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Evaluating capture stress in wild gray mouse lemurs via repeated fecal sampling: Method validation and the influence of prior experience and handling protocols on stress responses ☆

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ABSTRACT

Reliable measurements of physiological stress are increasingly needed for eco-physiological research and for species conservation or management. Stress can be estimated by quantifying plasma glucocorticoid levels, but when this is not feasible, glucocorticoid metabolites are often measured from feces (FGCM). However, evidence is accumulating on the sensitivity of FGCM measurements to various nuisance factors. Careful species- and context-specific validations are therefore necessary to confirm the biological relevance and specificity of the method. The goals of this study were to: (1) establish and validate sampling methods and an enzymeimmunoassay to measure FGCM in the gray mouse lemur (*Microcebus murinus*); (2) explore causes of variability in the FGCM measurements, and; (3) assess the consequences of capturing and handling for free-living individuals by quantifying their stress responses via repeated fecal sampling within capture sessions. We further assessed the influence of different handling protocols and the animals' previous capture experience on the magnitude of the physiological response. Our validations identified the group-specific measurement of 11ß-hydroxyetiocholanolone as the most suitable assay for monitoring adrenocortical activity. The sample water content and the animal's age were found to significantly influence baseline FGCM-levels. Most captured animals exhibited a post-capture FGCM-elevation but its magnitude was not related to the handling protocol or capture experience. We found no evidence for long-term consequences of routine capturing on the animals' stress physiology. Hence the described methods can be employed to measure physiological stress in mouse lemurs in an effective and relatively non-invasive way.

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1. Introduction

The assessment of physiological stress is increasingly used in many contexts of evolutionary biology, ecology and conservation to assess the health and coping of individuals or populations with environmental challenges (Ricklefs and Wikelski, 2002; Romero, 2004; Wikelski and Cooke, 2006). To this end, the capturing of animals is often necessary for physical examinations or sample collection. However, capturing and handling themselves are known to cause a stress response (Fletcher and Boonstra, 2006; Romero and Reed, 2005) that may introduce significant bias to studies of other phenomena if unaccounted for (Reeder and Kramer, 2005). The level of invasiveness of the handling procedures can influence the level of capture stress experienced by the animal (Bennett et al., 2012; Garcia et al., 2000) and within an individual, the magnitude of the physiological response to capture may change over subsequent captures via habituation or sensitization to the stressor (Boonstra, 2013; Dickens et al., 2013; Fletcher and Boonstra, 2006; Garcia et al., 2000; Lynn et al., 2010; Romero, 2004; Walker and Dee, 2006). Relatively little is known about the impact of capturing and handling procedures on animals despite the potential consequences for the research outcome and the welfare of the animals involved.

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The physiological stress response – reflected by an increase in circulating glucocorticoid (GC) levels – facilitates appropriate reactions to and recovery from diverse challenges (Sapolsky et al., 2000). Capturing may cause an acute elevation in stress hormone output, but it might also alter the long term stress physiology of the animal (Boonstra, 2013; Clinchy et al., 2011) if a return to normal state is not achieved between capture events. While chronic stress may be adaptive in natural conditions in some circumstances (Boonstra, 2013; Crespi et al., 2013), chronically elevated GC levels are typically associated with compromised health, reproduction and survival of individuals (reviewed in e.g. Bonier et al., 2009; Romero, 2004). Therefore, capturing may adversely affect especially those individuals that already have high GC levels prior to the capture (Collins, 2001; Matthews et al., 2001) or have an impaired feedback system (e.g. due to old age) to facilitate the return back to baseline GC (Sapolsky et al., 1986).

The physiological stress response to capture can be measured via repeated blood sampling (e.g. Lynn et al., 2010; Romero and Reed, 2005, see also (Fletcher and Boonstra, 2006) or, alternatively, non-invasively using feces (Taylor, 1971). Fecal levels of GC metabolites (FGCM) reflect the baseline GC level at a delay of several hours and provide an integrated measurement of physiological stress levels over a period reflecting at minimum the animal's gut passage time (Harper and Austad, 2000; Sheriff et al., 2010; Touma and Palme, 2005). FGCM may also better indicate the levels of biologically active, unbound GCs than total GC levels measured from blood (Breuner et al., 2013; Sheriff et al., 2011, 2010; Touma and Palme, 2005).

Even though these advantages have led to the widespread use of FGCM in studies of natural populations, several caveats have recently been raised due to the sensitivity of FGCMs to potentially confounding factors (Goymann, 2012). The assessment of stress via FGCM measurements can be influenced by e.g. the ecological season (Huber et al., 2003; Romero, 2002), the animal's diet (Goymann, 2005), metabolic rate and gut bacterial community (Goymann, 2012) and, not least importantly, the treatment of the samples and the analyses performed (Goymann, 2005; Heistermann et al., 2006; Huber et al., 2003; Millspaugh and Washburn, 2004; Möstl et al., 2005; Sheriff et al., 2011; Shutt et al., 2012). Furthermore, the baseline GC level and the intensity of the endocrine stress response are known to vary among individuals based e.g. on their sex (Kudielka and Kirschbaum, 2005), age (Goncharova and Lapin, 2002; Sapolsky et al., 1987), or prior experiences (Fletcher and Boonstra, 2006; Garcia et al., 2000; Lynn et al., 2010; Walker and Dee, 2006). As a result of these complex interactions, FGCM data tends to be "noisy" and, to draw meaningful conclusions, it is necessary to conduct careful species-specific validations of the methods and an evaluation of factors potentially confounding FGCM levels (Buchanan and Goldsmith, 2004; Millspaugh and Washburn, 2004; Möstl and Palme, 2002; Romero, 2004; Sheriff et al., 2011; Touma and Palme, 2005).

In this study, we use FGCM measurements to quantify physiological stress in a small primate, the gray mouse lemur (*Microcebus murinus*). As GC excretion into feces has not been previously studied in the species, it was necessary to first select the most suitable assay for recording HPA axis activity and to validate the method. Biological validations have been suggested as a method alternative to an ACTH-challenge for quantifying the hormonal stress response (Goymann, 2005; Sheriff et al., 2011; Touma and Palme, 2005). Therefore we measured GC levels of wild and captive mouse lemurs before and after a known stressful event in three independent experiments. Based on these data, we selected an assay, assessed the lag-time to peak GC elevation and examined the influence of sample processing protocols on the FGCM measurements.

Following these validations, we examined stress responses to capture and handling of wild animals via repeated FGCM measurements. In a long-term monitored, routinely captured population of gray mouse lemurs (*Microcebus murinus*) (e.g. Dammhahn, 2012; Dammhahn and Kappeler, 2009; Kraus et al., 2008) some individuals voluntarily enter a trap up to 20 times per year and may be handled more than six times per year. The fact that mouse lemurs are easily re-trapped might suggest that the procedure is only minimally stressful to the individuals involved or that they habituate easily to trapping, in which case routine capturing may have few long-term consequences on their stress physiology. We hypothesized that the stress response to routine handling should be attenuated in animals with relatively frequent capture experiences and, if repeated capturing evokes chronic stress, this could translate to elevated baseline FGCM levels in the animals that are captured often. Additionally, the magnitude of the hormonal response should depend on the invasiveness of the handling regime the individual is subjected to (Bennett et al., 2012; Pitman et al., 1988). To evaluate these effects, baseline FGCM and the change from baseline to response level FGCM were measured via repeated fecal sampling during capture sessions.

