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Fecal glucocorticoid measurements and their relation to rearing, behaviour and environmental factors in the European pileated gibbon population (*Hylobates pileatus*)

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INHALTSVERZEICHNIS

SUMMARY

Fecal Glucocorticoid Measurements and their Relation to Rearing, Behaviour and Environmental Factors in the European Pileated Gibbon Population (*Hylobates pileatus*)

Quantifying effects of husbandry conditions on the physiology of zoo animals is an important part of assessing husbandry success. This study investigates fecal glucocorticoid (GC) levels of pileated gibbons (*Hylobates pileatus*) and its relationship to specific life-history variables and environmental factors. Following the validation of an enzymeimmunoassay for the measurement of 5-reduced 3α,11ß-dihydroxy cortisol metabolites to reliably assess GC output in the pileated gibbon, we collected fecal samples over several days from all 36 (22.14) European adult pileated gibbons located in 11 institutions and compared GC levels with respect to intrinsic individual parameters, husbandry, behaviour and breeding history. Hand-reared animals had higher GC hormone levels ($p=0.043$) and showed more behavioural abnormalities than parent-reared animals ($p<0.001$). Furthermore, non-reproducing gibbons living in a pair without infants had higher GC concentrations than gibbons living in a family ($p=0.039$). With respect to environmental factors, a large size of the inside enclosure ($p=0.011$) and the existence of visual protection from visitors ($p=0.003$) was associated with lower fecal GC output. The data indicate that rearing and housing conditions appear to be correlated to GC levels in pileated gibbons housed under captive conditions. This knowledge will hopefully support the future management of the species in captivity and thus lead to a more successful breeding of this endangered primate.

Key words: Hylobates pileatus, fecal glucocorticoids, housing conditions, hand-rearing, behavioural abnormalities.

ZUSAMMENFASSUNG

Glucocorticoid Werte im Kot im Vergleich zu Aufzucht, Verhalten und Umweltfaktoren in der europäischen Kappengibbon (*Hylobates pileatus*) Population

Die vorliegende Studie validierte die Glucocorticoid (GC) Messung im Kot von Kappengibbons (*Hylobates pileatus*) und vergleicht die gemessenen Werte der Europäischen Zuchtpopulation mit spezifischen individuellen Variablen und Umweltfaktoren. Nach einen ACTH Stimulationstest und einen induzierten Transportstress erwies sich ein Enzymimmunoassay für die Bestimmung des 3α,11β-dihydroxy-Cortisolmetaboliten als zuverlässigste Methode für die Messung von GC im Kot von Kappengibbons. Für die Vergleichsanalysen wurden über mehrere Tage der Kot von allen 36 (22.14) adulten europäischen Kappengibbons aus 11 verschiedenen Institutionen gesammelt, die GC Werte bestimmt und mit den gesammelten Tier- und Haltungsdaten statistisch verglichen. Handaufgezogene Tiere hatten höhere GC Werte ($p=0.043$) und zeigten mehr Verhaltensabnormalitäten als von den Eltern aufgezogene Tiere ($p<0.001$). Nicht-reproduzierende Gibbons, die in einem Paar ohne Nachwuchs lebten, hatten höhere GC Konzentrationen als Gibbons, die in einer Familie mit Jungtieren lebten ($p=0.039$). Bei den Umweltfaktoren war ein grosses Innengehege ($p=0.011$) und Gehege mit Sichtschutz ($p=0.003$) gegenüber von Besuchern mit tieferen GC Werten assoziiert. Diese Daten zeigen, dass Aufzucht- und Haltungsbedingungen mit den GC Werten von Kappengibbons in Menschenhand korrelieren. Dieses Wissen sollte das zukünftige Management der Spezies in Menschenhand unterstützen und zu einer erfolgreicheren Nachzucht dieser bedrohten Primatenart führen.

Stichworte: Hylobates pileatus, fäkale Glucocorticoide, Haltung, Handaufzucht, Verhaltensabnormaliäten.

Fecal Glucocorticoid Measurements and their Relation to Rearing, Behaviour and Environmental Factors in the European Pileated Gibbon Population (*Hylobates pileatus*)

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ABSTRACT

Quantifying effects of husbandry conditions on the physiology of zoo animals is an important part of assessing husbandry success. This study investigates fecal glucocorticoid (GC) levels of pileated gibbons (*Hylobates pileatus*) and its relationship to specific life-history variables and environmental factors. Following the validation of an enzymeimmunoassay for the measurement of 5-reduced 3α,11ß-dihydroxy cortisol metabolites to reliably assess GC output in the pileated gibbon, we collected fecal samples over several days from all 36 (22.14) European adult pileated gibbons located in 11 institutions and compared GC levels with respect to intrinsic individual parameters, husbandry, behaviour and breeding history. Age, sex and origin (wild vs. captive born) had no effect on GC levels. On the other hand, hand-reared animals had higher GC hormone levels and showed more behavioural abnormalities than parent-reared animals. Furthermore, non-reproducing gibbons living in a pair without infants had higher GC concentrations than gibbons living in a family, bachelor group or as singleton. 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 be correlated to GC levels in pileated gibbons housed under captive conditions. This knowledge will hopefully support the future management of the species in captivity and thus lead to a more successful breeding of this endangered primate.

