COMPARISON OF FECAL PRESERVATION AND EXTRACTION METHODS FOR STEROID HORMONE ANALYSIS IN WILD CRESTED MACAQUES

Gholib Gholib[1,2,3,4], Michael Heistermann[5], Muhammad Agil[6],Iman Supriatna[6], Bambang Purwantara[6], Taufiq Purna Nugraha[7], Antje Engelhardt[4,8]

[1]Graduate School of Reproductive Biology, Faculty of Veterinary Medicine, Bogor Agricultural University, Bogor, [2]Laboratory of Physiology Faculty of Veterinary Medicine University of Syiah Kuala, Banda Aceh, Jl Tgk Hasan Krueng Kalee No.4 Kopelman Darussalam, Banda Aceh 23111, Indonesia, [3]Seameo Biotrop (Southeast Asian Regional Centre for Tropical Biology), Bogor, [4]Junior Research Group of Primate Sexual Selection, German Primate Center, Göttingen, Germany, [5]Endocrinology Laboratory, German Primate Centre, Göttingen, Germany, [6]Department of Clinic, Reproduction, and Pathology, Faculty of Veterinary Medicine, Bogor Agricultural University, Bogor, [7]Laboratory of Reproduction Division of Zoology, Research Center for Biology, Indonesia Institute of Sciences (LIPI), [8] School of Natural Science and Psychology, Faculty of Science, Liverpool John Moores University, UK

Abstract

Since the non-invasive field endocrinology techniques were developed, several fecal preservation and extraction methods have been established for a variety of species. However, direct adaptation of methods from previous studies to a new species for instant in crested macaques should be taken with caution. We conducted an experiment to assess the accuracy and stability of fecal estrogen metabolite (E1C) and glucocorticoid

metabolite (GCM) levels in response to several pre-analytical parameters: (1) time lag between fecal preservation after sample collection; (2) long-term storage of fecal samples in 80% methanol (MeOH) at ambient temperature; (3) different degrees of feces drying temperature using a conventional oven; and (4) different fecal preservation techniques (i.e. freeze-drying, oven-drying, and field-friendly extraction method) and extraction solvents (methanol, ethanol, and commercial alcohol). The study used fecal samples collected from crested macaques (*Macaca nigra*) living in the Tangkoko Reserve, North Sulawesi, Indonesia. Samples were assayed using validated E1C and GCM enzyme immunoassays with self-made antibodies raised against estrone-3-glucuronide and 5β-androstane-3α,11β-diol-17-CMO-BSA, respectively. Levels of E1C and GCM in unprocessed feces stored at ambient temperature remained stable for up to eight hours of storage after which levels of both E1C and GCM changed significantly compared to controls extracted at time 0. Long-term storage in 80% MeOH at ambient temperature affected hormone levels significantly with levels of both E1C and GCM increasing after six and four months of storage, respectively. Drying fecal samples using a conventional oven at 50^{O}C, 70^{O}C and 90^{O}C did not affect the E1C levels, but led to a significant decline for GCM in samples dried at 90^{o}C. Different fecal preservation techniques and extraction solvent provided similar results for both E1C and GCM levels. Our results confirm previous studies that prior to application of fecal hormone analysis in a new species, several pre-analytical parameters should be evaluated for their effects on hormone metabolite stability. The results also provide several options for fecal preservation, extraction, and storage methods that can be selected depending on the condition of the field site and laboratory.

Keywords: Crested macaques, GCM, E1C, fecal preservation, extraction solvent

INTRODUCTION

Monitoring reproduction and stress physiology of wild and/or endangered animals living in their natural habitat can nowdays be done via the measurement of fecal steroid metabolites in non-invasively collected fecal samples (e.g. Hodges & Heistermann 2011). This is a suitable approach for field researchers including primatologists. The use of non-invasive methods such as fecal hormone analysis for endocrine assessment provides several advantages over traditional more invasive methods of hormone measurements. Fecal samples can be easily obtained without disturbing the study animals and do not put the animal in danger during capture, e.g. as for blood sampling (Sheriff et al. 2011). They may also provide an accurate assessment of stress hormone levels without the bias of capture-induced increases in stress hormones, i.e. glucocorticoids (Millspaugh & Washburn 2004; Touma et al. 2003).The feces of most vertebrate species contains metabolized forms of all major steroid hormones (e.g., progestins, estrogens, androgens, and glucocorticoids), which are secreted into the gut via the bile (Touma & Palme 2005; Heistermann et al. 2006; Möhle et al. 2002; Higham et al. 2012). These metabolites can be measured with hormone assay techniques.

Between the period of taking a fecal sample to subjecting it to hormone analysis, the concentrations of fecal hormone metabolites can change (Bielohuby et al. 2012) depending on how samples were treated. Previous studies have, for example, shown that the levels of steroid hormone in unprocessed fecal samples stored at ambient temperature might change over time and cause fluctuations in levels due to the activities of fecal bacteria (Mostl et al. 1999; Khan et al. 2002; Shut et al. 2012). Thus, fecal preservation, extraction, and storage play a critical role in fecal steroid analysis and methods should be

validated prior to application in each given species (Sousa & Ziegler 1998; Lynch et al. 2003; Beehner et al. 2004; Palme 2005).

