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# Non-invasive monitoring of fecal androgens in spotted hyenas (*Crocuta crocuta*)

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**Abstract**

Spotted hyenas (*Crocuta crocuta*) exhibit an array of behavioral and morphological characteristics that set them apart from other mammals: females are heavier and more aggressive than males, and females have external genitalia that closely resemble those of the male. Because androgenic hormones might mediate the expression of these traits, androgens are of great interest in this species. Past work on circulating androgens in wild hyenas has been limited, in part because of small sample sizes. In this study we validated a non-invasive method of monitoring variation in androgens by measuring total androgen metabolites in the feces of wild and captive spotted hyenas with an enzyme immunoassay. HPLC analysis revealed multiple immunoreactive androgen metabolites in fecal extracts from both males and females. LHRH challenge in three male and two female hyenas in captivity caused an increase in fecal androgens one to three days after LHRH injection. Furthermore, presence of bone in the diet did not affect fecal androgen concentrations in captive female hyenas. In wild spotted hyenas, time of day of fecal deposition, time elapsed between deposition and freezing of the sample, and time elapsed between freezing and extraction did not systematically affect fecal androgen concentrations. Finally, in wild hyenas, fecal androgen patterns mirrored plasma testosterone patterns in that adult immigrant males had higher concentrations than adult natal males, and pregnant females had higher concentrations than lactating females. These methods can therefore be used in future studies addressing relationships among fecal androgens, social status, reproductive state, and behavior in spotted hyenas.


*Keywords:* Fecal androgen metabolites; Spotted hyena; *Crocuta crocuta*; LHRH challenge; Non-invasive

## 1. Introduction

During fetal development, female spotted hyenas (*Crocuta crocuta*) are exposed to unusually high levels of androgens, and females are heavily masculinized in various aspects of their morphology and behavior (Glickman et al., 1987, 1992; Lindeque and Skinner, 1982; Licht et al., 1992). The external genitalia of females closely resemble those of males in that the clitoris is elongated to form a fully erectile pseudopenis, and the vaginal labia are fused to form a pseudoscrotum (Frank et al., 1990; Matthews, 1939; Neaves et al., 1980). Although adult females may weigh more than adult males, the sexes are monomorphic with respect to other adult body size measurements (Hamilton et al., 1986; Kruuk, 1972; Matthews, 1939; Van Horn et al., 2003). Females are socially dominant to adult breeding males (Kruuk, 1972; Smale et al., 1993, 1997), and females are more aggressive than adult males (Frank, 1986; Hamilton et al., 1986; Monaghan and Glickman, 1992; Szykman et al., in press).

Spotted hyenas live in social groups called clans. Adult male and female clan members have separate stable linear dominance hierarchies (Frank, 1986; Holekamp and Smale, 1990; Kruuk, 1972; Tilson and Hamilton, 1984), and both male and female offspring

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"inherit" their mother's social rank (Holekamp and Smale, 1991; Smale et al., 1993). Most males eventually disperse from their natal clans between the ages of 24 and 76 months (East and Hofer, 2001; Frank, 1986; Henschel and Skinner, 1987; Smale et al., 1997; Van Horn et al., 2003) and attempt to immigrate into new clans in search of mating opportunities (Engh et al., 2002; Holekamp and Smale, 1998). In males of many animal species, androgens vary according to social rank, aggressive behavior or dispersal status (e.g., rhesus monkeys: Rose et al., 1971, 1972; red deer: Lincoln et al., 1972; elephants: Jainudeen et al., 1972; primates: Bernstein et al., 1974; ground squirrels: Holekamp et al., 1984; vervet monkeys: Steklis et al., 1985; rabbits: Farabollini, 1987; birds: Wingfield et al., 1987; olive baboons: Alberts et al., 1992; macaques: Zumpe and Michael, 1996; cattle: Lunstra et al., 1978; spotted hyenas: Holekamp and Smale, 1998). Availability of a method for assessing relationships among androgens, behavior, and social rank in free-living spotted hyenas would therefore allow us to address a wide array of intriguing questions.

Matthews (1939) first proposed that the behavioral and morphological masculinization of the female spotted hyena might be associated with androgens, but this question was not addressed systematically until 40 years later. Although considerable headway has been made in hormone studies utilizing plasma samples from captive hyenas (see Drea et al., 1998, 2002; Glickman et al., 1998, 1992; Licht et al., 1992, 1998; Place et al., 2002; Yalcinkaya et al., 1993), the study of circulating androgen levels in free-ranging spotted hyenas has yielded conflicting results (Goymann et al., 2001a). For example, Racey and Skinner (1979) found that mean plasma testosterone (T) levels did not differ between the sexes, whereas Frank et al. (1985) concluded that adult males had significantly higher levels of serum T than adult females, and suggested that variation in T within each sex is related to social status. The most recent study addressing plasma androgen levels in wild spotted hyenas indicated that adult females had lower plasma T and dihydrotestosterone (DHT) levels than adult males, but androstenedione (A4) levels did not differ between the sexes (Goymann et al., 2001a).

