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Fecal Glucocorticoid Measurements and Their Relation to Rearing, Behavior, and Environmental Factors in the Population of Pileated Gibbons (*Hylobates pileatus*) Held in European Zoos

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Abstract Pileated gibbons (*Hylobates pileatus*) are rated as endangered according to the International Union of Conservation of Nature (IUCN) Red List. The captive population suffers from poor breeding success and is threatened to become overaged. Although several factors are likely to contribute to the poor breeding success, one in particular may be chronic stress associated with prolonged periods of high glucocorticoid (GC) output. We investigated fecal GC levels of pileated gibbons (*Hylobates pileatus*) and their relationship to specific life-history variables and environmental factors. After validation of an enzyme immunoassay for the measurement of 5-reduced 3α,11β-dihydroxy cortisol metabolites to assess GC output reliably in pileated gibbons, we collected fecal samples over several days from all 36 European adult pileated gibbons located in 11 institutions and compared

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GC levels to intrinsic individual parameters, husbandry, behavior, and breeding history. Age, sex, and origin (wild vs. captive born) had no effect on GC levels. However, unnaturally reared gibbons had higher GC levels and showed more behavioral abnormalities than parent-reared individuals. Further, nonreproducing gibbons living in a pair without infants had higher GC concentrations than gibbons living in a family, bachelor group, or as singletons. With respect to environmental factors, a large size of the inside enclosure and the existence of visual protection from visitors was associated with lower fecal GC output. The data indicate that rearing and housing conditions appear to correlate to GC levels in pileated gibbons housed under captive conditions. It is hoped this knowledge will support the future management of the species in captivity and thus lead to a more successful breeding of this endangered primate.

Keywords Behavioral abnormalities · Enclosure size · Fecal cortisol metabolites · Hand-rearing · *Hylobates pileatus* · Pileated gibbon · Stress · Visitors

Introduction

Pileated gibbons (*Hylobates pileatus*) are one of the up to 16 distinct gibbon species (Thinh *et al.* 2010) living in the tropical rain forests of eastern Thailand, western Cambodia, and southwest Laos. The species is rated as endangered according to the IUCN (International Union of Conservation of Nature) Red List and the wild population, estimated to consist of *ca.* 49,000 individuals in Thailand and Cambodia (Phoonjampa and Brockelmann 2008), is predicted to decline further in the near future owing to extensive habitat destruction and forest fragmentation as well as hunting for the pet trade. Pileated gibbons are thus under constant threat and can be considered underprotected in the wild. As part of conservation activities, efforts have been made to breed the species in captivity since 1957. Today, the total captive population of pileated gibbons, which is managed by coordinated breeding programs in the United States (Species Survival Plan [SSP]) and Europe (European Endangered Species Programme [EEP]), consists of *ca.* 107 individuals worldwide, housed in *ca.* 21 institutions. Although the global captive population of this monogamous species has generally increased over the last decades, breeding the species in captivity is still suboptimal because the population shows a low reproductive rate and, moreover, the sex ratio of the adults as well as of the offspring born is skewed toward males (International Pileated Gibbon Studbook 2008).

The factors underlying the low breeding success of the species in captivity are probably numerous, including high infant mortality, behavioral disorders (Skyner *et al.* 2004), and health-related problems. In addition, an increased level of stress, i.e., due to suboptimal housing conditions or incompatibility of the breeding pair/group, may contribute to the lack of breeding in many of the captive pileated gibbon groups. It is well known that chronically elevated stress hormone (glucocorticoid [GC]) levels can suppress reproductive function, alter animal behavior, and increase susceptibility to disease by disrupting immune functions (Cameron 1997; Carlsdead *et al.* 1993; Dobson and Smith 2000; Heistermann *et al.* 2004; Sapolsky *et al.* 2000;

Wielebnowski *et al.* 2002). The assessment of stress hormone levels and the intrinsic and socioenvironmental factors that influence GC output in the potential breeding individuals are important for developing a better understanding of whether chronic stress might also contribute to the problems encountered with breeding pileated gibbons in captivity. However, to date such information is not available for captive pileated gibbons or any other gibbon species.

The measurement of fecal GCs has been established as a valuable noninvasive tool to evaluate effects of individual and environmental factors on adrenocortical activity in captive-housed individuals of different species (Barbosa and da Silva Mota 2009; Carlstead and Brown 2005). In the case of nonhuman primates, these studies have shown that GC output can be influenced by intrinsic factors such as age, sex, and reproductive status (Rangel-Negrin *et al.* 2009; Stoinski *et al.* 2002; Ziegler *et al.* 1995) as well as by environmental (extrinsic) factors such as the presence of visitors (Chamove *et al.* 1988; Cooke and Schillaci 2007; Davis *et al.* 2005), suboptimal housing conditions (Boinski *et al*. 1999), and social factors, e.g., group structure or social status (Abbott *et al.* 2003). To this end, our overall aim here was to examine to what extent these variables also influence adrenocortical activity in captive-housed pileated gibbons and thereby to provide information on which future captive management decisions can be based. Because to date no noninvasive method has been reported in the literature for assessing adrenocortical activity in gibbons based on fecal samples, we initially established and validated such a method for monitoring GC output. Using the newly established methodology, our more specific objectives were then 1) to characterize adrenocortical activity in all adult males and females of the captive EEP population of pileated gibbons and 2) to investigate the potential influence of individual and life-history variables as well as environmental factors on GC levels. The information generated should help to identify the nature and significance of factors influencing stress hormone output in pileated gibbons, information that not only is important to understand better the general stress physiology of the species but may also provide the basis to evaluate the quality of housing conditions and identify potential factors associated with impaired breeding of the species under captive conditions.