The most common way to preserve and extract fecal samples is by freezing them at -20^{o}C followed by lyophilization (freeze-drying) and extracting fecal steroid hormone using laboratory 80% ethanol or methanol (Hodges & Heistermann 2011, Palme et al. 2013). However, freezing fecal samples at -20^{o}C is not always possible when the study is conducted in a remote area with limited or no electricity. In addition, the lyophilizers are expensive and not always accessible in many laboratories, particularly often not in primate habitat countries. As an alternative, several fecal preservation techniques have been developed such as drying samples using in solar or conventional oven or on silica (Terio et al. 2002; Khan et al. 2002; Pettitt et al. 2007), or storing the fecal samples in ethanol or other chemicals (e.g., sodium azide, ascorbic acid, 1-4 sacco lactone carpol and natrium chloride;Wasser et al. 1988; Nugraha et. al 2016). Alternatively, hormone are extracted from feces immediately after collection (Weingrill et al. 2004; Ziegler and Wittwer 2005; Shutt et al. 2012; Nugraha et al. 2016). The common solvent extraction used to extract steroid hormones from feces is laboratory grade ethanol or methanol (Hodges & Heistermann 2011; Palme et al. 2013). However in remote areas, these chemicals are not always accessible, and ordering or shipping them is often difficult as the shipping of flammable liquids such as ethanol or methanol restricted by aviation regulation. As an alternative, common alcohol such as commercially available medical alcohol (commercial alcohol) can be easily acquired in small pharmacies even in rural areas.

Although the previous studies provided valuable information on the suitability of different techniques of preservation and solvent extraction for hormone analysis, none

has tested the reliability of all in one experiment. Therefore, the relative value of each of the techniques still remains unclear. Moreover, whether these methods can be applied across species is also unclear. As part of a study to monitor reproduction and stress physiology of crested macaques, we conducted an experiment to compare different techniques of fecal preservation and solvent extraction using fecal samples of crested macaques. The crested macaque can only be found at the North-eastern tip of Sulawesi, Indonesia and has been categorized as critically endangered (IUCN Red list 2017). Testing different fecal preservation methods and solvent extractions is an important prerequisite for endocrinological studies in crested macaques or other species of interest living under remote field condition with no or limited access to electricity, or researcher who have not access to a sophisticated laboratory equipment such as freeze-drying machine (Shutt et al. 2012; Nugraha et al. 2016).

In this study, we conducted several experiments to validate the accuracy and stability of fecal estrogen (E1C) and glucocorticoid metabolite (GCM) measurements in response to pre-analytical parameters. First, we performed an experiment to investigate the effect of placing unpreserved fecal samples for up to 10 h after defecation at ambient temperature before freezing them. Second, we conducted a long-term storage experiment of feces in 80% methanol (MeOH) for up to 12 months that simulated a condition when a study is conducted in a remote area where immediate freezing of fecal samples is not possible. Third, we tested the suitability of preserving fecal E1C and GCM levels using oven drying of the feces at different temperatures (50^{O}C, 70^{O}C, and 90^{O}C. Finally, we compared three different fecal preservation methods (freeze-drying, oven drying, and field friendly extraction) in combination with three different extraction solvents (80% MeOH, 80% ethanol (EtOH), and 80% commercial alcohol (propanol or isopropyl

alcohol or rubbing alcohol, Brataco®, Indonesia) for both E1C and GCM to investigate whether levels of hormone change as a function of these combinations.

MATERIALS AND METHODS

Study Site, Animals and Time; sample collection

Fecal samples were collected from June to September 2014 from 10 free-ranging females of a group of crested macaques (Rambo 1) living in the Tangkoko Reserve, North Sulawesi, Indonesia (1°33' N, 125°10' E) during focal animal sampling, as well as fortuitously. For a detailed description of the study site (see Neumann et al. 2010). Samples were collected straight after defecation, homogenized and processed as described below.

Experiment 1. Testing the Effect of the Time Lag between Defecation and Fecal Preservation on E1C and GCM Levels

In order to test whether the time from collection to the preservation influences the stability of E1C and GCM levels in feces, fresh fecal samples (N=7) were collected from animals directly after defecation. Feces were homogenized and ~3-5g feces of each sample was placed in a polypropylene tube and preserved immediately in the freezer at -20^{O}C (control sample). The remaining feces was left on the ground at ambient temperature, exposed to air but protected from rain. An aliquot of 3-5 g was then taken after 2,4,6,8, and 10 hours, respectively and also stored at -20^{O}C as the test groups (Table 1). For hormone analysis, the samples were thawed, freeze-dried and extracted using the freeze-drying method described in experiment 4 (see below). Following extraction, fecal extracts from all groups were assayed together for E1C and GCM levels.

