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Modifications of a field method for fecal steroid analysis in baboons

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Abstract

By extracting steroid metabolites from feces, researchers can track endocrine activity noninvasively in free-ranging animals. Sample preservation is a critical component of such methods because steroid metabolites rapidly decompose. Here, we describe a method for preservation, field extraction, and radioimmunoassay of steroid metabolites (estradiol, progesterone, glucocorticoids, and testosterone) from the feces of wild female baboons (*Papio* spp.). This method is a modification of that developed by Stavisky [Socioendocrinology: noninvasive techniques for monitoring reproductive function in captive and free-ranging primates. PhD, Emory University, 1994.], which employs reversed-phase octadecylsilane cartridges to extract steroids from feces. In addition to providing physiological validation for this method, we examine variation in steroid concentration across different (1) collection times (morning vs. afternoon), (2) methanol extraction treatments (homogenized vs. hand-mixed), and (3) solid-phase extraction times (2 vs. 10 h after collection). We then examine the stability of sample storage at ambient and subzero temperatures to determine whether storage time significantly alters steroid concentrations. Our results show that hormone concentrations do not differ between morning and afternoon samples, homogenization yields significantly higher fecal steroid concentrations, and fecal steroids are stable in a methanol/acetone solution for up to 10 h. When stored at ambient temperatures, only glucocorticoid metabolites had some degradation over a period of up to 40 days. However, when stored at −10 °C, no significant steroid changes were observed for up to 400 days. This method is particularly suited for behavioral research because it permits delays between sample collection and sample processing, thus allowing behavioral observations to continue.
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1. Introduction

Monitoring hormone profiles through the use of fecal steroid assays offers a noninvasive and potentially long-term means of assessing adrenocortical [2–8], testicular [9–14], and ovarian [15–19] hormones in many vertebrate taxa. Because feces can be collected without capturing or interfering with animals, methods that use fecal steroid metabolites have become increasingly popular in both captive and free-ranging studies [20]. In combination with behavioral observations, researchers are now able to investigate many of the proximate physiological mechanisms that underlie animal behavior.

The primary problem with fecal steroid analysis arises from the breakdown of steroids by gastrointestinal bacteria and exogenous microbes. Fecal samples must be preserved within hours of evacuation to avoid the decomposition of steroids [21,22]. Frozen storage of fecal samples minimizes bacterial metabolism and is the preferred method under controlled laboratory conditions [23]. Methods for preserving fecal steroids in field settings, however, vary widely and include immediate freezing [21,24], storage in ethanol followed by freezing [18,25,26], storage in ethanol followed by lyophilization [27,28], oven drying [29], or extracting steroids from the fecal matrix and containing them in a stable environment [1,30,31].

Determining the most suitable method for a given study population involves several considerations. Fecal sample collection may interrupt the collection of behavioral data, particularly for methods that require sample processing shortly after defecation (i.e., freezing or oven drying). Therefore, if behavioral data are simultaneously collected, a method that requires minimal immediate fecal processing is most suitable. Another consideration is the availability of equipment, such as an oven, a freezer, or a freeze dryer.

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Many remote field sites do not provide easy access to such equipment. Finally, the chosen method must be validated for the species in question. Although emphasis has been placed on validating the immunoassay antibodies for a given steroid and species, some of the largest potential sources of error can occur during sample collection and storage prior to immunoassay. Evaluating the factors that distort or bias fecal steroid concentrations is necessary to demonstrate the validity of any method. Comparatively few studies have tested how factors such as time of day, storage time, and sample mixing can affect fecal steroid recovery and accuracy [16,21,27–29,32–35].

Here, we describe a method for preservation, field extraction, and radioimmunoassay (RIA) of fecal steroids [estradiol (E_2), progesterone (P_4), glucocorticoids (GC), and testosterone (T)] from the feces of wild female baboons (*Papio* spp.). This method is a modification of that developed by Stavisky [1] that employs reversed-phase octadecylsilane (C18) cartridges to extract steroids from feces [30]. The objectives of our study were to (1) develop a fecal preservation method that tolerates considerable time delays between sample collection and sample processing, (2) determine the effect on steroid concentrations of several collection factors (collection time, homogenization, and extraction time), and (3) determine the effects of storage over time at both ambient and subzero temperatures.

2. Materials and methods

All fecal samples analyzed in this study were collected from wild female hybrid baboons (*Papio hamadryas anubis* × *Papio hamadryas hamadryas*) living in the Awash National Park of Ethiopia. A detailed description of the Awash hybrid zone in general [36,37] and this group in particular [38–40] can be found elsewhere. All fecal samples ($n = 844$) were collected over a period of 11 months from 25 adult females at the rate of ~ 1 sample/female/week.

