycle and two subzero temperatures for storage up to 60 days. Although the present experimental design is likely not applicable to most primate field stations, which usually have no cryopreservation methods or are subjected to more than one freeze-thaw cycle, captive studies often require the collaboration of national/international facilities to ship samples. Considering the current demands for dry ice and the lack of ultracold freezers in some research/animal facilities, the results show that overnight shipping with ice packs is a viable alternative option for transporting samples, even if it results in sample thawing. Moreover, these data show that DHEAS levels remain stable in fecal extracts stored at freezer temperatures (−20°C or −80°C) for at least 2 months.

In summary, this study described and validated a new EIA to measure DHEAS levels from gibbons and siamang feces. This method is noninvasive and can be used for other species to monitor chronic stress, and can aid in the development of strategies to improve animal conditions in captivity. In addition, these preliminary data showed that DHEAS levels are stable after one freeze-thaw cycle, and an ultracold freezer is not better than a regular freezer (−20°C) for fecal extract storage if assayed within 2 months. The hormonal concentrations measured in feces represent a window of 24–48 h of hormonal response and can be useful to monitor welfare, gestation, as well as development, and senescence in wild and captive gibbons and siamangs.

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CONFLICT OF INTEREST

The author declares no conflict of interest.

DATA AVAILABILITY STATEMENT

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

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