Key words: pileated gibbon; *Hylobates pileatus*; fecal glucocorticoids; stress; enclosure size; visitors; hand-rearing; behavioural abnormalities

INTRODUCTION

Pileated gibbons (*Hylobates pileatus*) are one of the 12 distinct gibbon species living in the tropical rainforests 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 about 49'000 animals in Thailand and Cambodia (Phoonjampa & Brockelmann, 2008), is believed to decline further in the near future due 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 US (Species Survival Plan; SSP) and Europe (European Endangered Species Programme; EEP), consists of about 107 animals worldwide, housed in about 21 institutions. Although the global captive population in the two breeding programs generally increased over the last decades, breeding the species in captivity is still suboptimal as the population shows a low reproductive rate and, moreover, the sex ratio of the adults as well as of the offspring born is skewed towards 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, behavioural disorders (Skyner, Amory, & Hosey, 2004), and health-related problems. Additionally, 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) levels can suppress reproductive function, alter animal behaviour and increase the susceptibility to disease by disrupting immune functions (e.g. Cameron, 1997; Carlsdead, Brown, & Seidensticker, 1993; Dobson & Smith, 2000; Heistermann, Ademmer, & Kaumanns, 2004; Sapolsky, Romero, & Munck,

2000; Wielebnowski, Fletchall, Carlsdead, Busso, & Brown, 2002). The assessment of stress hormone levels and the intrinsic and environmental factors that influence glucocorticoid output in the potential breeding animals, represents an important aspect towards 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 nor any other gibbon species.

The measurement of faecal glucocorticoids (GC) has been established as a valuable non-invasive tool to evaluate effects of individual and environmental factors on adrenocortical activity in captive-housed animals of different species (e.g. Barbosa & da Silva Mota, 2009; Carlstead & Brown, 2005). These studies showed that adrenocortical function and associated glucocorticoid output can be influenced by intrinsic factors such as gender and reproductive status. Several environmental factors have also been shown to affect glucocorticoid secretion in captive primates. Here, the presence of visitors (Chamove, Hosey, & Schaetzel, 1988; Cooke, Schillaci, & 2007; Davis, Schaffner, & Smith, 2005), suboptimal housing conditions (Boinski, Swing, Gross, & Davis, 1999) and social factors e.g. group structure or ranking (Abbott et al., 2003), have all been reported to influence stress hormone levels in captive non-human primates .

The aim of the present study therefore was i) to characterize adrenocortical activity in all adult males and females of the captive EEP population of pileated gibbons and ii) to investigate the potential influence of individual and life-history variables as well as environmental factors on glucocorticoid levels. As to date no non-invasive method has been reported in the literature for assessing adrenocortical activity in the study species or any other gibbon species based on fecal samples, a second major aim was to establish and validate such a method for monitoring glucocorticoid output. The information generated should help to identify the nature and significance of factors influencing stress hormone output in the pileated gibbon, information that is not only important to better understand 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.

MATERIAL AND METHODS

Animals and individual characteristics

Study subjects were all 36 adult pileated gibbons (22 males; 14 females) of the European captive breeding population. The animals were housed in 11 institutions and 18 "groups", either as pairs, bachelor or family groups or singletons (Table 1). Information about the animal´s basic characteristics such as gender, age, origin (wild-born/captive-born), rearing history (hand-reared/parent-reared) and breeding success was provided by the international studbook.

The mean age (end of 2009) was 18.5 years (range: 5-50 years) in study males and 17.6 years (range: 5-35 years) in study females. Of the 36 animals, 13 were wild-born and 23 were bred in captivity. While the rearing history of the captive-born animals is known, that of the wild-born animals is mostly missing. Since, however, wild-born gibbons in zoos originate mainly from rescue centres, these animals were considered to be hand-reared because they are usually taken from their mother at a very young age and kept as pets with little or no contact to other gibbons. Thus, of the 36 animals of the study, 17 were hand-reared, while 19 were parent-reared.

For every animal, keepers were asked to assess the general behaviour of their animals to determine if certain animals behaved "abnormally". The following behavioural patterns were evaluated: 1) aggressive behaviour toward visitors/keepers (yes/no), 2) attached behaviour toward humans (yes/no), 3) fearful of conspecifics (yes/no), 4) not interested in conspecific (ignorant) (yes/no), 5) aggressive behaviour toward conspecifics (yes/no) and 6)

stereotypies (yes/no). If the answers for one animal contained more than one "yes", it was categorized to the group with "abnormal behaviour".

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

Environmental variables

All institutions were visited by the same person during 2009 to record data on housing conditions of the study subjects as well as to facilitate fecal sample collection (see below). Specifically, the following parameters were recorded: 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 the author), 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 one institution, which had only an indoor enclosure for its gibbons, all enclosures consisted of an indoor and outdoor area. Height and volume of the enclosures were measured either on site or calculated 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 metres (range: 2-6 metres). Outside enclosures had an average volume of 500.2 m^3 (range: 72-2260 m^3) and an average height of 4.4 metres (range: 2.5-6 metres). Five enclosures had an outside area on an island. Two of the 18 enclosures had no public access.

