

Effects of storage treatment on fecal steroid hormone concentrations of a rodent, the Cape ground squirrel (*Xerus inauris*)

B.A. Pettitt^{a,*}, C.J. Wheaton^b, J.M. Waterman^a

^a Department of Biology, University of Central Florida, Orlando, FL 32816, USA

^b Department of Education and Science, Disney's Animal Kingdom, Lake Buena Vista, FL 32830, USA

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Abstract

Fecal steroid analysis is an increasingly common non-invasive technique used in both captive and field studies to measure an animal's approximate hormonal levels and corresponding physiological state. Fecal collection in the field necessitates storage and transportation methods that will prevent the degradation of hormonal metabolites by fecal bacteria. To determine the most stable and therefore preferred method of storage, 48 fecal samples were collected from six captive female Cape ground squirrels (*Xerus inauris*). Each sample was randomly divided into three sub-samples to be processed for storage through freezing, drying, or preservation in ethanol. Frozen samples were stored at -20°C , dried-treated samples were desiccated in a conventional oven at 40°C for 4 h, and alcohol-treated samples were preserved in 3 ml of 95% ethanol. Samples were stored for 330 days followed by enzyme immunoassay analysis (EIA) to determine their progesterone and estrone conjugate (E_1C) concentrations. Validations were performed to establish that the progesterone and E_1C assays accurately measure fecal progesterone and estrone conjugate concentrations and were sensitive enough to detect biologically meaningful differences in these steroid metabolite concentrations in female *X. inauris*. Validation results showed a significant difference in progesterone concentrations of gravid females compared to sub-adults and non-gravid females. There was also a significant difference in estrone conjugates between sub-adult and adult females. Duration of storage time did not affect progesterone or estrone metabolite concentrations after being frozen for 3 months. Storage treatment results showed no significant difference between frozen and dried samples, but a significant difference was found between frozen and ethanol samples in both progesterone and estrone conjugate concentrations demonstrating that drying feces provides a reliable method for long-term preservation of fecal steroid concentrations and is the better alternative when freezing is not a viable option.

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1. Introduction

Fecal steroid analysis is an increasingly popular non-invasive technique due to its enormous potential for answering wide-ranging questions in endocrinology (Schwarzenberger et al., 1996; Whitten et al., 1998; Brown et al., 1997). Combined with behavioral data, endocrine analysis can provide valuable information concerning the physiological mechanisms mediating numerous behaviors that ultimately influence a species' viability, reproduction and

life history. Application of fecal-based investigations in monitoring gonadal function has become popular since this method removes the necessity of obtaining blood samples from the study subjects. Assessing reproductive profiles requires repeated sample collection so the appeal of fecal steroid analysis (especially in free-ranging species) is apparent when considering that stress from frequent handling can affect hormone secretion (Hamilton and Weeks, 1985; Millsbaugh et al., 2002; Wasser et al., 2000). Studies examining reproduction in free-ranging species using fecal steroid analysis have involved taxa as diverse as muriquis, *Brachyteles hypoxanthus* (Strier and Ziegler, 2005); tule elk, *Cervus elaphus nannodes* (Stoops et al., 1999); deer mice,

* Corresponding author. Fax: +1 407 823 5769.

E-mail address: pettittbeth@yahoo.com (B.A. Pettitt).

Peromyscus maniculatus (Harper and Austad, 2004); black rhinoceros, *Diceros bicornis minor* (Garnier et al., 1998); and spotted owls, *Strix occidentalis* (Washburn et al., 2004).

Monitoring endocrine function by fecal analysis can be susceptible to problems resulting from high individual variability in reproductive hormone concentration. Steroid hormones are subject to metabolism by the liver as well as specific intestinal bacteria before they are eliminated via feces (Taylor, 1971; Palme et al., 1996). High intra-individual variation, possibly due to fluctuations in rates of metabolism and re-absorption of steroid hormone and amount of gut bacteria within an individual across time, may make it difficult to detect slight differences between individuals when examining patterns and cycles of multiple group members. Researchers must attempt to control for any variation due to methodological techniques, including preservation and storage procedures, which may alter hormone concentrations to avoid misinterpretation of the data (Lynch et al., 2003).

Fecal collection in the field is especially vulnerable to artificial fluctuations in steroid hormone concentration resulting from less than ideal fecal storage and transportation methods. Optimal storage techniques minimize degradation of steroid hormone metabolites by naturally occurring fecal bacteria, bacterial enzymes (Woods, 1975), temperature (Schlenker et al., 1999) and ultraviolet light (Matkovics, 1972). Microbial transformation of steroid hormone metabolites can occur within hours after defecation (Mostl et al., 1999; Wasser et al., 1988) due to the often high bacterial loads found in fecal matter. Leaving fecal samples untreated can alter fecal steroid concentration in a species-specific manner (increased: baboon: Wasser et al., 1988; black rhinoceros: Galama et al., 2004; decreased: cow: Masunda et al., 1999; Schlenker et al., 1999). Such findings suggest that the direction of fecal steroid concentration changes may be influenced, in part, by digestive strategy (carnivore, omnivore, forestomach or caecocolic fermenter). Decreases in temperature are known to drastically reduce the growth rate of bacteria and Schlenker et al. (1999) found that lower temperatures were found to slow the decline in concentrations of both estrogen and progesterone in cattle feces being stored at 30 and 5°C. Ultraviolet radiation (e.g. prolonged sun exposure) has also been found to degrade hormones by inducing steroid transformation (Matkovics, 1972) resulting in a modified hormone concentration.

In order to minimize degradation of the fecal steroid, it is typically recommended to store fecal samples at sub-zero temperatures until endocrine analysis can be performed (Whitten et al., 1998). While freezing is the preferred method under controlled laboratory conditions and recent studies have recommended the use of a newly developed field method of fecal steroid hormone extraction (separating steroids from fecal material by solid phase extraction: Ziegler and Wittwer, 2005), these techniques require either additional skill and/or equipment in the field making them not readily employable by most field researchers. Thus,

efficacy tests of alternative fecal storage methods for a wide variety of taxa are needed. With the exception of Beehner and Whitten (2004), previous studies investigating alternative field preservation or storage methods have focused on short-term effects of storage treatments and are limited to primates, carnivores, artiodactyla, and perissodactyla (baboon: 180 days, Khan et al., 2002; 30 days, Lynch et al., 2003; 400 days, Beehner and Whitten, 2004; cheetah: 7 days, Terio et al., 2002; white-tailed deer and elk: 7 days, Millspaugh et al., 2003; black rhino: 180 days, Galama et al., 2004; and giraffe, black rhino, dama gazelle, mountain goat: 90 days, Neumann et al., 2002). Terio et al. (2002) recommended ethanol as the best alternative preservation method for maintaining fecal progesterone and estrogen metabolite concentrations in cheetah feces for periods of up to 2 weeks. These results however were inconsistent with previous findings in baboon feces (Khan et al., 2002; Lynch et al., 2003) which were found to have altered steroid concentrations in ethanol-treated fecal samples. While Lynch et al. (2003) suggests freezing fecal samples in 95% ethanol at –20°C for up to 2 weeks as the best alternative preservation method, they also obtained results capable of detecting reproductively significant events (i.e. pregnancy) with samples stored in 95% ethanol in a charcoal refrigerator. Galama et al. (2004) recently found drying feces in a solar box cooker and mixing feces in 80% MeOH are both effective methods to maintain absolute and relative progesterone concentrations for black rhino fecal samples for up to 180 days. Drying fecal samples can, however, result in a loss of steroid hormone in some species (Ziegler and Wittwer, 2005).

