Non-invasive assessment of fecal glucocorticoid and androgen metabolites in the pygmy hippopotamus (*Choeropsis liberiensis*)

Gabriella L. Flacke [a,b,*], Linda M. Penfold [c], Franz Schwarzenberger [d], Graeme B. Martin [a], César A. Rosales-Nieto [e], Monique C. J. Paris [f,g,h]

[a]UWA School of Agriculture and Environment, University of Western Australia, 35 Stirling Highway, Crawley 6009, Australia

[b]Department of Animal Health, Zoo Miami, 12400 SW 152nd Street, Miami FL 33177, USA

[c]South East Zoo Alliance for Reproduction & Conservation, 581705 White Oak Road, Yulee FL 32097, USA

[d]Department of Biomedical Sciences – Endocrinology, University of Veterinary Medicine (Vetmeduni Vienna), Veterinärplatz 1, 1210 Vienna, Austria

[e]Department of Agricultural Sciences, Texas State University, 601 University Drive, San Marcos, TX 78666, USA

[f]Institute for Breeding Rare and Endangered African Mammals (IBREAM), Edinburgh EH3 6AT, United Kingdom

[g]Mammal Research Institute and Centre for Neuroendocrinology, University of Pretoria, Department of Zoology and Entomology, Pretoria 0084, South Africa

[h]College of Public Health, Veterinary and Medical Sciences, James Cook University, Townsville 4811, Australia

* Corresponding author at: Zoo Miami Department of Animal Health, 12400 SW 152nd Street, Miami, Florida, 33177, USA. Email: gabriella.flacke@miamidade.gov

Highlights

- We established enzyme immunoassays (EIAs) to measure fecal hormones in pygmy hippos.
- Fecal glucocorticoid metabolites (FGMs) were best assessed by the 5α-3β,11β-diol-CM EIA.
- Females experiencing chronic illness and conspecific aggression had higher FGMs.
- In males, fecal androgen metabolite concentrations are highest over spring-summer.
- Adult males have higher fecal androgen levels than adult females and juvenile males.

Abstract

The pygmy hippopotamus (*Choeropsis liberiensis*) is an endangered species endemic to the Upper Guinea Forest ecosystem in West Africa. We have limited information concerning the species' reproduction and well-being under managed care. We therefore developed non-invasive methods for characterizing gonadal androgen and adrenal hormone profiles in pygmy hippos using fecal samples collected from 12 males and 12 females housed in North

American zoological institutions. We aimed to: 1) identify and validate enzyme immunoassays (EIAs) for measuring metabolites of corticosteroids and testosterone in feces; and 2) test whether gonadal activity is correlated with previous breeding history, season or type of housing. For glucocorticoids, several EIAs for measuring metabolites were investigated. A group-specific EIA exhibiting cross-reactivity with 11,17-dioxoandrostane (DOA) metabolites of cortisol most clearly reflected adrenocortical activity in response to pharmocological challenge with adrenocorticotropic hormone (ACTH) in both males and females. However, day-to-day concentrations of this metabolite in the feces of pygmy hippos that did not undergo ACTH challenge were near the detection limits of the assay, making this EIA impractical for assessing glucocorticoid activity in this species. Another group-specific EIA, exhibiting cross-reactivity with 5α-pregnane-3β,11β,21-triol-20-one, produced biologically relevant data and evidence of an appropriate response to pharmacological challenge with exogenous ACTH. The testosterone metabolite assay C196 (Arbor Assays, Ann Arbor, Michigan, USA) also produced biologically coherent data: adult males exhibited the highest mean androgen metabolite concentrations (477 ng/g), followed by adult females (259 ng/g) and juvenile males (160 ng/g). Proven breeding males had higher, but not significantly different, mean concentrations (472 ng/g) to unproven males (352 ng/g; $P = 0.400$). Similarly, adult males housed outdoors year-round in subtropical climates exhibited higher, but not statistically different mean concentrations (554 ng/g) to males in temperate climates that were housed indoors at least part of the year (412 ng/g; $P = 0.208$). There were, however, significant differences in mean concentrations among seasons for adult males, with higher values in spring (546 ng/g) and summer (542 ng/g) than in autumn (426 ng/g) and winter (388 ng/g, $P = 0.003$). In conclusion, we identified EIAs for the measurement of fecal metabolites of androgens and glucocorticoids that can be used for further studies to monitor gonadal activity in male pygmy hippos and adrenocortical activity in both sexes. We also identified a seasonal trend in male gonadal activity in this species under managed care in North America. Finally, our findings highlight an important consideration when using non-invasive methods for evaluating fecal cortisol metabolites: ACTH used for pharmacological validation of an EIA does not necessarily equate to biological relevance.

Keywords: ACTH challenge; Cortisol; Fecal steroid hormone metabolites; Enzyme immunoassay (EIA); Pygmy hippo; Testosterone

1. Introduction

The pygmy hippopotamus (*Choeropsis liberiensis*) – hereafter referred to as pygmy hippo – is endemic to a small area within the Upper Guinea Forest ecosystem in West Africa. The species is classified as Endangered by the International Union for the Conservation of Nature (Ransom et al., 2015) and is identified by Programme EDGE (https://www.edgeofexistence.org) as a priority for conservation action, being ranked 21st worldwide among mammals (Isaac et al., 2007)**.** There is no information concerning reproductive biology in wild pygmy hippos, but some general aspects are known from animals under managed care. However, at several zoological facilities worldwide, numerous breeding pairs have failed to reproduce and others have repeatedly experienced perinatal calf mortality or stillbirth (Flacke et al., 2016, Steck,

2021). These issues have limited the success of *ex situ* breeding programs and have the potential to reduce genetic diversity of the managed population in the long-term.

In a recent review, Mason (2010) identified a number of species under managed care that experience behavioral abnormalities and altered reproductive success in comparison to their wild counterparts, potentially as a result of chronic stress. The adverse effects of chronic stress on reproductive health are associated with the increased production of glucocorticoids and their resulting impact on the hypothalamic-pituitary–gonadal axis (Moberg et al., 2000, Rivier and Rivest, 1991, Sapolsky et al., 1992 Tilbrook et al., 2000, Wingfield and Sapolsky, 2003). Negative outcomes can include abnormal expression of sexual behavior, irregular ovulatory patterns, and inhibited production of gonadal hormones. It is therefore important to develop species-specific methods for endocrine monitoring of reproductive health and wellbeing in wildlife species under managed care, even as zoological institutions strive to mimic natural environmental, dietary, and social conditions.

The measurement of fecal metabolites of gonadal and adrenal hormones is one of the most commonly applied methods for monitoring endocrine responses in a variety of domesticated and wildlife species (Brown, 2018, Keay et al., 2006, Kersey and Dehnhard, 2014, Millspaugh and Washburn, 2004, Palme, 2019, Palme, 2005, Schwarzenberger, 2007, Touma and Palme, 2005, Wasser et al., 2000). However, even closely related species produce unique repertoires of hormone metabolites of unknown cross-reactivity, so it is essential to perform laboratory validation and to demonstrate species-specific biological relevance of each enzyme immunoassay (EIA) when using these non-invasive techniques (Millspaugh and Washburn, 2004, Palme, 2019, Palme, 2005, Touma and Palme, 2005). Many factors can cause variations in hormone metabolism and excretion among species and individuals, including age, sex, diet, digestive physiology, degree of enterohepatic circulation, and microbial flora in the gastrointestinal tract. Thus, specific EIAs that measure native hormones will often exhibit limited cross-reactivity with the diverse spectrum of metabolites, particularly in herbivores because, compared to carnivores, they have a longer gastrointestinal transit time and more extensive metabolism during foregut or hindgut fermentation (Goymann, 2012, Millspaugh and Washburn, 2004, Palme, 2019, Palme, 2005, Palme et al., 2005, Touma et al., 2003, Touma and Palme, 2005).

To address this issue, several laboratories have developed broad-spectrum EIAs that cross-react with hormone metabolites of similar chemical structure, thereby increasing the ability to detect a larger number of compounds. However, the native glucocorticoids (cortisol, corticosterone) and sex steroids (testosterone, estrogen, progesterone) are derived from the same precursor hormone, cholesterol, so their metabolites can have relatively similar structures (Payne and Hales, 2004). For example, most testosterone metabolites and some glucocorticoid metabolites exhibit a common androstane structure that only differs by one functional group (Ganswindt et al., 2003, Palme, 2019, Palme, 2005, Palme et al., 2005, Touma and Palme, 2005). Consequently, EIAs used to assess fecal glucocorticoid metabolites (FGMs) could exhibit cross-reactivity with androgen metabolites, complicating interpretation of endocrine patterns, as has been demonstrated for the male domestic dog (Schatz and Palme, 2001) and the male African elephant (Ganswindt et al., 2003). It is therefore critical to demonstrate both physiological validation and biological relevance of each EIA for the species

under consideration (Bashaw et al., 2016, Millspaugh and Washburn, 2004, Palme, 2019, Palme, 2005, Touma and Palme, 2005).

