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Comparison of Fecal Storage Methods for Steroid Analysis in Black Rhinoceroses (*Diceros bicornis*)

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Many field studies and conservation programs for wildlife species include noninvasive endocrine monitoring of gonadal function. Freezing fecal samples immediately after collection until further analysis is often not a viable option for researchers in remote areas. Phase 1 of this study was designed to compare different methods of preserving fecal samples over several time periods (30, 90, or 180 days) in order to determine which method provided the most accurate and reliable technique for measuring fecal progestagens. Fecal samples were collected from two female black rhinoceroses (*Diceros bicornis*) housed at Disney's Animal Kingdom, Lake Buena Vista, FL. We compared three storage methods: 1) storing fecal samples without processing or preservatives (untreated), 2) storing an aliquot of fecal sample in 80% methanol (MeOH), and 3) drying the fecal sample in a solar box cooker prior to storage. Control samples (day 0) were collected and extracted, and then stored at −20°C until they were analyzed. Phase 2 of the study was designed to examine the effects of long-term storage (up to 180 days) on fecal progestagen profiles that reflect reproductive activity (pregnancy and estrous cycles). In samples obtained from a pregnant female and stored for 30 days, there were no significant differences in fecal progestagen concentrations between the three treatment conditions. However, the mean concentrations of progestagens ($\pm$ SE) in untreated samples increased significantly from 8.3 $\pm$ 0.3 μg/g wet weight feces at day 0 to 17.7 $\pm$ 5.1 μg/g feces at day 90, and 17.8 $\pm$ 4.7 μg/g feces at day 180. Samples that were collected from a pregnant female and stored in 80% MeOH or dried in the solar box correlated with controls ($r = 0.86$ and 0.87, respectively; $P < 0.05$) at day 180. In contrast, samples that were stored without preservatives for 180 days did not correlate with controls ($r = 0.35$, $P > 0.05$). Progestagen concentrations from samples of the estrous cycling female showed similar results. In conclusion, fecal samples dried in a solar box cooker or stored in 80% MeOH maintained absolute and relative progestagen concentrations for at least 180 days when they were stored outdoors and exposed to the climatic conditions of central Florida. Both methods can have significant applications for

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the study of reproductive events in areas where access to electricity is limited. Zoo Biol 23:291–300, 2004.

Key words: preservation of samples; fecal steroids; progestagens; enzyme-immunoassay

INTRODUCTION

In the past two decades, considerable advances have been made in validating techniques for the noninvasive endocrine monitoring of gonadal function in zoo and wildlife species [for reviews see Lasley and Kirkpatrick, 1991; Schwarzenberger et al., 1996; Brown et al., 1997]. As a result, an increasing number of studies on free-ranging wildlife are incorporating noninvasive endocrine monitoring of reproduction into their study designs (e.g., *Brachyteles arachnoides* [Strier and Ziegler, 1994]; *Pongo pygmaeus* and *Pan troglodytes* [Knott, 1997]; *Diceros bicornis minor* [Garnier et al., 1998]; and *Cervus elaphus nannodes* [Stoops et al., 1999]).

Endocrine monitoring of gonadal function requires repeated sample collection. Typically, fecal or urine samples are collected and stored frozen until the endocrine analysis is performed. Although freezing samples immediately after collection may be an optimal means of preventing bacterial metabolism of the hormone metabolites, it is often not a viable option for researchers in the field. Study sites of free-ranging animal species may be far away from an electric power source and refrigeration equipment. Furthermore, the final shipment of samples to a laboratory for endocrine analysis may take many days, and there is no way to ensure that the samples will remain frozen if the shipment is detained for processing at international borders.

Only a limited number of studies have investigated alternative methods for "long-term" storage of fecal samples in the field. Terio et al. [2002] showed that concentrations of quantifiable steroid hormones in cheetah feces remained stable when the feces were preserved in ethanol and stored at room temperature for 14 days. These results are consistent with previous findings in macaque (*Macaca nemestrina*) and baboon (*Papio cynocephalus*) feces [Wasser et al., 1988].

