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Katarina Jewgenow · Frank Goeritz · Katrin Neubauer · Joerns Fickel · Sergej V. Naidenko

Characterization of reproductive activity in captive male Eurasian lynx (*Lynx lynx*)

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Abstract This study characterizes the reproductive activity of male European lynx kept in Central Russia. Four captive adult males were subjected to an electroejaculation prior to (November), during (March) and after (June) the breeding season. Concentration, motility and morphological integrity of spermatozoa as well as testis diameter and testosterone levels in serum were evaluated. Additionally, fecal samples were collected for 2 years to determine the fecal testosterone secretion. Testis sizes and serum testosterone concentrations were characterized by little changes with highest levels in March (2.98 mm^3; 1.96 ng/ml testosterone) and lowest in June (2.34 mm^3; 0.75 ng/ml testosterone). In faeces, the highest testosterone concentrations were measured in February followed by a second increase in May. The volume of ejaculates and percentages of motile and intact spermatozoa reached the maxima in March. By performing two-male mating experiments, we could prove multiple paternity within three litters. Paternity analysis of litter also revealed that 26 of 31 cubs (84%) were sired from the same male, independently from being the first or second mating partner of the respective female. This particular male showed the most developed and activated reproductive tract and also had the best semen quality, which seems to be important for sperm competition.

Keywords Lynx · Teratozoospermia · Mating experiment · Paternity · Seasonality

K. Jewgenow (✉) · F. Goeritz · K. Neubauer · J. Fickel
Institute for Zoo and Wildlife Research,
Alfred-Kowalke-Straße 17,
10315 Berlin, Germany
e-mail: Jewgenow@izw-berlin.de

S. V. Naidenko
A. N. Severtzov Institute of Ecology and Evolution,
Leninsky pr.33,
119071 Moscow, Russia

Introduction

The genus *Lynx* consists of four species: the Eurasian lynx (*Lynx lynx*), the Canadian lynx (*L. canadensis*), the bobcat (*L. rufus*) and probably the most endangered felid species (CITES App. I)—the Iberian lynx (*L. pardinus*). Lynxes are distributed over the Northern Hemisphere in Eurasia 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. In some parts of its range, the lynx is very rare. In the central European part including Switzerland and Germany, lynxes do not exist in sustainable populations, in spite of several strong efforts to reintroduce them (Breitenmoser and Breitenmoser-Würsten 1990).

All four species have some general features in common, which are typical for the socioecology of most *Felidae*. Lynxes live solitary (Heptner and Sludskii 1972; Breitenmoser et al. 1993) and have large home ranges, whose size differs between the sexes (females 170 km^2, males 250 km^2). In wild-living lynx, the dispersion of females, rather than that of prey, may underline the spacing of males. Usually, two to three female home ranges are encompassed within that of one male (Breitenmoser et al. 1993; Schmidt et al. 1997).

Most data gained on lynx reproduction are based on skinned carcasses collected from trappers. The mating period in lynx is described to be from January to April, in dependence on latitude. The majority of ovulations occur in February and March. Conception rate determined by placental scars is about 2.5 embryos per uterus. Parturition takes place after 70 days of gestation (Naidenko and Erofeeva 2004) during late May and early June (Kvam 1991).

In males, there is some indication that the animals are seasonal breeders according to their testis size, which increases in tendency from February to May (Kvam 1991). The whole seasonal pattern of reproduction, however, is unknown due to the lack of material out of hunting season.

Furthermore, no data on sperm production and testosterone levels during annual cycle are available.

Therefore, the aim of the study was to characterize the seasonal reproductive activity of captive male Eurasian lynxes living in Central Russia. Quantity and quality of semen were estimated in relation to serum and fecal testosterone concentrations as well as to testis size during three consecutive years in four adult males. Additionally, the fertility of three males was examined in mating experiments.

Materials and methods

Animals Four adult males were examined prior to (November), during (March) and after (June) the breeding season. They were housed together with ten females at the scientific field station "Tchernogolovka" of the A.N. Severtzov Institute, situated 50 km northeast from Moscow. There, the animals are kept within six enclosures of 74 m^2 each and in one large fenced enclosure of 7,500 m^2 that is a part of the natural mixed forest providing a seminatural environment. The animals were immobilized and subjected to an ultrasound examination and electroejaculation as described before (Hildebrandt et al. 2000). To reduce disturbance, the assessment was performed only once a year in June 2002, November 2003 and March 2004, respectively. Fecal samples were collected weekly from November to May and monthly from June to October throughout a 2-year period. In March (prospective mating season), the frequency of collection was increased to two to three times a week.

