Non-invasive monitoring of physiological stress in the western lowland gorilla (*Gorilla gorilla gorilla*): Validation of a faecal glucocorticoid assay and methods for practical application in the field

Kathryn Shutt[1]; Joanna M. Setchell[1]; Michael Heistermann[2]

[1]Department of Anthropology, Durham University, DH1 3LE, Durham, UK

[2]German Primate Centre, Reproductive Biology Unit, Kellnerweg 4, 37077 Gottingen, Germany.

Correspondence: Kathryn Shutt (Department of Anthropology, Durham University, DH1 3LE, Durham, UK) k.a.shutt@durham.ac.uk, + 44 (0) 7791339559

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SUMMARY

1). Enzyme-immuno-assays (EIAs) allow stress hormone output to be monitored via measurement of fecal-glucocorticoid metabolites (FGCMs) in many vertebrates. They can be powerful conservation tools which allow acquisition of otherwise-unobtainable physiological information from both captive animal breeding and endangered wild animals in remote forest habitats, such as great apes. However, methods for hormone measurement, extraction and preservation need to be adapted and validated for remote field settings.

2). In preparation for a field-study of western lowland gorillas (*Gorilla gorilla gorilla*) in the Central African Republic we used samples from captive gorillas collected around opportunistic stressful situations to test whether four different glucocorticoid EIAs reflected adrenocortical activity reliably and to establish the lag-time from the stressor to peak excretion. We also validated a field extraction technique and carried out storage experiments to establish a simple, non-freezer-reliant method to preserve FGCMs in extracts long-term. Finally, we conducted field experiments to determine the rate of FGCM change over 28 days when samples in alcohol cannot be extracted immediately and over 12 hours when faeces cannot be preserved immediately in alcohol, and used repeat samples from identified individuals to test for diurnal variation in FGCMs.

3). Two group-specific assays measuring major cortisol metabolites reliably detected the predicted FGCM response to the stressor, whereas more specific cortisol and corticosterone assays were distinctly less responsive and thus less useful. Our field extraction method performed as well as a laboratory extraction method and FGCMs

in dried extracts stored at ambient temperatures were more stable than those at -20C over one year.

4). Hormones in non-extracted feces in alcohol were stable up to 28 days at ambient temperatures. FGCMs in un-fixed gorilla feces deteriorated to almost 50% of the original values within 6 hours under field conditions. We detected no diurnal variation of FGCMs in samples from wild gorillas.

5). Our study highlights the importance of thorough biological and immunological validation of FGCM assays, and presents validated, practical methods for the application of non-invasive adrenocortical monitoring techniques to field conservation contexts where it is crucially needed.

INTRODUCTION

The vertebrate stress response involves the release of glucocorticoids (cortisol and corticosterone into the bloodstream which, in conjunction with accompanying physiological and behavioural responses, enables vertebrates to cope with threatening or demanding situations (Breazile 1987). Chronic stress, associated with prolonged periods of elevated glucocorticoid (GC) concentrations, however, has been shown to interfere with numerous physiological processes critical to individual health and survival, including immune and reproductive function and disease resistance (Selye 1955; Rivier & Rivest 1991; von Holst 1998; Wingfield & Sapolsky 2003; Muehlenbein 2009). It is, therefore, important to monitor and reduce possible sources of chronic stress in the management of captive breeding and the conservation of wild animal populations.

Enzyme-immuno assays (EIAs) for faecal glucocorticoid metabolite (FGCMs) measurements have proven highly valuable in this context, as they provide reliable information about FGCM output and thus help monitor physiological stress non-invasively in animals under both captive and wild conditions. EIAs are used frequently to investigate the potential links between stress and animal behaviour (Whitten, Brockman & Stavisky 1998), reproductive biology (Lasley 1985; Lasley & Kirkpatrick 1991; Peter, Kapustin & Critser 1996), and animal welfare (Romano *et al.* 2010; Pirovino *et al.* 2011). They also have important applications for addressing conservation issues (Foley, Papageorge & Wasser 2001; Millspaugh & Washburn 2004; Cockrem 2005; Wasser & Hunt 2005).

There are, however, several potential problems associated with the application of EIAs to new species and field settings. FGCM assays must be validated for each species (Bahr *et al.* 2000; Touma & Palme 2005; Heistermann, Palme & Ganswindt 2006) as must methods to preserve the fecal samples (or the steroid hormones therein) in the field (Ziegler & Wittwer 2005). Additionally, the effects of sampling limitations on the FGCM levels found in faeces [e.g., post-defecation degradation due to aged samples or environmental effects; (Mostl *et al.* 1999; Washburn & Millspaugh 2002) and diurnal variation in individual FGCM output (Sousa & Ziegler 1998; Raminelli *et al.* 2001; Beehner & Whitten 2004) should be assessed and considered in the final analyses. Thus, before these techniques can be used to their full potential in wild animal populations, the most suitable field sampling protocols for a given study population need to be developed (Washburn & Millspaugh 2002).

Most steroid hormones, including GCs, are heavily metabolized in the liver and are secreted through the bile into the gastrointestinal tract before they are eliminated from the body via excretion into the urine and/or feces (Brownie 1992; Mostl, Rettenbacher & Palme 2005). Species metabolise cortisol (and other steroids) differently resulting in a wide range of metabolites being present in the feces (Palme *et al.* 1996; Bahr *et al.* 2000; Wasser *et al.* 2000). Consequently, assays for FGCMs must be thoroughly validated physiologically, biologically and immunologically for each species to ensure biologically meaningful results (Buchanan & Goldsmith 2004; Touma & Palme 2005; Heistermann, Palme & Ganswindt 2006). Further, there are substantial differences in the rate of FGCM excretion in feces between species and

sexes (Palme et al., 2005). For example, larger bodied primates such as chimpanzees (*Pan troglodytes*) show peak excretion rates after approximately 24 hours (Bahr et al., 2000) whereas the peak for owls (*Strix occidentalis caurina)* has approximately a 12 hour lag-time (Wasser et al., 2000, for review see Palme et al., 2005). Knowledge of the delay to fecal excretion is crucial in determination of the experimental setup and biologically meaningful interpretation of results (Palme 2005).

In addition to analytical and physiological validation of the assay procedure itself, it is equally important that fecal samples are processed and stored in a way that ensures the stability of hormone levels long-term if they cannot be extracted immediately and analysed in the laboratory using established methods. The gold standard storage method for faecal samples is simple freezing, as this stabilizes levels of FGCMs (and other steroid hormones) over long periods of time (Hunt & Wasser 2003; Palme 2005; Herring & Gawlik 2009). However, many field sites are in remote locations in developing countries without access to electricity, and where it is not possible to keep faecal samples at sub-zero temperatures. Researchers have tried to overcome this problem using a variety of fecal storage methods, including preservation in ethanol and/or drying the feces (Brockman & Whitten 1996; Foley, Papageorge & Wasser 2001; Khan *et al.* 2002; Terio *et al.* 2002; Hunt & Wasser 2003; Galama, Graham & Savage 2004; Pettitt, Wheaton & Waterman 2007). While some of these methods have proven useful in stabilizing fecal steroids in the short- and long-term in some species, they have proven ineffective in others. One solution to overcome a potential "fecal storage effect" is to extract hormones from the faeces immediately *in-situ*, and to preserve the extracts so that microbial activity - the most

likely reason for alterations in steroid levels when feces are stored in alcohol or dried - is minimized.

Various extraction/storage techniques have been developed, but the efficiency of these techniques in extracting the hormones to be measured, and the reliability of the storage methods used to stabilize the FGCMs long-term varies with the species and context (Beehner & Whitten 2004; Wielebnowski & Watters 2007; Freeman *et al.* 2010; Pappano, Roberts & Beehner 2010; Santymire & Armstrong 2010). For example, while storing fecal extracts in solid-phase-extraction cartridges is highly efficient for preserving gelada (*Theropithecus gelada*) FGCM levels in for up to three weeks (Pappano, Roberts & Beehner 2010), it results in a decline of FGCM levels of about 80% within 30 days of storage in feces from African wild dogs (*Lycaon pictus pictus*) (Santymire & Armstrong 2010). Field-friendly extraction and storage methods should therefore also be validated for each species and context.

