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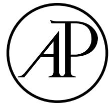

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A matter of time: evaluating the storage of fecal samples for steroid analysis

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Abstract

The extraction and immunoassay of fecal steroids is an increasingly common technique, used in both captive and field studies to provide an approximation of an animal's circulating concentration of hormones through non-invasive methods. Storage of fecal samples is of critical concern because fecal bacteria metabolize fecal steroids within hours after deposit. Ethanol is often used as a preservative for fecal samples stored for several hours at room temperature. We examined the stability of fecal estrogen (fE) and glucocorticoid (fGC) metabolites from baboon (*Papio cynocephalus*) samples in a 95% ethanol solution at ambient temperature and at −20 °C over the course of six months, to determine the effect of storage on steroid concentrations. As measured by radioimmunoassay, fE metabolite concentrations increased by 122% at 90 days and fGC metabolite concentrations increased by 92% at 120 days. After peaking, both hormones declined to near initial concentrations by 180 days in ambient temperature samples. In samples stored at sub-zero temperatures, fGC metabolite concentrations showed a similar but dampened pattern, while fE metabolite concentrations exhibited small and variable changes with no consistent trend. We discuss explanations for the dynamic pattern of changing fecal metabolite concentrations and offer practical and analytical guidance to field workers for situations in which ideal conditions for stabilizing hormones are not available. © 2002 Elsevier Science (USA). All rights reserved.

Keywords: Fecal steroids; Estrogens; Glucocorticoids; Storage; Ethanol; Baboon

1. Introduction

Fecal steroid measurement is a valuable non-invasive tool for assessing reproduction, environmental stress, and aggression in populations of captive and free-living animals (reviewed in Whitten et al., 1998). In captive settings, fecal samples are collected and frozen immediately (Graham and Brown, 1996; Hamilton et al., 2000; Sousa and Ziegler, 1998; Wallner et al., 1999). Fecal samples, as with most biological samples, are most stable over time when stored at sub-zero temperatures (Whitten et al., 1998). When a cooling mechanism is available, cold storage is the preferred method for fecal sample preservation in the field. Some methods for preserving feces in field conditions include immediate freezing in liquid nitrogen (Creel et al., 1997; Wasser et al., 1988), storage in ethanol in a −20 °C freezer (Strier and Ziegler, 1997; Wasser, 1996; Wasser et al., 1997), and field extraction, followed by storage of the extract at sub-zero temperatures (Lynch et al., 2002; Stavisky et al., 1995; Strier et al., 1999). However, liquid nitrogen, dry ice, or freezers are not always available in the field.

If the feces are not treated with preservatives or kept in cold storage, naturally occurring bacteria and bacterial enzymes in feces decompose steroid metabolites within hours after defecation (Moestl et al., 1999; Wasser et al., 1988). Wasser et al. (1988) demonstrated that ethanol stabilizes fecal estrogens and progestins over a period of 21 h at ambient temperatures, presumably by killing bacteria and inactivating their associated enzymes. Subsequently, ethanol either alone or with sodium azide has been used as a preservative for short-term ambient temperature storage of fecal samples (Strier and Ziegler, 1997; Wasser, 1996; Wasser et al.,

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1997; Ziegler et al., 2000) or long-term storage for 5 months (Cavigelli, 1999) to 3.5 years (Curtis et al., 2000). Other ambient temperature field storage techniques include oven-drying samples (Brockman and Whitten, 1996; Brockman et al., 1998). After oven-drying, estradiol and testosterone showed complete stability after 3 weeks, while progesterone showed less stability (Brockman and Whitten, 1996). Comprehensive tests of the efficacy of these storage methods have not been conducted (Whitten et al., 1998), resulting in a scarcity of practical information about viable storage techniques under field conditions (Table 1).

Our goal in this study was to determine the effects of storage over the course of 180 days, on a well-mixed pool of feces collected from a captive group of baboons. Here, we present results from a study that examines the long-term stability of fecal estrogen and glucocorticoid metabolites from baboon samples in an ethanol solution at ambient temperature (25 °C) and at sub-zero temperatures (−20 °C).