Materials and Methods

Subjects and Individual Characteristics

We studied all 36 adult pileated gibbons (22 males, 14 females) of the European captive breeding population. The subjects were housed in 11 institutions and 18 groups, either as pairs, bachelor or family groups, or singletons (Table I). Information about the subjects' basic characteristics such as sex, age, origin (wild-born/captive-born), rearing history (unnaturally reared/parent-reared), and breeding success was provided by the international studbook. All females were in a nonpregnant state during the sampling period.

The mean age (end of 2009) was 18.5 yr (range: 5–50 yr) in focal males and 17.6 yr (range: 5–35 yr) in focal females. Of the 36 subjects, 13 were wild-born and 23 were bred in captivity. Whereas the rearing history of the captive-born individuals

Table I Number of pileated gibbons (*Hylobates pileatus*), number of enclosures, and group structure in all investigated European facilities (end of 2009)

Facility	Sex distribution (male.female.infant)	Number of groups	Group structure
1	1.1.2	1	Family
2	1.0	1	Single
3	3.1	2	Pair; bachelor
4	1.0	1	Single
5	4.1.2	2	Family; single
6	1.1	1	Pair
7	1.2.1	1	Family
8	1.1	1	Pair
9	1.1.2	1	Pair
10	4.2.2	3	Family; pair; bachelor
11	5.3.5	4	Family; pair; bachelor

is known, that of the wild-born animals is mostly missing. However, because wild-born gibbons in zoos originate mainly from rescue centers, we considered them to be unnaturally reared because they are usually taken from their mothers at a very young age and kept as pets with little or no contact with other gibbons. Thus, of the 36 focal individuals, 17 were unnaturally reared and 19 were parent-reared.

For every subject, we asked keepers to assess the general behavior of their gibbons to determine if certain individuals behaved "abnormally." Although we are aware that it is difficult to determine and assess an individual's degree of abnormal behavior under captive conditions without detailed behavioral observations (which were, however, not possible in the scope of this study), we used the quality of key behaviors to distinguish what we thought would be a more normal from a more abnormal behavioral pattern. In this respect, we evaluated the following behavioral patterns: 1) aggressive behavior toward visitors/keepers (yes/no), 2) attached behavior toward humans (yes/no), 3) fearful of conspecifics (yes/no), 4) not interested in conspecific (ignorant; yes/no), 5) aggressive behavior toward conspecifics (yes/no), and 6) stereotypies (yes/no). If the answers for an individual contained >1 "yes," we categorized it in the group with "abnormal behavior."

All subjects were clinically healthy at the time of fecal sample collection and no medication potentially influencing GC levels was administered during sample collection. The diet in the different zoos was similar, consisting mainly of fruits and vegetables, and except in 2 zoos all gibbons received primate pellets on a daily basis. Behavioral enrichment was provided through hidden food in most facilities, and 1 zoo conducted regular clicker training.

Environmental Variables

M. Pirovino visited all institutions during 2009 to record data on housing conditions of the focal individuals as well as to facilitate fecal sample collection. Specifically, we recorded the following parameters: 1) the volume of the inside enclosure (m^3), 2) the volume of the outside enclosure (islands: the height of the trees was estimated by

M. Pirovino), 3) visual protection from visitors/keepers (present/absent), 4) and 5) protection from other gibbons (apart from cage mates; all gibbon species included) in visual range (yes/no) and in hearing distance (yes/no), and 6) the number of swinging/climbing elements per enclosure (inside/outside).

Except for 1 institution, which had only an indoor enclosure for its gibbons, all enclosures consisted of an indoor and an outdoor area. We either measured height and volume of the enclosures on site or calculated them from construction plans. Average total enclosure volume was 617.33 m^3 (range: 113–2818 m^3). Inside enclosures had an average volume of 117.1 m^3 (range: 10–616 m^3) and an average height of 3.1 m (range: 2–6 m). Outside enclosures had an average volume of 500.2 m^3 (range: 72–2,260 m^3) and an average height of 4.4 m (range: 2.5–6 m). Five enclosures had an outside area on an island. Two of the 18 enclosures had no public access.

Further, we evaluated group structure (family/pair/bachelor group/single) at the time of fecal sample collection. Owing to their high number of males in the captive population, males are kept not only in families or pairs but also in bachelor groups or as singletons.

Collection of Fecal Samples

From each focal subject, we generally collected morning fecal samples (between 07:00 h and 10:00 h) after we observed the individual defecating. Immediate access to fecal samples was not always possible because of safety regulations. We placed only samples uncontaminated with urine in plastic tubes and stored them frozen at −20°C within 2 h upon defecation. We observed no extreme activity or inactivity in any of our focal subjects during the sampling period, thus excluding an influence by this potential parameter on fecal GC levels. On mean, we collected 7.8 samples per individual (range: 2–36 samples), providing a total of 248 samples for GC analysis. We shipped samples frozen to the endocrine laboratory of the German Primate Centre for GC analysis.

