

RESEARCH ARTICLE

Testing Extraction and Storage Parameters for a Fecal Hormone Method

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Four experiments were conducted to test different aspects of a “field-friendly” fecal hormone extraction method that utilizes methanol extraction in the field followed by storage on C18 solid-phase extraction cartridges. Fecal samples were collected from geladas (*Theropithecus gelada*) housed at the Bronx Zoo, and the experiments were conducted in a laboratory setting to ensure maximum control. The experiments were designed to either simulate the conditions to which fecal samples are subjected during fieldwork or improve on an existing protocol. The experiments tested the relationship between fecal hormone metabolite preservation/recovery and: (1) the amount of time a sample is stored at ambient temperature; (2) the number of freeze/thaw cycles a sample undergoes; (3) the effectiveness of different extraction solutions; and (4) the effectiveness of different cartridge washes. For each experiment, samples were assayed by radioimmunoassay for fecal glucocorticoid (GC) and testosterone (T) metabolites. Results for each of the experiments were as follows. First, storage at ambient temperature did not affect hormone levels until 4 weeks of storage, with significant increases for both GC and T metabolites at 4 weeks. Second, hormone levels significantly decreased in samples after two freeze/thaw cycles for GCs and six freeze/thaws cycles for T. Third, for both GCs and T, hormone extraction using various methanol solutions was significantly higher than using 100% ethanol. Finally, using a 20% methanol solution to wash cartridges significantly increased GC levels but had no effect on T levels. These results suggest that, when utilizing C18 cartridges for fecal steroid storage, researchers should consider several methodological options to optimize hormone preservation and recovery from fecal samples. *Am. J. Primatol.* 72:934–941, 2010. © 2010 Wiley-Liss, Inc.

Key words: C18 cartridge; glucocorticoids; steroid; testosterone; *Theropithecus gelada*

INTRODUCTION

With the arrival of non-invasive hormone sampling, the past decade has yielded a multitude of studies documenting steroid hormone profiles across a wide array of vertebrate taxa [see reviews: Keay et al., 2006; Schwarzenberger et al., 1996; Wasser et al., 2000; Whitten et al., 1998]. Our ability to extract steroids from urine and feces (“excreta”) has opened up a physiological window into hormone–behavior relationships that was previously unavailable. However, only rarely are these methods able to directly measure the hormone in question. Most steroid hormones are metabolized within the liver and excreted (via bile) into the gastrointestinal tract and (via the kidney) into the urine. Additionally, some of the hormone metabolites in the gastrointestinal tract are reabsorbed and transported back into the bloodstream. As a result, steroid hormones, such as cortisol and testosterone, are virtually absent in urine and feces, and methods that measure these hormones from urine and feces are actually measuring downstream products of steroid metabolism.

Consequently, any method that extracts steroid hormones from “excreta” must be properly validated for each new species before reporting hormone measures [Palme, 2005; Touma & Palme, 2005]. In addition to analytical and physiological validation, however, other methodological concerns must be considered, such as how hormones are extracted from excreta and how and when samples are stored at sub-zero temperatures before assay. This is of particular concern for field researchers who are limited in their storage and transport capabilities

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of fecal samples [Ziegler & Wittwer, 2005]. For example, most studies that have examined the effects of variables such as storage techniques [Khan et al., 2002; Lynch et al., 2003], environmental conditions to which samples are exposed [Washburn & Millsbaugh, 2002], within-sample hormone variation [Millsbaugh & Washburn, 2003], and dietary variation [von der Ohe et al., 2004; Wasser et al., 1993] have found significant effects.

