

## Effect of Long-Term Preservation Methods on Fecal Glucocorticoid Concentrations of Grizzly Bear and African Elephant

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### Introduction

Fecal hormone analysis has become a powerful noninvasive tool for the study of animal endocrine status and stress physiology (Graham and Brown 1997; Wasser et al. 1997a; Whitten et al. 1998; Goymann et al. 1999; Möstl et al. 1999; Foley et al. 2001; Creel et al. 2002; Lynch et al. 2002; Morrow et al. 2002). Variation in field and storage conditions makes it essential to know whether fecal steroid concentrations change with the method and duration of sample storage. Lyophilization (freeze-drying) with subsequent storage at or below  $-20^{\circ}\text{C}$  is generally regarded as the most reliable method of long-term fecal hormone preservation (e.g., Wasser et al. 1988; Terio et al. 2002). However, lyophilizers are not widely available, and therefore investigators have turned to a variety of other fecal storage methods, including freezing at  $-20^{\circ}\text{C}$ , preservation in ethanol, and/or drying the feces with silica, ovens, solar radiation, fires, or other drying methods (Wasser et al. 1988; Whitten et al. 1998; Foley et al. 2001; Tecot 2001). Yet, some of these preservation methods may result in significant changes in immunoreactive hormone concentrations, varying with the preservation method, species, and hormone.

In this article, we report effects of several commonly used fecal preservation methods on immunoreactive glucocorticoid concentrations in feces of two species, African elephant (*Loxodonta africana*) and grizzly bear (*Ursus arctos horribilis*), over a 2-yr period. Five different preservation methods were chosen for comparison: no preservative, oven-drying at  $45^{\circ}\text{C}$ , silica-drying, 90% ethanol, and lyophilization, each with subsequent storage either at room temperature or in a  $-20^{\circ}\text{C}$  freezer. Con-

trols for the experiment consisted of lyophilized samples stored at  $-20^{\circ}\text{C}$ .

A critical issue in fecal hormone assays is whether the assay antibody is binding to the desired hormone(s). This is because parent hormones in plasma are often metabolized in the liver and gut to many different species-specific metabolites, most of which remain unidentified and can have unexpected antibody affinities (see Wasser et al. 2000 for discussion). From a practical perspective, it is not necessary to know the chemical identities of the metabolites that bind to the assay antibody, but it must be demonstrated that they are indeed metabolites of the hormone of interest. For fecal glucocorticoids, this is often accomplished with an adrenocorticotrophic hormone (ACTH) challenge to stimulate glucocorticoid secretion by the adrenal gland, followed by assay of fecal samples collected before and after the challenge. The assay antibody should detect a peak in excreted glucocorticoids after ACTH injection, following the species-specific excretion lag time (e.g., Graham and Brown 1996; Monfort et al. 1998; Goymann et al. 1999; Brown et al. 2001; Wielebnowski et al. 2002). Failure to detect a post-ACTH glucocorticoid peak indicates the assay is not suitable for fecal glucocorticoid metabolites in that species (e.g., Flood et al. 1992). An ACTH challenge on African elephant has previously been performed by Wasser et al. (2000) and established that the assay used in this study accurately detects the expected post-ACTH elevation in excreted glucocorticoids of African elephant. Since comparable data were unavailable for grizzly bears, our study includes an ACTH challenge on this species.

### Material and Methods

#### *Animals, Housing, and Diet*

Two grizzly bears (one male, one female) used in the ACTH challenge were housed at Northwest Trek (Eatonville, Wash.). The two grizzly bears (both male) and the African elephant (female) used in the fecal hormone preservation study were housed at the Woodland Park Zoo (Seattle). Grizzly bears at Woodland Park Zoo were provided an ad lib winter diet of Zupreem Omnivore Diet biscuits, Mazuri Leaf Eater biscuits, meat, apples, yams, carrots, grapes, romaine, celery, and kale; bears at Northwest Trek were provided with Zupreem Omnivore Diet biscuits, Blue Diamond dry dog food, apples, yams, carrots, and occasional fish. The African elephant was fed a daily diet of 36 kg timothy hay, 7 kg carrots, 3–5 kg other

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produce, and 8 kg Mazuri ADF-25 herbivore pellets, with supplemental vitamin E and biotin. All animal handling and animal experiments were done in accordance with local, state, and federal regulations for animal welfare.

#### *ACTH Challenge Experiment*

One adult male and one adult female grizzly bear were injected with porcine ACTH, a pituitary hormone that reliably causes a rapid increase in endogenous glucocorticoid secretion from the adrenal cortex (Norris 1996). The same pattern should be observed in feces, with the ~15-h excretion lag time typical for bears (Pritchard and Robbins 1990; Wasser et al. 2000). Both bears were injected intramuscularly with 2.2 IU/kg of ACTH dissolved in gel (Hadfield's Pharmacy, Edmonds, Wash.), administered by a trained veterinarian using a Telinject blow gun. The injections occurred on the morning of August 8, 2000.

All feces were collected beginning 48 h before injection and continuing for 100 h after ACTH injection. Fecal samples were well mixed, frozen at  $-20^{\circ}\text{C}$  for 1 wk, and freeze-dried for 5 d. Hormones were extracted and assayed the following week. The bears were housed separately during the 48 h after injection to ensure accurate identification of feces. Since precise excretion times were not always known (particularly feces from nighttime dens discovered the next morning), sample excretion times were estimated as the midpoint of possible excretion times for that sample.

