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Non-invasive measurement of fecal estrogens in the spotted hyena (*Crocuta crocuta*)

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Abstract

Fecal hormone analysis is a useful tool for frequent, non-invasive sampling of free-living animals. Estrogens fluctuate throughout life among reproductive states in female animals, and intensive repetitive sampling can permit accurate assessment of female reproductive condition. This type of repetitive sampling is difficult in large carnivores, including the spotted hyena (*Crocuta crocuta*). Patterns of estrogen secretion in captive and free-living hyenas are virtually unknown. Here we present validation of an enzyme-immunoassay to measure fecal estrogen (fE) concentrations in wild and captive spotted hyenas. Results from high-performance liquid chromatography indicate that an antibody specific for estradiol exhibits high immunoreactivity with our extracted samples. Fecal extract displacement curves paralleled our estradiol standard curve within the range of 20–80% antibody binding. Additionally, animals treated with luteinizing hormone-releasing hormone showed a measurable rise in fE concentrations. Finally, once we controlled for effects of time of day of sample collection from wild hyenas, patterns in fE concentrations resembled those in plasma estradiol, including higher levels of fE in mature than immature females, and higher levels of fE during late than early pregnancy. Together, these results suggest that fE concentrations reflect circulating estrogens in spotted hyenas.

Keywords: Spotted hyena; *Crocuta*; Fecal hormones; Estrogen; Reproductive states

1. Introduction

Knowledge about a female's reproductive state is essential to understanding aspects of her basic biology, including her fertility, behavior (Altmann et al., 2004; Maestripieri, 1999; Ramirez et al., 2004; Wise, 1974), nutritional requirements (Lambert et al., 2005; Schneider et al., 2000), and immune function (Gu et al., 2005; Hernandez-Gonzalez et al., 2006). Despite the importance of understanding reproductive cycles in females, there are many species for which we know virtually nothing about variation in reproductive status, particularly within free-living populations of large predatory mammals. Estrogen concentrations often vary predictably during female reproductive cycles, so the ability to measure estrogens offers one potential mechanism for monitoring ovarian status. Here our goal was to develop methods for non-invasive measurement of estrogens in free-living spotted hyenas (*Crocuta crocuta*).

Studies of the endocrinology of spotted hyenas to date have focused on androgenic hormones, so a great deal is known about circulating and excreted androgens in *Crocuta* (Dloniak et al., 2006, 2003; Glickman et al., 1998, 1992; Goymann et al., 2001; Licht et al., 1992, 1998; Van Jaarsveld and Skinner, 1991). By contrast, little is known about

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estrogenic hormones in this species. In a study of captive spotted hyenas between infancy and sexual maturity, Glickman et al. (1992) found that plasma estrogen concentrations begin to rise in females by 23 months of age, which is also when they may conceive their first litters in nature (Holekamp et al., 1996). It was further shown that plasma estrogen concentrations are higher in adult females than in adult males, and that mean plasma estrogen concentrations are higher in adult than sub-adult females (Glickman et al., 1992). Additionally, pregnant females have higher plasma estrogen concentrations than non-pregnant females, and plasma estrogen concentrations peak during late pregnancy (Licht et al., 1992). However, we know very little about the female's estrous cycle in this species, including the cycle length, and whether ovulation is induced or spontaneous, nor is anything known about patterns of estrogen secretion among wild hyenas.

Gaining information about the reproductive condition of wild female hyenas is difficult. There are no external indicators of changing reproductive state, such as genital swelling, color change, or other visual signs of ovulation often used by researchers studying free-living mammals. Even late pregnancy is difficult or impossible to detect by visual inspection in this species because female body mass can change by up to 18 kg at a single meal (e.g., Henschel and Tilson, 1988). Sampling blood from hyenas for measurement of steroid hormone concentrations requires invasive and potentially dangerous procedures, including isolation and anesthesia (Glickman et al., 1992), so multiple samples from the same individual are difficult to obtain in both wild and captive settings. Recent advances in non-invasive sampling methods for hormone analysis offer opportunities for more rigorous investigation of the endocrine correlates and control of reproduction in *Crocuta*. Fecal steroid analysis, in particular, offers an attractive alternative to blood sampling in both wild and captive animals. For collecting samples frequently, non-invasively, and without disrupting normal behavior of individuals or groups, techniques have been developed to assay fecal estrogen concentrations in a number of other mammalian carnivores (Brown et al., 1994; Monfort et al., 1997; Onuma et al., 2002; Shille et al., 1984; Young et al., 2001). A preliminary study in captive *Crocuta* detected measurable amounts of radiolabeled estrogen in fecal matter, and indicated that feces, rather than urine, should prove useful for sampling of excreted steroid hormones (Koretz, 1992).