Validation of Fecal GC Measurements

We determined the validity of a cortisol (CORT; Palme and Möstl 1997), corticosterone (CCST; Heistermann *et al.* 2006), and 2 group-specific enzyme-immunoassays against cortisol metabolites with a 3α,11-oxo (3α,11-oxo-CM; Möstl and Palme 2002) and 3α,11β-dihydroxy structure (3α,11β-dihydroxy-CM; Ganswindt *et al.* 2003) to assess adrenocortical function in pileated gibbons. All 4 assays have been successfully used to monitor GC output in various other primate and nonprimate species (Ganswindt *et al.* 2003; Heistermann *et al.* 2004, 2006; Wasser *et al.* 2000). For validation, we used 1) a physiological stimulation of the adrenal gland by injecting a synthetic adrenocorticotropic hormone (ACTH) preparation (a single dose of 50 IU Synacthen®, Novartis, Switzerland; *cf.* Heistermann *et al.* 2006) in 2 individuals (1 male, 1 female) and 2) a biological stressor (anesthesia in combination with a routine transport for gibbon exchange between zoos; travel time by plane and car *ca.* 10–12 h) in 4 other individuals (2 males, 2 females) to evaluate whether the induced increase in GC output is detected by the

different fecal GC assays. The ACTH administration was approved by the animal welfare committee of the Canton Zürich, Switzerland (No. 80/2008). Anesthesia was induced with 4–10 mg/kg ketamine (Narketan®, 10 ad us. vet., Vétoquinol, 3123 Belp, Switzerland) and 40–80 μg/kg medetomidine (Domitor®, Orion Pharma, 02101 Espoo, Finland) intramuscularly by blow pipe and was performed as a part of a routine preshipment examination and loading. We reversed the medetomidine hydrochloride with 200–400 μg/kg atipamezole (Antisedan® hydrochloride) and monitored subjects until they recovered in crates before shipment. From each individual, we collected daily fecal samples for 4–6 d before the treatment to establish pretreatment baseline GC levels and for 5 d thereafter to establish the GC response. From the ACTH-treated subjects, we collected every sample defecated within the first 72 h after ACTH injection with once daily samples for the next 2 d. From the transported individuals, we usually collected samples once daily. We stored fecal samples at −20°C and until shipping them frozen to the endocrine laboratory for analysis.

We processed and extracted all samples as described by Heistermann *et al.* (1995). Briefly stated, we lyophilized and pulverized the fecal samples and extracted an aliquot representing 0.05–0.08 g of fecal powder with 3 ml of 80% methanol by vortex-mixing for 15 min. After centrifugation of the fecal suspension, we recovered the supernatant and stored it at −20°C until hormone analysis. We analyzed fecal extracts for GC immunoreactivity by the 4 different aforementioned EIA systems as described in detail by Heistermann *et al.* (2004, 2006). Information on antibody characteristics, standards, and labels used as well as on other assay details, e.g., data on assay sensitivities, is given in Heistermann *et al.* (2006).

To assess the pattern of metabolites measured and thus characterize the specificity of the 4 GC assays, we performed a reverse-phase high performance liquid chromatography (RP-HPLC) on a male fecal extract representing peak GC response to transport using the procedure previously described by Möhle *et al.* (2002) and Heistermann *et al.* (2006). The HPLC system used also allowed us to evaluate whether the GC antibodies tested show a co-measurement of certain fecal androgens that may potentially be detected by antibodies raised against cortisol metabolites (Ganswindt *et al.* 2003; Heistermann *et al.* 2006; Möstl and Palme 2002). After HPLC, we measured each fraction in all 4 GC assays to generate the profiles of immunoreactivity.

Fecal GC Measurements

Based on the outcome of the validation tests, we finally analyzed fecal samples in the 3α,11β-dihydroxy-CM EIA. For this, we processed and extracted all samples as described in the preceding text. On extraction, we diluted extracts 1:50–1:500 (depending on concentration) and took duplicate aliquots to assay. Sensitivity of the assay was 1 pg/well. Fecal extracts from different individuals gave displacement curves, which were parallel to the 11β-hydroxyetiocholanolone standard curve. Intra- and interassay coefficients of variation of high- and low-value quality controls were 5.2% (high, $n=16$) and 7.5% (low, $n=16$) and 11.6% (high, $n=14$) and 15.8% (low, $n=14$), respectively. We express all hormone concentrations as μg/g dry fecal weight.

Statistical Analysis

We determined correlations of fecal GC levels measured by the 4 different EIAs with each other in each individual using the Pearson-Product-Moment correlation. To evaluate the impact of the different variables on GC output in the data set for zoo gibbons, we initially calculated the median and interquartile range of GC levels for each individual. Sex, rearing history, incidence of abnormal behavior, group structure (family/pair), visual protection from visitors, other gibbons in hearing distance, and other gibbons in visual range were nominal data and we used the *t*-test to compare the fecal GC concentrations between these groups. We applied multiple regression analysis to evaluate the influence of the inside and outside volume of the gibbon's enclosures (independent variable) on GC levels (dependent variable). In addition, we used a mixed-effect ANOVA to test the effects of the various parameters on median fecal GC concentrations of each individual. Main effects introduced to the model included categorical factors (sex, rearing history, abnormal behavior) and continuous factors (visual protection from visitors, inside and outside enclosure volume). Based on preliminary plots of single factors, only one interaction term was included (sex × age). To achieve a normal distribution of residuals and to meet the assumption of linearity in continuous predictor effect, we log-transformed the measure of enclosure volume before analysis. We carried out all analyses with STATISTICA (Statistika™ 8.0, StatSoft®) using a significance level (2-tailed) of $p<0.05$.