#### *Fecal Glucocorticoid Preservation Experiment*

For the fecal preservation experiment, fresh fecal samples from two adult male grizzly bears were collected and pooled over a 36-h period, the minimum time necessary to acquire sufficient fecal mass for the study. The pooled bear sample was kept at  $4^{\circ}\text{C}$  until the end of the 36 h and then processed and preserved. Fecal samples from a single adult female African elephant were collected over 4 h, pooled at room temperature, and processed immediately at the end of the 4 h. Possible effects of the lag time between excretion and preservation have not been investigated for these two species, but for other species, immunoreactive fecal glucocorticoids (for the assay used in this study) are stable for a day or more if kept chilled (e.g., Morrow et al. 2002). In general, the lag time from excretion to preservation in this study was comparable to or shorter than that achieved in most field studies of grizzly bear and African elephant.

For each species, the freshly pooled fecal mass was mixed with gloved hands continuously for 2 h to ensure even distribution of hormone throughout the sample pool (Wasser 1996). Each pooled fecal mass was then divided into 1,000 samples of ~15 g (bear) or ~25 g (elephant). In addition, 20 1-g samples from each species were placed immediately into a freeze-dryer until dry (24 h for these small samples) and extracted and assayed on the next day. These 20 samples provided a "time

zero" reference value for the initial glucocorticoid concentration for each species, allowing us to verify that our control group (lyophilized-freezer) did not change over time.

The 1,000 samples from each species were divided equally among the following five groups. (1) Lyophilized: samples placed in a freeze-dryer at  $-20^{\circ}\text{C}$  under vacuum for 5 d. (2) Unpreserved: samples placed in uncovered vials in a fume hood at ambient temperature ( $\sim 20^{\circ}\text{C}$ ) for 5 d. The fume hood produced mild air flow over the samples, approximating natural air flow in the field. (3) Oven-dried: samples placed in a top-vented laboratory drying oven at  $45^{\circ} \pm 3^{\circ}\text{C}$  until dry, with top vent open to allow moist air to escape. To reduce drying time, samples were flattened to increase surface area and placed in large-mouthed vials. (4) Silica-dried: samples sealed in plastic zip-type bags with desiccating silica beads. Samples were flattened to increase surface area, wrapped twice in paper coffee filters to prevent silica beads from adhering to feces, and fully buried in silica at a 4 : 1 v/v ratio of silica to feces. The silica was a 3 : 1 mixture of nonindicating silica beads to indicating silica crystals (Sigma-Aldrich Chemical, St. Louis). The latter change color when saturated with moisture. At 1 wk, all silica was discarded and replaced with fresh silica mixture. Thereafter, silica was changed for each sample whenever approximately 80% of the indicating silica had changed color. (5) Ethanol: samples fully submerged in 90% ethanol (10% water) at a ratio of 4 mL ethanol per ~1 g feces and sealed in jars with vapor-proof screw-top lids.

All five preservation groups remained in their initial preservative regime for 5 d. This 5-d period was the minimum length of time needed for the oven and silica to thoroughly dry their samples and also serves to approximate a "field transport" period during which samples in a field study might be collected, preserved, and transported from a remote field site back to a laboratory. For these first 5 d, all unpreserved, silica, and ethanol groups were kept at room temperature, whereas oven samples were in the  $45^{\circ}\text{C}$  drying oven and lyophilized samples were in the  $-20^{\circ}\text{C}$  lyophilizer.

At the end of the 5-d initial period, half the samples from each group were put in long-term storage at room temperature ( $\sim 20^{\circ}\text{C}$ ) and the other half in a non-frost-free freezer ( $-20^{\circ}\text{C}$ ; frost-free freezers were avoided because they produce periodic warming cycles). This produced 10 groups per species (five preservative methods  $\times$  two temperatures), each with 100 samples per group. The lyophilized group stored at  $-20^{\circ}\text{C}$  was our control group, selected on the basis of its widespread use in fecal endocrinology and on our experience in other ongoing studies on several species (e.g., African elephant, tree kangaroo, Alaskan moose, Steller sea lion) indicating that freeze-dried frozen samples maintain stable hormone concentrations for up to 1 yr or more (K. Gobush, J. Steenberg, K. Hunt, A. Trites, and J. Crouse, unpublished data).

Each group is hereafter referred to by the initial preservation method (lyophilized, unpreserved, oven, silica, or ethanol) fol-

lowed by the long-term storage temperature (room or freezer), for example, lyophilized-room, silica-freezer, etc.

#### *Time in Long-Term Storage*

Samples were removed from long-term storage for testing at nine time points after fecal collection: 15 d, 30 d, 60 d, 90 d (grizzly bear only), 120 d, 200 d (~6 mo), 270 d (~9 mo), 365 d (1 yr), and 730 d (2 yr). Ten subsamples from each of the 10 groups were selected randomly for analysis at every time point. To allow comparability of hormone levels on a nanogram per gram basis, all subsamples were freeze-dried for 5 d to remove any residual water (Wasser et al. 1988, 1993). Ethanol was first evaporated from the ethanol samples under an air stream overnight in a fume hood at room temperature. After freeze-drying, each subsample was pulverized and sifted through a steel mesh colander, and the resulting fecal powder was well-stirred before extraction. Samples that could not be pulverized (see "Results") were cut into pieces <2 mm wide.

#### *Hormone Extraction*

Steroids were extracted from feces with a methanol vortex method (Schwarzenberger et al. 1991) modified as follows. We weighed fecal subsamples of ~0.2 g to the nearest 0.0001 g and placed them in 16 × 125-mm borosilicate glass tubes. Exactly 2.00 mL of 90% methanol (10% water) was added to each tube. Capped tubes were vortexed for 30 min in a multitube pulsing vortexer (Glas-Col, Terre Haute, Ind.; speed 70, 1 pulse/s) and then centrifuged for 20 min at 1,400 g (2,200 rpm in a Sorvall RC-3B Plus centrifuge with a H-2000B swinging-bucket rotor). We transferred 1.0 mL of the supernatant (containing steroids) to a vapor-proof vial. Extracts were stored at -20°C until assay. Extraction occurred within 1 wk of the time point, and assays occurred within 1 wk of extraction. Recovery of added steroids with this extraction method is >90% for grizzly bear and African elephant feces (data not shown).

