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Original Research

Organ Profiles of the Antioxidant Defence System and Accumulation of Metals in *Helix aspersa* Snails

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Abstract

This paper studied the association between metals accumulation in selected tissues and the shell and antioxidant defence system in *Helix aspersa* snails.

Accumulation of Zn, Fe, Pb, Cd, and Mg was organ-specific and was the highest in the hepatopancreas. Hepatopancreatic catalase and glutathione peroxidase activities were the highest, whereas the highest glutathione reductase activity was recorded in the foot. Concentration of GSH was inversely proportional to hepatopancreatic accumulation of the metals. The metals accumulation did not affect hepatopancreatic lipids peroxidation, which means that the hepatopancreatic antioxidant defence was quite capable of eliminating the overproduction of reactive oxygen species evoked by metals.

Keywords: antioxidant defence, metals, *Helix aspersa*, lipid peroxidation, oxidative stress

Introduction

Metals accumulation by different species of land snails is still a subject of concern. It seems that studies on *Helix aspersa* can be helpful for understanding this process because of their ability to accumulate metals and simultaneously their limited ability to excrete the metals [1]. The metals can be highly reactive and they are toxic to most organisms [2] because of their ability to disturb various cellular processes, especially the functioning of cellular membranes [3, 4]. The toxic effect of metals appears to rely on generating reactive oxygen species (ROS) in biological systems. Reactive oxygen species are continuously generated in various cellular processes, but the state when the rate of their generation exceeds the rate of their decomposition is called oxidative stress.

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The antioxidant defence system plays a crucial role in animals' survival of periods of ROS overproduction. It consists of enzymatic and non-enzymatic components. In land snails activities of the antioxidant enzymes change in response to natural environmental stressors such as annual cycles of photoperiod, ambient temperature, and humidity [5-7]. Under physiological conditions snails are able to maintain a balance between production and neutralization of ROS, but pathological disturbances resulting from metals pollution lead to oxidative stress.

Antioxidant defence system have been studied in land snails in connection with annual cycles of photoperiod and temperature as well as with their estivation/arousal cycles [5-9]. Moreover, metal concentration in gastropods has also been investigated [10], but only few data are available concerning the antioxidant defence modulation in snails in response to metals exposure [2, 12, 13]. The specific aim of the present investigation was to check the association

between metals concentrations and the status of the antioxidant defence system in selected organs (the hepatopancreas, kidney and foot) of *H. aspersa* snails (the snails were purchased from a snail farm, because they do not live in natural habitat in Poland). Accordingly, the accumulation of zinc, nickel, copper, cadmium, iron, magnesium, and lead in the hepatopancreas, kidney, foot, and shell was evaluated and the organs' activities of antioxidant enzymes such as catalase (CAT), total glutathione preoxidase (GPX), selenium-dependent peroxidase (Se-GPX), and glutathione s-transferase (GST), and concentrations of reduced glutathione (GSH) as a non-enzymatic antioxidant, were also recorded. To estimate oxidative damage to the snails' organs, concentrations of thiobarbituric acid-reactive substances (TBARS), as a product of lipid peroxidation, were measured.

Materials and Methods

Animals

Adult specimens of the *H. aspersa* (Müller 1774) with well-defined *labium* on the shell, weighing between 24 g to 26 g, were used. They were purchased from an experimental farm (National Research Institute of Animal Production in Balice near Kraków). In the farm they were maintained outdoors in spacious garden boxes, where they had an opportunity to burrow and had free access to various vegetables. Because metals accumulation and antioxidant status of the organism cannot be assessed in the same animal (for technical reasons) the snails were divided into two groups (6 and 5 animals, respectively).

Chemicals

Cumene hydroperoxide, reduced glutathione (GSH), oxidised glutathione (GSSG), 5,5-dithio-bis (2-nitrobenzoic) acid (DTNB), 1-chloro-2,4-dinitrobenzene (CDNB), and etylenediaminetetraacetic acid (EDTA) were purchased from Sigma Chemical (Steinheim, Germany). Trichloroacetic acid (TCA), tris(hydroxymrthyl)aminomethane (TRIS), and disodium versenate dihydrate were purchased from Polskie Odczynniki Chemiczne (Gliwice, Poland). All other reagents were of analytical grade. CDNB was dissolved in ethanol and DTNB was diluted in methanol. The remaining solutions were dissolved in deionized water.

Sample Preparation

For sampling purposes snails were decapitated and after breaking their shells the hepatopancreas, kidney, and part of the foot were quickly (within 1-3 min) dissected out. For determination of metals concentrations a part of their shell was also taken. The samples were weighed and divided into two groups:

(i) five of them were frozen until they were used for assessment of protein content, catalase activity, reduced glutathione concentration, activities of glutathione-related enzymes, and TBARS concentrations

(ii) six samples were dried for measurement of metals concentrations

To determine antioxidant status of snails the organ samples were homogenized in potassium phosphate buffer at pH 7.4 using a Potter homogenizer with a Teflon piston at 200 rotations per minute. After centrifugation for 10 min. at 12,000 g, the supernatants were collected in Eppendorf tubes and were deep frozen and stored until they were used for the assessment of protein content, reduced glutathione and TBARS concentrations, and antioxidant enzyme activity.

