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SHORT COMMUNICATIONS

Effect of Industrial Pollution of the Environment on the Frequency of Abnormal Spermatozoa in the Bank Vole, *Myodes glareolus*

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Stability of mammal populations exposed to toxic impact largely depends on the efficiency of reproduction. Therefore, reproductive parameters allowing estimation of potential and actual fertility are widely used in ecotoxicology [1–4]. One of direct methods for the diagnosis of male fertility is to assess different parameters of sperm, including the concentration, mobility, and morphology of spermatozoa. In turn, morphological assessment involves analysis of not only the shape and size of normal spermatozoa but also of the spectrum and frequencies of their pathological changes (anomalies, or defects).

Since various structural defects of spermatozoa are indicative of their reduced fertilizing capacity [5], the screening of male gametes is relevant for many fields of biology and medicine [6, 7]. In rodents, sperm morphology has been analyzed with respect to its genetic variability [8–10], relationship with age and hormonal and social status [11–13], and feasibility for evaluating the influence of various environmental factors [14–16].

It is generally accepted that spermatozoa are sensitive to toxic exposure: as shown in numerous laboratory experiments, sperm quality (including morphological parameters) deteriorates under its effect [17–19]. However, opposite examples are also known: no differences in sperm quality from the control group were reveled in mice poisoned with aluminum [1]. Relevant studies on rodents from natural populations are few, and their results are contradictory: some authors report an increase in the number of defective spermatozoa after toxic exposure [3, 4], while others have observed no such changes [2]. Moreover, there is no information on variation in the proportion of abnormal spermatozoa in the majority of rodent species living in the wild.

The purpose of this study was to estimate the effect of industrial pollution on the frequency of abnormal spermatozoa in the bank vole with regard to animal age and the year of capture. The hypothesis to be tested was that the frequency of abnormal spermatozoa is higher in animals from strongly polluted than from background (clean) areas.

The bank vole (*Myodes glareolus* Schreber, 1780), one of the best studied small mammal species, is widespread in forest ecosystems of the Middle Urals. This study was performed in the zone of impact from the Middle Ural Copper Smelter (MUCS), a major plant that have been in operation since 1940. The main components of emissions from the MUCS include gaseous compounds of sulfur, fluorine, and nitrogen and industrial dust containing heavy metals (Cu, Pb, Zn, Cd, Fe, Hg, etc.) and metalloids (As).

In 2014 and 2016, adult male voles were collected using live and crush traps in background and impact plots located 20–20 and 1.5–5 km west of the MUCS, respectively, in fir–spruce forest biotopes. Forest ecosystems in background plots were in a relatively undisturbed state, whereas their degradation was observed in polluted plots, with phytocenoses being affected in the first place. Technogenic degradation involves the accumulation of pollutants in soil and plants, fragmentation of habitats, and impairment of microenvironmental conditions, which may lead to decrease in the abundance of small mammal communities and impoverishment of their species composition [20]. Detailed information on pollution levels in these plots was published previously [21, 22]. In particular, it was found that cadmium concentrations in the forest litter and the liver of bank voles from polluted plots exceeded the background values by factors of 7–10 and 12, respectively.

The animals were euthanized by cervical dislocation either immediately after capture or after keeping in a vivarium for 1–3 days at room temperature and natural photoperiod. They were kept in cages with bedding of wood shavings, moss, or hay and had free access to water and food (oats, carrots, and apples). Analysis was performed with epididymal spermatozoa collected postmortem, which were possible in view of the data that they retain functional activity for a long

Table 1. Data on the effect of animal age, year of capture, and pollution level on the proportion of abnormal spermatozoa in the bank vole (results of logit regression)

