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ORIGINAL ARTICLE

Validation of a field-friendly extraction and storage method to monitor fecal steroid metabolites in wild orangutans

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Abstract Measuring hormone metabolites from feces is the most often used method to assess hormonal status in wildlife. Although immediate freezing of fecal samples collected in the field is the best method to minimize the risk of degradation of hormones over time, this is often not possible in remote field sites. Therefore, alternative storage and preservation methods for fecal samples are required in these conditions. We conducted an experiment to investigate if fecal glucocorticoid (FGCM) and progesterone metabolite (pregnanediol-3-glucuronide; PdG) levels measured from samples that were extracted with a simple, field-friendly methodology correlate with those generated from frozen samples. We also evaluated whether storing fecal samples in alcohol is a suitable alternative to preserve FGCM and PdG concentrations long-term (i.e. over a 9-month period) at locations where fecal extraction is not feasible. Finally, we tested if the hormone concentrations in unpreserved fecal samples of orangutans change over 14 h when stored at ambient conditions, representing the maximum duration between sample collection and return to the camp. FGCM and PdG levels measured from samples that were extracted with the field-friendly method showed strong correlations with those generated from frozen samples, and mean levels did not differ significantly between these methods. FGCM concentrations showed no significant change compared to control samples when fecal samples were stored for up to 6 months in alcohol at ambient temperature and PdG concentrations even remained stable for up to 9 months of storage. FGCM concentrations of fecal samples kept at ambient temperature for up to 14 h post-defecation did not significantly differ compared to control samples frozen immediately after collection. These results provide the basis for the successful monitoring of the physiological status of orangutans living in remote natural settings, like those included in the Indonesian reintroduction programs.

Keywords *Pongo* spp. · Fecal glucocorticoids · PdG · Sample storage · Hormone degradation

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Introduction

Hormones play an important role in physiology, including the regulation of metabolism, reproduction, development, and the expression of behavior (Touma and Palme 2005; Yeo and Sawdon 2013). The classical method to measure hormone concentrations is to collect blood samples invasively. However, this method is impractical for stress-susceptible species, especially when studying potential stressors of animals in their natural environment (Brown et al. 1994; Goymann 2005). Over the last decades, therefore, non-invasive methods that measure hormones, or their metabolites excreted in feces, urine and saliva, have become popular tools to monitor hormonal profiles in

captive and free-ranging animals (e.g. Millspaugh and Washburn 2004; Hodges et al. 2010; Hodges and Heistermann 2011; Sheriff et al. 2011; Weingrill et al. 2011; Ozella et al. 2015; Marty et al. 2015). Measuring hormone metabolites in feces is the most popular of these methods because feces are easier to collect than urine or saliva, especially under field conditions. In addition, due to longer gut passage time, metabolite steroid concentrations in fecal samples can be regarded as values averaged over a longer period of time compared to saliva and urine samples, which is particularly advantageous in studies that investigate chronic stress in animals (Millspaugh and Washburn 2003; Palme 2005; Ashley et al. 2011; Weingrill et al. 2011). However, one major challenge that is sometimes neglected is the storage of fecal samples before the analyses take place in the laboratory. Enzymes produced by bacteria in the feces are known to alter the composition of steroid hormone metabolites in feces through degradation and, thus, these hormones are prone to further metabolism over time (Winter and Bokkenheuser 1978; Wasser et al. 1988; Terio et al. 2002; Khan et al. 2002; Palme 2005).

The recommended procedure for storing fecal samples prior to hormonal analysis is to freeze the samples at sub-zero temperatures immediately after collection (Hunt and Wasser 2003; Hodges and Heistermann 2011). However, immediate freezing is not always possible because fecal samples of captive animals can often be only collected after animals have been removed from the cage, or samples collected in the wild have to be first transferred to the camp, which may take several hours. For longer storage under field conditions, where freezers are not available, different methods such as drying of the samples or using chemical preservatives (e.g. ethanol and methanol) have been applied (Whitten et al. 1998; Khan et al. 2002; Terio et al. 2002; Lynch et al. 2003; Galama et al. 2004; Ziegler and Wittwer 2005; Fichtel et al. 2007). Furthermore, in recent years, simple protocols for extracting fecal samples and storing hormonal extracts directly at the field site without the need for electricity have been developed and provide an alternative way to preserve samples under remote conditions before they are transported to the laboratory (Shutt et al. 2012; Kalbitzer and Heistermann 2013; Murray et al. 2013). These field-friendly techniques make use of manually operated equipment (e.g. battery-driven sample homogenizer, hand-operated centrifuge) and simple watery solutions of alcohol (usually ethanol) to collect, extract and preserve the samples at the field site. Since different species metabolize hormones differently, have different diets, and different compositions of gastrointestinal tract microorganisms (Palme 2005), any preservation and storage method when applied under field conditions needs to be validated for every studied species (Khan et al. 2002; Hunt and Wasser 2003). This also needs

to be done for orangutans (*Pongo* spp.) for which a validation of a storage method for fecal hormones at ambient temperatures has not yet been carried out.