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

Collection of fecal samples

From each study subject, fecal samples were generally collected after an animal had been observed defecating. Samples were mainly collected in the morning between 7 and 10 a.m.. Only samples uncontaminated with urine were placed in plastic tubes and stored frozen at -20°C within two hours upon defecation. On average, 7.8 samples per animal (range: 2-36 samples) were collected, providing a total number of 248 samples for GC analysis. Samples were transported frozen to the endocrine laboratory of the German Primate Centre for glucocorticoid analysis.

Validation of fecal glucocorticoid measurements

We determined the validity of a cortisol (CORT), corticosterone (CCST) and two group-specific enzymeimmuno assays against cortisol metabolites with a 3α,11-oxo (3α,11-oxo-CM) and 3α,11ß-dihydroxy structure (3α,11ß-dihydroxy-CM) to assess adrenocortical function in pileated gibbons. All four assays have been successfully used to monitor GC output in various other primate and non-primate species (Ganswindt, Palme, Heistermann, Borragan, & Hodges, 2003; Heistermann et al., 2004; Wasser et al., 2000). For validation, we used i) a physiological stimulation of the adrenal gland by injecting a synthetic ACTH preparation (a single dose of 50 IU Synacthen, Novartis, Switzerland; c.f. Heistermann, Palme, & Ganswindt, 2006) in two animals (1 male, 1 female) and ii) a biological stressor (i.e. anesthesia in combination with transport) in four other individuals (2 males, 2 females) to evaluate whether the induced increase in glucocorticoid output is detected by the different fecal GC assays. From each individual, daily fecal samples were collected 4-6 days prior to

the treatment to establish pre-treatment baseline GC levels and for 5 days thereafter to establish the GC response. From the ACTH treated animals, every sample defecated within the first 72 hours after ACTH injection was collected with once daily samples for the next two days. From the transported animals, samples were usually collected once daily. Fecal samples were stored at -20°C and shipped frozen to the endocrine laboratory.

All fecal samples were processed and extracted as described by Heistermann et al. (1995). Briefly, fecal samples were lyophilized and pulverized and an aliquot representing 0.05-0.08 g of fecal powder was extracted with 3 ml 80% methanol by vortexing for 15 min. Following centrifugation of the fecal suspension, the supernatant was recovered and stored at –20°C until hormone analysis. Fecal extracts were analyzed for glucocorticoid immunoreactivity by the four different EIA systems mentioned above 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).

In order to assess the pattern of metabolites measured and thus characterize the specificity of the four 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 to evaluate whether the GC antibodies tested show a co-measurement of certain fecal androgens which may potentially be detected by antibodies raised against cortisol metabolites (see Ganswindt et al., 2003; Heistermann et al., 2006; Möstl & Palme, 2002). Following HPLC, each fraction was measured in all four GC assays to generate the profiles of immunoreactivity.

Faecal glucocorticoid measurements

Based on the outcome of the validation tests (see results), fecal samples were finally analyzed in the 3α,11ß-dihydroxy-CM EIA. For this, all samples were processed and extracted as described above. Fecal extracts were diluted 1:50-1:500 (depending on concentration) and duplicate aliquots were taken to assay. Sensitivity of the assay was 1pg/well. Fecal extracts from different animals gave displacement curves, which were parallel to the 11ß-hydroxyetiocholanolone standard curve. Intra- and inter-assay 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. All hormone concentrations are expressed in µg/g dry fecal weight.

Statistical analysis

Fecal GC levels measured by the four different EIAs were correlated with each other in each individual using Pearson-Product-Moment correlation. To evaluate the impact of the different variables on GC output in the zoo-animal data set, we initially calculated the median and interquartile range of GC levels for each animal. Gender, rearing history, degree of abnormal behaviour, group structure (family/pair), "visual protection from visitors", "other gibbons in hearing distance" and "other gibbons in visual range" were nominal data and a t-test was used to compare the fecal GC concentrations between these groups. Multiple regression analysis was used to evaluate the influence of the inside and outside volume of the gibbon´s enclosures (independent variable) on GC levels (dependent variable). In addition, a mixed-effect ANOVA was used to test the effects of the various parameters on median faecal glucocorticoid concentrations of each animal. Main effects introduced to the model included categorical factors (gender, rearing history, abnormal behaviour) and continuous factors (inside and outside enclosure volume). Based on preliminary plots of single factors, only one interaction term was included (gender x age). To achieve a normal distribution of residuals and to meet the assumption of linearity in continuous predictor effect, enclosure volumes

were log-transformed prior to analyses. Significance level (2-tailed) was set at $P < 0.05$. All analyses were carried out with STATISTICA (Statistika™ 8.0, StatSoft®, U.S.A.).