2. Methods

2.1. The study population

The gray mouse lemur (*M. murinus*) is a small-bodied primate (average body mass ~60–80 g) that inhabits dry deciduous forests in Western Madagascar. The species is nocturnal, arboreal, sexually monomorphic and solitary living. The study population in the forest segment "N5" in Kirindy forest, Western Madagascar, has been intensively monitored since year 2001 for the purposes of long-term data collection (see e.g. Dammhahn, 2012; Dammhahn and Kappeler, 2009; Kraus et al., 2008). All individuals of the study population are individually marked with a subcutaneous transponder chip (Trovan). The animals are trapped using live-catch Sherman traps baited with banana. Routine capturing is conducted on three consecutive nights ("capture session") monthly in March–May and September–November, in addition to which smaller scale captures may take place for the purposes of specific research projects.

2.2. Fecal sampling and field extraction

Upon each capture, fresh fecal samples were collected into polypropylene tubes from previously cleaned traps or when animals defecated during handling. The freshness of the feces was assessed based on the presence of a glossy surface on the pellets, since in the dry season the feces dry quickly after defecation. Because in most primates, a larger proportion of glucocorticoids are excreted via urine than via feces (Bahr et al., 2000; Wasser et al., 2000), any feces where urine contamination was suspected was not sampled. The time of day of collection and time lags to processing and extraction were recorded for each sample. Samples were extracted into ethanol in the field within 4 h of sample collection adapting a protocol described by Ziegler and Wittwer (2005) and modified by Shutt et al. (2012). Briefly, the freshest collected fecal pellets (total fecal mass of 0.15–0.8 g) were homogenized in a collection tube or on a petri dish with a metal rod, then a subsample of approximately 0.2 g (to the nearest 0.001 g) was weighed into an extraction tube and mixed with 2 ml of ~90% ethanol. For logistic reasons, the fecal suspensions were left to stand for 5–12 h, then vortexed for 2 min. Samples were finally centrifuged using a manually operated centrifuge (Hettich GmbH & Co. KG Tuttlingen, Germany) for 2 min (Shutt et al., 2012). The supernatant was poured into a 2 ml polypropylene tube, sealed with parafilm and stored in a dark container at slightly below ambient temperatures until export to Germany, where samples were stored at −20 °C until

hormone analysis. The remaining fecal matter was sun-dried to a constant mass to obtain an estimate of the water content of the feces. The repeatability of the field processing and extraction procedure was assessed by splitting 16 homogenized samples collected in the wild into two subsamples and treating them the same way.

2.3. Method validations

2.3.1. Biological validation 1: Sampling series of wild animals in temporary captivity

We determined the validity of cortisol (CORT; Palme and Möstl, 1997), corticosterone (CCST; Heistermann et al., 2006), and two group-specific enzymeimmunoassays (EIA) against cortisol metabolites with a 3α,11-oxo (11-oxoetiocholanolone: 3α,11-oxo-CM; (Möstl and Palme, 2002) and 3α,11β-dihydroxy structure (11ß-hydroxyetiocolanolone: 3α,11β-dihydroxy-CM; (Ganswindt et al., 2003) to assess adrenocortical activity. All four assays have been successfully used to monitor GC output in various primate and non-primate species (Ganswindt et al., 2003; Heistermann et al., 2006; Martínez-Mota et al., 2008; Pirovino et al., 2011; Shutt et al., 2012; Wasser et al., 2000; Weingrill et al., 2011), including other lemurs (Ostner et al., 2008; Fichtel et al., 2007). For validation, we used the response to capture-and-handling stress (previously used e.g. by Bosson et al. (2009), Dickens et al. (2009), Fletcher and Boonstra (2006)) in 4 individuals living in the camp area (thus being accustomed to human presence) to evaluate whether the increase in GC output is detected by the different fecal GC assays.

The animals were captured along paths in the field camp at sunset in October 2010 (2 males and 1 female) and in October 2012 (2 females; one female was used in both seasons). The captured individuals were handled briefly, first fecal samples were collected within 2 h of capture to determine pre-capture control levels and the animal was allowed to re-enter the trap and left in a safe area overnight. In the morning, the animals were handled for approximately 10 min for weighing, morphometric measurements and hair sampling. At dusk of the same day, the individuals were released into individual cages of approximately 1 m^3 that were kept inside a closed platform (for protection from predators) and furbished with branches, nest boxes and cover. Underneath each cage, plastic sheets were placed to facilitate collection of feces. The animals were held captive for 5 days. During this time they were fed with fruit and insects each morning and evening (approximately 12 h intervals) and all available fecal matter was removed from the cage at these occasions. At each collection, the freshest fecal pellets were homogenized and 0.2 g was extracted according to the protocol outlined in Section 2.1.

2.3.2. Biological validation 2: Translocation of captive animals

Data from a translocation event was used to further assess the suitability of the four GC assays when a group of 17 animals (living in 8 subgroups of 1–3 individuals) was transported from Biopark d'Archamps (Archamps, France) to the German Primate Centre. Three fecal samples were collected from each subgroup of animals in the 5 days before the transport to record baseline GC levels and for 6 days thereafter to establish the GC response. Sleeping boxes were checked every morning and feces, if present, was collected in polystyrene tubes and stored at −20 °C until further processing. Since animals within each subgroup shared nest boxes, we were unable to assign samples to specific individuals, thus, we assessed the stress-related changes in FGCM concentrations on the group level.

2.3.3. Biological validation 3: Repeated sampling of wild animals recaptured within a capture session

We collected repeated samples from wild individuals that were recaptured within capture sessions (see Section 2.1) to determine whether a capture-induced increase in the FGCM-levels could be seen in the days following a capture, and to assess the optimal delay to recording the maximum elevation in FGCM. Animals were sampled during handling on day 1 of the session, and subsequently samples were collected from animals recaptured one ("day 2", 24 h delay, $n = 21$), two ("day 3", 48 h delay, $n = 23$) or three ("day 4", 72 h delay, data from additional capture nights, $n = 26$) days after the first capture. For this analysis we only included animals that were not captured on any of the intermediate days to exclude the possibility of cumulative stress due to repeated captures. Based on the magnitude of the change in FGCM from day 1 to the subsequent recaptures (see Section 3.1.4), and data from the other two biological validation series, a sampling interval of two days (~48 h, day 1 to day 3 of the session) was chosen for the subsequent study on capture-and-handling stress (Section 2.4).

2.3.4. Hormone analysis and HPLC

The fecal samples collected from captive mouse lemurs were processed and extracted following Heistermann et al. (1995). In summary, we lyophilized and pulverized the feces and extracted an aliquot representing 0.04–0.06 g of fecal powder in 3 ml of 80% methanol in water by vortexing the suspension for 10 min. Following centrifugation of the fecal suspension at 3000 rpm for 10 min, we recovered the supernatant and stored it at −20 °C until analysis. We analyzed fecal extracts for GC immunoreactivity with the four aforementioned EIA systems as described by Heistermann et al. (2004, 2006). Information on antibody characteristics, standards, and hormone labels used as well as on other assay details, including assay sensitivities, is given in Heistermann et al. (2006). Intra- and inter-assay coefficients of variation of high- and low-value quality controls were <10% and <13%, respectively, for all four assays.