Our aim here was to develop and validate an enzyme-immunoassay (EIA) for measurement of fecal estrogens (fE) in wild and captive spotted hyenas. First, we identified the immunoreactive estrogen metabolites in hyena feces using high-performance liquid chromatography (HPLC). We then investigated assay parallelism, examined possible procedural covariates of estrogen concentrations, and assessed the biological validity of our assay by monitoring changes in estrogen concentrations following treatment with luteinizing hormone-releasing hormone (LHRH) of wild and captive hyenas. Finally, we compared patterns of estrogen concentration between feces and plasma among classes of hyenas that were expected to exhibit significant variation in estrogen (i.e., pregnant *vs.* non-pregnant females, mature *vs*. immature females).

2. Methods

2.1. Field study site, subjects, and sample collection

Field data were collected in the Talek region of the Masai Mara National Reserve, Kenya. Subjects were members of one large, stable social group (''clan'') that defended a territory of 62 km^2 (Boydston et al., 2001). Talek hyenas have been closely monitored since 1988, and clan members are usually observed intensively on at least 23 days each month. All clan members were individually recognized by their unique spot patterns, and sex was determined by the dimorphic glans morphology of the erect phallus (Frank et al., 1990). Ages of all individuals born into the clan since 1988 were known, and mother–offspring relationships were established on the basis of nursing associations. Age at first reproduction and full reproductive histories were known for all female members of the clan from 1988 to 2004 based on known dates of birth and weaning of cubs. Each female was assigned to one of the following three reproductive states each time she was observed.

2.1.1. Immature

Females were considered reproductively immature from birth until first conception. In the wild, we can confidently assign a date to each female's first parturition because of the extensive tearing and scarring of her phallus during the birth process (Frank and Glickman, 1994). Gestation in spotted hyenas lasts 110 days (Kruuk, 1972), so we could accurately estimate the date of first conception by counting backwards 110 days from the known date of first parturition.

2.1.2. Pregnant

Females were considered pregnant during the period between the birth of each litter and its estimated conception date. Birth dates were assigned based on the size, pelage, and behavior of cubs when they were first seen above ground, and were accurate to within ±7 days (Holekamp et al., 1996). Pregnancy was further divided into trimesters to analyze variation in hormone concentrations among trimesters. Early pregnancy was the period between conception and day 37 of gestation. Middle pregnancy was day 38 through day 74, and late pregnancy was day 75 through the day of parturition.

2.1.3. Lactating

The lactation interval in *Crocuta* is highly variable in length (from 7 to 24 months), as it is affected by food availability, social rank, and litter composition (twin *vs.* singleton litters) (Holekamp et al., 1996, 1999). Termination of lactation was determined here either by conception of the next litter, or by weaning or disappearance of the current litter, whichever came first. Wean dates were determined using behavioral observations of mother–infant conflicts over nursing, as described by Holekamp et al. (1996). Wean dates for which mother–infant conflicts were not observed were calculated as the day halfway between the last time the cubs were seen nursing and the next time dam and cubs were seen together, if these could be estimated to ±10 days.

2.1.4. Sample collection

Starting in 1990, each clan member was immobilized at least once for collection of physiological and morphological data. Individuals were anesthetized with Telazol (6.5 mg/kg body mass) administered in a plastic dart fired from a CO_2 rifle. All immobilizations took place between 06:30 and 09:00 hours, and were performed in accordance with Kenyan law and *NIH Guide for the Care and Use of Laboratory Animals*. Within 17 min of Telazol administration, a blood sample (10 ml) was taken from the jugular

vein in a heparinized vacutainer tube. Blood samples were centrifuged, and plasma was stored in liquid nitrogen until it could be shipped on dry ice to Michigan State University (MSU), where it was stored at −80 °C until radioimmunoassay.

Beginning in 1993, fecal samples were collected whenever a known hyena was observed to defecate. Fecal samples were collected either in the morning (06:00–09:00) or evening (17:00–20:00) into plastic bags within 30 min of excretion. Samples were maintained at room temperature after collection for up to 12 h, after which they were mixed and stored in liquid nitrogen until they were shipped on dry ice to MSU (Dloniak, 2004). Dloniak (2004) found that variation in time elapsed between collection and freezing of fecal samples does not significantly affect concentrations of other steroid hormones in the samples, and we assumed here this applied to fE as well. Samples were stored at MSU at minus 20 °C until extraction and enzyme-immunoassay.

