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Seasonal chenges in activity of males' reproductive system in Eurasian lynx

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ABSTRACT**.** Eurasian lynx is strongly seasonal in its reproduction. This seasonality in reproduction becomes apparent in spatial population structure and behavior of lynx males during the year. However it was not clear how are these changes correlated with physiological changes at the individual level. In this article we studied the changes in sperm quality and testosterone level in Eurasian lynx males over the year. It was shown that activity of reproductive system in Eurasian lynx males changed seasonally as well. Testosterone level increased in advance of the mating season and spermatogenesis "normalized" at the same period.

KEY WORDS: Eurasian lynx, seasonality, sperm quality, testosterone, reproductive biology.

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Сезонные изменения активности репродуктивной системы самцов евразийской рыси

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РЕЗЮМЕ. Евразийская рысь строго сезонна в размножении. При этом такая сезонность в размножении накладывает отпечаток на изменения пространственной организации и поведения самцов рыси в течение года. Однако неясно как эти изменения коррелируют с физиологическим состоянием животных. В данной статье рассмотрено изменения в качестве спермы и уровне тестостерона у самцов евразийской рыси в течение года. Отмечено, что активность репродуктивной системы у самцов рысей так же подвержена сезонным изменениям. К периоду гона в значительной степени возрастает уровень тестостерона в плазме крови самцов и происходит «нормализация» сперматогенеза.

КЛЮЧЕВЫЕ СЛОВА: евразийская рысь, сезонность, качество спермы, тестостерон, репродуктивная биология

Introduction

Unlike other felid species inhabiting temperate latitudes Eurasian lynx (*Lynx lynx*) reproduces only once per year during the short breeding season (Heptner & Sludskii, 1972; Stehlik, 2000; Naidenko & Erofeeva, 2004). Usually mating occurs in March and pregnant females give birth between May 10th and June 10th (Naidenko, 2005). Seasonality in reproduction is closely related with annual changes in spatial and social organization of Eurasian lynx populations.

Eurasian lynxes live solitary the major part of the year. Like for many other felid species, females with the kittens are the only stable social groups that exist for a long time till the dispersion of the young. Beside this contacts between animals are rare and occasional (Seidensticker at al., 1973; Poole, 1995). Home range of a male overlaps with home ranges of two-three females. The overlapping of home ranges may be different (up to 86%) (Davidov, 1983; Breitenmoser et al., 1993; Jedrzejewski at al., 1996).

However, males' activity changes in advance of the mating season. They start to make excursions outside their home ranges (Breitenmoser et al., 1993) searching for the potential mating partners. Communicative activity sharply increases before the mating season and this is very important for the species whose breeding period is very short. Marking activity of the males increases toward the beginning of mating season (Zheltukhin, 1982; Sokolov et al., 1995) and the density of chemical marks per home ranges is enhanced. The special system of spatial distribution of chemical marks (mainly urine) and highly informative content of chemical signals allow animals to obtain the detailed information about neighbors and transit animals (Naidenko, 2005). Acoustic activity also drastically increases toward the breeding season in both males and females of the Eurasian lynx (Rutovskaya & Naidenko, 2006; Erofeeva, 2010).

Vocalizations transmit information about physiological status of animals (Rutovskaya et al., 2009).

Reproductive seasonality in Eurasian lynx becomes apparent in spatial and social structure. However, it is unclear how the changes in individual animal behavior correlate with the changes in their physiology (hormonal status and sperm quality). Pioneer studies were conducted at our station (Jewgenow et al., 2006; Goritz et al., 2006). They described seasonal profile of testosterone metabolites level in feces, sperm quality of four Eurasian lynx males in mating and non-mating periods.

The aim of this study was to conduct complex analyses of the main reproductive parameters (testosterone level in blood plasma, testes volume, sperm quality) of Eurasian lynx males throughout the year and to compare their changes in different month and seasons.

Materials and methods

The study was conducted at the biological station "Chernogolovka" of A.N. Severtsov Institute of Ecology and Evolution which is located 50 km to the north-east of Moscow (56°00′ northern latitude, 38°22′ eastern longitude). Average annual temperature was +3.5…4.3° C, average temperature in July was +19°C, in January was –11°C.

