

Monitoring testicular activity of male Eurasian (*Lynx lynx*) and Iberian (*Lynx pardinus*) lynx by fecal testosterone metabolite measurement

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Abstract

The aim of the present study was to identify relevant fecal testosterone metabolites in the Eurasian lynx (*Lynx lynx*) using HPLC analysis and to evaluate the specificity of two testosterone immunoassays against these fecal metabolites. Finally, fecal hormone analysis was used to characterize seasonal reproductive activity of captive male Eurasian and Iberian (*Lynx pardinus*) lynx. Fecal samples from a male Eurasian lynx who received an i.v. injection of [³H]testosterone were subjected to HPLC analysis. All HPLC fractions were analyzed for radioactivity and androgen content by two testosterone immune assays (EIA and Testosterone-Immulite[®] kits, DPC Biermann, Germany). Furthermore, fecal samples from four Eurasian lynx males ($n = 174$) and three Iberian lynx ($n = 52$) were collected throughout the year and fecal testosterone metabolites were determined with Testosterone-Immulite[®] assay. HPLC separation of radiolabeled Eurasian lynx fecal extract indicated that the majority of testosterone metabolites are substances with a higher polarity than testosterone. Only minor proportion of radioactivity co-eluted with authentic testosterone and dihydrotestosterone. Enzymatic hydrolysis and solvolysis of the fecal extract were insufficient to liberate testosterone. After solvolysis relatively more activity was eluated the position of DHT, but the majority of metabolites remained unaffected. The EIA measured substantial amount of immunoreactivity, which corresponded with two radioactive peaks. Additionally, both immunoassays recognized two metabolites, which were only minor components according to their radioactivity. The Immulite assay was able to recognize a metabolite at the position of dihydrotestosterone. HPLC separation of Iberian lynx feces extracts revealed a similar metabolite pattern determined by EIA that were typical for Eurasian lynx fecal extracts. Simultaneous analyses of fecal samples with both testosterone assays provided comparative results for both lynx species (Eurasian lynx, $r^2 = 0.488$; $p < 0.001$; Iberian lynx, $r^2 = 0.85$, $p < 0.0001$). Thus, seasonal reproductive activity of male Eurasian lynx was demonstrated also by Immulite[®]-assay, confirming high testosterone levels during breeding season in March/April as previously documented with EIA. Preliminary results on testosterone measurements in Iberian lynx feces confirmed the suitability of the applied Immulite[®] test in this highly endangered species.

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1. Introduction

The genus *Lynx* includes four species: the Eurasian lynx (*Lynx lynx*), the Canada lynx (*Lynx canadensis*), the Bobcat (*Lynx rufus*) and the probably most endangered

felid species of the world, listed on CITES Appendix 1, the Iberian lynx (*Lynx pardinus*). Lynx is distributed over the Northern Hemisphere in Eurasia, Asia and America (Nowell and Jackson, 1996). In Europe, the recent distribution of Eurasian lynx stretches from the Northern part of Scandinavia up to the Southern boundary in Turkey and from the European region of Russia in the East to two isolated subpopulations in the French Pyrenees (Breitenmoser, 1991). In some parts of its range the lynx is

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very rare (Henriksen et al., 2005). In central Europe including Switzerland and Germany, lynx does not exist in sustainable populations, despite strong efforts to reintroduce them (Breitenmoser and Breitenmoser-Würsten, 1990).

The Iberian lynx, found only in Spain and Portugal, has declined from 100,000 at the beginning of the 20th century to about 100–120 individuals at present. Dam building, illegal hunting, accidental killing by snares and poison baits set for other animals, road kill and decimated populations of the European rabbit, the lynx's main prey, have led to the cat's downfall. According to a recent study carried out by World Wildlife Fund and the conservation organization SOS Lynx, there are only about 21–25 breeding females in two isolated populations in southern Spain (Ward, 2005). In the past there had been some disagreement between experts and organizations about the importance and direction of captive breeding of Iberian lynx. However, there is now broad agreement that a scientifically managed captive population is an essential part of Iberian lynx conservation programs.

All four lynx species have some general features in common, which are typical for the socioecology of most *Felidae*. Lynx live solitarily (Heptner and Sludskii, 1972; Breitenmoser et al., 1993). Most data gained on lynx reproduction are based on skinned carcasses collected from trappers (Parker and Smith, 1983; Kvam, 1991). The short breeding season of the Eurasian lynx lasts from January through to early April, in dependence on latitude (Naidenko and Erofeeva, 2004). Parturition takes place after 70 days of gestation (Naidenko and Erofeeva, 2004) during late May and early June (Kvam, 1991; Henriksen et al., 2005). A recent investigation on the Iberian lynx in a sub-population of the Donana National Park revealed that nearly all known births (89%) took place in March (Palomares et al., 2005). These data support that male Iberian lynx are strong seasonal breeders. Due to their highly endangered status, investigations of testicular dynamics and sperm production as described for the Eurasian lynx (Goeritz et al., 2006) cannot be studied in the captive Iberian lynx population. In the 2005 season only three males and five females were available for the captive breeding program (Vargas, 2006). Therefore, the non-invasive monitoring of hormonal testicular activity in feces will provide necessary information on male reproductive seasonality, which is important for future assisted breeding and gamete collection attempts in the Iberian lynx.