Based on the outcome of the two biological validation tests (see Sections 3.1.1 and 3.1.2), the two group-specific assays were deemed most suitable for monitoring FGCM output. In order to characterize the patterns of metabolites measured by these two assays and evaluate any co-measurement of certain fecal androgens which could potentially be detected by antibodies raised against cortisol metabolites (see Ganswindt et al., 2003; Heistermann et al., 2006), we performed reverse-phase high pressure liquid chromatography analysis (HPLC). HPLC was carried out as described by Möhle et al. (2002) and Heistermann et al. (2006) using a fecal extract generated from a sample from one of the wild male study subjects. This sample was collected shortly prior to the onset of the mating season when the male had enlarged testes and presumably high levels of androgens (Aujard and Perret, 1998). We measured each HPLC fraction in the two group-specific FGCM assays to generate profiles of immunoreactivity.

Based on the combined validation and HPLC results, the 11ß-hydroxyetiocholanolone (3α,11β-dihydroxy-CM) EIA was used for the analysis of all fecal samples. The EIA was performed as described in detail by Heistermann et al. (2004). Prior to hormone measurement, we diluted extracts 1:50–1:300 (depending on concentration) in assay buffer and took duplicate aliquots to assay. Sensitivity of the assay was 1 pg/well. Serial dilutions of fecal extracts gave displacement curves parallel to those obtained with the 11ß-hydroxyetiocholanonole standard. Intra- and inter-assay coefficients of variation of high- and low-value quality controls were 6.5% (high, $n = 16$) and 7.4% (low, $n = 16$) and 10.4% (high, $n = 24$) and 11.9% (low, $n = 24$), respectively. Hormone concentrations are given as ng/g fecal wet weight for samples from the wild, and ng/g dry weight for samples from captivity.

2.3.5. Testing post-defecation change in FGCM levels

The delay from defecation to sample processing has been shown to potentially affect FGCM levels (Millspaugh and Washburn, 2004; Möstl et al., 1999; Shutt et al., 2012) as a result of bacterial induced alterations on excreted metabolites. Dependent on field conditions and trapping designs it is not always possible to know the exact time of defecation or to immediately process samples. Since in our study, feces had to be stored unpreserved for up to 4 h after defecation (see Section 2.2), we tested whether FGCM levels change as a function of the time elapsed between defecation and sample preservation and extraction. For this, we recorded the time delays from collection to processing for all samples collected in the wild and tested statistically for time effects (from collection to preservation in ethanol; from collection to extraction; from preservation in ethanol to extraction). Additionally, we conducted a controlled degradation experiment in captivity at a mouse lemur breeding colony in Brunoy, France (MNHN; European Institutions Agreement No. 962773), where larger sample masses compared to the wild were obtainable. In total, we collected 11 fresh fecal samples (4 pooled samples from 2 to 3 individuals, individual samples from 7 animals) immediately after defecation and homogenized them well. Each sample was split into 2–7 0.2 g subsamples (depending on total sample mass obtained) which were placed in extraction tubes. 2 ml of 90% ethanol was added to one of the subsamples within one hour after defecation (time 0 control). The remaining samples were left at ambient temperature (ca. 22–24 °C) and ethanol was added to the samples at one to two hour intervals until 10 h after defecation.

After the addition of the ethanol, each subsample was briefly stirred in order to immerse the entire fecal sample in the solvent. All tubes were stored at ambient temperature until the following morning (within 18 h of sample collection), and samples were then extracted as described in Section 2.2, with two exceptions: the precipitate was allowed to settle instead of centrifuging the samples, and the pellets were oven-dried instead of sun-drying.

2.4. Physiological response to capturing and different handling regimes

The stress response to capture and handling in the wild study population was evaluated during five monthly 3-day capture sessions in the non-reproductive season (March–July 2012) (see Section 2.1). In each session, traps were set before dusk and closed at dawn. In March–May, traps were additionally monitored in the evening when a subset of the captured animals was subjected to brief handling on site of capture for a physical strength experiment. All captured animals were transported to the field camp. In the morning following the first night of the capture session ("day 1"), each individual was subjected to handling and fecal samples were collected for establishing the baseline FGCM level. At dusk, the animals were released at the capture location.

To assess the magnitude of the physiological stress caused by capture and handling, repeated samples were collected from the individuals that were captured on days 1 and 3 of the capture session ($n = 65$) and the change in FGCM from day 1 to day 3 was used to quantify the stress response. On day 1 each individual was subjected to one of three handling protocols: minimum handling, repeated handling or anesthesia (Table 1). On day 3, they were only let out of the trap into a fabric bag to facilitate fresh feces collection. Many individuals ($n = 38$) were also captured on day 2, intermediate to the two sampling events, but were not handled.

Anesthesia was avoided whenever possible to minimize any adverse effects of the chemical treatment and therefore, only unmarked individuals were anesthetized in order to insert a transponder and gain tissue samples. Hence, we cannot fully disentangle the effects of anesthesia, first time capture and age (presumably, the youngest individuals of the cohort are anesthetized) as the size and complexity of the data set did not permit a detailed analysis of the possible interactions of these factors.

The animals' ages (juvenile, <1 year or adult, >1 year old) and individual capture histories were drawn from the routine capture data. Lifetime capture experience was calculated as the total number of capture events from birth to June 2012 (median = 6, range: 1–95), divided by the age in years (range: 0–10) to adjust prior experience for differences in exposure to traps.

2.5. Statistical analysis

2.5.1. Validation of a fecal glucocorticoid assay

A linear mixed effects model (Pinheiro and Bates, 2000) was used to estimate post-defecation change in FGCM in untreated feces in the controlled experiment in captivity. The log-transformed sample FGCM was used as the response variable and FGCM at time 0 (control), number of hours after collection and their interaction were included as covariates, weighted by the number of samples in the series. Sampling series identity was added as a random effect.

For samples collected in the wild, the influence of lag times from sample collection to the different stages of processing (preservation in ethanol and extraction) on the sample FGCM level were calculated using linear regression, with fecal water content (see Section 2.5.2) included as a control variable. Protocol repeatability was assessed by calculating Spearman's correlation coefficients for 16 duplicate samples. The optimal lag time to recording the maximum hormonal response to capture and handling (change from day 1 to day 2, 3 and 4, with no intermediate capture events) was assessed using one-tailed paired *t*-tests.

2.5.2. Baseline FGCM and the stress response to capturing and handling

Linear mixed models (Pinheiro and Bates, 2000) were used to examine the factors causing variation in baseline FGCM and in the stress response to capturing and handling. All mixed models were built using the R-program package lme4, employing the function lmer with a Gaussian error distribution and an identity link (Bates et al., 2012), and individual identity was used as a random effect. Variables were log-transformed when necessary to meet assumptions of normality, homoscedasticity and linearity of relationships, which were assessed using residual plots. Model stability was confirmed by calculating variance inflation factors and by excluding data points one by one and comparing the resulting estimates and fitted values with the inclusive model. The *P*-values were obtained via Markov Chain Monte Carlo (MCMC)-estimation Baayen, 2011 or, for factorial variables with 3 or more levels, via likelihood ratio tests (LRT). The relationship between the individual's baseline FGCM level and the magnitude of the response was studied with simple linear regression, controlling for the possible influence of second day captures.

