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1 **COMPARISON OF FECAL PRESERVATION AND EXTRACTION**
2 **METHODS FOR STEROID HORMONE ANALYSIS IN WILD**
3 **CRESTED MACAQUES**

4
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18 Psychology, Faculty of Science, Liverpool John Moores University, UK

19 **Abstract**

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

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

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

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INTRODUCTION

51

Monitoring reproduction and stress physiology of wild and/or endangered animals

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living in their natural habitat can nowadays be done via the measurement of fecal steroid

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metabolites in non-invasively collected fecal samples (e.g. Hodges & Heistermann 2011).

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This is a suitable approach for field researchers including primatologists. The use of non-

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invasive methods such as fecal hormone analysis for endocrine assessment provides

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several advantages over traditional more invasive methods of hormone measurements.

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Fecal samples can be easily obtained without disturbing the study animals and do not put

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the animal in danger during capture, e.g. as for blood sampling (Sheriff et al. 2011). They

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may also provide an accurate assessment of stress hormone levels without the bias of

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capture-induced increases in stress hormones, i.e. glucocorticoids (Millspaugh &

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Washburn 2004; Touma et al. 2003). The feces of most vertebrate species contains

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metabolized forms of all major steroid hormones (e.g., progestins, estrogens, androgens,

63

and glucocorticoids), which are secreted into the gut via the bile (Touma & Palme 2005;

64

Heistermann et al. 2006; Möhle et al. 2002; Higham et al. 2012). These metabolites can

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be measured with hormone assay techniques.

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Between the period of taking a fecal sample to subjecting it to hormone analysis,

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the concentrations of fecal hormone metabolites can change (Bielohuby et al. 2012)

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depending on how samples were treated. Previous studies have, for example, shown that

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the levels of steroid hormone in unprocessed fecal samples stored at ambient temperature

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might change over time and cause fluctuations in levels due to the activities of fecal

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bacteria (Mostl et al. 1999; Khan et al. 2002; Shut et al. 2012). Thus, fecal preservation,

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extraction, and storage play a critical role in fecal steroid analysis and methods should be

73 validated prior to application in each given species (Sousa & Ziegler 1998; Lynch et al.
74 2003; Beehner et al. 2004; Palme 2005).

75 The most common way to preserve and extract fecal samples is by freezing them at
76 -20°C followed by lyophilization (freeze-drying) and extracting fecal steroid hormone
77 using laboratory 80% ethanol or methanol (Hodges & Heistermann 2011, Palme et al.
78 2013). However, freezing fecal samples at -20°C is not always possible when the study
79 is conducted in a remote area with limited or no electricity. In addition, the lyophilizers
80 are expensive and not always accessible in many laboratories, particularly often not in
81 primate habitat countries. As an alternative, several fecal preservation techniques have
82 been developed such as drying samples using in solar or conventional oven or on silica
83 (Terio et al. 2002; Khan et al. 2002; Pettitt et al. 2007), or storing the fecal samples in
84 ethanol or other chemicals (e.g., sodium azide, ascorbic acid, 1-4 saccolactone carpol
85 and sodium chloride; Wasser et al. 1988; Nugraha et al. 2016). Alternatively, hormone
86 are extracted from feces immediately after collection (Weingrill et al. 2004; Ziegler and
87 Wittwer 2005; Shutt et al. 2012; Nugraha et al. 2016). The common solvent extraction
88 used to extract steroid hormones from feces is laboratory grade ethanol or methanol
89 (Hodges & Heistermann 2011; Palme et al. 2013). However in remote areas, these
90 chemicals are not always accessible, and ordering or shipping them is often difficult as
91 the shipping of flammable liquids such as ethanol or methanol restricted by aviation
92 regulation. As an alternative, common alcohol such as commercially available medical
93 alcohol (commercial alcohol) can be easily acquired in small pharmacies even in rural
94 areas.

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

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

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

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

123

124

MATERIALS AND METHODS

125 **Study Site, Animals and Time; sample collection**

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

132 **Experiment 1. Testing the Effect of the Time Lag between Defecation and Fecal** 133 **Preservation on E1C and GCM Levels**

134 In order to test whether the time from collection to the preservation influences the
135 stability of E1C and GCM levels in feces, fresh fecal samples (N=7) were collected from
136 animals directly after defecation. Feces were homogenized and ~3-5g feces of each
137 sample was placed in a polypropylene tube and preserved immediately in the freezer at -
138 20°C (control sample). The remaining feces was left on the ground at ambient
139 temperature, exposed to air but protected from rain. An aliquot of 3-5 g was then taken
140 after 2,4,6,8, and 10 hours, respectively and also stored at -20°C as the test groups (Table
141 1). For hormone analysis, the samples were thawed, freeze-dried and extracted using the
142 freeze-drying method described in experiment 4 (see below). Following extraction, fecal
143 extracts from all groups were assayed together for E1C and GCM levels.