Results

Validation of Fecal GC Measurements

In absolute terms, highest levels of fecal GCs were measured via the 11oxo- and 11β-hydroxy-CM EIAs (peak value range: 3.4–33.4 μg/g; Table II), those measured via the CORT and CCST assays being generally much lower (peak value range: 0.08–1.36 μg/g; Table II). All six subjects responded to the treatment (ACTH injection or anesthesia/transport) with a clear increase in fecal GC levels (Fig. 1, Table II) and with very few exceptions (3 out of 16 cases), fecal GC levels determined via the 4 EIAs significantly correlate with each other in each of the 6 individuals (mean *r*-value range: 0.65–0.90; *p*-value range: 0.05 to <0.001).

For each assay, the magnitude of response varied between subjects; however, there was no obvious difference in terms of the magnitude of GC elevation and absolute levels measured according to the type of treatment, e.g., ACTH or transport (Table II). The magnitude of response also clearly differed across the 4 assays, with the CORT assay showing on average the lowest response (2–3-fold; Fig. 1), while the CCST and the two group-specific cortisol metabolite assays detected a more marked elevation (6–7-fold; Fig. 1). Timing of peak GC elevation was consistent across assays but varied between subjects (Table II). In most cases, however, we detected a peak response between 22 and 55 h (Table II; Fig. 1) and GC levels had usually returned to pretreatment baseline levels by d 5 (Fig. 1).

Table II Fecal GC concentrations (as detected via 4 different assays) in pileated gibbons (*Hylobates pileatus*) in response to an ACTH challenge or anesthesia/transport stress

Name	Treatment	3α, 11β-Dihydroxy-CM				3α, 11oxo-CM				CORT				CCST			
		Pre[c]	Peak[d]	Delta[e]	Lag[f]	Pre[b]	Peak[c]	Delta[d]	Lag[f]	Pre[b]	Peak[c]	Delta[d]	Lag[f]	Pre[b]	Peak[c]	Delta[d]	Lag[f]
Kiki[a]	ACTH	1.62	15.44	9.5	22.0	4.66	33.39	7.2	22.0	0.042	0.257	6.1	22.0	0.032	1.356	42.4	22.0
Yhinda[b]	ACTH	0.62	4.03	6.5	54.8	1.59	15.23	9.6	54.8	0.029	0.120	4.1	30.3	0.018	0.143	7.9	54.8
Serotine[b]	Transport	0.49	17.16	35.0	24–40	0.27	10.93	40.5	24–40	0.036	0.203	5.6	24–40	0.029	0.378	13.0	24–40
Yhinda[b]	Transport	1.32	7.65	5.8	38–48	1.78	4.93	2.8	72–96	0.041	0.704	17.2	72–96	0.053	0.938	17.7	72–96
Banyar[a]	Transport	0.41	8.33	20.3	40–84	1.17	14.57	12.5	40–84	0.023	0.106	4.6	40–84	0.014	0.119	8.5	40–84
Chamoa[a]	Transport	0.29	3.36	11.6	24–40	0.63	7.37	11.7	24–40	0.027	0.081	3.0	24–40	0.014	0.118	8.4	24–40

[a] Male.

[b] Female.

[c] Pretreatment levels in μg/g feces (see Materials and Methods).

[d] Peak levels in response to treatment in μg/g.

[e] *x*-fold increase of peak levels above pretreatment concentrations.

[f] Lag time in hours between injection of ACTH or sedation for transport and peak response. Because for transported subjects samples were collected only once daily, lag times were estimated, e.g., 24–40 h means that the peak concentration was recorded after 40 h, but could have occurred earlier because no samples were available between 24 and 40 h.

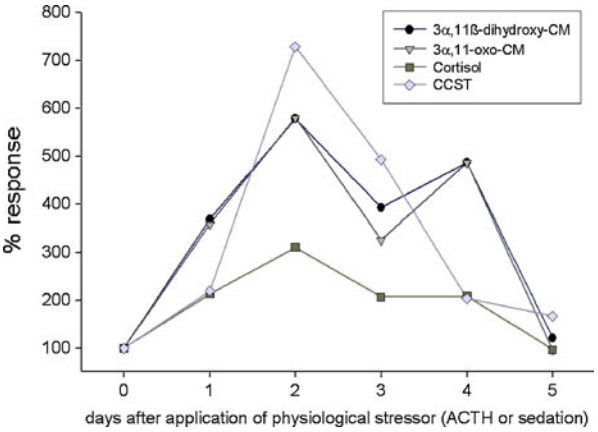

Fig. 1 Percentage of response in immunoreactive fecal GC levels to a physiological stressor in pileated gibbons (*Hylobates pileatus*). Data points represent median values calculated for 24-h intervals across the 6 individuals examined. Time 0 = time of ACTH injection or anesthesia.