Apart from analytical considerations, field sampling also involves other constraints and challenges which may affect the results of endocrine analysis. For example, it is rare to be able to sample each focal individual equally, or at the same time of day. Moreover, focal subjects may 'guard' their sample for long periods of time before the researcher can collect it without causing disturbance to them or their neighbours, or they may drop their contributions on the side of a high rocky outcrop or into a nest, allowing recovery of the sample only after a delay. Immunoreactive FGCM concentrations may begin to deteriorate or change immediately after defecation if they are not preserved in a suitable fixative (Mostl *et al.* 1999; Palme *et al.* 2004; Muehlenbein *et al.* 2012). It is, as yet, largely unclear to what extent this rate of change/deterioration is species-, temperature-, or time-specific. As a result, field sample sets can be limited to immediate fresh sample availability, or may be

affected by an unknown amount of exogenous FGCM variation when the samples cannot be immediately fixed, frozen, lyophilized or dried.

Finally, GC production is linked to adreno-corticotrophin releasing hormone (ACTH) circadian rhythms, where levels peak in the mornings and decline towards the evening (Rusak 1989; Chung, Son & Kim 2011). Steroid hormone levels found in blood serum, saliva, and excreted in urine often demonstrate circadian variation (Piro *et al.* 1973; Goodman *et al.* 1974; Coe & Levine 1995; Schlatt *et al.* 2008; Heintz *et al.* 2011; Kalsbeek *et al.* 2012), but the effect on FGCM output is not well studied and appears to be species-specific (Beehner & Whitten 2004). For example, Sousa and Ziegler (1998) found an increase in cortisol in the afternoon in feces of the common marmoset (*Callithrix jacchus*) whilst Beehner and Whitten (2004) reported no detectable effect of diurnal variation in FGCM output in baboons (Papio spp.). Thus, it is necessary to evaluate the extent of distortion that sampling constraints introduce to both ensure the validity of a method, and to account for any rates of FGCM change/deterioration in the final hormone results.

Approximately 95,000 western lowland gorillas (*Gorilla gorilla gorilla*) remain in the wild (International Union for the Conservation of Nature [IUCN] Red List 2012). The species is classified as critically endangered, as a result of the bush-meat trade, habitat destruction and disease (Mehlman 2009). All zoo-housed gorillas are lowland gorillas, and zoo efforts are based on maintaining healthy and genetically-robust breeding populations (Peel *et al.* 2005). In recent years, conservation efforts in habitat countries have turned to the habituation of gorilla groups for research and to draw in tourism revenue for conservation. Gorillas are, however, particularly sensitive to stress (McNeilage 1996), meaning that the impacts of methods used to

manage, conserve or research populations should be monitored carefully to ensure that the costs of such efforts to the individuals concerned do not outweigh the conservation benefits. To our knowledge, to date, published information on fecal GC output in western lowland gorillas is limited to only one study (Peel *et al.* 2005), in which the fecal cortisol assay used was not validated and results obtained were largely inconclusive. In preparation for a remote field study of free-living western lowland gorillas at the focus of an ecotourism project in the Central African Republic we set out to validate a suitable assay system for monitoring adrenocortical activity in gorillas based on fecal samples, validate a suitable extraction and storage method and to assess the effects of sampling constraints on FGCM measurements.

We tested four different FGCM enzyme-immuno-assays using samples from captive gorillas collected around opportunistic stressful situations (both physiological and biological). All four assays had been used previously to monitor FGCM output in other primate and non-primate species (Wasser *et al.* 2000a; Ganswindt *et al.* 2003; Heistermann, Ademmer & Kaumanns 2004; Heistermann, Palme & Ganswindt 2006). We also developed a 'field-friendly' extraction technique and validated its efficiency by comparing the results of the field method with those of currently accepted laboratory methods. We then conducted storage experiments on fecal extracts to assess the effects of different storage conditions and durations on FGCM levels, to establish a reliable method for long-term preservation of fecal extracts in the field where no freezer is available. Finally, our study required us to compare FGCM measures in completely wild, under-habituation, recently-habituated, and fully-habituated gorillas. This presented us with numerous sampling challenges, as the goal of collecting and extracting adequate fresh samples from identified

individuals could only be achieved with the fully-habituated gorilla group, whereas the recently-habituated groups' samples (although fresh and indentified to a minimum of age/sex class) could only be extracted three weeks later. Additionally, and because sampling wild gorillas in thick vegetation is very challenging, we maximized our potential sample size by collecting samples opportunistically at different times of the day.

Samples from groups under-habituation, and non-contacted, 'wild' gorillas were only available from their nests (usually defecated at around 05.30 am), which could take several hours to locate each day, or from opportunistic contacts. In order to have directly-comparable data-sets, we conducted experiments in the field to: determine the rate of FGCM change over 12 hours after defecation when faeces are not preserved immediately in alcohol; test whether FGCM concentrations alter as a function of storing feces for one month in alcohol under tropical conditions (e.g.,Khan *et al.* 2002; Hunt & Wasser 2003; Lynch *et al.* 2003) and whether FGCM measurements in repeat samples from individuals varied between morning and afternoon samples.

METHODS

Animals and sample collection

We asked ape keepers from three UK zoos to collect fecal samples from captive gorillas prior to six opportunistic routine veterinary and breeding management interventions that we predicted would be stressful for the gorillas. We obtained samples from three males around medical examinations (n=2) and relocations between zoos (n=2) and from a female around her social integration with two unknown but already bonded females (n=1) and later as a new silverback male joined all three females (n=1). For medical examinations and relocations a veterinarian sedated gorillas with mass-dependent doses of zoletil (a fixed-ratio combination of tranquillizer Zolazepam and the dissociative anesthetic Tiletamine) plus Zalopine, a physiological stressor for the animals. Anesthesia was not required for the social integrations, which involved social stress, and all animals were housed in their usual social groups, except when the various 'treatments' required short periods of isolation. The animals continued to receive a normal diet consisting of fruits, vegetables and leaves and water was available *ad libitum*.

Keepers collected fecal samples (range 1-15) for 1-6 days from each gorilla prior to exposure to the potential stressor to establish a pre-stress baseline FGCM level, and continued to collect samples for 3-9 days after the stressor to establish the FGCM response. During post-stress periods, the keepers collected all available samples including samples defecated overnight, noting any evidence of urine contamination. Fecal samples were stored at -20°C within 1 hour of collection.

Samples for our site-specific short-term storage, hormone degradation and diurnal variation controls were collected under completely natural field conditions. Sample collection took place between November 2010 to December 2011, at Bai Hokou study site (2º50'N, 16º28'E) in the Dzanga-Sangha Protected Areas in the Central African Republic, (for a more detailed description of the study site see Carroll, 1986). We worked with local BaAka trackers employed by the World Wildlife Fund (WWF) to collect opportunistic fecal samples from different groups of gorillas. We collected as soon as possible after defecation, noting any urine evidence of urine contamination.

Validation of FGCM measurements

We tested the ability of four different glucocorticoid assays to detect an increase in the gorillas' FGCM output in response to potential stressors: cortisol (CORT) (Palme & Möstl 1997), corticosterone (CCST) (Heistermann, Palme & Ganswindt 2006) and two group-specific enzyme-immunoassays against 5-reduced cortisol metabolites with a $3\alpha,11\text{ß-hydroxy}$ ($3\alpha,11$ß-dihydroxy-CM (Ganswindt et $al.$ 2003) and $3\alpha,11$oxo structure ($3\alpha,11$oxo-CM) (Möstl & Palme 2002).