2. Materials and methods

For validation of assays, we used freshly collected feces from wild- and captive-living baboons (*Papio cynocephalus*). For the storage experiment, to use a large quantity of feces from which many replicates could be analyzed across 180 days, we used approximately 1 kg of feces, which was collected from 5 to 7 captive adult male and female baboons. These individuals were members of a group of approximately 60 individuals that live in a large, semi-natural enclosure at Brookfield Zoo (Chicago Zoological Society). As a result of tubal ligation of females and vasectomies of males, no females in the colony were pregnant or lactating at time. Fresh samples were mixed thoroughly, frozen immediately, and sent on dry ice to Princeton University. The feces were thawed, mixed again and divided among 130 polypropylene vials in 4-g aliquots. Ten replicates were freeze-dried immediately and analyzed to provide baseline measurements for estrogen and cortisol metabolites. The remaining 120 samples were placed in 95% ethanol (2.5 ml ethanol:1 g feces). These samples were divided into two treatment groups: (1) storage at ambient temperature (on average 25 °C) or (2) storage at sub-zero temperatures (on average −20 °C). For the glucocorticoid and estrogen study, 10 replicates were freeze-dried and assayed at 30-day intervals for the first 120 days. For the glucocorticoid study, five replicates were freeze-dried and assayed at 15-day intervals between 120 and 165 days, while the number of replicates remained 10 and the interval remained at 30-days for the estrogen experiment. The greater frequency of assaying for glucocorticoids than estrogens resulted in smaller samples sizes per assay for the glucocorticoid study. We shortened the storage intervals to ensure that we observed the peak and decline in fecal steroid concentrations for the glucocorticoid study. Both experiments concluded at 180 days when the remaining 10 replicates were freeze-dried and assayed.

Processing of fecal samples. At Princeton University, ethanol was evaporated by placing each sample under a hood, at ambient temperature, overnight. Then the samples were freeze-dried, to ensure complete dryness, and sifted through fine mesh (40 mesh). About 0.2 g of each sample was extracted into 2 ml of 90% methanol by vortexing on a multi-pulse vortexer (Glas-Col, Terre Haute, IN; pulse rate 1/s, speed 70) for 30 min. The mixture was centrifuged for 20 min at a force of 1*g* and

Table 1
Reported length of time fecal samples that have been stored at ambient temperature and the preservatives used

Species		Maximum time ambient	Preservative	Reference
African wild dog	*Lycaon pictus*	None	Liquid nitrogen	Creel et al. (1997)
Baboon	*Papio cynocephalus*	None, stored on ice	Liquid nitrogen	Wasser et al. (1988)
Baboon	*Papio cynocephalus*	2 h	−20 °C	Stavisky et al. (1995)
Spotted owl	*Strix occidentalis*	2–5 h	90% EtOH	Wasser et al. (1997)
Ring-tailed lemur	*Lemur catta*	3 h	None	Cavigelli and Pereira (2000)
Hanuman langur	*Presbytis entellus*	5 h	90% EtOH	Ziegler et al. (2000)
Baboon	*Papio cynocephalus*	8 h	95% EtOH 0.2% NaN_3	Wasser (1996)
Muriqui	*Brachyteles arachnoides*	10 h[a]	95% EtOH	Strier and Ziegler (1997) (see newer technique, Strier et al., 1999)
Tule elk	*Cervus elaphus nanodes*	12–14 h	−20 °C	Stoops et al. (1999)
Baboon	*Papio cynocephalus*	21 h[b]	100% EtOH	Wasser et al. (1988)
Verraux's sifaka	*Propithecus verreauxi*	3 weeks[b]	Silica dried	Brockman et al. (1998)
Ring-tailed lemur	*Lemur catta*	5 months[a]	100% EtOH 3% NaN_3	Cavigelli (1999)
Mongoose lemur	*Eulemur mongoz*	3.5 years	96% EtOH	Curtis et al. (2000)

[a] Results from validation experiment unpublished (verified by personal communication).
[b] Validation experiment published in paper cited.

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the supernatant was transferred to a 2 ml polypropylene storage tube with O-ring caps to prevent evaporation. We assessed our extraction recovery of corticosterone, and estradiol by adding 10,000 cpm ^{125}I-labeled hormone to dry feces and incubating the mixture at ambient temperature for, 1 h prior to methanol extraction.

The methanol fecal extracts were stored at −20 °C until they were assayed. Fecal samples were assayed in duplicate, the results were averaged across duplicates, and hormone concentrations were expressed as nanograms of hormone per gram of dry fecal matter.