Experiment 2. Testing the Effect of Long-Term Storage of Feces in 80% MeOH at Ambient Temperature on E1C and GCM Levels

To test whether long-term storage of feces in alcohol at ambient temperature influences the stability of E1C and GCM levels, fresh fecal samples (N=8) were collected and homogenized. Each sample was divided into 8 aliquots (~0.5g feces each aliquot) which were placed in a tube containing 5ml 80% MeOH. One aliquot from each sample was extracted immediately as control using the field friendly extraction method (for details see Shut et al. 2011; Nugraha et al. 2016) and the extract stored at -20^{O}C until hormone analysis. The remaining aliquots were stored at ambient temperature and extracted after 1,2,4,6,8,10, and 12 months respectively as the test groups (Table 1) and extracts stored at -20^{O}C. After the end of the experiment, all fecal extracts from all the group were assayed together for E1C and GCM levels.

Experiment 3. Testing the Effectiveness of Oven Drying at Different Temperatures on E1C and GCM Levels

In order to evaluate the most suitable temperature for drying feces using a conventional oven, fresh fecal samples (N=14) were collected after defecation. Following homogenization, each sample was divided into three aliquots and placed into three different tubes (~5g feces/tube). Aliquots were then dried immediately after collection using a conventional aluminum oven which a probe connected with digital thermometer was placed inside the oven to control the temperature, and heated on top of a propane gas stove as used for cooking/baking in the field station. Aliquots were dried at 50^{O}C,70^{O}C, and 90^{O}C, respectively (Table 1) for 36 h. Dried feces was placed inside a plastic bag with a silica gel packet to prevent moisture and keep the sample dry and stored at room temperature until extraction.

Experiment 4. Testing The suitability of Different Fecal Processing Techniques and Extraction Solvents

Samples (N=6) were processed in three different ways: through 1) freeze-drying, 2) oven drying, and 3) field friendly extraction (Fig.1), and aliquots of each of these three procedures extracted with three different extraction solvents (80% MeOH, 80% EtOH, and 80% commercial alcohol (propanol or isopropyl alcohol or rubbing alcohol, Brataco®, Indonesia), respectively (Fig.1).

For the freeze-drying method, samples were lyophilized for 72 h using a freeze drying machine (Christ®, Gamma 1-20) at a temperature of -20 ^{o}C and a vacuum pressure of 1.030 – 0.630 mbar. Subsequently, dried fecal samples were pulverized using a pestle and mortar and sieved through a stainless steel strainer to separate the fecal powder from the fibrous material. Finally, an aliquot (i.e. about 50 mg, exact weight recorded) of the fecal powder was extracted with 3 ml of different solvents (80% MeOH, 80% EtOH, and 80% commercial alcohol). The solution was vortexed for 15 minutes in a 15 ml plastic tube using a multi-tube vortexer (Multi-Tube Vortexer, SMI®, USA). Following centrifugation at 3000 rpm for 10 minutes, the supernatant was decanted into 2 ml microtubes and stored immediately at -20^{o}C until hormone measurement.

For the oven drying method, samples were dried using a conventional oven at 50^{O}C (see the result in experiment 3). Dried samples were pulverized and extracted using the three different solvents in the same way as described for the freeze-drying method (see above).

For the field friendly extraction, fecal samples were extracted according to the procedure described by Shut et al. (2012) and Nugraha et al. (2016) using the three different solvents mentioned before. In brief, ~0.5g of fresh samples were placed into 15

ml tubes containing 5ml extraction solvent and mixed for 30 sec to produce a fecal suspension. Furthermore, samples were extracted by hand shaking the tubes horizontally for 2 min. Following centrifugation using a manually operated centrifuge (Hettich Handzentrifuge, Andreas Hettich GmbH & Co. KG, Germany) at high speed for 2 min, the supernatant was decanted into 2 ml microtubes and stored immediately at -20ºC until hormone measurement.

Hormone Analyses

Fecal estrogen metabolites were measured using an estrone conjugate (E1C) enzyme immunoassay (EIA) previously validated and used successfully for assessing reproductive status in female crested macaques (Higham et al. 2012). The assay utilizes an antiserum raised in a rabbit against estrone-3-glucuronide and estrone-3-glucuronide labeled with alkaline phosphatase was used as conjugate. The cross-reactivities of this assay as reported by Heistermann & Hodges (1995) are as follows: 100% with estrone-3-glucuronide,71% with estrone,17% with estrone-3-sulfate, 0.9% with estradiol, 0.2% with estradiol-3-sulfate,and < 0.1% with all other steroids tested. Prior to assay, fecal extracts were diluted in assay buffer (dilution 1:20 in 0.04 M PBS pH7.2). Duplicate 50 µl aliquots of fecal extracts were assayed along with 50 µl aliquots of blank, zero and standard (dose range 0.78-100 pg/well) on microtitre plates coated with sheep anti-rabbit IgG.50 µl labeled estrone-3-glucuronide and 50µl antiserum were then added to each well and the mixture incubated overnight at 4°C. Following incubation, the plates were washed four times with PBS washing solution (containing 0.05% Tween 20) and blotted dry. Subsequently, 150 µl phosphatase substrate solution (Sigma 104; 20 mg in 16 ml substrate buffer pH 9.8 containing 1 M diethanolamine and 0.1 M $MgCl_2$) was added to each well.