Fecal samples were shipped frozen to the endocrine laboratory of the German Primate Center where we processed and extracted them as described by Heistermann et $al.$ (1995). Briefly, we lyophilized and pulverized the feces and extracted an aliquot representing 0.05-0.08 g of fecal powder with 3 ml 80% methanol in water by vortexing for 10 minutes. Following centrifugation of the fecal suspension, we recovered the supernatant and stored it at –20°C until analysis. We analyzed fecal extracts for FGCM immune-reactivity using the four different EIA

systems described by Heistermann *et al.* (2004, 2006). Intra- and inter-assay coefficients of variation (CVs) of high- and low-value quality controls were <10% and <13%, respectively, for all four assays.

We performed reverse-phase high performance liquid chromatography (HPLC) to assess the pattern of metabolites measured and to characterize the specificity of the four GC assays tested. We chose a fecal extract from a male with a peak in FGCM output in response to stress of relocation and carried out HPLC using the procedure described by Möhle *et al.* (2002) and Heistermann *et al.* (2006). HPLC also allowed us to evaluate whether the FGCM antibodies co-measured fecal androgens which can also be detected by antibodies raised against cortisol metabolites (see Möstl & Palme 2002; Ganswindt *et al.* 2003; Heistermann, Palme & Ganswindt 2006). We measured each HPLC fraction in all four FGCM assays to generate profiles of immunoreactivity.

Experiment 1: Testing a field-friendly method for hormone extraction, and long-term preservation of fecal extracts

We compared two extraction methods using samples (n=29) collected for the FGCM assay validation tests from the two males who underwent anesthesia for medical examinations. We asked keepers to homogenize each sample well and to split it into two before freezing at -20°C. One set of samples was shipped frozen to the endocrine laboratory for processing using the laboratory extraction procedure described above ("laboratory extraction"). We incubated the other set in an oven at 40°C for 30 minutes until thawed, then mixed it again thoroughly using a spatula. We then weighed 0.5 g of wet feces into a 15 ml polypropylene tube (PPT) containing 5

ml 90% ethanol and carried out a field-friendly extraction method ("field extraction") based on the procedure described by Ziegler and Wittwer (2005). We shook the fecal-ethanolic suspension horizontally by hand for 5 minutes and allowed the fecal sedimentation to settle for 30-40 minutes standing on a bench. Following the separation process, we pipetted 1 ml of each extract into a 2 ml PPT and stored it at -20°C until shipment to the endocrine laboratory for GC analysis. We established FGCM content for extracts generated by both methods using the two group-specific GC assays, as they were deemed the most suitable for monitoring FGCM output during validation testing (see Results).

To test preservation of GCs in fecal extracts under different conditions for periods of between one month and one year we extracted 0.5 g of wet feces (n=12 samples, 6 for each sex) using the "field extraction method" (but using a centrifuge to separate the liquid from the faeces). We then divided the fecal extracts into 5 aliquots. We stored three 0.65 ml aliquots as liquids and dried two 0.2 ml aliquots overnight at 50°C. We subjected the aliquots to the following 5 conditions: i) storage as liquid in PPTs (Sarstedt 2.0ml safe-seal micro tube neutral flip cap Poly Propylene) at room temperature (RT, 21-23°C); ii) storage as liquid in a glass tube (75x12 mm) at RT; iii) storage dried in PPT at RT; iv) storage dried in a glass tube at RT and v) storage as liquid in a PPT at -20°C. We closed the glass tubes with fitted caps and wrapped all tubes (glass and PPT) with parafilm to minimize risk of evaporation. We determined FGCM levels for each sample immediately after extraction ("controls") and repeatedly after 1, 3, 6, 9 and 12 months of storage for each condition to assess any potential storage type- and time-dependent effect on FGCM stability. We determined FGCM content using the two group-specific assays. For this experiment, inter-assay CVs of high- and low-value quality controls

determined over the 12 months of analysis were 7.4% (high) and 14.2% (low) for the 3a,11ß-dihydroxy-CM assay and 12.7% (high) and 11.5% (low) for the 3a,11oxo-CM assay.

Experiment 2 - Testing short-term storage of feces in alcohol under field conditions

We collected large fecal samples (n=10; 8 animals) directly after defecation and homogenized them well. We split the feces into 15 portions of ~0.5 g and placed them in 4 ml 90% ethanol within 5 hours of collection. We extracted one portion immediately as described above ("day 0") and extracted the remaining 14 aliquots every other day until day 28. Following each extraction, we pipetted 0.5 ml of supernatant into 2 ml PPTs and evaporated the liquid by putting the tube(s) into a transparent fish-steamer placed in a light-reflective basin in the sun (an additional drying-down step based on the ideas of Terrio *et al.* [2002] and Galama *et al.* [2004]). Sample extracts dried within a range of 1-3 days depending on the amount of direct sunlight. We kept dried samples at ambient temperatures in the dark until shipment to the endocrine laboratory where they were kept at -20°C until analysis.

For analysis, we reconstituted samples in 0.5 ml 80% ethanol in water by sonication in a water bath for 5 minutes, followed by 30 seconds vortexing. We analyzed reconstituted extracts for FGCM levels using the 3a,11ß-dihydroxy assay. Inter-assay CVs for these measurements were 6.8% (high value quality control) and 13.2% (low value quality control).

Experiment 3 - Testing post-defecation FGCM change in un-preserved feces under field conditions

To test whether FGCM levels in feces change as a function of the time between defecation and sample preservation in alcohol, we collected 10 large fresh fecal samples (from 7 animals) immediately after defecation and homogenized them well. We placed ~0.5 g of each sample in a tube with 4 ml 90% ethanol whilst still in the forest to act as the time-0 sample. We left remaining feces at ambient temperature (on the ground exposed to air but no samples were subjected to rain) and removed and preserved an aliquot (~0.5g) in alcohol every two hours until 12 hours after defecation. Thereafter, we extracted each sample and dried down 0.5 mls from each sample in 2 ml PPTs as described above. We kept dried extracts at ambient temperatures in the dark until shipment to the endocrine laboratory for FGCM analysis using the 3a,11ß-dihydroxy assay. Inter-assay CVs for these measurements were 6.8% (high value quality control) and 13.2% (low value quality control).

Experiment 4 - Testing for diurnal effects on FGCM levels in feces

To test for a potential diurnal effect in excretion of FGCMs, we collected 15 pairs of morning (07:00 - 09:35) and afternoon (13:45 – 16:05) samples from eight gorillas. We put ~0.5g wet feces of each sample into 4 ml 90% ethanol, and subjected each sample to the field extraction procedure described above within 24 hours of collection. We kept dried extracts at ambient temperatures in the dark until shipment to the endocrine laboratory for FGCM analysis using the 3a,11ß-dihydroxy assay. Assay CVs for these measurements were <5% for both high and low value quality controls.

Data analysis

All hormone data are given as mass hormone per mass fecal wet weight, except for the validation tests, where lyophilized samples were used for extraction. FGCM levels in samples where urine contamination may have occurred did not differ obviously from adjacent uncontaminated samples so we used all samples in the analysis. To evaluate the correspondence between FGCM levels in extracts generated by the laboratory and field extraction method, we calculated the Spearman rank correlation coefficients for the set of samples tested.

For Experiment 1, we calculated the percentage change in FGCM levels for each of the five storage conditions and each storage duration (1, 3, 6, 9, and 12 months) as $(a_n-x_n)/x_n*100$, where a_n is the nth sample value in each condition/duration and x_n is the value at time point 0 of the nth sample. To analyze the overall main effects and possible interaction of time and condition on FGCM changes we first fit a 'General Linear Mixed Model for Repeated Measures' (GLMM-RM), with the best fit based on AIC Selection Criteria (ASC). The final model used unstructured variance and log-transformed FGCM values, as variance in the original values was high and not-normally distributed. We treated the repeated measure variable of "Time" as categorical, assuming variance from day 0. Following this we ran pair-wise comparisons on each condition separately using the fitted GLMM-RM model with post-hoc comparisons using Bonferroni tests. We again treated the variable "Time" as categorical assuming variance from 0 to locate where FGCM

levels significantly differed from day 0 values. We also carried out Spearman rank correlation tests to investigate whether FGCM concentrations in extracts stored for 12 months (the maximum period of storage) in the various conditions correlated with the control values measured directly after extraction. Finally, we calculated the coefficient of variation (CVs) for each of the 12 samples across the six measurements conducted over the whole experimental period for each condition and took the mean to assess how variation in sample values varied as a function of storage duration (which includes inter-assay variation) compared with inter-assay variation for our quality controls.