Physiological validation for an EIA measuring gonadal steroids can be demonstrated by an increase in testosterone or estrogen metabolite concentrations after an injection with a gonadal stimulating agent (e.g., gonadotropin-releasing hormone). Biological relevance can be demonstrated by correlating endocrine patterns with reproductive status – for example, greater level of androgen metabolites for an adult breeding male compared to a juvenile or a female, or an increase in progestogen metabolite concentration during pregnancy followed by a return to baseline after parturition (Brown, 2018, Comizzoli et al., 2009, Edwards et al., 2015a, Edwards et al., 2015b, Flacke et al., 2017, Kersey and Dehnhard, 2014, Schwarzenberger, 2007). To validate EIAs for measuring fecal cortisol metabolites, stimulation of the adrenal cortex via a pharmacological challenge with synthetic adrenocorticotropic hormone (ACTH) is a common approach. If the EIA is predominately measuring fecal metabolites of cortisol, it should detect a marked increase in concentrations following treatment, in accordance with gastrointestinal transit time for the species, followed by a return to baseline. It is also possible to evaluate biological relevance by measuring glucocorticoid metabolite concentrations before and after a known stressful event, such as anesthesia (e.g. Shutt et al., 2012) or translocation (Bashaw et al., 2016, Franceschini et al., 2008, Möstl et al., 2002, Palme, 2019, Touma and Palme, 2005, Wasser et al., 2000).

We have identified EIAs for non-invasive monitoring of estrogen and progestogen metabolites in female pygmy hippos (Flacke et al., 2017) but not methods for quantifying glucocorticoid metabolites and characterizing male endocrine patterns. Thus, the primary objective of the present study was to identify and validate EIAs that demonstrate biological relevance for measuring immunoreactive metabolites of gonadal androgens and glucocorticoids in pygmy hippo feces. Additional aims included characterizing patterns of androgen metabolites for males throughout the year to test for seasonality and the possible influence of other environmental factors, and to compare metabolite concentrations between proven breeding males and adult males that have not reproduced. We expect the results of this study to provide valuable tools and guidance for future investigations into the influence of husbandry variables on stress, welfare, and reproductive health in pygmy hippos under managed care.

2. Materials and methods

2.1. Animals and sample collection

Twelve male and 12 female pygmy hippos from 12 North American zoological institutions were included in this study (Appendix I). Ten of the males and all of the females were sexually mature (≥3 years) at the time sampling commenced. Fresh fecal samples for assessment of glucocorticoid metabolites were collected twice weekly for 1 year from each male and female hippo; a total of 2255 fecal samples were available for analysis. Fresh fecal samples for assessment of androgen metabolites were collected once weekly for 1 year from all 12 males and from 4 of the females; fecal androgen metabolites were analyzed in a total of 894 samples. Additional fecal sample collection for pharmacological validation of an EIA to assess glucocorticoid metabolites following an ACTH challenge is described in Section 2.2. Each zoological institution used the same protocol to collect fecal samples, and all samples were

stored at –20 °C until extraction for EIA. This research was conducted with the approval of the University of Western Australia Animal Ethics Committee (permits RA/3/500/67, RA/3/500/69, RA/3/100/1312) and the Zoo Miami Animal Care and Use Committee (project 2019-1).

2.2. ACTH challenge

We conducted an ACTH challenge in four adult male and four adult female pygmy hippos. An additional adult female, used as a control, had previously been trained to accept routine veterinary interventions while being offered a food reward. We therefore expected that this animal would show very little to no increase in glucocorticoid metabolites after receiving an injection. Initially, two males and two females were injected with 50 IU (approximately 0.2 IU/kg) short-acting synthetic ACTH (Cosyntropin 0.25 IU/mL, Sandoz Inc., Princeton, NJ, USA). Due to lack of clear and consistent results with the short-acting product, we performed an additional ACTH challenge for two males and two females using a higher dose of a long-acting product: 250 IU (approximately 1.0 IU/kg) sustained-release synthetic ACTH (Corticotropin 80 IU/mL, Wedgewood Pharmacy, Swedesboro, NJ, USA). The control female was injected with an equal volume of 0.9% sterile saline solution. Injections were given intramuscularly behind the ear, where the subcutaneous fat layer is thinnest, using an 18-gauge, 1.5-inch needle. Fecal samples were collected for 3 to 4 days before and 7 to 10 days after the ACTH or saline injection and stored at –20 °C until analysis. For the first 72 h after injection, all fecal material produced by each animal was collected (see Section 2.5); on all other days of the sampling period, only a single fresh sample was collected at approximately the same time each day.

2.3. Fecal hormone extraction

Fecal hormone metabolites were extracted at the Southeast Zoo Alliance for Reproduction & Conservation (SEZARC) laboratory, University of North Florida (Jacksonville, FL, USA), using methods previously described by Palme (2005) with the following modifications. Briefly, after manual homogenization of the sample, ~0.5 g wet fecal material was mixed with 4 mL methanol (reagent grade, Fisher Scientific, Fair Lawn, NJ, USA) and 1 mL reverse osmosis-purified water. Samples were then shaken in a Glas-Col Large Capacity Mixer (Glas-Col LLC, Terre Haute, IN, USA) for 20 min at 90 rpm followed by centrifugation for 10 min at 3100 rpm. Supernatant (200 µL) was transferred to 12 mm × 75 mm tubes (Perfector Scientific, Atascadero, CA, USA) for storage at –20 °C until analysis. Aliquots (0.25 mL) of the extracts were dried down and shipped to VetMed Uni, Vienna, Austria.

2.4. Enzyme immunoassays (EIAs)

Fecal extracts were analyzed in two separate laboratories to maximize the number of EIAs available for evaluating glucocorticoid metabolites. Initially italicized terms are those that we subsequently used to refer to the individual hormone assays throughout the study. Cross-reactivities for all of the assays are provided in Appendix II. All samples, controls, and standards were assayed in duplicate.

2.5. Laboratory A (SEZARC, Florida, USA)

Fecal extracts were analyzed via a double antibody method using microtiter plates (96-well; Santa Cruz Biotechnology, Santa Cruz, CA, USA) coated with 150 µL of 0.010 mg/mL goat anti-rabbit IgG (Arbor Assay, Ann Arbor, MI, USA). Samples were analyzed using a polyclonal cortisol antibody (*cortisol*: R4866, Coralie Munro, University of California, Davis, USA), a polyclonal corticosterone antibody (*CC*: CJM006, Coralie Munro, University of California, Davis, USA), and a testosterone antibody (*testosterone*: C196, Arbor Assays, Ann Arbor, Michigan, USA) together with the respective HRP conjugate. Serial dilution of pools of extracted feces from males and females generated parallel displacement curves to the standard curve. Samples were diluted 1:10, 1:50 and 1:50 for cortisol, CC and testosterone, respectively. Assay accuracy was assessed by using the standard spiked with a known amount of sample (2–1000 pg/well) and yielded 108% recovery for testosterone ($F_{1,17}$ = 20,270, $y = 1.0295x + 0.0006$, $r^2 = 0.9998$, $P < 0.001$). The cortisol and CC EIAs did not provide physiologically or biologically relevant results so were not further investigated at Lab A. Plates were read at 405 nm using an optical density plate reader (Dynex Technologies, Chantilly, VA, USA) The intra- and inter-assay coefficients of variation for all assays were < 10% (average 2.27%) and < 20% (average 5.22%), respectively. The minimum assay sensitivities, determined at 90–95% binding, were 3.17 pg/well for cortisol, 3.90 pg/well for CC, and 2.3 pg/well for testosterone. Data are reported as ng/g fecal wet weight.

2.6. Laboratory B (VetMed Uni, Vienna, Austria)

This laboratory analyzed fecal extract aliquots using group-specific EIAs with rabbit-origin polyclonal antibodies. Before analysis, sample extracts were reconstituted in 0.25 mL 80% methanol and diluted 1:10 in assay buffer. Microtiter plates were coated with goat anti-rabbit IgG. Antibodies used for steroid metabolite analysis were raised against the following steroids: *i*) cortisol (*CORT*: 4-pregnene-11β,17α,21-triol-3,20-dione, Palme and Möstl, 1997); *ii*) corticosterone (*CCST*: 4-pregnene-11β,21-diol-3,20-dione, Palme and Möstl, 1997); *iii*) *5α-3β,11β-diol-CM* (5α-pregnane-3β,11β,21-triol-20-one-CMO:BSA, Touma et al., 2003); *iv*) 11β-hydroxy-etiocholanolone (*3α,11β-dihydroxy-CM*; 5β-androstane-3α,11β-diol-17-one-CMO:BSA, Frigerio et al., 2004); *v*) 11-oxo-etiocholanolone-I (*11,17-DOA*; 5β-androstane-11,17-dione-3-HS:BSA, Palme and Möstl, 1997); *vi*) 11-oxo-etiocholanolone-II (*3α,11-oxo-CM*; 5β-androstane-3α-ol-11-one-17-CMO:BSA, Möstl et al., 2002). For all assays, the intra-assay coefficient of variation was < 10% and the inter-assay coefficient of variation was < 15%. The detection limits were 1.5 pg/well for CORT; 2.0 pg/well for CCST; 0.8 pg/well for 5α-3β-11β-diol-CM; 2.0 pg/well for 3α,11β-dihydroxy-CM; 3.0 pg/well for 11,17-DOA; and 3.0 pg/well for 3α,11-oxo-CM. Serial dilutions of fecal extracts yielded a displacement curve parallel to the standard curve for the four EIAs numbered *iii* – *vi*. Data are reported as ng/g fecal wet weight.