RESULTS

Validation of fecal glucocorticoid measurements

In absolute terms, highest levels of fecal GCs were measured by the 11oxo- and 11ß-hydroxy-CM EIAs (peak value range: 3.4-33.4 µg/g; Table 2), those measured by the CORT and CCST assays being generally much lower (peak value range: 0.08-1.36 µg/g; Table 2). All six animals responded to the treatment (ACTH injection or anesthesia/transport) with a clear increase in fecal GC levels (Fig. 1, Table 2) and with very few exceptions (3 out of 16 EIAs), fecal GC levels determined by the four EIAs significantly correlated with each other in each of the six individuals (mean r-value range: 0.65 to 0.90; P-value range: 0.05 to <0.001).

For each assay, the magnitude of response varied between animals; 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 2). The magnitude of response also clearly differed across the four 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 animals (Table 2). In most cases, however, peak response was detected between 22 and 55 hours (Table 2; Fig. 1) and GC levels had usually returned to pre-treatment baseline levels by day 5 (Fig. 1).

HPLC analysis indicated that in the CORT and the two group-specific assays, the majority (~90%) of immunoreactivity was detected as several distinct peaks between fractions 9 and 31 (positions were cortisol metabolites in the HPLC system elute, see Heistermann et al. (2006)), with little amounts of immunoreactivity measured after fraction 31 (positions were certain potentially cross-reacting androgen metabolites elute; Ganswindt et al., 2003;

Heistermann et al., 2006), suggesting 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 11ß-hydroxyetiocholanolone (fractions 24/25) and 11oxo-etiocholanolone (fractions 29/30) in the respective assays indicated that these two cortisol metabolites were abundant in pileated gibbon feces. In contrast, the HPLC profile measured by 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 by 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; see Heistermann et al., 2006), suggesting that this assay was highly non-specific and likely co-measured substances not derived from cortisol metabolism (Fig. 2b).

Faecal glucocorticoid analysis of the captive pileated gibbon population

Overall, animals 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 3 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 gender, age nor origin were significantly associated with fecal GC output in captive-housed piletaed gibbons. Significantly higher mean fecal GC concentrations were, however, found in hand-reared animals (1214.61 ± SD 861.25 ng/g) compared to parent-reared animals (703.63 ± SD 582.03 ng/g) (P=0.043) and animals showing behavioural abnormalities (1255.93 ± SD 884.54 ng/g) compared to those showing social behaviour more typical for the species (722.78 ± SD 585.98 ng/g) (P=0.036). Rearing condition and behaviour

were significantly associated (P<0.001); 82.4% (N=14) of the hand-reared animals showed abnormal behaviour compared to 5.3% (N=1) of the parent-reared animals.

Environmental variables and GC output

Fecal GC concentrations of animals in the 11 housing institutions showed substantial differences, with animals in certain locations (e.g. no. 11) showing consistently low levels while those in others (e.g. no. 10) showing markedly elevated concentrations (Figure 3). GC levels did not differ according to whether the study subjects were in visual range of hearing distance with other gibbons, but animals with visual protection from visitors had significantly lower GC concentrations (490.95 ± SD 402.95 ng/g) than animals without visual protection (1234.24 ± SD 800.66 ng/g) (P=0.003) (Table 3).

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. animals housed in smaller enclosures had higher GC levels than animals in larger enclosures. Specifically, gibbons housed in an inside enclosure <75 m^3 (N=22) had significantly higher GC concentrations (1283.7 ± SD 774.7 ng/g) than gibbons with an inside enclosure >75 m^3 (N=14; 412.64 ± SD 305.75 ng/g) (P<0.001). 75 m^3 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.

Group structure had also an effect on GC output. Animals living in a family (689.8 ± SD 677.3 ng/g) had significant lower GC concentrations than animals living in a pair without offspring (1321.3 ± SD 816.1 ng/g) (P=0.039) (Table 5). Among males, mean GC concentration of individuals living in a pair (N=6, 1210.83 ± SD 904.36 ng/g) was not significantly different compared to those found in males living in a family (N=7, 600.98 ± SD

622.29 ng/g), bachelor group (N=6, 733.92 ± SD 726.23 ng/g) or single (N=3, 715.96 ± SD 356.11 ng/g). Rearing history had also an apparent effect on stress hormone levels (Table 5). While parent-reared males had relatively low fecal GC concentrations when kept in groups as pairs (N=2, 444.9 ± SD 28.6 ng/g), family (N=5, 379.6 ± SD 218.1 ng/g) or as bachelors (N=3, 327.5 ± SD 154.5 ng/g), GC levels in hand-reared males were on average 3-4 times higher under all these conditions (pair without breeding success: N=4, 1593.8 ± SD 881.0 ng/g; family: N=2, 1154.4 ± SD 1129.5 ng/g; bachelor group: N=3, 1140.4 ± SD 893.9 ng/g). The females, which were only kept in a pair or family, had no significant difference in fecal GC concentration between these two groups or between hand- and parent-reared females kept in pair/family. If data of all animals are combined, hand reared individuals (1214.61 ± SD 861.25 ng/g) had significantly higher GC levels than parent-reared ones (703.63 ± SD 582.03 ng/g) (P=0.043).