2.1. Field processing of fecal samples

When a positively identified fecal sample was observed, the fecal sample was collected in a plastic cup and thoroughly mixed with a spatula. Next, ~ 0.5 g of the sample was placed in 10 ml of a methanol/acetone solution (100% methanol; 8:2) and the sample was immediately homogenized (~ 1 min) using a battery-powered homogenizer (BioVortexer, MidWest Scientific). Following homogenization, samples were capped tightly and stored at ambient temperature until processing.

Ten hours after the sample was collected and homogenized, 4.0 ml of the sample solution were filtered through a polytetrafluoroethylene (PTFE) syringeless filter (0.2 μm; catalogue AV125EORG, Whatman, Clifton, NJ) to remove particulate matter. The filter was then washed with 4.0 ml of methanol/acetone solution (100% methanol; 8:2). The filtrate was diluted 1:2 with distilled water and set aside while cartridges were primed. Sep-Pak Plus C18 cartridges (catalogue WAT020515, Waters Associates, Milford, MA) were primed according to the manufacturer's instructions using 2.0 ml of 100% methanol followed by 5.0 ml of distilled water. The filtrate was loaded onto the primed cartridge at a steady rate (~ 0.2 ml/s) using a syringe. The cartridge was washed with 2.0 ml of a sodium azide solution (0.1%) to further reduce degradation [41], placed in a sterile Whirl-Pak bag with 1.0 g of silica beads (a desiccant), and stored for up to 40 days at ambient temperature until shipped (via DHL) to the United States. On arrival, all samples were immediately frozen at − 10 °C. After allowing the residual fecal matter to dry completely (up to 2 weeks), we removed undigested seeds and recorded the dry weight of the residual fecal material.

The recovery of labeled steroids from fecal homogenates following (1) PTFE filtration and (2) solid-phase extraction was assessed by Stavisky [1] and Stavisky et al. [30]. Following PTFE filtration, recovery for E_2 was 91.9 ± 1.6% ($n = 5$) [1], P_4 was 95.4 ± 2.5% ($n = 6$), and cortisol was 92.4 ± 1.1% ($n = 4$; Stavisky and Whitten, unpublished data). Solid-phase extraction recovery for E_2 was 82.7 ± 1.8% ($n = 5$) [1], P_4 was 93.0 ± 3.9% ($n = 4$), and cortisol was 76.4 ± 2.0% ($n = 9$; Stavisky and Whitten, unpublished data). Additionally, Stavisky et al. [30] also assessed steroid recovery from samples processed in the field ("field-extracted") as compared with sample duplicates that were frozen in the field and later processed in the laboratory ("lab-extracted"). E_2 concentrations in the field-extracted samples were 115.0 ± 16.5% ($n = 22$) of lab-extracted duplicates, and P_4 concentrations were 72.6 ± 9.4% ($n = 22$) of lab-extracted duplicates. E_2 and P_4 concentrations obtained from field-extracted samples were correlated with the values obtained from the lab-extracted duplicates (E_2: $r = 0.994$, $y = 0.919x + 0.004$, $P < .01$, $n = 22$; P_4: $r = 0.669$, $y = 0.342x + 0.236$, $P < .01$, $n = 22$) [30].

2.2. Laboratory processing and RIA

Following sample storage at − 10 °C for up to 400 days, samples were incubated at room temperature for 1 h. Steroids were then slowly eluted from cartridges with a syringe and 3 ml of 100% methanol. Samples were frozen (− 80 °C) until the time of RIA. Immediately prior to RIA, aliquots of samples were evaporated under nitrogen and reconstituted 1:1 in working buffer (working buffer varied for RIA). Samples were assayed to determine the concentration of fecal E_2 (fE_2), fecal P_4 (fP_4), fecal GC (fGC), and fecal T (fT). For each RIA, we list any compounds that cross-react more than 0.1% with the antibody. All samples were run in duplicate and mean concentrations are expressed in ng/g.