#### *Glucocorticoid Assay*

We assayed all samples with a <sup>125</sup>I double-antibody radioimmunoassay (MP Biomedicals, formerly ICN Biomedicals, Costa Mesa, Calif.) that has been validated for fecal glucocorticoids of a variety of mammalian species, including African elephant (Wasser et al. 2000) and two ursids (Malayan sun bear, Wasser et al. 2000; grizzly bears, this study). This assay has been used extensively in our laboratory for African elephant and grizzly bear feces and shows good parallelism and accuracy with fecal extracts of both species (data not shown). Extracts were diluted fourfold in assay buffer for assaying. Interassay and intra-assay variation are both ~6% for this assay in our lab.

The antibody in this assay was raised in rabbits against cor-

ticosterone-3-carboxymethyloxime:BSA. It has high affinity for corticosterone and for various fecal metabolites of corticosterone, and though it has low affinity for pure cortisol (cross-reactivity 0.05%), it binds well to fecal metabolites of cortisol in several mammalian species (probably metabolites that have been dehydroxylated at C-17; see Wasser et al. 2000 for discussion). The manufacturer's reported cross-reactivities for common plasma steroids are desoxycorticosterone 0.34%, testosterone 0.25%, cortisol 0.05%, aldosterone 0.03%, progesterone 0.02%, androstenedione 0.01%, 5 $\alpha$ -dihydrotestosterone 0.01%, and <0.01% for all other tested steroids.

At each time point, the 100 samples (90 experimental samples and 10 control samples) were analyzed in two assays of 50 subsamples each, with the experimental and control groups evenly divided across assays. Note that 10 control samples were assayed at each time point to control for intra-assay and interassay variation. The two assays for the same time point were run in the same week, with assays using the same batches of reagents. However, the long-term nature of this study required that different batches of assay reagents be used at different time points. This inevitably produced some interassay variation, consistent across all fecal samples. To eliminate any effects of this interassay variation on data analysis, data were expressed as a percentage difference from the mean concentration of the control samples (lyophilized-freezer group) analyzed in the same assay.

#### *HPLC*

To confirm that freeze-drying preserves fecal glucocorticoids effectively, we analyzed two grizzly bear groups with high-performance liquid chromatography (HPLC). The grizzly bear control group was analyzed by HPLC immediately after freeze-drying at day 1, to establish the range of immunoreactive metabolites initially present in fresh feces. The lyophilized-room group was then analyzed at day 60 and compared with the control. (Funding constraints prohibited HPLC analyses of all groups.) For each analysis, 4.0 mL of pooled hormone extract was thoroughly dried, reconstituted in citrate buffer, washed by passing through a 0.2- $\mu$ m filter followed by a C-18 matrix column (Spice cartridge, Rainin Instruments, now Varian), and eluted with 5 mL of 80% methanol (see Shackleton 1986). Fecal glucocorticoids were separated using a reverse-phase C-18 column (Rainin Instruments, now Varian) with a gradient solvent system (1 mL/min) of 20%–30% methanol (0–10 min), 30%–40% (10–40 min), 40%–50% (40–55 min), 50%–80% (55–80 min), 80%–100% (80–85 min), and 100% (85–120 min; Wasser et al. 2000). Eluates were dried down, reconstituted in 200  $\mu$ L assay buffer, and assayed with the ICN corticosterone assay. In periodic testing of the HPLC system, tritiated steroids eluted no more than one fraction away from the expected fraction.

### Data Analysis

Hormone data were converted to nanograms immunoreactive hormone per grams dry fecal mass. ACTH challenge data were analyzed with one-tailed *t* tests on preinjection versus postinjection samples, with  $\alpha = 0.05$ . Fecal preservation data were converted to percentage difference from control samples and were then analyzed with repeated-measures ANOVA for each species separately, followed by Fisher's PLSD post hoc tests. Preservation methods identified as significantly different from the control group were then analyzed further with ANOVA on each time point separately, with the Bonferroni correction for multiple tests.

Since a major goal of this study was to identify preservation methods that did not differ from controls, we wished to assess our risk of Type II error (failing to detect a real difference between groups), particularly in the planned post hoc tests after ANOVAs. (Pilot studies led us to expect that the overall ANOVAs would nearly always be significant.) Statistical power analyses based on typical standard deviations observed in pilot studies indicated that our sample size (10 per group) should give a 99% probability of correctly detecting a real 20% difference between any two groups in our planned post hoc tests, a 76% probability of detecting a real 10% difference, and a 25% probability of detecting a real 5% difference, which is in the range of assay variation. Statistical tests on fecal preservation data were two tailed with  $\alpha = 0.05$ . Analyses were performed with Statview 4.5, JMP 4, or SPSS 11 for Macintosh.

### Results

#### ACTH Challenge Results for Grizzly Bear

In both male and female grizzly bears, the glucocorticoid assay successfully detected the expected increase in immunoreactive fecal glucocorticoid concentration after ACTH injection (Fig. 1). Both bears showed a sharp rise in fecal glucocorticoids within 15 h of ACTH injection. Peak fecal glucocorticoid concentrations occurred at 22 h (male) to 32 h (female) after injection. Immunoreactive fecal glucocorticoid concentrations were significantly higher in the 48 h after ACTH injection than in the 48 h before injection (female,  $t_5 = 2.686$ ,  $P = 0.0217$ ; male,  $t_6 = 2.404$ ,  $P = 0.0265$ ). Glucocorticoid levels returned to sustained preinjection levels by 63 h (male) to 87 h (female) after injection.