HPLC analysis indicated that in the CORT and the 2 group-specific assays, the majority (*ca.* 90%) of immunoreactivity was detected as several distinct peaks between fractions 9 and 31 and thus at positions where cortisol metabolites in our HPLC system elute (Heistermann *et al.* 2006). The presence of only small amounts of immunoreactivity measured after fraction 31 (positions were certain potentially cross-reacting androgen metabolites elute; Ganswindt *et al.* 2003; Heistermann *et al.* 2006) suggests a low degree of co-measurement of androgens in these three assays (Fig. 2a). Moreover, the presence of major peaks of immunoreactivity at the elution positions of 11β-hydroxyetiocholanolone (fractions 24/25) and 11oxo-etiocholanolone (fractions 29/30) in the respective assays indicated that these 2 cortisol metabolites were abundant in pileated gibbon feces. In contrast, the HPLC profile measured via the CORT assay showed a higher number of immunoreactivity peaks, with no clear peak at the position of authentic cortsiol (fractions 14/15; Fig. 2b). The HPLC profile of immunoreactivity detected via the CCST assay indicated a large number of peaks, many of which eluted beyond fraction 31, and thus at positions where cortisol metabolites are unlikely to elute (Heistermann *et al.* 2006), suggesting that this assay was highly nonspecific and likely co-measured substances not derived from cortisol metabolism (Fig. 2b).

Fecal GC Analysis of the Captive Pileated Gibbon Population

Overall, subjects varied substantially in their fecal GC levels, ranging from 181.8 ng/g feces to 3053.9 ng/g, with a mean of 958.3±SD 782.3 ng/g. Table III summarizes the data on statistical comparisons of fecal GC concentrations in relation to the various individual parameters and environmental variables examined.

Individual Parameters and Fecal GC Output Neither sex, age, nor origin was significantly associated with fecal GC output in captive-housed pileated gibbons (Table III). However, we found significantly higher mean fecal GC concentrations in unnaturally reared compared to parent-reared individuals and individuals showing behavioral abnormalities compared to those showing social behavior more typical for the species (Table III). Rearing condition and behavior were significantly

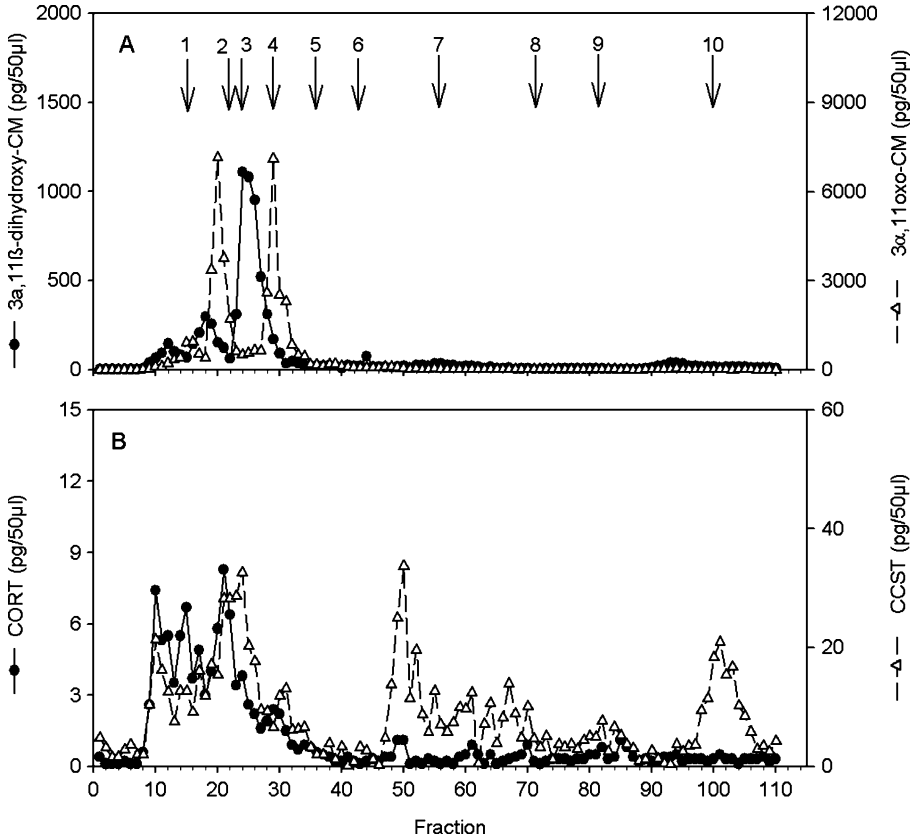

Fig. 2 HPLC profiles of immunoreactivity detected with **(A)** the 3α,11β-dihydroxy-CM and 3α,11oxo-CM EIA and **(B)** the cortisol (CORT) and corticosterone (CCST) EIA in a peak sample after adrenocortical stimulation in a captive male pileated gibbon (*Hylobates pileatus*). Arrows indicate elution positions of reference standards: 1) cortisol (fraction 14), 2) corticosterone (22), 3) 11β-hydroxyetiocholanolone (24), 4) 11-oxoetiocholanolone (29), 5) 5β-androstane-3,11,17-trione (36), 6) testosterone (43), 7) androstendione, dehydoepiandrosterone (55), 8) epiandrosterone, 5β-DHT, 5β-androstane-3β-ol-17-one (72), 9) 5β-androstane, 3α-ol-17-one (82), and 10) androsterone (100).

associated ($p<0.001$); 82.4% ($n=14$) of the unnaturally reared individuals showed abnormal behavior vs. 5.3% ($n=1$) of the parent-reared subjects.