2.7. Data analysis

Gastrointestinal transit time for pygmy hippos varies between animals, ranging from one to three days (Flacke et al., 2017). We therefore considered hormone metabolite levels measured in the feces to represent endocrine patterns from the previous 24–72 h.

2.7.1. ACTH challenge and glucocorticoid metabolite assay validation

We calculated baseline concentrations separately for each of the ten pygmy hippos for each glucocorticoid EIA using the mean of values for the samples collected before ACTH or saline injection. We subsequently calculated all concentrations for pre- and post-ACTH challenge or saline control as a percentage of each animal's baseline. We then compared results for all of the glucocorticoid metabolite EIAs to determine which assay exhibited the most relevant pattern for a pharmacologically induced response after ACTH challenge, and a lack of such response for the saline control. We also used Friedman's Rank test to determine if there was a significant difference for each hippo between the mean pre-injection baseline and the mean post-ACTH (first 72 h) metabolite concentrations.

In addition to the ACTH injections, we compared glucocorticoid metabolite concentrations over a year in fecal samples from seven females, subjectively classified by animal caretakers and veterinarians as 'healthy and not particularly stressed,' with data from two females considered to be chronically stressed. One of these females was subject to repeated episodes of conspecific aggression when placed with the male for attempted breeding; the other was a geriatric animal that required recurring medical intervention due to chronic health issues and advanced osteoarthritis. A monthly average for each EIA was calculated over the one year for the seven 'healthy' females and compared to the monthly average over the same period for the two 'stressed' females, and unpaired *t*-tests were also used to compare overall values for both categories.

2.7.2. Fecal androgen metabolites

Baseline androgen concentrations were defined as an average of the lowest 10% of the samples for each pygmy hippo and peak values were calculated as a percentage of each hippo's baseline. To demonstrate biological relevance for the testosterone EIA, we compared metabolite concentrations throughout the year for a proven breeding male, a geriatric male (aged ~ 40 years) held at a facility without a female and presumed to be reproductively senescent, and two juvenile males that were 1.5 years old at the start of the sampling period. Additionally, we compared mean concentrations for the 10 adult males in our study with the four adult females and the two juvenile males. Previous research demonstrated non-seasonality of reproduction in female pygmy hippos (Flacke et al., 2017), so to evaluate this parameter in adult males the data were also grouped by northern hemisphere season as follows: spring (March, April, May); summer (June, July, August); autumn (September, October, November); winter (December, January, February).

We used the SAS statistical package version 9.3 (2010). Each animal was considered an experimental unit. A Shapiro-Wilk normality test was used to indicate that the data were normally distributed. Data for testosterone concentration were analyzed using PROC-MIXED. The fixed effects included sex (adult male *vs* adult female), age (adult male *vs* juvenile male), adult male age (<10 years, between 10 and 20 years, and > 20 years), breeding history (proven *vs* non-proven adult male), housing (outdoor *vs* indoor for adult male), and season as described above. The sampling date with ID as a nested effect was included as a repeated measure. ID was included as a random effect. These analyses include n = 10 adult male hippos (n = 629 fecal samples), n = 4 adult female hippos (n = 173 fecal samples) and n = 2 juvenile

male hippos (*n* = 96 samples). Of the 10 adult males, seven were proven breeding males (*n* = 391 samples) and two were males that had not reproduced despite having access to a breeding-age female (*n* = 163 samples). An additional adult male had not been with a female and thus did not have an opportunity to breed. For seasonal analyses, five of the adult males were located in subtropical climates with year-round outdoor housing (*n* = 279 samples), whereas the other five adult males were located in temperate climates with indoor housing, either year-round or in colder weather (*n* = 350 samples). For all statistical analyses, values of $P < 0.05$ were considered significant.

Table 1. Fecal glucocorticoid metabolite (FGM) concentrations in pygmy hippos (n = 2 males, n = 2 females) for each EIA tested in the pharmacological challenge study using long-acting ACTH. i) cortisol (CORT: 4-pregnene-11β,17α,21-triol-3,20-dione, Palme and Möstl, 1997); ii) corticosterone (CCST: 4-pregnene-11β,21-diol-3,20-dione, Palme and Möstl, 1997); iii) 5α-3β,11β-diol-CM (5α-pregnane-3β,11β,21-triol-20-one-CMO:BSA, Touma et al., 2003); iv) 11β-hydroxy-etiocholanolone (3α,11ß-dihydroxy-CM; 5β-androstane-3α,11β-diol-17-one-CMO:BSA, Frigerio et al., 2004); v) 11-oxo-etiocholanolone-I (11,17-DOA; 5β-androstane-11,17-dione-3-HS:BSA, Palme and Möstl, 1997); vi) 11-oxo-etiocholanolone-II (3α,11-oxo-CM; 5β-androstane-3α-ol-11-one-17-CMO:BSA, Möstl et al., 2002).

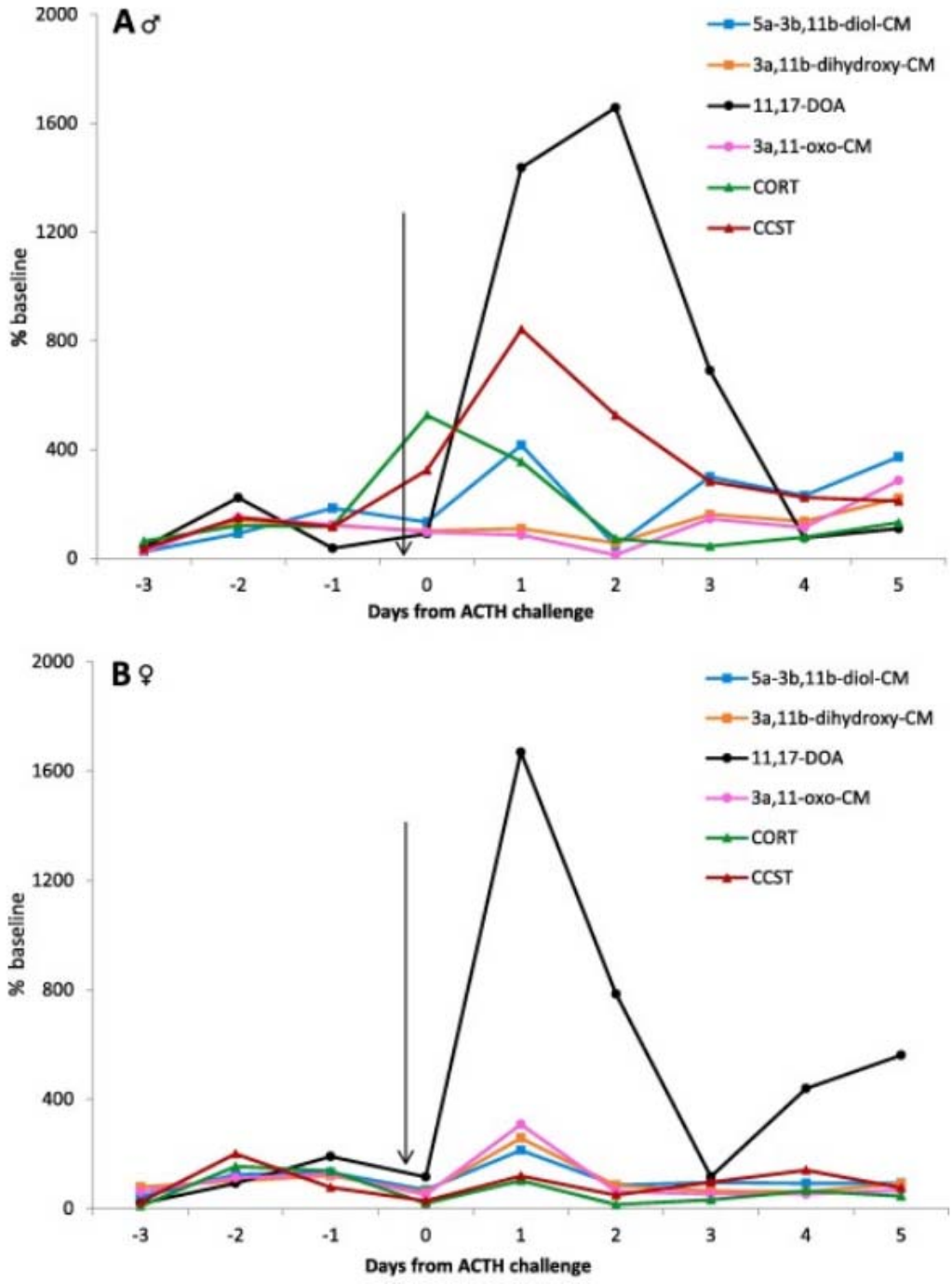
EIA	Sex	Mean (ng/g)	Range (ng/g)	Peak above baseline (%)
CORT	♂	4.4	0.8 – 16.1	604.5
	♀	8.2	1.1 – 22.3	172.1
CCST	♂	87.5	9.1 – 254.4	972.5
	♀	118.0	36.5 – 313.4	199.9
5α-3β,11β-diol-CM	♂	35.5	2.1 – 113.4	609.6
	♀	45.9	7.3 – 229.6	1269.9
3α,11β-dihydroxy-CM	♂	168.2	60.1 – 372.8	221.1
	♀	126.1	66.9 – 293.9	257.8
11,17-DOA	♂	58.9	0.6 – 279.3	1657.9
	♀	28.1	0.2 – 212.3	1668.5
3α,11-oxo-CM	♂	24.9	1.2 – 84.1	452.2
	♀	29.1	0.6 – 135.3	745.1