The aim of the present paper was: (1) to identify relevant testosterone metabolites in Eurasian lynx feces by radiometabolism study and (2) to evaluate the specificity of two testosterone immune assays, EIA and Immulite, against androgen metabolites separated by HPLC. Both immune assays were used (3) to characterize seasonal endocrine testicular activity of captive male Eurasian lynx living in central Russia. Our final aim was to prove whether the non-invasive technique established in the Eurasian lynx can be used to monitor seasonal testicular hormone activity in the Iberian species.

2. Materials and methods

2.1. Animals and experimental design

Four males and 10 females Eurasian lynx were housed at the scientific field station "Tchernogolovka" of the A.N. Severtzov Institute, situated 50 km north-east from Moscow (56°00' northern latitude, 38°22' eastern longitude). Average annual temperature varied from +3.5 to +4.3 °C, average temperature in July +19 °C, January –11 °C. The animals were kept within six enclosures (74 m²) and in one large fenced enclosure (7500 m²), that is part of the natural mixed forest providing a semi-natural environment. Each enclosure had an additional 1–4 cages (6–8 m² each), where some of the individuals were kept. The food diet consists of 1 kg of chicken meat daily with rare occasional additions (rats, rabbits). One day per week animals fasted. Animals were housed separately; males and females were combined just for the mating (Naidenko and Erofeeva, 2004). The animals reproduced every year with mating in March. Fecal samples were collected monthly throughout a two-year period from individual animals and stored at –20 °C within 1 h after defecation until analyses. From February to April (prospective mating season) the frequency of collection was increased to 1–2 times a week.

Fecal samples from three captive male Iberian lynx collected during one week in October 2004, January 2005, April 2005 and July 2005, respectively, were provided from the Iberian Lynx Captive Breeding Center (ILCBC, Spain). This center coordinates Iberian lynx captive breeding program at the El Acebuche Captive Breeding Center in Doñana's National Park in Southern Spain. At the start of 2005 breeding season, all captive Iberian lynx with reproductive potential (two males, three females) were located at this Breeding Center. The animals were kept in separate enclosures (1200 m²).

2.2. Processing fecal samples

All fecal samples were processed as described before (Kretschmar et al., 2004). In brief, wet fecal samples (0.5 g) were extracted for 30 min by shaking with 4.5 ml of 90% methanol. After centrifugation (15 min at 1200g) the supernatant was transferred into a new tube and diluted 1:1 with water. Aliquots of the fecal extracts were subjected either to HPLC analysis, or directly to the in-house testosterone EIA and the Testosterone-Immulite® assay.

2.3. Radiometabolism study

The radiometabolism study was performed in November 2005. A solution (0.25 ml) containing ~250 µCi [³H]testosterone (70–105 Ci/mmol, TRK921, Amersham Bioscience, UK) in ethanol was used. Sterile 0.9% NaCl solution (2.25 ml) was added to the radiolabeled solution and the total volume was injected into the cephalic vein of a 15-year-old male Eurasian lynx. Prior to injection, the animal was sedated by an i.m. injection with 3 ml of a mixture of Rometar (2% solution of xylazine hydrochloride) and ketamine hydrochloride (ratio 3:1). On the first day the animal was housed in a metabolic cage and released into its enclosure thereafter. Following radiolabeled injection, all excreted fecal samples ($n=5$) were collected separately for 4 days in plastic bags from the cage and the floor of the enclosure immediately after defecation and stored at –20 °C. Aliquots of each sample were extracted for testosterone determination and radioactivity counting. The second sample, collected on day 2 after injection, contained the highest amount of radioactivity (89%) and was used for HPLC analyses.

To prove whether testosterone or its metabolites were conjugated to glucuronides or sulfates, the fecal extract was also subjected to enzyme hydrolysis and solvolysis (see below) before HPLC separation. All radioactive counting was conducted in a Packard TRI-CARB 1900 TR liquid scintillation counter (Canberra-Packard GmbH, Germany).