A review of the existing literature on sex differences in circulating androgens in spotted hyenas (Goymann et al., 2001a) identified some of the variables that may contribute to differences among results from previous studies, including different blood collection procedures, various extraction and assay methods, and lack of knowledge about the reproductive or social status of individuals. Furthermore, the problem of small sample size has plagued all studies involving measures of circulating androgens in free-ranging spotted hyenas. Drawing blood from large carnivores in the wild involves immobilizing each sampled animal, and this invasive procedure represents a large effort that yields limited sample sizes. Multiple samples from the same individual are difficult to achieve by darting, as it can be stressful to the hyena (van Jaarsveld and Skinner, 1992) and conditions are often unsuitable for obtaining repeated measures from specific individuals. Fecal steroid hormone analysis offers an appealing alternative to blood sampling in order to answer questions about hormone–behavior relationships in the spotted hyena. This technique has recently been used to measure fecal androgens in an array of wild mammals (for example; African wild dogs: Creel et al., 1997; sifakas: Brockmann et al., 1998; muriquis: Strier et al., 1999; ringtailed lemurs: Cavigelli and Pereira, 2000; mongoose lemurs: Curtis et al., 2000; hairy-nosed wombats: Hamilton et al., 2000; meerkats: Moss et al., 2001; Japanese macaques: Barrett et al., 2002). Multiple fecal samples can be collected from an individual non-invasively, without disrupting normal behavior. Within a well-studied population, each sample can be placed in the context of the individual's known age, reproductive status, social rank, and observed behavior. In contrast to plasma samples, large numbers of fecal samples can often be collected from animals assigned to particular reproductive and social rank categories, yielding sample sizes appropriate for analyses of the relationships between these variables and androgens. Although Goymann et al. (2001b, 2003) have recently examined the relationship between ecological and social variables and glucocorticoid hormones excreted in hyena feces, no methods have been described to measure excreted androgens in this species.

Our aim here was to validate a fecal androgen enzymeimmunoassay (EIA) for use in spotted hyenas. After demonstrating assay parallelism and confirmation of immunoreactive androgen metabolites by High performance liquid chromatography (HPLC), we assessed the biological validity of our androgen EIA. We first injected captive hyenas with LHRH, expecting to see a post-injection increase in fecal androgens. We also determined whether variation in diet might affect fecal androgen levels by systematically manipulating the proportion of the diet derived from bone in captive hyenas, and then measuring excreted androgens in samples collected after feeding. Using archived frozen fecal samples collected from wild hyenas over the last decade, we inquired whether time of day of sample deposition, time elapsed before freezing the sample, or time elapsed between freezing and assay had any systematic effects on fecal androgen concentrations. Finally, we compared patterns of excreted and circulating androgens collected from particular groups of wild hyenas (immigrant vs. natal adult males, pregnant vs. lactating females) among which we expected to observe differences in plasma androgens.

## 2. Materials and methods

### 2.1. Captive study site, subject animals, and sample collection

All captive hyenas were housed at the University of California Berkeley Field Station for Behavioral Research. These individuals were of known age and reproductive status. Some hyenas were housed individually and others were housed in small groups (for more details see Berger et al., 1992). To identify feces produced by group-housed hyenas, their food was treated with food coloring. All fecal samples were collected between 0800 and 1200. These were immediately mixed thoroughly and stored in individual containers at −80 °C until extraction and assay.

LHRH challenges were conducted on five gonadally intact adult hyenas (three males and two females). Fecal samples were collected daily for seven days prior to treatment to establish baseline levels of fecal androgen excretion. On the day of LHRH challenge, animals were immobilized with ketamine and xylazine administered by blow dart, and anesthetized with isoflorane inhalant. Each hyena then received a single i.v. injection of gonadotropin releasing hormone (1 mg/kg LHRH, L-7134, Sigma Chemical Co., St. Louis, MO). Each hyena was allowed to recover from anesthesia and released back to its home enclosure. Fecal samples were collected on the day of challenge as well as for eight days after the challenge.

Although wild hyenas ingest highly variable amounts of meat and bone as they feed on ungulate prey, captive hyenas in this colony are normally fed a standard zoo carnivore mix (Nebraska Brand Feline Food, Central Nebraska Packing, North Platte, NE) and small amounts of bone every day (Berger et al., 1992). In order to assess whether variation in the amount of bone in the diet influences measurement of fecal androgens, we varied the bone content in the diet of five adult females. Two of these females were ovariectomized for another experiment. At the start of this experiment some individuals were fed only feline diet while others were fed a small amount of feline diet plus three or four sheep neck bones. After one to three days on their respective diets, diets were reversed in all subjects, and reversed again another one to three days later. Fecal samples were collected each day from all subjects. For statistical analysis, a given fecal sample represented the previous day's diet.