The plates were again incubated for 30–45 minutes by shaking in the dark at room temperature. Finally, absorbance was measured at 405nm on an automatic plate reader.

Fecal glucocorticoid metabolites (GCM) were measured using an 11ß-hydroxyetiocholanolone EIA (3α,11β-hydroxy-CM) previously validated for the use in crested macaques (Gholib 2011). An antiserum against 5β-androstane-3α,11β-diol-17-CMO-BSA was used in the assay with 5β-androstane-3α,11β-diol-17-CMO-DADOO-biotin as conjugate and 5β-androstane-3α,11β-diol-17-one (Sigma H8251) as standard. The cross-reactivities of this assay as reported by Ganswindt et al. (2003) are as follows: 100% with 5β-androstane-3α,11β-diol-17-one, 3.4% with 5β-androstane-3β-ol-17-one, 1.8% with 11-oxo-etiocholanolone and <0.1% with corticosterone, cortisol, 5α-androstane-3,11,17-trione, 5β-androstane-3,11,17-trione, testosterone, 5α-andro-stane-3,17-dione, 5β-androstane-3,17-dione, androstenedione,5β-androstane-3β-ol-17-one,5β-androstane-17-one, dehydro- epiandrosterone, and androsterone. Fecal extracts were diluted in assay buffer (dilution 1:100 in 0.04 M PBS pH7.2). Duplicate 50 µl aliquots of fecal extracts were assayed along with 50 µl aliquots of blank, zero and standard on microtitre plates coated with donkey anti-sheep IgG. Afterward, 50 µl conjugate and 50µl antibody were added to each well and the mixture incubated overnight at 4°C. Following incubation, the plates were washed four times with PBS washing solution (containing 0.05% Tween 20), blotted dry, and 150 µl (6 ng) of streptavidin-peroxidase (S-5512, Sigma Chemie, Germany) in assay buffer was added to each well. The plates were incubated at room temperature (RT) in the dark for 30 minutes, after which they have washed again four times. A substrate solution (150 µl, containing 0.025% tetramethyl-benzidine and 0.05% H_2O_2) was then added to each well. The plates were again incubated in dark at RT for 30–45 min after which the enzyme reaction was stopped by adding 50

µl 2M H2SO4 into each well. Finally, absorbance was measured at 450 nm on an automatic plate reader.

Intra-assay coefficients of variation (CVs) of high and low-value quality controls were 2.3% and 5.2% for E1C and 6.4% and 8.4% for 3α,11β-dihydroxy-CM, respectively (n=16). While, inter-assay coefficients of variations(CVs) of high and low-value quality control were 6.3% and 9.4% for E1C and 10.2% and 11.4% for 3α,11β-dihydroxy-CM, respectively (n= 6).

Statistical Analysis

All hormone data were calculated as mass hormone per mass dried fecal weight. Prior to statistical analysis, data were tested for normality distribution (Shapiro-Wilk test). For experiment 1, the proportion of change in E1C and GCM levels relative to time 0 h (control) of preservation was calculated as $(a_n/x_n)*100$, where a_n is the nth sample value in each duration and x_n is the value at time 0 (control) of the nth sample. A paired-samples t-test was then used to determine which groups were significantly different from the control. For experiment 2, the proportionate change in E1C and GCM levels for each storage duration or time group (1,2,4,6,8,10, and 12 months) was calculated as $(a_n-x_n)/x_n*100$, where a_n is the nth sample value in each duration and x_n is the value at time point 0 of the nth sample. Friedman Repeated Measure ANOVA on ranks was used to determine which groups were significantly different from the control. For experiment 3, Friedman Repeated Measure ANOVA on ranks was used to analyze for a potential difference of E1C and GCM levels in each treatment group. Post-hoc analysis using the Wilcoxon signed rank test was then performed where applicable for experiments 2 and 3. For experiment 4, a two-way ANOVA was used to determine whether there was statistically significant difference in E1C and FGCM levels depending on fecal

263 preservation and solvent extraction. All statistical tests were two-tailed and statistical
264 significance was set to $\alpha = 0.05$. All analyses were conducted using SPSS 20.

265 **RESULTS**

266 ***Experiment 1. Testing the Effect of the Time Lag between Defecation and Fecal***
267 ***Preservation on E1C and GCM Levels***

268 Mean E1C and GCM levels in feces stored at ambient temperature remained stable
269 for up to 6 h. After that, E1C levels increased gradually (mean=13.42%; SEM=8.20%;
270 N=7) when kept at ambient temperature for 8 h and were significantly higher than control
271 samples after 10 h (mean=16.78%; SEM=5.30%; t=-3.165; N=7; p=0.019; Fig. 2a). Mean
272 GCM levels in feces kept at ambient temperature also stayed stable for a period of 6h, but
273 in contrast to E1C decreased afterward. After 8h, they had dropped by 13.7%
274 (mean=13.70%; SEM=9.00%; N=7) and were significantly lower than control samples
275 after 10h (mean=25.8%; SEM=10.04%; t = 2.573; p=0.049; Fig. 2b).

