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Structural–functional aberrations of erythrocytes in the northern red-backed vole (*Clethrionomys rutilus* Pallas, 1779) that inhabits the zone of influence of the copper smelter (the Middle Ural)

Natal'ya A. Orekhova · Yulia A. Davydova · Georgii Yu. Smirnov

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Abstract Red blood cell parameters were assessed in a natural population of the northern red-backed vole (*Clethrionomys rutilus* Pallas, 1779) in the zone of influence of the Kirovgrad Copper Smelter along a gradient of pollution by heavy metals (Cu, Zn, Cd, and Pb) at three catching sites (polluted [Imp] and controls [Bg-1, and Bg-2]). The difference of the smelter area (Imp group of voles) from both background groups (Bg-1 and Bg-2) was proven by means of a set of 13 parameters in univariate and multivariate analyses. Among the detected erythrocyte disturbances, we noted the following: a decrease in activities of Na^+,K^+-ATPase and antioxidant enzymes (SOD, GSH-Px, and CAT); an increase in the concentration of lipid peroxidation products, in osmotic fragility, and in intravascular hemolysis; interruption of carbohydrate metabolism; and lowered oxygen-carrying capacity. A higher load of Cd ($p = 0.0009$) and possibly Pb ($p = 0.054$) in the Imp animals was confirmed by quantitation of heavy metals in the liver. Most erythrocyte parameters (11 out of 13) covaried with individual Cd load by obeying a semilogarithmic dependence; such a relation was not found for Cu, Zn, and Pb. A decrease in the growth rate of structural and functional erythrocyte aberrations ("resistance improvement") with increasing cadmium load is probably due to compensatory enhancement of the synthesis of metallothioneins in the liver and kidneys and hence a greater proportion of Cd bound to metallothioneins. Problems of differences/similarities in Cd-associated reactivity among the animals are discussed too, taking into account the catching sites (polluted [Imp] and controls [Bg-1, and Bg-2]) and reproductive-age (i.e., immature underyearlings, mature underyearlings, and individuals that overwintered). The persistence of differences in erythrocyte status observed by us between the Imp and background groups after normalization to Cd load may be due to the action of other (unexamined) adverse factors and calls for further ecotoxicological studies.

Keywords Copper smelter · Heavy metal load · Red blood aberrations · Cd- associated reactivity · Voles

Introduction

The constant increase in the amount of heavy metals (HMs) in the biosphere is among formidable factors of the ecological imbalance (Ali et al. 2019). HMs enter the environment during the combustion of fossil fuels (coal and oil) as part of vehicle exhaust fumes, as a consequence of activities of metallurgical

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N. A. Orekhova (✉) · Y. A. Davydova · G. Y. Smirnov
Institute of Plant and Animal Ecology, Ural Branch, Russian Academy of Sciences, 8th March Street 202, Yekaterinburg, Russia 620144
e-mail: naorekhova@mail.ru

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industries, and due to the widespread use of pesticides and fertilizers (Merian 1984; Spiegel 2002; Khan et al. 2004). HM-contaminated areas can be used as unique test sites to examine the effects on flora and fauna and to develop regulations in the field of environmental safety (Vorobeichik et al. 1994; Vodyanitskii 2013; Fairbrother et al. 2007).

There is no widely agreed criterion-based definition of a heavy metal. Different meanings may be attached to the term, depending on the context. In metallurgy, for example, a heavy metal may be defined on the basis of density and atomic mass (Morris 1992) whereas in physics the distinguishing criterion might be atomic number (Gorbachev et al. 1980) and a chemist or biologist would likely is more concerned with chemical behaviour (Hawkes 1997). Density criteria range from above 3.5 $g \times cm^{-3}$ to above 7 $g \times cm^{-3}$ (Duffus 2002). Atomic weight definitions can range from greater than 22 [starting with sodium] (Duffus 2002); greater than 50 [starting with vanadium] (Csuros and Csuros 2002) or more than 200, i.e. from mercury onwards (Baldwin and Marshall 1999). Atomic numbers of heavy metals are generally given as greater than 20 [calcium] (Duffus 2002) sometimes this is capped at 92 [uranium] (Lyman 1995). Most often, the term "heavy metals" is considered from a medical and environmental point of view. When included in this category, not only the chemical and physical properties of the element, but also its biological activity and toxicity, as well as the amount of use in economic activities, can be taken into account. Chromium, arsenic, cadmium, mercury, and lead have the greatest potential to cause harm on account of their extensive use, the toxicity of some of their combined or elemental forms, and their widespread distribution in the environment (Khan et al. 2011).

HMs are categorized into essential and nonessential depending on their role in biological systems. Essential HMs are required for certain biological processes: oxygen and electron transport [iron and copper]; complex syntheses and cell metabolism [cobalt]; hydroxylation [zinc]; enzyme regulation or functioning [vanadium and manganese]; glucose utilisation [chromium]; cell growth [nickel]; antioxidant functioning and hormone production [selenium] (Nieboer and Richardson 1980; Emsley 2011). A deficiency or excess of essential HMs leads to diseases or aberrations. Nonetheless, those lists may differ among groups of organisms such as plants, animals, and microorganisms (Aggett et al. 2022). Nonessential HMs do not perform a “useful” function and are considered potential toxic (in medical toxicology, they are referred to as “thiol poisons”). The term has particular application to cadmium, mercury and lead (Brathwaite and Rabone 1985), all of which appear in the World Health Organization’s list of 10 chemicals of major public concern. Other examples include in the toxic metal group both essential and non-essential HMs: chromium, cobalt, nickel, copper, zinc, silver, antimony and thallium ("10 chemicals of public health concern". www.who.int. Retrieved 2021-10-09). Studies point to three main reasons for HM toxicity [which causes damage to the structure of tissues (organs) and a loss of their functionality (Ercal et al. 2001; Tandon et al. 2003; Hartl, 2013; Rubino 2015; Mao et al. 2018)]: (1) the ability to inactivate vital proteins (mostly enzymes) because of affinity for thiol (–SH), amino ($–NH_2$), and carboxyl (–COOH) groups in their active centers; (2) replacement of elements such as calcium in bones or iron in red blood cells; (3) direct or indirect promotion of the production of reactive oxygen species (ROS), such as hydroxyl radical (HO·), superoxide radical ($O^{\cdot 2-}$), and hydrogen peroxide (H_2O_2), thereby leading to a state referred to as “oxidative stress”.

Animals from the Rodentia group, because they consume flora that reflects local soil and water contamination, are actively used in many regions as bioindicator species of environmental gradients of metal concentrations and their bioavailability (Zamani et al. 2020). In view of the high fecundity and rate of reproduction of Rodentia, the effects on this group of small mammals make it possible to predict the genetic hazard of technogenic pollution for a local population and to track down possible mechanisms of adaptation to the toxicological factor (Sheffield et al. 2001).

For the Ural region, the problem of monitoring of toxic effects is especially relevant because large industrial facilities (the Karabash Copper Smelter, Middle Ural Copper Smelter, and Kirovgrad Copper Smelter [KCS]) are located here, and their activity has led to the formation of HM pollution zones (Kaigorodova 2012; Vorobeichik and Kozlov 2012). In our present work, the zone of influence of the KCS was investigated, which is located in the Middle Ural in the city of Kirovgrad (Russia), ~ 72 km

north–north-west of Yekaterinburg. The duration of its impact on ecosystems is currently 108 years. The main components of emissions are gaseous compounds of sulfur, fluorine, and nitrogen (sulfurous anhydride predominates) as well as particulate matter with adsorbed HMs (e.g., Cu, Zn, Cd, Pb, Fe, and Hg), among which Pb predominates (Vorobeichik et al. 2006). An excess of such toxic substances as Cu, Zn, Pb, and Cd in soil, vegetation cover, and animal organisms suggests that ecosystems on these territories are still subject to chronic technogenic pressure (Vorobeichik et al. 2006).