Testis diameter Testis diameter was determined by the use of an orchimeter. The volumes of testes were calculated using the equation for a simple rotation ellipsoid accommodating the spherical and symmetrical shape of these organs. Additionally, the texture of testis tissue was assessed by transcutaneous ultrasound as described before (Goeritz et al. 1997).

Semen collection Semen collections were performed after each ultrasound assessment by electrostimulation, a method described previously for roe deer with some modifications (Goeritz et al. 2003). Briefly, a rectal probe (diameter, 2.7 cm) plated with three raised longitudinal electrodes was placed into the rectum above the prostate. Ten electrical stimulations were given using the Seager model 14 (Dalzell USA Medical Systems, The Plains, VA, USA). Semen was collected in sterile 2-ml vials (Eppendorf, Germany). The volume of ejaculates was estimated using a micropipette. For each sample, we estimated sperm concentration, percentage of progressively motile spermatozoa and urine contamination. Corresponding smears were prepared for the evaluation of the morphological integrity of 200 spermatozoa per sample after staining with Congo red, tannic acid and brilliant cresyl blue (Blottner et al. 1989).

Estimation of serum and fecal testosterone Serum testosterone was directly measured in a 20-μl sample using an Immulite automated analyser (DPC, Germany) with Testosterone Immulite kit. Fecal samples were extracted as follows: 0.5 g faeces was combined with 4.5 ml of 90% methanol, agitated for 30 min and centrifuged (15 min at 1,000×*g*). The supernatant was diluted 1:2 (v/v) with water. Aliquot portions of 20 μl were subjected to a testosterone enzyme immunoassay as described before (Goeritz et al. 1997). The testosterone concentrations are presented in nanograms per gram faeces. Means±standard deviations (SD) were calculated for each month, and differences between months were assessed by parametric Student–Newman–Keul's test. 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.).

Mating experiments and paternity analysis During 3 years, three of the four males were used for mating experiments with ten females. To simulate a multimale mating pattern, each female was allowed to pair with two males in a sequential order. During estrus, the female was paired for 4 h with the first male and, on the next day, for 4 h with the second one. Mating activities were recorded by permanent observation. "Male–female combinations" and "order of males" (first or second mating partner) were randomly chosen to test all six possible pairing combinations (Table 2) at least three times. Since some females denied mating with a particular male or did not conceive, some combinations of males are still missing or underrepresented. Blood or, in case of death, tissue samples were taken from each cub to determine paternity by microsatellite analysis using previously published cat-specific primers (Menotti-Raymond and O'Brien 1995). The PCR reaction was performed using the following protocol: 1× (95°C, 4 min), 30× (95°C, 30 s; 50°C, 30 s; 72°C, 45 s) and 1× (72°C, 30 min). Amplification products were separated on a Pop-6 matrix using an ABI A3100 automated sequencer.

Results

Most felids are polyseasonal reproducers. However, our morphological and hormonal data indicate that spermatogenesis in lynx is strongly seasonal. The testis volumes of lynxes changed moderately within an annual cycle with 2.98±0.8 cm^3 in March, 2.34±0.71 cm^3 in June and 2.59±0.6 cm^3 in November (Table 1). The tissue texture of testes was activated most in March and June, but not in November (see Goeritz et al. 2005). Blood serum testosterone concentration followed the same pattern with highest values in March and lowest values in June (Table 1). The maximal fecal testosterone concentration was measured in February (1,240±393 ng g^{-1} faeces). A second increase was documented in May (971±202 ng g^{-1} faeces). The lowest fecal testosterone concentrations were measured in January (Fig. 1). The volume of ejaculates, percentages of motile spermatozoa and intact sperm were also maximized during the breeding season in March. The overall number of sperm per ejaculate, however, was

Table 1 Testes volume and ejaculate parameter obtained in adult male European lynx prior to (November), during (March) and after (June) breeding season