### 2.2. Field study site, subject animals, and sample collection

Our field study site was the Talek area of the Masai Mara National Reserve in southwest Kenya. The subject population was one large, stable *Crocuta* clan inhabiting a home range of approximately 65 $km^2$ (Boydston et al., 2001). The Talek hyenas have been monitored intensively since June 1988, and all hyenas in the clan were identifiable based on each individual's unique spot pattern and other distinguishing marks. Sex was determined by the dimorphic glans morphology of the erect phallus (Frank et al., 1990). Ages of individuals born in the Talek clan were estimated to within seven days based on pelage, size, appearance, and behavior of cubs when first observed. Adult natal males were Talek-born males between 24 and 60 months of age that had not yet dispersed from the Talek home range. Immigrant males were adult males that had dispersed from natal clans elsewhere and had been present in the Talek clan for at least six months. Ages of immigrant males were estimated based on toothwear (Van Horn et al., 2003). Date of first appearance in the Talek clan was recorded for each immigrant male, and tenure was calculated as time elapsed since joining the clan (Holekamp and Smale, 1998). Female reproductive state was determined by behavioral observations or by assessment during immobilization. A female was pregnant if she gave birth to cubs within 110 days after sampling (Matthews, 1939), or if fetuses were observed in her uterine horns during immobilization, using a Hitachi portable ultrasound machine. A female was lactating if she was observed to nurse cubs around the time of sampling and/or milk could be expressed from teats when she was immobilized. Critical incident sampling (Altmann, 1974) of all observed aggressive and appeasement behaviors was used to determine social ranks of individuals. Social ranks were assigned based on a matrix of outcomes of dyadic agonistic interactions (Martin and Bateson, 1988), as described previously (Smale et al., 1993).

Fecal samples were collected either during early morning (0530–0900 h) or evening (1700–2000 h) observation periods. Samples were collected whenever a hyena defecated, upon direct and unambiguous observation. Samples were first collected into plastic bags at the site of defecation, and later approximately 6 ml of mixed sample were transferred to multiple 2 ml cryovials for freezing in liquid nitrogen. Ninety-four percent of the samples were frozen within 12 h of collection, and all samples were frozen within 48 h of collection. Samples were stored in liquid nitrogen until shipped on dry ice to the United States, where they were stored at −20 °C or colder until extraction and assay.

Between 1990 and 2002, 33 adult immigrant males, 13 adult natal males, 16 pregnant females, and 33 lactating females in the Talek clan were anesthetized with Telazol (2.5 mg/kg body mass) administered in a lightweight plastic dart fired from a $CO_2$ rifle. All immobilizations took place between 0630 and 0900 hours, when hyenas were found resting. Within 10–17 min of anesthetic injection, we drew a blood sample from each hyena's jugular vein into a heparinized vacutainer tube, and then

proceeded to take an array of body and dental measurements, as described elsewhere (Van Horn et al., 2003). Hyenas typically recovered from anesthesia within 60 min. All immobilizations were performed in accordance with Kenyan law and with NIH animal treatment guidelines. Blood was centrifuged at 1000*g* for 5 min, then plasma was drawn off and stored in liquid nitrogen until it was shipped on dry ice to the United States, where it was stored at −80 °C until radioimmunoassay.

### 2.3. Radioimmunoassay of plasma testosterone in wild hyenas

Duplicate aliquots of plasma from each sample from each wild hyena were assayed for total testosterone (T) using coated tube $I^{125}$ kits from Diagnostic Products Corporation (Los Angeles, CA), as described by Holekamp and Smale (1998). The T kit was previously validated for use with this species by demonstrating parallelism between serial dilutions of plain and T-spiked plasma and the standard curve generated using kit calibrators. The detection limit of the assay was 0.04 ng/ml. Cross-reactivity of the T anti-serum with 5-alpha-dihydrotestosterone was 3.3%, and was less than 0.1% with any other androgen. The mean coefficient of variation between T assays ($N = 11$) was 7.1%. The mean intra-assay coefficient of variation for high and low T control tubes run with each T assay was 6.8%.

### 2.4. High performance liquid chromatography of fecal samples

Fecal samples from six adult male and six pregnant adult female wild spotted hyenas were selected for two pools (male and female, respectively) to be subjected to HPLC for determination of androgen metabolites. Samples were collected between 1995 and 2001, and stored frozen until extraction. Each animal contributed approximately 0.5 g of feces to its sex-specific pool, and steroids were extracted with ethanol (see below). Samples were spiked with ~7000 cpm of $^3$H-testosterone, $^3$H-androstenedione, and $^3$H-dihydrotestosterone and air-dried. Samples were reconstituted in 500 μl of phosphate buffered saline (PBS; pH 5.0), sonicated, and vortexed to remove any particulates sticking to the surface of the glass tube. Fecal metabolites were first recovered by: (1) priming a Spice C18 sample preparation cartridge (Analtech, Newark, DE) with 3 ml of methanol followed by 3 ml of $dH_2O$; (2) loading the 500 μl PBS sample onto the cartridge; (3) pushing 5 ml of $dH_2O$ through the cartridge; and (4) pushing 5 ml of methanol through the cartridge, and collecting this portion into a 12 × 75 mm glass tube. The methanol portion was dried under air and the residue was resuspended in 300 μl of methanol. HPLC was conducted by injecting 50 μl of the reconstituted sample onto the column (Reverse Phase Microsorb MV 100 C18, 5 μm diameter particle size, Varian Analytical Instruments, Woburn, MA). A mobile phase of 45% acetonitrile in water over 80 min at room temperature was used at a rate of 1 ml/min. For determination of retention times of $^3$H reference standards, aliquots (100 μl) from each fraction were counted for radioactivity. The remainder of the fraction was dried down and resuspended in 250 μl PBS and analyzed by EIA to evaluate androgen immunoreactivity (see below).