Both orangutan species (*Pongo abelii* and *P. pygmaeus* spp.) are facing serious threats and experience serious declines in population size (Ancrenaz et al. 2008; Singleton et al. 2008). One of the current approaches, besides conservation of remaining populations, are reintroduction programs that establish new populations composed of reintroduced ex-captive or displaced animals (Soehartono et al. 2008). Rehabilitation and reintroduction is a potentially stressful process that may limit the success of such conservation activities, but so far no attention has been given to monitor the physiological stress associated with the processes involved and to evaluate the effect of this on the release success of the reintroduced animals (Teixeira et al. 2007; Jenni et al. 2015).

As part of a study to monitor the stress physiology and female reproductive state of orangutans involved in the Indonesian orangutan reintroduction program, we conducted a study to evaluate the suitability of field-friendly methodologies for extracting and preserving steroid hormone metabolites from orangutan fecal samples. This is an important prerequisite for the application of field endocrinology methods under remote field conditions where access to electricity and sophisticated laboratory equipment is usually not given (Shutt et al. 2012; Kalbitzer and Heistermann 2013). First, we tested the correspondence between concentrations measured from fecal samples extracted with a simple, field-friendly method and those extracted from conventionally frozen fecal samples for both fecal glucocorticoid metabolites (FGCMs) and fecal progesterone metabolites (pregnanediol-3-glucuronide; PdGs). Second, we carried out an experiment to investigate the suitability of long-term storage of feces in alcohol as a potential method for preserving FGCM and PdG concentrations for up to 9 months, thus simulating conditions where no freezer for sample preservation is available and even fecal extractions may be difficult or impossible to be performed. Finally, we conducted an experiment to investigate the effects of short-term storage of up to 14 h of fecal samples at ambient temperatures without preservation in alcohol, mimicking the potential effects involved in transporting fecal samples from the collection site to the camp prior to further processing.

Methods

Study animals and sample collection

Across the different experiments, fecal samples, all uncontaminated by urine, were collected from a total of 34

orangutans housed in two different institutions. Details on the exact numbers of animals involved in each experiment as well as the number of samples collected are given below for each experiment. Fecal sample collection and transport to the hormone laboratory within Indonesia were carried out in strict agreement within Indonesian and international regulations.

Experiment 1: comparing a field-friendly fecal extraction method with an established extraction protocol

To test the correspondence between the two extraction methods, samples were taken from Sumatran orangutans (*P. abelii*) housed at the Batu Mbelin Orangutan Care Centre. The orangutans were fed fruits and vegetables supplemented with infant formula milk for humans. For FGCM measurements, 17 fecal samples were collected from 3 individuals (two males and one female), while for PdG measurements, 17 fecal samples collected from 2 females were used. The fecal samples were collected within 2 h of defecation, immediately mixed thoroughly and any obvious non-fecal material was removed (e.g. undigested chunks of fiber and seeds). Mixed fecal samples were then aliquoted into two polypropylene tubes. The first tube was treated following the established preservation and extraction protocol described in Weingrill et al. (2011). In brief, about 2–3 g of material was placed in the tube and stored at −20 °C until shipment to the hormone laboratory for freeze-drying and extraction (see "Hormone analysis"). The second aliquot was subject to a field-friendly extraction protocol. Ca. 0.5 g of fecal material was transferred into a 15-ml graded polypropylene tube (PPT; Corning 430791) pre-filled with 4 ml of 80 % watery ethanol. The samples were then shaken by hand horizontally for 30 s to break up any fecal bolus and to form a fecal-ethanolic suspension (Ziegler and Wittwer 2005; Shutt et al. 2012). For extraction, the fecal suspension was shaken horizontally by hand for 2 min followed by subsequent centrifugation at 3000 rpm for 10 min. Although this was done with an electric centrifuge, in the field, a hand-operated centrifuge can be used (e.g. Hettich Handzentrifuge, Andreas Hettich GmbH & Co. KG, Germany; see e.g. Rimbach et al. 2013). Subsequently, 1.5 ml of the supernatants were transferred to 2-ml polypropylene microtubes which were sealed with parafilm and stored at −20 °C until shipment to the laboratory (ca. 3 months later) where FGCM and PdG concentrations were measured.