Masunda et al. [1999] stored cow fecal samples without preservatives for 2 days at room temperature, and observed an exponential decrease in the measured progestagen levels. In a similar study, Schlenker et al. [1999] stored cow feces without preservatives for up to 84 days at 5°C and 30°C. Estrogen and progestagen concentrations decreased more rapidly when they were stored at 30°C.

Although the use of ethanol for preserving samples is indeed promising, the practicality of using ethanol in the field may be an issue for some scientists. Local challenges involving access to remote field sites, transportation to and from the sites, costs, and the shipping of hazardous material may factor into the use of ethanol as a viable method to preserve samples in the field. The development of new methods to preserve feces, or modifications of techniques currently used in the laboratory (such as lyophilization [Brown et al., 1997; Terio et al., 2002]), may lead to practical solutions for field researchers.

The present study was designed to compare methods of preserving fecal samples from black rhinoceroses (*Diceros bicornis*) for reproductive hormone analysis, with the goal of developing methods that are applicable to field studies.

Phase 1 of the study was designed to compare different methods of preserving fecal samples over several time periods in order to determine which method provided the most accurate and reliable technique for measuring fecal progestagens. Aliquots of fecal samples were 1) stored untreated (i.e., with no processing or preservatives), 2) mixed with 5 ml of 80% methanol (MeOH), or 3) dried in a solar box cooker prior to storage. Phase 2 of the study was designed to examine the effects of long-term storage (up to 180 days) on fecal progestagen profiles that reflect reproductive activity (pregnancy and estrous cycles).

MATERIALS AND METHODS

Sample Collection

Fecal samples were collected from two female black rhinoceroses (*Diceros bicornis*; 7 and 5 years old, respectively, at the time of study) housed at Disney's Animal Kingdom, Lake Buena Vista, FL. The females were housed individually in an off-exhibit facility each evening, and remained indoors for approximately 14 hr before they were returned to the outdoor exhibit the next morning. The animal keepers collected fecal samples two to four times each week by 0600 hr. If first morning void samples were not observed, the keeper chose the freshest sample (i.e., the moistest and warmest).

Fecal Sample Processing

Phase 1: Comparison of different storage techniques and periods

In phase 1 of the study, we examined the effects of different storage treatments and periods on the fecal progestagen concentration. A single sample from a black rhinoceros in late pregnancy was collected, mixed well, and then divided into 48 0.5-g aliquots and subjected to the various storage treatment conditions. Twelve aliquots were extracted immediately after collection (control = day 0) and stored at -20°C in capped 8-ml glass evaporation-proof vials (glass screw-thread sample vials with Teflon fluorocarbon-resin-lined caps; National Scientific, Duluth, GA) until further analysis. The remaining 36 aliquots were equally divided over the three treatment groups described below (no preservatives, 80% MeOH, or dried). After 30, 90, or 180 days of storage outdoors, aliquots (n = 4/treatment group) were extracted and compared to aliquots extracted on day 0.

Phase 2: Stability of the fecal progestagen profile after 180 days of outdoor storage

In phase 2 we determined the effect of long-term storage (180 days) on maintaining the relative differences in fecal progestagen concentrations that are indicative of reproductive activity (e.g., ovulation and pregnancy). For an early-pregnancy profile, 20 samples were collected from a single female. The fecal samples were then divided into 0.5-g aliquots and allocated to each of the three different treatments (no preservatives, 80% MeOH, or dried; n = 5/treatment/subject). Five aliquots were extracted immediately after collection (control = day 0) and stored at -20°C in capped 8-ml glass evaporation-proof vials until further analysis. Treatment aliquots were stored outdoors and extracted after 180 days of storage.