For Experiments 2 and 3, we calculated the percentage change in FGCM levels relative to time 0 values or each of the extraction time points within each sample set as described above. We analyzed changes in FGCM levels as a function of short-term storage in alcohol (Experiment 2) or lag time between defecation and preservation of the sample (Experiment 3) using Friedman Repeated Measure ANOVA on ranks with post-hoc analysis using the Wilcoxon signed rank test where applicable. We also calculated Spearman rank correlation coefficients to determine whether FGCM values in feces stored in alcohol for 28 days (the maximum storage duration tested; Experiment 2) and samples left for 12 hours at ambient temperature before preserved in alcohol (the maximum delay to preservation tested, experiment 3) correlated with the control values irrespective of possible change in absolute hormone levels. In Experiment 3, we eliminated two outlier values (≥3.5 standard deviations above the mean of all other samples) from the dataset before analysis. We tested for a potential time-of-day effect on FGCM levels by comparing levels in the paired morning and afternoon samples using a paired t-test. All statistical tests were two-tailed and we considered results significant when p≤0.05.

RESULTS

Validation of FGCM measurements

The highest levels of fecal FGCMs were measured by the two group-specific cortisol metabolite assays (peak value range: 0.90-3.95 µg/g; Table 1) with those measured via the CORT and CCST assays being generally much lower (peak value range: 0.02-0.25 µg/g; Table 1). In all six cases, animals responded to the potential stressful event (medical examination/transport/social stressor) with an increase in FGCM levels (Table 1). However, the magnitude of response differed clearly across the four assays. Whilst the CORT assay showed no clear response in most cases and the CCST assay showed only a moderate response overall (Fig. 1), both group-specific assays showed a marked FGCM elevation in five of the six cases. In all six cases, the 3α,11ß-dihydroxy-CM assay showed a stronger response to the stressor than the 3α,11oxo-CM assay. The timing of FGCM peak elevation varied between cases and assays, but was more consistent for the two group-specific assays than for the more specific ones (Table 1). Peak response was usually detected between 43 and 68 hours after the stressor and FGCM levels had usually returned to pre-stress baseline levels by day 5 (Fig. 1).
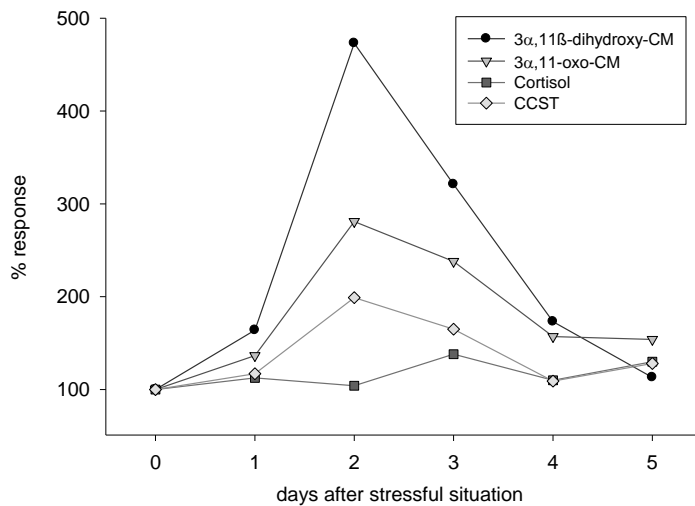
Figure. 1: Percentage response in immunoreactive fecal FGCM levels over baseline values in response to a stressful situation in lowland gorillas. Points represent median values calculated for 24-h intervals across 6 cases. Time 0 = onset of potentially stressful situation.

HPLC analysis for the two group-specific assays indicated that approximately 90% of immunoreactivity was detected as several distinct peaks between fractions 9 and 31 - positions where cortisol metabolites elute in our HPLC system. The low immunoreactivity after fraction 31, where potentially cross-reacting androgen metabolites elute (positions where certain possible cross-reacting androgen metabolites elute; Ganswindt *et al.* 2003; Heistermann, Palme & Ganswindt 2006a), suggests a low degree of co-measurement of these androgens in the two assays (Fig. 2). Moreover, the presence of the highest peaks of immunoreactivity at the elution positions for 11ß-hydroxyetiocholanolone (fractions 24/25) and 11oxo-etiocholanolone (fractions 29/30) in the respective assays indicated that these two cortisol metabolites were abundant in lowland gorilla feces. By contrast, and as

expected based on the validation results, HPLC indicated only low levels of immunoreactivity measured by the CORT and CCST assays (data not shown).
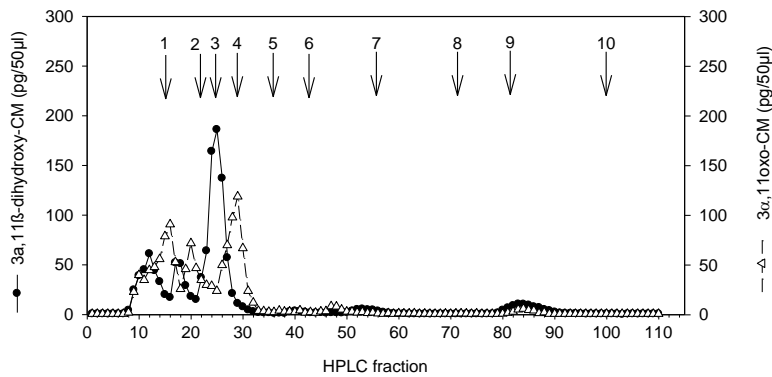

Fig. 2: HPLC immunoreactivity profiles detected using the 3a,11ß-dihydroxy-CM and 3a,11oxo-CM EIA in a peak sample of adrenocortical response to sedation in a male lowland gorilla. Arrows indicate elution positions of reference standards: 1) cortisol (fraction 14/15), 2) corticosterone (22), 3) 11ß-hydroxyetiocholanolone (24/25), 4) 11-oxoetiocholanolone (29/30), 5) 5ß-androstane-3,11,17-trione (36), 6) testosterone (42/43), 7) androstendione, dehydoepiandrosterone (55), 8) epiandrosterone, 5ß-DHT, 5ß-androstane-3ß-ol-17-one (72), 9) 5ß-androstane, 3α-ol-17-one (82/83), and 10) androsterone (100)

Experiment 1 - Testing a field-friendly method for hormone extraction, and long-term preservation of fecal extracts

Across all samples FGCM measurements from extracts generated using the "field extraction" method correlated strongly with those generated from extractions derived from