ID animal	Parameter	March 2004	June 2002	Nov 2003
L1 Pewez (13 years[a])	Testes volume (in cm^3)	1.9	1.5	1.9
	Motility	0%	n.d.	n.d.
	Volume (in μl)	470	<20	20
	Density (in 10^6 Sp/ml)	0.05	3.5	0
	Sperm number ($\times 10^6$)	0.02	0.07	0
	Intact sperm	4%	n.d.	n.d.
	Serum testosterone (in ng/ml)	2.60	0.63	0.84
L3 Psich (7 years[a])	Testes volume (in cm^3)	3.7	2.9	3.2
	Motility	90%	60%	55%
	Volume (in μl)	260	30	105
	Density (in 10^6 Sp/ml)	9.5	110	65
	Sperm number ($\times 10^6$)	2.47	3.30	6.83
	Intact sperm	29%	2%	13%
	Serum testosterone (in ng/ml)	1.30	1.40	2.60
L8 Phil (15 years[a])	Testes volume (in cm^3)	2.9	2.8	3.0
	Motility	90%	40%	5%
	Volume (in μl)	180	10	110
	Density (in 10^6 Sp/ml)	16.6	540	10
	Sperm number ($\times 10^6$)	3.00	5.40	1.10
	Intact sperm	17.5%	1.5%	2%
	Serum testosterone (in ng/ml)	3.20	0.23	1.30
L19 Zwetik (3 years[a])	Testes volume (in cm^3)	3.4		2.3
	Motility	50%		n.d.
	Volume (in μl)	200		5
	Density (in 10^6 Sp/ml)	4.2		60
	Sperm number ($\times 10^6$)	0.84		0.30
	Intact sperm	52.5%		2%
	Testosterone (in ng/ml)	0.73		0
Means	Testes volume (in cm^3)	2.9	2.4	2.6
	Motility	77%	55%	30%
	Volume (in μl)	278	20	60
	Density (in 10^6 Sp/ml)	8	218	45
	Sperm number ($\times 10^6$)	1.6	2.9	2.7
	Intact sperm	26%	2%	6%
	Serum testosterone (in ng/ml)	1.96	0.75	1.18

n.d. Not determined
[a]Age in 2004

relatively low with 2×10^6 cells and was not affected by season.

Mating of n=10 (2002), n=8 (2003) and n=7 (2004) females each with two adult males (in varying combinations and order) resulted in litters with one to four cubs. The paternity of cubs was examined in 14 litters with a total of 31 cubs (male/female ratio of 14:17). We could prove three cases of multiple paternity; each adult male was involved at least once. However, altogether, 26 cubs (84%) were sired from the same male ("Psich"), independently from being the first or second mating partner of the respective female (Table 2). Being the first male, "Psich" sired 14 out of 16 cubs, and as the second male, he fathered 12 of 13 cubs. "Psich" was the single sire in 10 cases; multiple paternity was observed twice, and in only one case he "lost the competition" against "Pewez." The sperm of "Psich" was superior in volume and in percentage of intact sperm in comparison to "Phil." The male "Pewez" was characterized by the biggest ejaculate volume but very low quality of sperm (Table 1).

Discussion

In contrast to most of the other feline species, lynxes have a quite narrow breeding season in March. All studied functional reproductive parameters of males (testis volume and tissue texture, testosterone secretion and sperm quality)