2.4. Hydrolysis and solvolysis

For hydrolysis, 100 µl of fecal extract was dried down, dissolved and hydrolyzed in 1 ml 0.05 M acetate buffer (pH 4.8) containing 4 µl β-glucuronidase.

ronidase and sulfatase from *Helix pomatia* (Boehringer, Mannheim, Germany) for 2 h at 37 °C. Steroids were then extracted twice with 2 ml *tert*-methylbutylether/petroleum ether (30:70, v/v), the extracts were combined, dried down at 50 °C, and reconstituted in 0.25 ml of 40% methanol. The solvolysis was performed according to Ziegler et al. (1996). Dried extract (100 µl) was dissolved in 500 µl of ethanol, then 100 µl of saturated NaCl and 50 µl of 1.4% trifluor-acidic acid were added, followed by 4 ml of ethyl acetate. The sample was vortexed and incubated overnight at 40 °C in a water bath. After adding 4 ml of ethyl acetate, the samples were agitated for 5 min and centrifuged (15 min at 1200g) to separate the aqueous and organic phase. The ethyl acetate phase (extract) was collected and neutralized by washing with 2.5 ml distilled water. After evaporation, the extract was resuspended in 40% methanol and stored at –20 °C until HPLC analyses.

2.5. HPLC

For separation and characterization of androgen metabolites, 50 µl of fecal extracts were loaded on a reverse-phase Ultrasep ES100/RP–18/6 µm HPLC column (4 × 150 mm, Sepserv, Berlin). A linear gradient was generated at a flow rate of 1 ml/min: 0–11 min, 60–75% methanol; 11–20 min, 75%; 20–25 min, 75–100% methanol. Fractions of 0.33 ml were collected at 20 s intervals over a period of 21 min and diluted with 1 volume of water, before 20 µl of the fractions were transferred into the assay systems. The elution positions of authentic testosterone and dihydrotestosterone (Sigma Chemie GmbH, Deisenhofen, Germany) on this column had been previously determined in separate HPLC runs.

2.6. Determination of fecal testosterone metabolites with enzyme immunoassay (EIA)

Fecal samples were analyzed for testosterone using in-house microtitre plate enzyme immunoassay procedures as described earlier (Kretzschmar et al., 2004). The antibody (provided by Prof. Meyer, Weihenstephan, Germany) was raised in rabbits immunized against 17 α -OH-testosterone-HS-RSA. The cross-reactivities of the antibody to different androgens were as follows: 4-androsten-17 β -ol-3-one (testosterone)—100%, 5 α -androstan-17 β -ol-3-one (dihydrotestosterone)—13.6%, 5 α -Androst-2-en-17 β -ol—1.5%, and <0.1% for 5 α -androstan-3 β -ol-17-one (epi-androsterone), 5 α -androstan-3 α -ol-17-one (androsterone), 5 α -Androst-2-en-17-one and 4-androsten-17 β -ol-3-one sulfate (testosterone sulfate). Testosterone-3-CMO-peroxidase was used as enzyme conjugate.

Serial dilutions of a fecal pool from Eurasian lynx gave parallelism to the standard testosterone with no differences in slopes ($p > 0.05$). Inter- and intra-assay coefficients of variation for two biological samples were 12.3% ($n = 11$) and 9.0% ($n = 8$), respectively.

2.7. Determination of fecal testosterone metabolites with Immulite-Testosterone assay

Fecal testosterone was measured with an automated chemiluminometric high sensitivity testosterone assay (Immulite, Diagnostic Products Corporation, Los Angeles, CA) using an Immulite[®] automated analyser (DPC Biermann, Germany). The Immulite 2000 is an automated, random-access immune assay analyzer with a solid-phase washing process and a chemiluminescence detection system. The solid phase is made up of a polystyrene bead enclosed within the Immulite test unit that is coated with a polyclonal rabbit antibody specific for testosterone (T). The fecal extract and an alkaline phosphatase-conjugated T reagent are simultaneously introduced into the test unit. During a 60-min incubation period at 37 °C with intermittent shaking, the T in the sample competes with the enzymelabeled T for a limited number of antibody binding sites on the bead. Unbound enzyme conjugate is then removed by a patented five-spin-wash technique. The chemiluminescence substrate, a phosphate ester of adamantyl dioxetane, is added and the test unit incubated for 10 min. The substrate is hydrolyzed by the alkaline phosphatase to an unstable anion. The decomposition of the anion yields a sustained emission of light. A single determi-

nation uses 25 µl of sample and the dynamic range of the Immulite-Testosterone assay is 0.14–15.86 ng/ml (0.5–55 nmol/liter). According to the manufacturer the functional sensitivity for the T assay on this system is 0.49 ng/ml (1.7 nmol/liter) and the average interassay coefficient of variation is 13.7% at a concentration of 4.27 ng/ml (14.8 nmol/liter). A test for parallelism was performed for both, feces from Eurasian and Iberian lynx. Serial dilutions of fecal pools ($n = 6$, each) resulted in similar testosterone concentrations confirming the parallelism of the assay. According to the manufacturer the cross-reactivity of the antibody to different androgens was as follows: testosterone—100%, 5 α -androstan-17 β -ol-3-one (dihydrotestosterone)—2.4%, androstenedione—0.8%, methyltestosterone—0.8%, 5 α -androstan-3 β , 17 β -diol—0.4%. The recovery of testosterone, which was added to fecal samples containing very low amount of endogenous testosterone metabolites, was $86 \pm 11\%$ for Eurasian (addition of 0.1 µg and 5 µg T to 1 g feces; $n = 6$) and $85 \pm 20\%$ for Iberian lynx feces (addition of 0.1 µg and 1 µg T per g feces; $n = 4$).