Fecal glucocorticoid radioimmunoassay (RIA). We used a modified Corticosterone Kit for Rats and Mice (ICN Diagnostics, Cat No. 07-120-102, Costa Mesa, CA). The primary antibody has high cross-reactivities with the major cortisol metabolites present in baboon feces (Goymann et al., 1999; Wasser et al., 2000). This antibody has been validated for use with the baboon; it detects a rise in cortisol metabolites after a baboon is presented with an ACTH challenge (Wasser et al., 2000).

We validated the radioimmunoassay by running dilutions of a fecal extract pool (1:2, 1:4, 1:8, 1:10, and 1:16) and comparing its slope to that of the standard curve. Samples were diluted 1:10 in assay buffer, prior to radioimmunoassay. Standards ranged from 0.125 to 5 ng/ml. Internal controls consisted of a pooled fecal sample diluted 1:10 in assay buffer, frozen in small aliquots, stored at −80 °C, and two controls provided with each kit, all of which were run in every assay. Intra- and inter-assay coefficients of variation (%CV = [mean/SD] * 100) were $4.4 \pm 1.0\%$ (mean ± SE) and 10.8 % for the fecal extract pool (~0.8 ng/ml), $2.5 \pm 0.5\%$ and 8.7% for a low concentration control, and $2.5 \pm 0.5\%$ and 9.8% for a high concentration control ($n = 7$ for all controls). Mean assay accuracy (observed/expected*100) was $103.2 \pm SE$ 5.1% ($n = 4$) and was assessed by running the 1.25 ng standard as a sample.

Fecal estrogens RIA. We used a modified Total Estrogen Kit (ICN Diagnostics, Cat. No. 07-140-202, Costa Mesa, CA). Wasser et al. (1994) found that in the female baboon, 10% of radio-labeled estradiol injected into an individual was excreted in the feces with the remainder excreted in urine. The original radio-labeled estradiol was excreted in the feces as estradiol (36%), estrone (44%), and estrone sulfate (20%; Wasser et al., 1994). The primary antibody in this kit cross-reacts 100% with estradiol-17β and estrone, 9.0% with estriol, 7.0% with estradiol-17α, and 2.5% with equilin (ICN Diagnostics).

Prior to analysis, fecal extracts were extracted using solid-phase Oasis cartridges (Waters, Milford MA, WAT094226; techniques modified by T. E. Ziegler, University of Wisconsin). Fecal extract (300 μl) was dried under nitrogen and reconstituted in 1 ml of 30% methanol. Cartridges were conditioned with 1 ml of 100% methanol, followed by 1 ml distilled water. The 30% sample was loaded onto the cartridge, followed by 1 ml of a 20% methanol rinse. The steroids were eluted off the column with 2 ml of 100% methanol, which was collected, dried under nitrogen, and reconstituted in 300 μl of 90% methanol to return the sample to its original volume in its original solute.

We validated the estrogen radioimmunoassay by running a serial dilution (1:20–1:1280) of baboon fecal extract pools and comparing the slope of the serial dilution to that of the standard curve. Samples were diluted 1:200 in assay buffer, prior to radioimmunoassay. Standards ranged from 5 to 200 pg/ml. Intra- and inter-assay coefficients of variation were 4.5 ± 1.4 % and 9.0% for a fecal extract pool (~60 pg/ml; $n = 7$). Assay accuracy was $89.5 \pm$ SE 3.9% ($n = 4$) and was assessed by running the 50 pg standard as a sample.

2.1. Statistical analysis

All data were log transformed to meet assumptions of normality. Parallelism between standard curves and serial dilutions of fecal extracts was determined by a test of the equality of two slopes (Neter et al., 1990). Variation of fecal glucocorticoid and estrogen concentrations across time was analyzed with analysis of variance (ANOVA), followed by Tukey pairwise comparisons. Significance levels for all tests were set at $P \leqslant 0.05$. Means are given with standard errors unless otherwise noted.

3. Results

3.1. Method validation

Methanol extraction recoveries were $85.0 \pm 0.5\%$ ($n = 10$) for corticosterone and $92.3 \pm 0.5\%$ ($n = 10$) for estradiol. Solid phase extraction recovery of estradiol was assessed by assaying 10 replicate samples before and after solid phase extraction, yielding a recovery of $89.1 \pm 1.32\%$.

The corticosterone standard curve was parallel to the displacement curve from the fecal extract serial dilution (Fig. 1A: Student's $t = 0.16$, $p = 0.87$). The total estrogens standard curve was parallel to the displacement curve from the fecal extract serial dilutions (Fig. 1B: Student's $t = 0.35$, $p = 0.73$).