3. Results

3.1. ACTH challenge

Samples from the ACTH challenge were initially analyzed in Laboratory A for both short- and long-acting ACTH preparations. However, neither the cortisol nor the CC assay detected relevant patterns of FGM concentrations that would suggest they could be used to detect a physiological stress response. The samples were subsequently re-analyzed in Laboratory B using six additional glucocorticoid metabolite assays, as described above. Overall results from these six EIAs is summarized in Table 1. The 11,17-DOA EIA demonstrated a pronounced physiological response post-ACTH challenge for both males and females that received long-acting ACTH, with concentrations increasing up to 1600% above baseline followed by a return to baseline within 3 to 4 days post-injection (Fig. 1A, 1B). For pygmy hippos that received short-acting ACTH, there was also an increase in 11,17-DOA concentrations above baseline on the day after injection, with a return to baseline the following day (Fig. 1C). The magnitude

and duration of response was markedly less pronounced for short-acting than long-acting ACTH, but patterns were otherwise comparable.



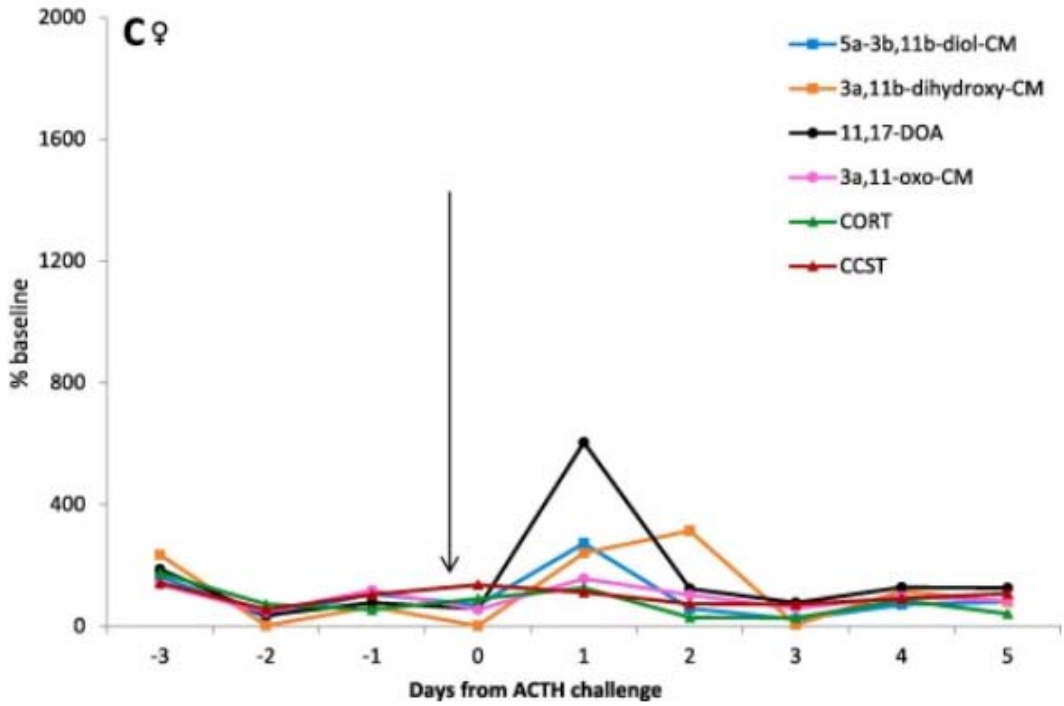


Fig. 1. Fecal glucocorticoid metabolite (FGM) profiles for three pygmy hippos, pre- and post-ACTH challenge, analyzed with six FGM antibodies: 5α-3β,11β-diol-CM; 3α,11β-dihydroxy-CM; 11,17-DOA; 3α,11-oxo-CM; CORT; and CCST. An arrow indicates the time of ACTH injection. The mean value is shown for Days 1–3 when multiple samples were collected. The 11,17-DOA assay shows the most pronounced response to ACTH in all cases. A) Adult male, long-acting ACTH; 11,17-DOA. B) Adult female, long-acting ACTH; C) Adult female, short-acting ACTH.

Based on these results, it was expected that the 11,17-DOA assay would most accurately reflect physiologically relevant FGM concentrations in pygmy hippo feces. However, further analysis of fecal samples not associated with ACTH challenge demonstrated that fecal steroid metabolite concentrations in the 11,17-DOA assay were too low to produce meaningful readings. These low concentrations were all in the upper end of the standard curves, above 80% binding, sometimes close to 100%, indicating that the assay was not sensitive enough to detect FGM concentrations at baseline levels.

The 5α-3β,11β-diol-CM EIA also showed the expected physiological response to ACTH challenge, although it was not as pronounced as with the 11,17-DOA EIA. Post-ACTH injection, 5α-3β,11β-diol-CM metabolite concentrations increased up to 600% above baseline for males and up to 1200% above baseline for females, with a return to baseline within 3 to 4 days post-injection for both sexes (Fig. 2). After the saline injection in the control hippo, there was no substantial increase in concentrations of 5α-3β,11β-diol-CM metabolites (Fig. 2).

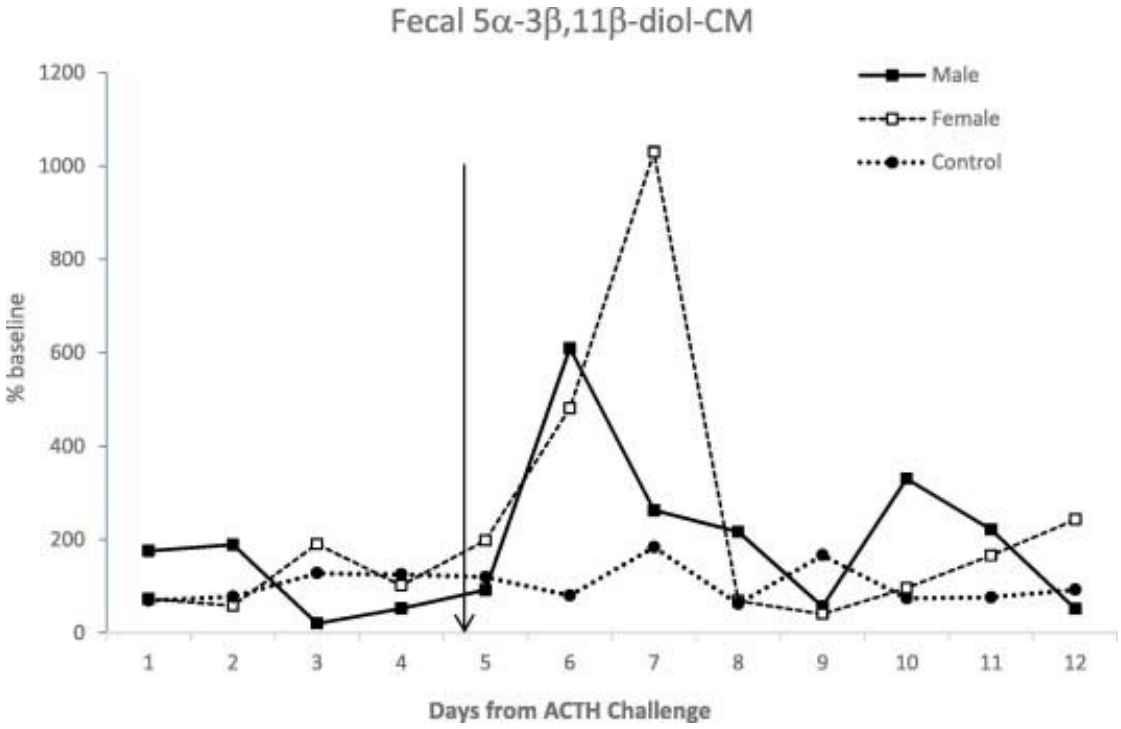


Fig. 2. Fecal concentrations of **5α-3β,11β-diol-CM** in pygmy hippos pre- and post-ACTH challenge with long-acting ACTH, or sterile saline as a control. An arrow indicates the time of ACTH injection. The mean value is shown for Days 1–3 when multiple samples were collected.