2.2.1. E_2 RIA

The E_2 RIA followed the microassay procedures developed by Worthman et al. [42] using reagents from the Pantex Direct ^{125}I Estradiol RIA kit for serum determinations (catalogue 174M, Pantex, Santa Monica, CA). The primary antibody in this kit cross-reacts 100% with estradiol-17β, 5.6% with estrone, 2.63% with ethynylestradiol, 1.9% with α-estradiol and 0.68% with estriol (Pantex). Working buffer was 0.1% gelatin phosphate-buffered saline (pH 7.4). The buffer was made by adding 0.1 g of gelatin to 100 ml of Dulbecco buffer (Gibco, Grand Island, NY) and incubating for 40 min at 45 °C. The E_2 standards provided with the Pantex kit were diluted 1:10 with buffer to give concentrations of 1–300 pg/ml. The first antiserum (rabbit-produced anti-E_2) was diluted 1:8, the second antiserum (goat anti-rabbit antibody) was diluted 1:4, and the controls (catalogue C-370-5-IA, Bio-Rad Laboratories, Anaheim, CA) were diluted 1:10 with buffer to yield high, middle, and low E_2 controls. ^{125}I E_2 tracer (20 μl) and diluted antiserum (500 μl) were added to aliquots (50 μl) of the diluted controls, diluted standards, and samples. After overnight incubation at room temperature, diluted second antiserum (500 μl) was added. The incubates were vortexed, incubated for 1 h at room temperature, and centrifuged for 1 h ($1500 \times g$) at room temperature. The supernatant was decanted and the radioactivity of the precipitate was determined by 10-min counts in a RIASTAR gamma counter (Packard, Downer's Grove, IL) with RIASMART and Expert QC software.

2.2.2. P_4 RIA

The P_4 RIA followed the microassay procedures developed by Worthman et al. [43] using reagents from the Pantex Direct ^{125}I Progesterone kit for serum determinations (catalogue 137, Pantex). The primary antibody in this kit reacts 100% with P_4, 0.5% with 17α-hydroxyprogesterone, and 0.1% with androstenedione. Working buffer was 0.1% gelatin phosphate-buffered saline (pH 7.4). The P_4 standards provided with the Pantex kit were diluted 1:5 with buffer to give concentrations of 0.04–16 ng/ml. The first antiserum (rabbit-produced anti-E_2) was diluted 1:6, the second antiserum (goat anti-rabbit antibody) was diluted 1:4, and the controls (Bio-Rad Laboratories) were diluted 1:10 with buffer to yield a high, middle, and low P_4 controls. ^{125}I P_4 tracer (100 μl) and diluted antiserum (100 μl) were added to aliquots (100 μl) of the diluted controls, diluted standards, and samples. After overnight incubation at room temperature, diluted second antiserum (100 μl) was added, and the incubates were vortexed, incubated for 1 h at room temperature, and centrifuged for 1 h ($1500 \times g$) at room temperature. Following decanting of the supernatants, the radioactivity of the precipitate was determined by 10-min counts in the gamma counter.

2.2.3. GC RIA

The GC RIA followed the instructions and used reagents from the ImmuChem double antibody corticosterone ^{125}I RIA kit (catalogue 07-120102, ICN Diagnostics, Costa Mesa, CA). The primary antibody in this kit reacts 100% with corticosterone, 0.34% with desoxycorticosterone, 0.1% with T, and 0.05% with cortisol. Although cortisol is the primary GC in primates, we selected the ICN Diagnostics corticosterone RIA for our study for several reasons. (1) In a recent study, Wasser et al. [4] demonstrated that the primary antibody in this kit had relatively high cross-reactivities to the major cortisol metabolites present in feces during peak excretion following both radiolabel infusion and adrenal activation. The authors concluded that the ICN Diagnostics corticosterone antibody may be a group-specific antibody, with cross-reactivities to multiple GC metabolites excreted in feces. (2) The authors reported that the ICN Diagnostics corticosterone antibody was superior to other antibodies for measuring GC metabolites in feces of a wide range of mammalian species. (3) The primary antibody in this kit has been previously validated for use in baboons [4,27].

Working buffer was phosphosaline gelatin buffer (pH 7.0) containing rabbit gamma globulins. The standards (25–1000 ng/ml), controls (human serum based), antiserum (rabbit-produced), and precipitant solution (goat anti-rabbit antibody) were not diluted for assays. Corticosterone ^{125}I tracer (200 μl) and antiserum (200 μl) were added to aliquots (100 μl) of the controls, standards, and samples. After 2 h of incubation at room temperature, precipitant solution (500 μl) was added, and the incubates were vortexed and centrifuged for 15 min ($1500 \times g$) at room temperature. Following decanting of the supernatants, the radioactivity of the precipitate was determined by 10-min counts in the gamma counter.