In this study, we test four different aspects of a “field-friendly” fecal hormone extraction method [Beehner & McCann, 2008; Beehner & Whitten, 2004; Beehner et al., 2009] to test several methodological concerns. Using fecal samples collected from geladas (*Theropithecus gelada*) housed at the Bronx Zoo, we designed experiments to either simulate the conditions that fecal samples are subjected to during fieldwork or improve on an existing protocol. Two methodological questions for field biologists who collect fecal hormone samples are: (1) how long can samples be stored at ambient temperatures prior to freezing? and (2) how many times can samples be thawed and refrozen? We store samples at ambient temperatures at our field site for variable time periods (from 1 day to 2 weeks) until they are transported to a freezer (located 1 hr away in a nearby town). Furthermore, this freezer is subjected to infrequent power outages that could cause our samples to thaw. Therefore, we are particularly interested in how these two parameters affect hormone content. Additionally, we wanted to examine the effectiveness of two parameters of our current protocol—mainly the solutions used for extraction and preservation—by comparing them with alternatives. For example, ethanol is much more widely available in developing countries than methanol and acetone. Therefore, we were interested to see whether ethanol could be used as a suitable replacement. In this study, we test the relationship between fecal hormone metabolite preservation/recovery and: (a) the time a sample is stored at ambient temperature; (b) the number of freeze/thaw cycles a sample undergoes; (c) the effectiveness of different extraction solutions; and (d) the effectiveness of different cartridge wash solutions. We examine each of these parameters with respect to both fecal glucocorticoid (GC) and testosterone (T) metabolites.

METHODS

Sample Collection

All fecal samples analyzed in this study were collected from captive geladas living at the Bronx Zoo (New York, NY). Fecal samples were collected from two adult males and two adult females during a period of 2 weeks. This research was approved by the Wildlife Conservation Society’s Institutional Animal Care and Use Committee (IACUC) and was

conducted in accordance with the American Society of Primatologists’ Principles for the Ethical Treatment of Non-Human Primates. Fecal samples for subsequent experiments were frozen immediately (-20°C) at the Bronx Zoo and transported on dry ice to the Core Assay Facility in the Department of Psychology at the University of Michigan. Steroid hormone metabolites (GCs and T) were later extracted from fecal samples using an extraction method and radioimmunoassay (RIA) that has been analytically and physiologically validated for use in geladas [Beehner & McCann, 2008; Beehner et al., 2009]. The protocol for fecal steroid extraction was identical for each experiment in this study. However, for each experiment, we modified one parameter of the protocol and compared results with the unmodified protocol, the “control” (see specific experiments below for details). Although we refer to the unmodified protocol as a “control”, we recognize that it is not a true control (with known hormone content) but rather a relative control (from which variation will be measured). The complete fecal extraction protocol can be found elsewhere [Beehner & McCann, 2008; Beehner et al., 2009], but we summarize it below.

Fecal Steroid Extraction

Before each experiment, frozen fecal samples were incubated at room temperature until thawed and then mixed thoroughly using a metal spatula (“fecal pool”). Because the experiments were conducted on different days, separate fecal pools were used for each experiment. To ensure we had enough feces per experiment, we combined several fecal samples together (matched for individual and time of collection) for each pool. (In the field, we also use a spatula to “homogenize” fecal samples—but each sample is from a single individual.) An aliquot of this fecal pool (~ 0.5 ml) was added to 3 ml of a methanol:acetone solution (8:2). Once in solution, the samples were vortexed for 40 sec and then centrifuged at 3,000 rpm for 10 min.

Immediately after centrifugation, 2.5 ml of fecal homogenate was filtered using a polytetrafluorethylene (PTFE) syringeless filter ($0.2\mu\text{m}$; Whatman, Clifton, NJ). The filter was then washed with 0.7 ml of the methanol:acetone solution. The filtered homogenate was diluted with distilled water (diH_2O , 7.0 ml), capped tightly, and mixed. Tubes containing the original fecal matrix were then set aside to air dry under a hood. Once completely dry, the fecal matrix for each sample was weighed (± 0.001 g) and recorded.

