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RESEARCH

Morphological parameters of hepatocytes in the European mole (*Talpa europaea*) and herb field mouse (*Sylvaemus uralensis*) under industrial pollution: Qualitative and quantitative assessment

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Abstract Morphological alterations of cells and tissues usually occur in biological organisms exposed to environmental contaminants, there by acting as a biomarker of environmental pollution, thus, making this study highly pertinent. The effect of industrial pollution on the qualitative and quantitative morphological parameters of hepatocytes (through histological analysis and cytomorphometry) was studied in two contrasting species of small mammals (*Talpa europaea* and *Sylvaemus uralensis*), taking into account the animal age (young and adult groups) and liver concentrations of heavy metals (Cu, Zn, Cd, Pb). Studies were performed in the regions exposed to emissions from two currently operating copper smelters: Middle Ural Copper Smelter (Middle Urals, *T. europaea* catching area) and Karabash Copper Smelter (Southern Urals, *S. uralensis* catching area). Seven morphometric parameters of hepatocytes were measured, of which two key parameters were selected by the method of principal components—the cell packing density and nuclear-cytoplasmic ratio (*N*/*C*). It was found that cell packing density in *T. europaea* from the impact zone decreased relative to the background area in young animals. At the same time, the differences in this parameter between the age groups from the background zone were leveled in the impact area of catching. The *N*/*C* ratio in *T. europaea* hepatocytes showed no correlation with either animal age or site of capture (background or impact area). In *S. uralensis*, both parameters, even taking into account the age, were found to be insensitive to indicate an effect of industrial pollution. Dystrophic changes (tested through histological analysis) in the liver tissue were revealed in all animal groups, but their frequency did not depend on any of the factors (age, zone) as well as the level of accumulation of toxic heavy metals (Cd, Pb). Morphometric parameters of hepatocytes have proved to be more reliable indicators of pollution, compared to the frequency of liver histopathology, due to lower subjectivity in their evaluation.

Keywords Liver · Histopathology · Cytomorphometry · Small mammals · Copper smelters · Heavy metals

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Introduction

In the last century, the multicomponent technogenic impact in ecosystems has become increasingly large scale, and its consequences are severe. Animals from natural populations that permanently live and/or feed within or near polluted areas are exposed to a complex mixture of chemical compounds that may produce independent, additive, or synergistic effects (Bellés et al., 2002; Cobbina et al., 2015; García-Barrera et al., 2012; Sexton & Hattis, 2007). Many studies report increased levels of heavy metal accumulation in the body of small mammals from industrially polluted areas (Camizuli et al., 2018; Fritsch et al., 2010; Kalisińska, 2019; Pankakoski et al., 1994; Suzuki et al., 2006). In different groups of mammals, micronutrient contamination has been shown to degrade the overall condition and reproductive abilities of parent animals and reduce offspring survival, even when the actual doses of contaminants were well below the lethal levels (Levengood & Heske, 2008; Miska-Schramm et al., 2014; Scheuhammer et al., 2007).

Morphology-based methods of studies on vertebrate organs and tissues are widely used to indicate industrial pollution and assess toxic risk for terrestrial and aquatic ecosystems (Au, 2004; Bartlow, 2019; Bernabò et al., 2017; Binkowski et al., 2013; Tête et al., 2014). Highly relevant methods include analysis for the frequency of histological patterns directly related to the health of animals from polluted environments (Damek-Poprava & Sawicka-Kapusta, 2003, 2004; Au, 2004; Salińska et al., 2012, 2013; Tête et al., 2014; Amuno et al., 2016). The main advantages of qualitative assessment of tissue characteristics are that this method is relatively sensitive, can reveal dose–response relationships, and produces interpretable results, i.e., it allows detecting of morphological alterations and assessing their relationship with the impact level. Its main drawbacks are subjectivity and insufficient formalization (Gibson-Corley et al., 2013; Hardisty & Brix, 2005; Holland & Holland, 2011).

Cytomorphometry can detect structural changes in cases where toxic substances act at a low concentration that cannot be detected by chemical analytical methods. Therefore, morphometric parameters can serve as cellular biomarkers of the early stages of stress caused by toxic factors, including industrial pollution (Gaidash & Klimatskaya, 2004; Sánchez-Chardi et al., 2009a; Sorensen, 1989; Visscher & Stifano, 1981). Cytomorphometry is used less frequently, compared with histopathological analysis, because this method is labor-intensive and reference parameter values have not been established for the majority of animal species.

Qualitative estimates of the frequency of histopathology and quantitative cytomorphometric data characterize the morphology of animal organs at different organization levels: at the tissue level and at the cell level. Using both methods together is a way to alleviate their drawbacks and compare the effects at different levels. In most cases, this approach is applied to humans and homogeneous samples of commercially important or laboratory animals (Chen et al., 1984; Gooneratne et al., 1980; Jarrar & Taib, 2012). In this study, we compared the results of qualitative and quantitative assessments of organ micromorphology in mammals from natural populations, which have been poorly studied in this respect (see Sánchez-Chardi et al., 2009a; Shinkarenko & Savchenko, 2015). The study objects of small mammals are traditional for ecotoxicological research in the liver, the most important detoxification organ (Treinen-Moslen, 2001), and hepatocytes, the most numerous multifunctional liver cells (Ham & Cormack, 1974).

We previously found out that spontaneous pathological changes occur in the liver of small mammals from undisturbed (background) areas (Davydova et al., 2017), which provides the possibility to compare their frequency at different levels of pollution. Moreover, we have shown that the morphometric parameters of cells differ between species, and the variability of hepatocytes should therefore be analyzed separately in each species. We also considered it necessary to take into account the age of animals as another important source of cell variability (O'Neil et al., 2013; Watanabe et al., 1978). Potential relationships between morphological changes in the liver and individual toxic load were evaluated by measuring the concentration of essential (Cu and Zn) and the most toxic (Cd and Pb) heavy metals (Andjelkovic et al., 2019; Kalisińska, 2019; Ostoich et al., 2020; Świergosz-Kowalewska, 2001; Wu et al., 2016). These elements are priority pollutants in the vicinity of Ural copper smelters (Mukhacheva et al., 2010; Vorobeichik & Kaigorodova, 2017).

The purpose of this study was to assess the effect of industrial pollution caused by copper smelting on the morphological parameters of hepatocytes in two highly different mammalian species, the European mole and herb field mouse, taking into account animal age. We expected that;

(i) morphometric parameters would be more informative than the frequency of histopathology, since the estimation of these parameters is less subjective (Gibson-Corley et al., 2013;

Hardisty & Brix, 2005; Holland & Holland, 2011);

(ii) toxic effects would be expressed more strongly in the mole than in the herb field mouse, since the former species is exposed to chemical pollutants to a greater extent and accumulates higher concentrations of these substances due to its mode of life (Hunter et al., 1987; Heske et al., 2003; Mukhacheva, 2004; Schipper et al., 2008).

On the whole, the morphological assessment of the microscopic appearance of the liver can provide information on the potential toxicity of a chemical (Hardisty & Brix, 2005). In turn, this information may provide an insight into the mechanism of toxicity and guide further study.

