

# REPEATED FREEZE-THAW CYCLES BUT NOT SHORT-TERM STORAGE OF FECAL EXTRACTS AT AMBIENT TEMPERATURE INFLUENCE THE STABILITY OF STEROID METABOLITE LEVELS IN CRESTED MACAQUES

Gholib Gholib<sup>1,2,3,4</sup>, Muhammad Agil<sup>2\*</sup>, Iman Supriatna<sup>2</sup>, Bambang Purwantara<sup>2</sup>, Michael Heistermann<sup>5</sup>, and Antje Engelhardt<sup>4,6</sup>

<sup>1</sup>Laboratory of Physiology, Faculty of Veterinary Medicine, Syiah Kuala University, Banda Aceh, Indonesia

<sup>2</sup>Department of Clinic, Reproduction, and Pathology, Faculty of Veterinary Medicine, Bogor Agricultural University, Bogor, Indonesia

<sup>3</sup>Seameo Biotrop (Southeast Asian Regional Centre for Tropical Biology), Bogor, Indonesia

<sup>4</sup>Junior Research Group of Primate Sexual Selection, German Primate Center, Leibniz-Institute for Primate Research, Göttingen, Germany

<sup>5</sup>Endocrinology Laboratory, German Primate Center, Leibniz-Institute for Primate Research, Göttingen, Germany

<sup>6</sup>Faculty of Science, School of Natural Science and Psychology, Liverpool John Moores University, United Kingdom (UK)

\*Corresponding author: [rhinogil@gmail.com](mailto:rhinogil@gmail.com)

## ABSTRACT

The objective of this study was to examine the effects of repeated freeze-thaw cycles and short-term storage of fecal extracts at ambient temperature on the stability of fecal glucocorticoid (fGCM) and estrogen metabolite (fEM) levels from crested macaques. In total 100 aliquots of fecal extracts from fecal samples collected from female crested macaques (*Macaca nigra*) living at the Tangkoko-Batuangus Nature Reserve, North Sulawesi were used. We performed two different experiments: (1) An experiment to investigate if levels of fGCM and fEM measured from fecal extracts that were exposed to two, four, six and eight repeated freeze-thaw cycles (test groups) differ to control samples (i.e. fecal extracts always stored frozen); (2) An experiment to evaluate whether storing fecal extracts at ambient temperature for two, four, six, and eight days (test groups) affects the levels of fGCM and fEM compared to the control group (i.e. fecal extracts frozen immediately). Results showed that hormone levels were significantly increased ( $P < 0.05$ ) after four freeze-thaw cycles for fGCM and after eight freeze-thaw cycles for fEM. By contrast, there was no significant difference ( $P > 0.05$ ) in levels of fGCM and fEM between the test groups and the control group in fecal extracts stored at ambient temperature. In conclusion, our data show that more than two and six repeated freeze-thaw cycles should be avoided when measuring fGCM and fEM in crested macaque fecal extracts, respectively. We also demonstrate that storing fecal extracts at ambient temperature is possible for at least 8 days without taking a risk of affecting the stability of fGCM and fEM levels.

Key words: crested macaques, estrogens, fecal hormone metabolites, freeze-thaw cycles, glucocorticoids

## ABSTRAK

Penelitian ini bertujuan menguji stabilitas konsentrasi metabolit hormon glucocorticoid (fGCM) dan estrogen (fEM) dari ekstrak feses setelah mengalami proses pembekuan-pencairan berulang dan penyimpanan singkat pada suhu ruang. Seratus aliquot ekstrak feses yang diperoleh dari feses monyet hitam sulawesi (*Macaca nigra*) yang hidup di Cagar Alam Tangkoko-Batuangus, Sulawesi Utara digunakan pada penelitian ini. Dua percobaan yang berbeda telah dilakukan: (1) sebuah percobaan untuk menguji konsentrasi fGCM dan fEM dari ekstrak feses setelah mengalami dua, empat, enam dan delapan kali (kelompok perlakuan) proses pembekuan-pencairan berulang yang berbeda dengan kontrol (ekstrak feses yang selalu dalam keadaan beku); (2) sebuah percobaan untuk mengevaluasi penyimpanan ekstrak feses pada suhu ruang selama dua, empat, enam, dan delapan hari (kelompok perlakuan) terhadap konsentrasi fGCM dan fEM jika dibandingkan kontrol (ekstrak feses segera dibekukan). Hasil penelitian menunjukkan bahwa konsentrasi hormon secara nyata meningkat ( $P < 0,05$ ) setelah mengalami empat kali proses pembekuan-pencairan berulang untuk fGCM dan delapan kali proses pembekuan-pencairan berulang untuk fEM. Tidak ada perbedaan yang nyata antara konsentrasi fGCM dan fEM kelompok perlakuan dengan kelompok kontrol dari ekstrak feses yang disimpan pada suhu ruang ( $P > 0,05$ ). Kesimpulan, pembekuan-pencairan berulang pada ekstrak feses lebih dari dua dan enam kali harus dihindari untuk pengukuran konsentrasi fGCM dan fEM pada monyet hitam sulawesi. Penyimpanan ekstrak feses pada suhu ruang selama delapan hari tidak berpengaruh terhadap kestabilan konsentrasi fGCM dan fEM.