276

277 ***Experiment 2. Testing the Effect of Long-Term Storage of Feces in 80% MeOH at***
278 ***Ambient Temperature on E1C and GCM Levels***

279 Long-term storage of feces in 80% MeOH significantly affected the stability of
280 E1C (χ^2=16.292, N=8, p=0.023) and GCM levels (χ^2=20.292, N=8, p=0.005). Post hoc
281 analysis test showed that E1C levels differed significantly from 6 months of storage
282 onwards, with levels being elevated compared to controls at all time points between 6 and
283 12 months of storage (Fig. 3a). GCM levels from feces stored in 80% MeOH at ambient
284 temperature remained stable for only up to 2 months after which levels also statistically
285 increased compared to the controls (Fig 3b). For both E1C and GCM, the data also

indicated a high variation of change at each time point indicated by the high standard errors of the means (SEM´s) recorded.

Experiment 3. Testing the Effectiveness of Oven Drying at Different Temperatures on E1C and GCM Levels

Drying fecal samples at three different temperatures using an oven drying procedure did not result in temperature-dependent significantly differences in the levels of E1C (χ^2=1.273, N=14, p=0.529). (Fig. 4a). In contrast, GCM levels were significantly affected by the drying temperature (χ^2=13.86, N=14, p=0.001; Fig. 4b). Post hoc analysis showed that GCM levels in feces dried at 90^{O}C were significantly lower compared to those dried at 50^{O}C (Z=-3.108, N=14, p=0.002) and 70^{O}C (Z=-2.856, N=14, p=0.004). GCM levels from feces dried at 50^{O}C and 70^{O}C were not significantly different (Z=-0.094, N=14, p=0.925).

Experiment 4. Testing The suitability of Different Fecal Processing Techniques and Extraction Solvents

Mean (±SEM) E1C and GCM levels in feces preserved with different techniques and using three different extraction solvents are presented in Table 2. There was no significant interaction between the different fecal preservation techniques and extraction solvents for E1C levels (F(4,45)=0.045, N=6, p=0.996) and GCM levels (F (4,45)=0.314, N=6, p=0.867). Different fecal preservation techniques did not affect the levels of E1C (p=0.947) and GCM (p=0.896). Similar to the result for the fecal preservation techniques, different extraction solvents also did not affect the levels of E1C (p=0.911) and GCM (p=0.924).

DISCUSSION

This study demonstrates the importance of validating pre-analytical parameters related to the preservation, extraction, and storage of feces for hormone analysis prior to the application to a new species, in our case the crested macaque. The results show that samples can be kept at ambient temperature for some time, but that then the levels of both estrogen and glucocorticoid metabolites start to vary from control samples. The data also showed that fecal samples of crested macaques could be preserved in 80% MeOH without major effect for up to 6 months for E1C measurements, but only for up to 2 months for GCM measurements. We also show that oven drying is a suitable alternative to preserve fecal samples for subsequent hormone analysis, but that drying temperature influences the levels of GCM, but not of E1C. Finally, our results demonstrate that a combination of three different fecal preservation methods and three different extraction solvents generate similar results in terms of E1C and GCM levels measured from crested macaque feces providing alternative methodologies for fecal hormone processing that can be applied under remote field conditions.

Interestingly, the changes observed in the two hormone metabolites after six and two hours storage in ambient temperature respectively were in opposite direction. Whereas E1C levels increased over time, GCM levels decreased. The reason for the opposite effect are not clear, but are likely induced by fecal bacteria. It has been shown previously that a microbial transformation of steroid metabolites can occur within hours after defecation and that these are particularly caused by bacterial enzymes (Mostl et al. 1999, Wasser et al. 1988, Palme 2005) leading to chemical changes (e.g., oxidation, deconjugation) of the steroid metabolites (Hunt & Wasser, 2003), and thus to higher or

lower binding affinities of the altered metabolites to the antibody depending on the assay used (Mostl et al. 1999; Washburn & Millspaugh, 2002).

Our results, particularly those of GCM, were similar to those of a previous study in the Western lowland gorilla, *Gorilla gorilla gorilla*, where levels of GCM also gradually decreased over time when samples were stored at ambient temperature post-defecation, with the first significant decrease recorded after 6 h of storage (Shut et al. 2012). In contrast, studies on cattle, horses, and pigs (Mostl et al. 1999), and orangutans, *Pongo sp.*, (Muehlenbein et al. 2012), reported already earlier increases in glucocorticoid levels when samples were stored unpreserved at ambient temperature. The differences in the results obtained from previous studies might be caused by differences in diet and gut microflora among species, which may affect the chemical nature of the metabolites in a species-specific manner (Hunt & Wasser, 2003; Goymann, 2012), or by experimental treatments such as hormone assays used and steroid hormones measured. This stresses the need of species-specific as validation of sample treatment procedures, as well as separate validation of metabolites measured and antibodies used in the assays (Nugraha et al. 2016). From a practical point of view, our data suggest that crested macaque fecal samples can be kept for up to 8 hours unpreserved without taking the risk of significant alterations in estrogen and glucocorticoid metabolite levels. Thus, under normal conditions, samples can be transported to camp for further processing without having to keep samples cold during transport.

