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

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A B S T R A C T

Enzymeimmunoassays (EIAs) allow researchers to monitor stress hormone output via measurement of fecal glucocorticoid metabolites (FGCMs) in many vertebrates. They can be powerful tools which allow the acquisition of otherwise unobtainable physiological information from both captive animals and 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 field settings. 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 established a simple, non-freezer-reliant method to preserve FGCMs in extracts long-term. We determined the rate of FGCM change over 28 days when samples cannot be extracted immediately and over 12 h when feces cannot be preserved immediately in alcohol. Finally, we used repeat samples from identified individuals to test for diurnal variation in FGCM output. Two group-specific assays measuring major cortisol metabolites detected the predicted FGCM response to the stressor reliably, whereas more specific cortisol and corticosterone assays were distinctly less responsive and thus less useful. We detected a lag time of 2–3 days from stressor to peak FGCM excretion. Our field extraction method performed as well as an established laboratory extraction method and FGCMs in dried extracts stored at ambient temperatures were as stable as those at −20 °C over 1 yr. 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 h under field conditions. We detected no diurnal variation in FGCMs in samples from wild gorillas. 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.

1. Introduction

The vertebrate stress response involves the release of glucocorticoids (GCs; cortisol and corticosterone) into the bloodstream which, in conjunction with accompanying physiological and behavioral responses, enables vertebrates to cope with threatening or demanding situations [3,58]. Chronic stress, associated with prolonged periods of elevated GC concentrations, however, interferes with numerous physiological processes critical to individual health and survival, including immune and reproductive function and disease resistance [40,55,62,67,78]. 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. Enzymeimmunoassays (EIAs) for fecal 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. EIAs are used to investigate the potential links between stress and animal behavior [43,75], animal reproductive biology [29,30,50], and animal welfare [53,57]. They also have important applications to conservation issues [9,13,35,70].

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 [1,21,66] as must methods for...
to preserve the fecal samples (or the steroid hormones therein) in the field [80]. Additionally, the effects of sampling limitations on the FGCM levels found in feces, such as post-defecation degradation due to aged samples or environmental effects [37,69] and diurnal variation in individual FGCM output [2,54,64], need to be assessed and considered in the final analyses. Thus, the most suitable field sampling protocols for a given study population need to be developed before these techniques can be used to their full potential in wild animal populations [69].

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 [5,39]. Species metabolize GCs (and other steroids) differently, resulting in the presence of a wide range of metabolites in the feces [1,45,71]. Consequently, FGCM assays must be thoroughly validated physiologically, biologically and immunologically for each species to ensure biologically meaningful results [6,21,66]. Further, there are substantial differences in the rate of FGCM excretion in feces between species [47], and therefore in observed peak-hormone excretion lag-times [e.g., 1,71 for a review see [47]]. Knowledge of the delay to fecal excretion is crucial in determination of the experimental setup and biologically meaningful interpretation of assay results [47].

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 it is not possible to extract and analyze them immediately in the laboratory or field. The gold standard storage method for fecal samples is simple freezing, as this stabilizes levels of FGCMs (and other steroid metabolites) over long periods of time [22,25,44]. However, many field sites are in remote locations where it is not possible to keep fecal 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 [4,13,15,25,28,51,65]. 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 feces 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. Additionally, once feces are extracted it is also important to know which storage medium is most suitable for storing hormonal extracts. For example, glass is generally accepted to be a more inert material than polypropylene, which may affect whether steroids – as non-polar compounds – stick to the material and to what extent they dissolve into solution if extracts are dried for long-term storage. Various field extraction and 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 [2,14,48,59,77] and should therefore be validated accordingly.