Administration of exogenous LHRH, acting via intermediate effects on the pituitary, stimulates secretion of steroid hormones from the gonads of hyenas (Ensley et al., 1982; Holekamp and Sisk, 2003; Place et al., 2002). Nine parous wild females were tested during late lactation (7.9–13.3 months after parturition) for steroid production in response to LHRH injection. Additionally, three wild adult females were administered saline to serve as handling and injection controls. After immobilization, blood samples were taken at 5-min intervals from the jugular vein for 45 min. Then, females were injected intravenously with LHRH (1 μg/kg LHRH, L-7134, Sigma Chemical CO., St. Louis, MO). Blood sampling then continued at 5-min intervals for 120 min. Supplementary doses of Telazol were administered as necessary throughout sampling to maintain deep anesthesia.

2.2. Captive study site, subjects, and sample collection

Captive data were collected from *Crocuta* in the colony maintained at the Field Station for Behavioral Research of the University of California, Berkeley. Subjects were housed individually or in small groups, and fed a standard zoo carnivore diet supplemented with bone. All fecal samples were collected between 08:00 and 12:00 h, mixed, and stored at −80 °C until extraction and enzyme-immunoassay.

Five gonadally intact adult captive hyenas (three males and two females) were injected with LHRH to induce the release of gonadal steroids. On the day of LHRH challenge, subjects were isolated, and then immobilized with ketamine (6.0 mg/kg body mass) and xylazine (1 mg/kg body mass) administered by blow dart. Each subject was administered an intravenous injection of LHRH, allowed to recover from anesthesia, and released back into its home enclosure. Fecal samples were collected daily for 7–8 days prior to LHRH injection, on the day of injection, and then for 7–8 days following injection.

2.3. Extraction and immunoassay of fecal samples

A total of 871 fecal samples were collected from known female Talek hyenas. Extraction of steroid hormones has been described elsewhere (Dloniak et al., 2006, 2003). Briefly, frozen samples were lyophilized (Labconco Freeze-Dry System 10-269), ground to a fine powder, and shaken overnight in 100% ethanol. Samples were then boiled and centrifuged, and the resulting supernatant was poured off into culture tubes while the remaining fecal pellet was discarded. Finally, the supernatant was allowed to evaporate. Extracted samples were then reconstituted in 1.0 ml phosphate-buffered saline (PBS; pH 5.0), sealed, and stored frozen at −20 °C until assay.

The number and proportion of estrogen metabolites in *Crocuta* feces were determined by HPLC analysis of fecal extracts from one pregnant and one non-pregnant wild female hyena using modifications of methods described in Brown et al. (1994). Extracted samples were spiked with 7000 cpm of [^{3}H]estrone (E_1) and [^{3}H]estrone sulfate (E_1S), and air-dried. Samples were then reconstituted in 500 μl of PBS and vortexed. A C18 sample preparation cartridge (Spice™ Cartridge; Analtech, Inc., Newark, DE) was primed with methanol (MeOH) and distilled water, and loaded with the total volume of sample in PBS. Distilled water and MeOH were pushed through the cartridge and collected into test tubes. The MeOH portion was allowed to evaporate and the residue was resuspended in MeOH. HPLC was then conducted by injecting 0.05 ml of the resuspended residue onto the column (Reverse Phase Microsorb™ MV 100 C18, 5 μm diameter particle size, Varian Analytical Instruments, Woburn, MA). Fecal extracts were recovered by a mobile phase gradient of 20% increasing to 80% MeOH in water over 80 min (1 ml/mm) at room temperature. A portion of each fraction (0.1 ml) was assayed for radioactivity to determine the retention times for the radiolabeled standards (E_1 and E_1S). The remainder of the fractions (0.9 ml) was allowed to evaporate until dry and reconstituted in 0.25 ml PBS. These samples were then assayed with an estradiol (E_2) EIA and an estrone conjugate (E_1C) EIA (French et al., 1996) to evaluate the immunoreactivity of the fractions.

Based on the results of these two assays (see Section 3), the E_2 EIA was chosen for use here, and all fecal samples were assayed for estrogens using a modified protocol previously described (Nunes et al., 2000). We used an E_2 antibody (R4972; Clinical Endocrinology Laboratory, University of California, Davis); other compounds cross-reactive with this E_2 antibody were estrone (3.3%), progesterone (0.8%), testosterone (1.0%), and androstenedione (1.0%). Absorbance was measured with a Dynex plate reader when optical density in B_0 wells reached 1.0. Precision of the assay was monitored by measuring two sets of hyena fecal extract pools. The intra-assay coefficients of variation for high and low pools were 3.66% and 3.30%, respectively. The interassay coefficients of variation were 11.96% and 14.71%, respectively ($n = 25$ plates). The 871 fecal samples were assayed over the course of two years. The correlation between 13 randomly chosen samples assayed repeatedly in each year was $r = 0.98$, demonstrating low inter-year variation.