Two potential nuisance factors: fecal water content (water%) and the time of day of sampling were included as control variables in the models. Fresh fecal mass was used instead of dry mass in samples collected from the wild to adjust for a higher measurement error in weighing very small dry sample masses. The water% (difference in mass between fresh and dried sample) showed high variability and strongly, negatively influenced the measured FGCM level ($\beta = -3.430$, SE = 0.613, $df = 130$, $X^2 = 30.510$, $P < 0.001$). Sample water content was therefore controlled for in all models. A part of the baseline samples were collected in the evening before the handling and all other samples in the morning during handling. As it is known from many species that the diurnal rhythms can cause variation on GC-levels over the course of the day (Bosson et al., 2009; Rimbach et al., 2013), we also included the time of day of sample collection in the baseline model.

Table 1
Description of the handling regimes used and associated sample sizes.

Regime	Procedure	~Handling time	Sample size
Minimum handling	Body mass measurement only. The animal is released from the trap into a fabric bag in the morning and weighed using a Pesola scale, then allowed to re-enter the trap.	2 min	47
Repeated handling	The animal was handled briefly for a physical strength experiment on the night before the routine processing and handled again in the morning according to the minimum handling regime.	5 min + 2 min	13
Anesthesia	Unmarked individuals were briefly anesthetized with 0.2 ml subdermally administered Ketamin-solution (Ketanest 100, Parke-Davis), then tissue sampled by cutting small pieces of the ears, equipped with a subcutaneous transponder microchip (Trovan) and hair sampled using pet clippers. Morphometrics and body mass were measured.	25 min	12

To study how the baseline FGCM was influenced by variables pertaining to the individual (age, sex, lifetime capturing experience) and the sampling event (month, time of day, water%) we used all baseline samples collected during the field season (March–July 2012, including also cases where the individual was not recaptured) in order to increase the sample size, resulting in $n = 277$ samples from 145 individuals.

To estimate the influences of lifetime capture experience and handling regime on the magnitude of the stress response to capture and handling, we created interaction terms of each of these variables with the sampling stage (baseline vs. response, *sensu* Liang and Zeger (2000) and Liu et al. (2009)) and modeled their influence on the sample FGCM level. From the baseline analyses (see Section 3.2) it was evident that water% explained a large part of the variation in the FGCM levels and was therefore included as a control. Furthermore, age and the potential cumulative effect of an intermediate capture between samplings (second day capture) were controlled for by including their interactions with stage. Due to missing values, the total number of cases included in the model was 55 observations from 28 individuals. To account for the low sample size and model complexity, we attempted to improve the model fit by removing the least significant interaction terms (above $P = 0.1$) one at a time based on LRT (Engqvist, 2005).

Since the influences of age, experience and regime cannot be separated in the anesthesia regime (only juvenile animals were anesthetized) we restricted the above response model to cases from the minimum and repeated handling regimes. Therefore, we constructed a separate model to assess the difference between the anesthesia and minimum regimes in juveniles only ($n = 53$ observations from 27 individuals), including regime and intermediate capture as interaction terms with stage, and water% as control. Capture experience was 0 for all anesthetized individuals and was therefore excluded from the model.

Although baseline values were not significantly influenced by the time of day of sampling (see Section 3.2), it was further examined in stress response models in order to control for possible time differences between the repeated sampling events in stress response models (evening samples $n = 6$). For this purpose the models were re-run after excluding the 6 cases of evening sampling. The exclusion did not affect the model outcomes and therefore all cases were included in the analyses and the time of day of sampling removed from the final models.

Means are given with standard errors and significance levels were set to $P = 0.05$ for all tests. All analyses were done in R version 2.15.1 (R Development Core Team, 2012).

3. Results

3.1. Validation of a fecal glucocorticoid assay

3.1.1. Biological validation 1

In absolute terms, the highest levels of fecal GCs were measured by the group-specific 11-oxoetiocholanolone assay whilst the lowest concentrations were detected by the CCST assay (Table 2; Fig. 1). Nevertheless, in all 5 cases of captured wild animals, all four assays measured a similarly strong response to the capture and handling as reflected by an 8–9-fold average increase in FGCM levels (Table 2; Fig. 1). FGCM levels started to rise within 24 h of the capture, with median lag times to peak FGCM response ranging from 38 to 50 h across the four assays (Fig. 1; Table 2). There was considerable individual variation in the timing of the peak FGCM response in all assays; however, the two group-specific assays were more consistent in this respect than the CORT and CCST assays (Table 2). FGCM levels usually returned to pre-stress levels by day 4 (Fig. 1).

3.1.2. Biological validation 2

The FGCM response to the translocation event was generally similar to that seen in the captured animals. Following translocation, a clear increase in FGCM levels was detected by the different assays with the exception of the CORT measurement which demonstrated a decrease (Fig. 2). The FGCM increase measured by the two group-specific EIAs was similar in magnitude (about 4-fold) and more pronounced than that of the CCST assay (about 2-fold). In terms of timing, both the start of the FGCM rise (on average at day 3 post-transport; Fig. 2) as well as the occurrence of peak levels (day 4 post-transport, Fig. 2) was delayed by about two days compared to the situation observed in the wild animals. This delay was most likely due to the fact that all animals were completely inactive and did not feed on the first day after arrival and remained very inactive with only small amounts of food consumed in the days thereafter. It is conceivable that metabolic rate and gut passage time were thus slowed down, resulting in a delayed hormone metabolism and excretion pattern. Additionally, the fact that animals were only sampled once per day likely contributed to the pronounced delay times in the GC responses observed.

3.1.3. HPLC

Since the two group-specific FGCM assays appeared to be of similar value for monitoring adrenocortical activity, we performed an HPLC analysis to characterize the immunoreactive metabolites present in the feces and measured by the two assays. HPLC immunoreactivity profiles indicated the presence of several distinct peaks between fractions 9 and 31 in both assays (Fig. 3), at positions where cortisol metabolites elute in our HPLC system (Heistermann et al., 2006). The presence of abundant immunoreactivity found at fractions 25 (11ß-hydroxyetiocholanolone EIA) and 30 (11oxoetiocholanolone EIA) at the elution positions of 11ß-hydroxyetiocholanolone and 11-oxoetiocholanolone standards, respectively, indicate that these two cortisol metabolites are abundant in the feces of grey mouse lemurs. In the 11oxo-etiocholanolone assay, however, the major peak of immunoreactivity was detected around fraction 50 at a position where in other primate species metabolites of testosterone elute (Möhle et al., 2002), indicating a substantial co-measurement of steroids not deriving from

Table 2
Fecal glucocorticoid concentrations (as detected by four different GC assays) in response to capture stress in wild mouse lemurs (biological validation 1).