Glucocorticoids. Fecal glucocorticoid concentrations varied with the number of days in storage for both the ambient temperature samples (Fig. 2A: $F = 27.75$, $p < 0.0001$) and −20 °C samples ($F = 20.82$, $p < 0.0001$).

Total estrogens. Fecal estrogen concentrations varied with the number of days in storage for both the ambient temperature samples (Fig. 2B: $F = 54.14$, $p < 0.0001$) and −20 °C samples ($F = 9.68$, $p < 0.0001$).

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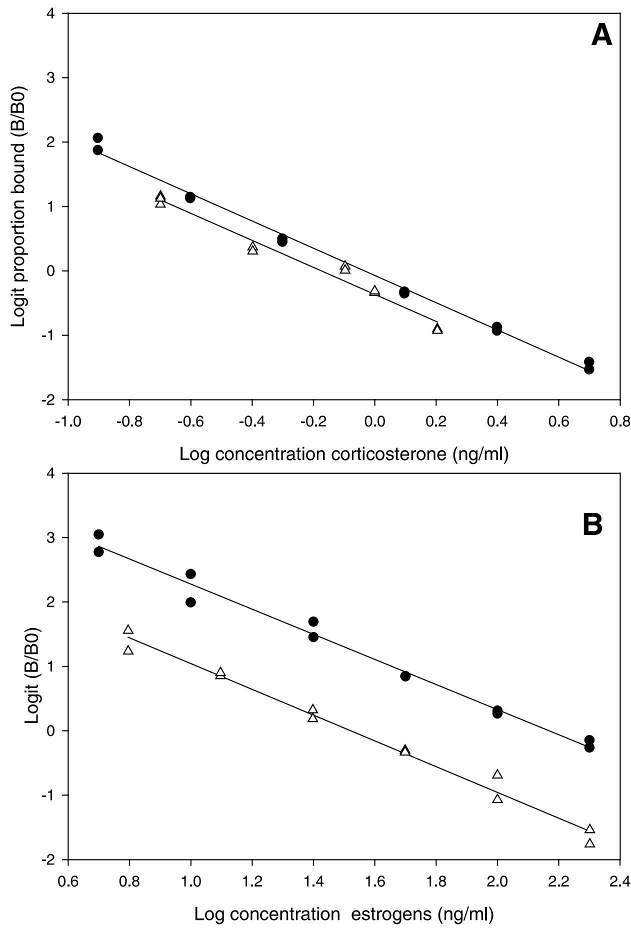

Fig. 1. (A) Log-logit transformed curves showing displacement of the binding of ^{125}I-labeled corticosterone to ICN corticosterone antibody by corticosterone standard (●), $y = -2.11x - 0.07$, $r^2 = 0.99$ and serial dilution of pooled fecal extract (△) $y = -2.09 - 0.36$, $r^2 = 0.99$. (B) Log-logit transformed curves showing displacement of the binding of ^{125}I-labeled estradiol to ICN total estrogen antibody by estradiol standard (●) $y = -1.95x + 4.80$, $r^2 = 0.99$, serial dilution of fecal extract pool (△) $y = -1.99x + 3.04$, $r = 0.98$.