Each pygmy hippo produced several daily post-ACTH samples (first 72 h post-injection) with values that were significantly higher than baseline. However, Friedman's Rank test did not show a significant difference between mean pre-injection baseline and post-ACTH metabolite concentrations for either the 11,17-DOA ($P = 0.132$) or the 5α-3β,11β-diol-CM ($P = 0.098$) EIA. Pairwise comparisons were not possible since only the day, not the time, was recorded for each sample, but the sum of ranks indicated that peak response occurred approximately 24 h post-ACTH injection for the 5α-3β,11β-diol-CM EIA in both males and females (Fig. 2).

Analysis of fecal samples from adult females using the 5α-3β,11β-diol-CM EIA produced the most biologically coherent results, with significantly lower mean FGM concentrations in the seven 'healthy and not particularly stressed' females than in the two 'chronically stressed' females ($P < 0.001$; Fig. 3). The mean concentration (±standard deviation) across the one-year period for the group of seven females was 248.5 ng/g (±129.0 ng/g). In contrast, the value was 528.7 ng/g (±222.6) for the female experiencing repeated conspecific aggression, and 876.3 ng/g (±292.8) for the geriatric female with chronic medical issues**.**

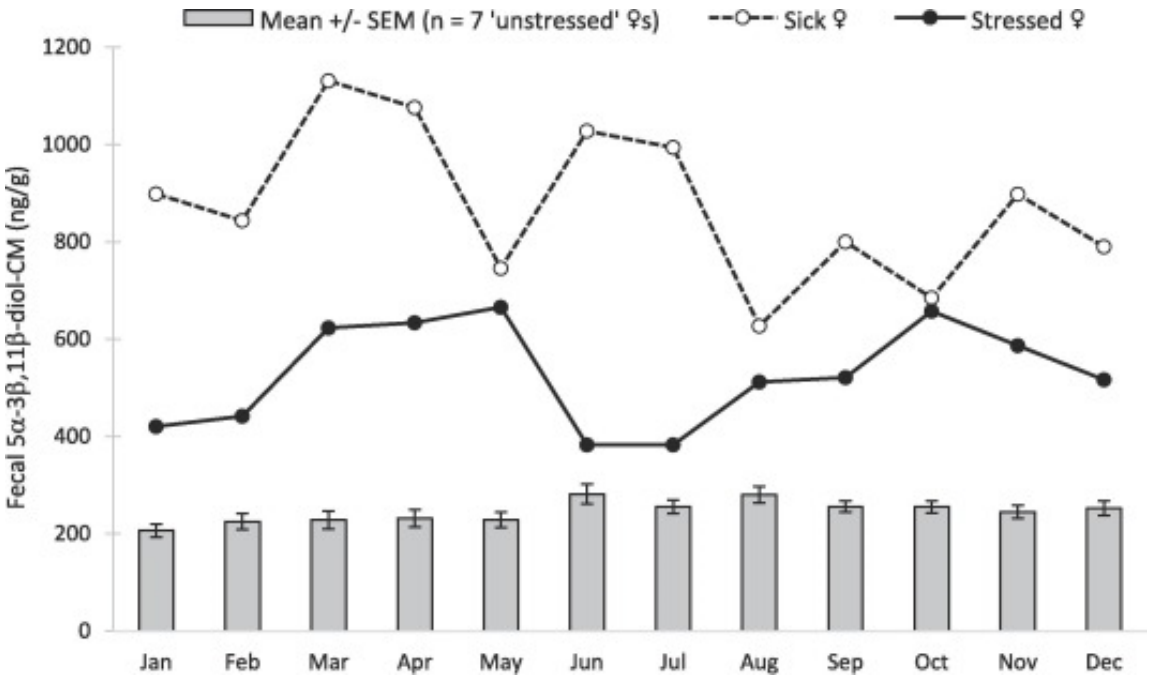


Fig. 3. Mean monthly fecal 5α-3β,11β-diol-CM concentrations over a one-year period for seven adult female pygmy hippos compared with one geriatric female that was sick during the sampling period (dashed line) and another adult female who experienced repeated conspecific aggression during introductions for breeding (solid line). Error bars represent standard error of the mean (SEM).

3.2. Reproductive patterns – Fecal androgen metabolites

Mean fecal androgen metabolite concentrations for each pygmy hippo, grouped by sex and age, are summarized in Table 2. The biological relevance of the testosterone EIA for measuring metabolites of gonadal androgens was supported by our comparison of metabolite concentrations throughout the year for a proven breeding male, a geriatric male, and two juvenile males (Fig. 4). As expected, the breeding male exhibited the highest concentrations throughout the year. The reproductively senescent male exhibited lower concentrations than the breeding male, but higher concentrations overall than the two juvenile males. Baseline concentrations ranged from 95 to 322 ng/g in the 10 adult males and from 56 to 123 ng/g in the 4 adult females; for the two juvenile males, baseline concentrations were 24 ng/g and 41 ng/g. Peak fecal androgen metabolite concentrations for adult males ranged up to 1860% above baseline, and peaks were evident throughout the year.

Table 2. Mean (±SD) fecal androgen metabolite (Testosterone; C196) concentrations in pygmy hippos, individually and grouped for adult males, juvenile males, and adult females. Adult males that have not reproduced despite being with a female are marked with a (*).

Age (years)	Sex	Studbook No.	Mean fecal androgen metabolites (ng/g)	Overall
40+	M	460	358 ± 103	Adult ♂ (n = 10) Mean
23	M	880	289 ± 124	477 ± 205 Range
20.5	M	902	674 ± 471	69–3146
20	M	919*	276 ± 155	
17	M	996	607 ± 450	
14.5	M	1053	746 ± 380	
12.5	M	1093*	372 ± 234	
10.5	M	1135	335 ± 171	
6	M	1241	267 ± 86	
3	M	1359	771 ± 591	
1.5	M	1422	228 ± 135	Juvenile ♂ (n = 2)
1.5	M	1423	51 ± 26	Mean 160 ± 114 Range 19–666
19	F	931	281 ± 105	Adult ♀ (n = 4) Mean
14	F	1063	268 ± 60	259 ± 21 Range
10	F	1147	242 ± 206	20–1094
3.5	F	1392	290 ± 215	

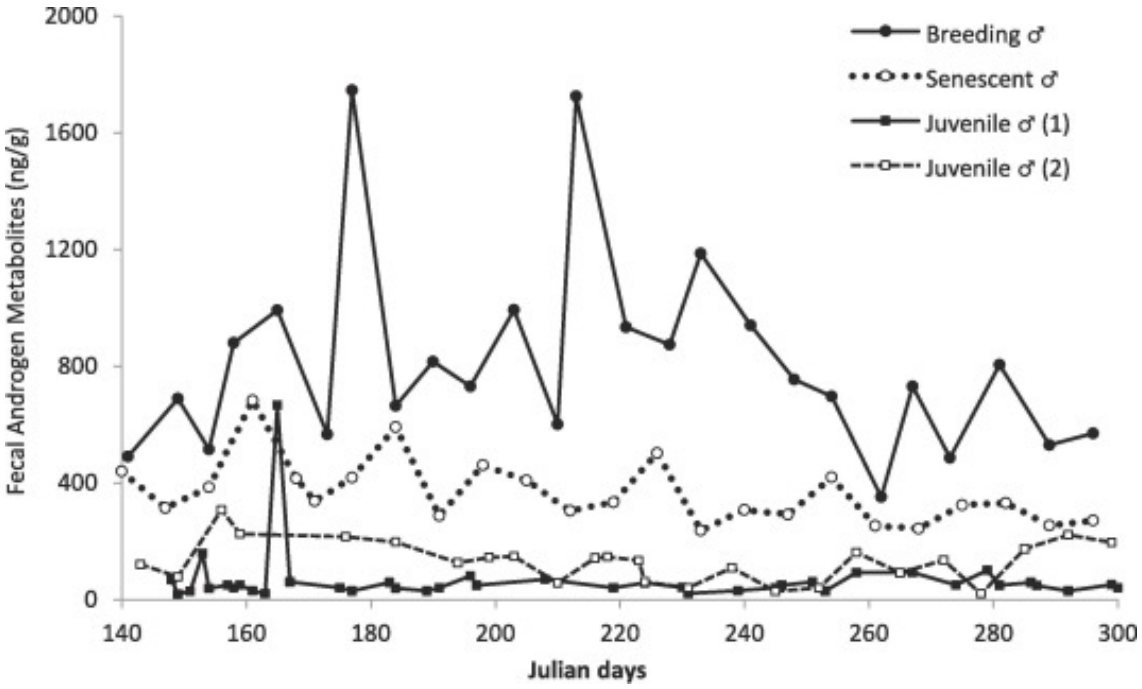


Fig. 4. Fecal androgen metabolite (Testosterone; C196) concentrations in an adult breeding male, a reproductively senescent male, and two juvenile male pygmy hippos. The breeding male exhibits the highest concentrations, while the two juvenile males both exhibit relatively low androgen metabolite levels. The senescent male, housed at a facility without a female, exhibits intermediate androgen metabolite concentrations. These results demonstrate biological relevance for the testosterone EIA for assessing gonadal androgens in pygmy hippos.