### 2.5. Extraction and assay of androgens from fecal samples

Approximately 0.5 g wet weight of each fecal sample were placed frozen in an open whirl-pak bag and lyophilized overnight (Labconco Freeze-Dry System 10-269). After lyophilization, each sample was ground to a fine powder with a mortar and pestle. In a 16 × 125 mm culture tube, 0.2 g of powdered feces were mixed with 5 ml absolute ethanol. Tubes were capped and vortexed, and then placed on an orbital shaker for 12–14 h. The samples were again vortexed to resuspend particulate matter from the side of the tube, placed in a block heater, and gently boiled for 20 min. The remaining suspension was centrifuged for 15 min at 1000*g* to pellet the solid fecal material. The ethanol supernatant was poured into a clean 12 × 75 mm culture tube and evaporated to dryness under compressed air in a warm-water bath. Samples were reconstituted in 1.0 ml PBS and stored frozen at −20 °C until assay.

Fecal androgens were assayed using a modified version of a previously described testosterone EIA (Nunes et al., 2000). The assay utilized a testosterone antibody (R156/7) and a testosterone conjugate (horseradish peroxidase; HRP) kindly provided by Dr. Bill Lasley and Coralie Munro of the University of California, Davis. When used with plasma, this antibody cross-reacts 57.4% with dihydrotestosterone, <0.3% with androstenedione, and <0.1% with androsterone, dihydroepiandrosterone, β-estradiol, and progesterone. Stock antibody was diluted 1:15,000 in bicarbonate coating buffer and T-HRP was diluted 1:15,000 in PBS. Testosterone standards (Sigma) ranged from 7.8–1000 pg per well in halving dilutions. Wells of microtiter plates (Nunc Maxisorp) were coated with 50 μl antibody, and diluted fecal sample extract (1:50 in PBS) and 50 μl T-HRP were incubated in duplicate wells for 2 h. Unbound hormone was removed by washing the plates, and 100 μl of substrate (ABTS-$H_2O_2$) were then added to each well. Plates were read on a Dynex plate reader when optical density in $B_0$ wells reached 1.0. Serial dilutions of pooled extracts from multiple males and females produced displacement curves that were parallel to the displacement curve produced by T standards.

Quality control in the assay was monitored by measuring T concentrations in two sets of pools on each plate. One pool was diluted to yield a high concentration of T in 50 µl (~30% binding) and the other to yield a low concentration of T (~70% binding). The inter-assay coefficients of variation were 7.8% (high pool) and 4.5% (low pool). The intra-assay coefficient of variation, based on these same pools, averaged $9.58 \pm 3.9\%$ ($n = 35$ assays).

### 2.6. Archived sample collection, processing and storage conditions

Conditions and time in storage can affect assessments of steroid hormone concentrations in fecal samples (Khan et al., 2002; Terio et al., 2002), and circadian variation has also been reported for some excreted steroids (Sousa and Ziegler, 1998). We therefore inquired whether time of collection or variation in processing and storage conditions systematically affected fecal androgen levels in archived samples from wild hyenas. We extracted and assayed 539 samples from 39 adult females and 302 samples from 32 adult immigrant males, collected between 1993 and 2001 in the Talek clan, for which time of day of sample and time elapsed between defecation and freezing had been recorded. We tested for effects of these variables, as well as time elapsed between freezing and assay, on fecal androgen levels measured in male and female hyenas separately.

### 2.7. Comparison of fecal and plasma androgens

A strong biological validation of a fecal hormone assay includes demonstration of differences in fecal hormone concentrations among groups that also vary in circulating hormone concentrations. In order to do this in spotted hyenas, we analyzed archived frozen fecal samples collected from the Talek clan between January 1993 and June 2001 that could be compared with plasma samples acquired during immobilizations. Fecal samples from 16 adult natal males, 25 immigrant males, 25 pregnant females, and 22 lactating females were accessed in order to compare patterns in fecal androgens with patterns in plasma testosterone among similar groups of immobilized hyenas. A single fecal sample for each hyena was selected for assay. Plasma T may vary with immigrant male tenure (Holekamp and Smale, 1998; Goymann, 2000), so we assigned each immigrant a tenure for the day on which his sample was collected. Tenure of males whose samples were used in the immigrant male group showed a random distribution between 6 and 36 months, so samples were not biased toward males with long or short tenure. Age estimates for immigrant males at time of fecal sampling ranged between 32 and 98 months.