The response of the body to any external factor may include a modification of the gas transport function of the blood; this function is necessary to maintain temperature homeostasis, osmoregulation, metabolic rate, and energy supply at the level of cells, tissues, and the body. In this regard, there are research articles about the monitoring of HM load and associated hematological effects in wild rodents living along a pollution gradient (Gorriz et al. 1996; Kovalchuk 2008; Ilyinskikh et al. 2011; Rogival et al. 2006; Tarakhty and Mukhacheva 2011 Nunes et al. 2001; Tete et al. 2015). Hematological parameters are considered early signs that do not necessarily indicate immediate damage to the organism (Tersago et al. 2004; Rogival et al. 2006).

The aim of the present work was to assess the influence of chemical pollution (taking into account individual HM load) on structural and functional characteristics of erythrocytes in the northern red-backed vole (*Clethrionomys rutilus* Pallas, 1779) that inhabits the KCS's zone of influence. To assess the impact of individual HM load on erythrocyte parameters, we determined the concentrations of priority (according to the content in emissions) pollutants (Cu, Zn, Cd, Pb) in the liver. The liver represents a primary site for toxicant storage because of its high metabolic potential and ability to clear xenobiotics from the blood (Hébert et al. 1993; Aburto et al. 2001; Świergosz-Kowalewska 2001; Andjelkovic et al. 2019).

Materials and methods

Study locations

The rodents were captured randomly in July–August 2019–2020 within the KCS's zone of influence at three sites in the gradient of HMs. The heavily polluted site (N 57.42; E 60.02) (hereafter: Imp site) is located 3.5 km west of the KCS in the vicinity of Kirovgrad. Differences of the Imp site from other gradient segments are explained by a high degree of the anthropogenic impact on typical southern taiga landscapes. As a consequence, there is soil degradation, a reduction and replacement of primary fir-spruce forests with derivatives, the presence of ruderal spaces, and the formation of a peculiar microclimate (warmer and more humid compared to adjacent territories). Concentrations of mobile forms of HMs in forest litter (mean ± standard error of the mean, μg g^{-1}) at the Imp site are known to be 764.18 ± 94.24 (Cu), 1503.34 ± 405.88 (Zn), 10.56 ± 1.47 (Cd), and 768.19 ± 70.94 (Pb) (Vorobeichik et al. 2006).

A site with nominally negligible level of pollution (N 57.37; E 59.77) (Bg-1) is located 18 km away from the KCS. It is characterized by low-mountain relief with elevation changes of 250–300 m and a maximum height of 699 m. The biotopes on which the study was performed are native fir-spruce tall-herb-fern forests. For the Bg-1 site, levels of HMs in forest litter are known to be 39.63 ± 2.68 (Cu), 398.380.45 ± 61.88 (Zn), 3.32 ± 0.35 (Cd), and 166.03 ± 11.65 (Pb) (Vorobeichik et al. 2006).

Another background site (N 57.53; E 59.77) (Bg-2) is 35 km away from the KCS; the levels of contamination of the forest litter known to be close to the regional background: 22.41 ± 1.70 (Cu), 268.89 ± 16.42 (Zn), 2.15 ± 0.04 (Cd), and 42.28 ± 3.05 (Pb) (Vorobeichik et al. 2006).

Catching, maintenance, and analysis of the voles

In this study, the northern red-backed vole (*Cl. rutilus* Pallas, 1779) was used [subfamilies Arvicolinae (= Microtinae) of the family Cricetidae (Kryštufek and Shenbrot 2022)]. It is widespread from Scandinavia to the Far East and inhabits zones of taiga and broad-leaved forests; in the north, it penetrates into the forest-tundra, and in the south, into insular forests and forest-steppes (Shenbrot and Krasnov 2005). The Middle Urals is regarded as the periphery of the geographic range of this species, where regular cyclical ups and downs in numbers are observed. In this paper, *Cl. rutilus* individuals were analyzed during the years of population growth (Kshnyasev and Davydova 2021).

For trapping, we used wooden live traps (210×88×85 mm) with a compartment for bait and a living chamber suitable for short-term stay of rodents under the conditions of the Middle Urals. After capture, the animals were transported to the laboratory for processing: measurements, weighing, determination of the mass of internal organs, and collection of biological samples. The rodents were kept for 1–3 days under laboratory conditions under natural light at room temperature. This acclimation of the animals helped to reduce the stress of transport and of the new environment. The animals were fed (without restrictions) oats, carrots, cucumbers, and apples; sawdust and hay served as bedding.

Captured animals were categorized into three reproductive-age groups, i.e., functional-physiological groups according to Olenev (2002). For this purpose, a set of parameters was used: body weight and size, the state of the gonads and uterus, and the presence of the thymus and dental roots. The following groups were analyzed, a total of 92 individuals: immature underyearlings (im; $n=49$), mature underyearlings (m; $n=22$), and the rodents that overwintered (ow; $n=21$).

Hematological tests (thirteen parameters)

For blood sampling, the animals were euthanized by decapitation. Trunk blood from each vole was collected immediately into tubes with sodium citrate as an anticoagulant (5.0% [w/v], 0.2 ml citrate/ml blood). Using an Abacus junior vet (Austria) hematology analyzer, the following parameters of RBCs were measured and calculated: hemoglobin concentration (Hb), the RBC count, mean corpuscular hemoglobin (MCHb = Hb/RBC count), mean corpuscular volume (MCV), and mean corpuscular hemoglobin content (MCHC = Hb/RBC count × MCV).

Next, the following biochemical parameters were assayed:

— (in RBCs) activities of antioxidant enzymes [glutathione peroxidase (GSH-Px: EC 1.11.1.9), catalase (CAT: EC 1.11.1.6), superoxide dismutase (SOD: EC 15.1.1)], lipid peroxidation (LP), carbohydrate metabolism [glucose-6-phosphate isomerase activity (GPI: EC 5.3.1.9)], and the sodium–potassium pump [Na^+,K^+-ATPase (NAKA: EC 3.6.3.9)] and osmotic fragility (OF);—(in blood plasma) concentrations of free hemoglobin (pfHb) and glucose (GLC).

Plasma was separated from erythrocytes by centrifugation at 1500 g for 15 min, frozen in liquid nitrogen, and stored at −80 °C until measurements. We washed the erythrocyte fraction from plasma and white blood cells by centrifugation in a tenfold volume of 0.9% NaCl. The erythrocyte suspension was prepared in phosphate-buffered saline (10 mM PO_4^{3-}, 137 mM NaCl, 2.7 mM KCl; pH 7.4). Laboratory RBC examination was carried out within 4 h of the sample collection.

Spectrometric methods were utilized to quantify analytes, enzymatic activities, and osmotic resistance of erythrocytes. All chemicals were purchased from Lachema (Czech Republic), Vital Diagnostics (Russia), PanReac AppliChem (USA) and Sigma-Aldrich (USA). The glucose test was performed with a glucose oxidase/peroxidase reagent and o-dianisidine dihydrochloride (Bergmeyer and Bernt 1974). Free hemoglobin concentration in blood plasma was measured by the azopyramic method, which consists of the oxidation of amidopyrine with hydrogen peroxide, followed by a reaction of the oxidation product with hydrochloric aniline (Johannsen and Zvyagina 1987). The catalyst of the reaction is hemoglobin. The magnitude of LP was expressed as a malondialdehyde (MDA) content determined by the TBARS (thiobarbituric acid reactive substances) method of Buege and Aust (1978). The concentration of MDA was calculated using an extinction coefficient of 1.56×10^{-5} M^{-1} cm^{-1} and expressed in moles of MDA per gram of Hb.