Assay parallelism was tested by measuring fecal estrogen concentrations in serial dilutions of samples from three different individuals. The slopes of the resulting displacement curves were compared to that of the standard curve using a test of slopes available in the program Prism (GraphPad Software Inc.).

2.4. Immunoassay of plasma samples

Plasma samples collected from free-ranging hyenas in the Talek clan were assayed for estradiol in duplicate using coated tube I^{125} radioimmunoassay (RIA) kits (Diagnostic Products Corp., Los Angeles, CA). Kit validation for use with *Crocuta* was accomplished by demonstrating parallelism between curves representing serial dilutions of pooled hyena samples, spiked samples, and standard curve calibrators included with the kit. The minimum detection limit for this assay was 7.4 pg/ml. The antibody is highly specific for estradiol and has minor cross-reactivity with other compounds: estriol 0.32%, estrone 10%, estrone-3-sulfate 0.58%, estradiol monosulfate 0.29%, estradiol propionate 0.70%, and DHEA, androstenedione, 5α-dihydrotestosterone, and testosterone, all at less than 0.001%. The mean intra-assay coefficient of variation was 7.85% and interassay coefficient of variation was 12.14% ($n = 23$ assays).

2.5. Statistical analysis

Data were log-transformed before analysis if not normally distributed. Procedural covariates (sample collection time and hyena identity) were analyzed with a step-wise regression allowing for each predictor variable to be measured while controlling for reproductive state. Significant predictors were then further investigated using within subjects analyses. Differences among reproductive states (immature, pregnant, and lactating) were evaluated using one-way analysis of variance (ANOVA). Along with test statistics, means and standard errors are presented. For further investigation of significant differences, two-tailed *t*-tests were used except when testing specific directional hypotheses based on expected differences in hormone concentrations between reproductive states, as when comparing reproductively immature and mature females, or pregnant and lactating females; in these cases one-tailed tests were used. To examine differences among trimesters of pregnancy, a post hoc Tukey's analysis was performed for both plasma and fecal samples. When log-transformed data

were not normally distributed, or sample size was very small (as in the case of the LHRH challenges), non-parametric tests were used. Significant differences were identified using $\alpha = .05$. All analyses were performed using SPSS for Windows (Version 12.0.0, SPSS Inc.).

3. Results

3.1. High-performance liquid chromatography

Immunoreactive E_2 was detected in fecal extracts at levels three to fourfold higher than immunoreactive E_1C in samples from both pregnant and non-pregnant females. We observed differences in immunoreactivity between samples for pregnant and non-pregnant females of roughly an order of magnitude (Fig. 1a and b). The highest concentrations of immunoreactive estrogen metabolites co-eluted with the [^{3}H]estrone standard (fractions 60–65), accounting for more than 74% of total immunoreactive estrogens. Also, a smaller peak in E_2 immunoreactivity was present in fractions 65–70, accounting for 14% of the total immunoreactivity. Thus, the E_2 antibody appears to detect multiple estrogen metabolites in the feces of female spotted hyenas, and may therefore be used as a measure of total fecal estrogens (fE).