### 2.8. Statistical analysis

All statistical treatment of data followed Zar (1996). Prior to statistical analysis, all data sets were tested for departures from normality and homoscedasticity. When such departures were detected, we employed the non-parametric equivalents of the appropriate parametric statistical tests. Analyses were considered significant when $P < 0.05$.

## 3. Results

### 3.1. HPLC analysis

HPLC fractions assayed with the testosterone EIA revealed the presence of six immunoreactive fecal metabolites in males and seven metabolites in females (Fig. 1). Three of the major peaks in both sexes were clearly associated with the elution of T, A4, and DHT, respectively. Highly polar fecal metabolites were noted in fractions 10–25, with two distinct peaks within these fractions for both males and females. One apolar peak

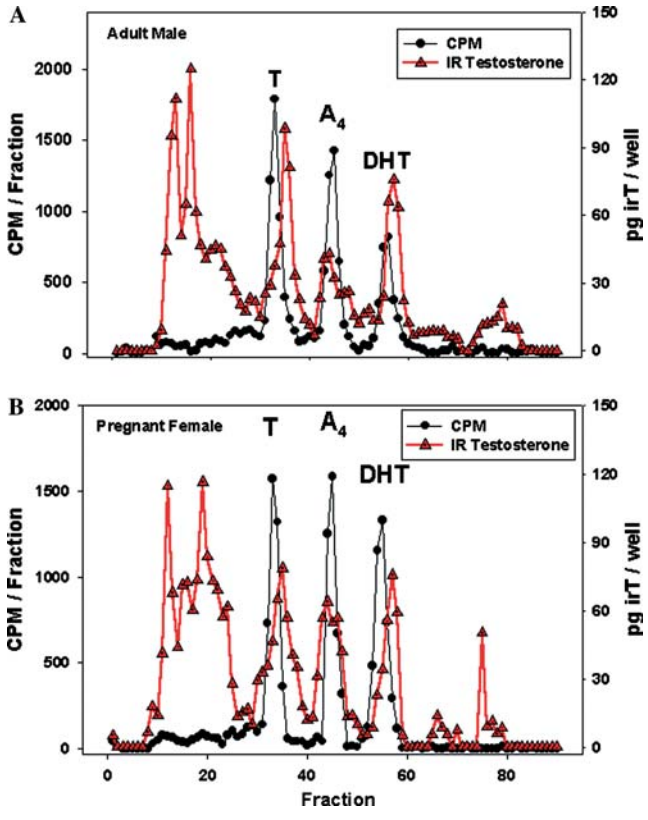


Fig. 1. HPLC profiles of immunoreactive androgen metabolites in separate male (A) and pregnant female (B) fecal extract pools. Elution of $^3$H-labeled testosterone [T], androstenedione [$A_4$], and dihydrotestosterone [DHT] expressed as counts-per-minute per fraction, and immunoreactive androgens measured by EIA expressed as pg irT per well.

was detected in males (fractions 72–83) and two apolar peaks in females (fractions 65–71 and 75–79), but these accounted for only a small proportion of the total immunoreactivity. Overall, patterns of immunoreactive androgen metabolites were strikingly similar in the fecal pools from males and females, although A4 and DHT were present at higher levels in females, relative to the T peak, than they were in males. Thus, our EIA has a broad ability to detect multiple androgen metabolites excreted in the feces of both male and female hyenas.

### 3.2. LHRH challenges

All five captive hyenas treated with LHRH responded physiologically to the challenge with an increase in fecal androgens from average baseline levels (Fig. 2, Table 1). The latency to peak androgen varied from one to three days post-LHRH, and averaged 2.4 days. Peak fecal androgen levels after challenge were significantly higher than average levels prior to challenge, but levels six to eight days after challenge were no longer different from initial baseline levels (Repeated measures ANOVA followed by Tukey test for multiple comparisons, $F = 7.32$, $p = 0.016$).

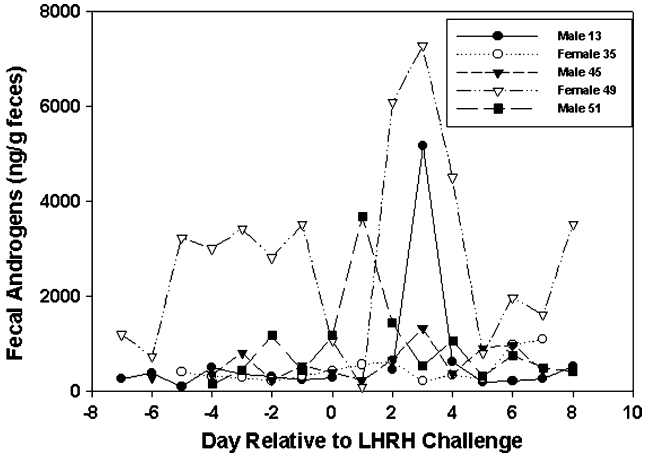


Fig. 2. Changes in fecal androgen excretion in response to LHRH challenge in captive spotted hyenas. LHRH was administered on day 0.