Apart from analytical considerations, field sampling also involves other constraints 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 remain near their sample for long periods of time before the researcher can collect it without causing disturbance to them or their neighbors, or they may defecate in almost inaccessible areas only allowing recovery of the sample after a delay (K.S. pers.obs). FGCM concentrations may begin to deteriorate or change immediately after defecation if samples are not preserved in a suitable fixative [37,41,47]. 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 fresh samples, 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 adrenocorticotropic hormone (ACTH) circadian rhythms, where levels peak in the mornings and decline towards the evening [8,59]. Steroid hormone levels found in blood serum, saliva, and excreted in urine often demonstrate circadian variation [10,17,18,27,52,60], but the effect on FGCM output appears to be species-specific [2,64]. 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 [68]. The species is classified as critically endangered, as a result of the bush-meat trade, habitat destruction and disease [34]. All zoo-housed gorillas are lowland gorillas, and zoo efforts are based on maintaining healthy and genetically-robust breeding populations [49]. 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 [33], 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, published information on fecal GC output in western lowland gorillas is limited to only one study [49], using an un-validated cortisol assay and with largely inconclusive results.

1.1. Aim and specific objectives

In preparation for a remote field study of free-living western lowland gorillas at 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 EIA's using samples from captive gorillas collected around opportunistic stressful situations. All four assays had been used previously to monitor FGCM output in other primate and non-primate species [16,19,21,71]. 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, in order to have directly-comparable data-sets for our long-term study of groups of gorillas in the wild, we conducted experiments in the field to: (i) determine the rate of FGCM change over 12 h after defecation when feces are not preserved immediately in alcohol; (ii) test whether FGCM concentrations alter as a function of storing feces for one month in alcohol under tropical conditions [e.g., 25,28,31] and (iii) whether FGCM measurements in repeat samples from individuals varied between morning and afternoon samples.

2. Materials and methods

2.1. Animals and sample collection

We asked ape keepers from three UK zoos (Twycross, Paignton and Belfast) to collect fecal samples from captive gorillas surrounding six opportunistic routine veterinary and breeding management interventions that we predicted would be stressful for the gorillas.
We obtained samples from two males around two medical examinations \((n=2)\) and relocations between zoos \((n=2)\) and from a female around her social integration with two unfamiliar, 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 anesthetized the gorillas with mass-dependent doses of a combination of Zoletil (Virbac, France; a fixed ratio combination of Zoletapam and Tiletamine) and Zolostine (Orion, Finland; Medetomidine). The procedures associated with anesthesia (isolation, darting, entry into and out of anesthesia) are considered to be disorientating \([77]\), and therefore a physiological stressor for the animals. Anesthesia was not required for the social integrations, which involved social stress between animals and are considered to be biological stressors. Throughout the study period 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 \((\text{range } 1-15)\) 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 early morning samples defecated in the night-cages, noting any evidence of urine contamination. If the latter was thought to have occurred \((n=3)\), where possible the keepers collected only an uncontaminated portion of the feces. Samples were stored at \(-20^\circ\text{C}\) within \(1\) h of collection. We shipped these fecal samples frozen to the endocrine laboratory of the German Primate Center where they were stored at \(-20^\circ\text{C}\) until analysis.

We collected samples for our site-specific short-term storage, hormone degradation and diurnal variation experiments between November 2010 to December 2011, at Bai Hokou study site/c0. We performed reverse-phase high performance liquid chromatography \((\text{HPLC})\) 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 previously \([21,36]\). 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 \([\text{see }16,21,38]\). We measured each HPLC fraction in all four FGCM assays to generate profiles of immunoreactivity.

Based on the results of this validation \((\text{see Section 4.1})\), we conducted all subsequent experiments using only the \(3\alpha,11\beta\)-dihydroxy-CM assay, as this was deemed most suitable for monitoring FGCM output.

### 2.3. 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 \(-20^\circ\text{C}\). We shipped one set of samples frozen to the endocrine laboratory for processing using the laboratory extraction procedure described in Section 2.2 \(\text{("laboratory extraction")}\). We incubated the other set in an oven at \(40^\circ\text{C}\) for \(30\) min 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 \((\text{PPT})\) containing \(5\) ml \(90\%\) ethanol and carried out a field-friendly extraction method \(\text{("field extraction")}\) based on the procedure described elsewhere \([80]\). In brief, we shook the fecal-ethanolic suspension horizontally by hand for \(5\) min and allowed the fecal sedimentation to settle for \(30-40\) min standing on a bench. Following the separation process, we pipetted \(1\) ml of each extract into a \(2\) ml PPT and stored it at \(-20^\circ\text{C}\) until shipment to the endocrine laboratory for GC analysis.