Noticeably missing among these studies are long-term fecal storage investigations addressing storage treatment effects in a more diverse array of species and the consideration of storage effects at the steroid hormone profile level. Within mammalia, rodents are the most diversified order and comprise the greatest number of species. Their diversity provides an invaluable tool for answering a limitless number of endocrine questions. In recent years, fecal steroid analysis has been used to investigate endocrine hormone concentrations in free-living deer mice (*Peromyscus maniculatus*) and southern red-backed voles (*Clethrionomys gapperi*: Harper and Austad, 2004), free-living midday gerbils (*Meriones hefidianus pall.*: Kuznetsov et al., 2004), captive oldfield mice (*Peromyscus polionotus*: Good et al., 2003, 2005), Belding's ground squirrels (*Spermophilus beldingi*: Mateo and Cavigelli, 2005) and laboratory mice and rats (Touma et al., 2004; Eriksson et al., 2004, respectively). With such analyses becoming more popular with rodents, especially free-living, the necessity of determining alternative fecal storage methods is apparent. Additionally, previous fecal steroid analysis studies have concentrated on the effects of storage techniques on measured hormone concentrations. An analysis of changes in hormone concentrations at the profile level (i.e. both high and low concentrations) due to storage effects is crucial for understanding the effects on cycling gonadal hormones.

Our goals in this study were to: (1) validate that progesterone and E₁C enzyme immunoassay analysis (EIA) accurately measure progesterone and estrone conjugate concentrations in feces of the African Cape ground squirrel (*Xerus inauris*, Family Sciuridae, Tribe Xerini), (2) demonstrate that the fecal progesterone and E₁C assays are sensitive enough to detect biologically meaningful differences in progesterone and estrone conjugate concentrations in sub-adult, non-gravid and gravid adult female *X. inauris*, (3) examine the effects of storage of samples stored at sub-zero temperatures on hormonal concentrations, (4) investigate the existence of diurnal variation in fecal progesterone concentrations and (5) determine the effects of fecal samples stored in alcohol or dried in a drying oven on hormonal concentrations.

2. Materials and methods

2.1. Fecal sample collection and extraction

2.1.1. Physiological validation

Fecal samples were collected from 11 free-ranging female Cape ground squirrels trapped at the S.A. Lombard Nature Preserve (SALNP) near Bloemhof, South Africa (27°35'S, 25°35'E) between May 26 and October 10, 2004. Two samples were collected from sub-adults, five from non-gravid adults and four from gravid adults. Females were considered adult if the nipples were dark and elongated, indicating that they had previously bred (Waterman, 1996). Gravid and non-gravid females were classified by examination of changes in the vulva, nipples, and weight as well as behavioral observations of estrus, and maternal isolation (Waterman, 1995). As well, pregnancy for each individual gravid adult was confirmed by emergence of viable offspring. All fecal samples were stored in a –20 °C freezer until transported back to the United States on dry ice. Collection of these fecal samples was part of a larger study that documented reproductive behaviors as well as pregnancies.

All fecal samples were extracted following Graham et al. (2001). Briefly, each sample was weighed out to exactly 0.50 g and placed in an 8-ml glass evaporation-proof vial (National Scientific, Pittsburgh, PA) into which 5 ml of 80% ethanol was added to extract the hormone. All vials were capped and placed on a shaker (Eberbach Corp., Ann Arbor, MI) overnight. Sub-samples were then centrifuged (2500 rpm, 30 min), and the supernatant was poured off and stored at –20 °C until EIA.

2.1.2. Storage time

Ten fecal samples were collected from 10 free-ranging adult female Cape ground squirrels trapped at SALNP between July 25 and 27, 2005. All fecal samples were stored in a –20 °C freezer for 1–3 days before being transported back to the United States on dry ice. Each fecal sample was thawed, mixed thoroughly and divided into four 0.50 g sub-samples. The first set of sub-samples from each of the 10 individuals was extracted immediately following the methods outlined above. Subsequent sets of sub-samples were extracted 1 and 3 months after initial extraction.

2.1.3. Time of day

We tested whether time of day affected progesterone metabolite concentrations by collecting 14 paired morning (8:00–11:00) and afternoon (11:00–15:00) fecal samples from nine free-living female Cape ground squirrels at SALNP between July and October 2004. All fecal samples were stored in a –20 °C freezer until transported back to the United States on dry ice where they were extracted following the methods outlined above.

2.1.4. EIA validation and storage treatment condition

Forty-eight fecal samples were collected from six captive adult female Cape ground squirrels housed at SALNP. Details of captive conditions are

reported in Bouchie et al. (2006). Fecal samples were collected between May 10 and June 22, 2003 from a plastic tray below each individual cage. Each tray was covered with a sheath of 40% shade cloth to keep urine and fecal matter separate. Each fecal sample was randomly subdivided into 3–1 g sub-samples prior to being stored in alcohol, dried or frozen. Frozen sub-samples were immediately stored in a –20 °C freezer. Ethanol-treated sub-samples were preserved in 3 ml of 95% ethanol at room temperature (to mimic storage conditions expected in most field situations). Sub-samples for the dried-treatment were wrapped in aluminum foil, pressed flat, and dried in a conventional oven (Kelvinator S2620, Johannesburg, South Africa; 60 × 60 × 65 cm) at 40 °C for 4 h. To determine sufficient drying time, 28 test samples were placed in the conventional oven at 40 °C and weighed at 1 h increments until no more weight was lost. Using this method, we established that a 4 h drying time was sufficient to eliminate all fecal moisture. Dried-treated sub-samples were weighed before and after drying to control for differences in water content across treatments. Percent dry mass was obtained by dividing dry weight by initial (wet) weight. Percent dry mass was then multiplied by 0.50 g to determine final weight used for extraction. Frozen-treated fecal samples were transported back to the United States on dry ice, while ethanol and dried-treatment samples were transported under their assigned treatment conditions. All sub-samples were stored for 330 days.