Experiment 2: testing the suitability of long-term feces preservation in alcohol

For this experiment, fecal samples from 19 Bornean (seven females; ten males) and two Sumatran orangutans (one female; one male) housed at Ragunan Zoo, Jakarta, Indonesia, were collected in the early morning (one sample per individual). Same as at the Batu Mbelin Orangutan Care Centre, the orangutans were fed fruits and vegetables supplemented with infant formula milk for humans. We measured FGCM concentrations from all of these animals. PdG concentrations were measured from six females of the same sample pool (five Bornean orangutans and one Sumatran orangutan). Upon collection, each sample was mixed thoroughly (any obvious non-fecal material was removed) and divided into five portions of ca. 0.5 g. The tubes were then labeled as zero (0), one (1), three (3), six (6) and nine (9) months. All samples were processed and extracted using the field-friendly method described in experiment 1. While the control samples were extracted immediately and the extracts stored at −20 °C ("time 0"), the other fecal suspensions (months 1–9) were stored at ambient temperature without air conditioner (temperature range: 24–34 °C at 70–90 % of relative humidity) in a dark box inside a closed cabinet until they were extracted after 1, 3, 6 and 9 months, respectively, in the same manner as the control samples. The sample storage conditions were comparable to conditions encountered at the field sites. FGCM and PdG concentrations for each sample and storage period were determined to assess any potential effect of storage time on steroid metabolite stability.

Experiment 3: testing short-term post-defecation FGCM change in unpreserved feces under field conditions

To test whether FGCM concentrations in feces change over time between defecation and sample preservation, we collected 10 fecal samples (from 10 Sumatran orangutans housed at Batu Mbelin Orangutan Care Centre) immediately after defecation and homogenized them well using a wooden applicator. Any obvious non-fecal material was removed from the fecal matrix. Afterward, each sample was divided into 8 aliquots of ca. 2–3 g, which were each placed into 14-ml polypropylene plastic tubes. The lids were then closed and marked as control (0 h), 2, 4, 6, 8, 10, 12 and 14 h. Control samples were placed immediately in a −20 °C freezer and the remaining aliquots were placed at ambient temperature (24–34 °C, with 90 % relative humidity) in the shade to avoid direct sunlight and thus again at conditions similar to those found at the field sites. Every 2 h, the relevant aliquots were placed in the −20 °C freezer (from 2 h until 14 h after defecation). All samples were freeze-dried, extracted and analyzed at the end of the experiment. This experiment was restricted to FGCM measurements because the large changes in PdG concentrations following ovulation and during pregnancy were expected to be much higher than any potential changes

expected from storing feces unpreserved short-term (e.g. Shutt et al. 2012; Hulsman et al. 2011; Lynch et al. 2003). Therefore, even marked storage-dependent changes in fecal progestogen levels are unlikely to change their temporal pattern (Barelli and Heistermann 2009).

Hormone analysis

All fecal samples were sent to the Endocrinology Laboratory of the Faculty of Veterinary Medicine, Bogor Agricultural University, for hormone analysis. FGCM and PdG concentrations were analyzed using microtiter plate enzyme immunoassays (EIA) for 11β-hydroxyetiocholanolone (3α,11β-dihydroxy-CM; Ganswindt et al. 2003) and PdG (Heistermann and Hodges 1995), respectively, running each sample in duplicate. Both assays have been previously used successfully for assessing female reproductive status and adrenocortical activity from orangutan fecal samples (Marty et al. 2015; Weingrill et al. 2011). The FGCM assay (3α,11β-dihydroxy-CM) showed cross-reactivities with the following hormones: 5β-androstane-3α,11β-diol-17-CMO antibody (100 %), 3.4 % for 5β-androstane-3β-ol-17-one, 1.8 % for 11-oxo-etiocholanolone and <0.1 % for corticosterone, cortisol, 5α-androstane-3,11,17-trione, 5β-androstane-3,11,17-trione, testosterone, 5α-androstane-3,17-dione, 5β-androstane-3,17-dione, androstendione, 5β-androstane-3β-ol-17-one, 5β-androstane-17-one, dehydroepiandrosterone and androsterone (Ganswindt et al. 2003). The PdG assay showed cross-reactivities with the following hormones: 20α-hydroxyprogesterone 32 %, pregnanediol 22 %, 5α-pregnane-20α-ol-3-one 14 %, progesterone 0.5 % and <0.1 % for all other steroid hormones including cortisol and testosterone (Heistermann and Hodges 1995). Details of the individual assay procedures are described elsewhere (FGCM: Heistermann et al. 2004; PdG: Heistermann et al. 1995).

To calculate the dry weight of the fecal matter collected into tubes containing ethanol, the remaining fecal pellets in the tubes were dried in an oven at a temperature of 50° C until constant weight was reached. Samples stored frozen without ethanol in experiment 1 and 3 were processed and extracted as described elsewhere (Heistermann et al. 1995; Palme et al. 2013). Briefly, samples were lyophilized and pulverized, and an aliquot representing 0.05–0.1 g of fecal powder was extracted using 3 ml of 80 % watery methanol by vortexing for 10 min, followed by centrifugation. The recovered supernatant was then aliquoted into a 2-ml polypropylene microtube and stored at −20 °C until analysis. All hormone concentrations were expressed in ng/g dry fecal weight.