In addition to time to preservation, storage time is also known to affect the reliability of fecal steroid hormone metabolites even when a preservative is added (reference). Our data show that the levels of E1C and GCM in 80% MeOH remained stable for up to 4, and 2 months respectively, but increased significantly when stored for

longer. Changes in fecal estrogen (fE) and glucocorticoid (fGC) metabolite levels over time were also reported in the feces of baboons (*Papio cynocephalus*) stored in 95% ethanol at ambient temperature and at -20^{O}C (Khan et al. 2002). In that study, fE metabolite concentration increased by 122% at 90 days and fGC metabolite concentration increased by 92% at 120 days. In another study, fecal samples stored in ethanol at ambient temperature showed increased GCM concentrations of more than 1000% and 45% in African elephants (*Loxodonta africana*), and grizzly bear (*Ursus arctos horribilis*) after 4 months of storage (Hunt and Wasser 2003).

However, in contrast to these and our own study, long-term storage of orangutan feces in 80% ethanol for up to 9 months did not affect the levels of GCM and PdG (Nugraha et al. 2016). The reason for this might lie in the solvent used. In our experiment, we used methanol while the orang-utan samples were stored in ethanol. All other procedures applied were similar, such as the mass ratio of solvent-to-fecal sample of 8:1, and a complete suspension of the feces in the solvent during storage as well as the use of extraction methodology. The differences between these study are therefore again likely due to species differences and show that findings of such experiments should not be extrapolated from one species to the other. Thus, even when similar methods are applied this does not guarantee a similar outcome of results. As such, the effect of storing preserved samples in alcohol (MeOH or EtOH) for long-term periods on hormone metabolite concentrations should also be evaluated for each species if this is the method of choice for feces preservation.

Our results for the crested macaques also demonstrate a high inter-sample variation in the recorded change of individual samples in response to the storage, reflecting both increases and decreases to the same storage condition. The magnitude of this variation is

clearly higher than that recorded in other studies (e.g. Shutt et al. 2012, Nugraha et al 2016) where a similar experiment has been carried out. The reason for this is not clear, but the data suggest that storing fecal samples of crested macaques in alcohol even for periods not resulting in an overall significant change in hormone metabolite levels may introduce a level of inter-sample variation that is unwanted as it may affect results to a non-acceptable degree.

We also tested three different temperatures for drying fecal samples in a conventional oven. Our results show that levels of E1C in fecal samples dried at 50^{O}C, 70^{O}C, and 90^{O}C, did not differ significantly from each other. However, levels of GCM from fecal samples dried at 90^{O}C were significantly lower compared to levels of GCM from fecal samples dried at 50^{O}C and 70^{O}C. This finding suggests that glucocorticoid metabolites are more sensitive to high temperatures than estrogen metabolites. More generally, different steroid hormone metabolites seem to have different sensitivities to drying temperature. In a study on cheetahs, drying fecal samples using a solar oven and a conventional oven at 71^{O}C for 3 days resulted in alterations in the levels of steroid hormone metabolites except for androgens (Terio et al. 2002). At the same time, the effectiveness of drying seems to depend on the drying temperature which usually ranges from 40-80^{O}C (Ziegler and Wittwer 2005). There is thus obviously a trade-off between effectiveness of sample drying and sensitivity of the respective metabolite to heat. Determining the optimal drying temperature is thus important to avoid steroid metabolite loss or alteration.

Altogether, we have shown that when using the correct temperature, drying samples with a conventional propane gas oven is a useful and reliable method. This technique is particularly suitable for remote areas because it does not require electricity. Furthermore,

propane gas ovens can easily be found in many developing countries. The sample can then be stored in moisture-proof packages with desiccant before transported to the laboratory for hormone analysis (Ziegler & Wittwer 2005). Also, freeze-drying sample is a reliable method, but it needs electricity and the rather expensive freeze-dryer. However, where both methods are not easily feasible, it is nowadays also possible to extract samples still in the field straight after collection. Fecal extracts can be stored directly at ambient temperature as a liquid, dried, or stored in solid-phase extraction (SPE) cartridges without the risk of introducing a high level of variation (Shut et al. 2012; Kalbitzer & Heistermann2013). Given its suitability, field extraction is nowadays already widely used in wildlife studies (Murray et al. 2013; Rimbach et al. 2013; Kalbitzer et al. 2015). Our study now confirms the equal value of oven drying, freeze-drying and field extraction for hormone analysis of field samples.

Finally, we tested the reliability of different extraction solvents on steroid hormone measurements comparing laboratory grade alcohols (MeOH and EtOHO) to commercially available ones (propanol/isopropyl alcohol/rubbing alcohol). Extracting the samples with commercially available alcohol produced results that did not differ to those using high laboratory grade organic solvents for sample extraction. This suggests that commercial alcohol is a suitable alternative to the laboratory grade MeOH or EtOH usually used for fecal sample extraction. This is particularly good news for field workers in rural areas where commercial alcohol can easily be acquired in even small pharmacy stores. Moreover, this solvent is three times cheaper than the laboratory grade MeOH or EtOH.