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

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

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

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

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

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

175 For the freeze-drying method, samples were lyophilized for 72 h using a freeze
176 drying machine (Christ®, Gamma 1-20) at a temperature of -20 °C and a vacuum pressure
177 of 1.030 – 0.630 mbar. Subsequently, dried fecal samples were pulverized using a pestle
178 and mortar and sieved through a stainless steel strainer to separate the fecal powder from
179 the fibrous material. Finally, an aliquot (i.e. about 50 mg, exact weight recorded) of the
180 fecal powder was extracted with 3 ml of different solvents (80% MeOH, 80% EtOH, and
181 80% commercial alcohol). The solution was vortexed for 15 minutes in a 15 ml plastic
182 tube using a multi-tube vortexer (Multi-Tube Vortexer, SMI®, USA). Following
183 centrifugation at 3000 rpm for 10 minutes, the supernatant was decanted into 2 ml
184 microtubes and stored immediately at -20°C until hormone measurement.

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

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

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

198 **Hormone Analyses**

199 Fecal estrogen metabolites were measured using an estrone conjugate (E1C)
200 enzyme immunoassay (EIA) previously validated and used successfully for assessing
201 reproductive status in female crested macaques (Higham et al. 2012). The assay utilizes
202 an antiserum raised in a rabbit against estrone-3-glucuronide and estrone-3-glucuronide
203 labeled with alkaline phosphatase was used as conjugate. The cross-reactivities of this
204 assay as reported by Heistermann & Hodges (1995) are as follows: 100% with estrone-
205 3-glucuronide,71% with estrone,17% with estrone-3-sulfate, 0.9% with estradiol, 0.2%
206 with estradiol-3-sulfate,and < 0.1% with all other steroids tested. Prior to assay, fecal
207 extracts were diluted in assay buffer (dilution 1:20 in 0.04 M PBS pH7.2). Duplicate 50
208 µl aliquots of fecal extracts were assayed along with 50 µl aliquots of blank, zero and
209 standard (dose range 0.78-100 pg/well) on microtitre plates coated with sheep anti-rabbit
210 IgG.50 µl labeled estrone-3-glucuronide and 50µl antiserum were then added to each well
211 and the mixture incubated overnight at 4°C. Following incubation, the plates were washed
212 four times with PBS washing solution (containing 0.05% Tween 20) and blotted dry.
213 Subsequently, 150 µl phosphatase substrate solution (Sigma 104; 20 mg in 16 ml substrate
214 buffer pH 9.8 containing 1 M diethanolamine and 0.1 M MgCl₂) was added to each well.

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

217 Fecal glucocorticoid metabolites (GCM) were measured using an 11 β -
218 hydroxyetiocholanolone EIA (3 α ,11 β -hydroxy-CM) previously validated for the use in
219 crested macaques (Gholib 2011). An antiserum against 5 β -androstane-3 α ,11 β -diol-17-
220 CMO-BSA was used in the assay with 5 β -androstane-3 α ,11 β -diol-17-CMO-DADOO-
221 biotin as conjugate and 5 β -androstane-3 α ,11 β -diol-17-one (Sigma H8251) as standard.
222 The cross-reactivities of this assay as reported by Ganswindt et al. (2003) are as follows:
223 100% with 5 β -androstane-3 α ,11 β -diol-17-one, 3.4% with 5 β -androstane-3 β -ol-17-one,
224 1.8% with 11-oxo-etiocholanolone and <0.1% with corticosterone, cortisol, 5 α -
225 androstane-3,11,17-trione, 5 β -androstane-3,11,17-trione, testosterone, 5 α -andro-stane-
226 3,17-dione, 5 β -androstane-3,17-dione, androstenedione,5 β -androstane-3 β -ol-17-one,5 β -
227 androstane-17-one, dehydro- epiandrosterone, and androsterone. Fecal extracts were
228 diluted in assay buffer (dilution 1:100 in 0.04 M PBS pH7.2). Duplicate 50 μ l aliquots of
229 fecal extracts were assayed along with 50 μ l aliquots of blank, zero and standard on
230 microtitre plates coated with donkey anti-sheep IgG. Afterward, 50 μ l conjugate and 50 μ l
231 antibody were added to each well and the mixture incubated overnight at 4°C. Following
232 incubation, the plates were washed four times with PBS washing solution (containing
233 0.05% Tween 20), blotted dry, and 150 μ l (6 ng) of streptavidin-peroxidase (S-5512,
234 Sigma Chemie, Germany) in assay buffer was added to each well. The plates were
235 incubated at room temperature (RT) in the dark for 30 minutes, after which they have
236 washed again four times. A substrate solution (150 μ l, containing 0.025% tetramethyl-
237 benzidine and 0.05% H₂O₂) was then added to each well. The plates were again incubated
238 in dark at RT for 30–45 min after which the enzyme reaction was stopped by adding 50

239 μl 2M H_2SO_4 into each well. Finally, absorbance was measured at 450 nm on an
240 automatic plate reader.