Frozen sub-samples were extracted following Graham et al. (2001), with modifications as follows for the alcohol and dried-treatments. Frozen aliquots were thawed for 15 min, weighed out to exactly 0.50 g and placed in an 8-ml glass evaporation-proof vial (National Scientific, Pittsburgh, PA) into which 5 ml of 80% ethanol was added to extract the hormone. Alcohol-treated sub-samples already in 3 ml of 95% ethanol received an additional 2 ml of 57.5% ethanol to make a total of 5 ml of 80% ethanol to match the extraction protocol used for the dried and frozen samples. The exact mass of each sub-sample for alcohol treatment was recorded when each whole sample was sub-divided into aliquots. These weights were used, instead of the 0.50 g as in the frozen and dried-treated aliquots, in calculating progesterone concentrations. Dried-treated sub-samples were weighed out using the calculated final weight (see treatment section) and were placed in an 8-ml vial with 5 ml of 80% ethanol. Sub-samples were then extracted following the methods outlined above.

2.1.5. Spike recovery test

Fecal samples were collected from 10 free-ranging female Cape ground squirrels trapped at SALNP between February 27 and July 1, 2005. All samples were immediately frozen and transported back to the United States on dry ice for further analysis. For the estrone conjugate spike recovery, a large sample (<2.0 g) was collected from a sub-adult female, an adult female and an adult male. For the progesterone spike recovery, a large sample (<2.0 g) was collected from a sub-adult female and an adult female, while five 0.50 g adult male samples were pooled together for one large sample. All six large, previously frozen samples were thawed, thoroughly mixed and subdivided into four 0.50 g sub-samples subjected to four treatments (control, frozen, alcohol, dried). Control sub-samples received no hormone spike and were stored in a –20 °C freezer. Frozen, alcohol and dried sub-samples were each spiked with 250 ng estrone conjugate or 1000 ng progesterone and treated as described above. The dried sub-samples were dried in a laboratory oven (Precision Econotherm Lab oven, Woodstock, IL, 39 × 47 × 38 cm). After 1 week of storage, sub-samples were then extracted following the storage treatments methods outlined above.

2.2. EIA validations and assay protocols

2.2.1. EIA validations

Serial dilutions of a fecal extract pool from captive *X. inauris* were used to validate the enzyme immunoassay for fecal progesterone and estrone conjugates (1:8, 1:16, 1:32, 1:64, 1:128, 1:256, and 1:512). Parallelism between serial dilutions of fecal extracts and standard curves was determined by a test of equality of two slopes (Zar, 1996).

2.2.2. Progesterone EIA

Fecal extracts were diluted (1:51–1:150) in assay buffer (0.02 M Trizma, 0.30 M NaCl, 0.1% BSA, and 0.1% Tween 80) before analysis. We used a

progesterone EIA protocol previously outlined in Graham et al. (2001). The antiserum (Quidel clone no. 425) cross-reacts with 14 progesterone metabolites (see Table 1, Graham et al., 2001) and maintains a greater than 50% cross-reactivity with six of those metabolites. In brief, microtiter plates (Nunc; Fisher Scientific; Pittsburgh, PA) were coated with affinity purified goat anti-mouse γ -globulin (Sigma Chemicals, St. Louis, MO) dissolved in coating buffer (0.015 M Na_2CO_3 , 0.035 M NaHCO_3 ; pH 9.59) and incubated overnight at room temperature. Wells were emptied and refilled with a second coating buffer containing preservative (0.02 M Trizma, 0.30 M NaCl, 1.0% BSA, 0.01% NaN_3 ; pH 7.5) and stored at room temperature. Plates were used within one week of coating.

The progesterone EIA GAMG (affinity purified goat anti-mouse γ -globulin 50 $\mu\text{g}/\text{plate}$; Sigma-Aldrich, St. Louis, MO) coated plates were washed (0.04% Tween 20) three times and 50 μl of diluted samples, standards (progesterone Sigma-Aldrich, St. Louis, MO), and controls were dispensed. Biotinylated progesterone (100 μl ; 1:590,000; provided by F. Schwarzenberger, Vienna, Austria) was added to each well prior to the addition of 100 μl of mouse anti-progesterone antibody (1:440,000; Quidel clone #425 supplied by C. Munro, Davis, CA). Plates were sealed and incubated at room temperature overnight. After incubation, plates were washed and 200 μl streptavidin–peroxidase conjugate (1 μl in 24 ml assay buffer; Roche Diagnostics Co., Indianapolis, IN) was added to each well. Following 45 min room temperature incubation with the enzyme, plates were washed and incubated (30 min; room temperature) with 200 μl substrate solution (500 μl of 0.016 M tetramethylbenzidine in dimethyl-sulphoxide and 100 μl of 0.1752 M H_2O_2 diluted in 24 ml of substrate buffer (0.01 M $\text{C}_2\text{H}_3\text{Na}$; pH 5.0)). Stop solution (50 μl of 3 M H_2SO_4) was used to stop the enzyme reaction once the desired color was reached and the optical density was measured using an Emax plate reader (Molecular Devices, Sunnyvale, CA) with a test filter of 450 nm and a reference filter of 650 nm. All samples, controls, and standards were assayed in duplicate.

2.2.3. Estrone conjugates EIA

Fecal extracts were diluted (1:6–1:100) in phosphate buffer before analysis. We used an estrone conjugate (E_1C) EIA protocol previously outlined in Munro et al. (1991). The antiserum (R522) cross-reacts with estrone-3-glucuronide (100%), estrone-3-sulfate, (66.6%), estrone (238.0%), estradiol-17 β (7.8%), estradiol-3-glucuronide (3.8%) and estradiol-3-sulfate (3.3%). In brief, microtiter plates (Nunc; Fisher Scientific; Pittsburgh, PA) were coated with affinity purified antibody (stock solution, 10-fold dilution) dissolved in coating buffer (50 mmol/l bicarbonate buffer; pH 9.6). We coated the flat-bottom plates with 50 μl of the antibody coating solution per well, sealed and incubated overnight at room temperature. Plates were used within 2 days of coating.

The E_1C coated plates were washed (0.04% Tween 20) three times and 50 μl of phosphate buffer was immediately added to all sample and standard wells and 100 μl was dispensed into blank rows. Plates were allowed to sit for 15–20 m to equilibrate. We then added 50 μl of samples, controls and standards, followed by 100 μl of enzyme conjugate (1:20,000 working dilution) to each well, covered plates tightly and incubated overnight at room temperature. After incubation, plates were washed and blotted dry. Substrate solution (per liter, 50 mmol of citrate, 1.6 mmol of H_2O_2 and 0.4 mmol of 2,2'-azino-di-(3-exthylbenzthiazoline sulfonic acid) diammonium salt, pH 4.0) was prepared and 100 μl was added to each well. Plates were placed on a shaker until the desired color was reached (~50 min) indicative of the enzyme conversion of the substrate. Plates were read on an Emax plate reader (Molecular Devices, Sunnyvale, CA) with a test filter of 405 nm and a reference filter of 650 nm. All samples, controls, and standards were assayed in duplicate.