Although both study animals were pregnant during this phase of the study, we wanted to determine whether the dramatic concentration changes observed in estrous cycles would be observed under similar treatment conditions. We used 24 samples from one subject that had been collected prior to the onset of pregnancy. Since the stored fecal samples were smaller than the unstored samples, only one treatment per subject could be used. Under the assumption that freezing does not affect the progestagen content in feces, the samples were thawed and aliquoted for the three different treatments following the same procedure as described above.

Treatments

Treatment 1: no preservatives

An aliquot of fecal sample (0.5 g) was placed in a capped 8-ml glass evaporation-proof vial with no preservatives.

Treatment 2: 80% MeOH

An aliquot of fecal sample (0.5 g) was placed in 5 ml of 80% MeOH and stored in a capped 8-ml glass evaporation-proof vial. We used 80% MeOH as the extraction medium because it is routinely used in our laboratory and has been validated for use in this species [Graham et al., 2001].

Treatment 3: dried

An aliquot of feces (0.5 g) was placed in an open 8-ml glass evaporation-proof vial and dried outdoors in a solar box cooker (Kerr-Cole, Tempe, AZ). Solar box cookers have been used for various applications (e.g., plant drying) (A. Savage, personal communication), and our personal observations suggested that this inexpensive, easily-constructed item might have promising results for drying and preserving fecal samples in the field. The aliquots were weighed every hour and were considered to be dried when the weight remained stable for at least 2 hr. Generally, a 0.5-g wet-weight aliquot reduced to an average of 0.18 g dry weight. The solar box cooker ($60 \times 72 \times 28$ cm) maintained a temperature of $>45^{\circ}$C when it was placed in direct sunlight, and the samples were usually dry in 4–5 hr. Dried samples were removed from the solar box cooker, capped, and stored.

Storage Conditions

Treatment aliquots were placed in closed cardboard boxes that were stored outdoors (28.21.53N 81.35.17W) in a closed clear plastic container ($41.2 \times 28.7 \times 25.3$ cm) covered by a clear plastic bag. The outdoor temperatures during the first phase (23 April to 20 October 2000) ranged from an average minimum of 15°C to an average maximum of 32.7°C. The outdoor temperatures during the second phase (24 October to 7 June 2001) ranged from an average minimum of 8.8°C to an average maximum of 28.8°C. Relative humidity ranged from 50% to 87%.

Fecal Steroid Extraction

The fecal extraction protocol used was described previously by Graham et al. [2001]. In brief, 5 ml of 80% MeOH were added to the 0.5-g treatment aliquots

(except in treatment 2) in the 8-ml glass vial so that the steroid metabolites could be extracted. The treatment 2 aliquots had already received 5 ml of 80% MeOH. Treatment 3 aliquots were pulverized prior to the addition of MeOH. The vials were capped and placed on a shaker (Eberbach Corp., Ann Arbor, MI) for 12 hr. The samples were then centrifuged (at 2,400 rpm for 15 min), and the supernatants containing steroid metabolites were poured off and stored in evaporation-proof vials at $-20^{\circ}C$ until analysis. Extracts were diluted (1:4–1:100) in assay buffer (0.02 M Trizma, 0.30M NaCl, 0.1% BSA, and 0.1% Tween 80) before the enzyme-immunoassay (EIA) was performed.