Sensitivities of the two assays at 90 % binding were 1 pg for FGCM and 20 pg for PdG. Serial dilutions of sample extracts (1:50 to 1:800) from both male and female samples showed displacement curves that were parallel to the standard curve in the FGCM assay ($F = 0.0264$, $p = 0.8762$; $F = 0.2147$, $p = 0.6594$, for male and female samples, respectively), indicating no interference of the sample matrix on the FGCM measurement. For the PdG assay, serial dilutions of sample extracts (1:50 to 1:800) from both pregnant and cycling females showed displacement curves that were also parallel to the standard curve ($F = 3.625$, $p = 0.106$; $F = 0.6123$, $p = 0.4637$ for pregnant and cycling females, respectively). Intra-assay coefficients of variation (CVs), calculated from repeated measurements of high- and low-value quality controls, were <10 % for both assays (Weingrill et al. 2011; Marty et al. 2015). Inter-assay CVs, where applicable, were calculated for each of the three experiments separately. For experiment 1, inter-assay CVs were 8.8 % (high) and 11.8 % (low) for the FGCM measurements ($n = 2$ plates). Since for PdG all samples of experiment 1 were measured on one plate, inter-assay CV values cannot be reported. The within-assay precision of this plate assessed as the average CV calculated from the individual CVs for all the duplicates was 9.6 %. For experiment 2, FGCM inter-assay CVs were 7.4 % (high) and 13.8 % (low; $n = 6$ plates). Again, for PdG, all samples were measured on one plate only. The within-assay CV for this plate, assessed as described above, was 6.9 %. Finally, for FGCM measurements of experiment 3, inter-assay CVs were 12 % (high) and 14.6 % (low; $n = 3$ plates).

Statistical analysis

In experiment 1, Spearman rank correlation tests were performed to evaluate the correspondence between hormone concentrations measured in the reference group samples (frozen and freeze dried) and the samples collected into ethanol and extracted using the field-friendly method. A Wilcoxon signed-rank test was used to compare absolute hormone concentrations between the two treatments. In experiment 2, percentage changes in FGCM and PdG concentrations at each storage period (1, 3, 6 and 9 months) were calculated as $(a_n - x_n)x_n \times 100$, where "$a_n$" is the nth sample value at each duration and "x_n" is the value at time point 0 of the nth sample (Shutt et al. 2012). Friedman repeated measures analysis of variance on ranks was used to determine whether there was an effect of storage duration on FGCM and PdG levels compared to the control. In addition, Spearman rank correlation tests performed to investigate whether FGCM and PdG concentrations in extracts at each storage period (1, 3, 6 and 9 months) correlated with the control values. For both hormone measurements, CVs were calculated for each of the tested samples across the five measurements to evaluate

whether the respective variation observed was within the range of assay variation or more likely related to storage-dependent changes in hormone concentrations. In experiment 3, we analyzed changes in FGCM concentrations as a function of storage time between defecation and preservation of the sample using Friedman's repeated measures analysis of variance on ranks. We also calculated Spearman rank correlation coefficients to determine whether FGCM values in feces left for 14 h at ambient temperature before preservation correlate with directly preserved samples (i.e. samples stored frozen immediately after collection).

Results

Experiment 1: comparing a field-friendly fecal extraction method with an established extraction protocol

FGCM concentrations measured from fecal samples collected into ethanol and processed applying the field-friendly extraction method correlated strongly with concentrations measured from frozen samples that were freeze-dried and extracted using our established laboratory procedure ($r = 0.929$, $p < 0.0001$; Fig. 1a). Moreover, absolute FGCM levels measured did not differ significantly between the two treatments [frozen stored samples: mean $\pm$ standard error of the mean (SEM); 982.7 ± 222.7; ethanol-stored samples: 893.1 ± 226.1; Wilcoxon's signed-rank test $W = -43.000$, $p = 0.329$, $n = 17$; Fig. 1b]. Similar results were found for PdG where concentrations also showed a strong correlation between both

treatments ($r = 0.936$, $p < 0.0001$; Fig. 2a) and no significant differences between absolute levels (frozen stored samples: mean $\pm$ SEM; 6120.3 ± 1181.6; ethanol-stored samples: 5958.9 ± 1198.3; Wilcoxon's signed-rank test $W = 7.00$, $p = 0.890$, $n = 17$; Fig. 2b).

Experiment 2: testing the suitability of long-term feces preservation in alcohol

FGCM concentrations remained relatively stable across the 9 months of storage tested, with average changes recorded at any point in time not exceeding 17 % of the controls (Fig. 3a). However, time of storage had an overall significant effect on FGCM levels ($F = 12.842$, $p = 0.012$). Post-hoc analysis revealed that this effect was solely based on a significant change seen at 9 months of storage ($p = 0.029$), whereas values at 1, 3 and 6 months were not statistically different from the control values ($p = 1$, $p = 0.24$ and $p = 1$, respectively). Moreover, individual FGCM values at all storage periods correlated strongly and significantly with the time 0 control values ($r = 0.9$, $p = 0.000$ for all correlations). The average CV value across the 5 measurements of each sample over the 9 months of storage was 13.3 %, which is well within the range of our inter-assay variation.