2.3. Statistical analysis

To compare the mean progesterone and estrone conjugate concentrations between squirrels in different physiological states we used the Kruskal–Wallis test. We used a repeated measure analysis of variance to determine the difference in hormone metabolite concentrations at different storage time periods compared to values obtained from immediate assay

analysis (3 days following collection). We used Wilcoxon signed ranks test to compare progesterone concentrations from paired morning and afternoon fecal collections. Fecal progesterone and estrone conjugate concentrations among storage treatments were compared using repeated measures analysis of variance (Neter et al., 1990). We used simple contrasts to compare fecal progesterone and E_1C concentrations between each alternative treatment (alcohol and dried) and frozen treatments when the overall treatment effect was significant. We used a one-way analysis of variance to compare differences in recovery rates, followed by Tukey's LSD test. Samples stored at sub-zero temperatures were considered reference values as freezing is a validated method considered to maintain stable steroid hormone concentrations for up to 2 years (Hunt and Wasser, 2003). Linear regression and correlation analyses were performed to evaluate the correspondence between the reference group (frozen) and other treatment group sub-samples when all samples from each treatment were pooled together.

To determine similarity between peak patterns of progesterone and estrone conjugate profiles, we computed peak and baseline concentrations by an iterative process (Graham et al., 2002; Brown et al., 1999). We calculated the mean concentration of all samples and temporarily removed values greater than the mean plus 1.75 standard deviations (SD) from the data set. These values were considered significant elevations. We then recalculated the mean and repeated the removal process until no values were higher than the mean plus 1.75 SD. Baseline concentrations consisted of the remaining fecal progesterone or estrone metabolite values. Percent agreement between timing (day) of peaks from treatment profiles and reference profiles were calculated and compared using a Chi-squared test. When assumptions of statistical tests were not met, data were normalized using a natural log transformation. Analyses were performed with SPSS 11.5.0 for Windows (SPSS, Inc., Chicago, IL). Means are reported with SE unless otherwise noted. Statistical significance was set at $p < 0.05$ for all analyses except for Kruskal–Wallis tests where significance was set at $p < 0.10$.

3. Results

3.1. EIA validation

Serial dilutions of selected fecal samples produced displacement curves parallel to that of the E_1C and progesterone standard curves. The test of the equality of slopes gave a value of $t = -1.21$, $df = 7$, $p = 0.86$ and $t = -2.52$, $df = 8$, $p = 0.98$ for E_1C and P4, respectively (Fig. 1). Recoveries from two positive and one negative previously frozen samples spiked with a moderate concentration of progesterone and estrone conjugate standard averaged $83.2\% \pm 8.7$ (SD) and $56.7\% \pm 9.6$ (SD), respectively. The sensitivity of the progesterone assay was 11.3 ± 4.6 pg/well. Inter-assay coefficient of variation was 7.0% (20% binding) and 12.6% (60% binding) ($n = 12$ plates). Intra-assay coefficient of variation was 5.4% and 5.5% for low and high pools, respectively ($n = 12$ plates). The sensitivity of the E_1C assay was 5.4 ± 1.1 pg/well. Inter-assay coefficient of variation was 5.8% (38% binding) and 13.7% (83% binding) ($n = 9$ plates). Intra-assay coefficient of variation was 28.3% and 9.6% for low and high pools, respectively ($n = 9$ plates).

3.2. Physiological validation

Mean fecal progesterone concentrations differed among sub-adult, non-gravid adult and gravid adult female Cape

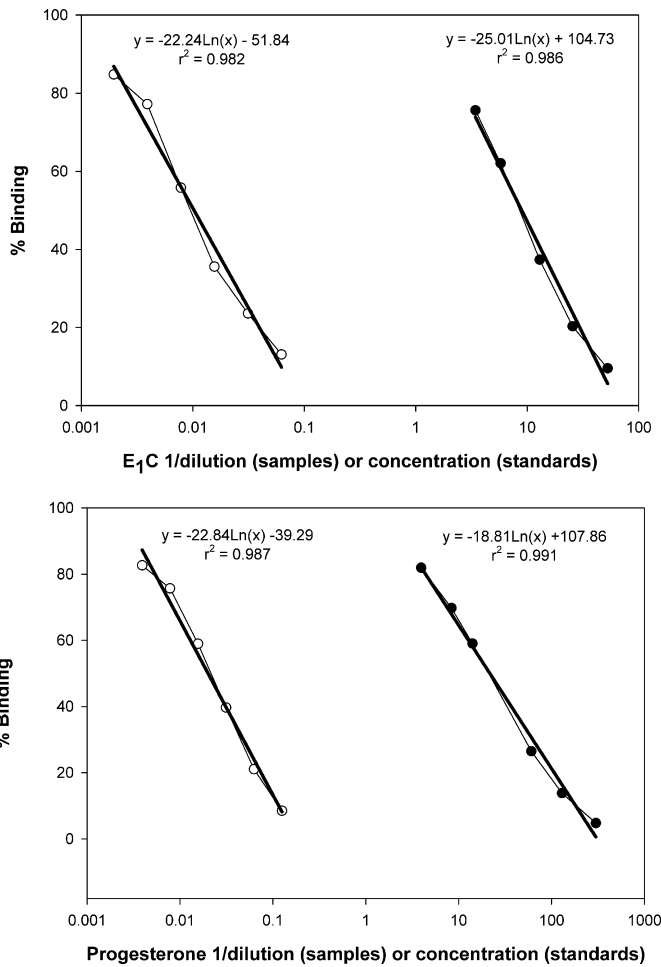


Fig. 1. Serial dilution results from a female Cape ground squirrel fecal extracts are presented. E₁C: The sample (open symbol) was diluted 1:16, 1:32, 1:64, 1:128, 1:256 and 1:512 in phosphate buffer and tested for binding with the estrone conjugate antibody in parallel with serially diluted standards (closed symbol) ranging from 3.125 to 50 pg/50 μ l. Regression equations derived from the linear portion of the curves produced almost identical r^2 values for the samples and standards (0.982 and 0.986, respectively). Progesterone: The sample (open symbol) was diluted 1:8, 1:16, 1:32, 1:64, 1:128, and 1:256 in assay buffer and tested for binding with the progesterone-antibody in parallel with serially diluted standards (closed symbol) ranging from 15.6 to 500 pg/50 μ l. Regression equations derived from the linear portion of the curves produced similar r^2 values for the samples and standards (0.987 and 0.991, respectively).