In summary, our results provide important information for researchers interested in field endocrinology, particularly in the monitoring of female reproductive hormones and

in stress physiology, by demonstrating several options of fecal preservation, extraction, and storage methods that can be selected depending on the field site and laboratory condition. Particularly at the same time, they form the methodological bases for the non-invasive studies of estrogen and glucocorticoid metabolites in feces of crested macaques.

ACKNOWLEDGEMENTS

We are grateful to all team members of the Macaca Nigra Project at Tangkoko for supporting data collection. We thank Mrs. Andrea Heistermann for her valuable help during hormone training and analysis in the laboratory of endocrinology at the German Primate Centre, Göttingen, Germany and to the head of the laboratory of hormone analysis at the Faculty of Veterinary Medicine, Bogor Agricultural University for hormone measurement. We gratefully acknowledge the permission granted by the Directorate General of Forest Protection and Nature Conservation (PHKA) and the Department of the Conservation of Natural Resources (BKSDA) in Manado and Indonesian Institute of Sciences (LIPI) to conduct research in the Tangkoko Batuangus Nature Reserve. This research was funded by the Ministry of Research, Technology and Higher Education, Indonesia and SEAMEO BIOTROP, Bogor Indonesia (060.20/PSRP/SPK-PNLT/III/2014). The authors declare that they have no competing interest. All applicable international, national, and institutional guidelines for the care and use of animals were followed.

REFERENCES

452 Beehner JC, Whitten PL (2004) Modification of a field method for fecal steroid analysis
453 in baboons. Physiol Behav 82: 269-277
454 Bielohuby M, Popp S, Bidlingmaier M (2012) A guide for measurement of circulating
455 metabolic hormones in rodents: Pitfalls during the pre-analytical phase. Molecular
456 Metabolism. 1:47–60
457 Ganswindt A, Palma R, Heistermann M, Borragan S, Hodges JK (2003) Non-invasive
458 assessment of adrenocortical function in the male African elephant (*Loxodonta*
459 *africana*) and its relation to musth. Gen Com Endocrinol 134:156-166
460 Gholib (2011) Non-invasive hormone monitoring: faecal androgen and glucocorticoid in
461 male crested macaques (*Macaca nigra*) in relation to seasonal and social factors.
462 Thesis. Bogor Agricultural University
463 Goymann W (2012) On the use of non-invasive hormone research in uncontrolled, natural
464 environments: the problem with sex, diet, metabolic rate and the individual.
465 Methods Ecol Evol 3:757-765
466 Heistermann M, Hodges JK (1995) Endocrine monitoring of the ovarian cycle and
467 pregnancy in the saddle-black tamarin (*Saguinus fuscicollis*) by measurement of
468 steroid conjugate in urine. Am J Primatol35:117-127
469 Heistermann M, Palme R, Ganswindt A (2006) Comparison of different enzyme
470 immunoassays for assessment of adrenocortical activity in primates based on fecal
471 analysis. Am J Primatol 68:257-273
472 Higham JP, Heistermann M, Saggau C, Agil M, Perwitasari-Farajallah D, Engelhardt A
473 (2012) Sexual signaling in female crested macaques and the evolution of primate
474 fertility signals. BMC EvolBiol12: 89-95

475 Hodges JK, Heistermann M (2011) Field Endocrinology: Monitoring Hormonal Changes
476 in Free-Ranging Primates. In: Setchell JM, Curtis DJ (eds)Field and Laboratory
477 Methods in Primatology: A Practical Guide, 2nd edn. Cambridge University Press,
478 Cambridge, pp 353-370
479 International Union for Conservation of Nature (IUCN) (2017) Crested Macaques
480 (*Macaca nigra*). http://www.iucnredlist.org. Accessed 5 January 2017
481 Hunt KE, and Wasser SK (2003) Effect of long-term preservation methods on fecal
482 glucocorticoid concentrations of grizzly bear and African elephant.
483 PhysiolBiochem Zool 76 (6):918-928
484 Kalbitzer U, Heistermann M (2013) Long-term storage effects in steroid metabolite
485 extracts from baboon (Papio sp.) faeces - a comparison of three commonly applied
486 storage methods. Methods Ecol Evol 4 (5):493-500
487 Kalbitzer U, Heistermann M, cheney D, Seyfarth R, Fischer J (2015) Social behavior and
488 patterns of testosterone and glucocorticoid levels differ between male chacma and
489 *Guinea baboons*. Horm Behav 75:100-110
490 Khan MZ, Altmann J, Isani SS, Yu J (2002) A matter of time: evaluating the storage of
491 fecal samples for steroid analysis. Gen Comp Endocrinol 128: 57-64
492 Lynch JW, Khan MZ, Altmann J, Njahira MN, Rubenstein N (2003) Concentration of
493 four fecal steroids in wild baboons: short-term storage condition and consequences
494 for data interpretation. Gen Comp Endocrinol 132:264-271
495 Millspaugh JJ, Washburn BE (2004) Use of fecal glucocorticoid metabolite measures in
496 conservation biology research: considerations for application and interpretation.
497 Gen Comp Endocrinol 138:189-199