To test preservation of GCs in fecal extracts under different conditions for periods of between \(1\) month and \(1\) yr we extracted \(0.5\) g of wet feces \((n=12\) samples, \(6\) for each sex) using the “field extraction method” \(\text{(but using a centrifuge to separate the liquid from the feces)}\). 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 \(50^\circ\text{C}\). We subjected the aliquots to the following five conditions: (i) storage as liquid in a PPT \((\text{SafeSeal Micro Tube; Ref. No. 72.695.200 from Sarstedt AG & Co. Nuernbrecht, Germany})\); at \(-20^\circ\text{C}\); (ii) storage as liquid in PPTs at room temperature \((\text{RT, }21-23^\circ\text{C})\); (iii) storage as liquid in a glass tube \((75 \times 12\) mm) at RT; (iv) storage dried in PPT at RT; and (v) storage dried in a glass tube at RT. We closed the glass tubes with fitted caps and wrapped all tubes \((\text{glass and PPT})\) with parafilm to minimize risk of evaporation. We determined FGCM levels for each sample immediately after extraction \(\text{("Time-0")}\) 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. For this experiment, inter-assay CVs determined over the \(12\) months of analysis were \(7.4\%\) \((\text{high value quality control})\) and \(14.2\%\) \((\text{low value quality control})\).

### 2.4. Experiment 2 – testing short-term storage of feces in alcohol under field conditions

We collected fecal samples \((n=10; 8\) animals\) directly after defection and homogenized them well. We split the feces into \(15\) portions of \(\sim 0.5\) g and placed them in \(4\) ml \(90\%\) ethanol within \(5\) h of collection. We extracted one portion immediately as described in Section 2.3 \(\text{("day 0")}\) 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 Terrio et al. [66] and Galama et al. [15]). 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 FGCM analysis, we reconstituted samples in 0.5 ml 80% ethanol in water by sonication in a water bath for 5 min, followed by 30 s vortexing. Inter-assay CVs for these measurements were 6.8% (high value quality control) and 13.2% (low value quality control).

2.5. Experiment 3 – testing post-defecation FGCM change in unpreserved feces under field conditions

To test whether FGCM levels in feces change as a function of the time between defection and sample preservation in alcohol, we collected 10 fresh fecal samples (from 7 animals) immediately after defection 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 (~0.5 g) in alcohol every 2 hrs until 12 h after defection. Thereafter, we extracted each sample and dried down 0.5 ml from each sample in 2 ml PPTs as described in Section 2.4. We kept dried extracts at ambient temperatures in the dark until shipment to the endocrine laboratory for FGCM analysis. Inter-assay CVs for these measurements were 6.8% (high value quality control) and 13.2% (low value quality control).

2.6. 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 ~0.5 g wet feces of each sample into 4 ml 90% ethanol, and subjected each sample to the field extraction procedure described in Section 2.2 within 24 h of collection. We kept dried extracts at ambient temperatures in the dark until shipment to the endocrine laboratory for FGCM analysis. Assay CVs for these measurements were <5% for both high and low value quality controls.

3. Data analysis

We give all hormone data as mass hormone per mass fecal wet weight, except for the validation tests, where we used lyophilized samples for extraction. We used all samples for analysis as there was no indication of an effect of urine contamination on FGCM levels in the three samples where contamination was noted but could not be avoided by collecting an uncontaminated portion of the feces. Furthermore, there was no evidence that samples collected at the first early morning check (i.e., samples potentially defecated overnight) differed in FGCM content from those collected later in the day. 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 \( \frac{\text{an} - \text{xn}}{\text{xn}} \times 100 \), where \( \text{an} \) is the nth sample value in each condition/duration and \( \text{xn} \) 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. Again, we 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 defection 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 h at ambient temperature before preserved in alcohol (the maximum delay to preservation tested, Experiment 3) correlated with the control values irrespective of a 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. Finally, for Experiment 4, 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 \( p < 0.05 \).