Animal breeding colony was formed in 1987 and included three adult males and three adult females of Eurasian lynxes that were captured in the wild in different regions of European part of Russia and Western Siberia. Taxonomically all animals belonged to the same subspecies: *L. l. lynx*. All the animals used for these experiments were the first-third generations born in captivity at the "Chernogolovka" station.

The Eurasian lynxes were kept in a complex of six tree-less enclosures (74 m^2 each) and in 12 small cages (6 m^2 each), also one cage (8 m^2) was attached to each tree-less enclosure. Each cage/enclosure had artificial den (wooden box 1.8×1×0.8 m) with the up-side covered by metal list and stayed on metal base (H=60 cm). Beyond the mating period animals were kept separately from each other excluding family groups (contained of mother with its kittens). Animals have access to water *ad libitum*. Daily ration included about 1 kg of chicken meat with additional vitamins.

An estimation of hormonal status of Eurasian lynxes

Blood sampling was conducted for adult Eurasian lynx males since 2002 until 2007. Altogether we collected 222 blood samples from six males over this period. Blood collection (1.5–2 ml) was conducted from the femoral vein in Eurasian lynxes immobilized by the standard method (Goritz et al., 2006). Blood samples were centrifuged at 6000 r/min during 20 minutes and the serum was transferred into a new tube. Serum was labeled, frozen and kept at –18°C. Serum samples were thawed just before measurements.

Testosterone concentration was estimated by enzyme immunoassay using "Immunotekh" kits (Moscow, Russia). Cross-reactivity of antibodies to testosterone was 9% to 5-dehydrotestosterone, 1% to 11-hydrooxitestosterone and 5-androstan-3,17-diol, less than 0.1% to all other tested steroids. Optical density (450 and 620 nm length wave) was measured with flat-bedded spectrofotometer Multiscan EX (ThermoElectron Corporation, Finland). Measurements were conducted in doubles and CV was calculated for the measurements. If CV was less than 5% the average optical density was taken for the analyses and if CV was more than 5% the measurements were repeated.

Sperm quality analyses

The study of seasonal changes in sperm quality in Eurasian lynx was conducted in 2002–2008. Sperm sampling was conducted in immobilized animals (four males) monthly (last week of the month) by electroejaculation. Ejaculates were collected with P-T Electronics (Oregon, USA) electroejaculator. Before the procedure the rectum was washed. Rectal probe (2.7 cm in diameter) with gel for ultrasound examination (Helteck, Russia) with three longitudinal electrodes was inserted into the rectum above the prostate. Electric impulses (2 V) were used with the constant increase of voltage till 5 V. Ejaculates were collected with clean and warmed (37°C) Eppendorf tube. Sperms motility was detected right after ejaculate collection as described below. Two microliters of ejaculate were placed with micropipette on warm (37°C) slide under the microscope with magnification 200 (10×20). Motility was estimated visually with the precision of 10%. Sperm concentration was estimated with Goryaev camera under 400 magnification (10×40) counting sperms number in five squares, situated on diagonals, and estimating average number. If sperms concentration was too high the ejaculate was diluted with physiological solution. Based on average sperm number in each square the average sperm concentration per one milliliter was calculated. Ejaculate volume was estimated. Sperms smear was prepared for the estimation of sperm morphology. Percentage of normal (intact) sperms was estimated under the magnification 400 (10×40). Morphology of 200 sperms on each smear was described. All pathology types of acrosomes, necks and tails were counted as it was previously described for the domestic cat (Wildt, 1991). The height, altitude and depth of each testis were measured with the electronic trammel and testes volume was calculated ($V=4/3\pi R^3$). Total volume of testis was calculated as a sum of volumes of both testes. Motility of sperms; percentage of morphologically intact sperms; ejaculate volume; sperm concentration were used as sperm quality characteristics for each ejaculate.