ground squirrels ($\chi^2 = 7.57$, $p = 0.02$; Kruskal–Wallis). Gravid adult females had significantly higher fecal progesterone concentrations than either sub-adult or non-gravid adult females ($\chi^2 = 4.50$, $p = 0.03$ and $\chi^2 = 6.00$, $p = 0.01$ for sub-adult and non-gravid, respectively). There was no significant difference between mean fecal progesterone concentration of sub-adult and non-gravid adult females ($\chi^2 = 1.35$, $p = 0.25$). Mean fecal estrone conjugate concentrations differed among sub-adult, non-gravid and gravid adult females ($\chi^2 = 4.66$, $p = 0.10$). Adult females, both gravid and non-gravid, had significantly higher fecal estrone conjugate concentrations than sub-adult females ($\chi^2 = 3.75$, $p = 0.053$ and $\chi^2 = 3.43$, $p = 0.06$ for non-gravid and gravid, respectively) (Fig. 2).

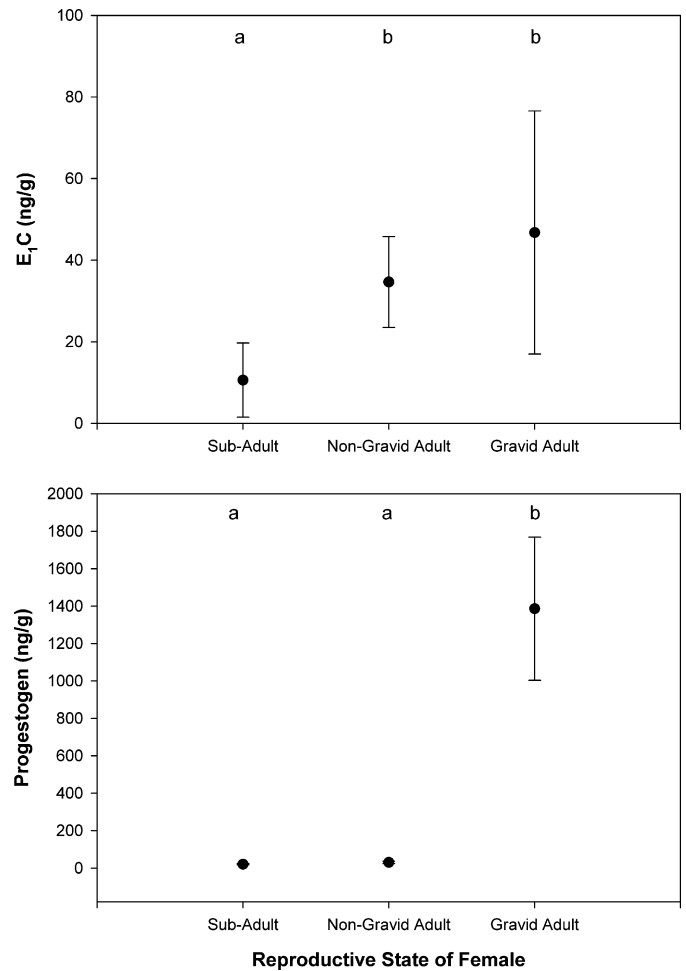


Fig. 2. Physiological validation of fecal estrone conjugate and progesterone concentrations among sub-adult ($n = 2$), non-gravid adult ($n = 5$) and gravid ($n = 4$) female Cape ground squirrels. Mean concentration \pm SE. Means with different letters above are significantly different ($p < 0.05$).

3.3. Storage time experiment

Duration of storage time did not affect absolute amount of progesterone and estrone metabolite concentrations ($F_{2,20} = 2.34$, $p = 0.122$ and $F_{2,20} = 3.181$, $p = 0.063$, respectively; repeated measures ANOVA). There was no difference in fecal progesterone concentrations between 0 and 1 or 0 and 3 months of frozen storage time ($F_{1,10} = 0.58$, $p = 0.46$ and $F_{1,10} = 2.82$, $p = 0.12$, respectively). There was no difference in fecal estrone conjugate concentrations between 0 and 1 month ($F_{1,10} = 0.47$, $p = 0.51$), or between 0 and 3 months ($F_{1,10} = 4.31$, $p = 0.07$).

3.4. Time of day

Paired comparisons showed that there was no diurnal variation in fecal excretion of progesterone in female Cape ground squirrels. Fecal samples collected in the early morning versus early afternoon did not differ in their progesterone concentrations ($Z = -1.036$, $p = 0.30$; Wilcoxon signed

ranks test), indicating the comparison of concentrations between samples collected at variable time periods is acceptable.

3.5. Alternative storage treatment experiment

Storage treatment affected absolute amount of fecal progesterone and estrone metabolite concentrations ($F_{2,10}=10.74$, $p=0.003$ and $F_{2,10}=5.96$, $p=0.02$, respectively). There was no difference in fecal progesterone concentrations between frozen and dried samples ($F_{1,5}=1.90$, $p=0.23$). While dried-treated fecal samples maintained a similar fecal estrone conjugate concentration compared to frozen samples, there existed a slight, though non-significant trend of decreased values ($F_{1,5}=5.70$, $p=0.06$). Fecal samples stored in 95% ethanol had significantly higher fecal progesterone concentrations than frozen samples ($F_{1,5}=9.84$, $p=0.03$) and significantly lower fecal estrone metabolite concentrations ($F_{1,5}=6.74$, $p=0.048$). The ethanol storage treatment increased fecal progesterone concentrations by greater than 20% in 55% of the samples (Fig. 3).

When fecal samples from all individuals were pooled together, there was no difference in the slopes of the regression lines from a plot of dried versus frozen-treated samples and the from a plot of alcohol versus frozen-treated samples for either log-transformed progesterone or estrone conjugate concentrations ($t=-0.17$, $df=90$, $p=0.57$ and $t=0.73$, $df=90$, $p=0.23$, respectively). The correlation between log-transformed fecal progesterone concentrations from dried-treated and corresponding frozen samples was significantly higher than for ethanol-treated samples ($Z=2.05$, $df=47$, $p=0.04$). The correlation between log-transformed fecal estrone conjugate concentrations from dried-treated and frozen samples tended to be higher than for ethanol-treated samples, although not significantly ($Z=1.75$, $df=47$, $p=0.08$) (Fig. 4).