Overall, the PROC-MIXED analysis showed a significant difference in androgen metabolite concentrations between individual adult male pygmy hippos ($F = 12.25$, $df = 9$, $P < 0.001$). However, when adult males were grouped by age (<10 years, between 10 and 20 years, and > 20 years), concentrations were similar across each age group ($F = 0.14$, $df = 2$, $P = 0.872$). Concentrations were higher for adult males ($n = 10$; 477 ng/g) than for juvenile males ($n = 2$; 160 ng/g) ($F = 4.62$, $df = 1$, $P = 0.055$), and tended to be higher in adult males than in adult females ($n = 4$; 259 ng/g), although the difference was not significant ($F = 3.18$, $df = 1$, $P = 0.095$), possibly due to the smaller sample size for females.

We did not find a significant difference between androgen metabolite concentrations in proven adult breeding males ($n = 7$; 472 ng/g) and adult males that had not reproduced despite being with a female ($n = 2$; 352 ng/g) ($F = 0.80$, $df = 1$, $P = 0.400$). Additionally, concentrations were similar for adult males housed outdoors year-round ($n = 5$; 554 ng/g) and those housed indoors year-round or in colder weather ($n = 5$; 415 ng/g) ($F = 1.87$, $df = 1$, $P = 0.208$). However, androgen metabolite concentrations differed significantly among the seasons of the year for adult males ($F = 4.79$, $df = 3$, $P = 0.003$). Mean concentrations in spring (546 ng/g) were greater than those in autumn (426 ng/g; $P = 0.009$) and winter (388 ng/g; $P = 0.011$). Similarly, mean concentrations in summer (542 ng/g) were also greater than those in autumn ($P = 0.004$) and winter ($P = 0.008$). Concentrations were similar for spring and summer ($P = 0.827$) and for autumn and winter ($P = 0.963$) (Fig. 5). Data from these analyses are summarized in Table 3.

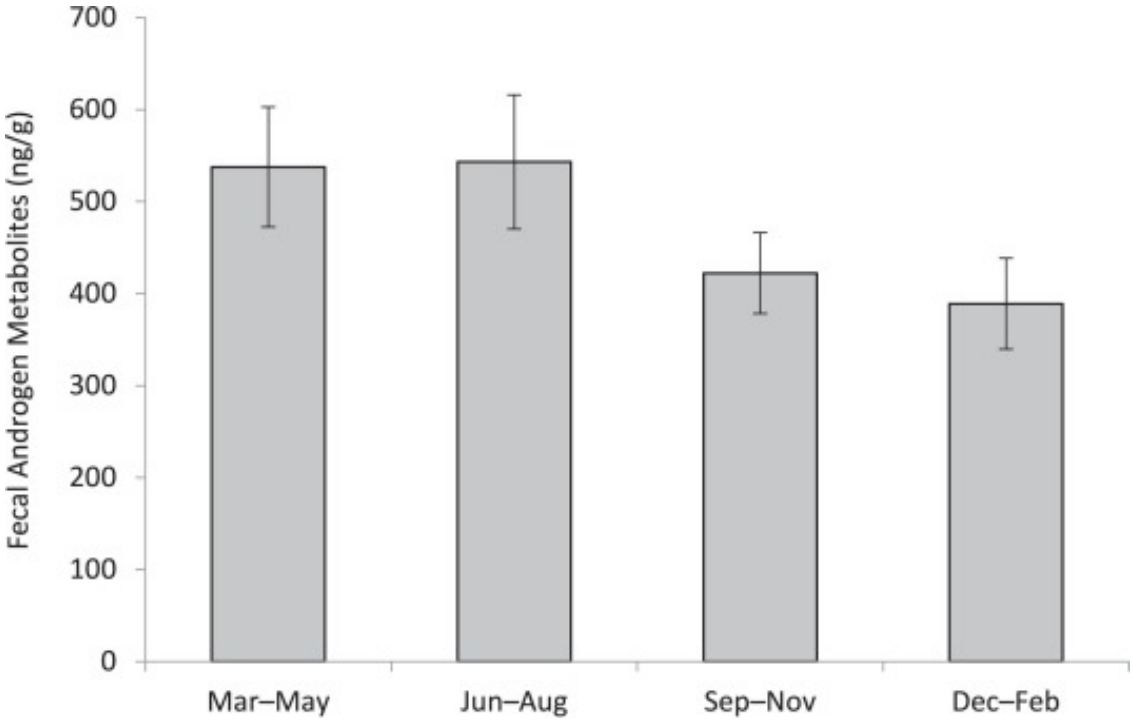


Fig. 5. Mean concentrations of fecal androgen metabolites (Testosterone; C196) in adult male pygmy hippos ($n = 10$) by northern hemisphere season. Concentrations in spring (Mar–May) and summer (Jun–Aug) are significantly higher than concentrations in fall (Sep–Nov) and winter (Dec–Jan). Error bars represent 95% confidence intervals of the mean.

Table 3. Mean (±SEM) fecal androgen metabolite (Testosterone; C196) concentrations of adult male, juvenile male, and adult female pygmy hippos. The effect for adult age group compares only adult males and combines male data for season, reproductive history, and housing. The effect for age compares adult and juvenile males and combines adult male data for age, season, reproductive history, and housing. The effect for sex compares adult males and females and combines male data for housing, reproductive history, season, and age. The effect for reproductive history compares only proven and unproven adult males and combines male data for housing, season, and age. The effect for housing compares only adult males and combines male data for season, reproductive history, and age. The effect for season compares only adult males and combines male data for housing, reproductive history, and age.

Adult ♂ age group (years)		
< 10	3	487 ± 32.7
Between 10 and 20	4	474 ± 21.3
> 20	3	470 ± 26.7
P value		0.872
Age		
Adult ♂	10	477 ± 15.2
Juvenile ♂	2	160 ± 14.8
P value		0.055
Sex		
Adult ♂	10	477 ± 15.2
Adult ♀	4	259 ± 14.1
P value		0.095
Reproductive history*		
Proven	7	472 ± 18.1
Unproven	2	352 ± 16.5
P value		0.400
Housing		
Outdoor all year	5	415 ± 16.5
Indoor some/all of the year	5	554 ± 26.8
P value		0.208
Season (northern hemisphere)		
Spring	10	546 ± 34.8
Summer	10	542 ± 35.8
Autumn	10	426 ± 21.5
Winter	10	388 ± 25.0
P value		0.003

*One adult male hippo was excluded from the analysis because he did not have contact with any females and thus did not have breeding opportunities.

4. Discussion

4.1. Fecal glucocorticoid metabolites and ACTH challenge

Results of this study show that the 5α-3β,11β-diol-CM EIA is appropriate for assessment of glucocorticoid metabolites in fecal samples for the pygmy hippo. The group-specific antibody exhibiting cross-reactivity with 5α-3β,11β-diol-CM metabolites of cortisol not only demonstrated an appropriate response to the ACTH challenge in both males and females, but also produced biologically relevant data that reflected expected patterns of FGMs in the two chronically stressed *vs*. seven healthy females. Although there is some overlap between the two groups in the absolute values for 5α-3β,11β-diol-CM concentrations, the monthly mean for the 'chronically stressed' animals was consistently above the range for the 'healthy and

not particularly stressed' animals (Fig. 3). Other herbivore species where this assay has been shown to be biologically relevant for measuring FGMs include eastern grey kangaroos (*Marcopus giganteus*) and red deer (*Cervus elaphus*) (Fanson et al., 2017, Huber et al., 2003).

Even though the 11,17-DOA EIA indicated a pronounced response to ACTH challenge, it was deemed to be biologically less relevant and not practically applicable for routine monitoring of glucocorticoids in pygmy hippos as it was not sufficiently sensitive to determine FGM concentrations in fecal samples. Several studies describe FGMs with an 11,17-DOA structure in the feces of a number of other herbivore-ungulate species, especially ruminants, including giraffe (*Giraffa camelopardis*), roe deer (*Capreolus capreolus*), African buffalo (*Syncerus caffer*), chamois (*Rupicapra rupicapra*) and domestic cattle (*Bos taurus*) and sheep (*Ovis aries*), and the biological relevance of this EIA for measuring a physiological stress response has been established for most of these species (Bashaw et al., 2016, Dehnhard et al., 2001, Ganswindt et al., 2012, Hadinger et al., 2015, Möstl et al., 1999). Although we demonstrated that the pygmy hippo, a non-ruminant foregut fermenter, also produces metabolites of cortisol with an 11,17-DOA structure, our data show that metabolites with a 5α-3β,11β-diol-CM structure are generally present in higher concentrations and are thus biologically more appropriate for assessment of FGMs in this species. Our study therefore again emphasizes the need to validate both the physiological and biological relevance of an EIA for assessing FGMs, as highlighted in previous reviews on this technique (Millspaugh and Washburn, 2004, Palme, 2019, Touma and Palme, 2005), and demonstrates the value of testing several assays as more than one may exhibit relevance (Bashaw et al., 2016, Fanson et al., 2017). Our results also highlight the importance of an appropriate experimental design (magnitude of stimulus, frequency of sample collection) in order to maximize the detection of meaningful changes in FGMs.