#### Fecal Glucocorticoid Preservation Experiment

Fecal preservation methods produced marked changes from controls in fecal glucocorticoid concentrations for both elephant and grizzly bear (Fig. 2). Both species exhibited significant main effects of preservation method and time and a significant interaction of preservation method  $\times$  time ( $P < 0.0001$  for all tests; elephant: preservation method,  $F_{9,90} = 123.5$ ; time,

$F_{7,90} = 33.3$ ; interaction,  $F_{7,9,63} = 27.3$ ; grizzly: preservation method,  $F_{9,90} = 209.6$ ; time,  $F_{7,90} = 36.0$ ; interaction,  $F_{7,9,63} = 8.9$ ).

*Preservation Methods Similar to Controls.* Only three methods maintained stable glucocorticoid concentrations relative to the control in either species (Fig. 2; Tables 1–3). The unpreserved-freezer group was not significantly different from the control group in either elephant or grizzly bear. The lyophilized-room group was similar to the control group in elephant but not in grizzly bear. However, post hoc analyses of each time point for grizzly bear show that the lyophilized-room group was not different from controls for any individual time point, and the overall significant difference appears primarily because of the 730-d time point (Table 3). Finally, the silica-freezer group was similar to the control in elephant but not in grizzly bear. All other methods were significantly different from the controls in both species (Table 1). Interestingly, most changes in the elephant samples were increases in glucocorticoid concentrations compared with controls, whereas in the grizzly bear, with the exception of the ethanol-room group, most changes were decreases compared with controls (Fig. 2).

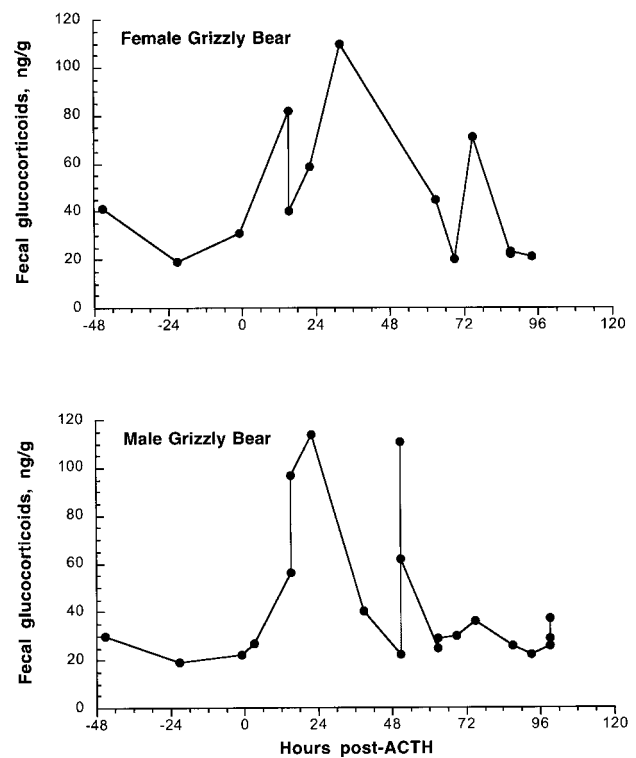


Figure 1. Immunoreactive fecal glucocorticoid concentrations of two grizzly bears before and after injection with adrenocorticotrophic hormone (ACTH).