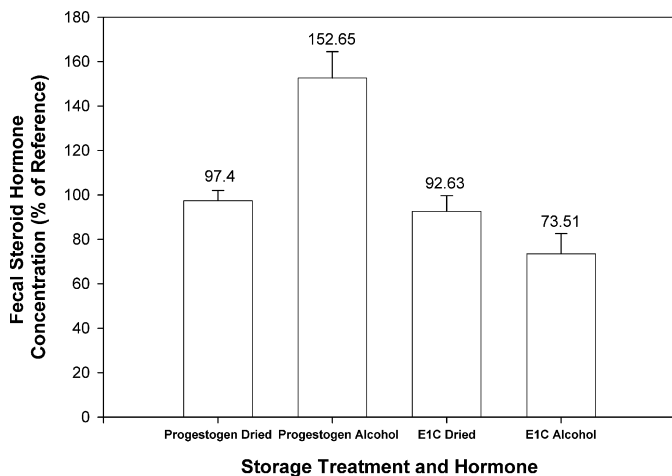


Fig. 3. Mean concentration \pm SE for progesterone and estrone conjugate concentrations as percent of frozen reference samples for Cape ground squirrel fecal samples ($n=48$) subjected to 330 days of storage following oven-drying or 95% ethanol.

Storage treatment also affected the recovery rate of a known amount of progesterone, but not estrone conjugate added to the previously frozen fecal sample prior to treatment and extraction ($F_{1,2}=6.91$, $p=0.03$ and $F_{1,2}=9.47$, $p=0.04$, respectively). For the progesterone spike, there was no difference in the recovery rates between frozen ($80.8\% \pm 3.5$) and dried samples ($76.2\% \pm 3.9$; Tukey's LSD, $p=0.35$), however the mean recovery rate for alcohol-treated samples ($92.5\% \pm 8.0$) was significantly higher than the mean frozen sample recovery rate ($p=0.04$). Average recovery rates from previously frozen stored samples spiked with estrone conjugate were $46.59\% \pm 14.01$ (frozen), $57.64\% \pm 13.86$ (dried) and $65.74\% \pm 22.14$ (alcohol). The low recovery rate may indicate the presence of an interfering substance.

Graphing progesterone profiles of both treatments and reference samples for each squirrel revealed a stronger similarity in peak pattern between frozen and ethanol-treated

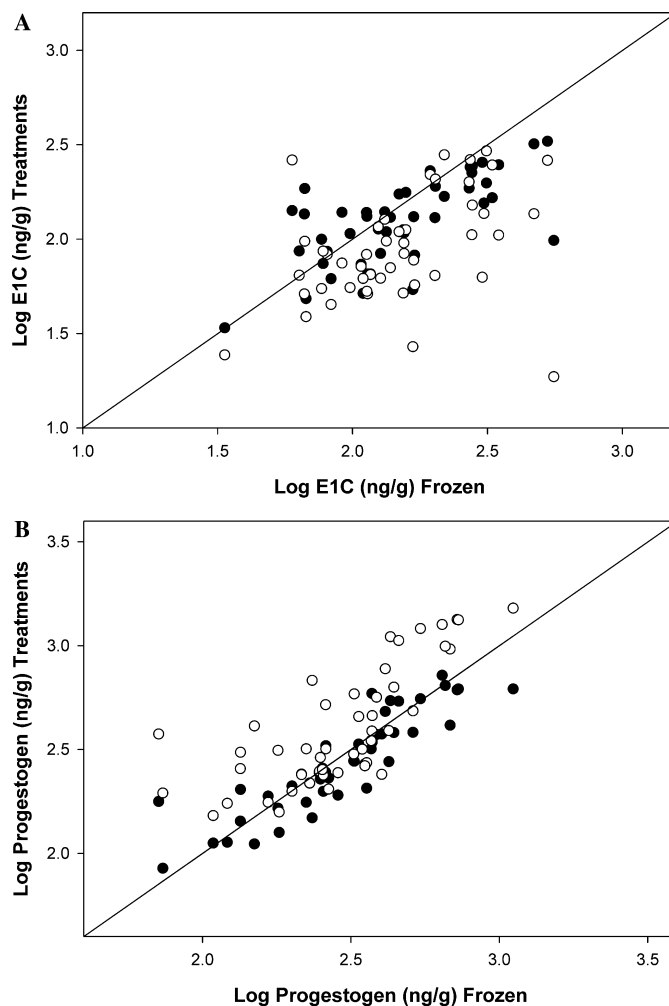


Fig. 4. Scatter-plot graphs of pooled, log-transformed fecal estrone conjugate (A) and progesterone (B) concentrations of fecal samples from captive *X. inauris*. Open circles (\circ) indicate log-transformed fecal hormone metabolite concentrations for alcohol-treated samples and filled circles (\bullet) indicate log-transformed fecal hormone metabolite concentrations for dried-treated samples. Solid line indicates slope = 1. E1C: (\circ) $y=0.435x+1.003$, $R^2=0.41$; (\bullet) $y=0.559x+0.880$, $R^2=0.67$. P4: (\circ) $y=0.830x+0.557$, $R^2=0.75$; (\bullet) $y=0.809x+0.443$, $R^2=0.89$.

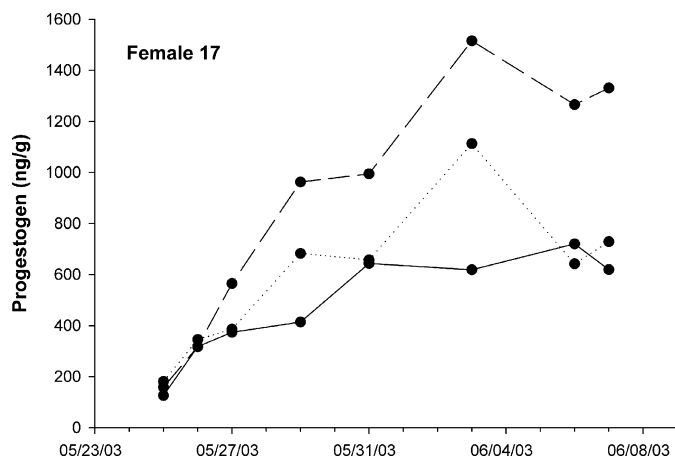


Fig. 5. Progestogen profile of an adult captive female *X. inauris* (female 17) for fecal samples subjected to freezing, drying and preservation in alcohol ($n = 8$ for each treatment) collected during early summer 2003 in Bloemhof, South Africa. Profile is shown from frozen samples (dotted line), alcohol-treated samples (dashed line) and dried-treated samples (solid line).

samples than frozen and dried-treated samples. In particular, female 17 exhibits parallel rise and fall in frozen and alcohol progestogen profiles (Fig. 5). However, once baseline concentrations were determined, dried-treated samples showed a significantly higher percentage of agreement in timing of peaks with corresponding frozen samples than ethanol-treated samples did with corresponding frozen samples (87.2% versus 66.0% agreement, $\chi^2 = 5.93$, $p = 0.03$; Chi-squared test). Additionally, ethanol-treated samples produced false peaks (ethanol samples peaked when frozen samples did not) 94% of the time when ethanol and frozen samples disagreed, while false peaks occurred 64% of the time when dried-treated samples disagreed with frozen samples (Table 1).