Kata kunci: monyet hitam sulawesi, estrogen, hormon metabolit, pembekuan-pencairan berulang, glukokortikoid

## INTRODUCTION

Non-invasive hormone measurements from feces have become an increasingly popular technique, especially for studying the physiology of wild or free-ranging animals (Hodges and Heistermann, 2011). The use of fecal samples provides several advantages over more traditional invasive methods such as using blood samples for hormone analysis. Feces can be easily obtained without disturbing the study animals and its collection does not put the animal in danger as no capture or restraint is required (Sheriff *et al.*, 2011). In addition, fecal hormone analysis may also provide a more accurate assessment of stress as it avoids a

potential bias caused by the increase of glucocorticoid levels as the result of capture or sedation (Millsbaugh and Washburn, 2004). Moreover, it also provides a feasible option for longitudinal studies such as monitoring of reproduction (Higham *et al.*, 2012). Therefore, the non-invasive approach is a preferable option for endocrinological studies in field research.

Feces of most vertebrate species contain metabolized forms of all major steroid hormones such as progestins (Engelhardt *et al.*, 2004), estrogens (Muren *et al.*, 2017), androgens (Ostner *et al.*, 2008), and glucocorticoids (Heistermann *et al.*, 2006). These metabolites are secreted via the bile into the gut and feces (Hodges and Heistermann, 2011) and can be

measured through hormone assay techniques. However, analyzing hormone metabolites from fecal samples is not as easy as it seems (Heistermann, 2010). There are several aspects concerned with pre-analytical factors that need to be considered prior to analysis, such as time of sample collection (Turriani *et al.*, 2016), handling/processing (Kalbitzer and Heistermann, 2013), preservation/storage (Shutt *et al.*, 2012; Nugraha *et al.*, 2016) and assay validation (Gholib *et al.*, 2016).

A critical issue for studies of non-invasive hormone analysis in wild animals is storage of fecal samples. The ideal storage method for fecal samples is by freezing them at  $-10^{\circ}$  C or lower (Hodges and Heistermann, 2011) because this can minimize degradation of fecal steroid by fecal bacteria (Goymann, 2012). However, freezing the samples is difficult to achieve when the study is conducted in remote locations with no or only limited access to electricity. To overcome this limitation, researchers used alternative preservation techniques, such as storing the fecal samples in alcohol or by drying them using oven or silica (Khan *et al.*, 2002; Pettitt *et al.*, 2007). However, this was not always successful as hormone levels still showed alterations after long-term storage. To overcome this problem, other researchers extracted the fecal samples immediately after collection using a “field friendly extraction“ method (Shutt *et al.*, 2012; Kalbitzer and Heistermann, 2013; Nugraha *et al.*, 2016). Ziegler and Wittwer (2005) assumed that once the hormone metabolites are extracted from the feces, the levels will remain stable even if the extracts are stored at ambient temperature.

Freeze-thaw cycles, repeated freezing and re-thawing, is one of the issues for the sample storage that may alter hormone levels. Bielohuby *et al.* (2012) stated that storage temperatures and the number of freeze and re-thawing cycles can be critical for accurate measurement of metabolic hormones. Previous studies reported that freeze-thaw cycles can indeed influence the stability of hormone levels. For example, in gelada baboons (*Theropithecus gelada*), hormone levels were significantly decreased in fecal extracts after being exposed to two freeze-thaw cycles for glucocorticoid metabolites (GCs) and six freeze-thaw cycles for testosterone (T) metabolites (Pappano *et al.*, 2010). Similarly, a study in rodents showed that serum insulin was decreased after ten freeze-thaw cycles (Bielohuby *et al.*, 2012). In contrast, in human serum and plasma, three cycles of freezing and re-thawing had no effect on the hormone levels (androstenedione, follicle-stimulating hormone/FSH, luteinizing hormone/LH, progesterone, estrone, estradiol, and testosterone), even after 6 or 10 cycles, the hormone levels changed less than 5% (Comstock *et al.*, 2001). Generally, these data suggest that frequent freezing and re-thawing cycles should be avoided (Bielohuby *et al.*, 2012). However, in developing countries power outages are frequently occurring and often result in unintended freeze-thaw cycles. Therefore, it is important to evaluate the effects of freeze-thaw cycles for hormone metabolite measurements from fecal extracts of crested ma-

caques, because so far there are no data available on this issue.

As part of a study to monitor the stress physiology and female reproductive state of wild crested macaques (*Macaca nigra*), we conducted pre-analytical experiments to test the stability of glucocorticoid and estrogen metabolites in crested macaque fecal extracts subjected to repeated freeze-thaw cycles and short-term storage at ambient temperatures. For this purpose, we collected fecal samples from females of crested macaques, the most endangered endemic macaque from Sulawesi, Indonesia. This species has been categorized as critically endangered (IUCN Red List, 2017) with the current population consisting of only about 2000 individuals in their natural habitat (Palacios *et al.*, 2012). Providing a methodological basis for fecal storage for hormone analysis is useful to support further studies in this species, particularly those aiming to monitor stress physiology and female reproductive state as part of conservation related efforts. The specific objectives of this study were to examine the stability of levels of fecal glucocorticoid metabolites (fGCM) and fecal estrogen metabolites (fEM) in the fecal extracts of crested macaques that were exposed to repeated freeze-thaw cycles for up to 8 times and stored at room temperature for up to 8 days.

## MATERIALS AND METHODS

### Collection of Fecal Samples

All fecal samples analyzed in this study were collected from wild crested macaques living in their natural habitat at Tangkoko-Batuangus Nature Reserve, North Sulawesi ( $1^{\circ}33'$  N,  $125^{\circ}10'$  E). Fecal samples were collected from 6 adult females. All individuals studied were from a group called Rambo 1. This group has previously been studied by Neumann *et al.* (2010) and was therefore well habituated and the studied individuals could be recognized individually. Fecal samples were collected opportunistically shortly after defecation. All samples were homogenized using a wooden stick, a dried small branch. Afterward, seeds and indigestible fibers were removed. Finally, fecal samples were placed into a tube and frozen immediately at  $-20^{\circ}$  C in the field camp. All samples were finally transported to the Hormone Laboratory at the Bogor Agricultural University using a cool box containing commercial ice packs (thermafreeze®).