After priming Sep-Pak Plus C18 cartridges (Waters Associates, Milford, MA) according to the manufacturer’s instructions (2.0 ml 100% methanol followed by 5.0 ml of diH_2O), the sample filtrate was loaded onto the cartridge at a steady rate (~ 0.2 ml/sec) using a syringe. Cartridges were then

washed with 2.0 ml of a 0.1% sodium azide solution (a preservative), placed in a sterile Whirl-pak bag with ~2.0 g of silica beads (a desiccant), and immediately frozen (-20°C).

After cartridges were stored frozen for at least 2 weeks, they were incubated at room temperature for 1 hr. Steroids were eluted with 2.5 ml of methanol (100%) using a syringe. After elution, samples were frozen for at least 1 week until the time of RIA. Samples were reconstituted in working buffer before RIA (buffer varied depending on the hormone assayed). For RIAs, all standards were run in triplicate, and all samples were run in duplicate.

Radioimmunoassays

All samples were assayed for GC and T metabolites using modified protocols for commercially available RIA kits (Corticosterone ^{125}I RIA Kit; MP Biomedicals, Solon, OH, and Testosterone ^{125}I RIA kit, Diagnostics Systems Laboratories, Webster, TX). Because both female subjects were ovariectomized and not cycling [McCann, personal communication], we did not examine ovarian hormones in these experiments. The primary antibody from the corticosterone kit cross-reacts 100% with corticosterone, 0.34 % with desoxycorticosterone, 0.10% with testosterone, and 0.05% with cortisol (cross reactivity of the antiserum with other specific steroids is less than 0.03%). The primary antibody from the T kit cross-reacts 100% with testosterone, 6.6% with 5α -dihydrotestosterone, 2.2% with 5-androstane- 3β , 17β -diol, 1.8% with 11-oxotestosterone, 0.9% with androstenedione, and 0.6% with 5β -dihydrotestosterone (cross reactivity of the antiserum with other steroids is less than 0.5%). Primary antibodies from both kits have been previously validated for use in geladas and were shown to be parallel, precise, and accurate [Beehner & McCann, 2008; Beehner et al., 2009]. For the GC assay, inter-assay coefficients of variation (CVs) for a high (20% binding), middle (50% binding), and low (80% binding) fecal pool were 18.84%, 13.82%, and 7.02%, respectively ($N = 4$). The intra-assay CV for a middle fecal pool was 7.50% ($N = 10$). For the T assay, inter-assay CVs for a high, middle, and low fecal pool were 7.01, 10.79, and 14.81%, respectively ($N = 3$). The intra-assay CV for a middle fecal pool was 6.72% ($N = 10$).

Experiment 1—Testing How Long Samples can be Stored at Ambient Temperature

Fifty aliquots (“samples”) from a previously frozen homogenized fecal pool (i.e. 3–4 individual feces combined) were extracted as described above. After loading samples onto the cartridges, samples in the control group ($N = 10$) were frozen immediately. Samples in the test groups ($N = 10$, for each group) were stored at ambient temperature (23°C) for

1–4 weeks in a dark location (Table I). Each test group was subsequently frozen after 1, 2, 3, and 4 weeks of storage at ambient temperatures. After all the cartridges were frozen for at least 2 weeks, samples from all the groups were eluted and assayed together.

Experiment 2—Testing How Many Times Samples can be Thawed and Refrozen

Fifty aliquots (“samples”) from a second homogenized fecal pool were extracted as described above. After loading samples onto the cartridges, all 50 samples were frozen immediately. Samples in the control group ($N = 10$) remained frozen until the time of assay. Samples in the test groups ($N = 10$ for each group, except the final test condition where one sample was spilled) were thawed (incubated at 23°C for 24 hr) and subsequently refrozen one to six times (Table I). We conservatively chose 24 hr for the incubation period because this time period probably represents the maximum time that samples might be “thawed” under field conditions (if an outage occurs, power is generally restored within a day). After all freeze/thaw cycles were complete, all groups were eluted and assayed together. Note that all samples must be thawed to be eluted. For this experiment, we only count the number of times samples were *refrozen* (i.e. 0 times for the control, and 1, 2, 4, and 6 times for the test groups).