Statistical data analysis

We used Microsoft Excel and Statictica 6.0 to conduct statistical data analysis. We used non-parametric

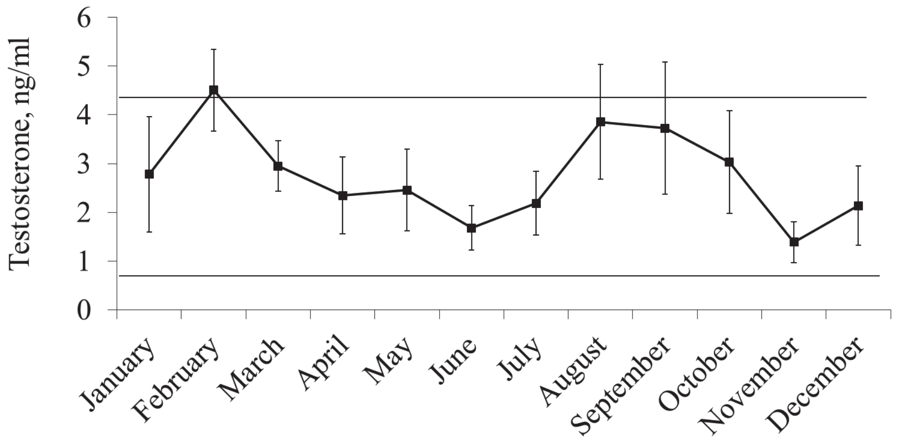

Figure 1. Seasonal changes in testosterone level in lynx blood serum in lynx males (mean±SE) (n=6). Horizontal lines indicate the average annual value ± 2SD.

statistics, because the distribution was statistically different from normal distribution (Kolmogorov-Smirnov test, $p<0.05$). The data are presented as mean and standard errors of the mean (mean±SEÌ).

To find out changes in sperm quality and testes volume we analyzed seasonal means. We described these changes for spring (February–April, because it is mating period in Eurasian lynx), summer (May–July), autumn (August–October), winter (November–January). We described mating of Eurasian lynxes at our station just in March (Erofeeva, Naidenko, 2004), so we considered here March as "mating period" and February as "premating period" We compared mean sperm quality parameters and testes volumes in different seasons by Friedman ANOVA. Data are presented on graphs as means and standard errors (mean±SE).

Results

Changes of activity of reproductive system of Eurasian lynx males during the year

Hormonal changes

Testosterone concentration in Eurasian lynx blood serum changed significantly throughout the year (Fig. 1). Average testosterone level varied from 1.7 to 4.5 ng/ml. Testosterone level was the highest in February just in advance of breeding season (4.5±0.8 ng/ml), about 170% of average yearly testosterone level in Eurasian lynx males. Testosterone increased in autumn as well. In August testosterone level in Eurasian lynx males was 3.9±1.4 ng/ml, in September — 3.7±1.1 ng/ml. However testosterone level only in February was significantly higher than the mean value over the year (M±2SD).

Changes in sperm quality

Eurasian lynx males had significant seasonal differences in testes volume (Friedman ANOVA, T=10.8; df=3; $p<0.05$). Testes volume was maximal in winter (6.33±0.51 $cm^{3)}$ and spring (6.21±0.29 cm^3) (Fig. 2) and smaller in summer (5.42±0.22 cm^3) and autumn (4.51±0.21 cm^3). Such way, testes volume started to increase before spring and reached its maximum already in winter (November–January).

Ejaculate volume significantly differed between seasons (Friedman ANOVA, T=8.7; df=3; $p<0.05$). Seasonal changes in ejaculate volume were similar with changes in testes volume (Fig. 3). The largest ejaculate volume was reported in winter (50.7±5.5 µl). In spring ejaculate volume was smaller — 38.2±10.8 (SE) µl, and it was at its minimum in summer (13,3±3,9 µl) and in autumn (21.4±5.8 µl).

Sperms concentration also varied significantly during the year (Friedman ANOVA, T=9,9; df=3; $p<0.05$). Concentration of spermatozoa was highest in summer: 490.8±177.7 mln/ml (Fig. 4); in spring sperms concentration was much lower: 40.2±19.9 mln/ml. The same pattern was found for sperms number per ejaculate. Sperms number per ejaculate was 1.2±0.7 mln in spring, and in non-breeding season varied from 3.7±3.1 mln up to 7.5±2.8 mln. However these differences were not significant.