EIA

Fecal progestagen concentrations were quantified with the use of a progestagen EIA that was previously validated for black rhinoceros feces [Graham et al., 2001]. In brief, microtiter plates (Nunc; Fisher Scientific) were coated with affinity purified goat anti-mouse gamma globulin (Sigma Chemicals, St. Louis, MO) dissolved in coating buffer (0.015M Na_2CO_3, 0.035M $NaHCO_3$; pH 9.6) and incubated overnight at room temperature. The wells were emptied and refilled with a second coating buffer containing preservative (0.02M Trizma, 0.30M NaCl, 1.0% NaN_3, 1.0% BSA; pH 7.5) and stored at room temperature. The plates were used within 2 weeks of coating. For the EIA, the coated plates were washed (0.02% Tween 20), and diluted samples, standards, and controls were dispensed. Biotinylated progesterone (1:1,500,000; F. Schwarzenberger, Vienna, Austria) and antibody (Quidel clone #425, 1:400,000; final purification by C. Munro, Davis, CA) were added to the wells. The plates were incubated overnight at room temperature. After the plates were incubated with the antibody and biotinylated label, they were washed and incubated (45 min at room temperature) with streptavidin-peroxidase conjugate (Roche Molecular Biochemicals, Indianapolis, IN). After the plates were incubated with the enzyme and washed, a substrate solution (0.5 ml of 0.016M tetramethylbenzidine in dimethylsulphoxide and 100 μl of 0.175M H_2O_2 diluted in 24 ml of 0.01M $C_2H_3O_2Na$; pH 5.0) was added to each well. The enzyme reaction was stopped with 50 μl of stop solution (3M H_2SO_4) following substrate incubation (45 min at room temperature), and the optical density was measured at 450 nm. Progesterone was used as the standard, and serial dilutions of fecal extracts gave displacement curves parallel to that of the standard curve. Figure 1 depicts the serial dilutions of the fecal extracts. The CL#425 monoclonal antibody was produced against 4-pregnen-11-ol-3, 20-dione hemisuccinate: BSA and cross-reacts with various fecal progesterone metabolites [Graham et al., 2001]. Recoveries from one positive and two negative samples spiked with a moderate concentration of progesterone standard indicative of a normal luteal phase averaged 89.5% $\pm$ 0.9 (SD). The sensitivity of the assay was 15 pg/well. The interassay coefficients of variation were 12.2% ($\sim$30% binding) and 11.9% ($\sim$70% binding; $n = 21$ plates).

Data Analysis

In phase 1 of the study, treatment means at each time point were compared with the controls by analysis of variance (ANOVA) followed by Dunnett's multiple-comparisons test. Levene's homogeneity-of-variance test was performed to ascertain whether the variances of the treatment means at the different storage time periods were equal. The one-way ANOVA test was performed to ascertain whether there

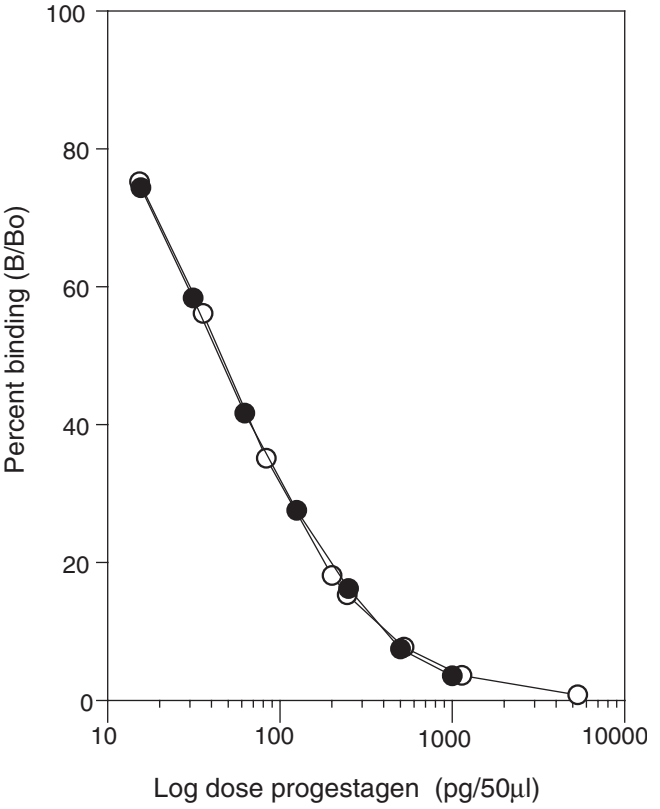

Fig. 1. Serial dilution results from female black rhino fecal extract are presented. The sample (○; open circles) was diluted 1:16 to 1:2,048 in assay buffer and tested for binding with the progestagen antibody in parallel with serially diluted progesterone standards (●; closed circles) ranging from 1,000 to 15.6 pg/50 µl with a slope of 1.018. Regression equations derived from the linear portion of the curves produced almost identical R^2 values for the samples and standards (0.9985 and 0.9947, respectively).

were any differences between the mean hormone concentrations of the various storage periods within one treatment group.