Materials and methods

Study areas

Studies were performed in the Russia's regions exposed to emissions from two currently operating copper smelters. Moles were collected at the vicinity of the Middle Ural Copper Smelter (MUCS; Sverdlovsk oblast, the Middle Urals), herb field mice, near the Karabash Copper Smelter (KaCS; Chelyabinsk oblast, the Southern Urals) (Fig. 1). The smelters have much in common: both are point polluters that, in the study period, have produced comparable amounts of emissions with a similar spectrum of priority toxicants, including sulfur dioxide (SO_2), metals (Cu, Zn, Pb, Cd, Fe, Hg, etc.), and metalloids (As). Moreover, there are comparable levels of heavy metal accumulation in different components of the biota around both smelters (Table S1). Since both smelters have long been in operation (MUCS, since 1940; KaCS, since 1910), technogenic geochemical anomalies have been formed in their vicinities. In the vicinity of MUCS, for example, the contents of metals and other elements in the soil are 10–100 times higher than their background levels (Vorobeichik & Kaigorodova, 2017). Although both smelters were reconstructed and emissions have been reduced during the past decades, they still have a strong impact on ecosystems (Mukhacheva, 2017; Mukhacheva et al., 2010).

Trapping plots were established in zones with different degrees of damage to ecosystems, namely, the conditionally clean background zone (Bg) and heavily polluted impact zone (Im) at distances of 20–30 and 1–4 km from the polluter, respectively. However, the impact plots for the mole were established 5–10 km west of the polluter since this species was absent in the close vicinities of the smelters, with the so-called

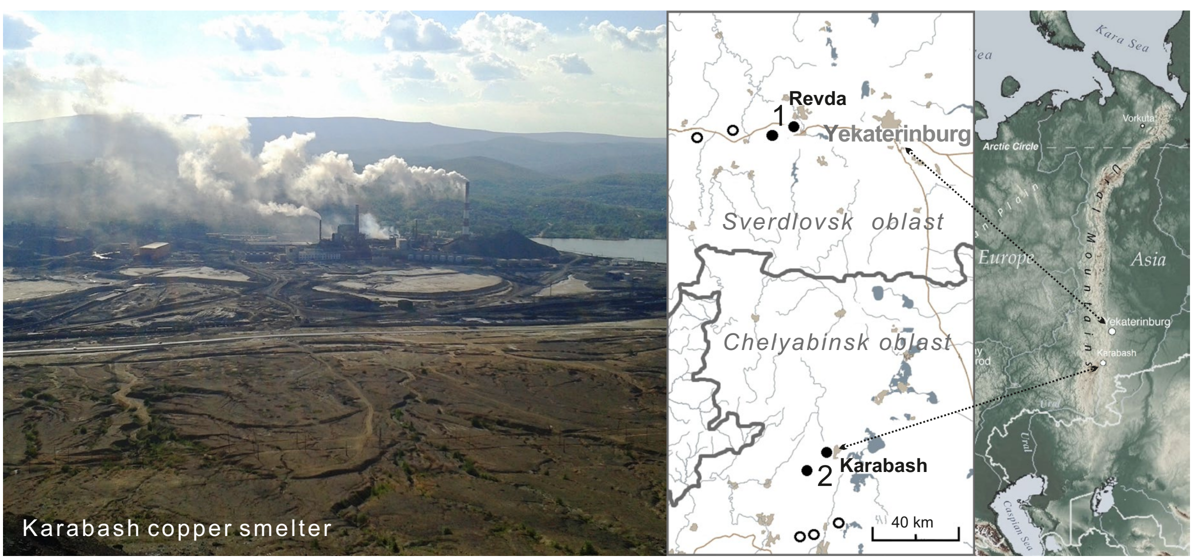

Fig. 1 Locations of sampling plots in the vicinities of (1) MUCS and (2) KaCS: white circles, background plots; black circles, impact plots

mole desert being formed there. The main factor responsible for the disappearance of moles from in these heavily polluted areas is the reduction of their food resources (Nesterkova, 2014; Vorobeichik & Nesterkova, 2015).

Zones with different pollution levels were distinguished based on the data of geobotanical relevés and the contents of heavy metals in the forest litter. Forest ecosystems in unpolluted plots were in a relatively undisturbed state. As the distance to the polluter decreased, a gradual transformation of various environmental parameters was observed, including an increase in the contents of metals in biological substrates (forest litter, soil, plants, and depot organs of small mammals), a decrease in soil pH, fragmentation of habitats, deterioration of microenvironmental conditions, and reduction of biodiversity and productivity of herbaceous and tree layers (Mukhacheva et al., 2010; Trubina et al., 2014; Vorobeichik & Kaigorodova, 2017). An important consequence of this transformation is a decrease in the abundance of small mammal communities and impoverishment of their species composition (Mukhacheva, 2021; Mukhacheva et al., 2012).

Trapping was carried out in fir–spruce and birch forest biotopes in the region of MUCS and in pine–birch forest biotopes in the region of KaCS.

Choice of animal species

Species selected for study—the European mole (*Talpa europaea* L., 1758) and herb field mouse (*Sylvaemus uralensi*s Pallas, 1811)—represent different orders of mammals (Insectivora and Rodentia) and differ from each other in life span, habitat, mode of life, social structure, reproductive features, type of feeding, and metabolic rate.

The mole leads an exclusively underground way of life, moving mainly within the limits of its home range (approximately 2300 m^2 in size, extending for about 50 m) (Macdonald et al., 1997). The life span of a mole reaches 6 years (Nesterkova, 2019). This animal is an obligate zoophage, with its diet consisting mainly of earthworms (Deparma, 1951; Godfrey & Crowcroft, 1960). The herb field mouse, in contrast, lives on the ground and actively moves both within individual home ranges and over long distances (up to 9 km) (Grigorkina & Olenev, 2018), has a life span of 1 year, and shows granivorous feeding habits, with its diet consisting mainly of plant seeds and fruits (Bol'shakov et al., 2006).

In addition, it is known that small insectivores (Insectivora) accumulate more toxicants than rodents (Rodentia) (Hunter et al., 1987; Schipper et al., 2008) and voles (Arvicolinae) than in mice (Murinae) (Heske et al., 2003). We previously studied heavy metal concentrations in the body and food of 12 insectivore and rodent species from five genera living in the Middle and Southern Urals (Mukhacheva, 2004). According to the levels of metal accumulation, these genera be arranged in the following series: *Talpa* > *Sorex* > *Microtus* > *Clethrionomys* > *Sylvaemus*. Thus, the choice of these species is explained by very high differences in heavy metal accumulation between them also.

Animal sampling

Moles and rodents were captured with Falkenstein–Popov's wire traps (Deparma, 1951) and wooden treadle-type live traps, respectively, which were checked several times a day (Table 1). The animals were collected alive to exclude the effect of cadaverous autolysis. According to censuses taken in the study years, the abundance of moles in the background plots varied from 1.4 to 11.4 tunnels per km route, and that of herb field mouse, from 1.8 to 3.0 ind./100 trap–days. The animal abundance in the impact plots was significantly lower, sometimes dropping almost to zero. Moreover, the abundance of herb field mice in the vicinity of MUCS was much lower than that of moles (only 0.3–0.4 ind./100 trap–days), which does not allow us to obtain representative samples of each species from this area. It should also be noted that moles and mice were collected from different impact plots, where the concentrations of pollutants in substrates were also different. Taking this into account, comparisons between the two species were made using samples from the vicinities of different copper smelters, giving special attention to the basic similarity of the two copper-smelting industries and the contrasting environmental conditions in the background and impact areas inhabited by the test species.