### 3.3. Effect of bone in diet

The presence or absence of bone in the diet did not affect mean androgen concentrations in feces collected the next day from adult female hyenas in captivity. No differences in fecal androgens were found when hyenas switched from a meat plus bone diet to one consisting of only meat (meat plus bone = 625.74 ± 381.02 ng/g, meat = 614.41 ± 191.10 ng/g; Wilcoxon paired sample test: $Z = 0.944$, $p = 0.35$) or the reverse (meat = 839.96 ± 186.12 ng/g, meat plus bone = 1218.10 ± 529.58 ng/g; Wilcoxon paired sample test: $Z = 0.404$, $p = 0.68$).

### 3.4. Archived sample collection, processing and storage conditions

Variation in sample collection time, processing methods, and storage conditions had no systematic influence on measured concentrations of fecal androgens. Although there was considerable variation in hormone concentrations, there were no pronounced effects of time of day of deposition on androgen levels. Mean levels in morning samples did not differ from evening samples in adult immigrant male hyenas, whether levels were analyzed within ($t$ tests; $t$'s<2.30, $p$'s>0.06) or across individuals (am = 652.64 ± 193.88 ng/g, pm = 509.62 ± 165.38 ng/g; paired sample $t$-test; $t = 0.552$, $p = 0.592$). Although two adult females had significantly different morning and evening mean values, they differed in opposing directions, and there was no overall difference in morning and evening levels within individual females (all other t tests; $t$'s<3.64, $p$'s>0.06) or across all females (am = 1325.336 ± 136.311 ng/g, pm = 1125.739 ± 125.392 ng/g; paired sample $t$-test; $t = 1.456$, $p = 0.161$). Likewise, the latency to process and freeze samples was not associated with significant variation in excreted androgen. The number of minutes elapsed between sample collection and freezing was not correlated with fecal androgen levels in either females ($R_p = -0.057$; $F = 1.742$; $p = 0.187$) or males ($R_p = -0.082$; $F = 1.998$; $p = 0.158$). Finally, long-term storage of samples while frozen was not associated with systematic changes in fecal androgen. The number of days frozen until extraction and assay (ranging from 46 to 2673 days) was

Table 1
Individual and mean response patterns to LHRH challenge in five captive spotted hyenas

| Hyena ID | Baseline androgen | Maximum androgen | % Change | Days to maximum |
|---|---|---|---|---|
| Male 13 | 298.46 ± 48.84 | 5167.0 | 1733.89 | 3 |
| Female 35 | 325.71 ± 37.45 | 629.35 | 193.32 | 2 |
| Male 45 | 431.61 ± 84.45 | 1331.67 | 308.82 | 3 |
| Female 49 | 2371.88 ± 414.03 | 7281.50 | 307.09 | 3 |
| Male 51 | 673.01 ± 209.24 | 3671.11 | 545.47 | 1 |
| All | 820.14 ± 393.52 | 3616.13 ± 1224.39 | 440.92 | 2.4 ± 0.89 |

Androgen values indicate mean ± s.e.m. and are expressed as ng androgen per g lyophilized feces.

not correlated with fecal androgen levels in either males ($R_p = 0.024$; $F = 0.169$; $p = 0.681$) or females ($R_p = 0.092$; $F = 0.824$; $p = 0.115$).

### 3.5. Comparisons of patterns in fecal androgens with patterns in plasma T

Differences in fecal androgens mirrored those in plasma T in wild males and females. Mean plasma T and fecal androgens both varied with dispersal status in adult males (Fig. 3A). Adult immigrant males had higher plasma T levels than adult natal males (Mann Whitney $U$ test: $U = 97.5$, $p = 0.0043$), as well as higher fecal androgen concentrations (Mann Whitney $U$ test: $U = 125$, $p = 0.045$). Mean fecal androgens and plasma T also varied with reproductive status in adult females (Fig. 3B). Pregnant females had higher fecal androgen concentrations (Mann Whitney $U$ test: $U = 104$, $p = 0$), and higher plasma T values than did lactating females (Mann Whitney $U$ test: $U = 58.5$, $p = 0$). In addition, we tested whether either category of adult males differed from pregnant females in either plasma T or fecal androgen concentrations. Immigrant males had significantly higher concentrations of both plasma T and fecal androgens than both adult natal males and pregnant females, which did not differ from each other (ANOVAs followed by Tukey tests: plasma T, $F = 12.54$, $p = 0$; fecal androgens, $F = 10.99$, $p = 0$).