our established laboratory procedure (3α,11ß-dihydroxy-CM: r=0.79, p<0.001; 3α,11oxo-CM: r=0.80 p<0.01; n=29; Fig. 3).
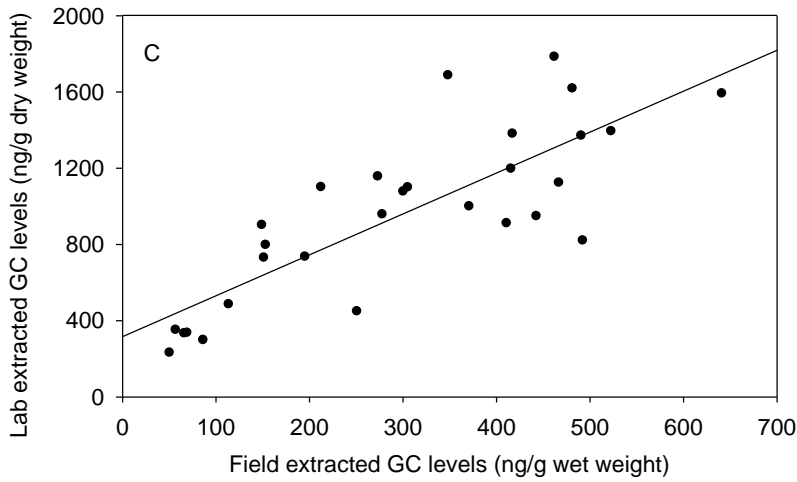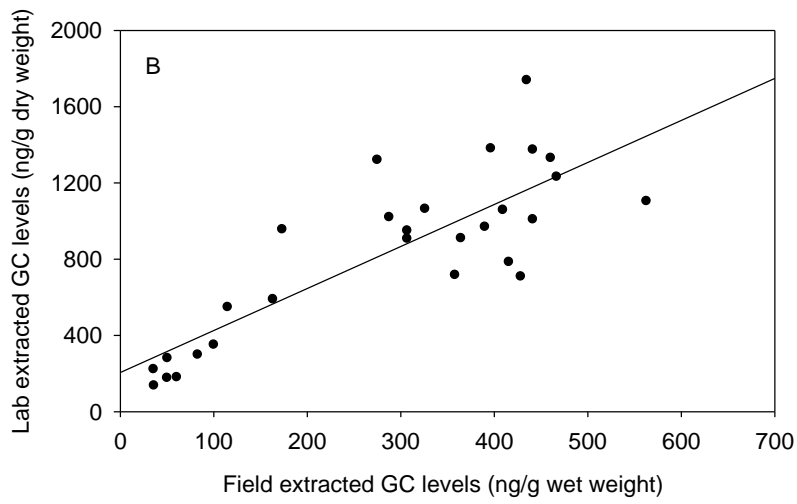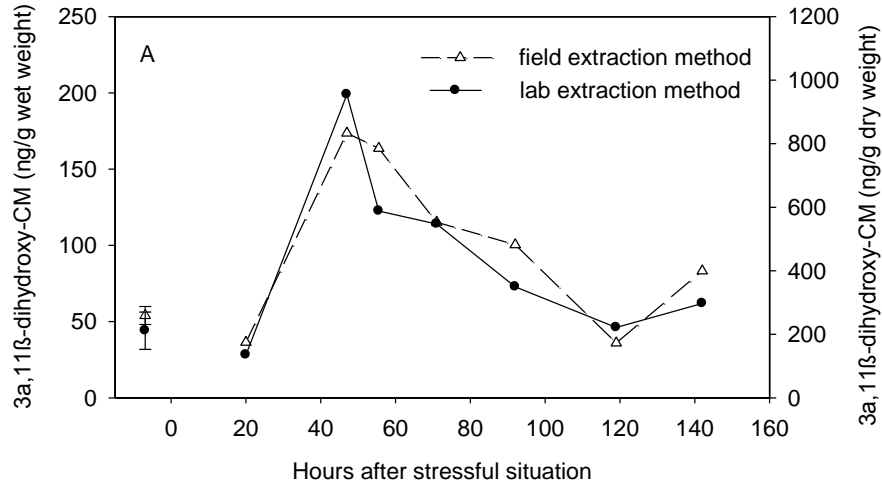
Fig. 3: Example of (A) 3α,11ß-dihydroxy-CM response profile to a stressful situation in a male lowland gorilla as determined from fecal extracts generated using the standard laboratory procedure and our simple field extraction method. Scatter plots show the correlations between FGCM levels generated by the two extraction methods for (B) 3α,11ß-dihydroxy-CM and (C) 3α,11oxo-CM (n=29 samples).

Our storage experiment revealed that for each storage condition, FGCM levels measured in the two group-specific assays stayed relatively stable over the 12 months of storage. as indicated by the findings that i) mean changes in FGCM concentrations at each condition and storage duration did not exceed ±20% of the controls (Fig. 4) and ii) the relative rank orders of samples during the experimental period remained very stable (Fig. 5).
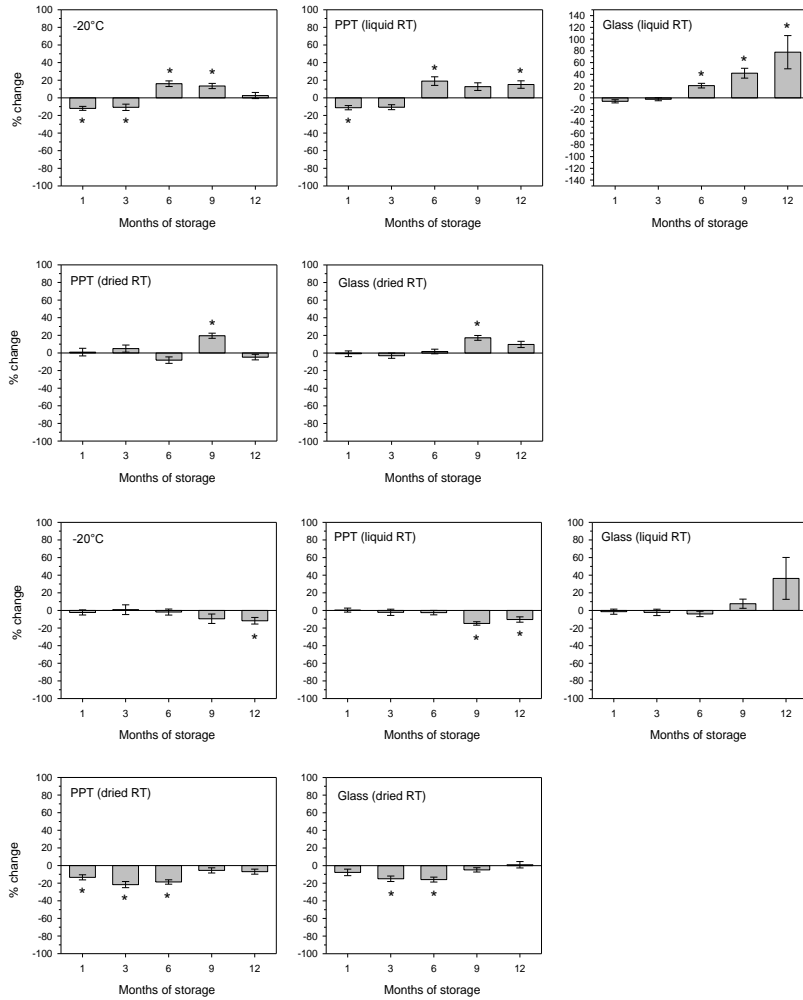
Fig. 4: Percentage change in 3α,11ß-dihydroxy-CM (top five graphs) and 3α,11oxo-CM (bottom five graphs) levels in relation to samples analyzed immediately (time 0) for fecal extracts stored under various conditions for 1-12 months (* = indicates where p<0.05 compared to time 0 in the GLMM-RM model for each condition).
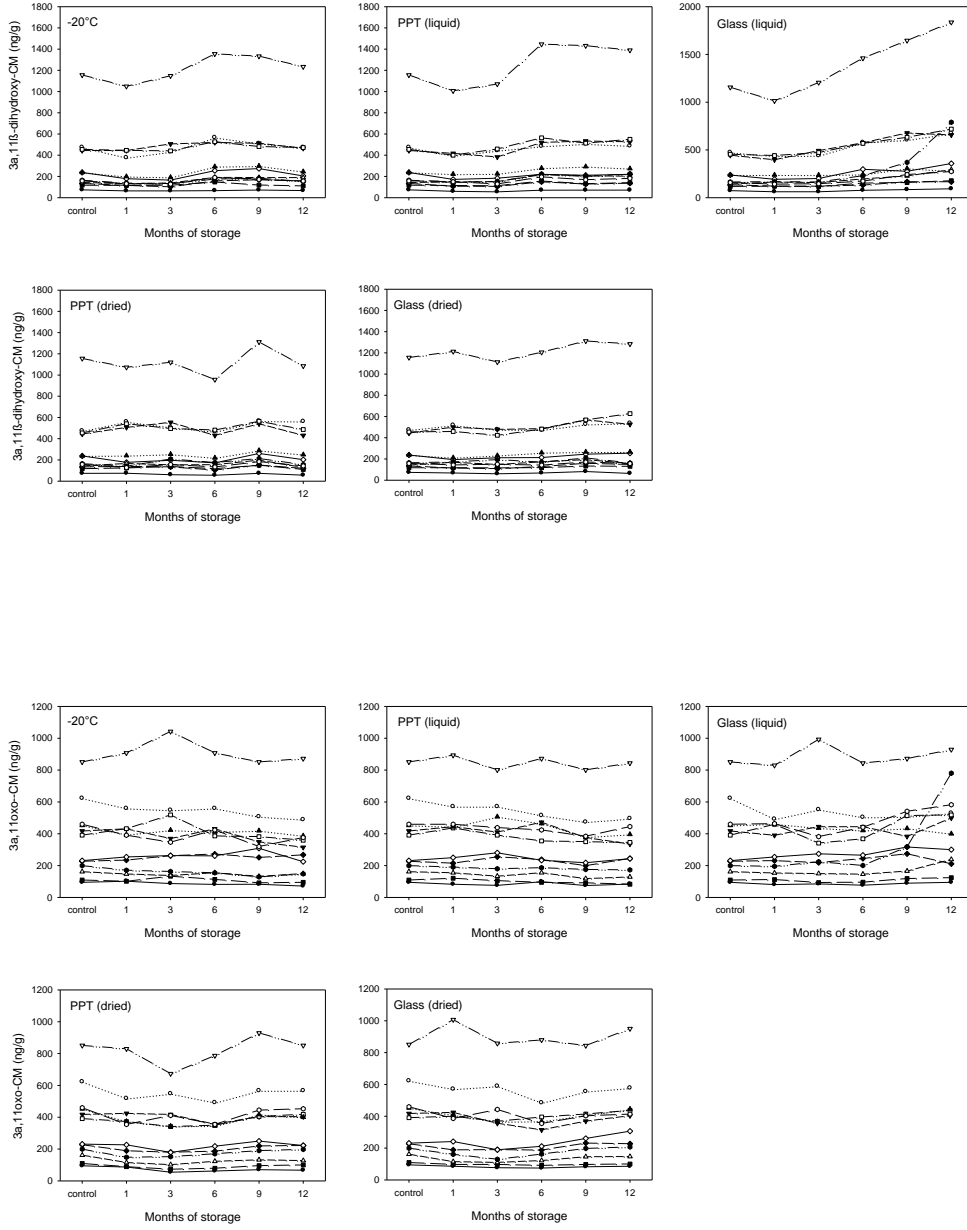
Fig. 5: 3α,11ß-dihydroxy-CM (top) and 3α,11oxo-CM (bottom) levels in individual fecal extracts (n=12) stored under various conditions for 1-12 months compared to control values analysed immediately after extraction (time 0).