2.2.4. T RIA

The T RIA followed the microassay procedures developed by Beall et al. [44] using reagents from the Equate RIA ^{125}I Testosterone Kit for serum determinations (catalogue 616-100, SolidPhase, Portland, ME). The primary antibody in this kit reacts 100% with T, 1.7% with dihydrotestosterone, and 0.06% with estradiol-17β. All other compounds tested for cross-reactivity yielded less than 0.01% cross-reactivity. Working buffer was 0.1% gelatin phosphate-buffered saline (pH 7.4). The T standards provided with the Equate kit were diluted 1:10 with buffer to give concentrations of 1.2–100 ng/dl. The first antiserum (rabbit-produced) was diluted 1:4, the second antiserum (goat anti-rabbit antibody) was diluted 1:2, and the controls (male and female controls from Equate kit, high and low controls from Bio-Rad Laboratories) were diluted 1:10 with buffer. ^{125}I testosterone tracer (50 μl) and diluted antiserum (100 μl) were added to aliquots (10 μl) of the diluted controls, diluted standards, and samples. After overnight incubation at room temperature, diluted second antiserum (500 μl) was added, and the incubates were vortexed, incubated for 20 min at

room temperature, and centrifuged for 1 h (1500 × *g*) at 4 °C. Following decanting of the supernatants, the radioactivity of the precipitate was determined by 10-min counts in the gamma counter.

2.3. RIA validation

We validated all RIAs by running serial dilutions of a fecal extract pool and comparing the slope of expected dose versus percent bound to the slope of the standard curve for each RIA. Table 1 lists the regression statistics for serial dilutions. Mean assay sensitivity (defined as the quantity of unlabeled steroid required to inhibit binding of tracer by an amount equal to 2 S.D. below the mean in the absence of unlabeled steroid), accuracy (assessed by running one of the standards as a sample), and recovery (assessed by adding incremental known quantities of steroid to samples and measuring the incremental increase in concentration) are also listed in Table 1. Internal controls were run in every RIA and consisted of a pooled fecal sample and two immunoassay controls (high and low) either provided with the kit (ICN Diagnostics) or purchased separately (Bio-Rad Laboratories). Intraassay and interassay coefficients of variation (CV) for each RIA are listed in Table 1.

While we use RIA kits that employ fairly specific antibodies, there is no reason that the method of steroid hormone preservation, extraction, and storage described here would not also work for more group-specific antibodies. We selected and validated antibodies based on their commercial availability, the availability of quality control data and testing procedures from the manufacturer, and the ability to compare our data to other studies using the same antibodies.

2.4. Physiological validation

To assess whether the Pantex E_2 and P_4 antibodies reflected gonadal function, we compared fE_2 and fP_4 levels across four categories of female baboons: nonpregnant (cycling) and first, second, and third trimesters of pregnancy. In all cases, pregnant females had significantly higher ovarian hormone levels than nonpregnant females. Furthermore, non-pregnant females ($n = 18$) had the lowest P_4 and E_2 levels followed by first trimester ($n = 14$), second trimester ($n = 10$), and third trimester ($n = 5$) females, respectively. ANOVA followed by a Tukey's multiple comparisons test indicated that all differences between reproductive stages were significant [P_4: $F(3,43) = 11.85$, $P < .01$; E_2: $F(3,43) = 113.02$, $P < .01$].

Although Wasser et al. [4] physiologically validated the ICN Diagnostics corticosterone antibody for yellow baboons (*Papio hamadryas cynocephalus*), showing that it detects a rise in GC metabolites levels following an ACTH challenge, we wanted to validate it for this population of hybrid baboons. To do so, we used two trapping

Table 1
Validation of fecal steroid RIA in baboons (*Papio* spp.)

RIA (manufacturer)	Sensitivity	Accuracy ± S.E.% (*n*)	Log-logit serial dilution regression equation (*n*)	Parallelism	Recovery ± S.E.% (*n*)	Intraassay CV (%)	Interassay CV (%)		
						Fecal extract pool (*n*)	Fecal extract pool (*n*)	High control (*n*)	Low control (*n*)
E_2 (Pantex)	0.58 pg/ml	95.11 ± 2.06 (7)	$y = -1.70x + 0.66$ (4); $r^2 = .99$, $P < .01$	Yes	82.38 ± 9.14 (6)	5.12 ± 0.46 (6)	1.73 ± 0.15 (6)	2.85 ± 0.62 (6)	2.59 ± 0.09 (6)
P_4 (Pantex)	0.03 ng/ml	118.47 ± 11.54 (9)	$y = -1.18x - 0.81$ (4); $r^2 = 1.00$, $P < .01$	Yes	104.74 ± 2.34 (8)	2.61 ± 0.01 (6)	5.52 ± 0.02 (6)	6.74 ± 0.07 (6)	6.93 ± 0.03 (6)
Corticosterone (ICN Diagnostics)	0.71 ng/ml	97.08 ± 3.18 (6)	$y = -0.90x + 0.89$ (5); $r^2 = .97$, $P < .01$	Yes	96.58 ± 3.94 (5)	8.98 ± 5.19 (6)	12.14 ± 7.29 (6)	7.73 ± 13.40 (6)	3.54 ± 1.92 (6)
T (Equate)	0.16 ng/dl	118.11 ± 8.89 (7)	$y = -0.72x - 0.81$ (4); $r^2 = .99$, $P < .01$	Yes	105.88 ± 3.15 (6)	2.90 ± 0.78 (6)	6.18 ± 1.44 (6)	5.93 ± 1.32 (6)	3.83 ± 0.05 (6)