2.8. Analysis of data

All hormone measurements were duplicates. The results were expressed as immunoactive testosterone metabolites in µg/g of fecal wet weight.

For each male, means \pm standard error (SEM) were calculated for each time period and differences were assessed by multiple ANOVA. Samples analyzed by both test systems, EIA and Immulite, were tested for linear correlation (Pearson r). Calculated p values <0.05 were considered to be significantly different. The statistical procedures were performed with the software program InStat Version 3 (Graphpad Software Inc.).

3. Results

3.1. HPLC analysis

HPLC profiles of radiolabeled testosterone metabolites in Eurasian lynx feces in combination with data on elution positions of immune reactivity determined in both immunoassays are shown in Fig. 1A and B. The extract of male lynx feces is composed of several radiolabeled metabolites. The majority was detected in fractions 14–18, 21–23, 29–32, 45–48 (double peak) and 61–62. Two minor radioactive peaks co-eluted with testosterone and dihydrotestosterone at fraction 36 and 45, respectively. The broad-shouldered peak at fractions 14–18 indicates a cluster of polar metabolites. Hydrolysis (not shown) and solvolysis of the samples did not change the elution pattern of the polar metabolites, only after acid treatment (solvolysis) the double peak at in fraction 45–48 increased relatively from 14% to 26% of eluted radioactivity on the expense of the polar fractions (Fig. 1A). All further analyses of fecal samples were carried out on untreated extracts.

Both immune assays detected the testosterone immunoactivity (Fig. 1B) consisting of two major peaks (fractions 24–25 and 28–29), which were not identical with distinct radiometabolite peaks, apparently covered by non-immunoactive metabolites. Two additional immune reactive peaks co-eluting with major peaks of radioactivity (fractions 17–19 and 21–22) were detected only with the EIA. None of the two assays showed substantial immune reactivity associated with the elution position of testosterone. The Immulite system, however, found a significant amount of immune reactivity