Storage condition did not have an overall significant effect on FGCM values as measured by both assays (3α,11ß-dihydroxy-CM: $F_{(3, 44.0)} = 0.012$, $p = 0.998$; 3α,11oxo-CM: $F_{(3, 44.0)} = 0.027$, $p = 0.994$), but time did have a significant effect (3α,11ß-dihydroxy-CM: $F_{(5, 44.0)} = 37.290$, $p<0.001$; 3α,11oxo-CM: $F_{(5, 44.0)} = 11.462$, $p<0.001$). This resulted in an overall significant interaction effect of time and storage condition on FGCM values for both assay measures (3α,11ß-dihydroxy-CM: $F_{(15, 44.0)} = 17.619$, $p<0.001$; 3α,11oxo-CM: $F_{(15, 44.0)} = 13.244$, $p<0.001$). Time also had an overall significant effect in each condition (3α,11ß-dihydroxy-CM: liquid -20C: F=15.05; liquid PPT RT: F=59.43; dried PPT RT: F=100.70; dried glass RT: F = 31.06; all at 5,11.0, $p<0.001$; 3α,11oxo-CM: liquid -20C: F=6.45; liquid PPT RT: F=29.63; dried PPT RT: F=62.66; dried glass RT: F = 20.58; all at df 5,11.0, $p<0.005$). However, post-hoc analysis showed that there was no consistent pattern of significant FGCM level changes across conditions or in relation to the duration of storage (Fig 4), i.e., within condition 4 'PPT Dried RT' as measured by the 3a, 11ß-dihydroxy-CM assay, values for month of storage 3 is higher than for month 6 which is lower than month 9. There was one exception here: the "Liquid Glass RT" condition showed a more linear increase and higher variation from controls in both assay measures in months 9 and 12 (Fig 4). Visual inspection of these samples suggested that this change was most likely due to increased evaporation in at least two samples. Although the correlations between extracts stored in this condition for 12 months were still highly correlated with control values (3α,11ß-dihydroxy-CM: $r_s=0.84$, $p<0.0005$; 3α,11oxo-CM: $r_s=0.76$, $p<0.004$), we removed this condition from the analysis as it no longer reliably reflected true changes

in FGCM levels. We report all results accordingly. Correlations for all other storage conditions between 12 months and 0 control values were much stronger (r_s ≥0.94, p<0.0001 for both assays).

Mean CV values across the 6 measurements of each sample over the 12 months of analysis ranged 9.6-25.2% for 3α,11ß-dihydroxy-CM and 9.5-16.4% for 3α,11oxo-CM. For both FGCM measures the highest CV values were for the removed condition "Glass liquid RT". All others were <14% and thus well within the range of our inter-assay variation.

Table 2: Mean 3α,11ß-dihydroxy-CM levels (ng/g) in fecal extracts (n=12) stored under various conditions for 1-12 months compared to values immediately after extraction.

Storage condition	1 mo	3 mo	6 mo	9 mo	12 mo
Immediate analysis 316.4					
-20°C	282.4	297.2	370.3	359.5	327.5
PPT (liquid)	278.6	289.2	377.0	366.5	367.2
Glass (liquid)	291.2	318.0	390.3	444.1	526.4
PPT (dried)	323.4	295.6	289.3	375.3	311.2
Glass (dried)	322.3	331.6	326.6	369.6	359.6

[a] for details of conditions see Methods

Table 3: Mean 3α,11oxo-CM levels (ng/g) in fecal extracts (n=12) stored under various conditions for 1-12 months compared to values immediately after extraction

Storage condition	1 mo	3 mo	6 mo	9 mo	12 mo
Immediate Analysis 351.4					
-20°C	343.7	357.7	346.4	318.5	312.7
PPT (liquid)	355.1	345.7	342.3	303.5	318.2
Glass (liquid)	343.6	349.3	337.5	377.7	433.9
PPT (dried)	310.8	289.6	293.7	343.7	335.8
Glass (dried)	340.4	314.5	303.9	334.2	358.4

[a] for details of conditions see Methods

Experiment 2 - Testing short term storage of feces in alcohol under field conditions

Mean FGCM levels in feces stored for 2-28 days in ethanol before extraction were usually slightly (but non-significantly) higher than control values extracted at time 0, with a mean increase of 8.2% (range: -6.2-20.8%) within the 4 week experimental period (x^2=22.407, p=0.071). FGCM levels measured after 28 days of storage, however, showed a strong and significant correlation with the values immediately after extraction (r_s=0.83, p<0.001, see Fig. 6).
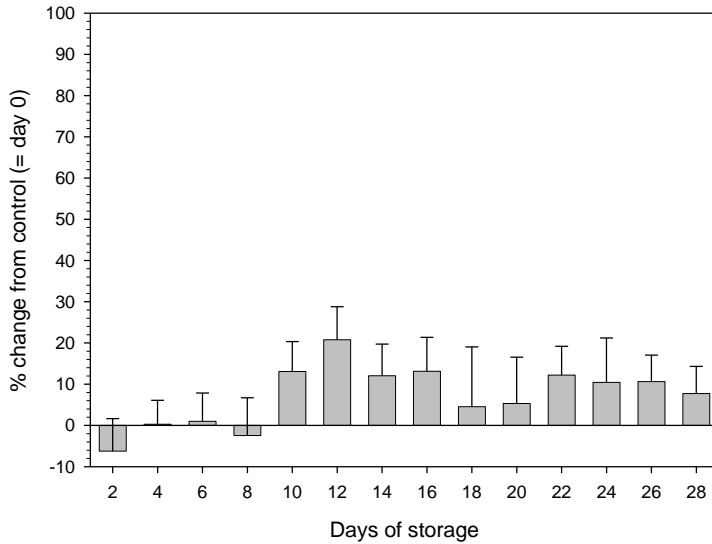
Fig. 6: Percent change relative to time-0 values in 3α,11ß-dihydroxy-CM concentrations of fecal samples stored for up to 28 days in 90% ethanol. Values represent mean +SEM (n=9).