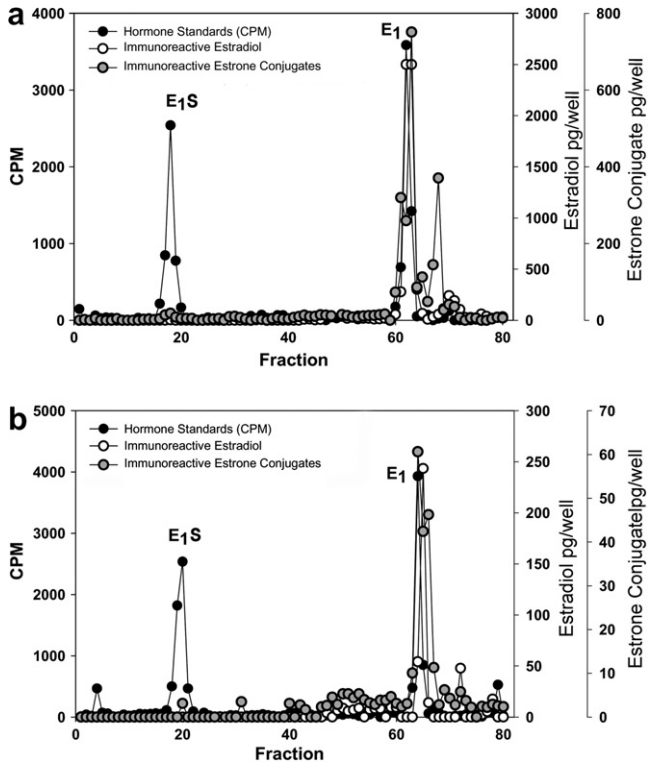

Fig. 1. HPLC results from (a) pregnant and (b) non-pregnant female hyenas. Fractions were assayed for immunoreactivity with both estradiol (open circles) and estrone conjugate (gray circles) enzyme-immunoassays, and immunoreactivity is expressed on the right-hand axes. Elution of radiolabeled standards (black circles), estrone (E_1) and estrone sulfate (E_1S), are expressed as counts-per-minute (CPM) per fraction on the left-hand axis.

3.2. Fecal EIA parallelism

Serial dilutions of three hyena samples were plotted along with standards as relative dose *vs.* percent bound. Fecal extract curves did not differ significantly from the standard displacement curve within the range of 20–80% antibody binding ($F_{3,8} = 2.06$, $P = 0.18$; Fig. 2). Of our assayed fecal samples ($n = 871$), 83% fell within these binding parameters, and samples falling outside this range were diluted accordingly and re-analyzed.

3.3. LHRH challenge experiments

Estradiol was measured in repeated plasma samples drawn from nine free-living females challenged with LHRH administration during late lactation, and from three saline-treated control females. For each challenged individual, the sample with the highest concentration of E_2 prior to LHRH administration (pre-injection peak, 17.76 ± 2.44 pg/ml) was compared to its sample with the highest E_2 concentration after LHRH administration (post-injection peak, 21.71 ± 2.21 pg/ml). Peak E_2 concentrations were significantly higher after than before LHRH administration (paired $t_8 = -6.19$, $P < 0.001$; Table 1). By contrast, the three females injected with saline demonstrated no rise in plasma E_2 (Wilcoxon matched pairs test: $z_3 = -5.35$, $P = 0.59$). Thus, treatment with LHRH was followed by a significant rise in circulating E_2 in lactating female spotted hyenas.

Five captive hyenas (Table 1) were also challenged with LHRH. For each individual, the sample with the highest fE prior to LHRH administration (484.18 ± 364.09 ng/g) was compared to peak fE concentrations after LHRH administration (1437.26 ± 773.33 ng/g). Peak fE concentrations were significantly higher after than before LHRH administration (Wilcoxon matched pairs test: $z_5 = 2.02$, $P = 0.04$). Mean latency to peak fE post-challenge was 2.0 ± 0.31 days. Thus, as with plasma E_2, LHRH administration was followed by a measurable increase in fE, but with an average time lag of two days.

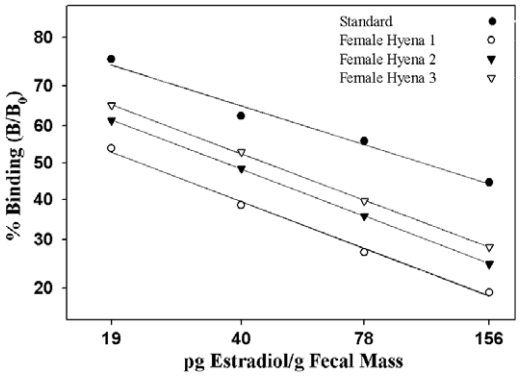

Fig. 2. Relative dose *vs.* percent of antibody bound for standards and serial dilutions of samples from three hyenas. Slopes of all curves are parallel in this range (20–80% binding; $P = 0.18$).

Table 1
Individual and mean (±standard errors) response patterns to LHRH challenge

Hyena ID	Pre-injection peak	Post-injection peak	% Pre-injection peak	Minutes to maximum
Free-living hyena plasma estradiol (pg/ml)				
BM	7.8	14.23	182.40	58
COCH	26.3	29.51	109.70	24
DJ95	15.4	21.66	140.65	64
JAB	22.7	28.52	125.64	114
PT	18.4	19.27	108.42	67
SX	18.5	22.59	122.11	19
UA94	13.4	15.35	114.55	109
UA96	8.5	13.7	161.18	60
WHO	28.2	30.58	108.44	19
Mean	17.76 ± 2.44	21.71 ± 2.21	129.93 ± 8.87	59.33 ± 11.81
Hyena ID	Pre-injection peak	Post-injection peak	% Pre-injection peak	Days to maximum
Captive hyena fecal estrogen (ng/g feces)				
Female 35	129.16	174.99	135.49	2
Female 49	1935.52	3480.34	179.81	2
Male 13	226.69	3175.10	1400.66	2
Male 45	84.06	191.82	228.18	3
Male 51	45.49	164.07	360.64	1
Mean	484.18 ± 364.09	1437.26 ± 773.30	460.96 ± 237.94	2.0 ± 0.31