Factor	Proportion of abnormal spermatozoa					
	with head defects			with tail defects		
	b (SE)	Wald $X^2(1)$	Odds ratio [95% CI]	*b* (SE)	Wald $X^2(1)$	Odds ratio [95% CI]
b_0	−3.69 (0.181)	418.4***		−1.20 (0.072)	278.4***	
Age	0.58 (0.165)	12.5***	1.79 [1.30–2.50]	−0.14 (0.064)	4.6*	1.14^{-1} [1.30–1.01]$^{-1}$
Year of capture	−0.23 (0.122)	3.5	1.26^{-1} [1.60–0.99]$^{-1}$	−0.25 (0.056)	20.2***	1.29^{-1} [1.43–1.15]$^{-1}$
Pollution level	−0.33 (0.162)	4.0*	1.38^{-1} [1.90–1.01]$^{-1}$	−0.21 (0.066)	9.7**	1.23^{-1} [1.40–1.08]$^{-1}$

b_0, reference group (young of the year from background plots, 2014); *b,* regression coefficient; SE, standard error; *p,* significance level; CI, confidence interval; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

time and that the ratio of normal and defective spermatozoa remains unchanged for several days after death [23].

With regard to a series of morphological characters, male voles were divided into two ager groups: overwintered individuals and young of the year. Animals included in analysis had no signs of the involution of reproductive organs: testis weight was at least 250 mg, and the weight of seminal vesicles, at least 125 mg. In the background plots, 18 overwintered voles and 1 young of the year were trapped in 2014, and 13 overwintered voles and 8 young of the year in 2016; in the polluted plots, 1 and 5 ind. in 2014 and 4 and 2 ind. in 2016, respectively (a total of 52 ind.).

Sperm morphology was analyzed in smear preparations of unstained cells from the epididymal cauda fixed with 96% ethanol, which were examined and photographed under a Leica DM1000 LED microscope with a Leica DFC 295 digital camera (Leica Microsystems, Germany) at ×400 magnification. Spermatozoa were divided into three groups: normal, (without deformation of structural components), with defects of the head (including deformation of the acrosome), and with defects of the tail (different variants of loops and hairpin bends). For each animal, 200 cells were analyzed in each of 15–30 microscopic fields chosen at random to calculate the proportions of normal and defective spermatozoa.

Correlations between different defects were evaluated by calculating Spearman's rank correlation coefficient. A search for possible predictors of pathology in the development of spermatozoa (animal age, year of capture, pollution level) was performed using generalized linear models (GLM) for dichotomous characters (multiple additive logit regression). Odds ratios (OR) and their (CI) confidence intervals were expressed as exponentiated logarithms of logit regression coefficients, or logarithms of OR: exp(lnOR) or 1/exp(lnOR). Statistical analysis was performed using the Statistica 8.0 package, with one animal being taken as a statistical unit.

No correlation was revealed between tail defects and head defects: for the total sample, $r = 0.02$, $p = 0.88$; for the background samples, $r = -0.07$, $p = 0.67$; for the impact samples, $r = 0.33$, $p = 0.29$. The frequency of spermatozoa with a coiled tail proved to depend on every factor included in analysis: it was lower in overwintered voles than in young of the year by a factor of 1.1; in 2016 than in 2014 by a factor of 1.3; and in animals from impact than background plots by a factor of 1.2 (Table 1, Fig. 1). Head defects occurred 1.8 times more frequently in overwintered voles than in young of the year and 1.4 times more frequently in animals from background than impact plots, but no between-year differences in their frequency were revealed. Despite formal statistical significance, the observed effects are very weak, with OR close to unity; therefore, the probability of revealing abnormal spermatozoa relying on some of these factors is low.