Experiment 3 - Testing post-defecation FGCM change in un-preserved feces under field conditions

FGCM levels in feces stored at ambient temperature for up to 12 hours before preservation in alcohol showed a significant and strong decline over time (χ^2=24.376, p=0.001; Fig. 7). On average, concentrations decreased by about 17% within the first two hours and declined progressively further to reach a plateau at approximately 50-55% of their original concentration by 8 hours (Fig. 7) after which levels remained stable. Post-hoc analysis revealed that FGCM concentrations in samples stored for 6 hours or more before

preservation were significantly lower than those measured in samples preserved directly after defecation (all $p < 0.05$). Despite this change in absolute concentrations, FGCM levels measured in samples that spent 12 hours at ambient temperature correlated strongly and significantly with the time-0 values ($r_s = 0.77$, $p < 0.01$).
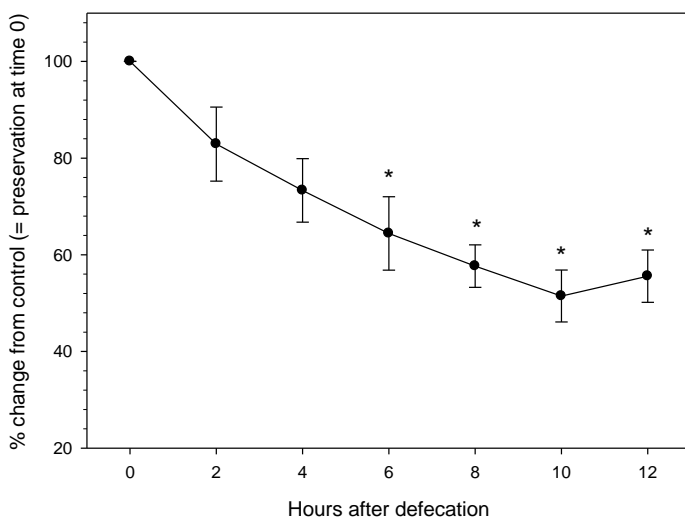

Fig. 7: Percentage change of 3α,11ß-dihydroxy-CM concentrations of fecal samples from values preserved and extracted immediately, in those stored for up to 12 hours at ambient temperature before being preserved in 90% ethanol. Values represent mean +SEM (n=10) relative to time 0. *p<0.05 compared to time 0-values.

Experiment 4 - Testing for diurnal effects on FGCM levels in feces

Comparison of FGCM concentrations in samples collected in the morning hours versus afternoon hours indicated no statistical difference (morning samples: mean ± SD: 114.1±52.0 ng/g; afternoon samples: 121.5±67.9 ng/g; N=15, t=-0.625; P=0.542).

DISCUSSION

We demonstrated the validity of a group-specific EIA for 5-reduced cortisol metabolites with a 3α,11ß-dihydroxy- and 3α-11oxo structure for monitoring the physiological stress response from feces in the western lowland gorilla, and established methods to extract and preserve FGCM concentrations long-term under tropical field conditions where freezing is not possible. We also showed that samples stored in 90% ethanol can be stored up to 28 days prior to extraction in tropical conditions without altering FGCM concentrations. In addition, we demonstrated that FGCM concentrations decrease almost linearly over the course of 12 hours when feces are not preserved immediately, and that FGCM concentrations do not show diurnal variation in wild western lowland gorillas. Our study therefore provides important new information for field researchers interested in using fecal hormone analysis techniques to monitor endocrine status in their study species.

EIA Validation of FGCM measurements

The two group-specific assays measuring major cortisol metabolites detected the predicted FGCM response to stressors reliably, whereas the two more specific cortisol and corticosterone assays were distinctly less responsive. The characteristics in terms of magnitude of response and time course detected by the two group-specific measurements were within the range of those reported in other studies on primates (Whitten *et al.* 1998;

Heistermann, Palme & Ganswindt 2006) and non-primate species (Wasser *et al.* 2000;

Young *et al.* 2004) and indicate that peak FGCM output responses in the lowland gorilla

can generally be predicted 2-3 days after exposure to a stressor. Our finding that the group-

specific cortisol metabolite assays were superior to the two more specific ones is in line with

findings from many other studies comparing the suitability of diverse fecal FGCM assays in

reflecting the stress response (Palme & Mostl 1997; Ganswindt *et al.* 2003; Palme 2005;

Heistermann, Palme & Ganswindt 2006; Fichtel *et al.* 2007; Pirovino *et al.* 2011; Weingrill *et

al.* 2011). Of the two cortisol metabolite assays, the $3\alpha,11\text{ß-dihydroxy-CM}$ assay appears to

have a higher biological sensitivity compared to the $3\alpha,11\text{oxo-CM}$ assay, showing a

stronger response to the stressor in all animals. As such we recommend using the $3\alpha,11\text{ß-}$

dihydroxy-CM assay to assess FGCM output in the gorilla.

**Experiment 1 - Testing a field-friendly method for hormone extraction, and long-term
preservation of fecal extracts**

Our simple extraction technique using hand-shaking samples in 90% ethanol recovered

FGCMs from feces reliably, providing information on relative FGCM level changes similar to

that generated with established laboratory methods. Having a validated option to extract

feces in the field is increasingly important as it removes the risk of unknown alterations in

hormone concentrations when feces are stored in alcohol for prolonged periods of time

(Khan *et al.* 2002; Hunt & Wasser 2003; Daspre *et al.* 2009), and also allows the researcher

to collect fecal samples quickly whilst following wild animals without the need for lengthy or

complicated treatments. The best solvent for extracting hormones from feces may be

species- and hormone-specific (Palme & Möstl 1997; Pappano, Roberts & Beehner 2010),

but several studies have reported high extraction efficiency for steroids using ethanol at 80-

100% (Palme & Möstl 1997; Mateo & Cavigelli 2005; Freeman *et al.* 2010; Santymire & Armstrong 2010). The strong correlation between field- and laboratory-extracted FGCM values in our study supports this contention indirectly, and we recommend ethanol for extraction of feces in the field as it is often readily available in primate habitat countries. Our data also suggest that simple hand-shaking of samples for a constant amount of time, as suggested by Ziegler and Wittwer (2005), is sufficient to obtain reliable results, although use of a battery-powered homogenizer might increase hormone extraction efficiency slightly (Santymire & Armstrong 2010) especially if feces are hard in texture.

As it is uncommon to have the necessary laboratory facilities available to analyse sample-extracts in the field, researchers need simple but appropriate methods to preserve them long-term under field conditions. Our storage experiments showed that FGCMs (measured by either of the two group-specific assays) are generally stable over 12 months (the longest period tested) when stored in liquid or dried forms at ambient temperatures, and that storage at -20°C did not appear to be better than storage at higher temperatures. Although post-hoc pair-wise comparisons revealed a number of significant differences in hormone concentrations at different months when compared to time-0 values within conditions, there were no linear or otherwise predictable patterns over time within any of the conditions. Furthermore, the percentage change in hormone values between time periods were always ≤ 20%, the relative rank orders of samples remained stable over time and FGCM values after 12 months of storage strongly correlated with those at time 0. As the variation in repeated sample measurements across the 12 months was within the range of inter-assay variability, we believe that any significant differences within conditions are likely to be an artefact of assay variation and do not reflect true changes in FGCM concentrations. Such changes to FGCM levels in feces can occur, due to, for instance, activity of extracted bacterial enzymes or chemically-induced changes in metabolite

structure (e.g., due to oxidation processes). However, such effects would normally result in a more directional change of hormone levels similar to that found when fecal material is stored long-term in alcohol (Khan *et al.* 2002; Hunt & Wasser 2003; Daspre *et al.* 2009) and may also be temperature-dependent, being more pronounced in samples stored at higher temperatures when compared to frozen samples (Khan *et al.* 2002; Hunt & Wasser 2003). Our results did not support either prediction, however.