The GPI activity was tested by means of conversion of glucose-6-phosphate (G6P) to fructose-6-phosphate (F6P). To this end, G6P disodium salt hydrate (Sigma-Aldrich, USA) was injected into a sample and was incubated for 30 min at 37 °C. F6P concentration was measured before and after incubation on the basis of the color reaction of F6P with resorcinol (Roe and Papadopoulos 1954). CAT activity was assessed with the help of H_2O_2 and an ammonium molybdate solution (Goth 1991). GSH-Px activity was determined using NADPH (Sigma-Aldrich, USA) and 5,5′-dithio-bis(2-nitrobenzoic acid) (Paglia and Valentine 1967). NAKA activity in erythrocyte membranes was determined from the rate of accumulation of inorganic phosphorus (P_n) in the incubation medium during ATP hydrolysis and was calculated as the difference between total and Mg^{2+}-dependent ATPase activity. For this purpose, an aliquot of a

suspension of erythrocyte shadows [prepared by the method of Dodge et al. (1963)] was introduced into a medium (30 mM Tris–HCl buffer, pH 7.4) containing ions at various concentrations: NaCl, 100 mM; KCl, 10 mM; and $MgCl_2$, 2 mM as well as 2 mM ATP and 1 mM EDTA (Kazennov et al. 1984). When determining the activity of Mg^{2+}-ATPase, aside from the indicated components, an inhibitor of NAKA (1 mM ouabain) was added to the incubation medium. The enzyme was incubated at 37 °C for 15 min, the reaction was stopped by the addition of a solution of 20% trichloroacetic acid, protein precipitation was carried out by centrifugation at 3500 rpm for 10 min, and the supernatant from the shadow suspension was employed to quantify P_n using ascorbic acid as a reducing agent (Chen et al. 1956). The enzymatic activities (GPI, CAT, GSH-Px, and NAKA) were expressed in katal, namely, moles of a substance produced or consumed per second in an enzymatic reaction (i.e., katal = mol s^{-1}) (NC-IUB 1979). This value was normalized to grams of Hb.

SOD activity in the extract was determined from this enzyme's ability to inhibit photochemical reduction of nitro blue tetrazolium in the presence of riboflavin and L-methionine, measured as absorbance of the reaction mixture at 560 nm (Giannopolitis and Ries 1977). The enzymatic activity was expressed in arbitrary units (AU) per gram of Hb using the formula: $A_{SOD} = \log (OD_c/OD_t)/(\log C_p)$, where OD_c and OD_t are optical density of the control and test sample, and C_p is Hb concentration (g) in the sample.

OF is the proportion of hemolysis that occurs when a sample of RBCs is subjected to osmotic stress by placement in a hypotonic solution. OF was measured via 30 min incubation of erythrocytes in phosphate-buffered hypotonic saline with different concentrations of sodium chloride (C_{NaCl} from 0.85% to 0.15%). The parameter was defined as the C_{NaCl} at which 100% hemolysis of the test sample takes place (Mukherjee 2017).

Measurement of metal concentrations in the liver (four parameters)

Not all animals were analyzed for heavy metals. Sampling for chemical analysis (n = 52) was carried out randomly. Liver tissue samples were dried at 75 °C to air-dry mass. Then, the samples were crushed and weighed on a KERN-770 analytical balance (Germany) with an accuracy of 0.01 mg. Aliquots of ~ 100 mg were placed in Teflon vials containing 7 ml of 65% HNO_3 (ultra high purity) mixed with 1 ml of deionized H_2O, incubated for 30 min, and ashed in an MWS-2 microwave oven (Berghof, Germany). The sample volume was then adjusted to 10 ml with deionized H_2O. HMs were quantitated on an atomic absorption spectrometer (ContrAA 700 vario Analytik Jena, Germany) by flame (for Cu and Zn) or electrothermal (for Cd and Pb) atomization. The concentrations were expressed in μg g^{-1} dry liver mass. The quality of measurements was assessed by means of an international standard (bovine liver BCR-185R; European Commission, Belgium). The recovery percentage averaged 81.5% for Cu, 83.6% for Zn, 94.0% for Cd, and 95.0% for Pb.

Statistical analysis

These calculations were performed in STATISTICA software version 8.0 and STATGRAPHICS version 8.0 (StatSoft 2007). Statistical significance of 2-tailed differences among the groups (Bg-1, Bg-2, and Imp) according to nonparametric methods (Kruskal–Wallis *H* test and Mann–Whitney *U* test) was evaluated; these tests do not require normally distributed data. To identify a cluster of analyzed groups of animals, using a set of intercorrelated 13 hematological variables (R_{xy} shown in Supplement 1), principal component (PC) analysis was performed, which reduces the total dataset's dimensionality (Mather and Koch 2011). The variation of the resulting trait (Y: PC-1 and PC-2) depending on the variation of a factor trait (X: HM concentration) was estimated using equations of three functions: linear ($Y = b_0 \pm b_1 \times X$), semilogarithmic ($Y = b_0 \pm b_1 \times \lg X$), and exponential ($Y = b_0 \times b_1^x$ or $\lg Y = b_0 \pm b_1 \times X$). To select the most adequate regression model, their residual variances (SS_{res}) were compared. SS_{res} is defined as a percentage of total variance ($SS_{res}/SS_{tot} \times 100$) (StatSoft 2007). In our opinion, the preservation of "sensitivity" to the effects of pollutants is proved the adequacy of the linear function; adequacy of the exponent model was indicated by an increase in "sensitivity", whereas adequacy of the semilogarithmic model was reflected by an increase in the proportion of individuals resistant to exposure.

We chose the level of statistical significance (*p*) of 0.05, which is widely accepted in biological research.

To statistically characterize differences in absolute values of regression coefficient b_1 among the three groups, we used the 95% confidence interval (*CI*) for b_1 values. The differences between groups in this coefficient were assumed to be statistically significant when 95% *CI*s did not overlap. The 95% *CI* was calculated as $b_1 \pm SE \times t_{0.05}$, where the latter takes into account df (n − 2). In all the analyses, the statistical unit was the individual.

To take into account a possible influence of sex, statistical preprocessing of the available data was conducted. No significant differences were found between males and females (Supplement 2).

Results

Comparison of the three catching sites by hematological parameters: results of the univariate analysis

Hematological parameters of peripheral blood in *Cl. rutilus* from the background (Bg-1, Bg-2) and Imp sites are presented in Table 1. Separation of the impact group (Imp), implying simultaneous differences from the two background groups (Bg-1 and Bg-2), was determined by means of all the above parameters. The parameters did not have significant differences between the background sites. Based on hematological characteristics, the northern red-backed vole in the chemically contaminated area has a higher concentration of hemoglobin (Table 1, entry No. 11: 169% relative to the Bg-1,2 value) and glucose (Table 1, entry No. 12: 144%) in blood plasma. In addition, peripheral blood of the Imp animals is characterized by a smaller number of erythrocytes (entry No. 1: 77%), their larger volume (entry No. 3: 115%), and a higher Hb content (entry No. 2: 110%), with a low activity of antioxidant enzymes (entry No. 5: 36%, No. 6: 62%, and No. 10: 41%) and Na^+,K^+-pump (entry No. 13: 64%). OF of RBCs is high (entry No. 9: 72%), along with high intensity of LP (entry No. 8: 188%) and GPI activity (Table 1, entry No. 7: 228%).

Comparison of the three catching sites by hematological parameters: results of the multivariate analysis

Variation in the set of studied parameters among the catching sites (Bg-1/Bg-2/Imp) is presented in the plane of two components (PC-1 and PC-2) (Fig. 1). The Imp group is fairly well separated, but the magnitude of shifts in PC-1 is significantly greater as compared to PC-2 (Fig. 1a). The largest distance between the groups was observed for *Cl. rutilus* that overwintered (ow), and the smallest for immature underyearlings (im) (Fig. 1b).