Trapped animals were kept in a vivarium for 1–3 days at room temperature and natural photoperiod to level off differences in their body condition. Moles were placed in special plastic containers with soil and forest litter and fed earthworms. Herb field mice were supplied with wood shavings for bedding and fed oats and carrots. Food and water were

Table 1 Sampling areas and sample structure and sizes

Species	Copper smelter	Years	Age group	Number of animals*	
				Back-ground zone	Impact zone
Talpa europaea	MUCS	2008–2010	Young	6/1	2/5
			Adult	2/4	2/4
			Total	8/5	4/9
Sylvaemus uralensis	KaCS	2012–2014	Young	7/7	3/11
			Adult	3/1	3/1
			Total	10/8	6/12

*Numbers of animals with normal / pathological liver. The absolute age of young and adult *T. europaea* varied within the ranges of 1.5–5 and 12–73 months, respectively

provided ad libitum. To perform external examination and autopsy, the animals were anesthetized with ether and sacrificed by cervical dislocation.

The samples of each species included individuals of different age group and sex (Table 1; Table S2). We distinguished them into two age groups, young and adult, based on odontological and morphometric parameters (body length, weight, and the length to weight index) and the state of the reproductive system. Animals trapped in the year of birth were classified as young, and animals born in the previous year or earlier (in rodents, overwintered animals) were included in the adult group. The absolute age of moles was determined by annual growth layers in teeth according to Klevezal (2017) (Fig. S1). A subsample of moles for histological analysis of the liver ($n = 26$) was selected from the total sample ($n = 127$) using a random number generator, taking into account the two age groups, while that of the herb field the entire sample ($n = 36$) was used for this purpose. Samples of the liver for chemical analysis (as a rule, fragments of lateral lobes) and histological analysis (fragments of medial lobes not adjoining gallbladder) were taken simultaneously. The former were dried, and the latter were fixed in 10% formalin.

Moreover, the large amount of data on elements accumulation (Cu, Zn, Cd, Pb) in the liver are available for the two species from the regions of MUCS and KaCS over the period of 2007–2014 (*T. europaea*, $n = 209$; *S. uralensis*, $n = 245$; below, referred to as extended samples). These data were used for analyzing individual toxic loads.

Chemical analysis

Samples of the liver tissue from each animal (*T. europaea*, $n = 26$; *S. uralensis*, $n = 35$) were dried at 75 °C to the air-dry state, ground, and weighed on a KERN-770 analytical balance (Germany) with an accuracy of 0.01 mg. Aliquots of the liver powder (about 100 mg) were placed in Teflon bombs containing 7 mL of 65% HNO_3 (ultra high purity) mixed with 1 mL of deionized H_2O, incubated for 30 min, and incinerated in an MWS-2 microwave oven (Berghof, Germany). The sample was then diluted with deionized H_2O, adjusting its volume to 10 mL. The heavy metals concentrations (µg/g dry weight) were measured in ContrAA 700 vario atomic absorption spectrometers (Analytik Jena, Germany) with flame (for Cu and Zn) or electrothermal (for Cd and Pb) atomization. Analysis was performed in the Laboratory of Ecotoxicology of Populations and Communities, Institute of Plant and Animal Ecology (Yekaterinburg, Russia), certified for technical competence (certificate No. ROSS RU0001.515630). The quality of measurements was estimated with reference to the International Standard CRM 185R (bovine liver). The average extraction percentages were 93.2% for Cu, 99.8% for Zn, 114.2% for Cd, and 94.4% for Pb. The respective detection-limit concentrations in the flame atomization variant were 0.03 for Cu and 0.015 µg/mL for Zn; in the electrothermal atomization variant, 0.0008 for Cd and 0.0025 µg/mL for Pb. If the element concentration was below the detection limit (*nd*), the value equal to half of the detection limit was used for statistical analysis. The heavy metals concentrations in the extended samples were analyzed similarly.

Micromorphological analysis

Tissue samples were embedded in paraffin, and standard Sects. 5–7 µm thick were stained with Mayer's hematoxylin and eosin and examined under a Leica

DM1000 LED microscope with a Leica DFC 295 color digital camera (Leica Microsystems, Germany).

Qualitative assessment

All the samples were examined to visually determine whether the liver tissue was in a normal or pathological state (Table 1). The most frequent pathology was parenchymal dystrophy indicative of disturbances in cell metabolism and accompanied by degeneration of hepatocytes (the presence of granules, coagulations, vacuoles, or lipid droplets in the cytoplasm, deformed nuclei, etc.) (e.g., see Thoolen et al., 2010) (Fig. 2; Fig. S2). In extreme cases of liver dystrophy, pycnosis and lysis of the nuclei and anucleate hepatocytes were observed. To avoid the effect of confirmation bias, the assessment was independently performed by two specialists in a blind way.

Quantitative assessment

In view of the functional heterogeneity of liver tissue (Gebhardt & Matz-Soja, 2014; Gumucio, 1989; Lamers et al., 1989), morphometric analysis was performed in the central zones of the liver lobules in order to unify measurements. It is considered that liver cells located around the central vein of the lobule are relatively poorly supplied with oxygen and nutrients (Jungermann & Kietzmann, 1996; Kietzmann, 2017) and are therefore more sensitive to various factors. Measurements were made in microscopic images (magnification 630×, 10–20 images per animal) using ImageScope M software (http://www.microscop.ru).

Parameters measured in mononuclear hepatocytes (100 cells per animal) included the maximum (a) and minimum (b) diameters of the nucleus and the area of the cell (S_{cell}) in projection on the image plane. The results were used to calculate the areas of the nucleus ($N = a/2 \times b/2 \times \pi$) and cytoplasm ($C = S_{cell} - N$) and the nuclear–cytoplasmic ratio (N/C). Variation in the nuclear and cell size (in medical terms, anisokaryosis and anisocytosis) was estimated by calculating the interdecile range. The hepatocyte packing density and the proportion of binucleate cells in the liver parenchyma were evaluated in 10 microscopic fields per sample (one field ≈ 10,000 μm^2) (Fig. 2).

Data analysis

The results were processed statistically with Statistica v. 8.0 (StatSoft Inc., 2007), AtteStat v. 13.1 (Gaidyshev, 2015), and R-project software packages (R Core Team, 2019), with an animal being taken as a statistical unit. Correlations between different morphometric parameters of hepatocytes were evaluated using Pearson's linear correlation coefficient (r), and the nonparametric Mann–Whitney U test was used to compare these parameters in normal and pathological liver. The most informative morphometric parameters were chosen based on principal component analysis.

Differences in the frequency of liver pathology between animal groups were evaluated by Pearson's χ^2 contingency tables. Sparse tables (with frequencies of no more than 5 in some cells) were tested for homogeneity by Simonov–Tsai statistics, with the results being used to determine the validity of approximation by χ^2. In case of problems with this approximation, the Freeman–Halton extension of Fisher's exact test was applied.