Our finding that keeping extracts frozen is not better than storing them at ambient temperatures is remarkable and contrasts to current thinking that freezing is the 'golden standard' for steroid preservation (Touma & Palme 2005; Ziegler & Wittwer 2005; Hodges & Heistermann, 2011). This may be extremely useful for field researchers working in remote conditions without a freezer.

> **Comment [KS1]:** Kat NB put in ref

Additionally, we show that FGCM levels in dried extracts stored in simple polypropylene (PPT) tubes were no different from those stored in glass tubes, a material generally considered more inert and therefore better suited for keeping steroids long-term. This finding is also very valuable for field researchers as plastic is lighter than glass and less prone to breakage, providing a better option for shipment of samples from field sites to laboratories. Use of dried extracts in PPT was also shown to stabilize FGCMs from African wild dog feces for up to six months (Santymire & Armstrong 2010). To our knowledge however, our study is the first to validate the use of dried fecal extracts for one year; again highly valuable for researchers in remote places wishing to include yearly seasonality effects in studies of FGCMs from animal species as it overcomes the need to analyse samples during fieldwork. Furthermore, we are confident that dried sample extracts can be stored beyond 12 months without causing significant changes to FGCM content as FGCM levels in liquid extracts re-analysed after 18 months of storage at ambient temperatures remained equally stable (data not shown).

Whilst our storage experiments show that hormones are stable in both liquid and dried forms even at ambient temperatures, drying the ethanol extract in the field may be preferable for several reasons. First, drying likely inactivates any bacterial enzyme activity (for which the presence of water is essential), thus preventing the risk of biologically induced alterations (e.g. deconjugation) that may change hormonal structures. Second, drying removes the possibility of evaporation and subsequently falsely inflated hormone values over time, which, as our results suggest, is a real risk when alcohol is stored in liquid form long-term. Third, using dried samples removes potential problems associated with transportation/exportation of alcoholic solutions from field sites. Drying alcoholic extracts should be easily possible under most field conditions, particularly as only a small volume of extract (e.g. 0.5 ml or less) is needed for hormone analysis. However, when researchers prefer storing extracts in liquid form, we would recommend to bring samples to a fridge or freezer periodically (if this is possible) in order to reduce the risk of solvent evaporation and a resultant change in hormone levels.

Using field extraction by hand-shaking in combination with drying small volumes of extract in plastic tubes which can be stored long-term at ambient temperatures therefore offers a simple and reliable method for preserving hormones under remote or tropical conditions. We envisage that this method applies not only to fecal FGCM levels in the gorillas tested here, but also to studies of other hormones and animal species (see for instance Santymire & Armstrong, 2010).

Experiment 2 - Testing short term storage of feces in alcohol under field conditions

We found that hormone values in samples stored for up to 28 days in alcohol did not differ from values of immediately extracted samples. This finding is similar to that reported for

baboons (*Papio cynocephalus*) (Khan *et al.* 2002; Lynch *et al.* 2003) and grizzly bears (Hunt and Wasser, 2003) but different from the results of a storage experiment conducted with elephant feces in which FGCM levels rose after two weeks of storage (Hunt and Wasser 2003). Although we did observe some variation in mean FGCM levels between days within the month, there was no predictable trend for increasing/decreasing FGCM content over time. The small changes observed may again be due to assay variation, or to the uneven spread of metabolites in feces (Wasser *et al.* 1996), as although we homogenized the sample well, gorilla feces are very large and can be hard, making them difficult to mix. Our findings mean that immediate fecal extraction after collection is not necessary for obtaining reliable results for gorilla FGCM levels. This is a particularly valuable implication as field conditions often prevent immediate or regular processing of samples after defecation.

Experiment 3 - Testing post-defecation FGCM change in un-preserved feces under field conditions

The results of our hormone change experiment show an almost linear pattern of hormone degradation over 12 hours, with the most pronounced decay between zero and four hours, and levels stabilizing at around 50% of the original concentration from 6 hours onwards. To date, few studies have investigated hormone change in feces between defecation and fixation, but studies of cattle, horses and pigs (Mostl *et al.* 1999) and orangutans (*Pongo pygmaeus morio*) (Muehlenbein *et al.* 2012) also found a significant change in FGCM levels within a few hours when samples were stored unpreserved at ambient temperature. In these studies however, FGCM concentrations increased rather than decreased as we report here for gorilla feces. However a decrease in concentrations was also found in brown hyaena (*Hyaena brunnea*, Hulsman *et al.* 2011*)*. Differences in

experimental treatments (e.g. samples stored in plastic tubes vs. samples left exposed to real environmental conditions) may partly account for the different effects seen, although chemical alteration (e.g. oxidation or deconjugation) of the metabolites due to species-specific gut flora activity, which would be more immunoreactive in farm animals and orangutans and less in the gorilla or hyaena, is a more likely explanation for the differences in findings (Mostl *et al.* 1999; Washburn & Millspaugh 2002). These results highlight the importance of controlling for possible sources of exogenous FGCM change and show that fecal samples should be preserved as soon as possible after defecation to minimize the risk of sample degradation. For gorilla studies, our data specifically imply that FGCM concentrations in samples collected from nests, where exact defecation time is usually unknown, are likely to be underestimated. If, however, information on the time between defecation and collection can be obtained, our finding of an almost linear degradation pattern would allow a corrective factor to be applied to estimate more realistic final hormone values.

Experiment 4 - Testing for diurnal effects on FGCM levels in feces

We found no differences in mean FGCM levels between morning and afternoon samples. This finding is to be expected for the gorilla which has relatively consistent feeding patterns (and therefore likely FGCM excretion rate) throughout the day interrupted by regular periods of rest in the wild (KS pers. obs). Our data are consistent with those from other studies of larger-bodied mammals (Wasser *et al.* 1993; Schwarzenberger *et al.* 1996; Ostner, Kappeler & Heistermann 2008) where gut passage-time is comparatively long as for the gorilla. In contrast, diurnal differences in FGCM levels are often seen in smaller-bodied species (Sousa & Ziegler 1998; Beehner & Whitten 2004; Sheriff *et al.* 2009; Kalliokoski *et al.* 2012) and, more generally, in animal urine (e.g. Robbins & Czekala 1997;

Muller & Lipson 2003) due to faster and more frequent excretion rates. Given that the time
of day did not affect fecal FGCM levels in our study of lowland gorillas, we suggest that
fecal samples for FGCM analysis do not need to be collected during a specific time-window,
but can be collected throughout the day. This is of high practical value as it potentially
allows researchers to follow more animals and gather larger sample sets within restricted
periods of time.

CONCLUSIONS

Overall, our results further support the use of FGCMs for long-term studies of the correlates
of FGCMs in animals as they reflect an integration of the hormone over a longer period
rather than the shorter-term fluctuations found in serum and urine. Here, we validated a
system to monitor FGCMs in the critically endangered western lowland gorilla species. The
ability to non-invasively monitor adrenocortical activity in gorillas is of major value in captive
animal breeding and welfare-management strategies. Researchers studying wild
populations can also use this tool to monitor natural and human-derived effects on FGCMs
which may affect health and reproduction. Methods for non-invasive fecal hormonal
monitoring are generally not field-friendly and ways to adapt and validate them to the field
need testing for each animal species. Here we provide general methods by which fecal-
hormonal monitoring can be applied to a variety of field conservation contexts and wild
animal species where arguably, it may be most crucially needed.

ACKNOWLEDGEMENTS

This research was conducted with permission and in accordance with the Zoos' research protocols and adhered to the legal requirements of the UK. We adhered to the research protocols defined by the Dzanga-Sangha Protected Areas Direction and all research was approved by the Durham University Life Sciences Ethical Review Process Committee.

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