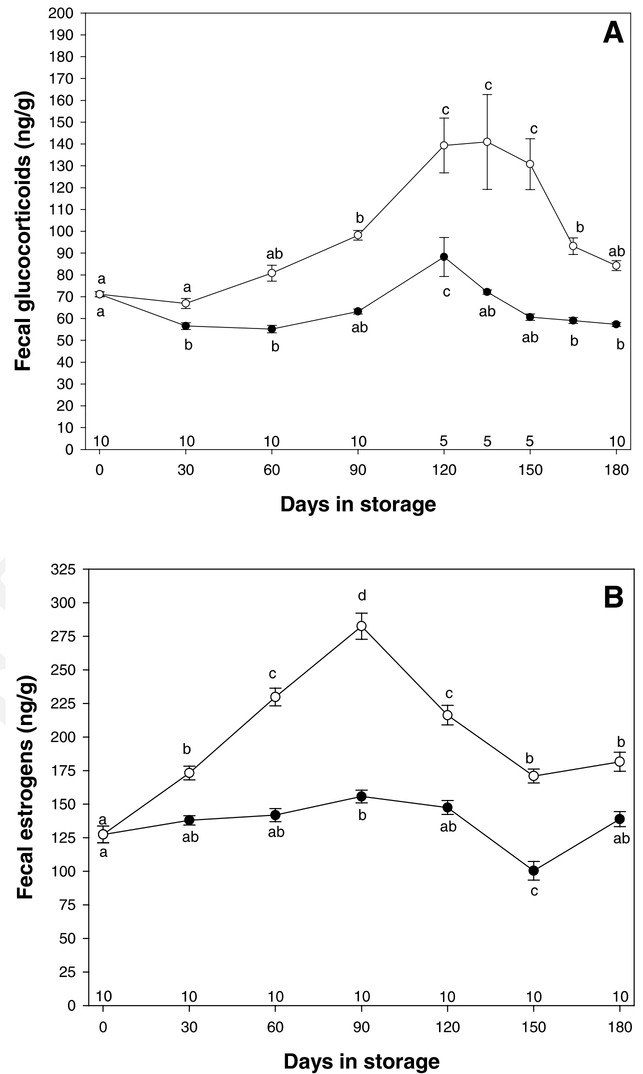

Fig. 2. (A) Change in fecal glucocorticoid metabolite concentration over time in captive baboon feces stored in ethanol at −20 °C (closed circles) and 25 °C (open circles). (B) Change in fecal estrogens as a function of storage time in ethanol at −20 °C (closed circles) and 25 °C (open circles). Statistical differences and similarities between groups are indicated with letters (Tukey's studentized range test, $p < 0.05$). For example, initial concentrations of fGCs are not significantly different from concentrations measured after 30, 60, and 180 days of storage at ambient temperature. The number of replicates at each time point is indicated above the x-axis.

3.2. Magnitude of variability over time vs assay variability

Inter-assay and intra-replicate variabilities are unlikely explanations for the patterns of dynamic change in either the fGC or fE studies. First, the maximum percent change among quality control samples was lower than the percent change observed among unknown samples stored at ambient and sub-zero temperatures (Table 2). Second, while inter-replicate variability was low at most time periods for both studies, inter-replicate variability at peak fGC concentrations was 5–9 times higher than the variability observed at other points in time. The increased variation at these time points is probably caused by subtle differences between the replicates, so that some were rising and others were falling at these points in time. The increased variation during peak concentrations cannot be attributed to methodological vagaries because the quality controls in these assays indicated high precision. Intra-assay variation during peak concentrations, days 120, 135, and 150, was 4.2 ± 1.2%.

4. Discussion

Both the fGC and fE results showed marked changes over time in fecal samples stored at both ambient and sub-zero temperatures. This previously unappreciated variability has important implications for field storage

Table 2
Percent variation in fGC and fE concentrations observed among quality control samples and storage experiment samples

Maximum percent change	fGC		fE	
	Ambient	Subzero	Ambient	Subzero
Quality control pools	12%	12%	13%	13%
Storage samples				
Initial vs Peak	92%	17%	122%	22%
Initial vs Nadir	6%[a]	29%	N/A[b]	21%

[a] Nadir glucocorticoid concentrations among ambient temperature samples were not significantly different from initial concentrations.
[b] Fecal estrogen concentrations never fell below initial concentrations among ambient temperature samples.

254 of samples for hormone analysis. Below, we discuss four options for researchers who wish to work with fecal steroids that were collected in difficult field situations.

257 First, these findings suggest rapid freezing of samples when possible, and if freezing is not possible, keeping samples at room temperature for no longer than 30 days if the hormones of interest are glucocorticoids, and for a shorter period if estrogens are the focus. If freezers are available, long-term storage of fecal samples in ethanol at −20 °C is more preferable than storage at room temperature; however, ideally it should not exceed 90–120 days.

265 Both the glucocorticoid and estrogen experiments might be interpreted as suggesting that ambient temperature storage for six months or longer may be advisable because the final concentrations of fGCs and fEs were approaching those of initial concentrations. However, before this strategy is employed one would need to validate, for the species and hormones of interest, whether steroid metabolites measured after six months at ambient temperature storage are an accurate reflection of plasma steroid levels at initial sampling time. Validation is necessary because molecules cross-reacting with the antibody after six months may not be indicative of the physiological state of the animal at the time of sampling if chemical changes occurred within the sample.

280 The findings of the present study strongly point to the value of future studies of storage during the first month. These experiments would be conducted under actual field conditions, for each hormone of interest, and for samples that vary in initial hormone concentration. Such an experiment would validate the maximum storage duration and temperature regime that naturally heterogeneous samples can withstand without compromising one's results.