This study also verifies the advantage and importance of using a long-acting ACTH product for challenge studies, especially in herbivores. Although an increase in FGMs was also noted with the short-acting ACTH product, it was noticeably less pronounced in duration and amplitude than when the long-acting product was used (Fig. 1). In species with voluminous gastrointestinal tracts, and thus greater fecal mass and more frequent defecation (e.g. larger herbivores), it is recommended to use a sustained-release, long-acting ACTH product so that short-term changes in adrenal status are not diluted, rendering them more difficult to discern via FGM analysis (Wasser et al., 2000).

Sex differences between ACTH response were noted in that the duration of response was longer in males than females for both the 11,17-DOA and 5α-3β,11β-diol-CM EIAs (Fig. 1, Fig. 2). Both pairs of male and female pygmy hippos injected with long-acting ACTH were housed at the same facility, fed the same diet, comparable in body condition, exposed to the same environmental factors, and neither pair was in a breeding situation. The temporal difference in response to the same stressor may therefore be linked to sex, but since numbers of hippos were small, further research is necessary to determine if there are consistent sex differences.

4.2. Male reproductive patterns

The testosterone EIA that we used for measuring androgen metabolite concentrations in pygmy hippo feces produced data that are biologically coherent. While it was expected that

adult males would have higher androgen metabolite concentrations than juvenile males or females, it was interesting to note that the values were similar in the eight proven breeding males and the two males that had not successfully reproduced. However, we were unable to correlate androgen metabolite concentrations with other measures of male reproductive potential, such as testicular size or ejaculate quality. The relationship between androgen concentrations and these measures of reproductive function has been investigated in a handful of non-domestic species, and the patterns are variable (Brown et al., 2001, Comizzoli et al., 2009, Edwards et al., 2015a, Swanson et al., 2003, Wildt et al., 1993). Two previous studies examining ejaculates from ten pygmy hippos in European zoos did not test whether gonadal hormones or semen quality differed between proven and unproven males (Saragusty et al., 2010, Saragusty et al., 2012).

Some of the variation in androgen metabolite concentrations we observed among the 10 adult males is likely attributed to the fact that each male was housed at a different zoological facility, fed a different diet, and subjected to different environmental and husbandry conditions. Their age did range from three to >40 years, but did not appear to be a factor affecting gonadal androgen activity. Similarly, the type of housing also did not significantly affect androgen metabolite concentrations. Pygmy hippos that are housed outdoors year-round in subtropical climates (see Appendix I) do not experience large temperature fluctuations, but the same is likely to apply to those that are housed indoors (in heated spaces) in colder weather, possibly explaining the similar annual androgen patterns in the two groups.

The seasonal differences in androgen metabolite concentrations (Fig. 5) were not anticipated given this species is non-seasonally polyestrus under managed care (Flacke et al., 2017) and births occur in all months of the year in zoos across the globe (Steck, 2021). In the absence of comparable information for wild populations of pygmy hippos, we cannot conclude if the seasonality of androgen concentrations is reflective of normal biology for this species. Ecological life history traits largely support year-round reproduction in the wild because the species is endemic to a very limited region of tropical West African rainforest near the equator where temperature and light cycle remain relatively constant throughout the year (Verschuren, 1983, CEPF, 2000, Unep-wcmc, 2011). The range of latitudes in the natural habitat of this species (Guinea, Ivory Coast, Liberia, Sierra Leone) is between 5° and 8°N. It is therefore possible that the seasonal differences in androgen metabolites we observed might be attributable to variable day length and ambient temperature at non-tropical latitudes.

4.3. Future research

It is important to further investigate potential links between zoo-specific husbandry variables, chronic stress and reproductive patterns in pygmy hippos, and we have identified EIAs that can assist in conducting such studies. Moreover, we still need to address several questions concerning the reproductive biology of the male, including whether the seasonal androgen pattern applies to the *ex situ* population in general and to wild populations. It would additionally be useful to assess androgen concentrations in conjunction with other measures of reproductive function, primarily ejaculate characteristics and mating behavior. Clearly, non-invasive endocrine monitoring can also provide a useful tool for determining

reproductive patterns for wild pygmy hippos, although there are many logistical challenges, including being able to identify individuals.

4.4. Concluding remarks

Measuring hormone metabolites in fecal samples is valuable for identifying and monitoring both gonadal and adrenal activity in free-ranging populations and in wildlife under managed care. For the pygmy hippo, this study has been a step forward by validating non-invasive techniques for characterization of reproductive endocrine patterns in the male and assessment of glucocorticoid metabolites for both sexes. We are now equipped to investigate the dynamics of FGMs during potentially stressful situations, and the possible effects on reproductive health, wellbeing and welfare for pygmy hippos in the *ex situ* population. In addition, the 5α-3β,11β-diol-CM assay may prove to be useful in the future for monitoring FGMs as an indicator of stress in wild populations, particularly in response to anthropogenic environmental changes as a result of extensive logging, mining, and bush-meat hunting in this species' West African rainforest habitat.

Funding

This work was supported by the American Association of Zoo Veterinarians Wild Animal Health Fund; the Center for Conservation of Tropical Ungulates; Omaha's Henry Doorly Zoo and Aquarium; the University of Western Australia (UWA), Convocation Postgraduate Research Travel Award; UWA Postgraduate Student's Association Fieldwork and Data Collection Award; and a UWA Graduate Research School PhD Completion Scholarship.

CRediT authorship contribution statement

Gabriella L. Flacke: Conceptualization, Methodology, Formal analysis, Investigation, Writing – original draft, Visualization, Project administration, Funding acquisition. **Linda M. Penfold:** Validation, Resources, Writing – review & editing. **Franz Schwarzenberger:** Validation, Formal analysis, Resources, Writing – review & editing. **Graeme B. Martin:** Writing – review & editing, Supervision, Funding acquisition. **César A. Rosales-Nieto:** Formal analysis. **Monique C. J. Paris:** Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

We would like to express our gratitude to all of the institutions that participated in this study: Baton Rouge Zoo; Center for Conservation of Tropical Ungulates; Giraffe Ranch; Gladys Porter Zoo, Brownsville; Jackson Zoo; Louisville Zoo; Lincoln Park Zoo, Chicago; Lowry Park Zoo, Tampa; Oklahoma City Zoo; Omaha's Henry Doorly Zoo and Aquarium; and Zoo Miami. Our special appreciation goes out to the husbandry staff that collected the samples; without their help and dedication we would not have been able to conduct this research. We also thank Cayman Adams, Sarah Allred, Kim Daly-Crews, Saleha Khan, Elke Leitner, Lara Metrione, Paige

Pickering, Dr. Kibby Treiber, and Kayla Weller for technical support, and Dr. Rupert Palme for providing the cortisol metabolite EIAs.

Short summary

The pygmy hippopotamus (*Choeropsis liberiensis*) is endangered in the wild and we have limited information concerning the reproduction and welfare of this species under managed care. In this study, we validated enzyme immunoassays for measuring fecal metabolites of gonadal androgens and glucocorticoids in pygmy hippos that can now be used to further characterize the biology of both wild and managed populations.

Data availability

Data will be made available on request.

References

Bashaw, M.J., Sicks, F., Palme, R., Schwarzenberger, F., Tordiffe, A.S.W., Ganswindt, A., 2016. Non-invasive assessment of adrenocortical activity as a measure of stress in giraffe (*Giraffa camelopardalis*). BMC Vet. Res. 12, 235.

Brown, J.L., 2018. Comparative ovarian function and reproductive monitoring of endangered mammals. Theriogenology 109, 2–13.

Brown, J.L., Bellem, A.C., Fouraker, M., Wildt, D.E., Roth, T.L., 2001. Comparative analysis of gonadal and adrenal activity in the black and white rhinoceros in North America by non-invasive endocrine monitoring. Zoo Biol. 20, 463–486.

CEPF, 2000. Ecosystem Profile – Upper Guinean Forest Ecosystem of the Guinean Forests of West Africa Biodiversity Hotspot.

Comizzoli, P., Crosier, A.E., Songsasen, N., Szykman Gunther, M., Howard, J.G., Wildt, D. E., 2009. Advances in reproductive science for wild carnivore conservation. Reprod. Domest. Anim. 44, 47–52.

Dehnhard, M., Clauss, M., Lechner-Doll, M., Meyer, H.H.D., Palme, R., 2001. Non-invasive monitoring of adrenocortical activity in roe deer (*Capreolus capreolus*) by measurement of fecal cortisol metabolites. Gen. Comp. Endocrinol. 123, 111–120.

Edwards, K.L., Shultz, S., Pilgrim, M., Walker, S.L., 2015a. Male reproductive success is correlated with testosterone in the eastern black rhinoceros (*Diceros bicornis michaeli*). Gen. Comp. Endocrinol. 213, 40–49.

Edwards, K.L., Shultz, S., Pilgrim, M., Walker, S.L., 2015b. Irregular ovarian activity, body condition and behavioural differences are associated with reproductive success in female eastern black rhinoceros (*Diceros bicornis michaeli*). Gen. Comp. Endocrinol. 214, 186–194.

Fanson, K.V., Best, E.C., Bunce, A., Fanson, B.G., Hogan, L.A., Keeley, T., Narayan, E.J., Palme, R., Parrott, M.L., Sharp, T.M., Skogvold, K., Tuthill, L., Webster, K.N., Bashaw, M., 2017. One size does not fit all: Monitoring faecal glucocorticoid metabolites in marsupials. Gen. Comp. Endocrinol. 244, 146–156.