### Lyophilisation and Pulverization of Fecal Samples

Prior to hormone extraction, fecal samples were lyophilised and pulverized according to the procedure described by Gholib (2011). In brief, fecal samples were lyophilised for 72 hours using a freeze dryer machine (Christ®, Gamma 1-20) at a temperature of  $-40^{\circ}$  C and a vacuum pressure of 1.030-0.630 mbar. Afterwards, 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, the fecal powder was put in a tube and stored at  $-20^{\circ}$  C until extracted.

### Fecal Steroid Extraction

Fecal samples were extracted according to the procedure described by Gholib (2011). An aliquot of about 50 mg of fecal powder (exact weight was recorded) of each sample was extracted in 3 ml of 80% methanol (MeOH) in water. The fecal-methanol suspension was vortexed for 10 minutes in a 15 ml plastic centrifuge tube using a multi-tube vortexer (Multi-Tube Vortexer, SMI<sup>®</sup>, USA). Following centrifugation at 3000 rpm for 10 minutes, the supernatant was decanted into 2 ml microcentrifuge tubes. Fecal extracts were then processed depending on the experiment performed (see below).

### Experiment 1 - Testing How Many Times Fecal Extracts can be Thawed and Refrozen

To test the effects of freeze-thaw cycles of fecal extracts on the stability of fecal steroid hormone levels, 10 fecal samples were extracted as described above. Each fecal extract was then divided into 5 aliquots and filled into 1.5 ml Eppendorf Safe-Lock tubes (total 50 aliquots; 0.5 ml per tubes), closed tightly and sealed with parafilm to minimize the risk of evaporation. All tubes with fecal extracts were subsequently stored frozen at -20° C. Later, those aliquots were subjected to the following treatments: (i) aliquots remained frozen at -20° C until the time of hormone analysis, control group (N= 10); and (ii) for test groups, aliquots were exposed to freeze-thaw cycles for two, four, six, and eight times (N= 10 for each group) (illustrated in Figure 1, Table 1). Fecal extracts were thawed for six hours by placing the tube in a room without an air conditioner. The average temperature during this study was 27.6±0.7° C (range 26.7-28.7° C). Afterwards, fecal extracts were refrozen for 24-28 hours prior to rethawing. After all freeze-thaw cycles were completed, all fecal extracts were assayed together for measuring the levels of fGCM and fEM (see hormone analysis). Note that all fecal extracts had to be thawed prior to hormone analysis. However, for this experiment, the number of freeze-thaw cycles was counted when fecal extracts were refrozen (i.e. 0 times for the control group and 2, 4, 6, 8 times for the test groups).

### Experiment 2 - Testing Short-Term Storage of Fecal Extracts at Ambient Temperature

To test the effect of short-term storage of fecal extracts at ambient temperature (AT) on the stability of fecal steroid hormone metabolites, 10 fecal samples were extracted as described above. Each fecal extract was then divided into 5 aliquots and filled into 1.5 ml Eppendorf Safe-Lock tubes (total 50 aliquots; 0.5 ml per tubes), closed tightly and sealed with parafilm to minimize the risk of evaporation. After loading fecal extracts into the tubes, those aliquots were subjected to the following treatments: (i) aliquots were frozen immediately at -20° C which served as a control group (N=10); and (ii) for test groups, aliquots were stored at AT for two, four, six and eight days (N= 10 for each group; Table 1). The average temperature during this study was 26.9±1.2° C (range 24.2-28.0° C). After all treatments had been performed, all fecal extracts were assayed together for measuring the levels of fGCM and fEM (see hormone analysis).

### Hormone Analysis

Determination fecal hormone metabolite concentrations was performed using a direct competitive enzyme immunoassay technique. For fEM analysis, an estrone conjugate (E1C) assay was used. This assay has been previously used successfully for assessing female reproductive status in crested macaques (Higham *et al.*, 2012) and other macaque species (O'Neill *et al.*, 2004). Hormone analysis was performed as described by Heistermann and Hodges (1995). Prior to analysis, fecal extracts were diluted in assay buffer (dilution 1:20 in 0.04 M PBS pH7.2). In brief, 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/50 µl) on microtitreplates coated with sheep anti-rabbit IgG. Afterwards, 50 µl phosphatase-labelled estrone-3-glucuronide and 50 µl antiserum 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 phosphatase substrate solution (Sigma 104; 20 mg in 16 ml substrate

**Table 1.** Experiments performed and samples sizes for each

	N	
	fGCM	fEM
<b>Experiment 1</b>		
Number of freeze-thaw cycles of fecal extracts		
Control (0 time, always frozen)	10	10
2 times of freeze-thaw cycles	10	10
4 times of freeze-thaw cycles	10	10
6 times of freeze-thaw cycles	10	10
8 times of freeze-thaw cycles	10	10
<b>Experiment 2</b>		
Time fecal extracts were stored at ambient temperature		
Control (frozen immediately)	10	10
2 days	10	10
4 days	10	10
6 days	10	10
8 days	10	10

buffer, pH 9.8 containing 1 M diethanolamine and 0.1 M MgCl<sub>2</sub>) was added to each well. The plates were again incubated for 30-45 minutes by shaking in the dark at room temperature depending on the color change. Finally, absorbance was measured at 405 nm on an automatic plate reader.