Factors of variation in the selected morphometric parameters were evaluated using generalized linear models (GRM/GLM) for continuous data, which allowed the effect of each factor to be estimated with regard to the effects of other factors (Draper & Smith, 1966). Sex-dependent variation in the parameters of hepatocytes was not analyzed because of insufficient sample sizes. The effects of factors "age" and "zone" were assessed separately for each species.

In most cases, the distribution of morphometric parameters of hepatocytes and heavy metals concentrations were close to lognormal. Descriptive statistics such as the arithmetic mean, standard deviation or error, and minimum and maximum values were calculated for these parameters. Factors influencing heavy metal accumulation in the liver were evaluated using different models of ANOVA, including the randomization test that is insensitive to deviations from normality and uniformity of variances (Good, 2006). Element concentrations were log-transformed. The correlation between element concentration and morphometric parameters of hepatocytes was evaluated using Pearson's correlation coefficient and that between element concentration and liver dystrophy, using Spearman's correlation coefficient (R).

The false discovery rate (FDR) in multiple comparisons of statistical hypotheses was controlled using the Benjamini–Yekutieli procedure (Benjamini & Yekutieli, 2001).

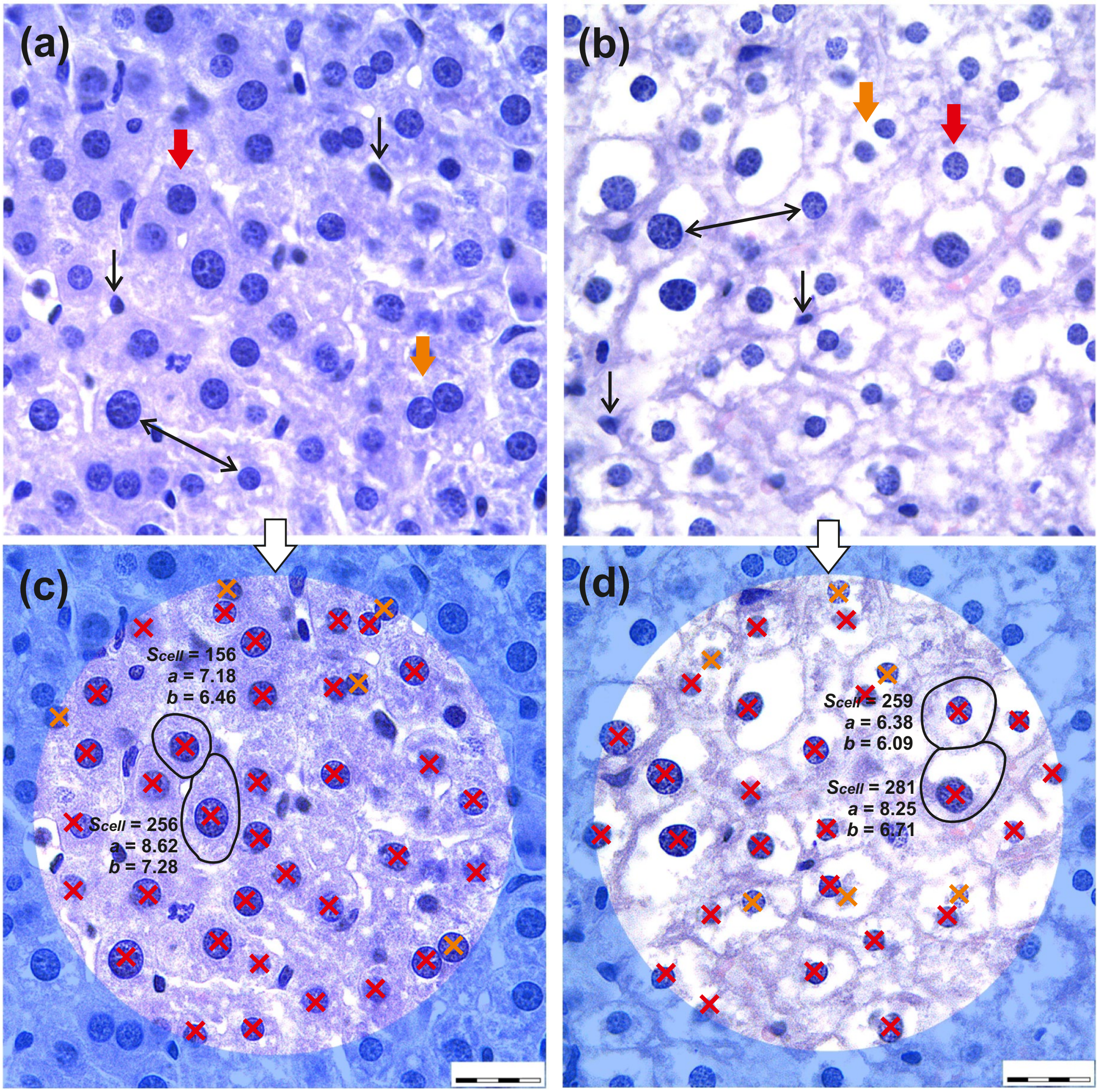

Fig. 2 Histological structure of **a** normal and **b** dystrophic liver in *Sylvaemus uralensis*: mononuclear hepatocyte (red arrow), binucleate hepatocyte (orange arrow), anisokaryosis (double-headed arrow), and Kupffer cells (black arrow). Below **c** and **d** are the same microscopic fields (≈ 10,000 μm.^{2}) with the examples of measurements of cell area (S_{cell}) and the maximum (a) and minimum (b) diameters of the nucleus and different hepatocytes (red and orange crosses) (hematoxylin–eosin; scale bar, 20 μm)

Results

Heavy metal concentrations

The concentrations of different metals were similarly correlated with each other in both species. Copper concentrations showed a correlation with those of zinc (for *T. europaea*, $r=0.7$; for *S. uralensis*, $r=0.6$, $P<0.0001$) and lead (for *T. europaea*, $r=0.5$, $P=0.006$; for *S. uralensis*, $r=-0.4$, $P=0.017$). Zinc concentrations also showed a correlation with lead (for *T. europaea*, $r=0.5$, $P=0.009$; for *S. uralensis*,

$r = -0.4$, $P = 0.014$). Cadmium showed no correlation with any of the metals.

In the moles analyzed for liver micromorphology (Table S3), the dependence of cadmium accumulation on factors "age" and "zone" was observed: cadmium concentrations were higher in adult than in young animals ($F_{(1, 26)} = 76.5$, $P < 0.001$) and in animals from the impact zone than in those from the background zone ($F_{(1, 26)} = 8.3$, $P = 0.010$). The effect of the interaction of factors "age" and "zone" was not significant ($F_{(1, 26)} = 0.9$, $P = 0.361$), although in young animals from the impact zone, the cadmium load increases by 1.7 times, and in adults one by 2.5 times. Concentrations of Zn, Cu, and Pb did not depend on any of the factors "age" and "zone" (Table S3; Table S4).

In herb field mice, copper accumulation proved to depend on factor "age," and zinc accumulation, on factor "zone." Copper concentrations were also higher in adult than in young animals ($F_{(1, 35)} = 19.9$, $P < 0.0001$), and zinc concentrations were higher in animals from the background zone than in those from the impact zone ($F_{(1, 35)} = 5.6$, $P = 0.024$) (Table S3; Table S4).