Spectrum of sperm defects. All classifications of pathological changes in spermatozoa are based on their localization to certain structural elements of the cell. It is considered that the causes of these changes are different: defects of the head (the nucleus and acrosome) such as changes in size and shape, condensation or fragmentation of chromatin result from atypical spermiogenesis and apoptosis, and tail defects are caused by genomic mutations [7]. Despite the common "topographic" principle, no unified classification of sperm pathology has been proposed as yet. Wyrobek and Bruce [6] distinguished six classes of spermatozoa with various head and tail defects. Many authors included in analysis only head defects in rodent spermatozoa, distinguishing three [9], four [13], or five types [8] of such defects. The level of detail in classifying sperm defects is sometimes very high: for example, 17 types of head and tail defects were distinguished in Syrian hamsters [24]. The WHO classification of human sperm pathologies was also applied to small rodents [25]. Some authors proposed a transitional category of "quasi-normal" cells to avoid diffi-

culties in differentiating between normal and defective spermatozoa [26].

We considered only two categories of pathologies, head and tail defects, which allowed our results to be compared with data by other authors who used different classifications. In addition, a simplified classification without detailed differentiation between defects may be applicable to mammals in which the shape of sperm head is less complex (e.g., to small insectivores).

The problem of classifying sperm defects is related to discussion of the prognostic significance of sperm morphological characters for estimating potential fertility [27]. The majority of authors consider that head defects leads to reduced fertilizing capacity and developmental defects in the offspring [28–30]. However, Burruel et al. [26], taking into account the data that all necessary genes and enzymes are preserved in defective spermatozoa, conclude on this basis that morphological defects of sperm head are not indicative of fatal pathology of male gametes. On the other hand, Kishikawa et al. [27] revealed chromosomal aberrations in 15% of morphologically normal spermatozoa of BALB/c mice. Certain skepticism in relation to the prognostic value of sperm morphological characters among reproductologists is reflected in the decreased significance assigned to the proportion of normal spermatozoa as a reference parameter [29].

As shown by Osadchuk and Kleshchev [10], male fertility can be maintained at an optimal level due to compensatory variation in the parameters of epididymal sperm: in CBA/Lac mice with a low concentration of spermatozoa, their mobility was increased and the proportion of defective cells among them was lower. Therefore, the entire set of sperm parameters should be taken into consideration for a more comprehensive analysis of fertility.

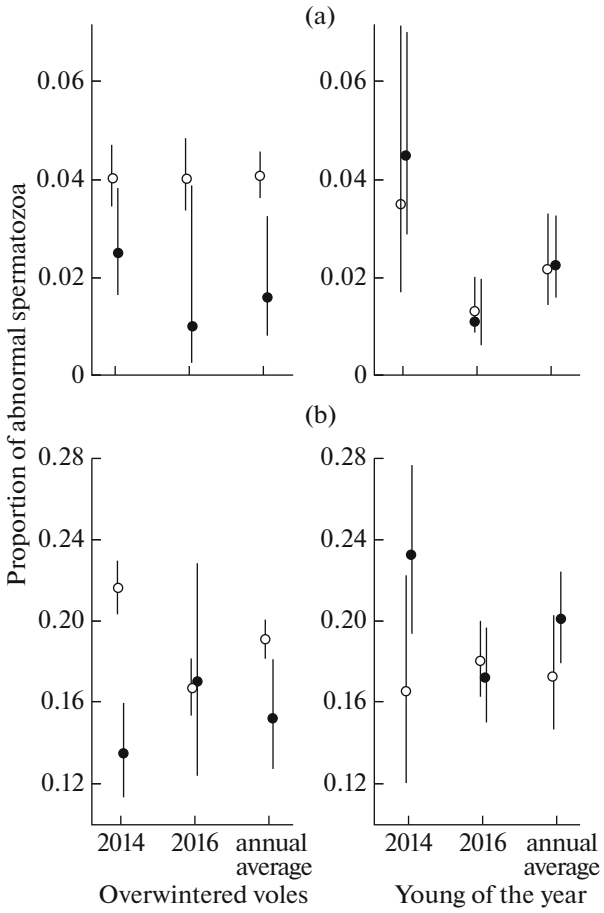

Fig. 1. Proportions of abnormal spermatozoa (a) with head defects and (b) tail defects (average values with confidence intervals) in bank voles from background plots (white dots) and polluted plots (black dots) in the vicinities of MUCS.