In phase 2 of the study, a linear regression analysis was performed to evaluate the correspondence between the control group and the treatment aliquots stored outdoors for 180 days.

RESULTS

Phase 1

There were no differences in fecal progestagen concentrations between the control group (day 0) and the three treatment groups after 30 days of storage (Fig. 2). Following 90 and 180 days of storage, fecal progestagen concentrations in samples stored with no preservatives were significantly ($P<0.05$) different from the controls. Mean concentrations ($\pm$SE) increased from 8.3 ± 0.3 µg/g feces at day 0 to 17.7 ± 5.1 µg/g feces at day 90 and 17.8 ± 4.7 µg/g feces at day 180. Samples that were

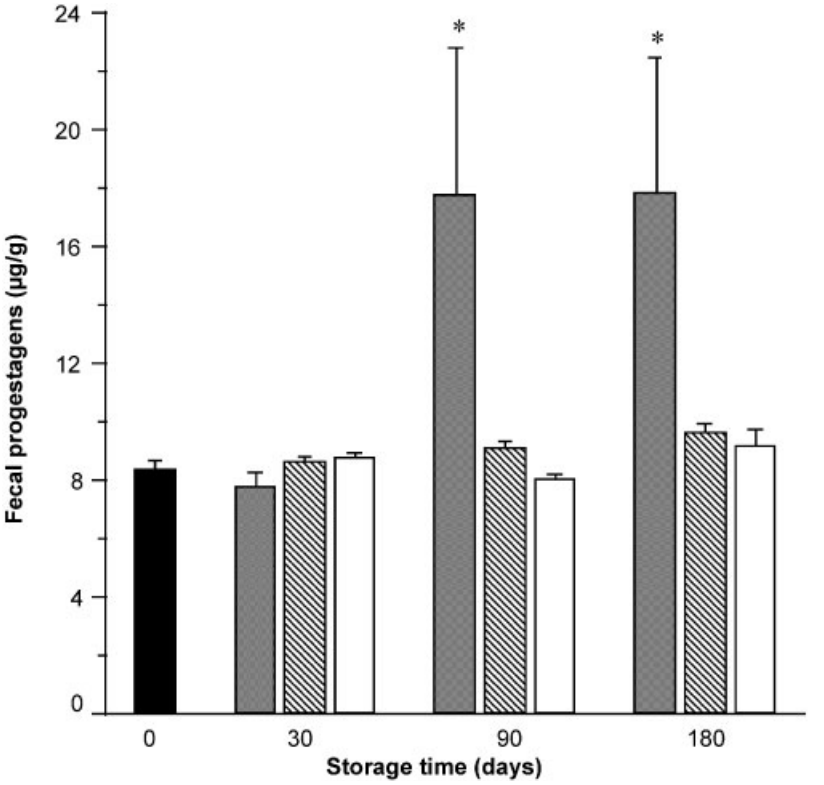

Fig. 2. Mean fecal progestagens (n = 4) ± SE for a pregnant female (*Diceros bicornis*) over various periods of outdoor storage (30, 90, or 180 days): (▩) fecal samples stored without preservatives, (▧) samples stored in 80% MeOH, and (□) samples dried in a solar box cooker before they were stored outdoors. ■ Control fecal aliquots (n = 12) that were immediately extracted and stored at −20°C. The * indicates statistical significance ($P < 0.05$).

dried or stored in 80% MeOH did not have significantly different fecal progestagen concentrations from controls at 90 or 180 days.