Individual	3α,11ß-dihydroxy-CM[B]				3α,11oxo-CM[B]				Cortisol				CCST			
	Pre[a]	Peak[b]	Delta[c]	Lag[d]	Pre[a]	Peak[b]	Delta[c]	Lag[d]	Pre[a]	Peak[b]	Delta[c]	Lag[d]	Pre[a]	Peak[b]	Delta[c]	Lag[d]
06DF B6BB[A]	0.21	1.68	8.0	62.5	0.50	4.50	9.0	62.5	0.42	1.44	3.4	38.8	0.12	0.87	7.4	62.5
06E9 B40D	0.10	1.11	11.1	50.3	0.20	2.06	10.3	38.3	0.51	6.13	12.0	38.3	0.09	1.37	16.1	38.3
06E9 CDDE	0.12	3.12	27.0	50.3	0.09	7.47	79.3	38.3	0.17	2.06	12.1	26.3	0.06	0.52	8.5	15.8
06DF B6BB[A]	1.23	8.90	7.2	45.5	2.51	9.36	3.7	45.5	0.46	1.94	4.2	45.5	0.36	1.41	3.9	45.5
CAMP F1	0.02	0.13	7.6	34.5	0.05	0.44	8.8	34.5	0.11	0.99	9.0	69.5	0.06	0.86	14.3	69.5
Median	0.12	1.68	8.0	50.3	0.20	4.50	9.0	38.3	0.42	1.94	9.0	38.3	0.09	0.87	8.5	45.5

[a] Baseline levels (i.e. levels within 2 h after capture) in µg/g wet feces (see Section 2).
[b] Peak levels in response to capture in µg/g wet feces.
[c] x-fold increase of peak levels above baseline concentrations.
[d] Lag time in hours between time of capture and peak response.
[A] Animal was captured twice (in 2010 and 2012).
[B] Group-specific assay.

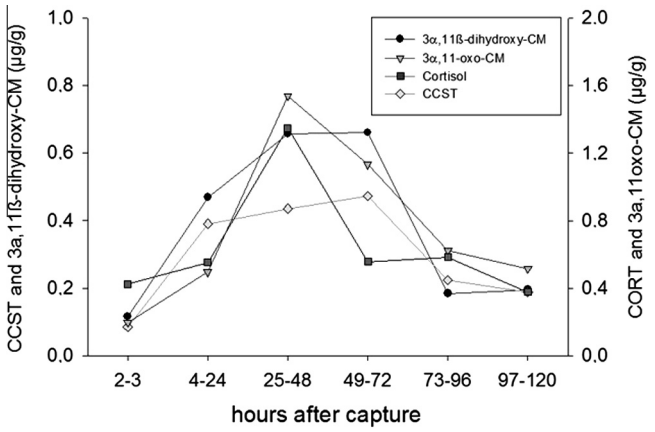

Fig. 1. Change in immunoreactive FGCM levels as measured by four glucocorticoid EIAs in response to capture and handling. Data points represent median values calculated for 24 h intervals across the 5 cases examined.

cortisol in the 11-oxoetiocholanolone EIA. Based on the combined results from validations 1 and 2 and the HPLC data, we selected the 11ß-hydroxyetiocholanolone assay for all further analyses.

3.1.4. Biological validation 3

Paired samples from repeated captures within a capture session (change from day 1 to day 2, 3 or 4) revealed a significant elevation from baseline FGCM at each time interval, with a 2-day lag (samples from day 1 and day 3) showing the strongest and most consistent effect (Table 3, Fig. 4), confirming the results on GC-response time lags derived from biological validation 1 (camp animals, Sections 2.3.1 and 3.1.1). Consequently, the day 1 to day 3 change in FGCM was used to quantify the stress response to capture and handling in the subsequent experiment.

3.1.5. Fecal sampling method

The protocol repeatability assessment revealed a highly significant correlation between the FGCM levels measured in duplicate subsamples (for ng/g fresh feces $r_S = 0.92$, $P < 0.001$, $n = 16$). In samples collected from the wild ($n = 289$), FGCM results were not significantly influenced by differences in the time lags from collection to preservation in ethanol (range: 0.5–4 h, $\beta = -0.138$, SE = 0.126, $t = -1.090$, $P = 0.276$), from collection to extraction (6–14 h, $\beta = 0.194$, SE = 0.125, $t = 1.558$, $P = 0.120$) or from preservation in ethanol to extraction (5–12 h, $\beta = -0.147$, SE = 0.127, $t = -1.158$, $P = 0.248$).

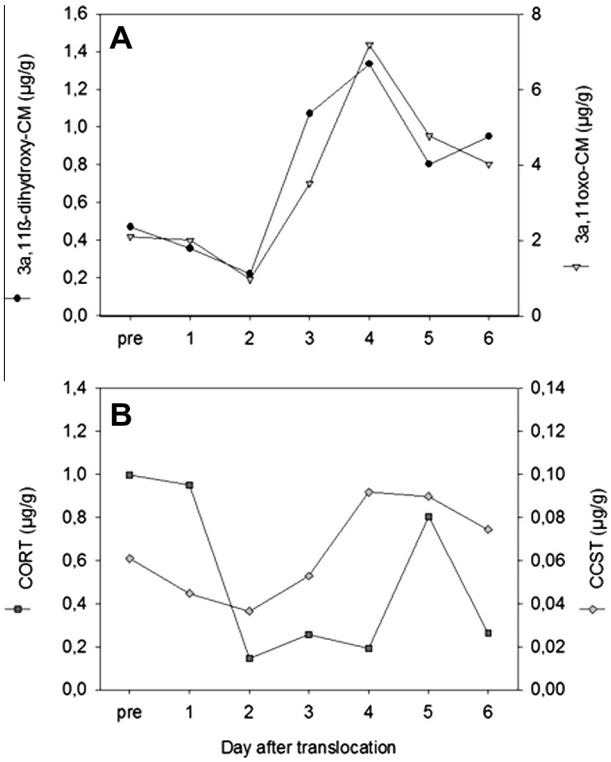

Fig. 2. Change in immunoreactive FGCM levels as measured by (A) the two group-specific assays and (B) the cortisol and CCST assays in response to translocation. Data points represent median values calculated for 24 h intervals across the 8 groups of animals examined. Pre = pre-transport baseline FGCM levels.

3.1.6. Post-defecation changes in FGCM

The profiles of change in FGCM over time (Fig. 5) illustrate that the delay from sample collection to preservation in ethanol did not induce statistically significant changes in the sample FGCM (baseline FGCM(t0): $\beta = 0.950$, SE = 0.027, $t = 34.93$, $P < 0.001$, hours to ethanol added: $\beta = 0.118$, SE = 0.046, $t = 2.57$, $P = 0.292$, interaction: $\beta = -0.020$, SE = 0.006, $t = -3.13$, $P = 0.121$).