The PdG concentrations showed no significant change from the control samples throughout all four storage periods ($F = 6.400$, $p = 0.171$). As for FGCM, the average PdG concentrations at each storage period did not exceed 16 % of the controls (Fig. 3b) and at all storage periods; PdG values correlated strongly and significantly with time 0 control values (month 1, 3 and 9: $r = 1$, $p = 0.000$;
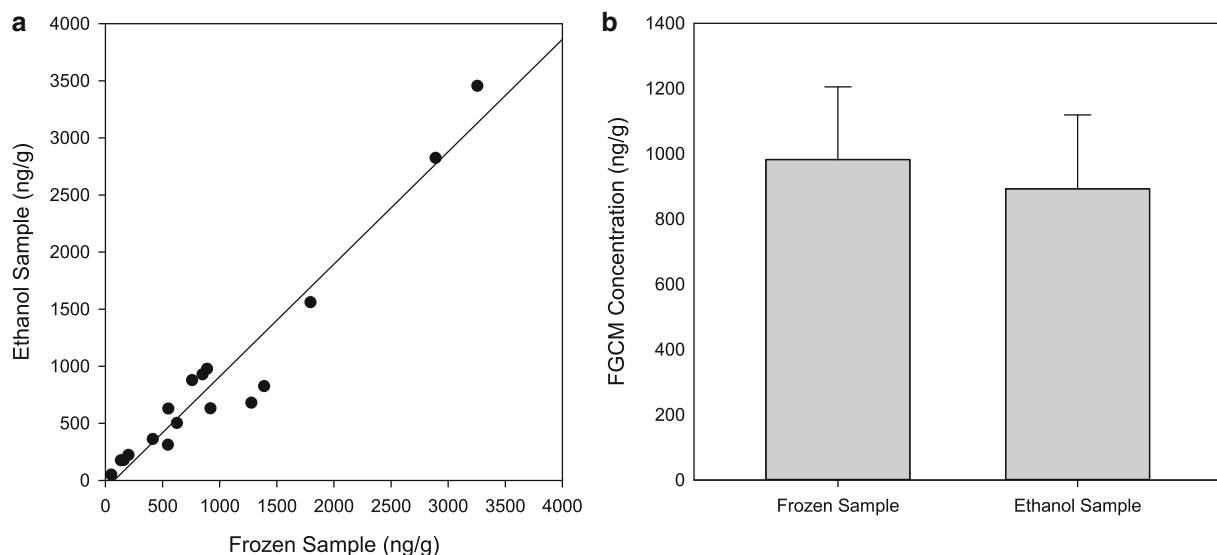