Judging by the close relation between PC values and hematological data (Table 2), the main contribution to the variation of PC-2 (13.8% of the total variance) is made by the RBC count and MCHC (bold-faced in Table 2). The remaining parameters form the second factor (62.1% of the total variance).

HM load and its relation with hematological data

According to our findings (Table 3), the accumulation of metals (Cu, Zn, Cd, and Pb) is not associated with reproductive-age status at the background sites (Bg-1: H [2, N = 14] = 1.22–5.10, $p = 0.08$–0.54; Bg-2: H [2, N = 20] = 0.84–7.76, $p = 0.02$–0.65) and at the Imp site (H [1, N = 18] = 0.03–1.50, $p = 0.21$–0.86). In total, the background groups do not differ in the concentrations of cadmium ($U_{78} = 124$; $p = 0.57$), zinc ($U_{78} = 121$; $p = 0.51$), and lead ($U_{78} = 120$; $p = 0.48$). There is a difference in the concentration of copper (Bg-2 > Bg-1; $U_{78} = 67$; $p = 0.01$). Cadmium load was higher in the Imp area by 4.8-fold ($U_{201} = 132$; $p < 0.00001$) with respect to the background. At the Imp site, zinc concentration remained at the level of control values ($U_{201} = 262$; $p = 0.39$). The level of the copper at the Imp site was 1.6 times higher relative to Bg-1 ($U_{74} = 39$; $p = 0.0009$). The concentration of lead was also higher but without significance ($U_{201} = 206$; $p = 0.054$).

Table 4 presents the results of the analysis of the adequacy of the tested models (linear, semilogarithmic, and exponential) in assessing the dependence of PCs' variation on the variation of individual HM load. In terms of the standardized regression coefficient (β), it can be confidently stated that the variation of hematological parameters is determined, first of all, by the cadmium load. According to the percentage of residual variance (SS_{res}), preference should be given to the model based on the semilogarithmic function (for PC-1) or linear function (for PC-2).

Furthermore, a comparison of Cd- associated reactivity of hematological status among the catching sites indicated the absence of statistically

Table 1 Hematological parameters of peripheral blood in *Cl. rutilus* from the background (Bg-1 and Bg-2) and KCS (Imp) sites

No	Parameters	Catching site	Median (Q1-Q3)	*p* values (2-tailed comparison)		
				a/b	a/c	b/c
1	RBCs count, $\times 10^{6}$ μL^{-1}	Bg-1 (a)	9.5 (9.2–10.4)	0.407	**0.000**	**0.000**
		Bg-2 (b)	9.3 (8.3–9.9)			
		Imp (c)	7.3 (6.7–8.0)			
2	MCHb, $\times 10^{-12}$ g per RBC	Bg-1	14.2 (13.7–15.0)	0.759	**0.000**	**0.000**
		Bg-2	13.8 (13.5–14.7)			
		Imp	15.6 (14.6–16.3)			
3	MCV, $\times 10^{-15}$ L per RBC	Bg-1	38.0 (37.0– 39.0)	1.000	**0.000**	**0.000**
		Bg-2	37.0 (37.0– 39.0)			
		Imp	43.0 (42.0– 44.0)			
4	MCHC, g dL^{-1}	Bg-1	36.7 (34.9–39.1)	1.000	**0.002**	**0.019**
		Bg-2	37.7 (34.7–38.8)			
		Imp	34.9 (33.6–36.3)			
5	GSH-Px activity, katal g^{-1} Hb	Bg-1	28.3 (20.5–40.7)	1.000	**0.000**	**0.000**
		Bg-2	32.7 (25.6–37.6)			
		Imp	10.9 (4.6–15.8)			
6	CAT activity, mkatal g^{-1} Hb	Bg-1	798.9 (679.1–955.4)	0.936	**0.000**	1.000
		Bg-2	884.4 (790.6–915.6)			
		Imp	524.0 (389.1–666.2)			
7	GPI activity, μkatal g^{-1} Hb	Bg-1	31.7 (22.9–43.7)	1.000	**0.000**	**0.000**
		Bg-2	38.2 (35.4–42.8)			
		Imp	79.9 (59.6–86.8)			
8	LP, nmol MDA g^{-1} Hb	Bg-1	32.4 (17.5–56.1)	1.000	**0.000**	**0.000**
		Bg-2	32.5 (15.7–41.3)			
		Imp	61.2 (52.2–71.9)			
9	OF, C_{NaCl} at 100% hemolysis	Bg-1	0.26 (0.23–0.30)	1.000	**0.000**	**0.000**
		Bg-2	0.26 (0.23–0.29)			
		Imp	0.36 (0.30–0.39)			
10	SOD activity, AU g^{-1} Hb	Bg-1	5.3 (3.5–6.1)	1.000	**0.000**	**0.000**
		Bg-2	5.3 (4.2–6.2)			
		Imp	2.2 (1.7–4.2)			
11	pfHb, mg dL^{-1} plasma	Bg-1	37.0 (24.4–46.3)	1.000	**0.006**	**0.006**
		Bg-2	33.6 (18.9–50.0)			
		Imp	59.8 (31.2–73.5)			
12	GLC, μmol L^{-1} plasma	Bg-1	4.0 (3.2–4.5)	1.000	**0.000**	**0.000**
		Bg-2	3.9 (3.2–4.3)			
		Imp	5.7 (4.8–6.3)			
13	NAKA activity, μkatal P_n g^{-1} Hb	Bg-1	100.8 (83.6–112.4)	1.000	**0.000**	**0.000**
		Bg-2	104.2 (83.6–118.2)			
		Imp	65.5 (44.4–100.1)			

Numbers of animals (*n*): Bg-1 (35), Bg-2 (25), and Imp (32).

Bold type stands for a *p*-values less than 0.05

significant differences in the severity of the response of 11 out of 13 parameters (forming PC-1) to the same dose of a pollutant in animals from the background sites and Imp site (Fig. 2a). 95% CIs for regression coefficients b_1 of the three plots overlap, i.e., $b_1 \pm \mathrm{SE} \times 2.18$ [Bg-1] $\approx b_1 \pm \mathrm{SE} \times 2.10$ [Bg-2] $\approx b_1 \pm \mathrm{SE} \times 2.10$ [Imp]. Differences in Cd-associated reactivity between the background and

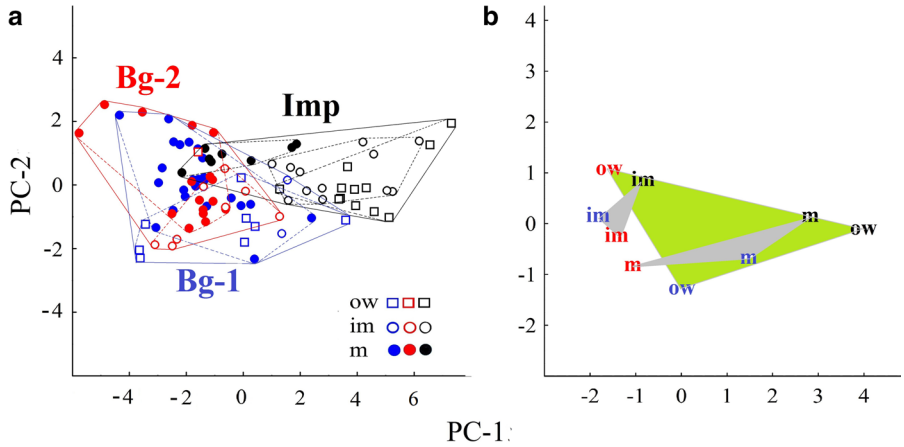

Fig. 1 Positioning of the three groups (Bg-1, Bg-2, and Imp) of *Cl. rutilus* in the plane of the two principal components (PC-1 and PC-2) based on the 13 hematological parameters of peripheral blood (see Table 2) **a** scatter plot **b** median values for nine groups: Imp, Bg-1,2 (im,m,ow)