Teratospermia was described for Eurasian lynx males. Percentage of morphologically intact sperms did not exceed 40%. Only one male featured 56% of morphologically normal sperms during premating period (in February). Totally the ratio of morphologically normal and abnormal sperms varied seasonally. We found significant seasonal differences in percentage of normal sperms (Friedman ANOVA, T=7.8; df=3; $p<0.05$). The percentage of morphologically normal sperms increased in winter and spring (Fig. 5). In spring the percentage of morphologically normal sperms was 36.8±2.8%, in summer it decreased to 31.6±3.5%. In autumn this parameter reached its minimum (23.3±3.1%) and in winter it started to increase again (28.0±1.5%).

The same seasonal differences were described in sperms motility (Friedman ANOVA, T=9.9; df=3; $p<0.05$). Sperms motility was maximal during premat-

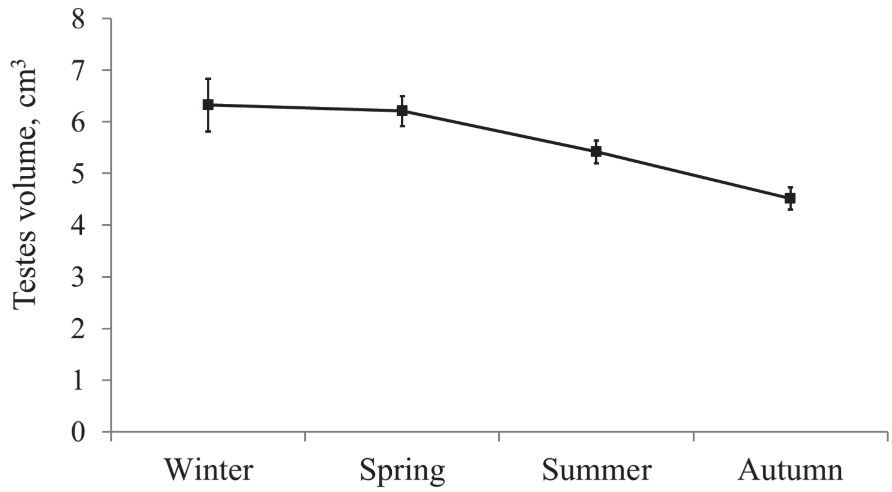

Figure 2. The volume of the testes in lynx males in different seasons (mean±SE) (n=6).

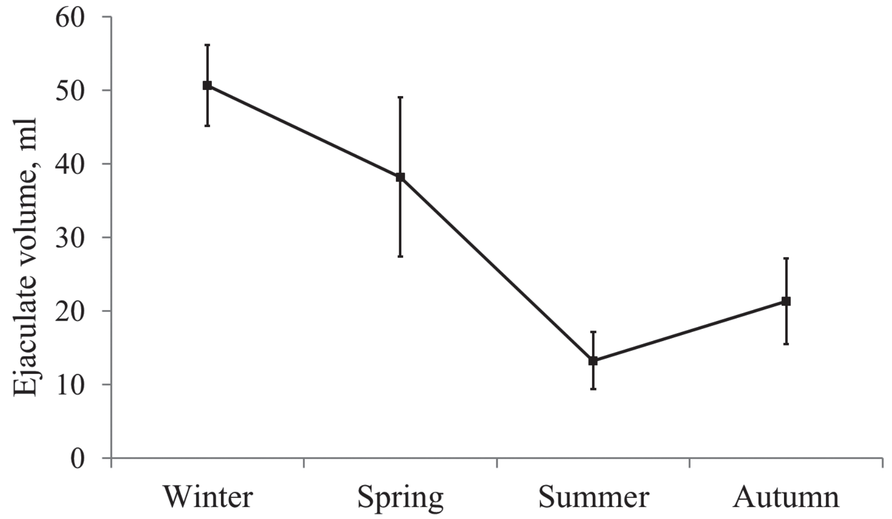

Figure 3. The volume of ejaculate in lynx males in different seasons (mean±SE) (n=6).