3.4. Comparison of plasma and fecal estrogen concentrations

3.4.1. Procedural covariates

To evaluate procedural covariates associated with our sampling technique and to avoid pseudoreplication, a step-wise regression was used to determine how much variation in fE concentrations could be explained by collection time (morning, 06:00–09:00, or evening, 17:00–20:00) and individual identity, after reproductive condition had been controlled. The full model yielded an $R^2 = 0.07$, $F_{3,536} = 14.09$, $P < 0.001$. Reproductive state (immature, pregnant, and lactating) explained a significant portion of the variance in fE ($P < 0.001$). Collection time was a significant predictor over and above the variance explained by reproductive state ($P = 0.009$), whereas hyena identity was not ($P = 0.22$). The effect of collection time was further examined by pairing morning and evening samples for 35 individuals. Concentrations of fE were significantly higher in morning than evening samples ($t_{34} = 4.19$, $P < 0.001$). Therefore all subsequent analyses utilized only morning samples.

3.4.2. Age

To examine how fE varies with age between 11 and 35 months, we assigned females that had not yet given birth to a specific 4-month age interval, and calculated the percent of these females whose feces contained more than 100 ng/g fE within each interval (Fig. 3). The threshold of 100 ng/g was chosen because it exceeded average fE concentrations from pre-reproductive females. Concentrations of fE were averaged for females sampled multiple times within a given age interval. These data suggest that fE begins to rise at approximately 23 months of age in free-ranging females.

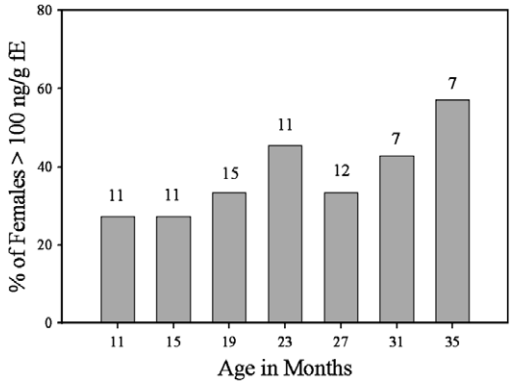

Fig. 3. Percentage of nulliparous females sampled in each 4-month age interval whose mean fecal estrogen concentrations exceeded 100 ng/g feces. Numbers over bars represent females sampled in each age group. Multiple samples collected from individual females within age interval were averaged.

3.4.3. Reproductive state

During immobilizations conducted in the wild from 1990 to 2004, we collected 136 blood samples from 89 females in known reproductive condition. Plasma E_2 concentrations were averaged for females sampled multiple times when they were in a single reproductive state. A one-way between subjects ANOVA indicated a significant effect of reproductive state (immature, pregnant, or lactating) on plasma E_2 ($F_{2,118} = 138.88$, $P < 0.001$). Plasma E_2 concentrations were low in immature females (5.29 ± 0.42 pg/ml) and lactating females (6.44 ± 0.77 pg/ml), and highest in pregnant females (137.79 ± 36.88 pg/ml). We next looked within individuals by conducting paired *t*-tests on samples from females that were sampled multiple times when they were in different reproductive states. Plasma E_2 concentrations were significantly higher

in females sampled after than before reproductive maturity ($t_{23} = -3.53$, $P < 0.001$; Fig. 4a), and concentrations were also higher during pregnancy than lactation ($t_7 = 4.67$, $P < 0.001$; Fig. 4b). Within pregnancy, plasma E_2 increased across trimesters ($F_{2,2} = 15.53$, $P < 0.001$) (Fig. 5). A post hoc Tukey's test (HSD = 0.20) showed that plasma E_2 concentrations were significantly higher in late pregnancy than during either the early or middle trimesters.