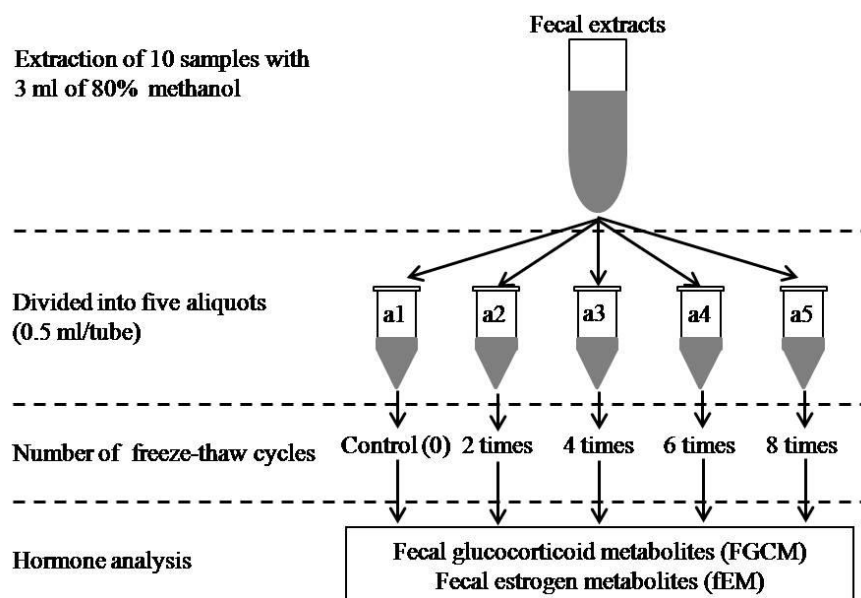
For fGCM analysis, an assay for the measurement of 11 $\beta$ -hydroxyetiocholanolone (11 $\beta$ -hydroxy-CM), a major metabolite of cortisol in macaque feces (Heistermann *et al.*, 2006; Girard-Buttoz *et al.*, 2009) was used. This assay has been previously validated for the use in crested crested macaques (Gholib, 2011). Hormone analysis was performed as described by Gholib (2011). Prior to analysis, fecal extracts were diluted in assay buffer (dilution 1:100 in 0.04 M PBS pH 7.2). Duplicate 50  $\mu$ l aliquots of diluted extracts were assayed along with 50  $\mu$ l aliquots of blank, zero and standard (0.6-78 pg/50  $\mu$ l) on microtitreplates. Afterwards, 50  $\mu$ l biotin-labelled hormone and 50  $\mu$ l antibody were added to each well and the mixture incubated overnight at 4 $^{\circ}$  C. Following incubation, the plates were washed four times with PBS washing solution (containing 0.05% Tween 20), blotted dry, and 150  $\mu$ l (6 ng) of streptavidin-peroxidase (S-5512, supplied by 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 were washed again four times. A substrate solution (150  $\mu$ l, containing 0.025% tetramethylbenzidine and 0.05% H<sub>2</sub>O<sub>2</sub>) was then added to each

well. The plates were again incubated in the dark at RT for 30-45 min depending on the color change. Finally, the enzyme reaction was stopped by adding 50  $\mu$ l 2M H<sub>2</sub>SO<sub>4</sub> into each well. 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 2.1% and 2.3% for E1C and 9.4% and 9.2% for 11 $\beta$ -hydroxy-CM, respectively (n=16). Inter-assay coefficients (CVs) of variation of high and low value quality control were 3.2% and 2.9% for E1C and 10.7% and 9.3% for 11 $\beta$ -hydroxy-CM, respectively (n=4).

### Data Analysis

For experiment 1 and 2, mean fEM and fGCM levels were calculated for each group. Afterwards, the percentage change in fEM and fGCM levels relative to the levels of the respective controls was calculated as  $(a_n - x_n)/x_n * 100$  within each sample set, where  $a_n$  is the  $n$ th sample value in each experimental group and  $x_n$  is the control value of the  $n$ th sample. Friedman Repeated Measure Anova on ranks was used to determine whether tests groups overall differed from the control group. Post-hoc analysis using the Wilcoxon signed rank test was then performed to assess which of the test groups differed significantly from the control group. All statistical tests were two-tailed and statistical significance was set at  $\alpha = 0.05$ . All analyses were performed using SPSS 20.0 for Windows (SPSS, Inc., Chicago, IL).



**Figure 1.** Flow chart illustrating the procedures of the freeze-thaw cycle experiment

**Table 2.** Levels of fGCM and fEM (mean $\pm$ SEM) from fecal extracts exposed to repeated freeze-thaw cycles

Number of freeze-thaw cycles	Hormone levels (ng/g dry feces)	
	fGCM	fEM
Control (0)	601.55 $\pm$ 69.08	214.33 $\pm$ 39.89
2	620.57 $\pm$ 69.68	218.58 $\pm$ 40.10
4	650.64 $\pm$ 77.42*	225.71 $\pm$ 42.22
6	678.06 $\pm$ 72.15**	231.41 $\pm$ 42.89
8	655.51 $\pm$ 72.95*	232.13 $\pm$ 42.36**

\* indicates a significant difference to the control group which \* = P<0.05 and \*\* = P<0.01