seasons carried out by the Awash National Park Baboon Research Project [45] as a stress challenge to trapped individuals. Prebaiting traps with corn created a situation where a high-calorie resource provoked aggressive interactions at a much higher rate than observed during normal foraging [40]. This stress challenge continued for 2 weeks while fecal and other data were collected on this group. For each female, we compared trapping period fGC concentrations with basal (pretrapping) levels. A Wilcoxon Signed Ranks Test indicated that trap sample fGC levels were significantly higher than basal sample fGC levels ($Z = -3.42$, $P < .01$, $n = 19$).

Like ovarian hormones, T changes with reproductive state, significantly rising with the onset of pregnancy. Because T is a precursor for estrogens, the ovaries increase T production during pregnancy in response to the need for increased estrogens [46]. To validate the Equate T antibody, we compared T levels from pregnant females ($n = 25$ females) with T levels of nonpregnant (i.e., cycling and lactating) females ($n = 20$ females). fT was significantly higher in pregnant than in nonpregnant females [$F(1,43) = 11.98$, $P < .01$].

2.5. Treatment groups

First, we wanted to check for possible circadian effects on fecal steroid concentrations. Serum and urinary steroids certainly show diurnal variation [47–53]; however, the results from fecal steroid studies are less consistent, and it seems to be related to the particular target species. Hence, a methodological question arises as to whether fecal samples can be collected throughout the day or whether they must be restricted to the morning only. To address this question, we collected two samples from the same female on the same day, one in the early morning and one in the early afternoon. We collected two daily samples from 20 different females. Seven "pairs" (i.e., a morning sample and an afternoon sample) were collected during the rainy season, and 13 pairs were collected during the dry season to control for seasonal differences in diet. Paired samples were assayed for fE_2, fP_4, fGC, and fT. All storage conditions for these pairs were identical.

Second, to determine whether homogenization using the battery-powered vortexer aided in methanol extraction of fecal steroids, we collected seven samples (from seven different individuals) in duplicate, homogenizing one with the vortexer (~ 1 min) and mixing the other one by hand (~ 1 min). GCs and T were assayed for these seven pairs. All storage conditions for these pairs were the same.

Wasser et al. [21] demonstrated that ethanol stabilizes fecal estrogens and progestins over a period of 21 h at ambient temperatures, presumably killing bacteria and inactivating their associated enzymes. To determine whether fecal steroids are also stable for extended amounts of time in the methanol/acetone solution, we collected 20 samples in duplicate (from 20 different individuals). All duplicates were collected and homogenized in the methanol/acetone solution. The first sample was processed (i.e., filtered and solid-phase extracted) 2 h after collection and the duplicate was processed 10 h after collection. All 20 pairs were assayed for fE_2, fP_4, fGC, and fT. Storage conditions for all duplicates were the same.

To determine whether steroid concentrations changed in relation to the number of days that samples were stored at ambient temperature prior to freezing, we examined steroid concentrations for all samples as a function of number of storage days. The length of time samples were stored at ambient temperature ranged from 4 to 39 days (including time spent in transit by air to the United States). There were 11 separate sample shipments (~ 1/month for 1 year). For logistical reasons, the trips to Addis Ababa (to mail samples to the United States) during the rainy season were less frequent; therefore, most of the samples stored for the longest period of time (35–39 days) were rainy season samples. Moreover, samples collected during the rainy season also had significantly lower fGC concentrations [40]. Therefore, the samples stored for the longest period of time may have lower fGC concentrations not due to steroid degradation but rather because the entire shipment had a lower fGC mean. To avoid this bias, samples within shipments were standardized by subtracting the shipment hormone mean from each sample value. Ovarian hormone samples were split by reproductive state (pregnant/nonpregnant). Ambient storage temperatures before shipping ranged from 16.4 to 36.7 °C. Although temperatures were higher on average during the dry season than during the wet season, daily temperature fluctuations in the ANP always were greater than yearly fluctuations [40,54].