Fig. 1 Comparison between FGCM values of samples ($n = 17$) collected into ethanol and extracted applying the field-friendly extraction method and frozen samples that were extracted using our established laboratory procedure. **a** Correlation between the two data sets; **b** Mean + SEM of frozen and ethanol-stored samples
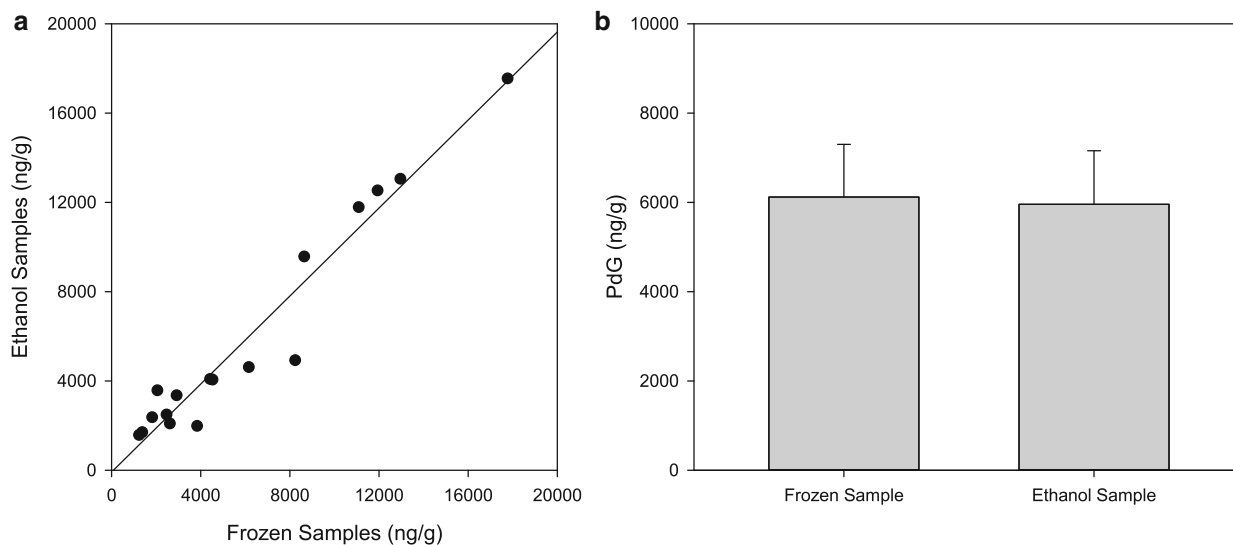
Fig. 2 Comparison between PdG values of samples ($n = 17$) collected into ethanol and extracted applying the field-friendly extraction method and frozen samples that were extracted using our established laboratory procedure. **a** Correlation between the two data sets; **b** Mean + SEM of frozen and ethanol-stored samples
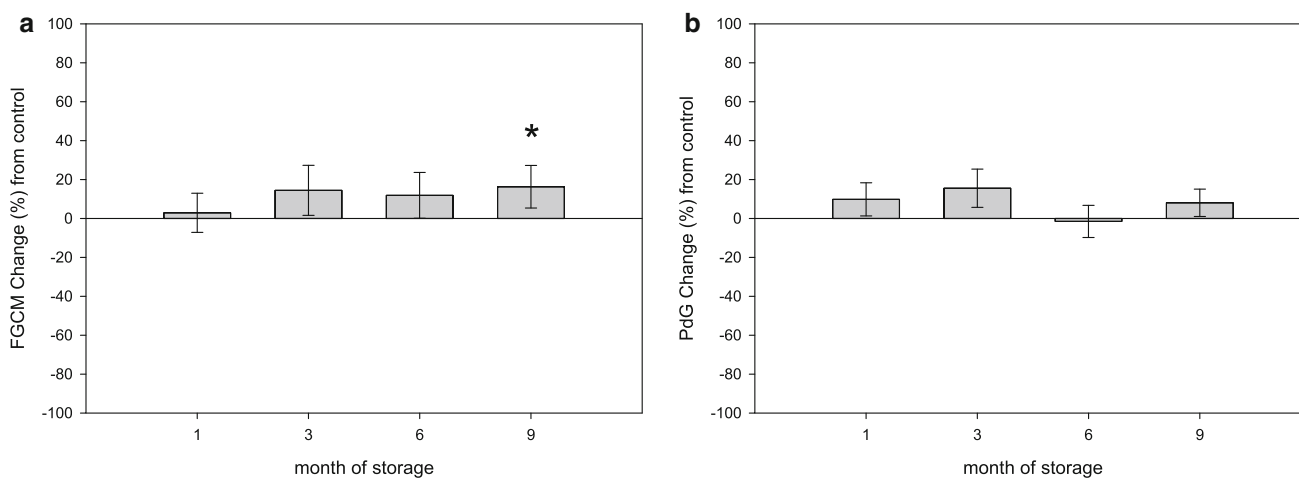

Fig. 3 Percent change in FGCM (**a**) and PdG (**b**) concentrations relative to time 0 control values in fecal samples stored for up to 9 months in 80 % ethanol. Values represent the mean ± SEM (*$p < 0.05$, see text; FGCM: $n = 19$; PdG: $n = 6$)

month 6: $r = 0.9$, $p = 0.037$). The average CV value across the 5 measurements of each sample over the 9 months of storage was 12.0 %, which is again within the range of assay variation.

Experiment 3: testing short-term post-defecation FGCM change in unpreserved feces under field conditions

FGCM concentrations in feces stored at ambient temperature for up to 14 h before being frozen showed no significant change over time compared to the original concentration at time point "0" (sample preserved directly after defecation; $F = 1.2431$, $df = 7$, $p = 0.9899$, $n = 10$; Fig. 4). Values at all storage times correlated strongly and significantly with

the time 0 control values (mean $r = 0.770$, range 0.624–0.842, $p < 0.05$ for all correlations).

Discussion

We evaluated the suitability of a field-friendly extraction and preservation methodology to assess glucocorticoid (FGCM) and progesterone metabolite (PdG) concentrations from orangutan feces. We demonstrate that a simple extraction method that can be easily performed under field conditions generates data similar to those generated by a well-established laboratory procedure. We also show that preservation of orangutan feces in 80 % ethanol for periods of up to 9 months does not markedly affect FGCM and
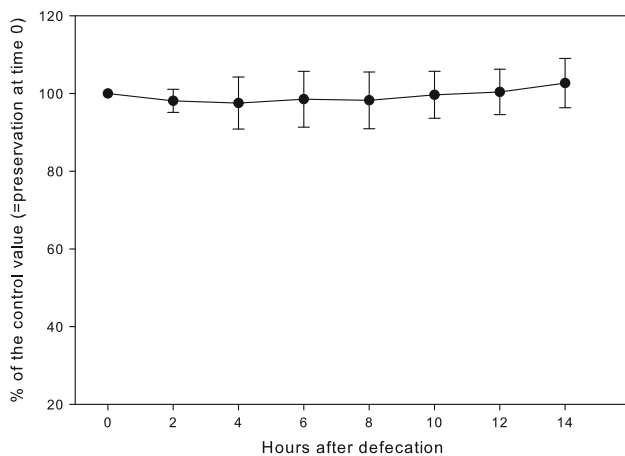
Fig. 4 FGCM concentrations of fecal samples stored for up to 14 h at ambient temperature. Values represent percentages as the mean ± -SEM ($n = 10$) relative to the control value at time 0 (=100 %)