Frequency of sperm defects. Published data on the ratio of normal and defective spermatozoa show a wide scattering of its values in rodents even in the absence of toxic influences. For example, about 14% of spermatozoa with head defects were revealed in normal CBA mice [8], and the proportion of such cells found in mice of 13 inbred lines varied from 5 to 20% [31].

It has been found that the proportion of abnormal spermatozoa is correlated with animal age and physiological (hormonal) status [11, 13]. In particular, the proportion of abnormal epididymal spermatozoa in Syrian hamsters proved to decrease almost threefold upon transition from puberty to maturity [11], and the proportion of spermatozoa with head defects in bank voles kept under laboratory conditions varied from 3 to 16%, with the highest values recorded in the youngest and oldest animals aged 1.5 and 12–15 months, respectively [13]. Our results concerning the frequency of head defects agree with published data: they occurred more frequently in overwintered voles than in young of the year, but tail defects were more frequent in the latter group. Different directions of age-related changes in the proportions of head and tail defects may be due to the difference in their genesis. It is noteworthy that their frequencies differently depended on the year of capture (a kind of substitute for parameters such as ecological conditions and population density): the frequency of head defects did not vary between years, unlike the frequency of tail defects.

Species specificity of the spectrum and frequency of defective spermatozoa has been revealed in five rodent species: *Myodes glareolus, Phodopus campbelli, Ph. sungorus, Apodemus agrarius,* and *Acomys cahirinus*, with the highest and proportions of abnormal cells (less than 10 and more than 90%) being characteristic of *A. cahirinis* and *M, glareolus,* respectively [25].

Toxicological studies also provided evidence for high variation in the proportion of abnormal spermatozoa in rodents. This proportion in CF-1 mice increased from 3.7% in the control to 9.7% upon lead poisoning [19], and in Swiss mice, from 9% in the control to 21% upon aluminum poisoning [1]. The proportion of spermatozoa with coiled tails (86%) was

found to be several times higher than that of cells with head defects (14%) [1], which is comparable to our data. Notable differences between the frequencies of the two types of defects may be regarded as additional evidence that they have different etiology.

Miska-Schramm et al. [3, 4] observed "classical" dose effects in experiments with bank voles from a laboratory colony: the proportion of head defects increased from control 22% to 43% upon aluminum poisoning and to 54% upon copper poisoning. Species specificity of changes in the frequency of abnormal spermatozoa was revealed in one of a few studies on rodents from natural populations [2]: their frequency in the control was higher in yellow-throated mice than in bank voles, and significant differences in this frequency between animals from background and polluted areas were observed only in the former species.

Our results are opposite to what was expected: the probability of finding animals with abnormal spermatozoa in the sample from strongly polluted plot proved to be lower than in the background sample. A negative correlation between the frequency of defects and the level of toxic exposure was also observed in our previous studies on the frequencies of splenomegaly [32] and nephropathies [33] in the bank vole. This phenomenon may be attributed to natural factors whose effect is more powerful than that of pollution (infections, invasions, autoimmune diseases, etc.) or to more rapid elimination of animals with defective sperm in polluted than in background areas.

Thus, spermatozoa with tail defects can always be found in the epididymal cauda, whereas those with head defects occur more rarely or may be absent. These groups of defects are not correlated with each other, and differences in their frequencies and "responses" to factors of animal age and the year of capture indicate that the causes of these defects are different. The frequency of spermatozoa with head defects depends on animal age, and that of cells with tail defects, on both age and the year of capture. The frequencies of both defects depend on pollution level, but animals with abnormal spermatozoa unexpectedly proved to be more frequent in the control than in polluted areas. However, the observed effects are very weak and can be regarded as random. It is apparent that further studies are needed to elucidate this issue, which should be performed on significantly larger samples, including those of other species and animals from different localities, and should also involve analysis of not only morphological parameters of spermatozoa but also of their concentration and mobility. Despite the negative conclusion about the test hypothesis, the results of this study make it possible to estimate the magnitude of changes in the proportion of abnormal spermatozoa depending animal age, year of capture, and pollution level in one of small mammal species widely used in ecological research.