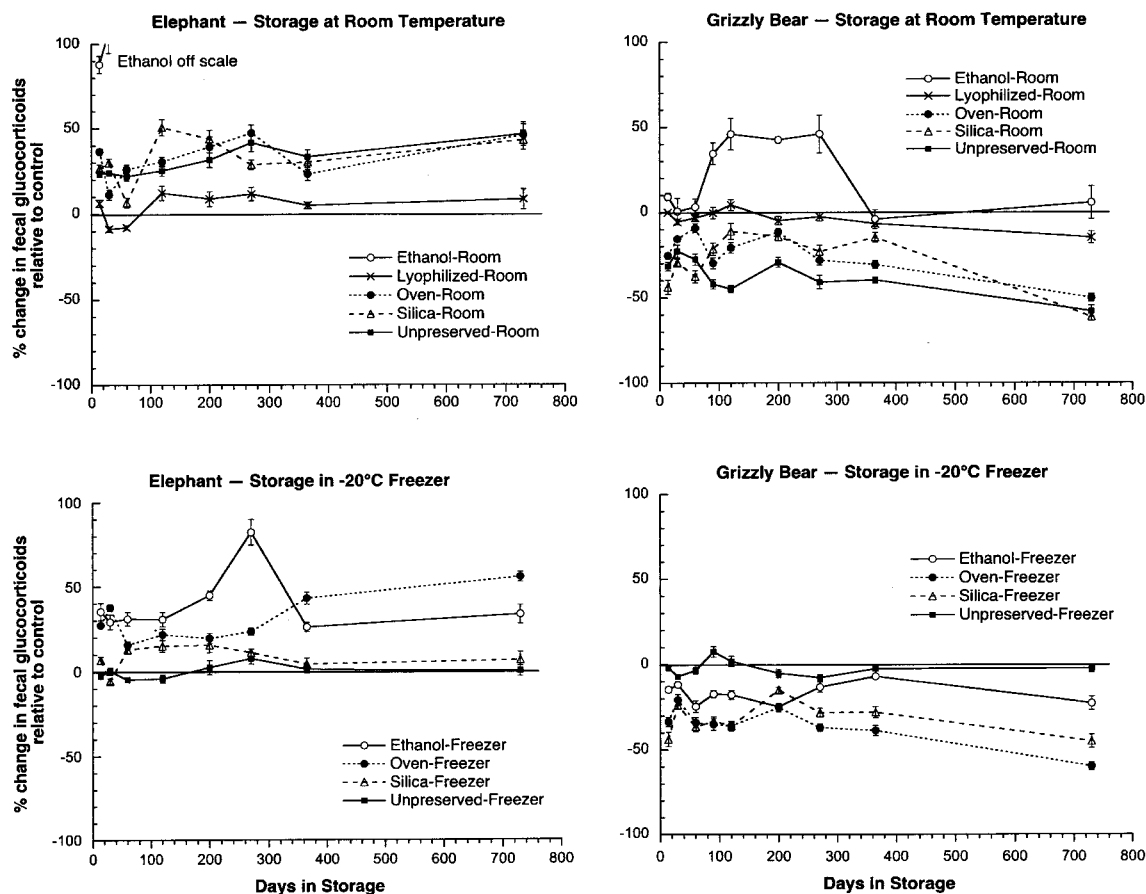


Figure 2. Effects of preservation method and temperature on immunoreactive fecal glucocorticoids in subsamples of a pooled African elephant fecal sample (*left*) and a pooled grizzly bear fecal sample (*right*). Subsamples were stored in various preservation methods at room temperature (*top*) or a  $-20^{\circ}\text{C}$  freezer (*bottom*). Note that the elephant room-temperature ethanol group is off scale; this group is redrawn at an adjusted scale in Figure 3, which also includes corresponding data on grizzly bear to illustrate temporal consistency across species. Data are expressed as percentage change relative to lyophilized frozen control. Points represent means  $\pm$  SEM ( $n = 10$  for each point).

#### Preservation Methods Differing from Controls: African Elephant.

In the African elephant, the ethanol-room group exhibited a dramatic  $\sim 1,400\%$  elevation in immunoreactive glucocorticoid concentrations over the control at 200 d and then declined to a level of  $\sim 200\%$ – $300\%$  above controls during the second year (Fig. 3). This group was significantly different from controls at every time point. The oven-room, silica-room, and unpreserved-room groups in elephant had variable results in the first several months but generally produced 20%–40% elevations in fecal glucocorticoids, although these differences were not always significant at every time point (Fig. 2; Table 2). The ethanol-freezer group showed a pattern similar to its room-temperature ethanol counterpart (Figs. 2, 3) but on a much smaller scale, with a peak at 270 d at  $\sim 80\%$  higher than controls. The oven-freezer group, like its room-temperature counterpart, developed a persistent  $\sim 20\%$  elevation in hormone concentrations. This increased to  $\sim 40\%$  by 1 yr (Fig. 2).

#### Preservation Methods Differing from Controls: Grizzly Bear.

In the grizzly bear, as in the elephant, the ethanol-room group showed a marked rise in immunoreactive fecal glucocorticoids beginning at 120 d, but the increase was not as extreme as in elephant (Figs. 2, 3). For the first 60 d, in fact, the bear ethanol-room group was statistically indistinguishable from the controls but began rising at 90 d, peaked at  $\sim 45\%$  above normal, and finally declined to control levels again by 1 yr (Figs. 2, 3; Table 3). The ethanol-freezer group in bear, in contrast to all our other ethanol groups, showed a transitory and mild decline of 20%–30%, starting at 60 d and returning to control levels at 1 yr (Fig. 2). The bear unpreserved-room group, and all bear oven and silica groups at both temperatures, showed erratic declines early on and reached declines of  $\sim 50\%$  below control levels by 1 yr. These groups were statistically different from controls at almost every time point (Table 3).

Table 1: *P* values from Fisher's PLSD tests after repeated-measures ANOVA on effect of preservation method on fecal glucocorticoid concentrations

	Elephant	Grizzly Bear
Lyophilized-room	(.7770)	.0378
Unpreserved-room	.0459	<.0001
Unpreserved-freezer	(.9972)	(.2055)
Oven-room	.0366	<.0001
Oven-freezer	.0477	<.0001
Silica-room	.0367	<.0001
Silica-freezer	(.5854)	<.0001
Ethanol-room	<.0001	<.0001
Ethanol-freezer	.0128	<.0001

Note. African elephant and grizzly bear were tested in different analyses. Significance level is  $\alpha = 0.05$ ; nonsignificant *P* values are in parentheses.

**Additional Observations.** Oven-dried samples at both temperatures, and silica and unpreserved groups at room temperature, often became very hard and/or gummy, especially for grizzly bears. Bear feces became particularly hard when preserved with oven-drying or silica-drying, requiring us to use hammers, chisels, and serrated knives to break them up for hormone extraction. Many silica samples at room temperature also developed visible mold, despite frequent changes of the indicating silica before the silica completely changed color.

**Stability of the Control Group.** The lyophilized-freezer control groups appeared to maintain stable glucocorticoid concentrations over the entire 2-yr study, as indicated by comparisons with the original time zero reference values. For elephant, the initial glucocorticoid concentration was  $19.71 \pm 0.94$  ng/g (mean  $\pm$  SD) for the original 20 time zero subsamples, and the mean glucocorticoid concentration for the elephant lyophilized-freezer group across all time points was  $20.33 \pm 1.74$  ng/g (mean  $\pm$  SD), ranging from  $18.26 \pm 1.73$  ng/g (day

60) to  $23.64 \pm 1.74$  (day 30). For grizzly bear, the initial glucocorticoid concentration was  $26.10 \pm 0.95$  (mean  $\pm$  SD), and the mean glucocorticoid concentration for the control group across all time points was  $27.40 \pm 1.78$  (mean  $\pm$  SD), ranging from  $25.99 \pm 3.40$  ng/g (day 90) to  $30.61 \pm 2.22$  ng/g (day 200). The average deviation of the control groups from the original time zero value was 6.01% for elephant and 5.77% for grizzly bear, consistent with the expected 6% assay variation for this assay. Thus, it appears that the variation that did occur in these control groups was likely entirely because of assay variation.