## RESULTS AND DISCUSSION

**Experiment 1 - Testing How Many Times Fecal Extracts can be Thawed and Refrozen**

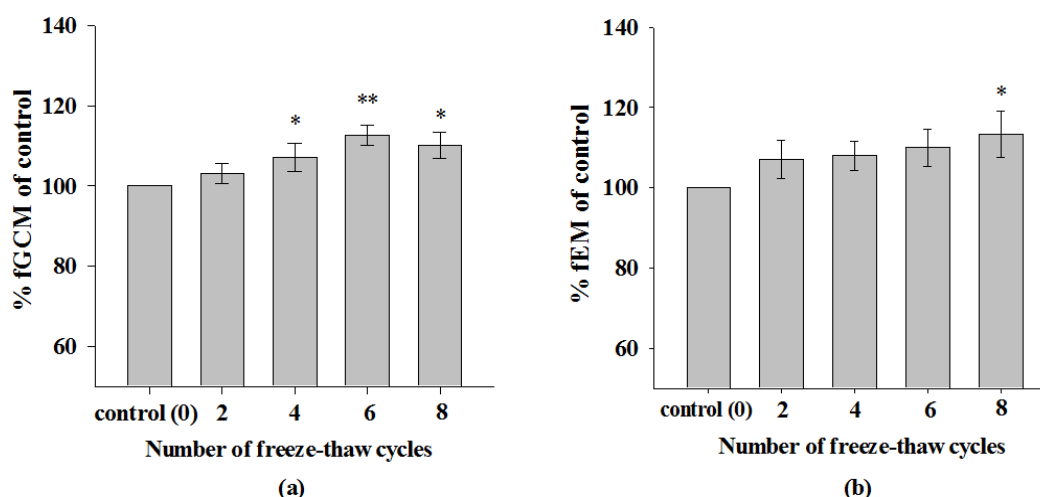
Fecal extracts exposed to repeated freeze-thaw cycles showed increased levels of both fGCM and fEM (Table 2). For fGCM, there was a significant difference in levels between test groups and the control group ( $\chi^2(4)=16.412$ ,  $P<0.01$ ). Post hoc analysis showed that the observed increase in fGCM levels was statistically significant when fecal extracts were exposed to four ( $Z=-2.191$ ,  $P<0.05$ ), six ( $Z=-2.803$ ,  $P<0.01$ ), and eight ( $Z=-2.090$ ,  $P<0.05$ ) freeze-thaw cycles (Table 2). There was not a significant difference in fGCM levels of fecal extracts exposed to two freeze-thaw cycles compared to the control ( $P>0.05$ ). On average, the percentage changes of fGCM levels were  $3.96\pm 2.51\%$ ,  $7.61\pm 3.31\%$ ,  $13.73\pm 2.60\%$ , and  $9.58\pm 3.12\%$  relative to the control after exposure to two, four, six and eight freeze-thaw cycles, respectively (Figure 2a).

There was also a significant difference in the levels of fEM between the test groups and the control group ( $\chi^2(4)=10.051$ ,  $P<0.05$ ). Post hoc analysis revealed that a significant increase in fEM levels was observed only for fecal extracts exposed to eight freeze-thaw cycles ( $Z=-2.803$ ,  $P<0.01$ , Table 2) with differences in fEM levels between the control group and fecal extracts exposed to two, four, and six freeze-thaw cycles being not significant ( $P>0.05$ ). On average, the percentage changes of fEM levels were  $7.03\pm 4.66\%$ ,  $7.99\pm 3.61\%$ ,  $10.02\pm 4.68\%$ , and  $13.30\pm 5.69\%$  relative to the control group after exposure to two, four, six and eight freeze-thaw cycles, respectively (Figure 2b).

Based on these results, the percentage changes in fGCM and fEM levels in all test groups were always less than 15% compared to the control group. However, the effects of freeze-thaw cycles were more pronounced for fGCM compared to fEM. Fecal extracts can be exposed for up to two freeze-thaw cycles for fGCM and up to six freeze-thaw cycles for fEM, suggesting that fEM are more resilient to freeze-thaw cycles than fGCM.

The reason why hormone levels increased after exposure to repeated freeze-thaw cycles (Figure 2a, 2b) is not entirely clear. It is possible, however, that fecal extracts left at high temperature ( $27.6\pm 0.7^\circ\text{C}$ ) during the thawing process are subject to an increased degradation of hormone metabolites that resulted in more metabolites cross-reacting with the antibody of the respective assay used (Pappano *et al.*, 2010). Alternatively or in addition, repeated freeze-thaw cycles may result in an increased evaporation risk of the alcoholic solvent, despite the fact that extracts were stored in save-lock tubes. However, high temperature differences between the freezer and environment may facilitate evaporation due to a higher gas pressure (Kalbitzer and Heisterman, 2013). This could lead to the fecal extracts to be more concentrated which consequently would result in higher metabolite concentrations.

