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

K. Jewgenow [a,*], S.V. Naidenko [b], F. Goeritz [a], A. Vargas [c], M. Dehnhard [a]

[a] *Leibniz-Institute for Zoo Biology and Wildlife Research, PF 601103, D-10252 Berlin, Germany*
[b] *A.N. Severtzov Institute of Ecology and Evolution, Moscow, Russia*
[c] *Iberian Lynx Captive Breeding Center, ILCBC, Spain*

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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 [^{3}H]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.

Keywords: Lynx; Testosterone; Non-invasive; Fecal metabolites; Seasonality

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

* Corresponding author. Fax: +49 30 5168612.
E-mail address: jewgenow@izw-berlin.de (K. Jewgenow).

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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 subpopulation 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 m^2) and in one large fenced enclosure (7500 m^2), that is part of the natural mixed forest providing a semi-natural environment. Each enclosure had an additional 1–4 cages (6–8 m^2 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 m^2).

2.2. Processing fecal samples

All fecal samples were processed as described before (Kretzschmar 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 1200*g*) 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 [^{3}H]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-glucu-