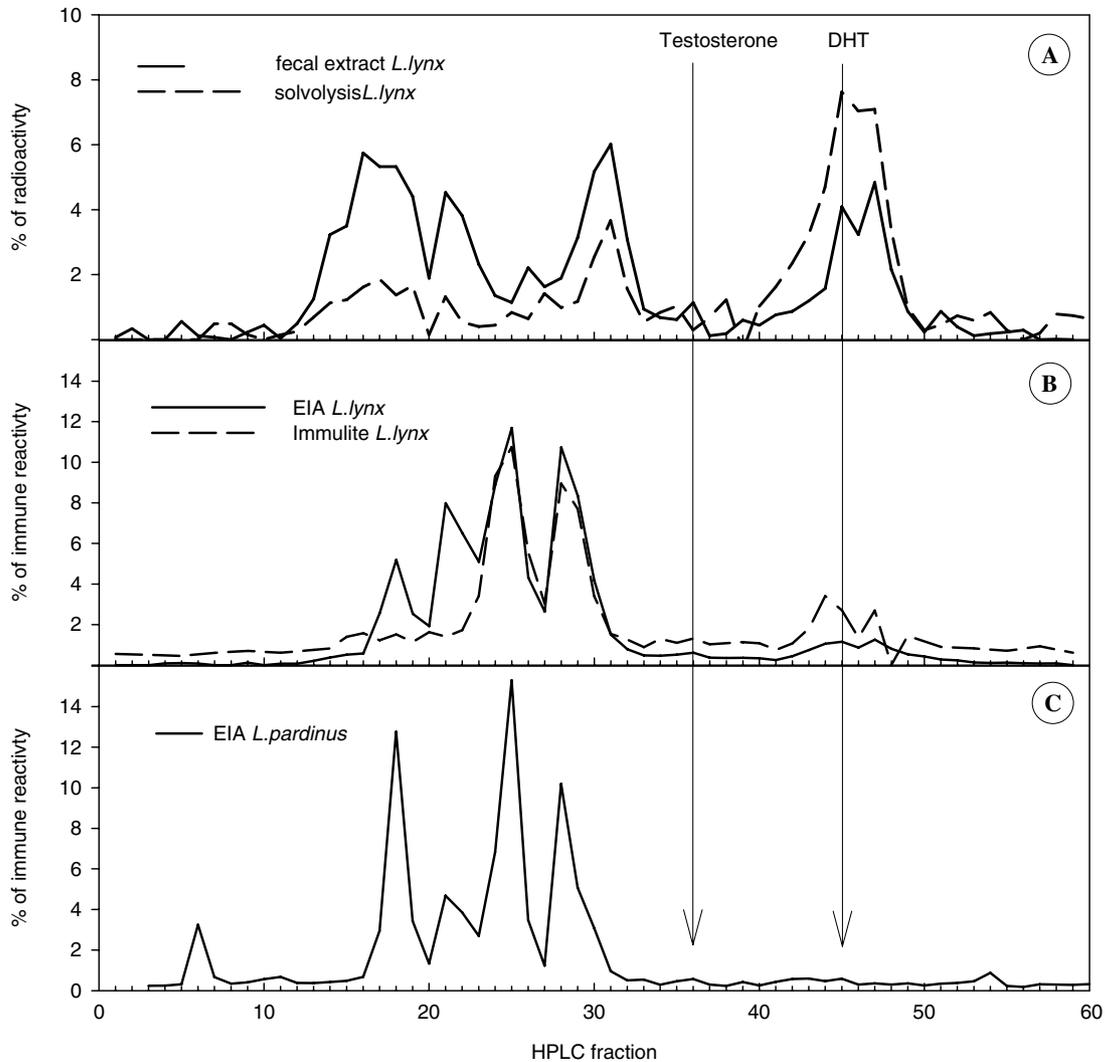


Fig. 1. (A) Testosterone radiometabolite study in male Eurasian lynx. HPLC separation of a fecal extract was performed with a linear gradient of methanol–water. Black line shows the radioactivity in each fraction (% of overall measured dpm). Dashed line shows the radioactivity in the fractions of the same extract after solvolysis. (B) Testosterone immunoactivity within the HPLC fractions of radiolabeled feces extract determined with EIA (black line) and Immulite (dashed line) assays. The immunoactivity is presented in % of overall eluted testosterone activity. (C) HPLC fraction of an Iberian lynx feces extract. Testosterone immunoactivity was determined with EIA. The immunoactivity is presented in % of overall eluted testosterone activity. Arrows indicate the elution positions of testosterone (T) and dihydrotestosterone (DHT).

associated with the position of dihydrotestosterone, and the respective peak of radioactivity.

We also investigated immunoactive testosterone metabolites in a fecal samples from Iberian lynx (Fig. 1C). The HPLC fractions were analyzed only with the EIA. Four major peaks at the same elution positions as in Eurasian lynx were obtained (fractions 17–19, 21–22, 24–25, 28–29).

3.2. Comparative measurements of EIA and Immulite

To assess the suitability of the Immulite-Testosterone assay for monitoring androgen secretion in feces of Eurasian and Iberian lynx, samples obtained from three male Eurasian ($n=174$) and three male Iberian lynx ($n=52$) were analyzed with both assays (Fig. 3). A linear correlation between the testosterone concentrations indicate a high concordance of both

assay systems (Eurasian lynx: Pearson correlation of $r^2=0.595$, $p<0.001$; Iberian lynx: $r^2=0.85$ and $p<0.0001$ (Fig. 2)). The Immulite assay detected a twice amount of testosterone metabolites compared to EIA.

3.3. Seasonal endocrine testicular activity

There were no differences between males ($p>0.05$) and between years ($p>0.05$) in fecal testosterone metabolite excretion. Because of limited size for each month, the samples were combined bimonthly before, during and after breeding season, and trimonthly during the non-breeding period. Fig. 3 presents means \pm SEM determined by the Immulite-Testosterone assay in four captive Eurasian males. In all four males, similar pattern of fecal testosterone metabolite concentration was obtained with highest