PdG concentrations. Finally, we demonstrate the stability of FGCM in feces kept unpreserved at ambient temperature for up to 14 h.

Experiment 1: comparing a field-friendly fecal extraction method with an established extraction protocol

Our result of a strong correlation between FGCM levels generated from samples processed with the field-friendly extraction method and our established procedure (preservation by freezing samples at −20 °C, followed by freeze-drying and extraction) confirms similar findings for the gorilla (Shutt et al. 2012) and preliminary data for orangutans (Marty et al. 2015). They also extend those findings by demonstrating that the field-friendly extraction methodology can be applied successfully to PdG measurements, suggesting that this simple methodology is potentially applicable to reliably extract metabolites of steroid hormones of different origin (i.e. adrenal and gonadal). In contrast to our study, absolute FGCM levels in the study of Marty et al. (2015; measured with the same assay) differed significantly between the two extraction methods. The reason for this discrepancy is not entirely clear, but may be related to the fact that in our study, samples collected into alcohol were thoroughly homogenized and were extracted relatively soon after collection (i.e. within a couple of hours), whereas in the study of Marty and colleagues, the feces were stored non-homogenized and extracted months later. Thus, these samples might have been subject to time-dependent alterations in steroid metabolite concentrations (Khan et al. 2002; Hunt and Wasser 2003, but see below). We, therefore, recommend that fecal samples of wild animals that have been collected into alcohol should be extracted within short time

periods following their collection (as in Rimbach et al. 2013; Kalbitzer et al. 2015) unless it has been tested for how long natural samples can withstand the storage in alcohol without compromising one's results (as, for example, in Khan et al. 2002; Shutt et al. 2012; this study). However, the strong correlation between the two storage/extraction methodologies would allow comparing values generated by the two different methods even if the absolute values differ using a simple regression formula. Thus, we are allowed to directly compare values from different study sites when both methods have been used (Marty et al. 2015). In our planned long-term study, this may be necessary, for example, when fecal samples of an animal can be frozen in the rehabilitation station and the field-friendly storage/extraction method has to be applied at the remote release sites.

Experiment 2: testing the suitability of long-term feces preservation in alcohol

Our long-term preservation experiment demonstrates that concentrations of FGCM in feces suspended in alcohol remain stable for up to 6 months of storage, while those of PdG were stable until the end of the experiment (9 months of storage). The change detected at month 9 of storage was statistically significant for FGCM. However, the percentage changes in FGCM and PdG levels at any time period were always <17 % compared to the controls and, thus, any change recorded was within the range of the respective assay variation. Therefore, we believe that the small changes in concentrations recorded were simply due to assay variability (see also Shutt et al. 2012; Kalbitzer and Heistermann 2013) rather than reflecting real storage-induced changes in metabolite levels that far exceed assay variation (as e.g. in Khan et al. 2002; Hunt and Wasser 2003). Concentrations measured in our FGCM and PdG assays also correlated strongly with the control samples at all months of storage, indicating that, irrespective of any change in absolute levels, the relative differences in hormone values across individual samples also remained stable over time.

Our results showing that hormone metabolite concentrations in ethanol-stored feces of orangutans remain stable for a minimum of 180 days of storage is remarkable as many previous studies in other species have shown greater changes (i.e. 50 to >1000 %) over the same or even shorter time period (e.g. Khan et al. 2002; Hunt and Wasser 2003). Apart from potential species differences in the vulnerability of fecal hormones to such storage effects (e.g. Hunt and Wasser 2003), differences in the methodologies applied may have also contributed to the differences in findings. For example, Khan et al. (2002) and Hunt and Wasser (2003) used a solvent-to-sample mass ratio of 2.5:1