Table 2 Pearson's coefficient of correlation (R_{xy}) between PC values (Y) and hematological parameters (X) in terms of the three groups

Parameters	PC-1	PC-2
RBCs	– 0.44	**– 0.76**
MCHb	**0.86**	– 0.29
MCV	**0.94**	0.05
MCHC	– 0.12	**– 0.92**
GSH-Px	**– 0.76**	0.25
CAT	**– 0.83**	0.00
GPI	**0.89**	0.00
LP	**0.87**	– 0.03
OF	**0.90**	– 0.16
SOD	**– 0.83**	– 0.14
pfHb	**0.79**	– 0.03
GLC	**0.82**	– 0.05
NAKA	**– 0.73**	– 0.11

Bold type stands for a *p*-values less than 0.05

Imp animals are statistically significant only in two parameters (RBCs and MCHC, forming PC-2) (Fig. 2b): $b_1 \pm SE \times 2.18$ [Bg-1] $\approx$ $b_1 \pm SE \times 2.10$ [Bg-2] $> b_1 \pm SE \times 2.10$ [Imp]. Nevertheless, the difference in hematological status between the Imp and background groups persisted after the adjustment for the cadmium content, i.e., $b_0 \pm SE \times 2.10$ [Imp] $> b_0 \pm SE \times 2.04$ [Bg-1,2].

We also analyzed the effect of Cd among the reproductive-age groups without taking into account the influence of the place of material collection. The dependence of PC-1,2 on the load for all groups proved to be more adequately described by the semi-logarithmic function (Table 5).

The degree of reactivity of 11 out of 13 parameters (explaining the variation of PC-1: see Table 2) to cadmium load was found to be more pronounced for sexually mature underyearlings and animals that overwintered as compared to immature rodents: $b_1 \pm SE \times 2.13$ [im] $< b_1 \pm SE \times 2.03$ [m, ow] (Fig. 3a). Differences in the reactivity among the groups were not confirmed by PC-2 (Fig. 3b).

Discussion

By looking at our results, one can confidently associate the degree of structural and functional aberrations of erythrocytes in *Cl. rutilus* primarily with its cadmium load. Cadmium (in contrast to Pb and Hg) is a serious food chain contaminant owing to higher rates of soil-to-plant transfer through the metabolic pathway of plants' essential nutrients, such as Zn and Fe (McLaughlin and Singh 1999).

In the bloodstream, the main targets for cadmium are albumins, erythrocytes, and transferrins (Nordberg et al., 2007). Then, this element accumulates in the liver and kidneys through strong bonds with the sulfur (S) contained in metallothionein (MT) molecules (Satarug 2018; Tinkov et al. 2018). Cadmium, which occurs in biological systems as the free divalent Cd^{2+} cation (not bound in Cd–MT complexes), exerts acute and chronic toxicity, according to the current understanding, through indirect effects that involve DNA damage, ROS formation, carcinogenesis, and the induction of apoptosis (Joseph 2009;

Table 3 Concentrations of HMs in the liver (μg g^{-1} dry mass) of the northern red-backed vole (*Cl. rutilus*) from the background (Bg-1 and Bg-2) and Imp sites at different ages (i.e., reproductive status: im, m, or ow); im: immature underyearlings; m: mature underyearlings; ow: rodents that overwintered

Catching site	Reproductive-age status (*n*)	HM content: median (Min–Max)			
		Cu	Zn	Cd	Pb
Bg-1	im (6)	10.2 (1.1–20.0)	84.5 (64.4–128.8)	0.3 (0.2–1.2)	1.7 (0.3–3.0)
	m (2)	5.9 (4.4–7.4)	86.5 (79.2–93.7)	1.1 (0.7–1.5)	1.6 (1.6–1.6)
	ow (6)	9.0 (3.4–10.1)	62.7 (52.4–90.3)	0.2 (0.1–1.6)	3.3 (0.4–8.5)
	Total (14)	8.9 (1.1–20.0)	75.1 (52.4–128.8)	0.3 (0.1–1.6)	1.6 (0.3–8.5)
Bg-2	im (10)	10.9 (8.2–13.2)	83.2 (62.4–100.2)	0.4 (0.3–0.8)	1.9 (0.5–4.3)
	m (8)	14.8 (5.6–16.6)	78.2 (41.9–84.4)	0.3 (0.1–0.8)	3.0 (1.0–6.4)
	ow (1)	13.0	86.8	0.5	1.4
	Total (19)	12.0 (5.6–16.6)	80.8 (41.9–100.2)	0.4 (0.1–0.8)	2.2 (0.5–6.4)
Imp	im (1)	10.5	79.7	0.3	1.4
	m (10)	14.3 (8.1–18.7)	82.6 (37.6–114.7)	1.4 (0.7–7.9)	1.8 (0.2–9.0)
	ow (8)	14.4 (11.8–19.7)	86.4 (68.8–105.5)	2.4 (0.9–12.0)	5.3 (0.9–9.6)
	Total (19)	14.4 (8.1–19.7)	82.6 (37.6–114.7)	1.7 (0.7–12.1)	4.2 (0.2–9.6)

Rani et al. 2014). Today, the damaging effect of toxic doses of cadmium on bone (itai-itai disease) and excretory, reproductive, and cardiovascular systems is known (Staessen et al. 1999; Järup 2002; Inaba et al. 2005; Thompson and Bannigan 2008; Fagerberg et al. 2012). In our case, studies on the effect of cadmium ions on blood cells, in particular erythrocytes, deserve special attention (Garty et al. 1986; Das et al., 1987; Hamada et al. 1998).

When released into the blood, cadmium mostly settles in erythrocytes via passive transport (Garty et al. 1986). Direct effects of Cd^{2+} are mediated by its high affinity for hemoglobin; for this reason, the latter successfully competes for cadmium binding with all bioligands, except for MTs (Vorobieva 1984; Siegel and Siegel 1993). Probably, the disturbance of hemoglobin protein chain structure by cadmium ions leads to compensatory upregulation of this protein in cells, which is necessary to ensure oxygen homeostasis. Indeed, in our assays, an increase in cadmium load was accompanied by an increase in the content of hemoglobin in cells (MCHb) and, as a consequence, their volume (MCV: see Fig. 2, Table 2). It should be noted that an increase in RBC size is not always effective for proper execution their functions. A disproportionate increase in MCV (110% of the Bg-value) relative to MCHb (115% of the Bg-value) in erythrocytes from the Imp animals led to a lower amount of hemoglobin per unit volume (MCHC at 94% of the Bg value). This parameter is a measure of the average oxygen-carrying capacity of the RBCs circulating in the body.

It is thought that Cd^{2+} can get integrated into both the outer and inner monolayer of the membrane, thereby contributing to alterations in the cytoskeleton (membrane proteins and/or lipids) of RBC with the formation of acanthocytes, spherocytes, and schistocytes (Nazima et al. 2016). The participation of cadmium in the modification of erythrocyte membranes and changes in the curve of osmotic fragility has also been noted in earlier reports (Garty et al. 1986; Kunimoto et al. 1986; Hamada et al. 1998). In our case, an increase in cadmium load led to significant growth of RBC osmotic fragility (C_{NaCl}: Imp = 0.36%; Bg = 0.26%; see also Fig. 2, Table 2) along with signs of stronger oxidation of membrane lipids (see LP in Table 1). It is believed that the accumulation of secondary LP products (MDA predominates among them) and their interaction with amino groups of the lipoprotein complex of erythrocyte membranes causes cell rigidity (García et al. 2000; Thirunavukkarasu and Sakthisekaran 2003). In general, this phenomenon causes a decrease in the "critical volume" of the cell upon exposure to a hypotonic solution. Deterioration of membrane elasticity of RBCs is possible after Cd-induced LP activation (Gill et al. 1989). It is suspected that this pollutant acts on this membrane indirectly through suppression of the erythrocyte antioxidant system (Gill et al. 1989).