TABLE I. Experiments Performed and Samples Sizes for Each

	N	
	T	GC
<i>Experiment 1</i>		
Time stored at ambient temperature		
Control (frozen immediately)	10	10
1 week	10	10
2 weeks	10	10
3 weeks	10	10
4 weeks	10	10
<i>Experiment 2</i>		
Number of freeze/thaw cycles		
Control (never frozen and thawed)	10	10
1 Freeze/thaw	10	10
2 Freeze/thaws	10	10
4 Freeze/thaws	10	10
6 Freeze/thaws	9	9
<i>Experiment 3</i>		
Extraction solution		
Control (MeOH:Acetone, 8:2)	10	10
MeOH:H ₂ O (9:1)	10	10
MeOH:H ₂ O (8:2)	10	10
EtOH	10	10
<i>Experiment 4</i>		
Cartridge wash		
Control (0.1% sodium azide solution)	10	10
20% MeOH	10	10

Experiment 3—Testing the Effectiveness of Different Extraction Solutions

Forty aliquots (“samples”) from a third homogenized fecal pool were divided into four groups. Samples from the control group ($N = 10$) were homogenized in a MeOH:acetone (8:2) solution as described above. Samples from the remaining three groups ($N = 10$ per group) were homogenized in solutions of MeOH:H₂O (9:1), MeOH:H₂O (8:2), or EtOH, respectively. The rest of the extraction protocol was as described for the four groups. After loading samples onto the cartridges, samples were immediately frozen for 2 weeks, and all groups were eluted and assayed together.

Experiment 4—Testing the Effectiveness of Different Wash Solutions

Twenty aliquots (“samples”) from a fourth homogenized fecal pool were extracted as described above with one exception. After loading samples onto the cartridges, samples in the control group ($N = 10$) were washed with a 0.1% sodium azide solution (2 ml). Samples in the test group ($N = 10$) were washed with a 20% MeOH solution (2 ml). All samples were immediately frozen for 2 weeks, and both the groups were eluted and assayed together.

Data Analysis

For experiments 1 and 2, percent change was calculated as $((a_n - x)/x)$, where a_n is the n th sample value in each experimental group and x is the control mean. Nonparametric statistics were used in all analyses due to non-normality of data and relatively small sample sizes for each group. Specifically, Mann-Whitney U tests were used to determine which groups were significantly different from the control. Statistical significance was set at $\alpha = 0.05$, and all analyses were conducted using SPSS 17.0.

RESULTS

Experiment 1—Testing How Long Samples can be Stored on C18 Cartridges at Ambient Temperature

Samples stored at ambient temperature remained stable for up to 3 weeks. However, samples kept at ambient temperature for 4 weeks had significantly higher hormone content for both GC (Mann-Whitney U test: $U = 17.0$, $P < 0.05$) and T metabolites (Mann-Whitney U test: $U = 19.0$, $P < 0.05$; Fig. 1A,B).

Experiment 2—Testing How Many Times Samples Stored on C18 Cartridges can be Thawed and Refrozen

Samples exposed to repeated freeze/thaw cycles exhibited decreased levels of hormone content for