Table 2 presents the results for the expanded samples from the MUCS and KaCS regions (*T. europaea*, $n = 209$; *S. uralensis*, $n = 245$), which turned out to be more significant for testing the heavy metal loads differences in animals from the background and impact zones. So, the weak effect of the interaction of factors "age" and "zone" was manifested in *T. europaea* by cadmium load ($F_{(1, 209)} = 3.9$, $P = 0.051$) (Table S5): it increases by 2.3 times in young animals individuals and 3.8 times in adult one (Table 2). The cadmium load is also higher by 2.5 times in *S. uralensis* from the impact zone ($F_{(1, 209)} = 17.4$, $P < 0.0001$), regardless of age (Table 2; Table S5). In addition, there were differences in the concentrations of zinc ($F_{(1, 209)} = 24.3$, $P < 0.0001$) and lead ($F_{(1, 209)} = 9.7$, $P = 0.002$) in *T. europaea* from the background and impact zones, regardless of the age group (Table S5). Zinc increases by 1.3 times, and lead by 1.5 times (Table 2).

Frequency of histopathology

Pathological changes in the liver tissue were revealed in all animal groups, but their frequency did not depend on any of the factors: for *T. europaea*, χ^2_{age} $(1) = 1.5$, $P = 0.82$; χ^2_{zone} $(1) = 2.5$, $P = 0.70$; for *S. uralensis*, χ^2_{age} $(1) = 3.9$, $P = 0.20$; χ^2_{zone} $(1) = 1.8$, $P = 0.46$.

Individual levels of heavy metal accumulation in *T. europaea* showed no correlation with the frequency of histopathology: $R = -0.1$ to -0.4, $P = 0.48 - 0.18$. In *S. uralensis*, the same was observed for cadmium

Table 2 Heavy metal concentrations (μg/g dry weight) in the liver of *Talpa europaea* and *Sylvaemus uralensis*

Element	Zone	Species			
		Talpa europaea ($n = 209$)		*Sylvaemus uralensis* ($n = 245$)	
		Young	Adult	Young	Adult
Cu	Bg	35.3 (18.2) [70] 14.2–96.9	29.3 (14.5) [35] 9.9–90.4	13.2 (5.1) [61] 6.9–31.1	12.8 (4.8) [50] 5.7–30.3
	Im	34.9 (19.8) [70] 11.5–102.2	39.5 (16.4) [33] 16.0–84.7	12.9 (4.8) [101] 7.0–33.3	14.0 (5.1) [33] 9.4–32.2
Zn	Bg	128.6 (52.2) [70][a] 68.2–282.8	113.6 (34.7) [35][a] 66.0–226.9	94.7 (19.2) [61] 66.7–157.3	93.3 (14.2) [50] 51.3–124.5
	Im	153.4 (60.5) [70][b] 67.8–376.3	156.6 (43.8) [33][b] 86.0–229.4	91.5 (18.7) [101] 66.7–157.3	90.6 (16.8) [33] 67.4–130.1
Cd	Bg	10.0 (5.9) [71][aA] 1.2–25.7	69.0 (42.8) [35][aB] 1.2–175.7	0.5 (0.4) [61][a] *nd* –1.9	0.6 (0.4) [50][a] 0.1–2.2
	Im	22.7(13.2) [70][bA] 0.9–61.7	264.3 (175.5) [33][bB] 25.6–618.3	1.2 (2.4) [101][b] 0.01–14.4	1.5 (1.5) [33][b] 0.2–6.4
Pb	Bg	3.3 (1.9) [71][a] 0.2–9.5	2.9 (1.8) [35][a] 0.2–7.6	2.5 (1.5) [60] *nd* –6.0	2.8 (1.7) [50] *nd* –8.0
	Im	5.3 (3.9) [70][b] 0.4–22.3	4.0 (2.1) [33][b] 0.01–8.7	2.9 (2.8) [98] 0.01–16.7	3.3 (2.9) [33] 0.3–12.6

Above, arithmetic mean with standard deviation (in parentheses) and the number of samples [in brackets];below, the minimum and maximum values; nd, the value below the detection limit. Different letters indicate statistically significant differences ($P < 0.05$; lowercase and uppercase letters are for pollution zones and age groups,respectively) and lead ($R=-0.02$ to -0.1, $P=0.45-0.90$), while the accumulation of essential elements was negatively correlated with the frequency of histopathology: for copper, $R=-0.53$, $P<0.01$; for zinc, $R=-0.42$, $P=0.01$. Since this correlation was statistically significant, we analyzed the dependence of copper and zinc accumulation in the herb field mouse liver on factors "age" and "zone" (Table S6). The copper concentrations were higher in adult than in young animals and in animals with normal liver than in those with dystrophic liver. The dependence of copper concentration on the pollution zone lacked statistical significance (Fig. 3), as also did the effects of both factors on zinc accumulation in the liver of *S. uralensis* was.

Morphometric parameters of hepatocytes

Hepatocytes were analyzed for the parameters listed in Table 3. Some of them were similarly correlated with each other in both species. For example, a negative correlation was revealed between the cell area and cell packing density (for *T. europaea*, $r=-0.8$; for *S. uralensis*, $r=-0.7$, $P<0.001$), and the area of the nucleus showed a positive correlation with the *N/C* ratio (for both species, $r=0.7$, $P<0.001$). Other parameters either showed no correlation with each other or correlation between them differed depending on species. On the whole, morphometric parameters of hepatocytes were less correlated in *T. europaea* than in *S. uralensis* (Table S7).

Some parameters of hepatocytes in dystrophic liver differed from normal. In particular, the cell area in *T. europaea* was increased ($U=27$, $P=0.041$), and the *N/C* ratio in *S. uralensis* was decreased ($U=50$, $P=0.008$), compared to those in normal liver (Table S8).

Individual levels of elements accumulation were not correlated with morphometric parameters of hepatocytes. However, there was an exception: *N/C* ratio in *S. uralensis* hepatocytes was correlated with copper concentration ($r=0.7$, $P=0.02$) (Table S9).

Principal component analysis (PCA) was performed for two parameters with the highest factor loadings that did not correlate with each other: hepatocyte packing density (PC1) and the *N/C* ratio (PC 2). Taken together, PC1 and PC2 accounted for 69.0% of the total variance in *T. europaea* and for 78.4% in *S. uralensis* (Table S10).

The *N/C* ratio in *T. europaea* hepatocytes showed no correlation with either animal age or impact zone ($F_{(3;\ 26)}=1.2$; $P=0.3259$). In contrast, hepatocyte packing density proved to be lower in adult than in young animals and in the impact than in the background zone ($F_{(3;\ 26)}=9.8$; $P=0.0003$), with the interaction of factors "age" and "zone" also having a significant effect (Fig. 4a; Table S11).