289 Second, for some questions and sample distributions, the effects of storage time on fecal steroid concentrations may also be controlled during data analysis. The small error bars at each time point in the laboratory study suggest that one strategy would be to analyze and compare samples that have been subjected to similar storage regimes. Similarly, one could analyze steroid concentrations relative to the average concentration for the month in which they were collected thereby generating residual values that can be compared. This approach needs to be taken with considerable attention to decide what values to average to generate meaningful residual data for comparisons, such as whether to pool data for males and females or across age classes in the average. The validity of particular analytic strategies will depend on the particular questions, reasonable assumptions, sample sizes, and sampling regimes in each instance.

307 Third, if long-term storage of fecal samples in ethanol is unavoidable, then researchers will need to validate their chosen storage method for each hormone of interest. Our findings underscore the importance of thorough validation of methods as a precursor to any work conducted with fecal steroids. Future work on field techniques might include long-term validation of other storage techniques, such as oven drying, which has been shown to be stable over three weeks for some hormones (Brockman and Whitten, 1996), field extraction into aqueous solutions (Lynch et al., 2002; Strier et al., 1999), or the addition of preservatives to the ethanol.

319 Fourth, the source of changes occurring during long-term storage may need to be identified to determine exactly what actions are most appropriate to ameliorate storage effects. Several possible explanations for the changes reported here illustrate the potential importance of identifying the source of changes. Stability studies of glucocorticoids conducted in feces (Wasser et al., 1988), urine (Brown et al., 1995), and pure preparations (Isaksson et al., 2000) report steroid deterioration under conditions of ambient temperature storage. The decline in fGC and fE concentrations after 120 or 90 days, respectively, may be caused by deterioration of the metabolites. Paradoxically, however, our results show *increasing* fecal hormone concentrations over a considerable time period.

334 The observed pattern of increasing concentrations may be an artifact of using a group-specific antibody as opposed to a highly specific cortisol or estradiol antibody. A group-specific antibody cross-reacts with a family of metabolites derived from the parent hormone (Palme and Moestl, 1997; Schwarzenberger et al., 1997; Wasser et al., 2000), while a highly specific antibody cross-reacts only with the parent hormone itself. Evidence that group-specific antibodies show different sensitivities to changing glucocorticoid or estrogen metabolites over time is provided by this study and three others. Studies using highly specific antibodies demonstrated deteriorating urinary cortisol metabolites (Brown et al., 1995) and fecal estrogen metabolites (Wasser et al., 1988) when samples were stored at ambient temperature. In contrast, studies using group-specific antibodies (Palme and Moestl, 1997) showed increasing fecal cortisol (Moestl et al., 1999, this study)

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and estrogen (this study) metabolite concentrations in samples stored at ambient temperature.

While the use of a group-specific antibody may have made the observed changes greater, we do not suggest that exclusive use of highly specific antibodies is necessary for fecal steroid measurements. Rather, the decision to use a group-specific or highly specific antibody should be determined by the hormone of interest and the steroid metabolism pathways in the study species. Storage effects should be prevented through storage regimes that are determined in part by what is chemically occurring within the samples over time. Below, we discuss three chemical explanations for our results, noting that these explanations are not mutually exclusive. In Table 3, we present predictions for temporal and HPLC analyses of samples subjected to a storage study similar to this one. We also propose practical solutions for the laboratory or field to ameliorate storage effects given each scenario.

First, the observed pattern may be caused by the ethanol solution increasingly extracting metabolites from the feces at ambient temperatures. This hypothesis questions the assumption that spiking samples with pure corticosterone or estradiol preparations prior to extraction is an accurate measure of the extraction efficiency of all cortisol or estradiol metabolites. It is possible that some immunoreactive metabolites have lower extraction efficiencies than pure preparations. Immunoreactive metabolites with normally low extraction efficiencies might have a higher extraction efficiency when stored in ethanol for several months at ambient temperature. These metabolites would be deposited on the surface of the fecal material during ethanol evaporation step and freeze-drying process, and would go into solution more easily during the extraction step than if the sample had not undergone long-term storage in ethanol (see Section 2).