Environmental Variables and GC Output Fecal GC concentrations of subjects in the 11 housing institutions showed substantial differences, with individuals in certain locations, e.g., no. 11, showing consistently low levels but those in others, e.g. no. 10, showing markedly elevated concentrations (Fig. 3). GC levels did not differ according to whether the focal subjects were in visual range of hearing distance with other gibbons, but individuals with visual protection from visitors had significantly lower GC concentrations than those without visual protection (Table III).

The volume of the inside enclosure showed a significant negative correlation with GC output (multiple regression analysis, $R=0.421$, $n=36$; $p=0.011$), i.e., subjects

Table III Comparison of mean fecal GC concentration ± standard error (SE) in relation to sex, origin, rearing, behavior, and protection from visitors in captive pileated gibbons (*Hylobates pileatus*) using *t*-test (n=36)

Variable	Mean ± SE fecal GC (ng/g)		p value
Sex	Male (n=22)	Female (n=14)	0.219
	819.24±151.71	1142.42±219.67	
Origin	Wild born (n=13)	Captive born (n=23)	0.376
	1,096±263.18	859.01±133.61	
Rearing	Hand-reared (n=17)	Parent-reared (n=19)	0.043
	1214.61±208.89	703.63±133.53	
Behavior	Normal (n=21)	Abnormal (n=15)	0.036
	722.78±127.87	1255.93±228.39	
Visual protection from visitors	Protection (n=14)	No protection (n=22)	0.003
	490.95±107.69	1234.24±170.70	
Gibbons in visual range	No gibbons visible (n=23)	Gibbons visible (n=13)	0.300
	844.68±157.59	1122.29±213.37	
Gibbons in hearing distance	No gibbons audible (n=12)	Gibbons audible (n=24)	0.218
	833.21±135.86	1168.36±264.30	

housed in smaller enclosures had higher GC levels than those in larger enclosures. Specifically, gibbons housed in an inside enclosure <75 m³ (n=22) had on average 3 times higher GC concentrations (1283.7±SD 774.7 ng/g) than gibbons with an inside enclosure >75 m³ (n=14; 412.6±SD 305.8 ng/g; p<0.001). 75 m³ is the minimum size for an inside enclosure according to the Swiss animal welfare law (Schweizerische-Tierschutzverordnung 2009). The number of climbing/swinging elements in the inside and outside enclosures depended on the volume of the enclosures and was therefore not separately used for further analyses.

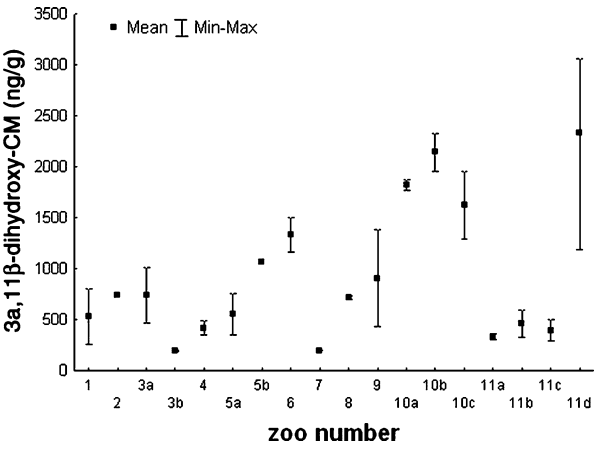

Fig. 3 Fecal GC concentrations of adult pileated gibbons (*Hylobates pileatus*) (n=36) in the 18 enclosures. The number on the *x*-axis corresponds to a single institution and the letters to different enclosures in each institution.

Group structure had also an effect on GC output. Individuals living in a family had significant lower GC concentrations than those living in a pair without offspring (p=0.038; Table IV). Among males, mean GC concentration of individuals living in a pair was not significantly different compared to those found in males living in a bachelor group or single (Table V). Rearing history had an apparent effect on stress hormone levels (Table V). Whereas parent-reared males had relatively low fecal GC concentrations when kept in groups as pairs, family, or as bachelors, GC levels in unnaturally reared males were on average 3–4 times higher under all these conditions (Table V). The females, which were kept only in a pair or family, had no significant difference in fecal GC concentration between these 2 groups or between unnaturally and parent-reared females kept in a pair/family (Table V). If data of all subjects are combined, unnaturally reared individuals had significantly higher GC levels than parent-reared ones (p=0.043; Table III).

Individual and Environmental Factors Combined The volume of the inside enclosure had the strongest effect and was the only significant predictor of GC levels in this model (Table IV).

Discussion

This is the first study determining adrenocortical activity in the pileated gibbon and evaluating the potential impact of individual and environmental factors on GC output in this species. The results of this cross-sectional study indicate that life-history factors, such as rearing history, and several environmental factors, such as group structure and enclosure size, significantly affect fecal GC concentrations. The data therefore suggest that rearing and housing conditions are important components determining an individual's GC output, which in turn may affect the welfare and breeding success of the species in captivity.