Variation in fE among reproductive states resembled that seen in plasma E_2. From 1993 to 2005, 295 morning fecal samples were collected from 56 females. A one-way between subjects ANOVA indicated a significant effect of reproductive state on fE ($F_{2,294} = 56.57$, $P < 0.001$). Fecal estrogen concentrations were lowest in immature females (150.27 ± 32.95 ng/g), higher in lactating females (368.91 ± 37.25 ng/g), and highest in pregnant females (1235.73 ± 229.24 ng/g). This result was confirmed by paired *t*-tests within females sampled in multiple reproductive states. Females had significantly higher fE concentrations after than before reaching reproductive maturity ($t_{21} = -6.49$, $P < 0.001$; Fig. 4a), and fE concentrations within females were higher during pregnancy than lactation ($t_{19} = 3.23$, $P = 0.002$; Fig. 4b). Finally, fE rose during the course of gestation ($F_{2,75} = 3.22$, $P = 0.045$; Fig. 5). A post hoc Tukey's test (HSD = 362.13) comparing trimesters revealed that late trimester fE concentrations were significantly higher than early fE concentrations, but middle trimester fE concentrations were not significantly different from either early or late trimester concentrations.

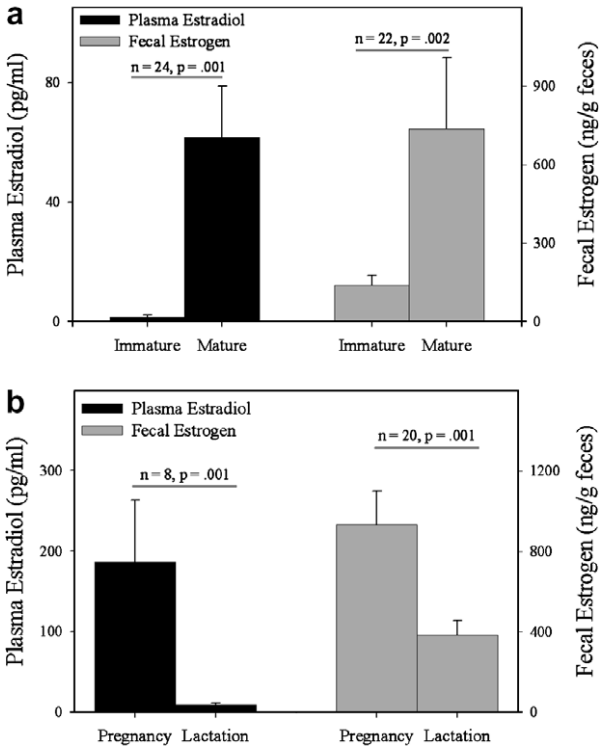

Fig. 4. Within-female comparisons between (a) reproductively immature and mature states, and (b) pregnant and lactating states for plasma estradiol (black) and fecal estrogens (gray). Means and standard errors are presented, and significance was tested using paired *t*-tests. Note that the scales represented on the two *y*-axes differ between sampling techniques.

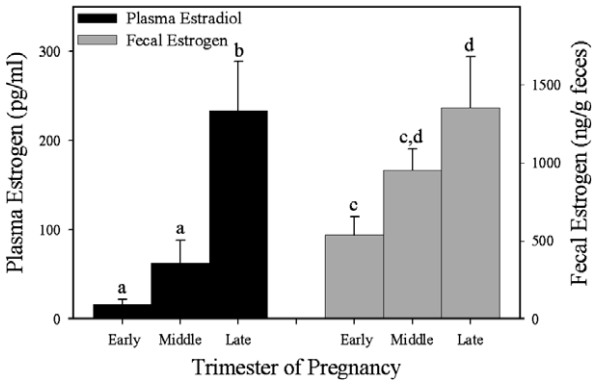

Fig. 5. Estrogen concentrations in plasma (black) and fecal material (gray) across trimesters of pregnancy, averaged across females. Significant differences among mean (±SEM) plasma estradiol and fecal estrogen concentrations are indicated by different letters, and were tested using Tukey's post hoc test. Note that the scales represented on the two *y*-axes differ between sampling techniques.

4. Discussion

The aim of this study was to validate a non-invasive technique for measuring estrogens in spotted hyenas. Our results show that biologically significant variation in fecal concentrations of estrogen metabolites can be assessed using the E_2 EIA in both wild and captive hyenas. Furthermore, estrogen concentrations in feces show the same patterns of variation as those in plasma. Fecal hormone analysis offers means for collecting samples repeatedly and frequently from individuals without disturbance of normal behavior, and should therefore help future researchers to answer a number of questions about the developmental and reproductive biology of this species.