Finally, to determine whether steroid concentrations changed in relation to the number of days that samples were stored at −10 °C prior to RIA, we examined steroid concentrations of cycling females as a function of number of storage days. We used only cycling females because P_4, E_2, and T are known to exhibit incremental increases and decreases over time in pregnant and lactating females, respectively. Furthermore, because behavioral aggression and fT increased during the wet season while fGC decreased [40], we used only dry season samples to control for differences in diet and behavior. Dry season samples included samples taken at the beginning and the end of the study period and therefore encompassed the entire range of storage days.

2.6. Statistical analysis

Wilcoxon Signed Ranks Tests were used in all paired comparisons. Variation of fecal steroids across time was analyzed using least squares linear regression as well as quadratic and cubic regression. All tests were two tailed and significance levels were set at $P \leq .05$.

3. Results

3.1. Morning versus afternoon samples

Paired comparisons indicated that there were no differences in fecal steroid concentration for morning and afternoon fecal samples for all steroid hormones (fE_2: $Z = -0.52$, $P > .05$; fP_4: $Z = -0.97$, $P > .05$; fGC: $Z = -0.67$, $P > .05$; fT: $Z = 0.34$, $P > .05$). For fE_2, fP_4, and fGC, afternoon samples were slightly higher, but the magnitude of the rise from morning to afternoon samples did not exceed 9.5%; for fT, the afternoon samples dropped by 0.04%. Similarly, there were no differences in steroid concentration for morning and afternoon samples when split into wet season samples (fE_2: $Z = -0.17$, $P > .05$; fP_4: $Z = -0.85$, $P > .05$; fGC: $Z = -0.51$, $P > .05$; fT: $Z = -0.17$, $P > .05$) and dry season samples (fE_2: $Z = -0.87$, $P > .05$; fP_4: $Z = -0.52$, $P > .05$; fGC: $Z = -0.52$, $P > .05$; fT: $Z = -0.66$, $P > .05$).

3.2. Homogenization versus nonhomogenization

Homogenized samples yielded higher fecal steroid recovery than nonhomogenized samples. A paired comparison indicated that fGC concentration of homogenized samples was 21.82% higher than that of nonhomogenized samples ($Z = -2.37$, $P < .05$; Fig. 1). fT was 16.36% higher in homogenized than nonhomogenized samples ($Z = -1.18$, $P > .05$), but this difference was not significant.

3.3. Time delay to solid-phase extraction (at ambient temperature)

Paired comparisons between samples solid-phase extracted 2 and 10 h after collection indicated no differences in fecal steroid recovery of all steroid hormones (fE_2: $Z = -0.261$, $P > .05$; fP_4: $Z = -1.14$, $P > .05$; fGC: $Z = -1.01$, $P > .05$; fT: $Z = -1.53$, $P > .05$).

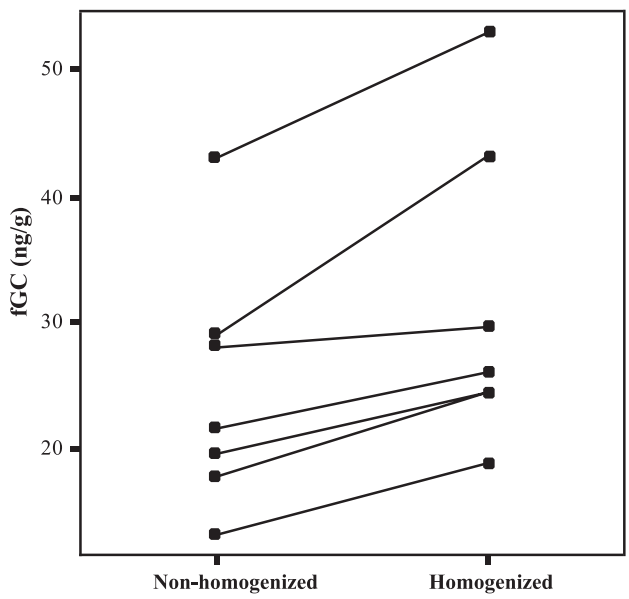

Fig. 1. fGC concentrations (ng/g) for nonhomogenized and homogenized duplicate samples ($n = 7$).