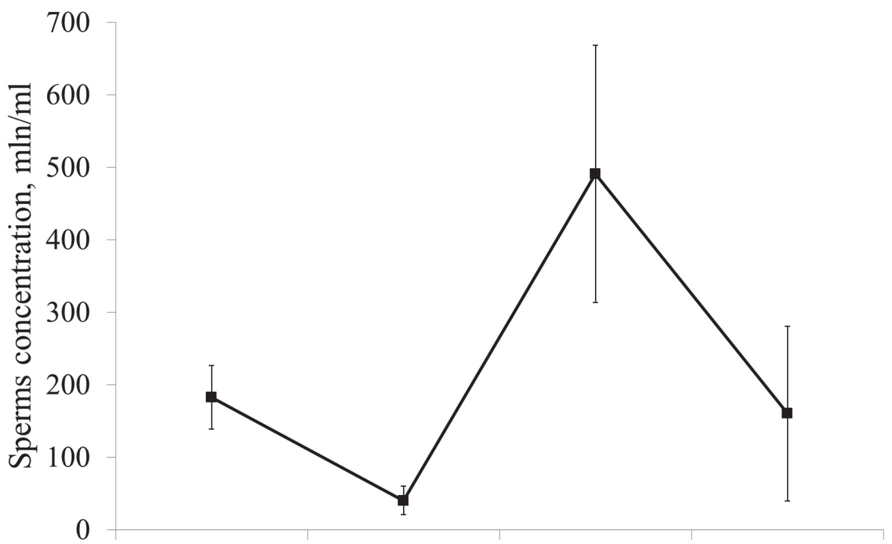

Figure 4. Sperm concentration in lynx males' ejaculate in different seasons (mean±SE) (n=6).

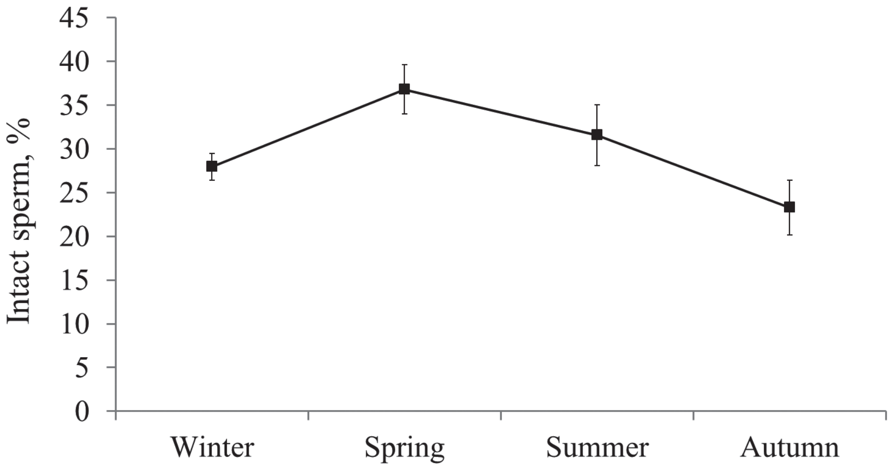

Figure 5. The average percentage of morphologically intact sperm in lynx males in different seasons (mean±SE) (n=6).

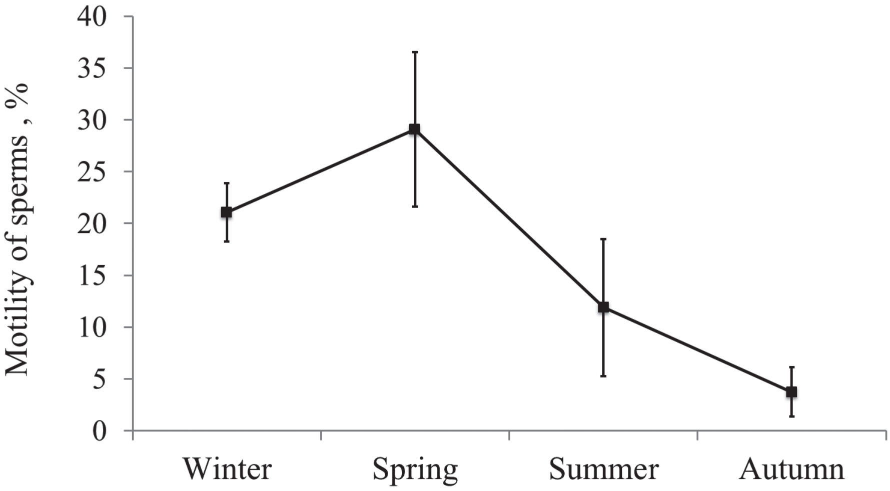

Figure 6. Motility of sperms in the ejaculate of lynx males in different seasons (mean±SE) (n=6).