Individual and environmental factors combined

The effect of individual (gender, age, gender*age, rearing, behaviour) and environmental (volume inside, volume outside, visual protection from visitors) factors on fecal GC concentration were analysed in a mixed-effects ANOVA. The volume of the inside enclosure had the strongest effect and was the only significant predictor of GC levels in this model (Table 4).

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 glucocorticoid concentrations. The data therefore suggest that rearing

and housing conditions are important components determining an individual's GC hormone level, 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 glucocorticoids as a measure of physiological stress in gibbons which is of primary importance before any application (Heistermann et al., 2006; Touma & Palme, 2005). In the current study, both group-specific cortisol metabolite assays appeared to be clearly superior over the two more specific ones designed to measure cortisol and corticosterone in blood, as indicated by substantially higher levels of immunoreactivity measured, a more specific measurement, and a generally higher response to the applied stressor. In addition, HPLC immunoreactivity peaks co-eluting with 11ß-hydroxyetiocholanolone and 11-oxoetiocholanolone standards indicated the presence of 3α,11ß-dihydroxylated GCMs and 11,17-dioxoandrostanes, both of which have also been reported as abundant fecal cortisol metabolites in other primate and non-primate species (e.g. Ganswindt et al., 2003; Heistermann et al., 2006; Ostner, Heistermann, & Schulke, 2008; Palme & 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 many other studies (e.g. Heistermann et al., 2006 for review; Palme, Rettenbacher, Touma, El-Bahr, & Möstl, 2005; Wasser et al., 2000). Concerning the two cortisol metabolite assays, both appeared to be equally valid for assessing GC output in the piletaed gibbon. For practical reasons, we chose the 11ß-hydroxyetiocholanolone (3α,11ß-dihydroxy-CM) assay which has already been successfully used to monitor adrenocortical activity in a number of other primate species (e.g. Fichtel, Kraus, Ganswindt, & Heistermann, 2007; Girard-Buttoz, Heistermann, Krummel, & Engelhardt, 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 study subjects. Our data show that sex, age and

origin (wild vs captive-born) of the animal had no influence on GC levels, a finding which, with respect to the effect of gender and age, is in line with results found in other primate species. Interestingly, and in contrast to reports from studies of other primates, the GC levels in captive-housed pileated gibbons were unaffected by the origin of the animals, suggesting that all animals irrespective of sex, age and origin responded to current husbandry conditions in a similar way.

In contrast, rearing history appeared to be of remarkable importance how housing conditions will be percept in a later stage of life. The hand-reared animals in the present study exhibited not only markedly higher fecal glucocorticoid levels than parent-reared animals, but showed also more abnormal, non-social behaviour. In contrast to the influence of rearing experience on physiology, i.e. adrenocortical activity, later in life, the influence of rearing on adult behaviour is very well documented. Behavioural abnormalities toward conspecifics or humans in hand-reared primates are often related to a lack of adequate social learning and an imprinting on humans in early infancy. It is unlikely that hand-reared animals experience differences in the functional development of the HPA axis compared to normally reared animals. Therefore, individuals missing the species-typical socialization process during infant life might be as adults overwhelmed by social demands from their conspecifics, which in turn may lead to chronically elevated GC levels as found in our study. 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. Rearing conditions should be therefore carefully evaluated and the presence of at least another young conspecific is recommended when the decision for hand-rearing is made.

Apart from intrinsic factors and life-history variables, many environmental factors have been shown to influence the behaviour and GC output in primates and other species (Barbosa & da Silva Mota, 2009; Boinski et al., 1999; Carlstead & Brown, 2005; Carlstead, Brown, & Seidensticker, 1993; Rangel-Negrin, Alfaro, Valdez, Romano, & Serio-Silva,

2009). In our pileated gibbon subjects, GC levels were 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 animal 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 where 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. Since 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 is of high importance for the welfare of the animals. 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 as gibbons living in such relatively large enclosures exhibited significantly lower GC levels than animals living in smaller cages suggested to be sufficient by the American Zoo and Aquarium Association (AZA, 1997). This result is not surprising given that a larger enclosure with a certain height provides animals with more space to express their species-typical movements (brachiation) and allow them to hide from the potentially stressful presence of visitors, factors which both likely influence the welfare of the individuals (see below). However, whether it is actually the mere size of the enclosure or its increased complexity, well-known to improve the welfare of primates living under captive conditions (reviewed in Buchanan-Smith, Prescott, & Cross, 2004; Hosey, 2005) or both that was mainly responsible for leading to lower GC output in our study subjects is not clear especially since the number of swinging and climbing elements in the enclosures correlated directly with the size of each enclosure. However, what our results

indicate is that sufficient space and an enriched enclosure design that allows expression of species-typical behaviour is of importance to improve the welfare of gibbons in captivity.