### HPLC

The bear control group at day 1 exhibited a prominent peak of immunoreactivity in fraction 79 and a small peak in fraction 3, probably reflecting a solvent front (Fig. 4). The lyophilized-room bear group at day 60 exhibited a similar pattern with a solvent front in fraction 4 and a prominent peak in fraction 80. When aligned by the solvent front, the two HPLC profiles are nearly identical (Fig. 4). The fecal metabolite in fraction 79–80 is not known but is slightly less polar than pure corticosterone. Pure corticosterone normally elutes in fractions 74–75 and pure cortisol in fractions 67–68 (Wasser et al. 2000).

### Discussion

The results from the ACTH challenge suggest that fecal glucocorticoid measures provide a reliable measure of adrenal activation in African elephants (Wasser et al. 2000) and grizzly bears (this study). However, the reliability of this measure also depends on how samples are preserved at the time of collection. The most reliable preservation methods may also vary between species. In this study, preservation methods had marked and varied effects on immunoreactive glucocorticoid concentrations in feces of both African elephant and grizzly bear. The methods that maintained the most stable fecal hormone concentrations, as compared with the control group (lyophilized-freezer), in-

Table 2: *P* values from post hoc tests on African elephant fecal glucocorticoid levels at each time point

	14 d	30 d	60 d	120 d	200 d	270 d	365 d	730 d
Preservation group:								
Ethanol-freezer	<.0001	<.0001	<.0001	(.1325)	(.7036)	<.0001	.0034	(.0406)
Ethanol-room	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
Oven-freezer	<.0001	<.0001	(.0070)	(.2825)	(.8668)	(.1050)	<.0001	.0009
Oven-room	<.0001	(.1108)	<.0001	(.1274)	(.7439)	.0017	(.0085)	.0061
Silica-room	<.0001	<.0001	(.2461)	(.0144)	(.7134)	(.0484)	<.0001	(.0093)
Unpreserved-room	<.0001	<.0001	.0002	(.2052)	(.7905)	.0058	.0003	.0054

Note. *P* values are from Fisher's PLSD comparisons of each group to the control group after ANOVA on each time point. Lyophilized-room, silica-freezer, and unpreserved-freezer groups were not included in these ANOVAs because they were previously shown not to be significantly different from controls. Significance level is  $\alpha = 0.00625$  with the Bonferroni correction for multiple tests. Nonsignificant *P* values are in parentheses.

Table 3: *P* values from post hoc tests on grizzly bear fecal glucocorticoid levels at each time point

	14 d	30 d	60 d	90 d	120 d	200 d	270 d	365 d	730 d
Preservation group:									
Ethanol-freezer	.0002	(.0158)	<.0001	.0011	.0032	<.0001	(.0468)	(.0838)	.0003
Ethanol-room	(.0170)	(.9118)	(.5015)	<.0001	<.0001	<.0001	<.0001	(.2815)	(.3783)
Lyophilized-room	(.9858)	(.2429)	(.4738)	(.9231)	(.4733)	(.1058)	(.6598)	(.0902)	(.0150)
Oven-freezer	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
Oven-room	<.0001	.0016	(.0458)	<.0001	.0006	.0003	<.0001	<.0001	<.0001
Silica-freezer	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
Silica-room	<.0001	<.0001	<.0001	<.0001	(.0574)	<.0001	.0007	.0004	<.0001
Unpreserved-room	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001

Note. *P* values are from Fisher's PLSD comparisons of each group to the control group, after ANOVA on each time point separately. The unpreserved-freezer group was not included in these ANOVAs because it was previously shown not to be significantly different from controls. Significance level is  $\alpha = 0.0056$  with the Bonferroni correction for multiple tests. Nonsignificant *P* values are in parentheses.

dependent of species, were lyophilization followed by storage at room temperature (also confirmed by HPLC) and unpreserved feces frozen at  $-20^{\circ}\text{C}$ . This latter result is encouraging since many fecal hormone studies rely on "simple freezing" for long-term sample preservation. Our data confirm that simple freezing (in a non-frost-free freezer) will reliably preserve immunoreactive fecal glucocorticoids for up to 2 yr in both species. To our knowledge, this is the first confirmation of the reliability of simple freezing for fecal hormone preservation over periods longer than 6 mo.

There were marked species differences in the effect of certain preservation methods, likely as a result of the inherent differences in diet and gut flora in these two species (the African elephant is a hindgut-fermenting herbivore, whereas the grizzly bear is an omnivore). For example, silica- and oven-drying methods tended to produce increases in fecal glucocorticoid concentrations for elephant but decreases for grizzly bear. These differences illustrate the importance of testing various preservation methods for the species of interest before initiating fecal hormone studies. It should also be noted that although the African elephant in our study had a naturalistic diet and normal fecal appearance compared with wild elephants, the same was not the case for grizzly bears, whose feces were denser and darker than most wild grizzly bear feces (S. Wasser, personal observation). This is likely related to the captive bears' diet, which included exotic fruits (e.g., seedless grapes) and processed biscuits, with less vegetation and more fresh meat than a typical wild diet (Servheen 1983; Hamer et al. 1991; McLellan and Hovey 1995). The sample hardening that we observed in some groups, and possible associated variations in hormone distribution and extraction efficiency, may thus be less a problem for wild bear scats. It is possible that similar diet-related differences, such as in fecal flora and/or fecal chemistry, may also affect the effectiveness of the different preservation methods for wild bear scats.