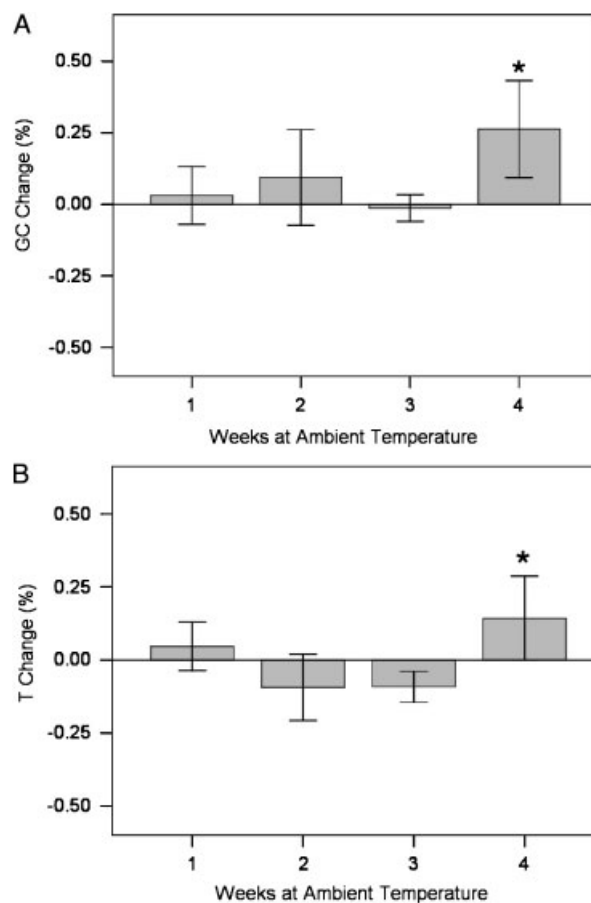


Fig. 1. Change in hormone content from control group ($\% \pm 2\text{SEM}$) for samples stored at ambient temperature (22°C) for 1–4 weeks for (A) fecal glucocorticoid (GC) metabolites and (B) fecal testosterone (T) metabolites. [$*P < 0.05$].

both GC and T metabolites. This effect was more pronounced for GC metabolites. Glucocorticoids from samples exposed to two (Mann-Whitney U test: $U = 24.0$, $P < 0.05$), four ($U = 20.0$, $P < 0.05$), and six ($U = 19.0$, $P < 0.05$) freeze/thaw cycles exhibited significantly less hormone content than controls (Fig. 2A). For T metabolites, a significant decrease in hormone content was observed only for samples exposed to six freeze/thaw cycles (Mann-Whitney U test: $U = 14.0$, $P < 0.05$; Fig. 2A,B).

Experiment 3—Testing the Effectiveness of Different Extraction Solutions

Extraction recovery was significantly different across the solutions tested for both GC and T metabolites. The MeOH:H₂O (8:2) solution extracted significantly more GC metabolites (Mann-Whitney U test: $U = 18.0$, $P < 0.05$) and less T metabolites (Mann-Whitney U test: $U = 13.0$, $P < 0.01$) than the control solution (MeOH:acetone). Additionally, 100% EtOH extracted significantly less GC (Mann-Whitney U test: $U = 0.0$, $P < 0.001$) and T metabolites

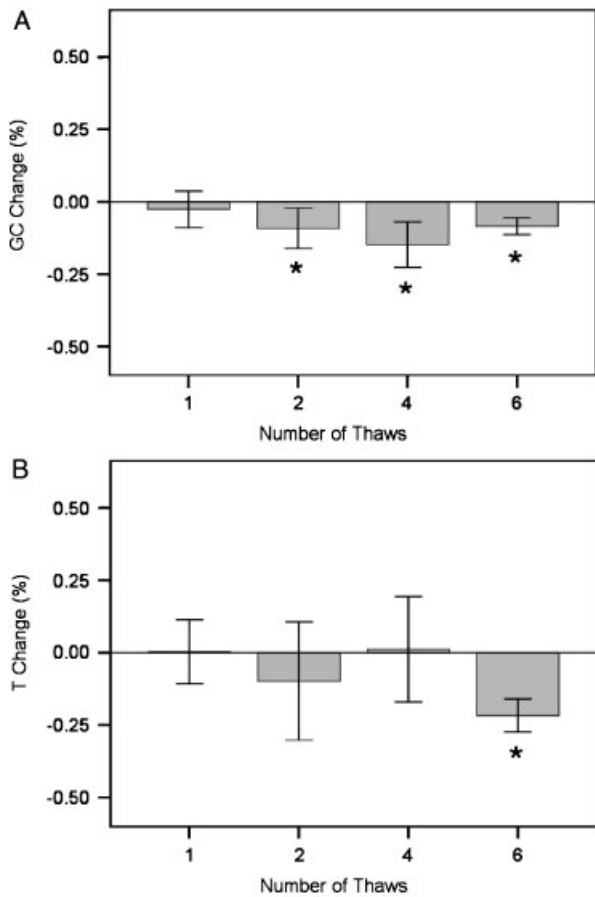


Fig. 2. Change in hormone content from control group (% \pm 2SEM) for samples undergoing one to six freeze/thaw cycles for (A) fecal glucocorticoid metabolites (GC) and (B) fecal testosterone metabolites (T) [* P < 0.05; ** P < 0.01].