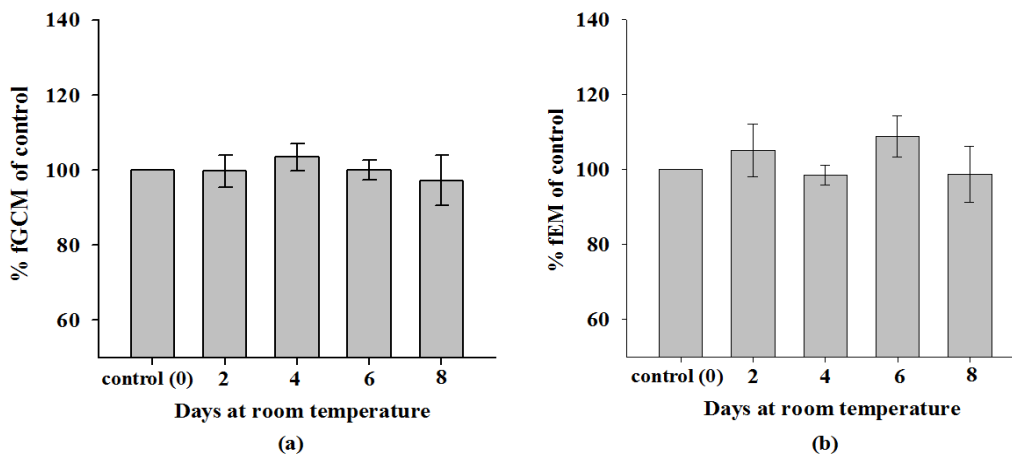
A similar study in gelada baboons (*Theropithecus gelada*) showed that fecal extracts stored on Sep-Pak Plus C18 (SPE) cartridges can be exposed to at least one freeze-thaw cycle for glucocorticoid metabolites (GCs) and up to four freeze-thaw cycles for testosterone (T) metabolites (Pappano *et al.*, 2010). In



**Figure 2.** Percentages of hormone levels in fecal extracts exposed to repeated freeze-thaw cycles in comparison to control. a= for fGCM, b= for fEM. Values represent mean $\pm$ SEM relative to control (= 100%). A significant difference is indicated by: \*=  $P<0.05$  and \*\* =  $P<0.01$  compared to the control group

**Table 3.** Levels of fGCM and fEM (mean $\pm$ SEM) from fecal extracts stored at ambient temperature for several days

Days at room temperature	Hormone concentrations (ng/g dry feces)	
	fGCM	fEM
Control (0)	613.51 $\pm$ 78.62	217.17 $\pm$ 38.80
2	592.46 $\pm$ 68.27	220.60 $\pm$ 37.80
4	629.86 $\pm$ 76.32	215.54 $\pm$ 39.89
6	620.07 $\pm$ 79.55	231.78 $\pm$ 40.35
8	631.28 $\pm$ 93.31	216.26 $\pm$ 41.35



**Figure 3.** Percentages of hormone levels of fecal extracts stored at ambient temperature for several days in comparison to control. a= for fGCM, b= for fEM. Values represent mean±SEM relative to control (= 100%)

contrast to our study, repeated freeze-thaw cycles in the study of Pappano *et al.* (2010) showed significantly decreased levels of hormone content for both GC and T metabolites. There are several reasons that might explain the different findings between our experiment and those of this previous study. First, different assays were used for GC measurements. Our experiment used a group-specific measurement of 5 $\beta$ -reduced cortisol metabolites using an 11 $\beta$ -hydroxyetiocholanolone EIA that has been previously validated by Gholib (2011) and used successfully for other primate species (e.g. Girard-Buttoz *et al.*, 2009; Nugraha *et al.*, 2016), while Pappano *et al.* (2010) used a commercial corticosterone radioimmunoassay. Second, our experiment used save-lock (Eppendorf®) tubes for storing fecal extracts, while the previous study used SPE cartridges for storing the fecal extracts. Finally, for thawing, we left the fecal extract at room temperature to thaw for 6 h, whereas in the previous study, samples were thawed by incubating them at 23° C for 24 hours. These differences in treatments might potentially lead to a different degree of chemical changes (e.g., oxidation, deconjugation) of the steroid metabolites (Hunt and Wasser, 2003), resulting in different 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). Moreover, different species have different diets and gut microflora which may affect potential degradation and the chemical nature of the metabolites in a species-specific manner (Hunt and Wasser, 2003; Goymann, 2012). Different assay antibodies may be more or less prone to detect these changes (Nugraha *et al.*, 2016).

From a practical point of view, our results suggest that repeated freeze-thaw of fecal extracts may compromise the stability of fGCM and fEM levels, although the changes in hormone metabolite levels recorded were relatively minor, i.e. not exceeding 15%. Thus, circumstances that may potentially cause repeated freeze-thaw cycles of samples designated for hormone analysis, such as frequent power outages in developing countries, should be avoided. This can easily be achieved, for example, by installing in each

laboratory a backup generator to supply electricity when there are power outages. In addition, each freezer should contain a blanket of ice packs, either commercially available ice packs or artificial ice packs, to maintain the temperature inside the freezer for several more hours during the power outage. Using an aliquot of fecal extract for several analyses at different times should be also averted or, at least, kept to a minimum. However, if several analyses should be performed from the same sample extract, this could be divided into several aliquots before freezing so that freeze-thaw cycles can be kept to a minimum number.

### Experiment 2 - Testing Short-Term Storage of Fecal Extracts at Ambient Temperature

The average fGCM and fEM levels for the test groups were slightly higher (Table 3) compared to the levels of the control group, but the differences were not statistically significant (fGCM:  $\chi^2(4)=0.904$ ,  $P>0.05$ ; fEM:  $\chi^2(4)=4.490$ ,  $P>0.05$ ). In relation to the control group, fGCM and fEM levels of the test groups changed between -1.93% to 4.49% and -1.51% to 8.84%, respectively (Figure 3a and 3b).

Levels of fGCM and fEM from fecal extracts stored at higher temperature (27.4±0.6° C) thus remained stable for up to 8 days (the maximum time tested in this experiment), with changes in levels varying no more than ±10% from the controls. This indicates that short-term storage of fecal extracts at ambient temperature is unlikely to induce alterations of fecal hormone metabolites which is in contrast to storing unprocessed feces in alcohol (Lynch *et al.*, 2003). Therefore, for short-term or even long-term fecal preservation, it is highly recommended to extract the feces as soon as possible after its collection and store the fecal extracts rather than storing the whole unprocessed feces in alcohol when feces cannot be frozen immediately (Kalbitzer and Heisterman, 2013).