3.2. Correlates of baseline FGCM

The percentage of water negatively influenced the FGCM level in a sample (Table 4). The overall baseline FGCM decreased slightly over the course of the season, but the overall influence of the

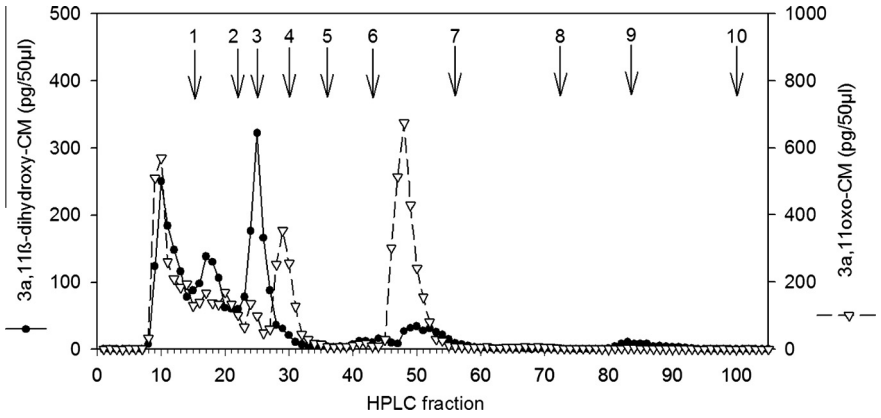

Fig. 3. HPLC profiles of immunoreactivity detected with the 3α,11β-dihydroxy-CM and 3α,11-oxo-CM EIA in a fecal extract of a wild male gray mouse lemur. Arrows and numbers indicate the associated elution positions of reference standards: (1) cortisol (fraction 14/15), (2) corticosterone (22/23), (3) 11ß-hydroxyetiocolanolone (24/25), (4) 11-oxoetiocholanolone (29/30), (5) 5β-androstane-3,11,17-trione (36), (6) testosterone (43), (7) androstenedione, dehydroepiandrosterone (55/56), (8) epiandrosterone, 5β-dihydrotestosterone (72), (9) 5β-androstane-3α-ol-17-one (82/83) and (10) androsterone (100).

Table 3
Differences between baseline and response FGCM level, measured by the 11ß-hydroxyetiocholanolone assay, at a delay of 1–3 days. Paired samples from recaptures within a capture session.

	Approximate time difference (h)	One-tailed paired *t*-test	*df*	*P*
Day 1 vs. day 2	24	$t = 3.449$	20	0.001
Day 1 vs. day 3	48	$t = 4.159$	27	0.0001
Day 1 vs. day 4	72	$t = 1.832$	16	0.043

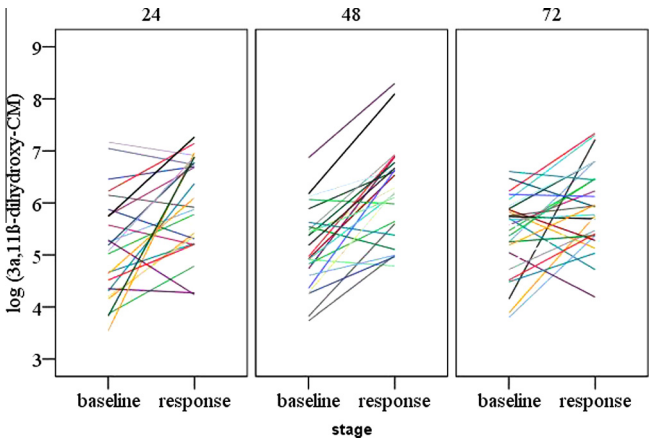

Fig. 4. Change in FGCM levels, detected by the 11ß-hydroxyetiocholanolone assay, from baseline (day 1) to a consecutive sample collected (A) 24, (B) 48 or (C) 72 h after the baseline sample. For statistics see Table 3.

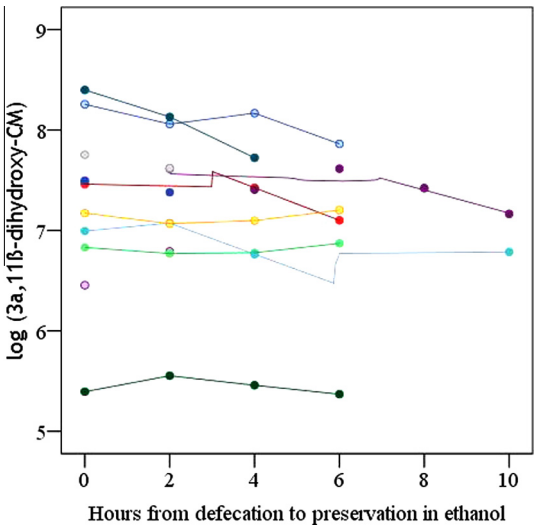

Fig. 5. Post-defecation changes over time in FGCM levels (detected by the 11beta-hydroxyetiocholanolone assay) in untreated feces, based on the experiment in captivity. Lines (95% loess smoothers) represent sampling series of fecal samples divided into several subsamples (points) that were preserved in ethanol at different delays after defecation.

month of capture was not statistically significant (month of capture: $X^2_4 = 6.962$, $P = 0.138$). Adults had on average higher baseline FGCM values than juveniles (Table 4), but the time of day of sampling, the animal's sex and previous capture experience had no significant influence on the FGCM level (Table 4).

3.3. Physiological response to capturing and handling

In the capture and handling experiment, an increase in the FGCM level from day 1 to day 3 was evident in 78% (61/78) of the sample pairs (3.30 ± 2.81-fold increase from baseline, $t_{paired} = 6.12$, $df = 77$, $P < 0.001$). The magnitude of the response was positively influenced by the baseline FGCM level ($\beta = 0.449$, SE = 0.144, $F_{2,45} = 9.406$, $P = 0.002$). The individuals that showed a decline from day 1 to day 3 had higher than average baseline FGCM concentrations and mainly represented the minimum handling regime (15/17 cases).

In the stress response model, the interaction terms *stage x regime* (LRT: $X^2_1 = 0.007$, $P = 0.934$) and *stage x experience* ($X^2_1 = 0.028$, $P = 0.867$) were the least significant terms and were removed from the model in this order. In the final model, the magnitude of the stress response tended to be slightly higher for the animals that were captured also on the intermediate day ($P = 0.068$, Table 5). The response strength was not significantly influenced by the animal's age, handling regime or capture experience (Table 5).

Table 4
Variables influencing the baseline FGCM levels (day 1 level). P-values are based on MCMC-tests. n = 277 observations from 145 individuals.

Fixed effects		Estimate	SE	*t*	*P*
Intercept		8.040	0.485	16.573	<0.001
Water%		−3.661	0.620	−5.906	<0.001
Time of day (Ref. Morning)		0.295	0.151	1.948	0.087
Month (Ref. March)	April	−0.355	0.199	−1.783	0.068
	May	−0.486	0.203	−2.392	0.008
	June	−0.519	0.232	−2.239	0.008
	July	−0.365	0.231	−1.583	0.067
Age category (Ref. Adult)		−0.404	0.136	−2.959	0.002
Sex (Ref. Female)		0.081	0.126	0.645	0.613
Capture experience		0.023	0.074	0.305	0.727

In the model for anesthesia vs. minimum handling (juveniles only), the *regime x stage* interaction was non-significant (LRT: $X^2_1 = 0.356$, $P = 0.551$). After removing the interaction term, the anesthesia main effect was significant (reference value: no anesthesia, $\beta = -0.662$, SE = 0.269, $t = -2.463$, $P = 0.009$), indicating that anesthetized animals had overall lower FGCM values than the minimum handling juveniles.

4. Discussion

In this study, we successfully established a group-specific 11ß-hydroxyetiocholanolone assay for monitoring the physiological stress response in the grey mouse lemur via fecal samples and applied this method to explore the influence of capture and handling on the hormonal stress response in wild animals. These data showed no evidence of habituation or sensitization of individuals as a response to frequent capture and handling. In addition, the results indicate that more invasive handling did not cause a stronger stress response in the animals. Our study provides important new information for field researchers interested in using fecal glucocorticoid analysis techniques to monitor adrenocortical activity in mouse lemurs and suggests that routine capturing by trapping and brief handling at monthly intervals does not cause chronic stress to animals.