4. Results

4.1. 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 (Table 1, 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 h after...
the stressor and FGCM levels had usually returned to pre-stress baseline levels by day 5 (Fig. 1 top).

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 [21]. The low immunoreactivity after fraction 31, where certain potentially cross-reacting androgen metabolites elute [16,21], suggests a low degree of co-measurement of these androgens in the two assays (Fig. 1 bottom). Moreover, the presence of the highest peaks of immunoreactivity

Table 1
Fecal glucocorticoid concentrations (as detected by four different assays) in response to various types of stressor in individual lowland gorillas.

<table>
<thead>
<tr>
<th>Animal</th>
<th>Stressor</th>
<th>3α,11ß-dihydroxy-CM</th>
<th>3α,11oxo-CM</th>
<th>Cortisol</th>
<th>CCST</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pre</td>
<td>Peak</td>
<td>Delta</td>
<td>Pre</td>
<td>Peak</td>
</tr>
<tr>
<td>Matadi</td>
<td>Relo</td>
<td>0.19</td>
<td>3.26</td>
<td>17.2</td>
<td>96</td>
</tr>
<tr>
<td>Matadi</td>
<td>Health</td>
<td>0.21</td>
<td>0.95</td>
<td>4.5</td>
<td>47</td>
</tr>
<tr>
<td>Boulaa</td>
<td>Relo</td>
<td>0.58</td>
<td>3.14</td>
<td>5.4</td>
<td>68</td>
</tr>
<tr>
<td>Oumbia</td>
<td>Health</td>
<td>0.98</td>
<td>1.78</td>
<td>1.8</td>
<td>43</td>
</tr>
<tr>
<td>Assante</td>
<td>Social 1</td>
<td>0.50</td>
<td>1.78</td>
<td>3.6</td>
<td>60d</td>
</tr>
<tr>
<td>Assante</td>
<td>Social 2</td>
<td>0.39</td>
<td>1.99</td>
<td>5.1</td>
<td>47</td>
</tr>
<tr>
<td>Median</td>
<td></td>
<td>0.45</td>
<td>1.89</td>
<td>4.8</td>
<td>54</td>
</tr>
</tbody>
</table>

* Male.
* Female.
* Relo = Relocation to another zoo; Health = health check; Social 1 = introduction to other females; Social 2 = introduction to a silverback male.
* Pre-treatment levels in µg/g feces (see Methods).
* Peak levels in response to stressor in µg/g.
* X-fold increase of peak levels above pre-treatment concentrations.
* Lag time in hours between occurrence of the stressor and peak GC response.
* No samples available after 60 h.

Fig. 1. Top: Percentage response in immunoreactive fecal FGCM levels from Time-0 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. Bottom: HPLC immunoreactivity profiles detected using the 3α,11ß-dihydroxy-CM and 3α,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) androstenedione, dehydroepiandrosterone (55), (8) episterosterone, 5β-DHT, 5β-androstane-3ß-ol-17-one (72), (9) 5β-androstane, 3α-ol-17-one (82/83), and (10) androsterone (100).
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).

4.2. 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 (r = 0.79, p < 0.001, n = 29). Our storage experiment revealed that FGCM levels stayed relatively stable over the 12 months of storage for each storage condition, as indicated by the findings that (i) mean changes in FGCM concentrations at each condition and storage duration excluding condition “Liquid RT” (see following discussion), did not exceed ±20% of the controls (Fig. 2) and (ii) at any storage duration FGCM values correlated strongly and significantly with 0 control values (r = 0.9, p < 0.0001 for all correlations).