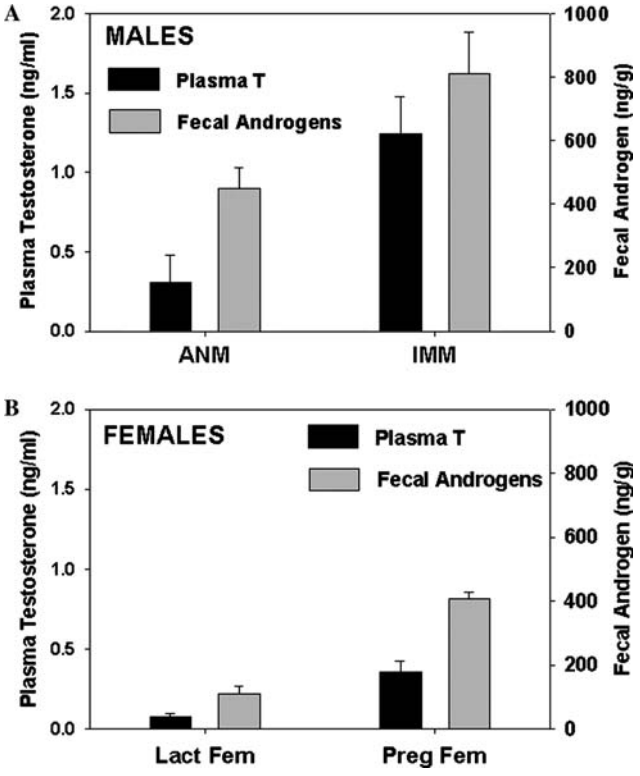


Fig. 3. Relationship between plasma and fecal hormone concentrations in male (A) and female (B) wild hyenas. ANM = Adult natal males; IMM = Immigrant adult males; Lact Fem = Lactating, non-pregnant adult females; Preg Fem = Pregnant adult females.

## 4. Discussion

The goal of this study was to determine the extent to which we can reliably measure androgens in the feces of spotted hyenas and make available a non-invasive measurement tool to investigate relationships between androgens and behavior in this species. Our results show that multiple fecal androgen metabolites can be reliably measured by use of our EIA in both male and female spotted hyenas. The assay measures variation in fecal androgen concentrations that is biologically significant, and we have also shown similar relationships in plasma and feces for both males and females. In addition, frozen archived samples can be analyzed without concern regarding variation in the amount of bone in the diet, the time of defecation, or the length of time the sample has been frozen.

We subjected male and female fecal sample pools to HPLC analysis to determine which androgen metabolites show immunoreactivity in our T assay system. The HPLC analysis revealed that both male and female spotted hyenas excrete multiple immunoreactive androgen metabolites, with markedly similar patterns for the two sexes. Three of the large peaks of immunoreactivity corresponded to the elution times for testosterone, androstenedione, and dihydrotestosterone. A broad peak of immunoreactivity was detected in early fractions, where more polar steroids are eluted. Similar broad, early peaks in metabolite excretion have been noted in studies of androgen excretion in marmosets (Möhle et al., 2002) and maned wolves (Velloso et al., 1998), and in estrogen and progestin excretion in felids (Brown et al., 1994). These peaks likely represent conjugated androgens (glucuronide and/or sulfate conjugates) that nonetheless cross-react with the T antibody (Möhle et al., 2002; Brown et al., 1996; J.L. Brown, pers. comm.). Our assay thus permits estimation of multiple androgen metabolites in spotted hyena feces.

Steroid hormone conversion and metabolism, excretion dynamics in the gut, variation in uptake of hormones by target tissues, binding proteins, and even defecation rate all undoubtedly influence the makeup of the pool of androgen metabolites in fecal samples. Although the exact makeup of the metabolite pool is highly variable, the other carnivore species sampled to date excrete multiple androgen metabolites, some of which remain unidentified (domestic cats: Brown et al., 1996; wild dogs: Monfort et al., 1997; cheetahs: Brown et al., 1998; maned wolves; Velloso et al., 1998; red wolves: Walker et al., 2002). In these species, the number of androgen metabolites that show immunoreactivity in a particular T or androgen assay is also quite variable.

In the case of the spotted hyena, we have shown that all of the detected fecal androgen metabolites show immunoreactivity in our T assay, in both males and females.

LHRH is a potent stimulator of the hypothalamic-pituitary-gonadal (HPG) axis, therefore the first step of the biological validation of our assay was to administer LHRH challenges to five captive adult spotted hyenas. As predicted, LHRH treatment led to substantial increases in excreted fecal androgen in the three males and two females tested in this study. There was an average 5-fold increase in excreted androgens across individuals, ranging from 2- to 17-fold. The timing of peak androgen excretion was somewhat variable in that three hyenas responded maximally after three days, one responded after two days, and one responded the day after injection with LHRH. Goymann et al. (1999) found similar variation in peak glucocorticoid excretion in feces of captive spotted hyenas after ACTH injection. While the average latency to peak excretion of fecal glucocorticoids after injection was 24 h, some hyenas exhibited peak excretion rates more than 48 h after injection. Variation in the efficiency of i.v. infusion, variable thresholds in sensitivity to LHRH, variation in dietary intake or fecal output during the course of the experiment, and individual differences in steroid hormone metabolism and excretion might all factor into the variation in the peak response to LHRH challenge. Regardless, the predicted response to LHRH challenge was seen in all five hyenas, showing that our EIA is capable of measuring physiologically induced elevations in androgen activity within individuals. In addition, we now know that changes in circulating levels of androgen are typically expressed in fecal samples one to three days later, which is important for future studies investigating relationships between behavior and fecal androgen levels in this species.