Table 4 PCs' values (Y) depending on individual HM load (X) in *Cl. rutilus* taking into account a catching site (Bg-1, Bg-2, and Imp): a standardized regression coefficient (β ± SE) and residual variance (SS_{res}, % of total) in the analysis of linear ($Y = b_0 \pm b_1 \times X$), semilogarithmic ($Y = b_0 \pm b_1 \times lgX$), and exponential ($lgY = b_0 \pm b_1 \times X$) functions

Catching sites	Models	HMs	PC-1			PC-2		
			β ± SE	*p*	SS_{res}	β ± SE	*p*	SS_{res}
Bg-1	$Y = b_0 \pm b_1 \times X$	Cu	− 0.16 ± 0.28	0.57	97	0.07 ± 0.29	0.82	99
	$Y = b_0 \pm b_1 \times lgX$		− 0.19 ± 0.28	0.51	96	0.01 ± 0.29	0.95	100
	$lgY = b_0 \pm b_1 \times X$		− 0.15 ± 0.28	0.61	98	0.11 ± 0.28	0.70	98
Bg-2	$Y = b_0 \pm b_1 \times X$		0.18 ± 0.23	0.45	97	− 0.21 ± 0.23	0.36	95
	$Y = b_0 \pm b_1 \times lgX$		0.14 ± 0.23	0.55	98	− 0.23 ± 0.23	0.33	95
	$lgY = b_0 \pm b_1 \times X$		0.12 ± 0.23	0.61	99	− 0.25 ± 0.23	0.29	93
Imp	$Y = b_0 \pm b_1 \times X$		− 0.03 ± 0.25	0.89	99	− 0.29 ± 0.24	0.23	91
	$Y = b_0 \pm b_1 \times lgX$		− 0.05 ± 0.25	0.83	99	− 0.31 ± 0.24	0.21	90
	$lgY = b_0 \pm b_1 \times X$		− 0.03 ± 0.25	0.91	99	− 0.29 ± 0.24	0.24	91
Bg-1	$Y = b_0 \pm b_1 \times X$	Zn	0.39 ± 0.26	0.16	84	0.43 ± 0.26	0.12	81
	$Y = b_0 \pm b_1 \times lgX$		0.43 ± 0.26	0.13	84	0.45 ± 0.26	0.10	79
	$lgY = b_0 \pm b_1 \times X$		0.42 ± 0.26	0.13	82	0.44 ± 0.26	0.11	81
Bg-2	$Y = b_0 \pm b_1 \times X$		0.18 ± 0.23	0.45	97	0.25 ± 0.22	0.29	94
	$Y = b_0 \pm b_1 \times lgX$		0.14 ± 0.23	0.55	98	0.28 ± 0.22	0.22	93
	$lgY = b_0 \pm b_1 \times X$		0.12 ± 0.23	0.61	99	0.29 ± 0.22	0.21	92
Imp	$Y = b_0 \pm b_1 \times X$		0.14 ± 0.23	0.54	98	0.08 ± 0.25	0.76	99
	$Y = b_0 \pm b_1 \times lgX$		0.17 ± 0.23	0.46	97	0.00 ± 0.25	0.99	100
	$lgY = b_0 \pm b_1 \times X$		0.18 ± 0.23	0.45	99	0.09 ± 0.25	0.72	100
Bg-1	$Y = b_0 \pm b_1 \times X$	Cd	0.66 ± 0.22	0.01	55	**0.65 ± 0.22**	**0.01**	**57**
	$Y = b_0 \pm b_1 \times lgX$		**0.81 ± 0.17**	**0.00**	**33**	0.63 ± 0.22	0.02	60
	$lgY = b_0 \pm b_1 \times X$		0.65 ± 0.21	0.01	55	0.62 ± 0.22	0.02	61
Bg-2	$Y = b_0 \pm b_1 \times X$		0.78 ± 0.15	0.00	39	**0.60 ± 0.19**	**0.00**	**76**
	$Y = b_0 \pm b_1 \times lgX$		**0.79 ± 0.14**	**0.04**	**37**	0.49 ± 0.20	0.03	63
	$lgY = b_0 \pm b_1 \times X$		0.77 ± 0.15	0.00	40	0.52 ± 0.20	0.02	73
Imp	$Y = b_0 \pm b_1 \times X$		0.89 ± 0.11	0.00	20	**0.79 ± 0.15**	**0.00**	**38**
	$Y = b_0 \pm b_1 \times lgX$		**0.94 ± 0.08**	**0.00**	**12**	0.75 ± 0.16	0.00	44
	$lgY = b_0 \pm b_1 \times X$		0.85 ± 0.13	0.00	25	0.77 ± 0.16	0.00	40
Bg-1	$Y = b_0 \pm b_1 \times X$	Pb	− 0.00 ± 0.29	0.97	100	− 0.09 ± 0.29	0.76	100
	$Y = b_0 \pm b_1 \times lgX$		0.24 ± 0.28	0.40	91	− 0.04 ± 0.29	0.90	100
	$lgY = b_0 \pm b_1 \times X$		0.00 ± 0.29	0.98	100	− 0.04 ± 0.29	0.88	100
Bg-2	$Y = b_0 \pm b_1 \times X$		0.43 ± 0.21	0.06	81	− 0.20 ± 0.23	0.40	96
	$Y = b_0 \pm b_1 \times lgX$		0.30 ± 0.22	0.20	91	− 0.20 ± 0.23	0.39	96
	$lgY = b_0 \pm b_1 \times X$		0.39 ± 0.21	0.08	86	− 0.19 ± 0.23	0.43	98
Imp	$Y = b_0 \pm b_1 \times X$		0.22 ± 0.24	0.38	95	0.05 ± 0.25	0.83	99
	$Y = b_0 \pm b_1 \times lgX$		0.32 ± 0.24	0.19	89	− 0.05 ± 0.25	0.85	100
	$lgY = b_0 \pm b_1 \times X$		0.21 ± 0.24	0.41	96	0.05 ± 0.25	0.85	99

Bold type stands for a *p*-values less than 0.05

In our study, a significant drop of the activity of membrane-bound Na^+,K^+-ATPase (NAKA, see Table 1) was observed in erythrocytes of the Imp animals. This enzyme in all types of cells carries out the ATP-dependent transfer of Na^+ and K^+ ions across the membrane against an electrochemical gradient (Takeuchi et al. 2008). Most likely, Cd inhibits the ATPase activity directly due to its binding to -SH groups in the active site of this enzyme or indirectly, via the LP activation. Primary LP products (lipid radical L·, lipid peroxide radical LOO, and lipid peroxide LOOH) modify cell membrane phospholipids, and these modifications can suppress Na^+,K^+-ATPase activity (Haviv et al. 2013; Cornelius et al. 2015). The Cd- associated disturbance of the cation transport (of three Na^+ ions out of the cell and simultaneously two K^+ ions into the cell) undermines enzymatic