Storage condition did not have an overall significant effect on FGCM values: (F (3, 44.0) = 0.012, p = 0.998), but time did have a significant effect (F (5, 44.0) = 37.290, p < 0.001). This resulted in an overall significant interaction effect of time and storage condition on FGCM values (F (15, 44.0) = 17.619, p < 0.001). Time also had an overall significant effect in each condition (liquid/C0 11β/C: F = 15.05; liquid PPT RT: F = 59.43; dried PPT RT: F = 100.70; dried glass RT: F = 31.06; all F (5,11.0), p < 0.001). However, pos-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 2). For example, in condition 4 “PPT Dried RT”, the value 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 months 9 and 12 (Fig 2). Visual inspection of these samples suggested that this change was most likely due to increased evaporation in at least two samples (i.e., the volume of these samples was visibly less than that of the other samples). Although the correlations between extracts stored in this condition for 12 months were still highly correlated with control values (r = 0.84, p < 0.0005), we removed this condition from the analysis as it no longer reliably reflected true changes in FGCM levels. We report all results accordingly.

Mean CV values across the 6 measurements of each sample over the 12 months of analysis ranged 9.6–25.2%. 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.

4.3. 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%) over the 4 week experimental period (χ² = 22.407, p = 0.071). The temporal pattern shown in Fig. 3 indicates that this rise in levels occurred mainly after day 8 of storage. FGCM levels measured after 28 days of storage, however, showed a strong and significant correlation with the values immediately after extraction (r = 0.83, p < 0.001, Fig. 3).

4.4. 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 h before preservation in alcohol showed a significant and strong decline over time (χ² = 24.376, p = 0.001; Fig. 4). 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 h, after which levels remained stable (Fig. 4). Post-hoc analysis revealed that FGCM concentrations in samples stored for 6 h 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 h at ambient temperature correlated strongly and significantly with the time 0 values (\( r_s = 0.77, p < 0.01 \)).

4.5. Experiment 4 – testing for diurnal effects on FGCM levels in feces

Comparison of FGCM concentrations in samples collected from the same individuals 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; \( N = 15 \), \( t = -0.625; P = 0.542 \)).

5. Discussion

We demonstrated the validity of two group-specific EIAs for the measurement of 5-reduced cortisol metabolites with a \( 3\alpha,11\beta \)-dihydroxy- and \( 3\alpha,11\alpha \)-oxo 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 with negligible alterations in FGCM concentrations. In addition,
we demonstrated that FGCM concentrations decrease almost linearly over the course of 12 h when feces are not preserved immediately, and that FGCM concentrations do not show diurnal variation in the monitored group of wild western lowland gorillas in the CAR. 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. Furthermore, excluding the “Liquid RT” condition, the percentage change in hormone values between time periods were always ≤20% and FGCM values strongly correlated with those at time 0 at all months of storage, indicating that the relative differences in FGCM levels across individual samples remained stable over time. 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 artifact 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 be predicted to result in a more directional change of hormone levels similar to that found when fecal material is stored long-term in alcohol [11,25,28] and may also be temperature-dependent, being more pronounced in samples stored at higher temperatures when compared to frozen samples [25,28]. Our results did not support either prediction, however.

5.1. 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 primate [21,76] and non-primate species [71,79] 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 assays is in line with findings from many other studies comparing the suitability of diverse fecal FGCM assays in reflecting the stress response [12,16,21,44,46,53,74]. Of the two cortisol metabolite assays, the 3α,11β-dihydroxy-CM assay appears to have a higher biological sensitivity than the 3α,11α-oxo-CM assay, showing a stronger response to the stressor in all animals. We, therefore, used this assay for our subsequent experiments and recommend using the 3α,11β-dihydroxy-CM assay to assess FGCM output in the lowland gorilla.

5.2. 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 method to extract feces in the field is important as it removes the risk of unknown alterations in hormone concentrations when feces are stored in alcohol for prolonged periods of time [11,25,28], 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 [47,48], but several studies have reported high extraction efficiency for steroids using ethanol at 80-100% [14,32,47,59]. 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 others [80], is sufficient to obtain reliable results, although use of a power- or chemically-induced changes in metabolite structure (e.g., 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 be predicted to result in a more directional change of hormone levels similar to that found when fecal material is stored long-term in alcohol [11,25,28] and may also be temperature-dependent, being more pronounced in samples stored at higher temperatures when compared to frozen samples [25,28]. Our results did not support either prediction, however.