Previous studies in primates reported that the stability of steroid metabolites in fecal extracts stored at ambient temperature varies between species. In baboons (*Papio sp.*), levels of estradiol, progesterone, and testosterone metabolites from fecal extract stored on SPE cartridges for up to 40 days were stable, but glucocorticoid levels

decreased slightly (Beehner and Whitten, 2004). A similar study in gelada baboons showed that fecal extracts stored on SPE cartridges were stable for up to 3 weeks for glucocorticoid and testosterone metabolites (Pappano *et al.*, 2010). However, a study conducted in lowland gorillas (*Gorilla gorilla gorilla*) by Shutt *et al.* (2012) showed that levels of glucocorticoids in fecal extracts were stable for up to 1 year when stored as a liquid in polypropylene tubes at 22° C. Although, our experiment tested only a brief period of up to 8 days of storing fecal extracts at ambient temperature it confirms that for shipping fecal extracts from the field site to the laboratory or from one laboratory to another laboratory, no freezing of the samples during transport is required. However, to keep the environmental temperature low during transport and thus help to keep any potential risk of alterations in hormone metabolite content low, the use of commercial ice pack, such as gel ice packs (e.g., thermafreeze, blue gel etc.) is nevertheless recommended. Further experiments are, however, needed to test for how long fecal extracts of crested macaques can be stored at ambient temperature without taking a risk of inducing changes to the hormone metabolites of interest.

## CONCLUSION

Fecal extracts exposed to repeated freeze-thaw cycles showed increased levels of fGCM and fEM with the effect being more pronounced for fGCM. By contrast, storing fecal extracts at higher room temperature for up to 8 days did not affect the stability of fGCM and fEM levels.

## ACKNOWLEDGMENTS

We acknowledge all team members of the Macaca Nigra Project at Tangkoko Nature Reserve for supporting data collection. We thank Mrs. Andrea Heistermann for her valuable help during hormone training and analysis in the Endocrinology Laboratory at the German Primate Centre, Göttingen, Germany. 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 the recommendation by the Indonesian Institute of Sciences (LIPI) to conduct research in the Tangkoko Batu Angus Nature Reserve. This research was supported by the Ministry of Research, Technology and Higher Education, Republic of Indonesia through Penelitian Disertasi Doktor 2017 (No:105/SP2H/LT/DPRM/IV/2017) and Research Grant of SEAMEO BIOTROP, Bogor, Indonesia, DIPA 2014 (No:060.20/PSRP/SPK-PNLT/III/2014).

## REFERENCES

Beehner, J.C. and P.L. Whitten. 2004. Modification of a field method for fecal steroid analysis in baboons. **Physiol. Behav.** 82:269-277.  
 Biellohuby, M., S. Popp, and M. Bidlingmaier. 2012. A guide for measurement of circulating metabolic hormones in rodents: Pitfalls during the pre-analytical phase. **Molecular Metabolism.** 1:47-60.