Phase 2

Fecal samples that were collected from the pregnant female and stored in 80% MeOH or dried correlated with corresponding control aliquots (r = 0.86 and 0.87, respectively; $P < 0.05$). In contrast, fecal aliquots stored without preservatives did not correlate with control samples (r = 0.35, $P > 0.05$) (Fig. 3). We found that 63% of the nonpreserved samples were covered with fungus, and in 88% of the samples there was a clear liquid (~260 µl) on the bottom of the vial.

A series of fecal samples collected from an estrous cycling female showed results similar to those obtained in the pregnant female (Fig. 4). Fecal samples stored in 80% MeOH or dried for 180 days correlated with the corresponding control aliquots (r = 0.96 and 0.95, respectively; $P < 0.05$). In contrast, fecal aliquots stored without preservatives for 180 days did not correlate with controls (r = 0.21; $P > 0.05$). Fungus was observed in 92% of the nonpreserved samples, and in 58% there was a clear liquid (~260 µl) on the bottom of the vial.

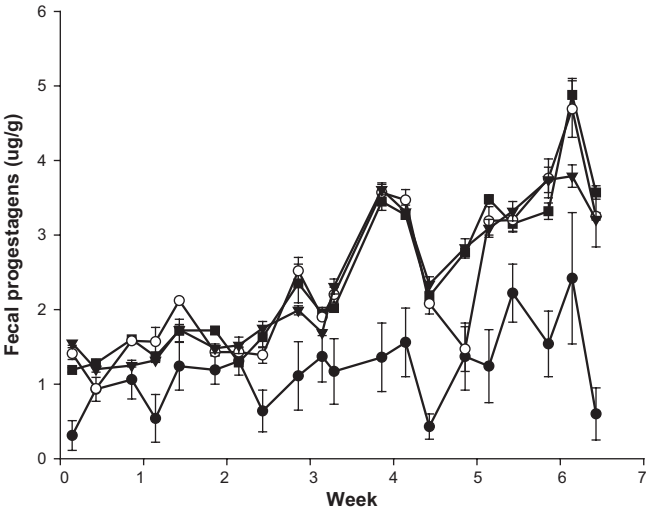

Fig. 3. Mean fecal progestagens (n = 5) ± SE during early pregnancy (*Diceros bicornis*) for treatment aliquots that were stored outdoors for 180 days: (●) fecal samples stored without preservatives, (○) samples stored in 80% MeOH, and (▼) samples dried in a solar box cooker before they were stored outdoors. (■) Control fecal aliquots that were immediately extracted and stored at −20°C.

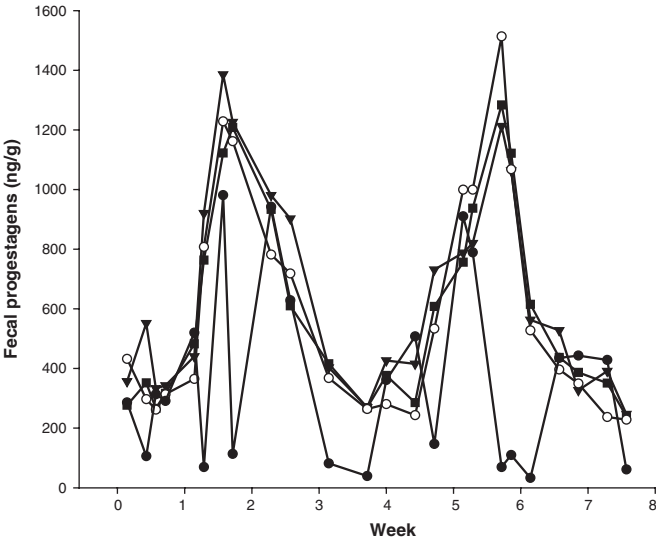

Fig. 4. Fecal progestagen concentrations during approximately two estrous cycles (*Diceros bicornis*) for treatment aliquots that were stored outdoors for 180 days: (●) fecal samples stored without preservatives, (○) samples stored in 80% MeOH, and (▼) samples dried in a solar box cooker before they were stored outdoors. (■) Control fecal aliquots that were immediately extracted and stored at −20°C.