Our finding that keeping extracts at ambient temperatures is equally good as storing them frozen is remarkable and contrasts with current thinking that freezing is the best method of steroid preservation [23,66,80]. Although we acknowledge the possibility that this may be a species-, or hormone-specific result, it may be extremely useful for field researchers working in remote conditions without a freezer. However, as lower temperatures clearly reduce the risk of evaporation, we recommend storing sample extracts in a cold environment where possible nevertheless.

Additionally, we show that FGCM levels in dried extracts stored in simple polypropylene (PPT) tubes were no different from those stored in glass tubes. 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 [59]. To our knowledge however, our study is the first to validate the use of dried fecal extracts for one year; again this is highly valuable for researchers in remote places with the need to include yearly seasonality effects in studies of FGCMs from animal species as it overcomes the need to analyze samples during fieldwork. Furthermore, we envisage that dried sample extracts can likely be stored beyond 12 months without causing significant changes in FGCM content.

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 also removes the possibility of evaporation and 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 a small volume of extract (e.g., 0.5 ml or less) is needed for hormone analysis. However, when researchers prefer to store liquid extracts, we recommend moving samples to a fridge or freezer periodically if possible, to reduce the risk of solvent evaporation and a resultant change in hormone levels.

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 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, [59]].

5.3. 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 significantly from values of immediately-extracted samples. This finding is similar to that reported for yellow baboons (*Papio cynocephalus*) [28,31], sifakas [12] and grizzly bears (*Ursus arctos*) [25] but different from the results of a storage experiment conducted with elephant feces in which FGCM levels rose after 2 weeks of storage [25]. Although we did observe some variation in mean FGCM levels between days within the month and a consistent small increase beginning after 8 days of storage, there was otherwise no clearly 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 [72], 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 to obtain 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.

5.4. 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 h, with the most pronounced decay between zero and four hours, and levels stabilizing at around 50% of the original concentration from 6 h onwards. To date, few studies have investigated hormone change in feces between defecation and fixation, but studies of cattle, horses and pigs [37] and Bornean orang-utans (*Pongo pygmaeus morio*) [40] also found a significant change in FGCM levels within a few hours when samples were stored unpreserved at ambient temperature. In these studies concentrations increased rather than decreased as we report here for gorilla feces. However, a decrease in concentrations was also observed in brown hyena feces (*Hyaena brunnea*) [24]. 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 result in more immuno-reactive compounds in farm animals and orang-utans and less immuno-reactive forms in the gorilla or hyena, is a more likely explanation for the differences in findings [37,69]. 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 specifically, our data imply that FGCM concentrations in samples collected from nests, where exact defecation time is usually unknown, are likely to be underestimated. If, however, the time between defecation and collection is known, our finding of an almost linear degradation pattern may allow a corrective factor to be applied to estimate more realistic hormone values.

5.5. 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 (K.S. Pers.obs). Our data are consistent with those from other studies of FGCMs in larger-bodied mammals [61,73] where gut passage-time is comparatively slow as seen in the gorilla. In contrast, diurnal differences in FGCM levels are more often seen in smaller-bodied species [26,63,64]. More generally, diurnal variation may be more detectable in the urine [e.g., [42,56]] than in feces, 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 allows researchers to follow more animals and gather larger sample sets within restricted periods of time.

6. Conclusions

Overall, our results further support the use of FGCMs in long-term studies of the correlates of stress 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. 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 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-hormone monitoring are not always field-friendly and ways to adapt and validate them in the field need to be tested for each species. Here we provide a validated EIA for the western lowland gorilla and general methods by which fecal hormone monitoring can be applied to a variety of field conservation contexts and wild animal species where, arguably, it may be most crucially needed.

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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.

References