Dietary variation may influence concentrations of excreted steroid hormones. For example, an increase in dietary fiber has a negative effect on progestogen excretion in adult female baboons (Wasser et al., 1993). While fiber is not necessarily of concern in carnivores, bone constitutes a variable proportion of the spotted hyena diet. Social rank affects nutritional status in female hyenas by means of priority of access to food (Holekamp et al., 1996), and higher ranking hyenas may be ingesting less bone than lower ranking hyenas. Finding variation in fecal androgens with the presence or absence of bone could force us to treat high and low ranking hyenas differently. Therefore, we tested whether the presence of bone had an effect on fecal androgen concentrations in captive females. Our results indicate no obvious effect of bone, therefore allowing us to treat high and low ranking hyenas similarly in future studies.

Slight variations in collection and storage conditions could also affect the ability to extract and detect androgen in hyena fecal samples. Hot weather and humidity could potentiate bacterial degradation of the hormone product. In the field, reluctance to disturb our study animals, behavioral sampling protocols, the presence of other large mammals such as lions, and the distance back to basecamp can make it difficult to quickly collect, process, and freeze samples. In addition, for this project, samples were collected opportunistically in both mornings and evenings. Other species exhibit diurnal variation in excreted hormone levels (e.g., marmosets: Sousa and Ziegler, 1998). However, we found no effect of variation in collection and storage conditions, and our data revealed no significant differences in fecal androgen concentrations in morning and evening samples, suggesting little circadian variation in the excretion of this class of steroids.

Steroid concentrations in fecal samples may change with long-term storage. For example, concentrations of fecal glucocorticoids in baboon feces stored in ethanol at either room temperature or −20 °C for 90–120 days differed from concentrations in the same samples measured after 180 days of storage (Khan et al., 2002). However, our data suggest that long-term frozen storage without the use of ethanol does not systematically affect androgen concentrations in spotted hyena feces. These findings are significant, because they suggest that minor variations in sample collection and processing, even delays of up to 48 h between deposition and freezing, do not affect fecal androgen concentrations systematically in this species. Furthermore, the ability to measure meaningful levels of androgenic steroids in samples that have been frozen for long periods of time allows us to retrospectively examine hormone–behavior-life-history relationships by accessing samples collected during the long-term study of the Talek hyenas that has endured for more than a decade.

In most mammals, males have significantly higher circulating T than females. Thus, a good test of the validity of a particular androgen assay is often the ability to differentiate males and females on the basis of excreted androgens. However, as described earlier, the demonstration of consistent sex differences in plasma androgens in free-ranging spotted hyenas has been problematic. In the LHRH challenges presented here, one female (#49) had higher baseline androgen than the three males (and had the highest post-LHRH levels), but LHRH-induced elevations in excreted androgens reduced the differences between this female and the three males. Inspection of the plasma T and A4 levels of this female, which were measured as part of another study (Place et al., 2002), showed that she had circulating T levels higher than the other female, but well below the levels of the males. However, this female had very high circulating A4 levels at the beginning of the experiment, and her plasma A4 levels 120 min after the LHRH challenge were five times higher than levels in the males.

Thus it appears that the relative concentrations of each of the androgen metabolites shown in Fig. 1 may vary among the endocrine responses of individual hyenas challenged with LHRH.

The results from our comparisons of plasma T and fecal androgens in wild hyenas provide valuable insight towards answering questions concerning sex differences in androgens in this species. Previous studies have shown that plasma T increases in captive females during pregnancy (Licht et al., 1992; Glickman et al., 1992). In one study of wild hyenas, no differences in plasma T were noted between pregnant and lactating females (Goymann et al., 2001a), but that sample included only four pregnant females. Here, using large numbers of both fecal and plasma samples from adult females in various reproductive states, we found that wild pregnant females do indeed show higher levels of both circulating T and excreted androgens than do lactating females. Also of significance is the fact that pregnant females had similar levels of androgens to those of adult natal males, but not to those of immigrant males. Lactating females had the lowest androgen levels among all groups of adults. This convincingly shows that it is important to account for female reproductive state and male dispersal status when comparing androgenic hormones in male and female hyenas.

In conclusion, we are confident that fecal androgen analysis will be a useful tool in future research with wild spotted hyenas. Our results indicate that we can measure biologically meaningful variation in fecal androgen concentrations among groups of hyenas known to vary with respect to concentrations of plasma androgens. Future studies can now focus on investigating the unusual relationships between sex, social status, reproductive state, aggression, sexual behavior and levels of excreted androgens in this species.

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