A major aim of this study was to validate a reliable assay for measuring fecal GCs as a measure of physiological stress in gibbons, which is of primary importance before any application (Heistermann *et al.* 2006; Touma and Palme 2005). In the current study, both group-specific cortisol metabolite assays appeared to be clearly superior over the 2 ones designed to measure cortisol and corticosterone in blood, as indicated by

Table IV Influence of different effects on fecal GC concentration in captive pileated gibbons (*Hylobates pileatus*) in a mixed-effects ANOVA (n=35 individuals)

Effect	F (df, total df) ratio	p value
Sex	F (1,25)=2.351	p=0.144
Age	F (1,25)=0.557	p=0.466
Sex * age	F (1,25)=1.147	p=0.299
Rearing	F (1,25)=1.586	p=0.225
Behavior	F (1,25)=0.272	p=0.609
Volume inside (log)[a]	***F* (1,25)=8.381**	***p*=0.010**
Volume outside (log)[a]	F (1,25)=0.196	p=0.664
Visual protection from visitors	F (1,25)=1.324	p=0.266

Bold type indicates $p<0.05$.

[a] The volume is log-transformed to linearity.

Table V Mean fecal GC concentration ± SE (ng/g) of captive male and female pileated gibbons (*Hylobates pileatus*) kept in different group structures (n=36 individuals)

Group structure	Mean ± SE fecal glucocorticoid (ng/g)				
	All subjects (n)	All males (n)	Hand-reared males (n)	Parent-reared males (n)	females (n)
Family	689.8±187.84 (n=13)	600.98±235.20 (n=7)	1154.39±798.66 (n=2)	379.62±97.52 (n=5)	793.41±319.44 (n=6)
Pair	1321.33±218.12 (n=14)	1210.83±369.20 (n=6)	1593.80±440.51 (n=4)	444.91±20.21 (n=2)	1404.19±281.63 (n=8)
Bachelor		733.92±296.48 (n=6)	1140.36±516.09 (n=3)	327.48±89.20 (n=3)	
Single		715.96±205.60 (n=3)	543.70±194.39 (n=2)	1060.49 (n=1)	

substantially higher levels of immunoreactivity measured, a more specific measurement, and a generally higher response to the applied stressor. In addition, HPLC immunoreactivity peaks coeluting with 11β-hydroxyetiocholanolone and 11-oxoetiocholanolone standards indicated the presence of 3α,11β-dihydroxylated GCMs and 11,17-dioxoandrostanes, both of which have also been reported as abundant fecal cortisol metabolites in other primate and nonprimate species (Ganswindt *et al.* 2003; Heistermann *et al.* 2006; Ostner *et al.* 2008; Palme and Möstl 1997). In contrast, native cortisol and corticosterone appeared to be present in only small amounts in the feces of pileated gibbons, a finding consistent with data from studies on many other species (Heistermann *et al.* 2006; Palme *et al.* 2005; Wasser *et al.* 2000). Concerning the 2 cortisol metabolite assays, both appeared to be equally valid for assessing GC output in pileated gibbons. For practical reasons, we chose the 11β-hydroxyetiocholanolone (3α,11β-dihydroxy-CM) assay, which researchers have successfully used to monitor adrenocortical activity in several other primate species (Fichtel *et al.* 2007; Girard-Buttoz *et al.* 2009; Ostner *et al.* 2008).

Using the established methodology, we were able to assess GC output in all adult animals of the EEP population and to examine the correlation between intrinsic and extrinsic variables to adrenocortical activity in our focal subjects. Our data suggest that sex, age, and origin (wild vs. captive-born) of the subject had no significant influence on GC levels, suggesting that GC output in response to current husbandry conditions was not obviously influenced by these intrinsic variables.

In contrast, rearing history appeared to be of remarkable importance in how housing conditions will be perceived in a later stage of life. The unnaturally reared gibbons in the present study exhibited not only significantly higher fecal GC levels than parent-reared animals, but also appeared to show more abnormal, nonsocial behavior. In contrast to the influence of rearing experience on physiology, i.e., adrenocortical activity, later in life, the influence of rearing on adult behavior is very well documented (Goin and Gust 1998; Meder 1988; Mootnick and Nadler 1997). Behavioral abnormalities toward conspecifics or humans in unnaturally reared primates are often related to a lack of adequate social learning and imprinting on humans in early infancy (Porton and Niebruegge 2006). Although prenatal stress can

affect an infant's neurobehavioral status (Coe *et al.* 2002, 2010), it is unknown how rearing history affects the functional development of the hypothalamic–pituitary–adrenal axis, but it is rather unlikely that unnaturally reared individuals experience differences vs. normally reared ones. Therefore, individuals missing the species-typical socialization process during infant life might be as adults overwhelmed by social demands from their conspecifics or their own social incompetence, which in turn may lead to elevated stress hormone levels as found in our study. Given the potential negative impact of elevated GC output on health, behaviour, and reproduction (Cameron 1997; Carlsdead *et al.* 1993; Dobson and Smith 2000; Heistermann *et al.* 2004; Sapolsky *et al.* 2000; Wielebnowski *et al.* 2002), this potential physiological effect of hand-rearing should be taken into account when decisions are made how to raise an infant not accepted by the mother. Therefore rearing conditions should be carefully evaluated, and the presence of at least another young conspecific is recommended when the decision for hand-rearing is made, despite the fact that peer-rearing is still much less than optimal, having significant and lasting costs (Goin and Gust 1998).