Additionally, female 17 exhibited a progestogen profile possibly indicative of ovarian activity (e.g. ovulation), though captive conditions removed the possibility of pregnancy. While reproductive cycle information is unclear for *X. inauris*, the fecal progestogen profile of female 17 involved an increase above baseline with progestogen concentrations from frozen reference samples remaining elevated for at least 10 days.

Examining E_1C profiles of both treatments and reference samples for each squirrel showed little difference in similarity in peak pattern between either treatment and reference samples. There was no significant difference between percent agreement of peak pattern between dried-treated and frozen samples and percent agreement between ethanol-

treated and frozen samples (87.2% versus 83% agreement, $\chi^2 = 0.34$, $p = 0.16$). When the timing of peaks did not agree between treatment and reference sample, dried-treated fecal samples showed false peaks 50% of the time, while alcohol-treated fecal samples showed false peaks 62% of the time (Table 1).

4. Discussion

4.1. Assay validation

Both P4 and E_1C enzyme immunoassays measured progestogen and estrone conjugate concentrations accurately and precisely in fecal samples of *X. inauris*. Previous studies of rodent endocrinology that used fecal steroid analysis (Harper and Austad, 2004; Kuznetsov et al., 2004; Good et al., 2003; Touma et al., 2004; Eriksson et al., 2004; Mateo and Cavigelli, 2005) have validated both radioimmunoassays and enzyme immunoassays for glucocorticoids, however our study is the first to validate both progestogen and estrone conjugate enzyme immunoassays for fecal samples from a rodent. As a result, these assays have the potential to be utilized as a non-invasive technique to answer a wide variety of reproductive questions of the largest, most diverse and most broadly studied order of mammals.

4.2. Physiological assay validation

Although detectable concentrations of progestogen were present in all age groups and all reproductive conditions, they noticeably increased during pregnancy. These results indicated that fecal progestogen steroid analysis can be useful in detecting pregnancy and possibly other reproductive events in *X. inauris*. While these samples were collected during peak breeding season, we expect similar results would be obtained as estrus occurs year-round in Cape ground squirrels (Waterman, 1996), eliminating effects of seasonality on hormone concentrations. The low levels of progestogen concentrations in non-gravid females may be influenced by small sample size that would reduce the probability of collecting luteal samples from cycling adults. Additionally, low progestogen concentrations may be indicative of a short luteal phase (Bouchie et al., 2006) or possibly induced ovulation. Further research into the source and regulation of progesterone secretion would benefit the understanding of progesterone profiles as more information is gained through hormone analysis.

Detectable concentrations of fecal estrone conjugates were also present in fecal samples of all age groups and all

Table 1

Percent agreement of timing of peaks between frozen and dried or alcohol-treated samples

	Dried				Alcohol			
	Agree (%)	Disagree (%)	False peak	Missed peak	Agree (%)	Disagree (%)	False peak	Missed peak
P4	87.2 (41/46)	12.8 (6/46)	67% (4/6)	33% (2/6)	66 (31/46)	34 (16/46)	94% (15/16)	6% (1/16)
E_1C	87.2 (41/46)	12.8 (6/46)	50% (3/6)	50% (3/6)	83 (39/46)	17 (8/46)	62.5% (5/8)	37.5% (3/8)

reproductive conditions with adult females (gravid and non-gravid) exhibiting significantly higher concentrations compared to sub-adult females. As sub-adults may have not yet developed a fully functioning reproductive system, lower concentrations of estrogen metabolites in feces of sub-adults may be expected. These results demonstrate the potential ability of fecal estrone conjugate analysis to provide evidence of sexual maturation. While this investigation did not reveal a difference in fecal estrone conjugate concentration between gravid and non-gravid adult females, a study with a larger sample size may elucidate such a distinction.

4.3. Storage time

Fecal progesterone and E₁C concentrations were relatively unaffected by storage at -20°C for up to 3 months, suggesting that freezing fecal samples has little impact on fecal progesterone and estrone conjugate measurements. A slight, although insignificant increase in fecal estrone conjugate concentrations did occur between 1 and 3 months of frozen storage suggesting further investigation into effects of freezing fecal samples for more than 3 months (e.g. 6, 9 and 12 months) is needed.

4.4. Time of day

Diurnal variation in fecal progesterone metabolite excretion appears to be species-specific (high morning concentrations in marmosets, *Callithrix jacchus*: Sousa and Ziegler, 1998 and tamarins, *Saguinus mystax*: Lottker et al., 2004; no diurnal variation in baboons, *Papio hamadryas*: Beehner and Whitten, 2004), necessitating the examination of variation in steroid hormone concentration in samples collected at varying times of day. While plasma steroid hormone concentrations are often higher in the morning (Steinetz et al., 1990), these circulating hormones are subject to various metabolic and dietary influences that may alter fecal steroid excretion. Therefore, our finding that fecal progesterone concentrations of Cape ground squirrels do not exhibit diurnal variation is not unexpected.

4.5. Storage treatment

Regarding both measured amount and relative (profile) hormone metabolite concentrations, our results demonstrate that drying feces provides a reliable method for long-term preservation of progesterone and estrone conjugate concentrations and is the better alternative when freezing is not a viable option. Oven-dried feces from Cape ground squirrels maintained stable fecal progesterone concentrations after long-term storage. While they tended to be slightly lower, absolute estrone conjugate concentrations from dried feces did not significantly differ from concentrations in frozen feces. As well, progesterone and estrone conjugate recovery rates did not differ between dried and reference samples. Our data illustrated that storage treat-

ment effects do not differ across different steroid hormone concentrations (i.e. there are not greater fluctuations due to treatment effects at higher or lower hormone metabolite concentrations). Our results were similar to those found in previous storage experiments. Drying black rhinoceros fecal samples in a solar box cooker did not affect the concentration of progesterone compared to control values (180 days, Galama et al., 2004). Similar results were found in a short-term study with drying sifaka fecal samples (3 weeks; Brockman and Whitten, 1996). Our results did differ slightly with findings from Terio et al. (2002) where they found the use of a solar or conventional oven to dry cheetah fecal samples resulted in alterations in both progesterone and estrogen metabolite concentrations.