DISCUSSION

This study demonstrates that drying feces from female black rhinoceroses in a solar box cooker, and mixing feces in 80% MeOH are both effective methods to

preserve samples for future progestagen analysis. The progestagen levels measured in the pregnant and estrous cycling females were similar to what has been observed in studies using traditional freezing of samples prior to analysis [Graham et al., 2001; Schwarzenberger et al., 1996]. Both storage methods appeared to prevent bacterial metabolism and maintain absolute and relative fecal progestagen concentrations for at least 180 days when the samples were stored outdoors and exposed to the climatic conditions of central Florida. A similar study using feces collected from a female cotton-top tamarin (*Saguinus oedipus*) showed similar results (W. Galama, personal communication).

In addition, this study also showed that the weight loss during the drying process of the 0.5-g aliquots did not affect the concentration of progestagens. The results from fecal samples stored in MeOH were also consistent with those obtained in cheetah fecal samples stored in ethanol for 14 days, as described by Terio et al. [2002]. However, the results from the cheetah fecal samples dried in a solar box oven were not consistent with our findings. Progestagen concentrations fluctuated when the cheetah feces were dried in a solar oven after they had been stored in ethanol for 14 days. The order of the processes (weighing, drying, and addition of ethanol/MeOH) or the drying time may have caused the differences in the results.

This study also shows that with the current methods, fecal processing and the extraction process can be less labor-intensive compared to previously described storage techniques [Wasser et al., 1988; Masunda et al., 1999; Schlenker et al., 1999; Terio et al., 2002]. However, the results of these various storage studies suggest that the storage and extraction methods should be part of the assay validation. Fecal samples that were not preserved showed marked differences in hormone concentrations and the ability to accurately reflect reproductive events. Such variations may have been caused by bacteria or other microorganisms in the feces transforming the available hormone metabolites so that they could not be detected by the progestagen assay used in this study. The high and/or fluctuating outdoor temperatures found in central Florida may have catalyzed the metabolism of the microorganisms. Schlenker et al. [1999] found that estrogen and progesterone concentrations in nonpreserved cow feces decreased more rapidly at 30°C than at 5°C when the samples were stored for 13 weeks. Various researchers [Masunda et al., 1999; Schlenker et al., 1998, 1999] also found variations in the hormone concentration and pH of feces that were stored untreated, and suggested that microbacterial-enzymatic processes influenced those changes. However, in the present study, the observation of fungus on the nontreated samples did not correlate with the level of the progestagen concentration. The biochemistry of progestagens found in excreted feces in relation to temperature has not received much attention in the literature.

Given the challenges faced by many field researchers in storing samples, it is our hope that the methods suggested here can be put to use. Cardboard solar box cookers are readily available and easy to construct, and may prove to be a good solution to the problem of preserving samples in the field. Once dried, the fecal samples can be stored in evaporation-proof vials until they are analyzed at a later stage. However, in areas where rain is prevalent and/or long periods of direct sunlight are limited, storing samples in 80% MeOH may be a more viable option. Once samples are weighed (0.5 g) and mixed with 5 ml of 80% MeOH, they can be stored for several months with no ill effects.

CONCLUSIONS

1. Black rhino fecal samples can be stored at outdoor temperatures for up to 180 days when dried or mixed with 80% MeOH without losing their ability to reflect physiological reproductive events.
2. There were no differences in fecal progestagen concentrations between control and fecal samples without preservatives after 30 days of storage. After 180 days of storage, untreated fecal samples did not demonstrate a significant correlation with control samples.
3. The use of low-cost yet efficient techniques (such as solar box cookers) to dry fecal samples, and/or the use of 80% MeOH as a preservative may have practical applications for the study of reproductive events in areas where access to resources is limited.

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