(Mann–Whitney U test: $U = 0.0$, $P < 0.001$; Fig. 3A,B) than the control solution.

Experiment 4—Testing the Effectiveness of Different Cartridge Wash Solutions

For GC metabolites, cartridges washed with different solutions demonstrated significant differences in hormone content with the 20% MeOH solution resulting in more GC metabolites than the control solution (0.1% sodium azide; Mann–Whitney U test: $U = 17.00$, $P < 0.05$). No differences were observed between the different wash solutions for T metabolites (Mann–Whitney U test: $U = 37.00$, $P = 0.326$).

DISCUSSION

The first two experiments aimed to simulate field storage conditions in a controlled laboratory setting. Other researchers report that extracted hormones are stable at ambient temperatures for up to 2 weeks if stored in 100% ethanol [Khan et al., 2002]. However, if samples could be stored for longer

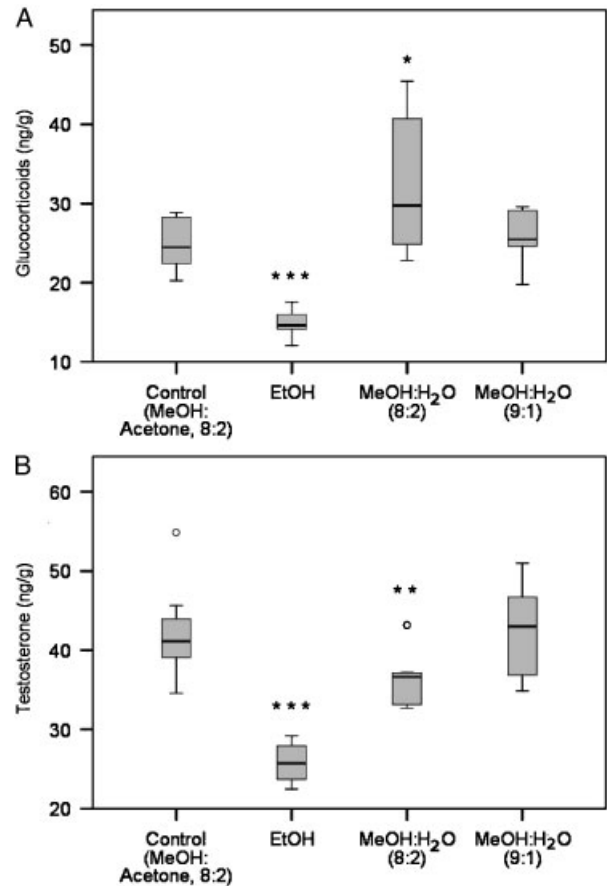


Fig. 3. Median values for samples extracted using four different solutions for (A) fecal glucocorticoid metabolites and (B) fecal testosterone metabolites [* P < 0.05; ** P < 0.01; *** P < 0.001]. Boxes represent interquartile ranges and open circles represent outliers (\pm 2SD).

periods of time at ambient temperature, then researchers would not need extra trips areas away from the fieldsite to ship or store samples. Furthermore, due to the potential for power outages (particularly in developing countries) and the necessity of shipping samples from a fieldsite to a laboratory, samples often undergo repeated freeze/thaw cycles. Therefore, the first two experiments sought to address these methodological concerns.