In addition, a large and well-equipped enclosure can help the animals to hide from the presence of a large number of visitors, which has resulted in avoidance behaviour, with animals retreating from visitors (Cooke et al., 2007; Skyner et al., 2004; Smith & 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 the pileated gibbon support the idea of an effect of visitors (and thus human disturbance) on increase of GC levels because gibbons with no visual protection from humans had significantly higher faecal GC levels than gibbons with the possibility to go out of sight of the visitors. However, as mentioned before, enclosure size, degree of enrichment and possibilities to hide from humans are all interconnected and therefore it is impossible to disentangle the influence of any of these factors on its own. Since results from Smith & Kuhar (2010) however 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 animals retreat if possible. We assume that pileated gibbons have similar demands regarding visual protection from visitors and thus, enclosure design should offer adequate retreat possibilities. Especially arboreal monkeys seem sensible to human unrestricted visual contact on the same height (Cooke et al., 2007; Davis et al., 2005; Smith & Kuhar, 2010). Lowering the height of viewing areas or to increase the height of cages were suggested to reduce the stressful effect of visitors.

An additional interesting finding was that successfully reproducing pairs had significantly lower faecal GC concentrations than animals 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, Harrold, & Kawakami, 1977), or whether low stress hormone levels rather are a consequence of

successful breeding due for instance 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 since high GC levels can impair reproductive function and fertility (e.g. Xiao, Xia-Zhang, & Ferin, 1999; Young et al., 2006), the absence of increased GC levels may be correlated 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 glucocorticoids over time as in some animals in the present study may be an indication of chronic stress, especially in combination with evidence of behavioural 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. Indoor enclosures above 75 m^3 and visual barriers to visitors will help to reduce adrenocortical activity.

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FIGURES

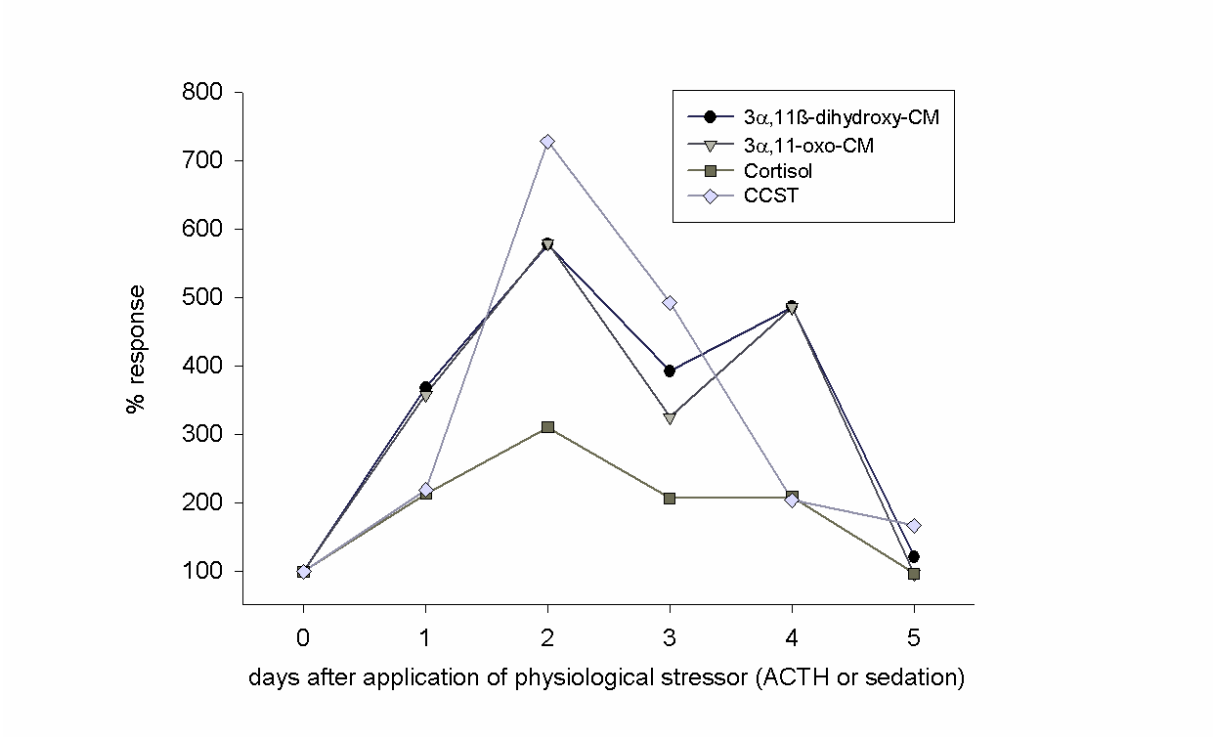

Fig. 1: Percentage of response in immunoreactive fecal glucocorticoid levels to a physiological stressor in pileated gibbons (*Hylobates pileatus*). Data points represent median values calculated for 24 hour intervals across the 6 individuals examined. Time 0 = time of ACTH injection or anesthesia.