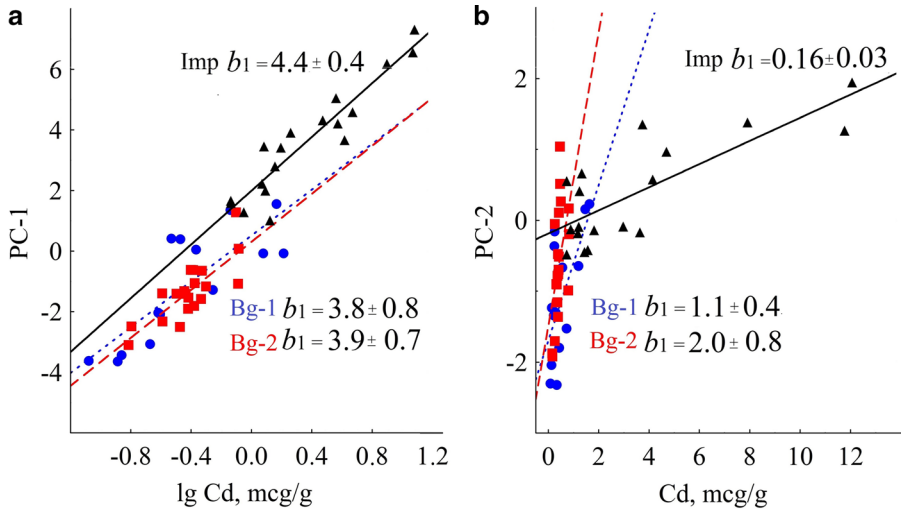

Fig. 2 Effects of individual Cd load (X) on the PCs (Y) in *Cl. rutilus* taking into account a catching site (Bg-1, Bg-2, and Imp). **a** PC-1 **b** PC-2; Correlation between PC-1,2 values and hematological parameters, see Table 2

Table 5 PCs' values (Y) depending on individual Cd load (X) in *Cl. rutilus* taking into account age (reproductive status: im, m, or ow): standardized regression coefficient (β ± SE) and residual dispersion (SS_{res}, % of total) in the analysis of linear ($Y = b_0 \pm b_1 \times X$), semilogarithmic ($Y = b_0 \pm b_1 \times lgX$), and exponential ($lgY = b_0 \pm b_1 \times X$) functions (im: immature underyearlings; m: mature underyearlings; ow: mature rodents that overwintered)

Reproductive-age status	Models	PC-1			PC-2		
		β ± SE	*p*	SS_{res}	β ± SE	*p*	SS_{res}
im	$Y = b_0 \pm b_1 \times X$	0.54 ± 0.22	0.03	71	0.27 ± 0.25	0.29	93
	$Y = b_0 \pm b_1 \times lgX$	**0.59 ± 0.20**	**0.01**	**65**	0.31 ± 0.24	0.22	90
	$lgY = b_0 \pm b_1 \times X$	0.54 ± 0.22	0.03	71	0.26 ± 0.25	0.31	94
m	$Y = b_0 \pm b_1 \times X$	0.83 ± 0.13	0.00	30	0.65 ± 0.12	0.00	58
	$Y = b_0 \pm b_1 \times lgX$	**0.97 ± 0.05**	**0.00**	**5**	**0.77 ± 0.15**	**0.00**	**41**
	$lgY = b_0 \pm b_1 \times X$	0.75 ± 0.15	0.00	43	0.60 ± 0.19	0.00	63
ow	$Y = b_0 \pm b_1 \times X$	0.77 ± 0.18	0.00	40	0.73 ± 0.19	0.00	46
	$Y = b_0 \pm b_1 \times lgX$	**0.95 ± 0.09**	**0.00**	**10**	**0.86 ± 0.14**	**0.00**	**26**
	$lgY = b_0 \pm b_1 \times X$	0.67 ± 0.20	0.00	55	0.67 ± 0.20	0.00	52

Bold type stands for a *p*-values less than 0.05

control of hydration, cellular volume, nutrient uptake, and cell fluidity.

Overall, Cd- associated alterations, namely, an increase in MCV/MCHb, OF, and LP and a decrease in NAKA activity—as metrics of structural anomalies of erythrocytes—worsen cell survival in the bloodstream. An increase in intravascular hemolysis during Cd accumulation was evaluated by us as a growing extracellular hemoglobin level in the plasma compartment (pfHb, see Table 1). From the reduced number of erythrocytes in peripheral blood of Imp rodents (RBC count, see Table 1), it can be concluded that greater erythrodieresis in *Cl. rutilus* because of technogenic load is not fully compensated by sufficient intensity of hematopoiesis. A decrease in the RBC count owing to toxic load has also been reported by other researchers. For instance, the decline of the relative proportion of RBCs (hematocrit) increases with blood levels of Cd (Rogival et al. 2006) in wood mice (*Apodemus sylvaticus* L.) along a geographic gradient of metal pollution. Laboratory experiments with intravenous administration of a $CdCl_2$ solution to Sprague–Dawley rats as well as *per os* administration of Cd to laboratory-bred bank voles (*Clethrionomys glareolus*) have revealed similar effects (Hiratsuka et al. 1996; Włostowski et al. 2000).

Redox-active metals (such as Cu and Fe) are known directly induce ROS production through Fenton and

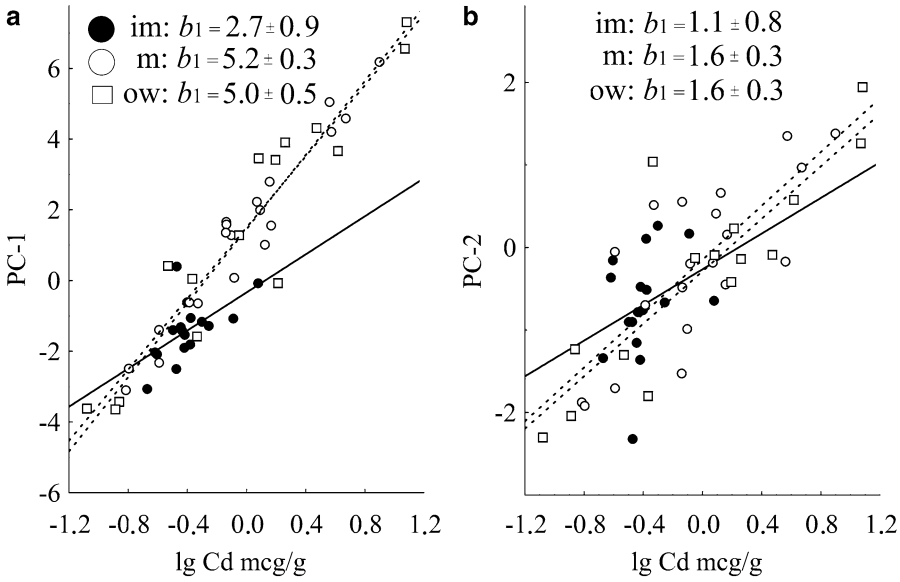

Fig. 3 Effects of individual cadmium load (X) on the PCs' (Y) of *Cl. rutilus* among reproductive-age groups: im – immature underyearlings; m – mature underyearlings; ow – mature voles that over-wintered. **a** PC-1 **b** PC-2; Correlation between PC values and hematological parameters, see Table 2

Haber–Weiss reactions. Nonetheless, non-redox-active metals (Cd, Pb and Zn) are still considered an effective pro-oxidant in the cell, presumably due to its ability to promote ROS production indirectly through suppression of antioxidant defenses (Valko et al. 2016). The damaging effects of thiol-binding metals have been documented in research on such biomarkers as antioxidant enzymes during monitoring of adverse effects of occupational exposure to lead and cadmium (Garçon et al. 2004; Kobal et al. 2004; Hambach et al. 2013). Assays performed on our subjects from the Imp site showed a pronounced pro-oxidant shift: inhibition of SOD activity (to 41% of the Bg value) and CAT activity (to 62% of the Bg value). This phenomenon is likely mediated by displacement of redox metals (such as Cu, Zn, and Fe) from active sites of SOD and KAT, resulting in the inactivation of these antioxidant enzymes. It should be pointed out that with respect to GSH-Px, we noticed the most pronounced inhibitory influence (to 36% of the Bg value). Significant effects of cadmium include its affinity for –SH groups, causing depletion of reduced glutathione (GSH) (Stohs and Bagchi 1995; Fortuniak et al., 1996; Stohs et al. 2001; Waisberg et al. 2003; Nemmiche 2017). Another important indicator of cellular redox status is the GSH/GSSG ratio (Rana et al. 2002; Cooper et al. 2011). GSH is regarded as the main defense mechanism against Cd^{2+} specifically bind its cysteine-thiol group the metal ion giving rise to a stable $[(GS-)n(Cd^{2++})]$ complex. Nevertheless, an equally important antioxidant function of this tripeptide should be mentioned, which is connected with its participation in the neutralization of H_2O_2 (i) and lipid hydroperoxides (LOOH) (ii) as a cofactor in detoxification reactions catalyzed by glutathione peroxidase (GSH-Px): (i) $GSH + H_2O_2 = GSSG + 2H_2O$; (ii) $2GSH + LOOH = GS\text{-}SG + LOH + H_2O$ (Deponte 2012). Thus, high metal-binding activity of GSH weakens its antioxidant function after exposure to high levels of cadmium.