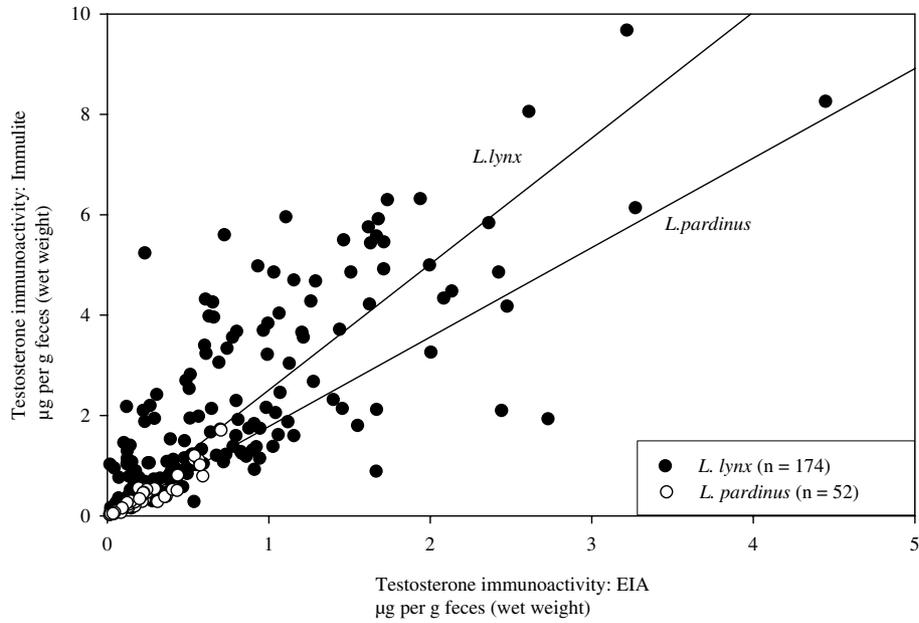


Fig. 2. Determination of immunoactive testosterone metabolites with EIA and Immulite assays in fecal samples collected from three male Eurasian lynx. Linear Pearson correlation between both methods is extremely significant with $r^2 = 0.488$ and $p < 0.0001$ ($n = 175$). Regressions between determined testosterone levels reveals a 2.2 higher hormone activity determined in Immulite in comparison to EIA ($y = 2.2x$).

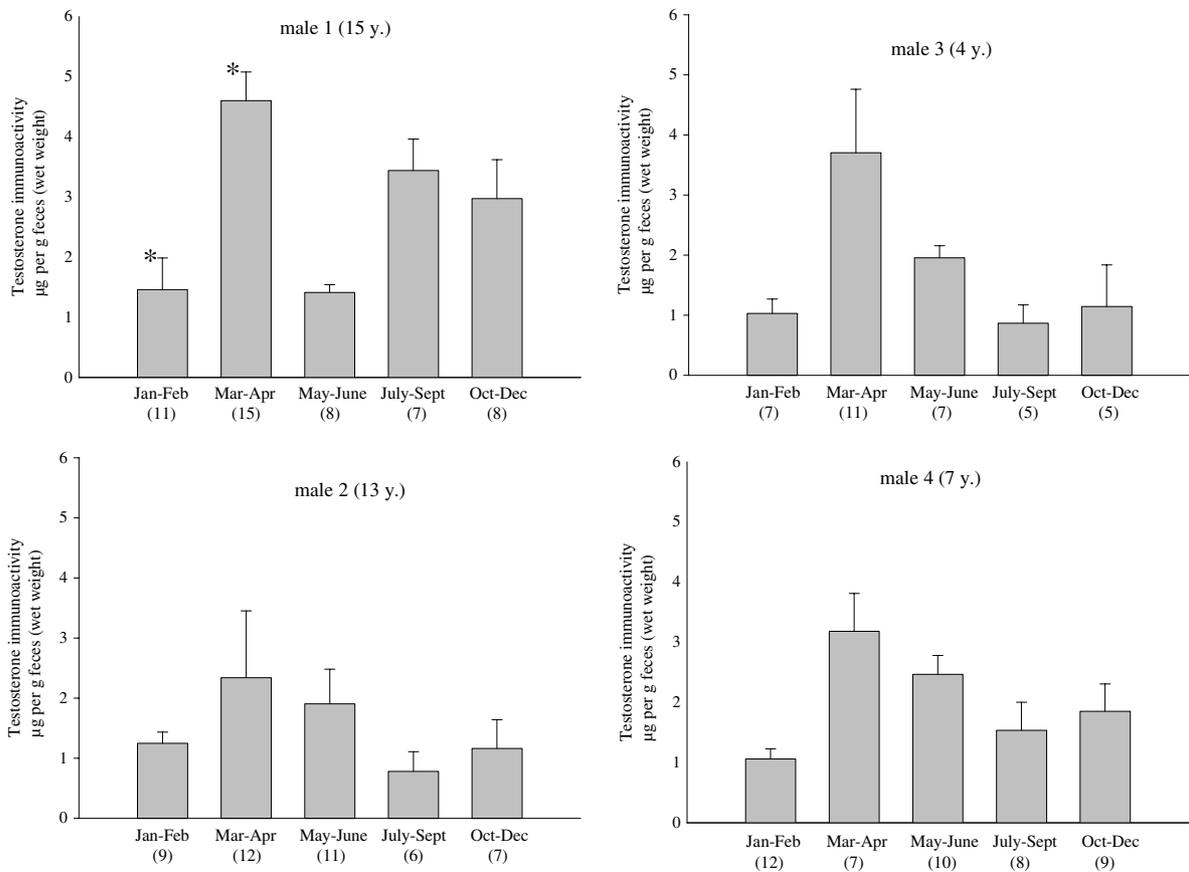


Fig. 3. Fecal testosterone metabolites determined by Immulite-Testosterone assay in four captive Eurasian males. Means \pm SEM are presented bimonthly for the period before and after breeding season (March–April) and for quarterly for the rest of the year. *Significant differences of testosterone content ($p < 0.05$).