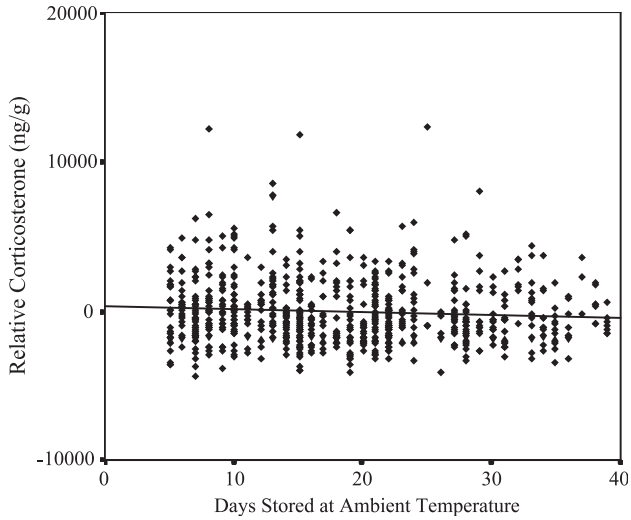

Fig. 2. fGC concentrations (ng/g) standardized by each shipment fGC mean (see text for details) for all samples as a function of days stored at ambient temperature prior to freezing ($r^2 = .06$, $P = .04$, $n = 728$, $y = 0.77x - 0.06$).

3.4. Time delay to freezing (at ambient temperature)

fE_2 and fP_4 concentrations did not vary with the number of days stored at ambient temperature prior to freezing for pregnant females (fE_2: $r^2 = .01$, $df = 283$, $P > .05$; fP_4: $r^2 = .00$, $df = 148$, $P > .05$) or nonpregnant females (fE_2: $r^2 = .00$, $df = 148$, $P > .05$; fP_4: $r^2 = .00$, $df = 283$, $P > .05$). Likewise, there was no relationship between fT concentrations and number of days stored at ambient temperature ($r^2 = .00$, $df = 719$, $P > .05$). fGC concentrations, however, were significantly correlated with storage time, with a gradual decrease in concentrations over time ($r^2 = .06$, $df = 727$, $P < .05$). The rate of decline was low, however, representing no more than 9.3% over 30 days of storage (Fig. 2).

3.5. Time delay to RIA (at −10 °C)

We assessed data using linear regression (least squares) to determine whether hormone concentrations increased or decreased over extended periods of time. None of the fecal steroids varied with the number of days stored at −10 °C prior to RIA for cycling females (fE_2: $r^2 = .00$, $df = 104$, $P > .05$; fP_4: $r^2 = .02$, $df = 105$, $P > .05$; fGC: $r^2 = .00$, $df = 91$, $P > .05$; fT: $r^2 = .04$, $df = 91$, $P > .05$). However, because one study found a nonlinear pattern to fecal steroid changes during storage at both ambient and subzero temperatures [27], noting a rise in fGC and fecal estrogens followed by a subsequent drop in fecal steroid concentrations over a 6-month period, we also assessed our data to determine if a curvilinear relationship existed. Our samples showed a high degree of variability between days because each sample was

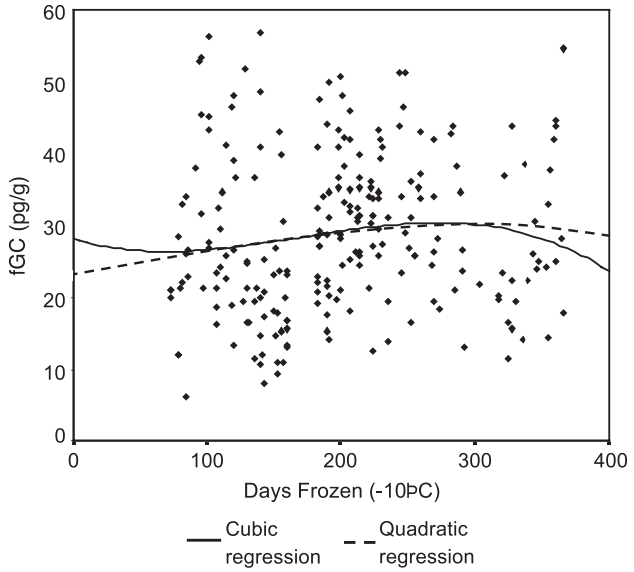

Fig. 3. fGC concentrations for samples (cycling females only) stored from 69 to 370 days at −10 °C (n=224). Lines represent nonsignificant quadratic (dotted) and cubic (solid) curvilinear relationships.

from one of several cycling females; however, neither quadratic (r^2=.01, df=223, P>.05) nor cubic (r^2=.01, df=222, P>.05) regression equations explained a significant proportion of the variability in fGC concentration (Fig. 3).