#### Nonfreezer Preservation Methods

Many field researchers must store fecal samples for weeks or months without access to a freezer (or a lyophilizer). However, none of the nonfreezer methods that we tested—silica-drying, oven-drying, or unpreserved—reliably maintained stable hormone concentrations compared with controls over extended time periods. Silica-drying and oven-drying produced erratic results during the first 6 mo and eventually showed pronounced changes in hormone concentrations (increases for elephant, decreases for grizzly bear). For the following reasons, we suspect that this may be due to the specific drying procedures used rather than drying per se: drying by lyophilization successfully

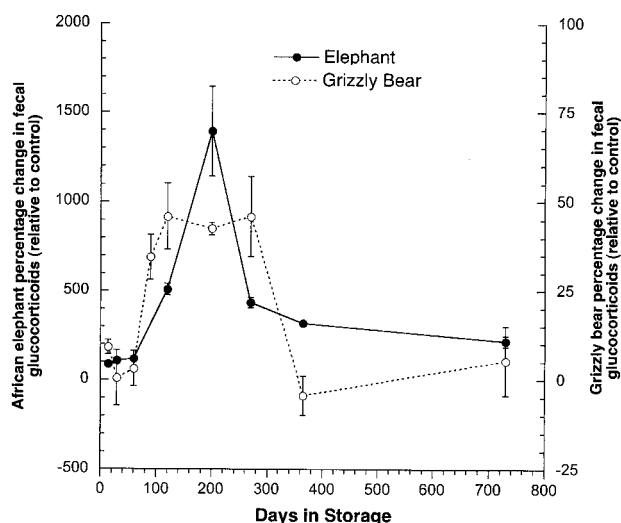


Figure 3. Effect of storage in ethanol at room temperature on immunoreactive fecal glucocorticoids of African elephant and grizzly bear. Note different Y-axes for the two species. Data are expressed as percentage change relative to lyophilized frozen control. Points represent means  $\pm$  SEM ( $n = 10$  for each point).

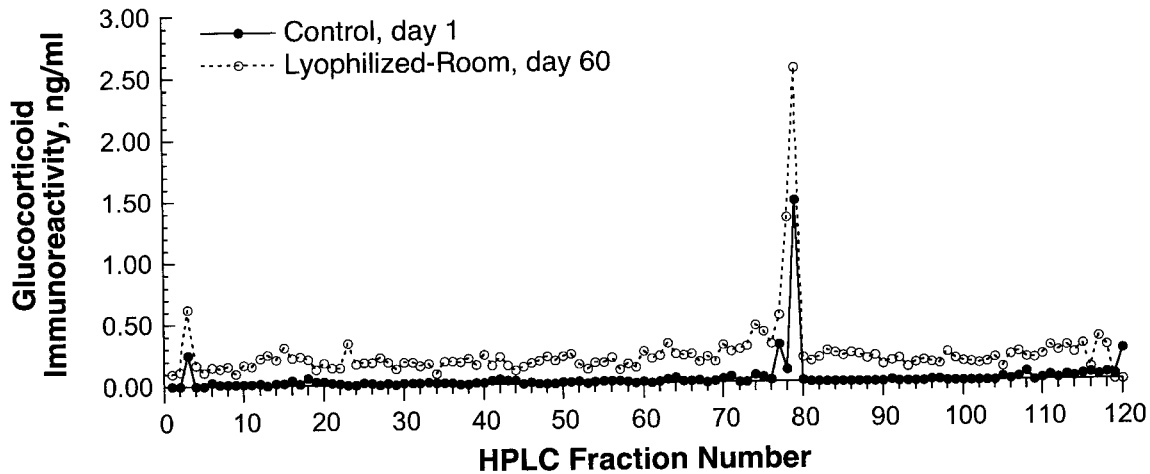


Figure 4. Fecal glucocorticoid immunoreactivity of high-performance liquid chromatography (HPLC) fractions from the grizzly bear control group at day 1 and the grizzly bear lyophilized-room group at day 60.

preserved samples of both species, and both silica- and oven-drying hardened the bear fecal samples so that they were difficult to pulverize. This hardening may explain the erratic results of the first few time points, especially in grizzly bears; hardened samples are difficult to mix, and thorough mixing is necessary to distribute hormones evenly in scat (Wasser 1996). Hardening of samples may also cause erratic extraction efficiencies and/or prevent the innermost portion of the sample from being adequately preserved.

There are several possible improvements of the silica- and oven-drying methods that are worth exploring. Pulverizing all oven or silica samples daily while they are drying may prevent sample hardening (K. Gobush, personal communication). Freezing silica samples on day 1 versus day 5 may improve preservation efficiency. Oven-drying samples at temperatures greater than 45°C may also produce improvements by drying samples more quickly, provided drying temperatures are not so extreme that they degrade the hormone of interest. Ultimately, one or more of the nonfreezer methods may be useful, especially when all samples in a research study are treated with the same protocol, since analysis of fecal hormone data generally concentrates on relative patterns rather than absolute levels.

#### Ethanol

Ethanol caused a large increase in immunoreactive glucocorticoids in samples stored at room temperature in both species, especially in the African elephant. Storage at  $-20^{\circ}\text{C}$  considerably dampened this effect in the elephant and eliminated it entirely in the grizzly bear. These increases had a distinct time course in both species, rising sharply between 3 and 9 mo, and then dropping again early in the second year. A similar pattern

has recently been reported for baboon feces, in which fecal estrogen and glucocorticoid concentrations increased  $\sim 100\%$  after storage in ethanol at room temperature, peaking at  $\sim 90$  d (estrogens) to  $\sim 120$ – $150$  d (glucocorticoids) and declining back to near normal levels by 6 mo (Khan et al. 2002). Consistent with our results, smaller increases were seen in baboon feces stored in ethanol at  $-20^{\circ}\text{C}$  than at room temperature. The causes of these changes are unknown, but these results collectively suggest that the ethanol-induced hormone rise and fall might be caused by progressive liberation of free hormones from lipid micelles (Yalkowsky 1999), followed by progressive reuptake beyond 9 mo as micelles reform. Micelles can produce slowed timed release of compounds, varying with temperature, pH, and polarity (Yalkowsky 1999). These characteristics might explain the across-species consistencies noted previously: 2–3 mo delayed elevation, followed by return toward, but not overshooting, control concentrations several months later and a substantial dampening effect of freezing. They might also explain the species differences in the magnitude of response.