Both test parameters in *S. uralensis* depended only on animal age: for the *N/C* ratio, $F_{(3;\ 36)}=5.0$; $P=0.0062$; for hepatocyte packing density, $F_{(3;\ 36)}=4.3$; $P=0.0123$. The *N/C* ratio in overwintered animals was higher than in young of the

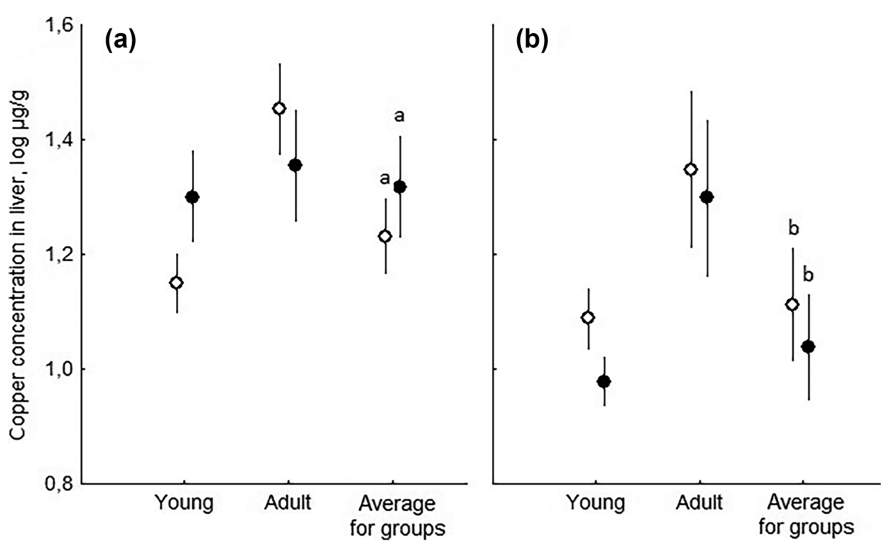

Fig. 3 Copper concentration (log µg/g, mean ± SE) in **a** normal and **b** dystrophic liver of *Sylvaemus uralensis* from the background zone (white circles) and impact zone (black circles). Different letters indicate statistically significant differences in the group average values ($P<0.01$)

Table 3 Morphometric parameters of hepatocytes (mean ± SE) in the normal liver of *Talpa europaea* and *Sylvaemus uralensis* from background (Bg) and impact zones (Im)

Parameter	Zone	Species	
		T. europaea ($n = 8 / 4$)*	*S. uralensis* ($n = 10 / 6$)
N (μm^2)	Bg	16.5 ± 1.57 [8.3–21.9]	33.9 ± 2.08 [26.4–43.6]
	Im	16.9 ± 3.17 [10.4–25.4]	35.4 ± 3.57 [26.3–49.8]
Anisokaryosis	Bg	5.6 ± 0.37 [4.4–7.5]	20.1 ± 1.68 [14.9–32.7]
	Im	6.6 ± 1.07 [3.5–8.1]	19.8 ± 2.07 [14.7–29.3]
S_{cell} (μm^2)	Bg	96.4 ± 5.53 [74.7–165.4]**	221.5 ± 8.24 [166.4–264.5]
	Im	121.0 ± 12.48 [93.2–152.9]	234.0 ± 23.88 [160.7–292.9]
Anisocytosis	Bg	39.2 ± 3.41 [28.7–61.3]	148.4 ± 10.80 [109.0–201.0]
	Im	54.2 ± 5.78 [39.1–67.2]	146.4 ± 19.89 [93.0–208.5]
N/C	Bg	0.21 ± 0.019 [0.13–0.26]	0.19 ± 0.001 [0.14–0.24]**
	Im	0.16 ± 0.017 [0.13–0.21]	0.19 ± 0.012 [0.15–0.22]
Cell packing density, cells/10^5 μm^2	Bg	501 ± 53.3 [245–702] [a]	260 ± 20.7 [161–352]
	Im	381 ± 47.1 [294–479] [b]	245 ± 28.7 [190–369]
Proportion of binucleate cells (%)	Bg	0.2 ± 0.08 [0.0–0.6]	31.6 ± 2.82 [17.5–41.9]
	Im	0.3 ± 0.10 [0.0–0.5]	30.0 ± 3.35 [21.6–42.9]

*Figures before and after the slash show the numbers of animals from the background and impact zones, respectively; figures in brackets show the minimum and maximum values of the parameter

**Values that differ significantly between normal and altered hepatocytes (Mann–Whitney U test, $P < 0.05$). Different letters indicate statistically significant differences between zones ($P < 0.05$)

year, while cell packing density was lower (Fig. 4b; Table S11).

The relationships between the studied factors and morphological parameters of the liver can be represented as a generalized scheme (Fig. 5).

Comparison of a qualitative and quantitative assessment of parameters of hepatocytes

We performed several statistical tests to determine how exactly the predictors and dependent variables are related in *T. europaea* and *S. uralensis* (Fig. 5; Table S12).

To assess the effect of industrial pollution on the morphology of hepatocytes, it seems logical and more efficient to use the full statistical models (GRM/GLM), including the entire set of studied predictors and dependent variables. However, morphometric parameters and the quality of hepatocytes are closely interrelated and are recursive variables that determine each other. There are "fines" for using recursive variables in regression models. For example, among a set of competitive models, the statistically optimal model is based on the minimum value of Mallows' C_p statistic, which is interpreted as an effective parameter. In our case, the optimal models did not include the factor "liver dystrophy" (Table S13); in some models, this factor was included but proved to be not significant. These results also allow us to consider that the qualitative assessment of hepatocytes is less informative, compared to cytomorphometry.

Discussion

Heavy metal concentrations

The dependence of heavy metal accumulation on animal age and the level of environmental pollution (with metal concentrations being higher in adults and in animals from impact zones) has been repeatedly documented for different species (Beernaert et al., 2007; Fritsch et al., 2010; Pankakoski et al., 1994; Pereira et al., 2006; Rogival et al., 2007). We have also observed this relationship for some metals in the samples studied and in extended samples. For example, adults of the European mole accumulated more cadmium both in impact and background zones than young

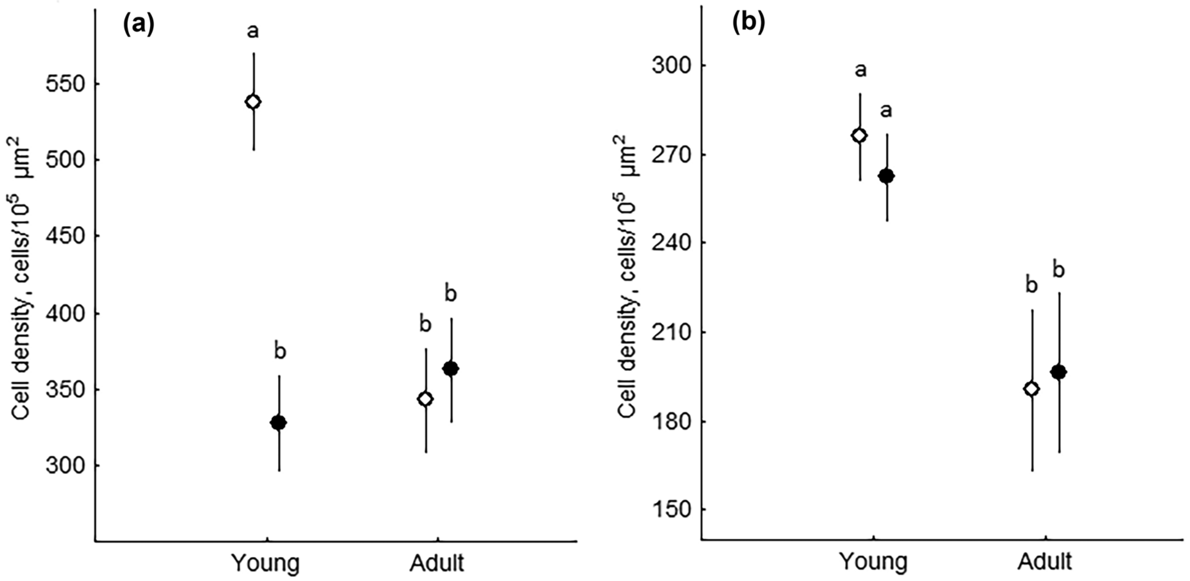

Fig. 4 Hepatocyte packing density (mean ± SE) in **a** *Talpa europaea* and **b** *Sylvaemus uralensis* from the background zone (white circles) and impact zone (black circles). Different letters indicate statistically significant differences between groups ($P < 0.01$)

one, which is primarily due to the longer exposure time of adult animals to pollutants (see Table 2; Table S3).