With respect to storage at ambient temperature, our results demonstrated that fecal hormone metabolites (both GCs and T) were stable at ambient temperature for up to 3 weeks, but by 4 weeks of storage both GC and T levels were significantly higher than the control group (Fig. 1). A previous study using the same method also reported that time spent at ambient temperature affected hormone metabolites across a similar time-frame [Beehner & Whitten, 2004]. However, the results from this previous study differed from the current one in two ways. First, only GCs (and not T) changed across storage time. Second, GCs *decreased* with time spent at ambient temperature, whereas in the current

study, both GCs and T *increased*. These differences might be due to methodological differences (a correlation study across multiple individuals versus a controlled study using a homogenized fecal pool) and location (field versus laboratory). Although our sample size was small ($N = 10$ per group), all aliquots all samples in each experiment were derived from a single homogenized fecal pool. Thus, assuming proper homogenization, samples in the current study should be identical with respect to hormone content. By contrast, the previous study (with a much larger sample size, $N = 728$) reported whether there was a trend toward increasing or decreasing hormone content based on samples derived from different individuals [Beehner & Whitten, 2004]. Thus, variation in the previous study could be due to individual differences in hormone levels, whereas variation in the current study must be due to experimental differences. Additionally, the extraction protocol used in this study was slightly modified from the previous one to increase hormone recovery (the new protocol increased recovery by an average of 34% [Beehner, unpublished data]). Finally, the ambient temperature across our experiments was always 22°C, while ambient temperature for the previous study ranged from approximately 22–35°C [Beehner, unpublished data].

At present, we are uncertain why hormone content increased (rather than decreased) after 4 weeks of storage. It is highly unlikely that contamination caused the increased hormone content, as all samples were stored individually in Whirl-pak bags. One possibility is that samples stored longer at ambient temperatures suffer increased hormone degradation that actually result in more metabolites cross-reacting with antibodies from the RIA kits. Resolving this issue will require high-performance liquid chromatograph (HPLC) to determine which metabolites are present at the various storage times. A second possibility is that “drier” samples (i.e. those stored longer at ambient temperature with the silica desiccant) yield higher hormone recoveries at the time of elution. We have some data to suggest that semi-“wet” samples (i.e. samples that are immediately frozen) do not elute properly from the cartridges [Beehner, unpublished data], and this could be largely attributed to the aqueous wash solution. Importantly, however, hormones stored at ambient temperatures for 1 day to 3 weeks exhibited no differences from the control group. A third possibility is that both gelada GC and T metabolites are conjugated when they are excreted in fecal samples. If prolonged storage at ambient temperature breaks steroid conjugates, then our control may be pseudo-reduced with respect to overall hormone content [Ziegler & Wittwer, 2005].

With respect to freezing and thawing, our results suggest that samples can be exposed to at least one freeze/thaw cycle for GCs and up to four

freeze/thaw cycles for T metabolites before hormone content is significantly different from control groups. Taken together, this suggests that T metabolites might be more stable on C18 cartridges than GCs are. Note, however, that we allowed all samples to incubate (“thaw”) for 24 hr, which may overrepresent how long samples in the field are left to thaw. It only takes about 1–2 hr for samples to fully thaw; thus, a shorter thaw period may result in a smaller change from the control. Although one freeze/thaw cycle does not seem to affect hormone content, we recommend that researchers using C18 cartridges to store fecal extracted hormones take pains to avoid freeze/thaw cycles altogether.