4.1. Validation of a fecal FGCM assay

A major aim of this study was to validate a reliable assay for quantifying fecal glucocorticoids as a measure of physiological stress in gray mouse lemurs which is of primary importance before any application (Buchanan and Goldsmith, 2004; Heistermann et al., 2006; Touma and Palme, 2005). Our tests of physiological validity suggest that at least three of the four glucocorticoid EIAs tested are generally suitable for the detection of changes in adrenocortical activity in the species through fecal GC metabolite analysis. However, the two group-specific assays appear to be more sensitive in detecting stress-related changes in glucocorticoid output as shown by a more consistent response across contexts compared to the CCST and, in particular, the CORT assay. Thus, the group-specific assays appear to be better suited for assessing adrenocortical activity in gray mouse lemurs.

Moreover, HPLC immunoreactivity peaks co-eluting with 11ß-hydroxyetiocholanolone and 11-oxoetiocholanolone standards indicated the presence of 3α,11ß-dihydroxylated GC metabolites and 11,17-dioxoandrostanes, both of which have also been reported as abundant fecal glucocorticoid metabolites in other primate and non-primate species (e.g. Ganswindt et al., 2003; Heistermann et al., 2006; Ostner et al., 2008; Palme and Möstl, 1997). Since group-specific glucocorticoid assays measure a broad spectrum of steroids, there is, however, a higher risk of co-measurement of androgen metabolites (Ganswindt et al., 2003; Schatz and Palme, 2001) compared to more specific assays. Our HPLC data indicate that, similar to findings in the chimpanzee (Heistermann et al., 2006), this is likely the case concerning the 11-oxoetiocholanolone assay which detected peak amounts of immunoreactivity at a position where in our HPLC system metabolites of testosterone (but not of cortisol) elute (Ganswindt et al., 2003; Heistermann et al., 2006; Möhle et al., 2002). The results also indicate a small potential for co-measurement of androgen metabolites by the 11ß-hydroxyetiocholanolone assay. For this study, however, this is likely of negligible importance given that the vast majority of samples of our capture stress experiments were collected when animals are reproductively quiescent and sex hormone levels are probably low (Aujard and Perret, 1998), thus FGCM levels in the experiments were unlikely affected by the animals' reproductive state. In contrast, the sample used for HPLC came from a male in prime reproductive condition (shortly before the onset of the short mating season, with maximum testes size) when androgen levels are clearly elevated (Aujard and Perret, 1998). For measuring FGCM in our samples, we therefore chose the 11ß-hydroxyetiocholanolone assay which has proven to be a versatile assay to monitor adrenocortical activity in many other species of primates (Fichtel et al., 2007; Ostner et al., 2008; Shutt et al., 2012; Weingrill et al., 2011; Heistermann et al., 2006; Martínez-Mota et al., 2008; Pirovino et al., 2011; Rimbach et al., 2013) and non-primates (Ganswindt et al., 2003; Möstl et al., 2002).

Our data from the capture-validation study as well as from the repeated capture experiment of wild animals indicate a 2-day delay in FGCM excretion from stressor to peak response, with levels starting to rise within 24 h of the stressful event. Although this time lag in fecal glucocorticoid excretion is within the range of those reported for many other primate species (Heistermann et al., 2006; Pirovino et al., 2011; Shutt et al., 2012; Weingrill et al., 2011; Whitten et al., 1998), it is distinctly longer than expected given the small body size of the species and a gut passage time (which usually determines the lag time between steroid secretion in blood and appearance of the metabolites in feces (Palme, 2005)) of less than 24 h (Raharivololona, 2009). The reason for this long delay in peak FGCM excretion is unknown, but may, at

Table 5
Variables influencing the FGCM response to capture and handling, final model. Interaction terms are indicated with "x". *P*-values are based on MCMC-tests. *N* = 55 observations from 28 individuals.

Fixed effects	Estimate	SE	*t*	*P*
Intercept	5.777	0.909	6.357	<0.001
Stage	0.464	0.410	1.133	0.335
Water%	−0.830	1.144	−0.726	0.476
Age category (Ref. Adult)	0.264	0.295	0.895	0.413
Intermediate capture (Ref. none)	−0.101	0.343	−0.294	0.782
Capture experience	−0.125	0.187	−0.671	0.514
Regime (Ref. Minimum)	0.334	0.282	1.187	0.253
Intermediate capture (Ref. none) x Stage	0.909	0.419	2.171	0.068
Age category (Ref. Adult) x Stage	−0.677	0.352	−1.922	0.118

least in part, be explained by cumulative stress of capture with up to 20 h restraint in the trap (Fletcher and Boonstra, 2006; Garcia et al., 2000) and subsequent handling and housing in a novel environment (validation animals). As suggested by our translocation data, the extent of activity and food consumption appears also to have a marked effect on the time course of FGCM excretion, probably by altering general metabolic rate and processes, which might affect hormone excretion patterns (Goymann, 2012; Morrow et al., 2002). There was no indication of reduced activity and feeding in our wild study animals, however. Finally, a pronounced and delayed enterohepatic recirculation of glucocorticoids prior to excretion of the metabolites into the feces (Lindner, 1972; Möstl and Palme, 2002; Symonds et al., 1994) may also contribute to the long lag time, although this remains completely speculative.

Collectively, these data suggest that peak FGCM output responses in the grey mouse lemur can generally be predicted to occur 1–2 days after exposure to a stressor. There was, however, considerable variation among individuals in the time to peak FGCM elevation. This variation was particularly evident for the CORT and CCST assay, whilst time lags for the two group-specific assays were more consistent, a finding also seen in lowland gorillas (Shutt et al., 2012). The reason for this is not clear but may be related to differing influences of factors affecting metabolic processes and the production of the various types of cortisol metabolites measured by the different assays. Further studies on stress responsiveness and GC-metabolism in the species, including an ACTH-challenge test and the assessment of post-peak decline in the FGCM in a larger number of animals will be required to identify the causes of the long delays and the heterogeneity of responses.

Most wild-caught individuals were able to return from the peak elevation to near the baseline within 2–3 days. Typically, habituation to captivity is assessed in time periods of months rather than days, particularly when behavioral aspects are studied and very few studies exist on the short term (physiological) habituation to captivity. One such study by Dickens et al. (2009) showed that wild-caught chukar partridge (*Alectoris chukar*) can return from stress response GC levels to near the baseline levels in approximately 9 days after capture and subsequent captivity. Short term studies such as ours provide evidence towards a more rapid physiological habituation than would be expected based on behavioral measures of habituation, which may reflect protective mechanisms against chronically elevated cortisol levels. There is some evidence that stabilizing conditions, such as an improvement in the predictability of food acquisition may decrease a stress response and could possibly reduce the time required to return to baseline when wild-caught animals are held in temporary captivity and fed at regular intervals (Romero, 2004). On the other hand, the diet in captivity differed from the natural diet which could potentially also influence the change from baseline FGCM levels to subsequent measurements (Behie et al., 2010; Dantzer et al., 2011; Goymann, 2005; Goymann, 2012; Millspaugh and Washburn, 2004) in our study. Overall, our study joins many others in stressing the importance of validation across contexts: we have shown that the delay from the stressor to recording the peak stress response via feces differed between experiments using captive and wild animals. This source of uncertainty should be considered when conducting GC research in conditions that differ from the validation setting.