Our samples stored in ethanol showed marked alteration in both fecal progesterone and estrone conjugate concentrations suggesting that ethanol does not provide a reliable long-term preservation method in this species. Over half of the ethanol-treated samples showed an increase in progesterone concentrations by over 20% and a decrease in estrone conjugate concentrations by over 30% compared to frozen-treated samples. Results from our spike recovery analysis provide further evidence of the amplification effects of fecal storage in alcohol, as progesterone recovery rates for alcohol-treated samples were significantly higher than reference samples. Due to the common occurrence of such increases in progesterone concentrations found in ethanol-treated fecal samples, we do not recommend the use of ethanol-treated samples for distinguishing reproductive states. Results from our ethanol-treated fecals were not consistent with previous findings in other species. Galama et al. (2004) and Terio et al. (2002) found that fecal samples stored in alcohol did not have significantly different fecal progesterone or estrone conjugate concentrations from controls. Lynch et al. (2003) reported that storing fecal samples in 95% ethanol at sub-zero temperatures resulted in no significant change from initial concentrations for both progesterone and estrogen metabolites. They did find a significant increase in fecal progesterone concentrations in samples stored in 95% ethanol in a charcoal refrigerator, however these samples still maintained the ability to distinguish reproductive states (gravid versus non-gravid) (Lynch et al., 2003). A study on baboon fecal steroid hormone storage found that fecal samples stored in ethanol over a six month period showed an increase in estrogen metabolite concentration for ~ 90 days, followed by a decrease to almost initial concentrations after ~ 180 days (Khan et al., 2002).

The direction of change (increases or decreases) in detectable fecal steroid hormone concentrations during long-term storage may be influenced by a combination of variables including digestive strategy, storage treatment, storage time and antibody specificity. Changes in the amount of hormone metabolites in a fecal sample are due to the influence these variables have on the metabolic transformation of the hormone. Before excretion, steroid hormones are metabolized by the liver and excreted into the urine and bile as conjugates to inactivate them and increase their water-solubility (Ziegler

and Wittwer, 2005; Adlercreutz et al., 1976). Once in the gut, hormone metabolites are deconjugated by intestinal microorganisms and bacteria and, if not reabsorbed, are excreted through the feces (Groh et al., 1993; Palme et al., 1996). Fecal hormones exist in both conjugated and unconjugated forms, although most species contain a higher percentage of conjugated than free steroids (Ziegler et al., 1996). As a result of this process, the form in which the metabolite is excreted influences how it is transformed during storage by the fecal microbes and bacteria (Bokkenheuser and Winter, 1980; Jarvenpaa et al., 1980).

Digestive strategy (i.e. carnivore, omnivore, forestomach or caecocolic fermenter) may influence fecal steroid concentrations as a result of differences in fecal bacteria loads and amount of dietary fiber. Low pH and short retention times limit the number and species of microorganisms, while neutral pH and long retention times are associated with an increase in number and species of microorganisms (Stevens and Hume, 1998), suggesting that fermenting herbivores likely have higher numbers of microorganisms in their feces than carnivores. Among herbivores, forestomach fermenters digest microbial cells while caecocolic fermenters typically lose microbial cells in the feces (Hume, 1999) resulting in higher microbial loads in caecocolic fermenters. Dietary fiber may also influence intestinal metabolism and retention time resulting in changes in steroid hormone concentrations.

Previous storage studies collectively suggest that storage treatment and time may indirectly affect the changes in hormone concentrations by the treatment's effect on the activity of fecal bacteria and microorganisms. Sub-zero temperatures, alcohol and lack of moisture can kill microorganisms or slow down microflora growth and may prevent further transformation of steroid hormones and hormone metabolites. Significant increases in steroid hormone concentrations may be due to the continued extraction of hormone metabolites by ethanol from fecal samples over time (Khan et al., 2002).

Antibody cross-reactivity may also influence changes in fecal hormone metabolite concentrations as the metabolites continue to be transformed during storage (Terio et al., 2002). The antibody developed for an immunoassay may be either a highly specific or a group-specific antibody. A highly specific antibody has been defined as cross-reacting with only the parent hormone (Khan et al., 2002), while a group-specific antibody typically has a high affinity for a set of steroid metabolites derive from the parent hormone (Wasser et al., 2000; Khan et al., 2002; Schwarzenberger et al., 1997). An artificial rise in hormone concentration may be a result of the antibody cross-reactivity of newly formed hormonal metabolites created by microbial transformation (Terio et al., 2002; Wasser et al., 2000).

Taken together, the above variables provide an explanation for the significant increase seen in the progesterone concentrations in the alcohol-treated fecal samples, as well as the mean recovery rate for the alcohol samples spiked with progesterone. Cape ground squirrels are herbivores and caecocolic fermenters indicating their feces likely contains

high microbial loads. As well, this study involved long-term storage and used a group-specific progesterone-antibody in the EIA analysis. As the high number of fecal bacteria continues to transform the progesterones into newly formed metabolites that cross-react with the group-specific antibody and the ethanol continues to extract these metabolites out over time, the measured concentration of progesterone through EIA analysis will increase.

The above explanation does not clarify however, the decreased concentrations observed in estrone conjugate concentrations of ethanol-treated fecal samples. The E₁C antibody used in this investigation cross-reacts with three main estrone conjugates, however it is possible that the bacteria found in Cape ground squirrel feces transforms estrone into metabolites that do not cross-react with this antibody. Consequently, the high fecal microbial loads may be reducing the number of cross-reacting estrone metabolites resulting in a decrease in the EIA measured estrone conjugate concentrations.

Our examination of relative hormone metabolite concentrations (temporal relationships as hormone concentrations naturally fluctuate) emphasizes the relevance of assessing efficacy of alternative storage methods at the profile level and the importance of determining a baseline for each treatment in order to identify similarity in peak pattern. While a visual inspection of peak patterns suggests that storage in ethanol provides an adequate alternative to freezing fecal samples, results from our baseline analysis revealed that the timing of peaks (i.e. potential reproductive events) from dried-treated samples more closely matched the peak pattern of frozen-treated samples. As well, ethanol-treated fecal samples are more likely to give false peaks than dried-treated samples. We encourage this type of comparison as part of the assay validation since studies addressing relative hormone concentrations are able to determine a storage treatment's effects on different concentrations of fecal hormone metabolite; a necessity when assessing biologically relevant events via rises and falls in fecal hormone metabolite concentrations. Examining hormone profiles as well as mean steroid hormone concentrations will provide a more comprehensive evaluation of the utility of possible alternative fecal preservation methods.

Knowledge of the effects of fecal storage is crucial prior to the onset of any fecal steroid analysis. Determination of the most ideal fecal storage method should become part of the assay validation procedure for each individual research project. The present study provides concrete evidence for storage effects for two fecal steroid metabolites in a rodent and a means by which storage treatments can be compared. Further application of this method will enhance our understanding of storage treatment effects on fecal steroid hormone concentrations and minimize the variability produced by improper preservation techniques for the species in question.

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