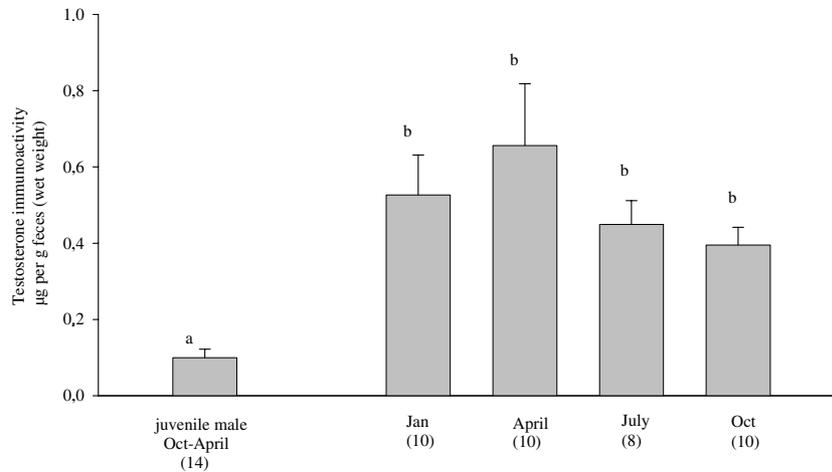


Fig. 4. Fecal testosterone metabolites determined by Immulite-Testosterone assay in three captive Iberian lynx males. Means \pm SEM are presented for fecal samples of the two adult males. The mean testosterone value for the juvenile male combined for all months was significantly under the level of adult males. a,b— different indices indicate a significant difference of testosterone content ($p < 0.05$).

concentrations during the breeding season (March–April) and low values in January/February were found. However, only in male one this difference was significant ($p < 0.05$).

Preliminary data on fecal hormone assessment in male Iberian lynx with the Immulite (Fig. 4) showed a distinct difference in the concentration of testosterone immune reactivity in samples of juvenile ($0.1 \pm 0.08 \mu\text{g/g}$, $n = 14$) and adult animals ($0.53 \pm 0.34 \mu\text{g/g}$, $n = 38$). Due to limited sample sizes, no significant difference was detectable between fecal testosterone concentrations in samples from the breeding (January: 0.53 ± 0.33 ; $n = 10$; April: 0.66 ± 0.51 ; $n = 10$) and the non-breeding season (July: 0.4 ± 0.12 ; $n = 8$; October: 0.4 ± 0.15 ; $n = 10$), although a tendency towards an increase in spring might be suggested.

4. Discussion

Few studies have monitored long-term seasonal changes in testicular activity of felids based on the measurement of peripheral blood testosterone levels (Kirkpatrick, 1985; Wildt et al., 1986; Byers et al., 1990; Tsutsui et al., 1990; Johnston et al., 1994; Morais et al., 2002). This is associated with difficulties conducting regular blood sampling procedures. They require handling, restraint and anesthesia of animals. In addition, testicular testosterone secretion is known to fluctuate in response to pulses of luteinizing hormone (Mattern et al., 1993; Lerchl and Nieschlag, 1995). Therefore, measurements in blood plasma reflect one point measurements affected by pulsatility and diurnal rhythms (Muller and Lipson, 2003; Mattern et al., 1993). Contrasting blood samples, the analysis of fecal metabolites provided an integrated measure of testicular activity over several hours decreasing the importance of considering time of day as a confounding variable in field studies of wildlife endocrine function. Therefore non-invasive approaches for assessing the dynamics of gonadal steroid secretion needed to be developed. Because reproductive (estradiol,

progesterone) and adrenal (cortisol) steroid metabolite excretion occurs mainly via the feces in cats (Brown et al., 1994; Graham and Brown, 1996; Morais et al., 2002), this study was designed to develop a non-invasive method for measuring fecal androgen metabolites in the Eurasian and Iberian lynx.

The radiometabolism study revealed the presence of various testosterone metabolites in Eurasian lynx feces. The nature of these substances could not be confirmed with the analytical methods used. A comparison of the unknown radiolabeled metabolites with elution positions of the standards revealed that testosterone and dihydrotestosterone (DHT) are not excreted in significant amounts in the feces. This has also been shown in the domestic cat (Brown et al., 1996). In other taxonomic groups, however, at least a part of the immune reactivity was shown to be authentic testosterone or DHT (Kretzschmar et al., 2004; Dloniak et al., 2004; Mooring et al., 2004; Möhle et al., 2002; Busso et al., 2005).