4.2. Post-defecation FGCM change in unpreserved feces

Our experiment illustrates that FGCM concentrations in grey mouse lemurs are generally unaffected when preservation of the sample in alcohol takes place within 10 h of defecation. To date, few studies have investigated hormone change in feces between defecation and fixation. Most of these studies demonstrated either an increase (cattle, horses and pigs (Möstl et al., 1999); Borneon orangutans (Muehlenbein et al., 2012), or decrease (brown hyena Hulsman et al., 2011; lowland gorilla (Shutt et al., 2012) in FGCM concentrations within a few hours when samples were stored unpreserved at ambient temperature. Our findings are therefore noteworthy and imply that immediate fecal preservation is not absolutely necessary to obtain reliable FGCM results for mouse lemurs (see also Rehnus et al., 2009 for mountain hares). This is of particular importance for studies in the wild where immediate immersion of the feces after defecation in ethanol may be difficult to accomplish. In our setting, most individuals are captured in the first hours of the activity period and the period between defecation to preservation of the sample in ethanol is largely standardized. However, as defecation time is often not accurately known, only fresh-looking feces were collected to minimize the time interval between defecation and fecal preservation and the risk of sample degradation. Confirming the findings from our captive experiment, no effect of the sampling-to-preservation period was found on FGCM results in the samples collected from wild animals.

4.3. Correlates of baseline FGCM

Adult wild mouse lemurs had significantly higher baseline FGCM levels than juveniles, which agrees with the trend found in humans, some non-human primates and rodents (Gunnar and Quevedo, 2007; Romeo, 2010; Sanchez et al., 2001; Sapolsky and Altmann, 1991; Sapolsky and Meaney, 1986; Sapolsky et al. 1987) and has been explained with the maturation and senescence of the adrenocortical system. The individual's sex or time of day of the sampling (morning vs. evening) had no influence on the FGCM levels. Uncontrollable variables such as diet, metabolic rates or bacterial communities may cause variation between individuals in the measured GC titres (Goymann, 2012) and could be partially responsible for the observed, substantial inter-individual variation. The water content of the feces significantly negatively influenced the baseline level. The influence of water content probably merely signifies the solid mass of the feces into which GC could be excreted or, alternatively, it may reflect the hydration status of the animal, which itself can cause a stress response (Kiss et al., 1994; Ulrich-Lai and Engeland, 2002).

4.4. Influence of handling stress on the hormonal stress response

As expected, the capture-and-handling protocol in general acts as a stressor, causing on average a threefold increase in FGCM levels from baseline within 2 days of the stressor. This is significantly lower than the responses recorded in the validation series conducted in the wild, where the peak GC elevation to stressors (capture, handling and subsequent temporary captivity) resulted, on average, in an 8-fold increase from baseline GC and the delay to peak FGCM response ranged between approximately 34–63 h after capture. The "trap happy" behavioral response (i.e. increased capturing probability) of most individuals in the population (Kraus et al., 2008) that leads to repeated voluntary trap entry may further indicate that the protocol causes them comparatively low levels of stress.

Individuals with higher baseline FGCM levels typically showed a higher response to the stressor than individuals with low baseline levels. This implies that also animals with higher baseline FGCM levels maintain the capacity to react adequately to stressful stimuli and do not show a desensitization of the HPA axis (associated with an attenuated stress response) as reported for animals that are severely or chronically stressed (Rich and Romero, 2005). High FGCM baseline levels, measured prior to an acute stressor, are typically interpreted as a sign of chronic stress (but see Cyr and Romero, 2009; Dickens et al., 2013), which in turn is known for its multiple detrimental influences on health, fitness and survival

(Crespi et al., 2013; Romero, 2004). The samples collected on the day of first capture were considered to represent the baseline level, but, since the animals could not be monitored prior to the captures, we cannot rule out the occurrence of prior natural stressors that may have elevated the pre-handling FGCM levels. Therefore the minority of cases that showed a decline in FGCM from baseline to day 3 likely reflect recovery from another stressor rather than a decline in GC level as a response to capture and handling. Contrary to our predictions, prior capture experience had no influence on the baseline FGCM level or on the magnitude of the stress response, which indicates a lack of habituation (or sensitization) to the repeated stressor. Few studies have previously explored the potential for habituation to repeated handling but the available studies (mainly from captive conditions, e.g. (Dobrakovová et al., 1993; Jones and Waddington, 1992)) suggest that repeated handling can lead to habituation when done regularly at relatively short intervals (several times per week). Longer intervals (several weeks) between stressors typically do not lead to habituation (e.g. Desportes et al., 2007; Dickens et al., 2013; Tort et al., 2001) since long delays between similar stressors may render the stressor too unpredictable for the animal to habituate to (Dickens et al., 2013; Fowler, 1999; Koolhaas et al., 2011). In our study population, the capturing is usually conducted at monthly intervals with seasonal breaks (reproduction and the high dry season) and handling is done on the first day, after which recaptured animals are only transported and fed, which may further decrease the predictability of the process for the animal. It has been suggested that habituation may also take the form of a more rapid recovery from the stressor even when the magnitude of the response remains similar (Sheriff et al., 2010) but unfortunately this possibility could not be addressed in our experimental design. Further support for the lack of habituation is suggested by the exploratory analyses of repeated measurements of the stress response (data not shown) for a small number of animals ($n = 9$) that could be repeatedly sampled in more than one capture session. While the sample size was insufficient for meaningful statistical analyses, these data suggested no systematic changes in the magnitude of the response within an individual from one capture session to another. The trapping frequency of an individual may be influenced by the exact location of its home range (e.g. center or edge of the study area) and it can be associated with certain personality traits, which may also influence the individual's stress levels (Koolhaas et al., 1999; Montiglio et al., 2012). However, explorative analysis of our (limited) data showed no patterns suggesting that individuals with lower baseline levels or lower stress responses on the first capture of the season would enter traps more frequently later on in the season.

Also in contrast to our predictions, the handling regime had no significant influence on the physiological response evoked at a two-day delay from the stressor, although lowest values were generally measured for animals in the anesthesia regime (probably due to these animals being the youngest of the cohort, or due to changes in metabolism induced by anesthesia) and highest for those individuals that were handled repeatedly. Handling is, however, only a short part of the protocol and likely contributes only partially to the variation in the response strength: individuals may experience differing levels of stress due to confinement in the trap and transport. This, along with some degree of cumulative stress induced by intermediate captures and the "noisy" data may mask any fine scale differences between the handling regimes.

5. Conclusions

The successful validation of a fecal glucocorticoid metabolite assay (11ß-hydroxyetiocholanolone) in grey mouse lemurs permitted us to use the routine recapturing of animals to measure stress induced by capture and handling without the need to draw blood or to restrain animals beyond the capture events. Routine capturing does not appear to induce chronic stress or lead to habituation in animals captured at monthly intervals. Bearing in mind the restrictions posed by the data, we also found no indications of the invasiveness of the handling influencing the magnitude of the stress response. Overall, the data suggests that regular capturing does not lead to long-term changes in the stress physiology that could interfere with the study of other phenomena in the species using a capture-and-handling design, or risk the well-being of the study animals. The methods we describe will be useful for the monitoring of health and overall physiological status of individuals or populations of wild mouse lemurs efficiently and in a relatively non-invasive manner.

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