Washburn and Millsbaugh (2002) showed that rainfall caused an increase in immunoreactive concentrations of cortisol metabolites in white-tailed deer (*Odocoileus virginianus*) feces. This suggests that water alone may have some of the same initial effects on measurable hormone concentrations as does ethanol. Consistent with this view, drying samples (e.g., freeze-drying in this study) appears to have a stabilizing effect on measurable hormone concentrations. The precise mechanisms underlying these changes are unknown at present, but changes in polarity and/or pH, possibly affecting micelle structure, may provide a common underlying thread in the temporal patterns of hormone concentrations associated with both water and ethanol.

A second possible explanation for increases in immunoreactive hormones is chemical alteration of hormones to a more

immunoreactive form, such as by oxidation or deconjugation, perhaps followed by continued degradation or reconjugation to progressively less immunoreactive forms. Washburn and Millsbaugh (2002) suggested bacterial metabolization of hormones as an explanation for the rainfall effects they observed. However, this explanation is unlikely to account for the effects of 90% ethanol since ethanol kills most bacteria. Moreover, if the subsequent ethanol-associated decline were due to degradation, we would expect hormone concentrations to eventually fall below control concentrations, particularly in room-temperature ethanol groups. This did not occur in elephant feces; it did occur in the grizzly bear ethanol-frozen group but not the grizzly bear ethanol-room group.

The previous two hypotheses are worth pursuing since they may eventually lead to protocols that allow ethanol to be used as an effective long-term preservative. For example, if the micelle hypothesis is correct, changing the polarity and/or pH of the preservation medium could facilitate liberation of all hormone bound to micelles at the time of extraction, eliminating the effects of preservation temperature and time. Both hypotheses could be tested by HPLC analyses of changes in the number, polarities, and/or amplitudes of metabolites in ethanol and by experimentally varying the polarity of the mobile phase during extraction.

The main advantage of pursuing this avenue of research is that ethanol has several benefits as a fecal preservative for remote fieldwork. First, ethanol provides an immediate fixative, stopping all bacterial action within the sample. Wasser et al. (1988) were among the first to demonstrate this on the basis of studies on baboon feces showing that ethanol stopped metabolization of fecal estrogens and progestagens within the first 24 h following excretion. Ethanol also prevented effects of urinary contamination of feces by blocking hydrolysis of any conjugated steroids contributed by urine. Second, ethanol is an excellent preservative of fecal DNA (Wasser et al. 1997b; Murphy et al. 2002), which is beneficial for studies conducting both genetic and endocrine analyses on the same samples.

Ethanol still appears usable for short time periods ( $\leq 60$  d) in several species, including perhaps the grizzly bear. Terio et al. (2002) recommended ethanol as the best preservation method for storage of cheetah feces for estrogens, androgens, progestagens, and glucocorticoids for periods of up to 2 wk. Lynch et al. (2003) recently obtained biologically meaningful progestagen and glucocorticoid patterns (e.g., detection of pregnancy with fecal progestagens, correlation of glucocorticoids with age class) with samples stored in ethanol for up to 4 wk. But clearly, our results and those of Khan et al. (2002) illustrate a need for caution when storing samples in ethanol for longer time periods.

Until the causes of the long-term, ethanol-induced hormone changes are addressed, we recommend that effects of ethanol first be tested on the species and hormones being examined and that its duration of use be determined accordingly. For

studies that have already used ethanol, comparison of relative hormone levels may still be informative (e.g., Foley et al. 2001; Lynch et al. 2003), particularly if storage time in ethanol is similar for all samples. However, comparison of absolute concentrations across different ethanol storage times should be avoided.

Finally, it must be emphasized that our study examined just one variable, preservation method, that affects fecal glucocorticoid concentrations. Many other factors may also have important effects on fecal glucocorticoid levels, most of which remain to be studied, for example, possible effects of gender, season, diet, and season  $\times$  diet interactions on glucocorticoid metabolism and excretion; effects of pregnancy and lactation in females; the lag time between excretion and sample collection; and exposure of samples to different field conditions before collection, such as ambient temperature, solar radiation, and rainfall. Although the demonstrated utility of fecal glucocorticoid assays to identify known stressors in real-world situations is very encouraging (e.g., Wasser et al. 1997a; Palme et al. 2000; Creel et al. 2002; Wielebnowski et al. 2002; Lynch et al. 2003), potentially confounding effects of these other factors should still be explored. It would also be useful to identify the major fecal glucocorticoid metabolites of our two species and others, which currently remain largely unknown for most mammals. Identification of these metabolites, along with their respective changes in response to storage or field conditions, may ultimately permit use of preservation methods that have useful field attributes (e.g., ethanol) but are not currently acceptable given our present knowledge.

In summary, for studies that do not have immediate access to a lyophilizer, simple freezing (storing samples in a non-frost-free  $-20^{\circ}\text{C}$  freezer) remains the easiest and most reliable storage method for fecal glucocorticoid hormones across species. For remote fieldwork, the ideal nonfreezing preservative method is yet to be determined. Modifications of some nonfreezing preservation methods may ultimately prove useful (e.g., mixing oven-drying or silica-drying samples during the drying process; testing higher oven temperatures; exploring the effects of ethanol liberation of hormones from micelles; investigating other alcohols such as methanol). In the interim, data analyses can concentrate on relative patterns of hormone concentrations, across samples preserved with the same method for the same time span. Overall, we recommend testing planned preservation methods on the hormones and species under study before proceeding with large-scale studies and, unless proven otherwise, using the same preservative method for the same time span for all samples in a study.

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