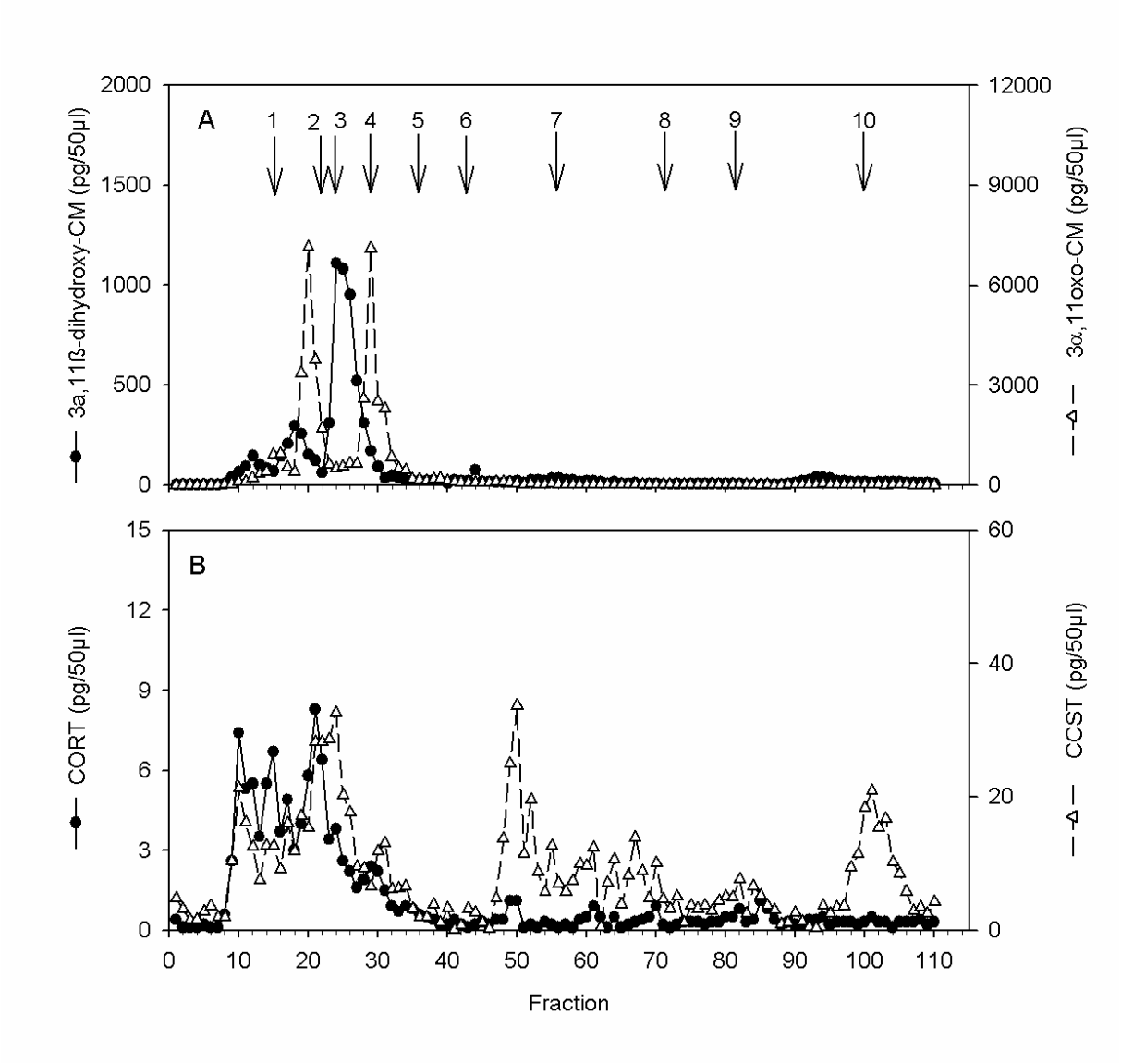

Fig. 2: HPLC profiles of immunoreactivity detected with (A) the 3α,11ß-dihydroxy-CM and 3α,11oxo-CM EIA and (B) the cortisol (CORT) and corticosterone (CCST) EIA in a peak sample following adrenocortical stimulation in a captive male pileated gibbon (*Hylobates pileatus*). Arrows indicate elution positions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