In addition to the intrinsic and life-history factors, several environmental factors also appeared to affect GC output in our pileated gibbon subjects. GC levels were, for instance, negatively associated with the size of the inside enclosure and visitors visibility. Both factors are likely to be interconnected because larger enclosures might allow the subject to have a greater physical distance to visitors and a better visual protection from visitors. The importance of enclosure size as a strong predictor of GC output in captive-housed pileated gibbons was substantiated by the results of the mixed-effects ANOVA in which the size of the inside enclosure remained highly significant, including all examined effects. In contrast, the volume of the outside enclosure did not have an effect on GC concentration, probably because outside enclosures were always large, whereas inside enclosures were usually much smaller and more variable in size. Because in institutions located in central Europe, gibbons require the inside enclosure for shelter from rain, sun, or cold and thus spend most of their time inside, an adequate size of this enclosure seems to be important for minimizing physiological stress and thus improving the overall well-being of the individuals. In this respect, it seems that the minimal size of 75 m^3 for housing gibbons recommended by the Swiss animal welfare act (Schweizerische-Tierschutzverordnung 2009) represents a useful guideline because gibbons living in such relatively large enclosures exhibited significantly lower GC levels than animals living in smaller cages suggested to be sufficient by the Association of Zoos and Aquariums (AZA 1997). This result is not surprising given that a larger enclosure with a certain height provides subjects with more space to express their species-typical movements (brachiation) and allows them to hide from the potentially stressful presence of visitors, factors that both likely influence the welfare of the individuals (Buchanan-Smith *et al.* 2004; Hosey 2005). Whether it is actually the mere size of the enclosure or its increased complexity or both that was mainly responsible for leading to lower GC output in our study subjects is not clear, especially because the number of swinging and climbing elements in the enclosures correlates directly with the size of each enclosure. However, what our results clearly suggest is that provision of sufficient space and an enriched enclosure design that allows expression of species-typical behavior leads to reduced stress hormone levels,

which, in our opinion, is an important component to facilitate the well-being of gibbons in captivity.

In addition, a large and well-equipped enclosure can help the subjects to hide from the presence of a large number of visitors, which has resulted in avoidance behavior, with individuals retreating from visitors (Cooke and Schillaci 2007; Skyner *et al.* 2004; Smith and Kuhar 2010), and in increased cortisol levels in other primates (Davis *et al.* 2005). Although the present study only indirectly examined the potential influence of visitors on GC concentration, our results for pileated gibbons support the idea of an effect of visitors, and thus human disturbance, on GC levels because gibbons with no visual protection from humans had significantly higher fecal GC concentrations than those that could hide from the sight of visitors. However, as mentioned previously, enclosure size, degree of enrichment, and opportunities to hide from humans are all interconnected and therefore it is impossible to disentangle the influence of any of these factors on its own without further experimental investigation. However, because results from Smith and Kuhar (2010) show that siamangs and white-handed gibbons spent more time in areas out of public view during large-crowd conditions, it is reasonable to assume that visitor presence provides a stressor from which individuals retreat if possible. We assume that pileated gibbons have similar requirements regarding visual protection from visitors, and thus enclosure design should offer adequate retreat possibilities. Arboreal monkeys seem especially sensitive to unrestricted visual contact with humans at the same height (Cooke and Schillaci 2007; Davis *et al.* 2005; Smith and Kuhar 2010). Lowering the height of viewing areas or increasing the height of cages was suggested to reduce the stressful effect of visitors.

An additional interesting finding was that successfully reproducing pairs had significantly lower fecal GC concentrations than individuals kept in pairs but without offspring. Whether this indicates that successful breeding is an outcome of low stress hormone levels, for instance due to better compatibility and stronger pair-bonding (Breznock *et al.* 1977), or whether low stress hormone levels are instead a consequence of successful breeding due, e.g., to a stress-reducing effect of the presence of offspring in combination with parental care, is not clear. However, given that a stable pair-bonding may act as a buffer against some types of stressors (DeVries 2002) and because high GC levels can impair reproductive function and fertility (Xiao *et al.* 1999; Young *et al.* 2006), the absence of increased GC levels is likely to correlate with successful reproduction.

Conclusion

To summarize, the present results showed that the measurement of fecal GC concentrations using a validated assay for the measurement of 5-reduced 3α,11βdihydroxy-cortisol metabolites is a valuable tool to evaluate adrenocortical activity in pileated gibbons. Increased concentrations of fecal GCs over time as seen in some individuals in the present study may be an indication of chronic stress, especially in combination with evidence of behavioral disorders or reproductive failure. Therefore, captive management of pileated gibbons should try to avoid hand-rearing or, when unavoidable, an early socialization process with conspecifics should

be attempted. The use of indoor enclosures >75 m³ in size and visual barriers to visitors are recommended because these are likely to help reducing the individual's physiological stress levels. This, in turn, should lead to improved well-being under captive conditions and thus may help to improve breeding success of this endangered primate species.

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