For the EIA developed here, we chose an antibody specific for E_2 because immunoreactivity of fractions from female hyenas was three to fourfold higher when assayed with the E_2 antibody than with an antibody specific for E_1C. Use of the E_1C antibody in the immunoassay would have rendered a large proportion of our samples below detectable limits, whereas all of our samples run with the E_2 EIA yielded measurable fE. Although E_2 was not run as a radiolabeled standard in our HPLC analysis, HPLC results from several felids; the taxonomic group closest to the hyenids (Bininda-Emonds et al., 1999) from which we have HPLC data from feces, reveal that elution of E_2 often overlaps with that of E_1 (Brown et al., 1994), the largest immunoreactive peak in fecal samples from both pregnant and non-pregnant hyenas seen here. Additionally, radiolabeled estrogens in the domestic cat are predominantly excreted as unconjugated E_2 and E_1 (Brown et al., 1994; Shille et al., 1984), and we presume because of their close taxonomic relationship to hyenas, these particular estrogens may also be important immunoreactive metabolites

in hyena feces. All of these factors suggest that an E_2-specific antibody represents an acceptable choice for measuring estrogen in hyena feces. With the understanding that other estrogens, and perhaps also novel metabolites, may be cross-reacting with this antibody in *Crocuta* samples, our assay permits measurement of total fecal estrogens, rather than fecal estradiol *per se*.

We assessed parallelism by comparing the slopes of curves generated by several diluted samples to the slope of the curve generated by the E_2 standards. Although these slopes were parallel only when antibody binding was between 20% and 80%, this assay should nevertheless be informative as long as this limitation is considered. The large majority of our samples fell within this range, and samples outside of this range could be assayed after appropriate dilution.

Circadian rhythms in fecal and urinary steroid hormones have been documented in many mammals (Bos et al., 1993; Sousa and Ziegler, 1998), so we examined time of day here as a procedural covariate. We found that, like fecal glucocorticoids (Dloniak, 2004), but unlike fecal androgens (Dloniak et al., 2003), concentrations of fecal estrogens in *Crocuta* samples collected in the morning are higher than in samples collected in the evening. This source of variation, once identified, was easily controlled by restricting analysis to morning samples.

The biological tests we conducted *in vivo* further validated our assay. After excluding evening fecal samples from our analyses, the same variation seen in plasma estradiol among age classes and reproductive states was observed in fE values. As in plasma, fE concentrations remained low until reproductive maturity, were highest during the final trimester of gestation, and were relatively low during lactation. Furthermore, although this requires further testing, our fE data offer a preliminary indication that estrogen concentrations in wild spotted hyenas began to rise late in the second year of life, as is also true in captive females, based on measurements of plasma estrogen (Glickman et al., 1992).

Our data suggest that, like plasma estrogens, fE concentrations rise in response to LHRH challenge, but not after saline injection. A previous study had shown that plasma LH, testosterone, and androstenedione concentrations in both males and females rise in response to LHRH administration (Place et al., 2002), but estrogen was not among the steroid hormones measured in that work. In the brown hyena (*Parahyaena brunnea*), a species closely related to the spotted hyena, non-invasively measured estrogens similarly appeared to rise after LHRH challenge in females (Ensley et al., 1982). Here captive hyenas of both sexes showed significant increases in fE concentrations after LHRH challenge, despite having highly variable baseline values. The variable responses observed in the two parous females may have been a consequence of differing stages of the ovulatory cycle at the time of the LHRH challenge. Endogenous hormone levels and ovarian status can greatly affect responses of the HPG axis to LHRH (Edwards et al., 1963; Hamilton and Armstrong, 1991; Jeffcoate, 1992), and Place et al. (2002) showed that the response of plasma LH to LHRH challenge was more variable in female than male *Crocuta*. Here, the three captive males, whose average baseline fE concentrations were lower than those in females, had post-injection fE concentrations roughly equal to those in the females. Estrogens in male mammals are produced in small amounts by the testes, and may also be aromatized from circulating testosterone in various other peripheral tissues (Nitta et al., 1993). However, the source of estrogens is not known for male spotted hyenas.

Use of the assay developed here for analysis of fecal estrogen should contribute to our understanding of reproduction and genital development in the female spotted hyena. There has been a keen interest in the endocrinology of this species and its ties to the unique characteristics of female dominance and genital masculinization; however, conventional methods of blood sampling for hormone analysis cannot be repeated with the frequency needed to explore these issues. Glickman et al. (unpublished data) have obtained preliminary data suggesting that estrogens may be involved in formation and development of the phallus in this species. Furthermore, we currently know virtually nothing about estrous cyclicity in any hyena species. By allowing for repetitive sampling from individuals in a non-invasive manner, it is our hope that availability of an assay for fecal estrogens will help answer these and many other questions about these fascinating animals.

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