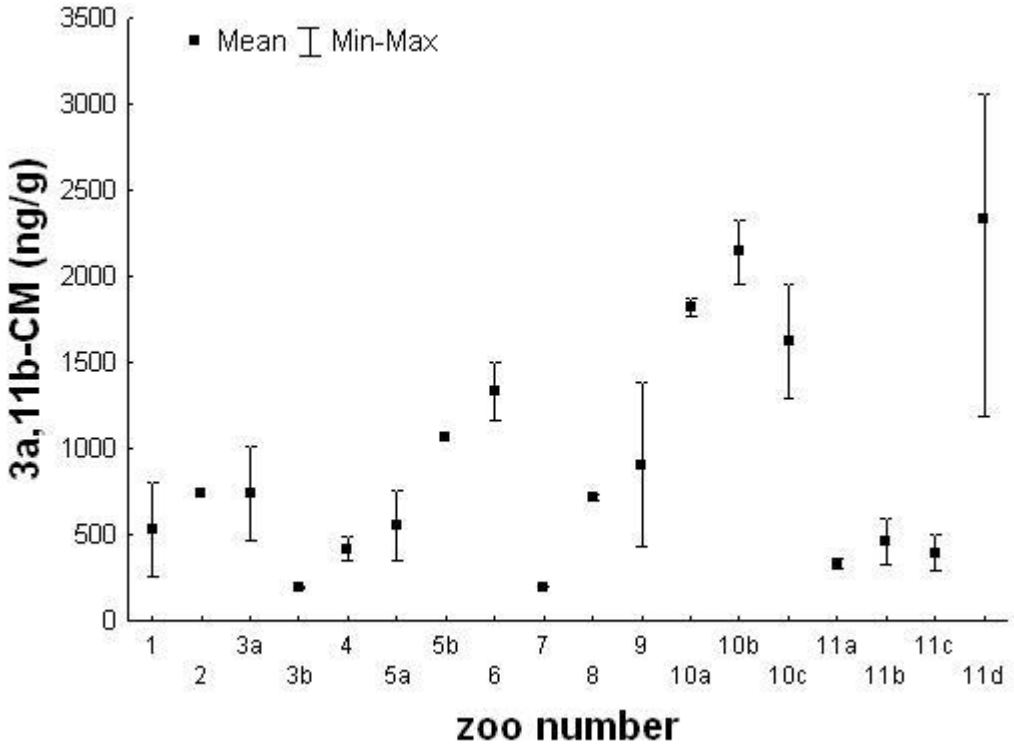

Fig. 3: Faecal glucocorticoid concentrations of adult pileated gibbons (*Hylobates pileatus*) (N=36) in the 18 enclosures. The number on the x-axis corresponds to one institution and the letters to different enclosures in each institution.

TABLES

Tab. 1: Number of pileated gibbons (*Hylobates pileatus*), number of enclosures and group structure in all investigated European facilities (end of 2009).

Facility	Sex ratio (m.f.i)	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

Tab. 2: Fecal glucocorticoid concentrations (as detected by four different assays) in pileated gibbons (*Hylobates pileatus*) in response to an ACTH challenge or anesthesia/transport stress.

Animal	Treatment	3α, 11ß-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] pre-treatment levels in µg/g feces (see Methods)

[d] peak levels in response to treatment in µg/g

[e] x-fold increase of peak levels above pre-treatment concentrations

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

Tab. 3: Mean ± SE fecal glucocorticoid concentrations in relation to gender, origin, rearing, behaviour and protection from visitors in captive pileated gibbons (*Hylobates pileatus*) (N = 36).

Sex	**Male**	**Female**	**p value**
	(n= 22)	(n= 14)	
	819.24 ± 711.56	1142.42 ± 821.91	p= 0.219
Origin	**Wild born**	**Captive born**	**p value**
	(n= 13)	(n= 23)	
	1096 ± 948.9	859.01 ± 640.78	p= 0.376
Rearing	**Hand-reared**	**Parent-reared**	**p value**
	(n= 17)	(n= 19)	
	1214.61 ± 861.25	703.63 ± 582.03	p= 0.043
Behaviour	**Normal**	**Abnormal**	**p value**
	(n= 21)	(n= 15)	
	722.78 ± 585.98	1255.93 ± 884.54	p= 0.036
Visual protection from visitors	**Protection**	**No protection**	**p value**
	(n= 14)	(n= 22)	
	490.95 ± 402.95	1234.24 ± 800.66	p= 0.003
Gibbons in visual range	**No gibbons visible**	**Gibbons visible**	**p value**
	(n= 23)	(n= 13)	
	844.68 ± 755.76	1122.29 ± 769.32	p= 0.300
Gibbons in hearing distance	**No gibbons audible**	**Gibbons hearible**	**p value**
	(n= 12)	(n= 24)	
	883.21 ± 665.58	1168.36 ± 915.57	p= 0.218

Tab. 4: Influence of different effects on fecal GC concentration in captive pileated gibbons (*Hylobates pileatus*) in a mixed-effects ANOVA (N=35 animals; bold type indicates P<0.05).

Effect	F (df, total df) ratio	p value
Gender	F (1,25)=2.351	p=0.144
Age	F (1,25)=0.557	p=0.466
Gender*Age	F (1,25)=1.147	p=0.299
Rearing	F (1,25)=1.586	p=0.225
Behaviour	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

[a] The volume is log-transformed to linearity

Tab. 5: Mean faecal glucocorticoid concentration ± standard error (ng/g) of captive male and female pileated gibbons (*Hylobates pileatus*) kept in different group structures (N= 36 animals).

Group-structure	**all animals**	**all males**	**hand-reared males** (n)	**parent-reared males** (n)	**females** (n)
Family	689.8 ± 677.28	600.98 ± 622.29	1154.39 ± 1129.47 (4)	379.62 ± 218.07 (2)	793.41 ± 782.48 (6)
Pair	1321.33 ± 816.14	1210.83 ± 904.36	1593.80 ± 881.02 (2)	444.91 ± 28.58 (5)	1404.19 ± 796.57 (8)
Bachelor		733.92 ± 726.23	1140.36 ± 893.90 (3)	327.48 ± 154.49 (3)	
Single		715.96 ± 356.11	543.70 ± 274.92 (2)	1060.49 (1)	

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