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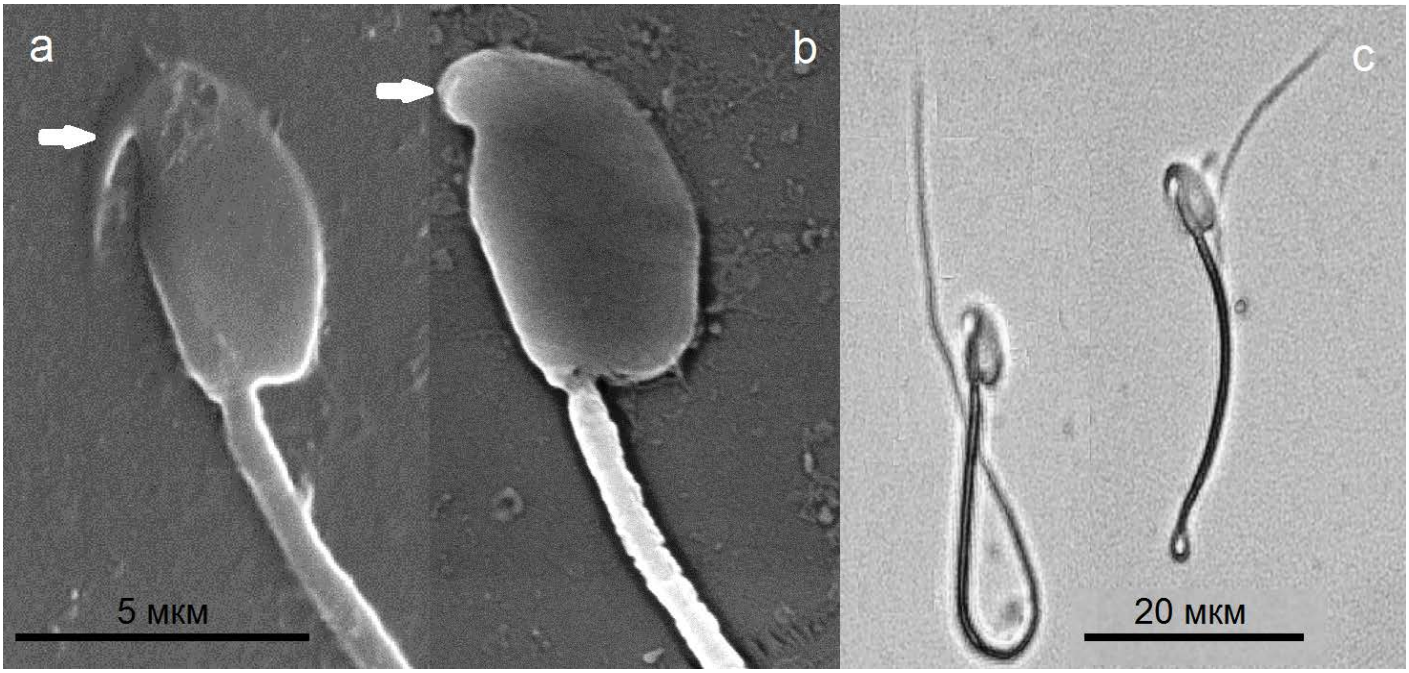

Figs a-c. Spermatozoa of the bank voles: a - with a normal head, b - with a head defect (swelling, acrosome deformation), c - with loop and hairpin bend. The arrows indicate the acrosome region of the sperm head. Figures a, b were obtained using scanning electron microscopy (VEGA \\ SBU, Tescan, Czech Republic).