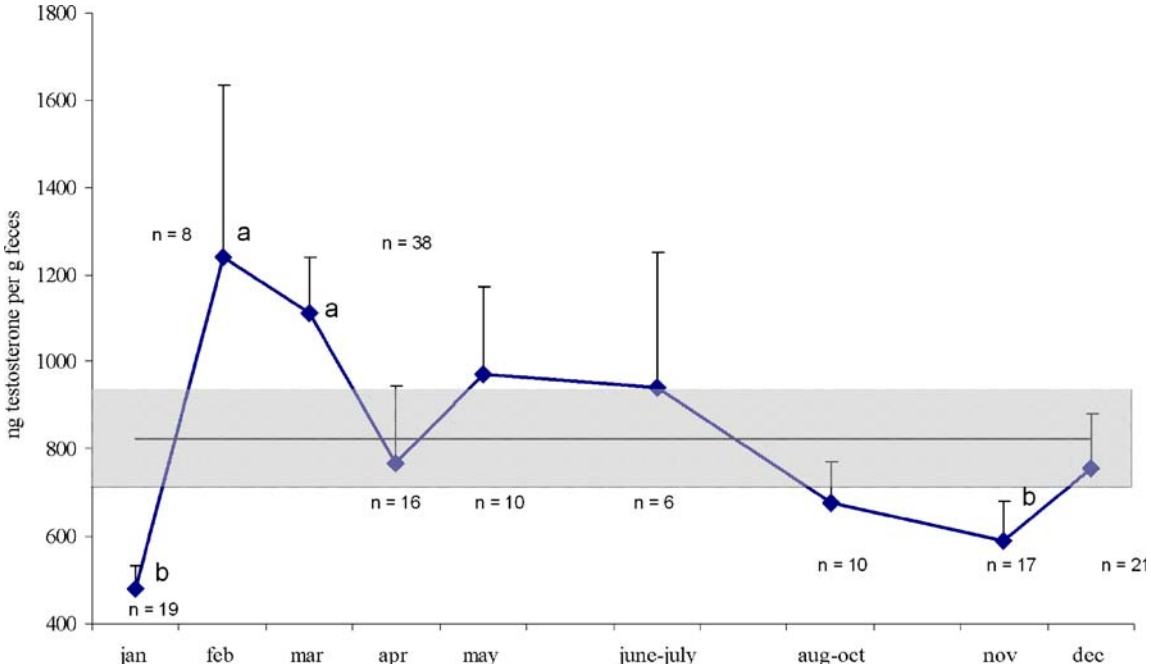

Fig. 1 Testosterone metabolites determined in the faeces of three lynxes over 2 years. *Dashed line* indicates the mean value of all samples (n=145)±2 SD (*grey bar*). Different indices (**a**, **b**) indicate significant differences of testosterone content (P<0.05)

reached their peak in March and decreased in June. There is some indication in nature that female lynx can be estrous again in May/June, e.g. if they lose their first litter very early. In such a case, they have a second litter in August or September. However, none of our females not having a litter showed estrous behavior in May/June (Naidenko, unpublished data). Referring to our males, sperm quality in June was poor, and testis activity as well as testosterone secretion was at a minimum. This low reproductive activity may be caused by closed housing of males with all females, most of which having offspring already.

All male lynxes investigated were found to ejaculate high proportions of pleiomorphic spermatozoa, which indicate that the lynx is also affected by teratozoospermia as described for other feline species (Pukazhenthi et al. 2001). However, all three adult lynx males proved to be fertile and sired offspring as shown by paternity analysis. The phenomenon of teratozoospermia is suggested to be linked to a reduction in genetic variability (Wildt et al. 1983, 1987). The high incidence of teratozoospermia in felids may also be caused by living conditions in captivity and/or the mating system. Our animals studied are genetically diverse but live very closely together. A suppression of male reproductive activity by the dominant male and/or permanent stress (e.g. caused by the proximity of females) cannot be excluded. In the wild, lynxes have vast home ranges and are able to keep distant to each other. The fact that in our mating experiments one male ("Psich") was superior to the two others may be attributed to its higher rank and better sperm quality. The male "Psich" showed the most developed and activated reproductive tract (Goeritz et al. 2005) and also showed the best semen quality, which seems to be important within multimale mating systems. Wild-living *Lynx*'s breeding system (Reynolds 1996) can be described as "dispersed promiscuous," which includes sperm competition. Sperm competition is the "competition within a single female between the sperm from two or more males for the fertilization of the ova" (Parker 1970). One evidence for sperm competition is the occurrence of multiple paternity in litter, as it was shown for felid species.

In conclusion, within our breeding group of European lynx, we found a pronounced breeding seasonality, restricted to the month March. Lynxes are also affected by teratozoospermia, a common phenomenon within feline species. Large ejaculate volumes may be advantageous in a multimale mating system. However, in our study, the breeding success eventually depended on sperm quality.

Table 2 Two male mating experiments in European lynx

First male	Second male	Number of litters	Paternity of cubs: first/second male
L1 Pewez	L3 Psich	4	1:5
L1 Pewez	L8 Phil	0	No offspring
L3 Psich	L1 Pewez	3	7:0
L3 Psich	L8 Phil	3	7:2
L8 Phil	L1 Pewez	1	1:1
L8 Phil	L3 Psich	3	0:7

During natural estrus, the first male was allowed to mate with the female for 4 h. On the next day, the second male was paired with the same female for 4 h. Paternity of cubs was analysed by cat-specific microsatellites

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