ing and mating period (29.1±7.4%) (Fig. 6). It decreased to summer (11.9±6.6%) and autumn (3.8±2.4%) and increased again to winter (21.0±2.8 %).

Discussion

In opposite to the most of *Felidae* species Eurasian lynx demonstrates strict seasonal pattern in reproduction. At the main part of its range breeding season occurs in Eurasian lynx in February–April (mainly in March) (Heptner & Sludskii, 1972; Stehlik, 1980), however parturition was reported for this species not only in May–June, but also in August–September (Kaczenscky, 1991; Stehlik, 2000). Detailed analysis of hormones profiles in male Eurasian lynx serum performed in our study revealed that the peak of testosterone occurred in premating-mating period (February–March). At the end of March testosterone level was much lower, so it decreased during and after the mating period. Based on fecal testosterone metabolites concentration (Jewgenow et al., 2006) we assume that it occurs right after the mating season. Such way, our data corroborate strict seasonal pattern in activity of reproductive system of Eurasian lynx males. Similar patterns of changes in testosterone level were described for the number of other felid species featured seasonality in their reproduction, i.g. Pallas' cat *Otocolobus manul* (Swanson et al., 1996). Some cases of Eurasian lynx reproduction beyond the breeding season (mating in May–June) were very rare (Kaczenscky, 1991; Stehlik, 2000). Our data on changes of motility of sperms and percentage of normal sperms assume that Eurasian lynx males reproduction (mating) the most probable in February–March (as it is). However, presence of alive sperm in any time of the year, higher sperm concentration in summer may provide a chance to reproduce in other seasons.

Obvious seasonality in activity of males' reproductive system was supported by significant seasonal changes in sperm quality and testes volume. An increase of testes volume toward the mating period was also described in other felids which reproduce seasonally (Swanson et al., 1996). In agreement with this report our observations indicate that some indexes of sperm quality increased toward the mating period.

Ejaculate volume increased to the mating season. However, even during the mating season this volume was smaller than in tomcats (about 100 μl) (Pukhazhenti et al., 2006; Jewgenow et al., 2007). These differences are even more striking if to take into account the drastic differences of body size in these animals. In big cats ejaculate volume varied from 1,8 ml (cheetah *Acinonyx jubatus*) till 3.4–7 ml (tiger *Panthera tigris*, leopard *Panthera pardus*, mountain lion *Puma concolor*) (Wildt et al., 1988). However, this index may be depended of the method of sperm collection (presence or lack of semen fluid in ejaculate may affect ejaculate volume and its concentration).