498 Möhle U, Heistermann M, Palme R, Hodges JK (2002) Characterization of urinary and
499 fecal metabolites of testosterone and their measurement for assessing gonadal
500 endocrine function in male nonhuman primates. Gen Comp Endocr 129:135–145
501 Möstl ES, Messmann EB, Robia C, Palme R (1999) Measurement of glucocorticoid
502 metabolite concentrations in faeces of domestic livestock. J Vet Med A 46 (10):621-
503 631
504 Muehlenbein MP, Ancrenaz M, Sakong R, Ambu L, Prall S, Fuller G, Raghanti MA
505 (2012) Ape conservation physiology: fecal glucocorticoid responses in wild Pongo
506 pygmaeus morio following human visitation. PLoS One 7(3):e33357
507 Murray CM, Heintz MR, Lonsdorf EV, Parr LA, Santymire RM (2013) Validation of a
508 field technique and characterization of fecal glucocorticoid metabolite analysis in
509 wild chimpanzees (*Pan troglodytes*). Am J Primatol 75:57-64
510 Neumann C, Assahad G, Hammerscmidt K, Farajallah DP, Engelhardt A(2010) Loud
511 calls in male Macaca nigra – a signal of dominance in a tolerant primate species.
512 Anim Behav 79: 187-193
513 Nugraha TP, Heistermann M, Agil M, Purwantara B, Supriatna I, Gholib G, van Schaik
514 CP, Weingrill T (2016) Validation of a field-friendly extraction and storage method
515 to monitor fecal steroid metabolites in wild orangutans. Primates 58 (2): 285-294
516 Palme R (2005) Measuring fecal steroids guidelines for practical application. Ann NY
517 Acad Sci 1046: 75-80
518 Palme R, Touma C, Arias N, Dominchin M, Lepschy M (2013) Steroid extraction: get
519 the best out of faecal samples. Wien Tierarztl Monatsschr 100: 238-246

Pettitt BA, Wheaton CJ, Waterman JM (2007) Effect of storage treatment on fecal steroid hormone concentrations of a rodent, the Cape ground squirrel (Xerus inauris). Gen Comp Endocrinol150: 1-11

Rimbach R, Heymann EW, Link A, Heistermann M (2013) Validation of an enzyme immunoassay for assessing adrenocortical activity and evaluation of factors that affect levels of fecal glucocorticoid metabolites in two New World primates. Gen Comp Endocrinol 191:13-23

Sheriff MJ, Dantzer B, Delehanty B, Palme R, Boonstra R (2011) Measuring stress in wildlife: techniques for quantifying glucocorticoids. Oecologia 166(4):869-887

Shutt K, Setchell JM, Heistermann M (2012) 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. Gen Comp Endocrinol179 (2):167-177

Sousa MBC, Ziegler TE (1998) Diurnal validation on the excretion patterns of fecal steroid in common marmoset (Callithrix jacchus) females. Am J Primatol 46:105-117

Terio KA, Brown JL, Moreland R, Munson L (2002) Comparison of different drying and storage methods on quantifiable concentrations of fecal steroids in the cheetah. Zoo Biol 21:215-222

Touma C, Palme R (2005) Measuring fecal glucocorticoid metabolites in mammals and birds: the importance of a biological validation. Ann NY Acad Sci 1046: 54–74.

Touma C, Sachser N, Möstl E, Palme R (2003) Effects of sex and time of day on metabolism and excretion of corticosterone in urine and feces of mice. Gen Comp Endocrinol 130: 267–278.

Washburn BE, Millspaugh JJ (2002) Effects of simulated environmental conditions on glucocorticoids metabolite measurements in white-tailed deer feces. Gen Comp Endocrinol 127:217-222

Wasser SK, Risler L, Steiner RA (1988) Excreted steroids in primate feces over the menstrual cycle and pregnancy. Biol Reprod 39:862-872

Weingrill T, Gray DA, Barrett L, Henzi SP (2004) Fecal cortisol levels in free-ranging female chacma baboons: relationship to dominance, reproduction state, and environmental factors. Horm Behav 45:259-269

Ziegler TE, Wittwer DJ (2005) Fecal steroid research in the field and laboratory: improved methods for storage, transport, processing, and analysis. Am J Primatol67 (1):159-174

Figure Legends

Fig. 1 Flow chart illustrating the experimental procedures of fecal preservation and solvent extraction.

Fig. 2 Proportion of change (mean±SEM) in (a) E1C and (b) GCM levels relative to control values (time 0, control) in feces kept for up to 10h at ambient temperature before being preserved. *$p<0.05$; $N=7$

Fig 3 Proportion of change (mean±SEM) in (a) E1C and (b) GCM levels relative to control values (samples extracted immediately, time 0) in fecal samples stored for up to 12 months in 80% methanol. *$p<0.05$, $N=8$

Fig. 4 Levels (mean ± SEM) of E1C (a) and GCM (b) dried with a conventional oven at 50^{O}C, 70^{O}C, and 90^{O}C. *$p< 0.05$, N=14

Table Legends

Table 1 Design of the different experiments conducted in this study and sample sizes for each

Table 2 Concentrations of GCM and E1C (mean, ±SEM) in fecal samples undergoing different preservation techniques and extracted from different solvents (N=6)