241 Intra-assay coefficients of variation (CVs) of high and low-value quality controls
242 were 2.3% and 5.2% for E1C and 6.4% and 8.4% for $3\alpha,11\beta$ -dihydroxy-CM, respectively
243 ($n=16$). While, inter-assay coefficients of variations(CVs) of high and low-value quality
244 control were 6.3% and 9.4% for E1C and 10.2% and 11.4% for $3\alpha,11\beta$ -dihydroxy-CM,
245 respectively ($n= 6$).

246 **Statistical Analysis**

247 All hormone data were calculated as mass hormone per mass dried fecal weight.
248 Prior to statistical analysis, data were tested for normality distribution (Shapiro-Wilk
249 test). For experiment 1, the proportion of change in E1C and GCM levels relative to time
250 0 h (control) of preservation was calculated as $(a_n/x_n)*100$, where a_n is the n th sample
251 value in each duration and x_n is the value at time 0 (control) of the n th sample. A paired-
252 samples t-test was then used to determine which groups were significantly different from
253 the control. For experiment 2, the proportionate change in E1C and GCM levels for each
254 storage duration or time group (1,2,4,6,8,10, and 12 months) was calculated as $(a_n-$
255 $x_n)/x_n*100$, where a_n is the n th sample value in each duration and x_n is the value at time
256 point 0 of the n th sample. Friedman Repeated Measure ANOVA on ranks was used to
257 determine which groups were significantly different from the control. For experiment 3,
258 Friedman Repeated Measure ANOVA on ranks was used to analyze for a potential
259 difference of E1C and GCM levels in each treatment group. Post-hoc analysis using the
260 Wilcoxon signed rank test was then performed where applicable for experiments 2 and 3.
261 For experiment 4, a two-way ANOVA was used to determine whether there was
262 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 ($\chi^2=16.292$, N=8, $p=0.023$) and GCM levels ($\chi^2=20.292$, 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

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

288 **Experiment 3. Testing the Effectiveness of Oven Drying at Different Temperatures on** 289 **E1C and GCM Levels**

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

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300 **Experiment 4. Testing The suitability of Different Fecal Processing Techniques and** 301 **Extraction Solvents**

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

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DISCUSSION

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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.

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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

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

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

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

358 longer. Changes in fecal estrogen (fE) and glucocorticoid (fGC) metabolite levels over
359 time were also reported in the feces of baboons (*Papio cynocephalus*) stored in 95%
360 ethanol at ambient temperature and at -20°C (Khan et al. 2002). In that study, fE
361 metabolite concentration increased by 122% at 90 days and fGC metabolite concentration
362 increased by 92% at 120 days. In another study, fecal samples stored in ethanol at
363 ambient temperature showed increased GCM concentrations of more than 1000% and
364 45% in African elephants (*Loxodonta africana*), and grizzly bear (*Ursus arctos horribilis*)
365 after 4 months of storage (Hunt and Wasser 2003).

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

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

382 clearly higher than that recorded in other studies (e.g. Shutt et al. 2012, Nugraha et al
383 2016) where a similar experiment has been carried out. The reason for this is not clear,
384 but the data suggest that storing fecal samples of crested macaques in alcohol even for
385 periods not resulting in an overall significant change in hormone metabolite levels may
386 introduce a level of inter-sample variation that is unwanted as it may affect results to a
387 non-acceptable degree.

388 We also tested three different temperatures for drying fecal samples in a
389 conventional oven. Our results show that levels of E1C in fecal samples dried at 50°C,
390 70°C, and 90°C, did not differ significantly from each other. However, levels of GCM
391 from fecal samples dried at 90°C were significantly lower compared to levels of GCM
392 from fecal samples dried at 50°C and 70°C. This finding suggests that glucocorticoid
393 metabolites are more sensitive to high temperatures than estrogen metabolites. More
394 generally, different steroid hormone metabolites seem to have different sensitivities to
395 drying temperature. In a study on cheetahs, drying fecal samples using a solar oven and
396 a conventional oven at 71°C for 3 days resulted in alterations in the levels of steroid
397 hormone metabolites except for androgens (Terio et al. 2002). At the same time, the
398 effectiveness of drying seems to depend on the drying temperature which usually ranges
399 from 40-80°C (Ziegler and Wittwer 2005). There is thus obviously a trade-off between
400 effectiveness of sample drying and sensitivity of the respective metabolite to heat.
401 Determining the optimal drying temperature is thus important to avoid steroid metabolite
402 loss or alteration.

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

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

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

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

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

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557 **Figure Legends**

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

561

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

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

569 Fig. 4 Levels (mean ± SEM) of E1C (a) and GCM (b) dried with a conventional oven at
570 50°C, 70°C, and 90°C. * $p < 0.05$, N=14

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589 **Table Legends**

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

593

594 Table 2 Concentrations of GCM and EIC (mean, \pm SEM) in fecal samples undergoing
595 different preservation techniques and extracted from different solvents (N=6)

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