4. Discussion

In combination with Stavisky et al.'s [30] work, this study has demonstrated the feasibility and validity of the fecal steroid extraction method for determining physiological function (adrenocortical, testicular, and ovarian) in free-ranging baboons. The RIAs employed here are sensitive, accurate, and precise, providing valid estimates of steroid hormones. Comparison with observational data showed that fGC metabolites exhibited the expected response to a stress challenge and that fE_2, fP_4, and fT reflected reproductive stages (increasing with stages of pregnancy).

There were no significant differences between morning and afternoon samples taken on the same day for any of the fecal steroids assessed in this study. Many steroid hormones exhibit diurnal variation in secretion. GCs, for example, show peak levels early in the morning and a nadir around midnight in humans and nonhuman diurnal primates [47–50]. T secretion has a rhythm that is related more to subject activity than photoperiodic effect; yet, in both human and nonhuman primates, plasma T concentrations do exhibit circadian patterns [49,51–53]. In three species of nonhuman primate, increases in serum cortisol metabolites were detected in urine ~5.5 h later [55], indicating that urinary and serum steroids are subject to the same circadian rhythms. Previous reports have suggested that morning sample collections provide the most accurate representation of urinary steroid concentrations [56]. However, there is a longer delay (26–48 h) between hormone secretion and detection in fecal steroids [55,57–59] because circulating steroids are first metabolized in the liver to conjugated metabolites before being excreted in the bile and, in some cases, are hydrolyzed to active and resorbable forms that can undergo enterohepatic recirculation before excretion [60,61]. Furthermore, differences in diet can drastically reduce or slow down the excretion of steroids in feces [33]. Therefore, it is not surprising that fecal steroid excretion in baboons did not exhibit a circadian rhythm. However, several studies of New World primates have found differences in circadian rhythms of fecal cortisol (capuchins and marmosets: Refs. [10,62]) and fP_4 (marmosets: Ref. [62]), a result that may be related to species differences in metabolism and diet.

Samples that were homogenized with a battery-powered homogenizer had higher hormone levels—presumably the result of increased hormone recovery from the fecal matrix. Many fecal steroid extraction methods recommend the use of a vortexer for optimal steroid recovery during a methanol or ethanol extraction step [4,27,63]. Manually shaking the sample in solution, while approximating homogenization to the naked eye, does not extract as much hormone into solution as mechanical homogenization. For field situations, we recommend a consistent, timed homogenization step of at least 1–2 min/sample.

Wasser et al. [21] demonstrated that fecal steroids were stable for up to 21 h when stored in ethanol at ambient temperatures. Our results suggest that a methanol/acetone solution also stabilizes fecal steroids for extended periods. Although we tested stability only up to 10 h (a convenient time for field extraction), there remains the possibility that fecal steroids might be stable for a longer period in this solution.

While fE_2, fP_4, and fT showed no changes when stored on the C18 cartridge at ambient temperatures, fGC showed limited degradation. Stavisky [1] found that fecal cortisol was stable when stored on C18 cartridges and frozen on the day of collection—a step that greatly enhanced steroid stability. In our study, steroid degradation was linear across time (Fig. 2), which facilitates the use of a correction factor once the change is quantified. However, we recommend that samples be frozen within 2 weeks to minimize steroid degradation and obviate the need for a correction factor. Once samples were frozen, there was no significant change in steroid concentrations. Rather, samples exhibited variable changes with no consistent trend across storage time, and this remained true for samples stored up to 400 days. These results are in contrast to a recent study on baboon fecal steroid storage [27] that found samples stored in ethanol at −20 °C exhibited an increase in fGC (up to 120 days) followed by a decrease in concentration (up to 180 days). The difference in fecal steroid stability at subzero temperatures between studies may, in part, be due to the different

storage media. Khan et al. [27] note that storage in ethanol may continue to extract metabolites from the feces over time, altering measured steroid concentrations. Dry storage of steroid hormones on the C18 cartridge may be more stable than wet storage.

Some drawbacks to the use of this method should be noted. It requires more time in the field for the filtration and solid-phase extraction steps, which are normally delayed until after shipping. Additionally, this method requires regular access to organic chemicals (methanol and acetone) and access to a freezer within 30 days and preferably within 2 weeks.

On balance, however, we feel that the method described here is particularly suited for behavioral research in remote locations for several reasons. First, this method allows an indefinite time lapse after sample collection so that an unassisted field researcher does not need to interrupt observation to process samples. Second, the initial preservation and methanol extraction step are combined, reducing time spent on sample processing. Third, immediate access to a freezer is not necessary. Fourth, this method separates steroids from fecal matrix on the same day as collection, reducing the steroid degradation that occurs in feces. Finally, the solid-phase column is available in the form of a small, lightweight cartridge, allowing for easy storage and transport.

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