and 4:1, respectively, while in our experiments the ethanol-to-feces mass ratio was much larger ($\sim 8{:}1$) and more comparable to solvent-to-sample mass ratios used in other research fields where fecal samples need to be preserved (e.g. parasitology). Moreover, in our experiment the feces was completely suspended in the ethanol and shaken thoroughly, thus maximizing contact between samples and preservative. This may have resulted in a more effective reduction of bacterial metabolization of hormones and/or liberation of hormones from fecal lipid micelles, both potentially involved in storage-induced alterations of fecal hormone concentrations (Möstl et al. 1999; Washburn and Millspaugh 2002; Hunt and Wasser 2003). Last but not least, it may be that different metabolites of the same parent compound differ in their vulnerability to storage-induced alterations. In support of this, using the same group-specific FGCM assay as in our study, Fichtel et al. (2007) also reported that FGCM levels remained stable in fecal samples of sifakas stored in ethanol at ambient temperature for up to 270 days. This may suggest that the glucocorticoid metabolites measured by the 11β-hydrox-yetiocholanolone assay might be less prone to storage-induced alterations than those measured by other FGCM assays (e.g. cortisol/corticosterone assays; Hunt and Wasser 2003), although studies testing this directly are lacking so far.

Overall, our results of highly stable FGCM and PdG levels in alcohol-suspended fecal samples stored long-term under field conditions is of great practical value given that opportunities for freezing are usually limited or non-existent at many orangutan field sites, where samples can only be transferred to a freezer from time to time. More generally, our findings and those of others (Khan et al. 2002; Hunt and Wasser 2003; Shutt et al. 2012; Kalbitzer and Heistermann 2013) underscore the importance of validating fecal storage and preservation methods as a prerequisite to any work conducted with fecal steroids under field conditions.

Experiment 3: testing short-term post-defecation FGCM change in unpreserved feces under field conditions

Storage of unprocessed fecal samples at ambient temperature for up to 14 h showed no significant change in FGCM concentrations in our experiment. This is contrary to other research carried out in orangutans and gorillas (Shutt et al. 2012; Muehlenbein et al. 2012). Muehlenbein et al. (2012) found that concentrations of FGCM in untreated orangutan fecal samples stored in closed tubes for 6 h at ambient temperature increase over time, with the increase starting after 3 h of storage. In contrast, Shutt et al. (2012) showed that FGCM levels in untreated gorilla fecal samples

exposed to the environment (but not subjected to rain) degraded over 12 h, with the first significant decrease recorded after 6 h. There are several methodological differences between our experiment and these previous studies that might explain, at least in part, the different findings. Muehlenbein et al. (2012), for example, used a cortisol immunoassay that was probably less suited to measure fecal glucocorticoid metabolites in feces of orangutans than our group-specific measurement of 5β-reduced cortisol metabolites using an 11β-hydroxyetiocholanolone EIA (Weingrill et al. 2011). Shutt et al. (2012) used the same assay for gorilla feces as we did, but exposed the fecal matter to completely natural conditions (although samples were protected from rain). The differences in treatments might potentially lead to a different degree of chemical alteration (e.g. oxidation, deconjugation) of the steroid metabolites of interest (Hunt and Wasser 2003), resulting in higher or lower concentrations of immunoreactivity measured due to alterations in binding affinities to the antibody depending on the assay used (Möstl et al. 1999; Washburn and Millspaugh 2002). Along the same lines, bacterial activity in feces can chemically alter steroid metabolites and their binding affinity shortly after defecation (e.g. Möstl et al. 1999). It is, therefore, likely that inherent differences in diet and gut microflora among species (or even different populations) affect the chemical nature of the metabolites in a species-specific manner (Hunt and Wasser 2003; Goymann 2012), and different assay antibodies may be more or less prone to detect these changes. Finally, fecal metabolites originating from different steroid hormones may also show different patterns of change in response to specific storage conditions. For example, glucocorticoids measured from baboon fecal samples placed in alcohol at room temperature are more stable over time than estrogens (Khan et al. 2002). Measuring PdG concentrations in our orangutan fecal samples would have been interesting in this regard, but we did not conduct this experiment with PdG because the manifold changes in PdG concentrations following ovulation and during pregnancy were expected to be much greater than any potential changes expected from storing feces unpreserved short-term.

From a practical point of view, our results indicate that storing orangutan fecal samples for up to 14 h at ambient temperature does not pose a risk to the stability of FGCM concentrations when measured by our validated 11β-hydroxyetiocholanolone assay. Thus, samples could be transported (e.g. to camp for further processing) after being collected without having to be kept in cold storage.

In summary, our study shows that extraction of FGCM and PdG from orangutan fecal samples can be conducted reliably using a simple methodology that does neither need sophisticated equipment nor access to electricity and is,

thus, suitable for use under remote field conditions. We also demonstrate that FGCM and PdG concentrations in orangutan feces are remarkably robust against changes when feces are kept unpreserved in the short-term (i.e. for up to 14 h post-defecation) or suspended in alcohol for long-term storage (i.e. for up to 6 months at least) at ambient temperatures. Overall, these results provide an important basis for applying adrenal and gonadal steroid hormone measurements in wild orangutans. Specifically, the use of the evaluated methodologies will allow the reliable monitoring of adrenocortical and reproductive activity of orangutans at remote field and reintroduction sites without electricity to run freezers.

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