Although, a radiometabolism study or a GnRH challenge is excluded in Iberian lynx, because of its highly endangered status and few individuals in captivity, HPLC analysis of Iberian lynx fecal extract revealed a similar pattern of four major immunoactive metabolites at the same positions as found in the Eurasian lynx (Fig. 1B and C). This might indicate no species differences in the composition of main immunoactive testosterone metabolites. The polar metabolites might be androgen sulfates or glucuronide mono-conjugates, as it was shown in earlier experiences in the chicken (Dehnhard et al., 2003). However, enzymatic hydrolysis failed to liberate testosterone and DHT, and failed to shift the high polar peak to a more apolar position as well. The finding of an exceptionally high proportion of non-hydrolyzable testosterone metabolites was similar to those observed for excreted steroids in the domestic cat (progesterone: Brown et al., 1994; testosterone: Brown et al., 1996; cortisol: Graham and Brown, 1996). It was suggested that these substances are hydrolysis-

resistant C₁₉-steroids conjugated at position 17 (Shackleton, 1986). Solvolysis procedures increased the portion of dihydrotestosterone, but also failed to liberate significant amounts of testosterone. This was in accordance with the findings of Mosbach et al. (1968) in the dog, where most of urinary methyltestosterone metabolites resided in an unidentified solvolysis resistant polar fraction.

Although the major testosterone metabolites in lynx were not identified, both immune assays (EIA and Immulite) were capable of detecting fecal androgen immune reactivity, however, with distinct specificity towards particular testosterone metabolites. Nevertheless, we found a significant linear correlation between both immune assays by measuring androgen content simultaneously in 174 Eurasian and 52 Iberian lynx fecal samples. Therefore, we conclude that both assays reflect the androgen secretion in lynx feces. The data also revealed that the sensitivity of the Immulite assay was twice as high as our testosterone EIA. In domestic cats (Brown et al., 1996) and other felid species (Morais et al., 2002) fecal testosterone levels were at the same range as in the Immulite assay (between 0.1 and 10 µg per g feces).

For biological validation of the immune assays, testosterone metabolites were determined throughout the annual cycle, which is characterized by a seasonality of sexual activity in Eurasian lynx males (Goeritz et al., 2006). In our previous study, we demonstrated that serum testosterone concentrations in male Eurasian lynx were characterized by seasonal fluctuation with its highest levels in March (1.96 ng/ml testosterone) and lowest in June (0.75 ng/ml testosterone) (Jewgenow et al., 2006).

The average testosterone metabolite excretion in this lynx study confirmed the above mentioned earlier results. In all four animals, the individual profiles revealed peak levels of testosterone metabolite secretion during March and April, which is consistent with the mating season of the Eurasian lynx (Kvam, 1991; Naidenko and Erofeeva, 2004). Low concentrations were obtained during January and February. Inconsistency between the animals exist during the period from May to December probably indicating a second increase against the end of the year. An increase several months before maximum in testis activity, may not be unusual. In the roe deer, a small testosterone rise was described in May, preceding the maximum of androgen production in August/September (Schams and Barth, 1982; Roelants et al., 2002) during rut season.

Our preliminary data on fecal hormone concentrations in captive Iberian lynx suggest that the Immulite assay is applicable in Iberian lynx, too. This basically may be traced back to the congruent metabolism of testosterone in both lynx species. The determination of fecal testosterone metabolites in a juvenile Iberian male reflected his gonadal immaturity by significant lower levels compared to the two adult males. The seasonal pattern of both adult males indicated a tendency towards increased testosterone secretion during spring time, however, more data are necessary to confirm this. Basic information on the reproductive charac-

teristics of this highly endangered lynx is very limited and, to our knowledge, no hormone profiles generated from long-term investigations encompassing the breeding season in male lynx had been published so far. Nevertheless, fundamental knowledge of basic reproductive physiology is essential to improve reproduction in captivity by assisted reproduction and gamete banking.

Although in recent years interest in fecal steroid assays as a non-invasive measure for monitoring reproduction in mammals has increased substantially, inconsistencies often exist between the analytical methods used. This complicates the compilation of a database of endocrine norms essential to evaluate the reproductive competence of animals. Thus, we propose to use the worldwide distributed Immulite automated immune assay analyzer (about 9700 instruments; P. Zwerenz DPC Biermann, personal communication) and the Immulite-Testosterone assay as a standard method to characterize testicular activity in lynx (and probably other felid) species based on fecal testosterone metabolites measurements. Zoos and research institutions, which are unable to run endocrine assays, may contact local Immulite operating institutions (like human hospital or endocrine labs) to gather information on testicular status of male felids.

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