In the European mole, the concentration of cadmium in the liver increases with age by a factor of 3–6 and is 3.8 times higher in animals from impact than from background areas (Nesterkova et al., 2014; Pankakoski et al., 1993), with its maximum concentration recorded in the liver of moles from the MUCS region (618 µg/g) approaching the upper limit of cadmium content in vertebrates. Cadmium concentrations in the *T. europaea* liver recorded in our studies are higher than those reported previously (Pankakoski et al., 1993), and the concentrations recorded in Muridae rodents (*Apodemus flavicollis* and *A. sylvaticus*) are comparable with the results obtained by other authors (Damek-Poprawa & Sawicka-Kapusta, 2003; Tête et al., 2014). In our samples, the concentrations of heavy metals in the liver were higher in *T. europaea* than in *S. uralensis* (by factors of 2–3 for copper, 1.5–1.7 for zinc, and 15–400 for cadmium) (Table 2). However, the levels of lead accumulation did not differ between the species and were relatively low (Table 2; Table S3), since this metal is not actively accumulated in the liver (Ostoich et al., 2020).

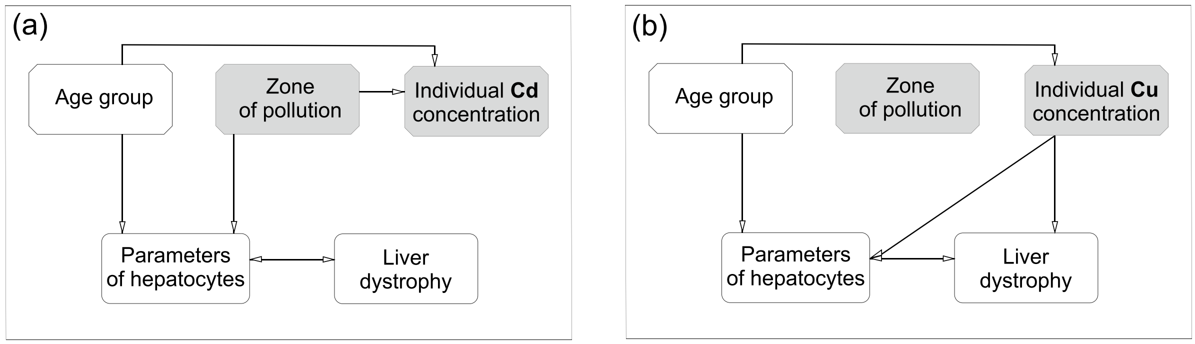

Fig. 5 Scheme of relationships between predictors (upper row) and dependent variables (lower row) in **a** *Talpa europaea* and **b** *Sylvaemus uralensis*. Arrows indicate statistically significant correlations

Expectedly, pollutants accumulation to such high levels should have a toxic effect. This primarily concerns the liver as a depot organ where up to 30% of body cadmium may concentrate (ATSDR Substance Priority List, 2022). Several authors have reported a correlation between the concentration of heavy metals and morphological changes in the liver, including alterations of hepatocytes (e.g., see Sánchez-Chardi et al., 2009a). However, we revealed such a relationship in only one case. In *S. uralensis* liver, the *N*/*C* ratio of hepatocytes was positively correlated with copper concentration (Table S9), while this correlation of the frequency of histopathology was negative (Fig. 3; Table S6), with copper accumulation being higher in adult animals with normal liver. The absence of correlation between heavy metal concentrations and morphological changes in various organs has been documented in studies on different species (Davydova & Mukhacheva, 2014; Pereira et al., 2006; Sánchez-Chardi et al., 2008). The authors explain this fact by high individual variation, the effects of synergistic or antagonistic interactions between pollutants, etc. All these factors may also be relevant in our case. Animal sex may also have a potential effect, which has not been considered in our study. Statistical models that include the factor of sex may reveal hidden patterns in the data at issue (Fritsch et al., 2010; Suzuki et al., 2006; etc.).

Frequency of histopathology

As noted above, the majority of studies analyzing histopathology of the liver in comparison with alterations of hepatocytes have been performed under laboratory conditions. For example, experiments on chronic lead poisoning of Wistar albino rats (*Rattus norvegicus*) revealed a contingency between the frequency of histopathology and the numbers of binucleate hepatocytes, cells with anisokaryosis, etc. (Jarrar & Taib, 2012). The exceptions known to us are the studies by Sánchez-Chardi et al. (2009a) and Shinkarenko and Savchenko (2015), who studied small mammals from polluted areas and compared alterations in morphometric parameters of hepatocytes and pathological changes in the liver tissue. The contingency of dystrophic changes in the liver with increase in the size of hepatocytes was also observed in two freshwater fish species from polluted lakes (Murzina et al., 2014).

Many authors have observed the relationship between the frequency of histopathology in animal organs and the level of environmental pollution (Damek-Poprawa & Sawicka-Kapusta, 2004; Tête et al., 2014; Ballová et al., 2020). However, the development of pathology does not always depend directly on toxic exposure. The relationship between toxic load and its effect may be nonlinear in animals living under multifactor conditions of natural environment (Au, 2004; Brumbaugh et al., 2010; Damek-Poprawa & Sawicka-Kapusta, 2003; Davydova & Mukhacheva, 2014; Pereira et al., 2006; Sánchez-Chardi et al., 2008). The absence of toxic effects is explained by insufficient sample size, subjectivity of methods, specific features of species and their diet (its multicomponent composition and variation in different areas), the influence of more powerful factors (infections, invasions, autoimmune diseases, etc.), patchiness of pollution, and rapid elimination of animals with pathology in chemically polluted areas. Many morphologists put emphasis on subjectivity in the qualitative assessment of the state of animal tissues, which is considered to be due primarily to the absence of universal criteria for such an assessment (Hardisty & Brix, 2005; Thoolen et al., 2010). Different approaches have been proposed to formalize this process, including conversion of qualitative morphological data into semiquantitative data (Gibson-Corley et al., 2013; Holland & Holland, 2011). One of these approaches—histopathology assessment in scores—has been used in studies on fish (Kessabi et al., 2014), small insectivores (Sánchez-Chardi et al., 2008, 2009a), rodents (Sánchez-Chardi et al., 2009b; Tête et al., 2014; Ballová, 2020), and lagomorphs (Amuno et al., 2016).