Flacke, G.L., Tkalčić, S., Steck, B., Warren, K., Martin, G.B., 2016. A retrospective analysis of mortality in captive pygmy hippopotamus (*Choeropsis liberiensis*) from 1912 to 2014. Zoo Biol. 35, 556–569.

Flacke, G.L., Schwarzenberger, F., Penfold, L.M., Walker, S.L., Martin, G.B., Millar, R.P., Paris, M.C.J., 2017. Characterizing the reproductive biology of the female pygmy hippopotamus (*Choeropsis liberiensis*) through non-invasive endocrine monitoring. Theriogenology 102, 126–138.

Franceschini, M.D., Rubenstein, D.I., Low, B., Romero, L.M., 2008. Fecal glucocorticoid metabolite analysis as an indicator of stress during translocation and acclimation in an endangered large mammal, the Grevy's zebra. Anim. Conserv. 11, 263–269.

Frigerio, D., Dittami, J., M¨ostl, E., Kotrschal, K., 2004. Excreted corticosterone metabolites co-vary with ambient temperature and air pressure in male Greylag geese (*Anser anser*). Gen. Comp. Endocrinol. 137, 29–36.

Ganswindt, A., Palme, R., Heistermann, M., Borragan, S., Hodges, J.K., 2003. Non-invasive assessment of adrenocortical function in the male African elephant (*Loxodonta africana*) and its relation to musth. Gen. Comp. Endocrinol. 134, 156–166.

Ganswindt, A., Tordiffe, A.S.W., Stam, E., Howitt, M.J., Jori, F., 2012. Determining adrenocortical activity as a measure of stress in African buffalo (*Syncerus caffer*) based on faecal analysis. African Zool. 47, 261–269.

Goymann, W., 2012. On the use of non-invasive hormone research in uncontrolled, natural environments: the problem with sex, diet, metabolic rate and the individual. Methods Ecol. Evol. 3, 757–765.

Hadinger, U., Haymerle, A., Knauer, F., Schwarzenberger, F., Walzer, C., 2015. Faecal cortisol metabolites to assess stress in wildlife: Evaluation of a field method in free-ranging chamois. Methods Ecol. Evol. 6, 1349–1357.

Huber, S., Palme, R., Zenker, W., Möstl, E., 2003. Non-invasive monitoring of the adrenocortical response in red deer. J. Wildl. Manag. 67, 258–266.

Isaac, N.J.B., Turvey, S.T., Collen, B., Waterman, C., Baillie, J.E.M., 2007. Mammals on the EDGE: Conservation priorities based on threat and phylogeny. PLoS One 2, e296.

Keay, J.M., Singh, J., Gaunt, M.C., Kaur, T., 2006. Fecal glucocorticoids and their metabolites as indicators of stress in various mammalian species: A literature review. J. Zoo Wildl. Med. 37, 234–244.

Kersey, D.C., Dehnhard, M., 2014. The use of non-invasive and minimally invasive methods in endocrinology for threatened mammalian species conservation. Gen. Comp. Endocrinol. 203, 296–306.

Mason, G.J., 2010. Species differences in responses to captivity: Stress, welfare and the comparative method. Trends Ecol. Evol. 25, 713–721.

Millspaugh, J.J., Washburn, B.E., 2004. Use of fecal glucocorticoid metabolite measures in conservation biology research: considerations for application and interpretation. Gen. Comp. Endocrinol. 138, 189–199.

Moberg, G.P., 2000. Biological response to stress: Implications for animal welfare. In: Moberg, G.P., Mench, J.A. (Eds.), The Biology of Animal Stress: Basic Principles and Implications for Animal Welfare. CABI Publishing, New York, pp. 1–21.

Möstl, E., Messmann, S., Bagu, E., Robia, C., Palme, R., 1999. Measurement of glucocorticoid metabolite concentrations in faeces of domestic livestock. Zentralbl. Veterinarmed. A 46, 621–631.

Möstl,, E., Maggs, J.L., Schr¨otter, G., Besenfelder, U., Palme, R., 2002. Measurement of cortisol metabolites in faeces of ruminants. Vet. Res. Commun. 26, 127–139.

Palme, R., 2005. Measuring fecal steroids: guidelines for practical application. Ann. N. Y. Acad. Sci. 1046, 75–80.

Palme, R., 2019. Non-invasive measurement of glucocorticoids: Advances and problems. Physiol. Behav. 199, 229–243.

Palme, R., Möstl,, E., 1997. Measurement of cortisol metabolites in faeces of sheep as a parameter of cortisol concentration in blood. Int. J. Mamm. Biol. Suppl. II 62, 192–197.

Palme, R., Rettenbacher, S., Touma, C., El-Bahr, S.M., Möstl, E., 2005. Stress hormones in mammals and birds: comparative aspects regarding metabolism, excretion, and non-invasive measurement in fecal samples. Ann. N. Y. Acad. Sci. 1040, 162–171.

Payne, A.H., Hales, D.B., 2004. Overview of steroidogenic enzymes in the pathway from cholesterol to active steroid hormones. Endocr. Rev. 25, 947–970.

Ransom, C., Robinson, P.T., Collen, B., 2015. *Choeropsis liberiensis* [WWW Document]. IUCN Red List Threat. Species 2015. https://doi.org/e.T10032A18567171.

Rivier, C., Rivest, S., 1991. Effect of stress on the activity of the hypothalamic-pituitary-gonadal axis: peripheral and central mechanisms. Biol. Reprod. 45, 523–532.

Sapolsky, R.M., 1992. Neuroendocrinology of the stress response. In: Becker, J.B., Breedlove, S.M., Crews, D. (Eds.), Behavioral Endocrinology. MIT Press, Cambridge, pp. 287–324.

Saragusty, J., Hildebrandt, T.B., Bouts, T., Göritz, F., Hermes, R., 2010. Collection and preservation of pygmy hippopotamus (*Choeropsis liberiensis*) semen. Theriogenology 74, 652–657.

Saragusty, J., Hermes, R., Hofer, H., Bouts, T., Göritz, F., Hildebrandt, T.B., 2012. Male pygmy hippopotamus influence offspring sex ratio. Nat. Commun. 3, 1–5.

Schatz, S., Palme, R., 2001. Measurement of faecal cortisol metabolites in cats and dogs: A non-invasive method for evaluating adrenocortical function. Vet. Res. Commun. 25, 271–287.

Schwarzenberger, F., 2007. The many uses of non-invasive faecal steroid monitoring in zoo and wildlife species. Int. Zoo Yearb. 41, 52–74.

Shutt, K., Setchell, J.M., Heistermann, M., 2012. Non-invasive monitoring of physiological stress in the Western lowland gorilla (*Gorilla gorilla gorilla*): validation of a fecal glucocorticoid assay and methods for practical application in the field. Gen. Comp. Endocrinol. 179, 167–177.

Steck, B., 2021. International Studbook for the Pygmy Hippopotamus 2020, 27th ed. Zoo Basel, Basel, Switzerland.

Swanson, W.F., Johnson, W.E., Cambre, R.C., Citino, S.B., Quigley, K.B., Brousset, D.M., Morais, R.N., Moreira, N., O'Brien, S.J., Wildt, D.E., 2003. Reproductive status of endemic felid species in Latin American zoos and implications for ex situ conservation. Zoo Biol. 22, 421–441.

Tilbrook, A.J., Turner, A.I., Clarke, I.J., 2000. Effects of stress on reproduction in non-rodent mammals: the role of glucocorticoids and sex differences. Rev. Reprod. 5, 105–113.

Touma, C., Palme, R., 2005. Measuring fecal glucocorticoid metabolites in mammals and birds: the importance of validation. Ann. N. Y. Acad. Sci. 1046, 54–74.

Touma, C., Sachser, N., Möstl, E., Palme, R., 2003. Effects of sex and time of day on metabolism and excretion of corticosterone in urine and feces of mice. Gen. Comp. Endocrinol. 130, 267–278.

Verschuren, J., 1983. Conservation of Tropical Rain Forest in Liberia – Recommendations for Wildlife Conservation and National Parks. Gland, Switzerland.

Wasser, S.K., Hunt, K.E., Brown, J.L., Cooper, K., Crockett, C.M., Bechert, U., Millspaugh, J.J., Larson, S., Monfort, S.L., 2000. A generalized fecal glucocorticoid assay for use in a diverse array of nondomestic mammalian and avian species. Gen. Comp. Endocrinol. 120, 260–275.

Wildt, D.E., Brown, J.L., Bush, M., Barone, M.A., Cooper, K.A., Grisham, J., Howard, J.G., 1993. Reproductive status of cheetahs (*Acinonyx jubatus*) in North American zoos: The benefits of physiological surveys for strategic planning. Zoo Biol. 12, 45–80.

Wingfield, J.C., Sapolsky, R.M., 2003. Reproduction and resistance to stress: When and how. J. Neuroendocrinol. 15, 711–724.

UNEP-WCMC, 2011. Taï National Park, Côte d'Ivoire. Cambridge, UK and Monrovia, Liberia.