The last two experiments tested the effectiveness of different extraction/wash solutions to optimize hormone recovery and/or capitalize on chemicals that are more easily obtained in developing countries (mainly, EtOH). The first of these (*Experiment 3*) tested how well different organic solutions extracted steroid hormones from the fecal matrix. We found no difference between the 100% organic control solution (MeOH:acetone; 8:2) and the 10% aqueous solution (MeOH:H₂O; 9:1). By contrast, the 20% aqueous solution (MeOH:H₂O; 8:2) extracted significantly *more* GC metabolites and significantly *less* T metabolites than the control. We suspect that the more aqueous extraction solution (MeOH:H₂O; 8:2) is better at extracting more polar metabolites (such as GCs or conjugated metabolites [for a fuller explanation, see Ziegler & Wittwer, 2005]), whereas the more organic solutions are better at extracting less polar metabolites (such as T and unconjugated metabolites). Surprisingly, 100% EtOH extracted significantly less hormone metabolites for both GC and T when compared with the control solution, possibly due to the stronger polarity of methanol ($\delta P = 12.3$) compared with ethanol ($\delta P = 8.8$). By contrast, a previous study of fecal GC metabolite extraction in Belding's ground squirrels (*Spermophilus beldingi*) demonstrated no difference in extraction efficiency between MeOH (80 and 100%) and EtOH (80 and 100%) solutions [Mateo & Cavigelli, 2005]. We suspect that the “best” extraction solution may be both species- and hormone-specific, and depends on the percentage of conjugated to unconjugated hormones present in excreta for a given species. For example, although most laboratories use at least a 30% solvent (ethanol or methanol), many use a mixture of solvent and aqueous solution to maximize extraction of both unconjugated steroids and conjugated steroids [Ziegler & Wittwer, 2005]. Although we do not yet know the percentage of unconjugated to conjugated hormones (GCs or T) for geladas, for baboons (*Papio cynocephalus*), a sister taxa to geladas, less than 20% of fecal cortisol metabolites consisted of hydrolysable conjugates [Wasser et al., 2000].

Finally, we examined the effect of two different wash solutions on hormone preservation (*Experiment 4*). Although there was no difference between a 100% aqueous sodium azide solution and an 80% aqueous methanol solution for T metabolites, we found that GC metabolites increased when the wash solution was less aqueous (80% aqueous). This difference might be due to the more polar structure of GCs, which may be partially eluted from the cartridge during a 100% aqueous wash. It is also possible that the absence of sodium azide, a known hormone preservative, results in greater degradation of GCs.

Although our results demonstrate that both GC and T metabolites are affected by manipulating various parameters of a fecal hormone extraction method, we do not know whether such patterns persist for ovarian hormones (estrogens (E) and progestins (P)). Fecal ovarian metabolites have been successfully used to determine reproductive parameters in wild populations [e.g. Beehner et al., 2006]. It will be important, however, to determine whether the storage and extraction differences examined here, can mask the cyclical hormone patterns for E and P. We have plans to use fecal samples from wild female geladas to investigate this area further.

Fecal hormone values are not “absolute” measures of circulating hormone levels, but rather *relative* measures, as they are downstream products of steroid hormone metabolism. Nevertheless, relative hormone measures still provide researchers with a mechanism for testing physiological hypotheses in wild animals. To maximize hormone extraction, preservation, and recovery, we recommend that similar methodological experiments be conducted for each species and hormone. With respect to chemical extractions and dry storage on cartridges, we recommend that researchers: (1) experiment with different percentages of organic to aqueous solutions to maximize hormone extraction, and (2) for long-term preservation in the field, use a 100% aqueous, sodium azide solution for the wash. With respect to sample storage, we recommend that researchers: (3) freeze samples 1–3 weeks after collection (allowing samples to sit for at least a few days with silica to allow the cartridge to dry), and (4) minimize freeze/thaw cycles to one cycle (at most) prior to the final thaw before eluting samples from the cartridges. We are currently exploring whether different HPLC-separated fractions of steroid hormone metabolites are differentially affected by freezing and thawing or prolonged ambient temperature storage. However, even in the absence of such controlled experiments, the most important methodological consideration with such non-invasive methods is consistency across samples and across time to keep such relative measures comparable.

Finally, all methodological considerations should take into account the expected biological patterns for

the species in question. In other words, if a researcher expects large differences between study groups (e.g. differences between sexes, reproductive stages, or times of the year), then biological patterns might easily override any methodological effects. However, if researchers are interested in subtle differences (e.g. differences in basal levels across same sex, same reproductive stage individuals), then methodological concerns will likely be of primary importance.

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