Comstock, G.W., A.E. Burke, E.P. Norkus, G.B. Gordon, S.C. Hoffman, and K.J. Helzsouer. 2001. Effects of repeated freeze-thaw cycles on concentration of cholesterol, micronutrients, and hormone in human plasma and serum. **Clinical Chemistry.** 47(1):139-142  
 Engelhardt, A., J.B. Pfeifer, M. Heistermann, C. Niemitz, J.A.R.A.M. van Hooff, and J.K. Hodges. 2004. Assessment of female reproductive status by male long-tailed macaques (*Macaca fascicularis*) under natural conditions. **Anim. Behav.** 67:915-924.  
 Gholib. 2011. Non-Invasive Hormone Monitoring: Faecal Androgen and Glucocorticoid in Male Crested Macaques (*Macaca nigra*) in Relation to Seasonal and Social Factors. **Thesis.** Faculty of Veterinary Medicine Bogor Agricultural University. Bogor.  
 Gholib, G., S. Wahyuni, O.K. Kadar, M. Adam, T.M. Lubis, Azhar, M. Akmal, T.N. Siregar, T. Armansyah, and T.P. Nugraha. 2016. Measurement of serum testosterone in kacang goat by using enzyme-linked immunosorbent assay (ELISA) technique: The importance of kit validation. **J. Ked. Hewan.** 10(1):33-36.  
 Girard-Buttoz, G., M. Heistermann, S. Krummel, and A. Engelhardt. 2009. Seasonal and social influences on fecal androgen and glucocorticoid excretion in wild male long tailed macaques (*Macaca fascicularis*). **Physiol. Behav.** 30:8-21.  
 Goymann, W. 2012. On the use of non-invasive hormone research in uncontrolled, natural environments: the problem with sex, diet, metabolic rate and the individual. **Methods Ecol. Evol.** 3(4):757-765.  
 Heistermann, M. 2010. Non-invasive monitoring of endocrine status in laboratory primates: Methods, guidelines, and applications. **Adv. Sci. Res.** 1:1-9.  
 Heistermann, M. and J.K. Hodges. 1995. Endocrine monitoring of the ovarian cycle and pregnancy in the saddle-black tamarin (*Saguinus fuscicollis*) by measurement of steroid conjugate in urine. **Am. J. Primatol.** 35:117-127.  
 Heistermann, M., R. Palme, and A. Ganswindt. 2006. Comparison of different enzymeimmunoassays for assessment of adrenocortical activity in primates based on fecal analysis. **Am. J. Primatol.** 68:257-273.  
 Hodges, J.K. and M. Heistermann. 2011. Field Endocrinology: Monitoring Hormonal Changes in Free-Ranging Primates. **In Field and Laboratory Methods in Primatology: A Practical Guide.** Setchell J.M. and D.J. Curtis (Eds.). Cambridge University Press, Cambridge.  
 Higham, J.P., M. Heistermann, C. Saggau, M. Agil, D.P. Farajallah, and A. Engelhardt. 2012. Sexual signalling in female crested macaques and the evolution of primate fertility signals. **BMC Evolutionary Biol.** 12:89.  
 Hunt, K.E. and S.K. Wasser. 2003. Effect of long-term preservation methods on fecal glucocorticoid concentrations of grizzly bear and African elephant. **Physiol. Biochem. Zool.** 76(6):918-928.  
 IUCN. International Union for Conservation of Nature. 2017. Crested Macaques (*Macaca nigra*). <http://www.iucnredlist.org>.  
 Kalbitzer, U. and M. Heistermann. 2013. Long-term storage effects in steroid metabolite extracts from baboon (*Papio sp.*) faeces - a comparison of three commonly applied storage methods. **Methods Ecol. Evol.** 4(5):493-500.  
 Khan, M.Z., J. Altmann, S.S. Isani, and J. Yu. 2002. A matter of time: Evaluating the storage of fecal samples for steroid analysis. **Gen. Comp. Endocrinol.** 128:57-64.  
 Lynch, J.W., M.Z. Khan, J. Altmann, M.N. Njahira, and N. Rubenstein. 2003. Concentration of four fecal steroids in wild baboons: short-term storage condition and consequences for data interpretation. **Gen. Comp. Endocrinol.** 132:264-271.  
 Millsbaugh, J.J. and B.E. Washburn. 2004. Use of fecal glucocorticoid metabolite measures in conservation biology research: considerations for application and interpretation. **Gen. Comp. Endocrinol.** 138:189-199.  
 Möstl, E., S. Messmann, E. Bagu, C. Robia, and R. Palme. 1999. Measurement of glucocorticoid metabolite concentrations in faeces of domestic livestock. **J. Vet. Med. A.** 46(10):621-631.  
 Muren, S. Kusuda, O. Doi, H. Naito and H. Hashikawa. 2017. Puberty, ovarian cycle, pregnancy, and postpartum ovulation in captive Sichuan golden monkeys (*Rhinopithecus roxellana*) based on changes in urinary and fecal gonadal steroid metabolites. **Theriogenology.** 87:179-186.  
 Neumann, C., G. Assahad, K. Hammerschmidt, D.P. Farajallah, and A. Engelhardt. 2010. Loud calls in male crested macaques, *Macaca nigra*: A signal of dominance in atolerant species. **Anim. Behav.** 79:187-193.

- Nugraha, T.P., M. Hesitermann, M. Agil, B. Purwantara, I. Supriatna, G. Gholib, C.P. van Schaik, T. Weingrill. 2016. Validation of a field-friendly extraction and storage method to monitor fecal steroid metabolites in wild orangutans. **Primates**. DOI:10.1007/s10329-016-0583-6.
- O'Neill, A.C., L.M. Fedigan, and T.E. Ziegler. 2004. Relationship between ovarian cycle phase and sexual behavior in female Japanese macaques (*Macaca fuscata*). **Am. J. Phys. Antropol.** 125:352-362.
- Ostner, J., P.M. Kappeler, and M. Heistermann. 2008. Androgen and glucocorticoid levels reflect seasonally occurring social challenges in male red fronted lemurs (*Eulemur fulvus rufus*). **Behav. Ecol. Sociobiol.** 62:627-638.
- Palacios, J.F.G., A. Engelhardt, M. Agil, J.K. Hodges, R. Bogia, and M. Waltert. 2012. Status of, and conservation recommendations for, the critically endangered crested black macaque *Macaca nigra* in Tangkoko, Indonesia. **Oryx**. 46:290-297.
- Pappano, D.J., E.K. Roberts, and J.C. Beehner. 2010. Testing extraction and storage parameters for a fecal hormone method. **Am. J. Primatol.** 72:934-941.
- Pettitt, B.A., C.J. Wheaton, and J.M. Waterman. 2007. Effect of storage treatment on fecal steroid hormone concentrations of a rodent, the Cape ground squirrel (*Xerus inauris*). **Gen. Comp. Endocrinol.** 150:1-11.
- Sheriff, M.J., B. Dantzer, B. Delehanty, R. Palme, and R. Boonstra R. 2011. Measuring stress in wildlife: techniques for quantifying glucocorticoids. **Oecologia**. 166(4):869-887.
- Shutt, K., J.M. Setchell, and M. Heistermann. 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. Endocrinol.** 179(2):167-177.
- Turriani, M., N. Bernabo, B. Barboni, G. Todisco, L. Montini, and P. Berardinelli. 2016. Circadian rhythm and stress response in droppings of *Serinus canaria*. **Vet. Med. Int.** DOI:10.1155/2016/3086353.
- Washburn, B.E. and J.J. Millspaugh. 2002. Effects of simulated environmental conditions on glucocorticoids metabolite measurements in white-tailed deer feces. **Gen. Comp. Endocrinol.** 127:217-222.
- Ziegler, T.E. and D.J. Wittwer. 2005. Fecal steroid research in the field and laboratory: improved methods for storage, transport, processing, and analysis. **Am. J. Primatol.** 67(1):159-174.