The main trait of the Eurasian lynx ejaculates was very few number of spermatozoa with normal morphology. Teratospermia in Eurasian lynx was described earlier (Jewgenow et al., 2006). For many cat species low sperm quality (high percentage of abnormal sperms) is typical and it related mainly to the low genetic diversity in felines population (Wildt et al., 1983, 1986, 1987a,b; O'Brien et al., 1987). It is possible, that high percentage of abnormal sperms in Eurasian lynx was also related to the low genetic diversity in this *Felidae* species. However, all experimental animals were born in captivity (first and second generation born from non-relative individuals) (Naidenko et al., 2007a) thus inbreeding effect on males sperm quality was excluded. Comparative analyzes of the sperm quality in captive born and wild caught animals could help to elucidate this contradiction. At the same time the analysis of genetic diversity on the Eurasian lynx range may help to describe genetic polymorphism of this species. Genetic polymorphism of Eurasian lynx populations was very low in the wild, for example in Scandinavia (Rueness et al., 2003; 2014) or Byalowieza Forest (Schmidt et al., 2009). Low percentage of morphologically intact sperms was evident throughout the most of the year. However this index increased toward the mating season up to 40%, and in some individuals even up to 56%. Moreover, sperm motility also increased toward the mating period. Both phenomenon were described for the first time. Obvious seasonal changes (and increase) of both parameters to the mating season correspond to the seasonal reproduction of Eurasian lynx. High percentage of morphologically abnormal sperms per ejaculate considered as the consequences of some abnormalities in spermatogenesis (Pukazhenti et al., 2006), and we can assume that spermatogenesis has normalize toward the mating season probably because of testosterone increase in Eurasian lynx males at this period. Sperm quality in two other Lynx species was estimated as well (Gañán et al., 2009a,b; 2010). The percentage of morphologically intact sperm was 23.7% in Iberian lynx (*Lynx pardinus*) (Gañán et al., 2009a) and did not differ in captive-hold and wild males (Gañán et al., 2010). In bobcat (*Lynx rufus*) this index was even lower (Gañán et al., 2009b), probably because the sampling was done out of mating season. That may be a reason of a lack of seasonal differences in this parameter of sperm quality in bobcat males. Another possible reason is that bobcat may have prolonged reproductive season in comparison of three other lynx species (Dehnhard et al., 2010). Earlier study also showed seasonal differences in intact sperm percentage in Eurasian lynxes with the maximum of 26% in March (Jewgenow et al., 2006). It did not provide any significant difference here because of small sample size. It is necessary to note that two males were very old (13 and 15 years) and they were excluded of analyses in present study.

Sperms concentration in opposite to other sperm quality parameters in Eurasian lynx males was maximal in summer $490.8\pm177.7*10^6$/ml and was much lower in mating period $40.2\pm19.9*10^6$/ml. In tomcats high sperm concentration is usually related with the high percentage of morphologically abnormal sperms (more than 60%) (Howard & Wildt, 1990). We did not find such correlation in Eurasian lynx, but the lowest sperm concentration was described for the mating period when the percentage of morphologically intact sperms was at the maximum. Sperm concentration in Eurasian lynx was comparable with concentrations known for other cat species (Far-East wildcat *Prionailurus bengalensis euptilurus* — 73 mln/ml; cheetah — 22 mln/ml; Bengal cat *Prionailurus bengalensis* — 37 mln/ml; tomcats — 108 mln/ml) (Howard & Wildt, 1990; Crosier et al., 2007; Pavlova, 2010; Glukhov, Naidenko, 2013). In Eurasian lynxes sperm concentration was estimated before 0–30 mln/ml, but highly increased with the decrease of ejaculate volume (up to 540 mln/ml) (Jewgenow et al., 2006). It seems that concentration was hardly dependent on the volume of prostate secret. To our opinion sperms number per ejaculate could be more relevant parameter to estimate sperm quality in these species. It was 1.6 mln/ejaculate in Eurasian lynx (mating season, Jewgenow et al., 2006) or 1.2–7.5 (this study), 3.3 mln/ejaculate in Iberian lynx (Gañán et al., 2009a), 10.0 mln/ejaculate in bobcat (Gañán et al., 2009b). It allow us to estimate the number of sperms in bobcat as the highest, in two other species it was approximately the same.

An increase of testosterone level was described in August–September in Eurasian lynx males (lower than to mating season) and was not followed by the increased intensity of spermatogenesis. The reasons of this testosterone peak are not clear. An increase of testosterone in August-September coincides with the increase of vocal activity in this period related with the beginning of kittens' active movements on their home range (Rutovskaya & Naidenko, 2006). Vocal activity of females in enclosures may be a reason of androgens increase in Eurasian lynx males in captivity. Similar changes of males' hormonal status at this time of the year were never described in zoos or in the wild.

Results on dynamic of reproductive parameters (testosterone level in blood plasma and sperm quality indexes) in Eurasian lynx males support clear seasonal pattern of reproduction in this species. Testosterone level increases and spermatogenesis normalizes (increase of percentage of intact sperms and sperms motility in ejaculate) toward the mating season. Testosterone increase in August-September was not related with changes in exocrine function of their reproductive system.

Acknowledgments

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