In our case, the frequency of histopathology proved to be little informative, which may be due to insufficient sample size and evaluation of liver dystrophy on a coarse binary scale, which did not allow us to reveal probable differences between animal groups. However, the observed relationship between morphometric parameters of hepatocytes and dystrophic changes (Table S8) provide evidence for the validity of our approach to the quantitative assessment of liver pathology and for the possibility of its diagnosis by means of cytomorphometry.

It should be noted that, since liver dystrophy is a consequence of metabolic disorders, we can further test our diagnosis using, for example, biochemical analysis of liver tissue. It is important that the copper concentration in the *S. uralensis* dystrophic liver was lower than in the normal liver (Fig. 3). In our opinion, this can be regarded as an additional characteristic of dystrophic changes caused by disturbances in cell metabolism.

Morphometric parameters of hepatocytes

Cytomorphometry of the liver in small insectivores and rodents can reveal features indicative of environmental pollution in their habitats, such as an increase in the nuclear volume of hepatocytes and the number of binucleate cells in the root vole (*Microtus oeconomus*) from the vicinities of an aluminum smelter (Gaidash & Klimatskaya, 2004) or in the thickness of hepatocyte plates in common voles (*Microtus arvalis*) inhabiting the territory of a coal mine (Shinkarenko & Savchenko, 2015). It should be noted that the authors of these studies have not taken into account natural factors of variation in the test parameters (animal sex and age). An exception is the aforementioned study by Sánchez-Chardi et al. (2009a) who took into account these factors when analyzing hepatocytes in white-toothed shrews (*Crocidura russula*) from areas polluted after an accident in a pyrite mine.

The ability of cells to respond even to low-level stress provides a basis for interpreting morphological changes as toxic effects. This approach is widely used in human and veterinary medicine and experimental research (Jarrar & Taib, 2012). For example, high values of *N*/*C* ratio, anisokaryosis, and anisocytosis are regarded as symptoms of pathological or compensatory changes in tissues (Nieburgs, 1967; Zhang et al., 2016). Stockhaus et al. (2004) proposed to evaluate different cytological parameters for their diagnostic value and, using logistic regression analysis, to define the key parameters for each histopathological diagnosis. For example, liver cells in dogs with hepatocellular carcinoma were characterized by a high *N*/*C* ratio, large cell diameters, increased numbers of nucleoli per nucleus, etc. (Stockhaus et al., 2004).

The results of our study provide evidence for differences in the parameters of hepatocytes between animals of different age groups (a decrease in cell packing density in adult animals of both species and an increase in the *N*/*C* ratio in *S. uralensis*) and between animals from background and impact zones (a decrease in hepatocyte packing density in *T. europaea*, see Table S11).

The size of human and animal hepatocytes normally changes with age (O'Neil et al., 2013; Watanabe et al., 1978). For example, hepatocyte volume in male Fischer 344 rats increases by 35–65% between the ages of 1 and 16 months and then decreases so that in old 30-month rats, it is similar to hepatocyte size in the youngest rats (Schmucker et al., 1978). Observations on mongrel white rats during early postnatal ontogeny (21–60 days) have revealed a decrease in hepatocyte packing density due to cell growth, with the cell section area increasing from 129.1 to 209.1 μm^2 (Kuznetsova & Khairullin, 2011). As shown by the same authors, hepatocytes cease to grow in size when their differentiation is complete. However, the increase in the size of hepatocytes revealed in our study cannot be explained by its normal age-related dynamics in view of high age differences between the samples of young and adult animals, especially in *T. europaea* (1.5–5 vs. 12–73 months), and the observed alteration of hepatocytes. This alteration is manifested in vacuolization of the cytoplasm, which is a result of infiltration, accumulation, and decomposition of various substances and indicates degenerative changes in the liver (e.g., see Thoolen et al., 2010). In turn, such degenerative (dystrophic) changes and cytoplasm vacuolization (their morphological indicator) in animals from polluted environments are regarded as a probable mechanism of ecobiochemical adaptation (Murzina et al., 2014).

Thus, we consider that alterations in hepatocytes accounted for the decrease in their packing density (= increase in size) observed in different groups of moles and herb field mice. In general, the state of hepatocytes in adult animals of both species was inferior to that in young animals, and their state in young moles was inferior in animals from the impact than from the background zone, although the frequency of histopathology did not differ between these groups.

Apparently, this is not accidental that differences in the parameters of hepatocytes have been revealed in moles from the impact zone. This species is zoophagous, and its diet (especially earthworms) contains higher amounts of toxic substances than vegetable food; its metabolic rate is high, while mobility is lower than in rodents, accounting for longer contact with pollutants (Godfrey & Crowcroft, 1960; Nesterkova, 2019; Nesterkova et al., 2014). Therefore, moles accumulate tens of times more toxic substances than do other species. The extremely high concentration of cadmium and other toxicants in the liver may be responsible for the alteration of hepatocytes indicative of disturbances in cell metabolism, which are more strongly expressed in adult animals and animals from polluted areas.

In *S. uralensis*, the relationships between individual concentrations of copper in the body, the *N*/*C* ratio of hepatocytes, and liver dystrophy are not correlated with the pollution zone. The variability of hepatocytes is dependent on age (a decrease in cell packing density in adult animals and an increase in the *N*/*C* ratio) and copper concentration (an increase in the *N*/*C* ratio in animals with a high copper concentration). In turn, the copper concentration and *N*/*C* ratio decrease in animals with the dystrophic liver.

In general, the observed differences in the relationships between morphological parameters of hepatocytes and relevant factors (animal age, pollution level, and individual metal concentration) appear to reflect specific features in the response of different species to heavy metal exposure and in the accumulation and effect of different metals (see Fig. 5).

Conclusions

Morphological parameters of hepatocytes in *Talpa europaea* and *Sylvaemus uralensis* from areas exposed to industrial pollution have been assessed with regard to animal age. The results confirm both our hypotheses. First, morphometric parameters have proved to be more reliable indicators of pollution, compared to the frequency of liver histopathology, due to lower subjectivity in their evaluation. Second, differences in morphometric parameters of hepatocytes related to the level of pollution have been revealed only in *T. europaea*, the species more vulnerable to chemical pollution than *S. uralensis.*

Hepatocyte packing, a meristic parameter that is counted rather than measured, is informative and can be used to analyze the state of liver tissue in large samples of animals and also to formalize the assessment of parenchymal liver dystrophy. The advantage of simultaneously using quantitative (cytomorphometry) and qualitative (frequency of histopathology) assessment of the state of tissues in organs is that the former method makes it possible to reveal relevant changes, while the latter can provide explanation to them.

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Author contribution All authors contributed to the study conception and design. The first draft of the manuscript and the drawing of charts were written by Yulia Davydova, and all authors commented on previous versions of the manuscript. The sample collection, data sorting, and experimental operation were completed by Dina Nesterkova and Svetlana Mukhacheva. All authors read and approved the final manuscript.

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Data availability The authors declare that (the/all other) data supporting the findings of this study are available within the article (and its supplementary information files).

Declarations

Ethics approval Not applicable.

Consent to participate Not applicable.

Consent for publication All authors agree to publish this research (including any individual details or images) in Environmental Monitoring and Assessment.

Competing interests The authors declare no competing interests.

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