According to our data, an increase in cadmium load is associated with an increase the glucose level (GLC) in blood plasma of northern red-backed voles. This finding is consistent with Cd being classified as a "hyperglycemic" metal (González-Villalva et al. 2016). A number of authors have linked blood hyperglycemia with Cd-induced inhibition of hepatic glycogen synthesis, damage to β-cells leading to reduced insulin production, or insulin resistance in target tissues (skeletal muscle and adipose tissue) diminishing glucose uptake (Ghafghazi and Mennear 1973; Ithakissios et al. 1975; Chapatwala et al. 1980; Bell et al. 1990). We were unable to investigate these phenomena because GLC enters the erythrocyte independently of insulin via a transporter located in the membrane. It is thought that the concentration of glucose in the intraerythrocyte medium is the same as that in blood plasma (Bohinski 1987). We observed an increase in GPI activity (to 228% of the Bg value), which catalyzes the isomerization reaction

G6P↔F6P (Cordeiro et al. 2003). In view of the foregoing, concentrations of G6P and/or F6P should go up, which, by the way, was documented elsewhere (Neville et al. 2020) when *Streptococcus pneumonia* was exposed to Cd^{2+}. The absence of an inhibitory effect of Cd on GPI activity is ascribed by a number of authors to the absence of –SH groups near the active site of this enzyme (Ramírez-Bajo et al. 2014). Given the finding of the decrease in the activity of GSH-Px (it requires GSH for manifesting its detoxifying role), the activation of GPI observed by us is most likely due to a decrease in G6P flux into the pentose phosphate pathway via suppression of the activity of NADP-dependent glucose-6-phosphate dehydrogenase (G6PD). The key enzyme of the pentose phosphate pathway, G6PD, provides reductive potential in the form of NADPH, which serves to regenerate the GSH pool: $GS\text{-}GS + NADPH + H^+ \rightarrow 2GSH + NADP^+$ (Bohinski 1987). A drop of G6PD activity in Cd-exposed rats has been shown (Khan and Kour 2007) probably also due to the formation of the Cd–SH complex via SH groups of this enzyme.

Therefore, we should list possible reasons for our observed Cd-induced shifts indicating structural and functional aberrations of *Cl. rutilus* erythrocytes along the gradient of toxic load: (1) an increase in osmotic fragility and a decline of membrane elasticity owing to LP activation; (2) a decrease in enzymatic (NAKA) control of hydration, cellular volume, nutrient uptake, and cell fluidity; (3) inactivation of antioxidant enzymes (SOD, GSH-Px, and KAT) controlling the level of hydrogen peroxide, superoxide radical, and lipid hydroperoxides; (4) diminishing G6P flux through the pentose phosphate pathway; this process depletes the pool of reduced forms of GSH; (5) greater erythrodieresis because of enhancement of intravascular hemolysis; (6) a decline of oxygen-carrying capacity by reason of a decrease in the hemoglobin amount per unit cell volume and erythropenia.

As the cadmium load went up (from 0.08 to 12 mg g^{-1} dry liver mass), we observed a decline of the growth rate of most of the tested erythrocytes aberrations (points 1–5 mentioned above), because of the semilogarithmic nature of the relation between hematological parameters and Cd concentration (see Table 4: PC-1). The improvement of resistance with increasing cadmium load is probably due to compensatory amplification of MT synthesis in the liver and kidneys and as a consequence the growth of the proportion of Cd bound to MT. We found no difference in Cd- associated reactivity of hematological parameters (which characterize points 1–5 mentioned above) between the Imp and background groups of *Cl. rutilus* (see Fig. 2a). The epigenetic nature of the observed effects in the Imp group, namely, a connection with hereditary changes in levels of MT gene expression, could be substantiated if $b_1 \pm SE \times 1.96$ [Imp] < or > $b_1 \pm SE \times 1.96$ [Bg-1,2].

The RBC count and MCHC characterizing oxygen-carrying capacity of the blood (point 6 mentioned above) are linearly dependent on Cd concentration but in a very narrow range of variation (see Table 4: PC-2). Most likely, both parameters are more dependent on the efficiency of the functioning of hematopoietic systems. Cadmium has cytotoxic effect not only on circulating blood cells but also on precursor ones in hematopoietic tissue (Lutton et al. 1984; Van Den Heuvel et al. 2001; Çelik et al. 2005; Çelik et al. 2009). Zhang et al. (2016) demonstrated that Cd provokes cell death and suppresses differentiation of erythroid progenitor cells while dramatically activating extramedullary erythropoiesis and causing considerable splenomegaly. It is quite possible that the lower Cd- associated reactivity of both parameters (the RBC count and MCHC) in the Imp animals (Fig. 2b) is due precisely to the mobilization of erythropoietic activity in the spleen. During the neonatal period of rodents, the spleen is a quite active hematopoietic site. In adulthood, not only does the spleen participate in the breakdown of RBCs, but it can also, in pathological conditions, play a role in hematopoiesis to help bone marrow compensate for the hemolysis taking place (Wolber et al. 2002).

It should also be noted that after normalization to toxic load (cadmium), the differences between the Imp and background groups in hematological status persisted (see Fig. 2a, b). Apparently, a certain percentage of hematological changes in Imp voles may be due to the action of other (unexamined) adverse factors, in addition to the Cd effect.

Our findings show that the group of immature underyearlings that do not mature in the year of their birth (im) has lower reactivity of most of hematological parameters (see Fig. 3a) to Cd as compared to the groups of breeding individuals (m and ow). It is possible that the higher resistance to technogenic stress in immature individuals is directly or indirectly related to physiological characteristics of this

able-bodied population segment, which possesses nonspecific resistance or tolerance to the action of extreme factors of natural origin: the greatest reduction in energy costs owing to the cessation of growth, delayed puberty, minimization of metabolism, and a low rate of aging processes (Olenev 2002).

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Author contributions Natal´ya A. Orekhova carried out hematological tests, statistical processing of data and their interpretation. Yulia A. Davydova and Georgii Yu. Smirnov were responsible for catching voles, maintenance, and analysis of reproductive-age groups. Natal´ya A. Orekhova and Yulia A. Davydova wrote the main manuscript text. All authors reviewed the manuscript.

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Data availability Baseline data analyzed in this study are presented in Supplementary Info File 3.

Declarations

Competing interests The authors declare no competing interests.

Conflict of interest The authors declare no conflicts of interest in relation to the work presented.

Ethical approval All procedures performed on the animals complied with the ethical standards of the Institute of Plants and Animals of Ecology, the Ural Branch of the Russian Academy of Sciences (Protocol No. 3 dated 18 December 2014).

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