Second, immunoreactive metabolites could be derived from the breakdown or deconjugation of multiply conjugated steroid metabolites into mono-conjugated or unconjugated metabolites. In the baboon, 11% of fecal cortisol metabolites are conjugated (Wasser et al., 2000), while 20% of estradiol is excreted as estradiol sulfate in feces (Wasser et al., 1994), making this a likely explanation for our results. The rate of deconjugation may be increased by storage in highly ethanolic solutions because conjugates are more stable in aqueous solutions (Ziegler, personal communication). That ethanol may be contributing to the observed chemical changes is supported by the observation of increased fGC concentrations, even in samples stored in ethanol at sub-zero temperatures.

Third, fecal metabolites related to the parent hormone may be oxidized in the ethanol solution. The formation of hydroxyl groups on fecal metabolites may result in increased affinity of these molecules to the primary antibody used in our assays (Moestl, personal communication). A group-specific antibody may have a greater affinity for these oxidized metabolites than a highly specific antibody. High rates of both deconjugation and oxidation may contribute to the increase in measured metabolites at the beginning of the experiment.

Despite inherent complications, some which have been identified in this paper, fecal steroid techniques are capable of answering a variety of physiological questions that otherwise might remain intractable in free-

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Table 3
Three hypotheses proposed to explain the chemical changes that may be occurring during long-term storage of fecal samples in ethanol and suggested practical solutions

Hypothesis		Temporal pattern	HPLC		Solution
			Fraction location	Fraction magnitude	
Extraction efficiency		Increasing over time, followed by a decline	Stable	Increasing	Extract samples ASAP after collection
Metabolite deconjugation	Before hydrolysis and solvolysis	Increasing over time, followed by a decline	Stable	Changing	Treat all samples with sequential hydrolysis and solvolysis[a]
	After hydrolysis and solvolysis	Constant, followed by a decline	Stable	Constant	
Metabolite oxidation		Increasing over time, followed by a decline	Changing	Changing	Limit the time samples that are stored in ethanol at room temperature[b]

Temporal pattern refers to predicted pattern of concentrations measured in samples assayed at time intervals. HPLC refers to the predicted results for each type of chemical change when samples are analyzed by high performance liquid chromatography and the subsequent fractions analyzed with RIA. Depending on the exact method, HPLC can generate 40 fractions. RIA determines which of the 40 fractions are immunoreactive and the relative concentration of immunoreactive compounds in each fraction. The location (e.g., fractions 2, 8, and 20 are immunoreactive, while the remaining 37 are not) and the magnitude (i.e., concentration) of fractions changes according to the chemical changess occurring in the sample.

[a] Sequential hydrolysis and solvolysis liberate steroids from their conjugate enabling the measurement of total fecal steroid concentrations (Ziegler et al., 1996; Ziegler et al., 1997a,b). Alternatively, conjugates, may be stabilized in the field by extracting fecal steroids into an aqueous solution (distilled water:ethanol::50:50, Lynch et al., 2002; Strier and Ziegler, 1997; Strier et al., 1999). However, further validation is needed to determine if ambient temperature storage of these extracts is feasible.

[b] A group-specific antibody may be more likely to detect this kind of chemical change than a highly specific antibody. Therefore, time limits on storage in ethanol may vary according to the type of antibody used in the RIA.

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living populations of large social animals. While storage effects may confound the ability to detect subtle physiological differences between individuals, fecal steroid techniques are capable of identifying large differences between study groups. For example, non-invasive fecal steroid techniques have been used (1) to study population dynamics (Berger et al., 1999), (2) to assess capture techniques (Creel et al., 1997), and (3) to examine the interface between hormones and behavior (Brockman and Whitten, 1996; Brockman et al., 1998; Cavigelli, 1999; Cavigelli and Pereira, 2000; Lynch et al., 2002; Scheibe et al., 1999; Strier and Ziegler, 1997, 2000; Strier et al., 1999). The ability to answer more fine-grained questions will be enhanced when field storage methods are improved to reduce or eliminate the variability introduced by various storage regimes.

While our results provide some guidance for field workers, they generate a number of additional questions. Some questions that warrant future attention are: (1) To what extent are the observed dynamics species-specific? (2) How does the initial concentration of these samples influence reaction dynamics? (3) Do temperature and initial concentrations interact at ambient temperatures and at temperatures below $-20\,^{\circ}$C? (4) Can this problem be ameliorated by using more specific antibodies, different preservatives, or extracting samples into aqueous ethanol? Field experiments will complement laboratory ones in answering these questions and enhancing the potential of non-interventive field sampling for physiological investigations.

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