Protein Concentration Assay

The protein concentration in the supernatants was estimated by Folin-Phenol methods described by Lowry et al., [14] using bovine serum albumin (Sigma Chemical, Steinheim, Germany) as a standard.

Antioxidant Enzymes Assays

Catalase (CAT) activity was determined according to Bartosz [15] by decomposition of 54 mM H_2O_2 in 50 mM phosphate buffer (pH 7.0) containing 20 µl of the enzyme extract in a total volume of 3 ml. Catalase activity was estimated at an ambient temperature of 25ºC by recording decreases in mixture absorbance for 3 minutes at 240 nm. Activity of catalase was expressed in U/g protein. The unit of catalase is defined as the reduction of 1µmol/l of the peroxide per minute.

Glutathione peroxidase (GPX) activity was determined as previously described by Chiu et al., [16], using cumene hydroperoxide for oxidation of NADPH. The reaction medium consisted of 700 µl of 50 mM TRIS-HCl buffer (pH 7.6), 100 µl of 1 mM GSH, 100 µl of the enzyme extract, and 100 µl of 0.05% (v/v) cumene hydroperoxide. The mixture was stabilized at 37ºC for 5 min. Then 1 ml of 20% (w/v) TCA was added and the samples were centrifuged. The supernatants were used for assessing enzyme activity. The reaction was initiated by adding 100 µl of DTNB diluted in methanol. The absorbance was recorded 5 minutes later at 412 nm. The blank sample was 50 mM TRIS-HCl buffer (pH 7.6).

Selenium-dependent glutathione peroxidase (Se-GPX) activity was determined according to the method described by Pagila and Valentine [17], using H_2O_2 as a substrate. The samples contained 2.63 ml of phosphate buffer (pH 7) with 13 mM of EDTA, 100 µl of NADPH, 10 µl of glutathione reductase (100 U/mg protein/ml), 10 µl of 1.125 M sodium azide, 100 µl of 0.15 M GSH, and 50 µl of the enzyme extracts. The reaction was initiated by the addition of 2.2 mM H_2O_2. Changes in the mixture absorbance were recorded at 340 nm for 3 minutes. Control samples contained all substances, but in place of the supernatants, 50 µl of water was added.

Glutathione reductase (GR) activity was determined by the method of Szczeklik [18] by following the consumption of NADPH per minute at 340 nm in 700 µl of a medium containing 480 µl of phosphate buffer (pH 7.5) and 100 µl of the assayed enzyme extract. The reaction was started by

the addition of GSSG. Changes in absorbance were monitored for 3 minutes.

Glutathione s-transferase (GST) activity was determined by the method of Habig et al., [19], based on the formation of the product of the reaction mixture between GSH and CDNB in phosphate buffer (pH 6.5) at 340 nm. The reaction was conducted at 25ºC and was initiated by the addition of 20 µl of the enzyme extracts and then changes in absorbance were followed for 3 min against the blank sample.

Non-Enzymatic Antioxidant Concentrations

Reduced glutathione (GSH) concentration was assayed according to the method described by Ellman [20], based upon the reaction of DTNB with sulfhydryl groups of GSH. Briefly, TCA, EDTA and the supernatants (prepared as described for the enzyme assays) in volumes 1:1:1 were centrifuged for 5 min at 10,000 g. Then, 200 µl samples of each supernatant were collected and used for quantification of the glutathione. The samples contained 2.3 ml of deionized water, 100 µl of EDTA and 300 µl of TRIS. The addition of 100 µl of DTNB started the reaction with thiols, which was analyzed spectrophotometrically at 412 nm. GSH concentration was expressed in µmols per gram of wet mass.

Lipid Peroxidation Assay

Measurements of concentration of TBARS were used to examine lipid peroxidation according to Bartosz [15], with modifications. Briefly, 250 µl of the supernatant was mixed with 1 ml of 15% (w/v) trichloroacetic acid in 25 mM HCl, and 0.37% (w/v) thiobarbituric acid in 25 mM HCl. Subsequently, samples were kept for 10 min at 100ºC, and then quickly cooled. After centrifugation for 5 min at 6500 g the supernatants were subjected to analysis at 535 nm. Two blank samples were used:

(i) without the supernatant

(ii) without thiobarbituric acid. TBARS concentration was expressed in mmols/g of wet mass.

Metals Concentration

For determination of metal concentrations the samples were dried at 105ºC. Then the samples were digested in HNO_3 and after complete dissolution they were diluted with water and neutralized with saturated $NaHCO_3$. Ammonium pyrrolidine ditiocarbaminate was added to each sample and they were shaken for 15 min. After the extraction, 2 ml samples were collected for measurement of metals concentrations. The metals content was estimated by AAS a BUCK 200A spectrophotometer with air-acetylene flame and appropriate hollow cathode lamps. Analytical efficiency of the measurements was checked using certified reference material (Bovine Liver BCR 185R) and analyzed in the same way as the samples. The recovery of the metals in this method ranges from 95 to 105%. Each measurement was repeated three times and the results were averaged. Moreover, standard curves were determined using standarized samples (Atomic Absorption Standard; BUCK Scientific, USA).

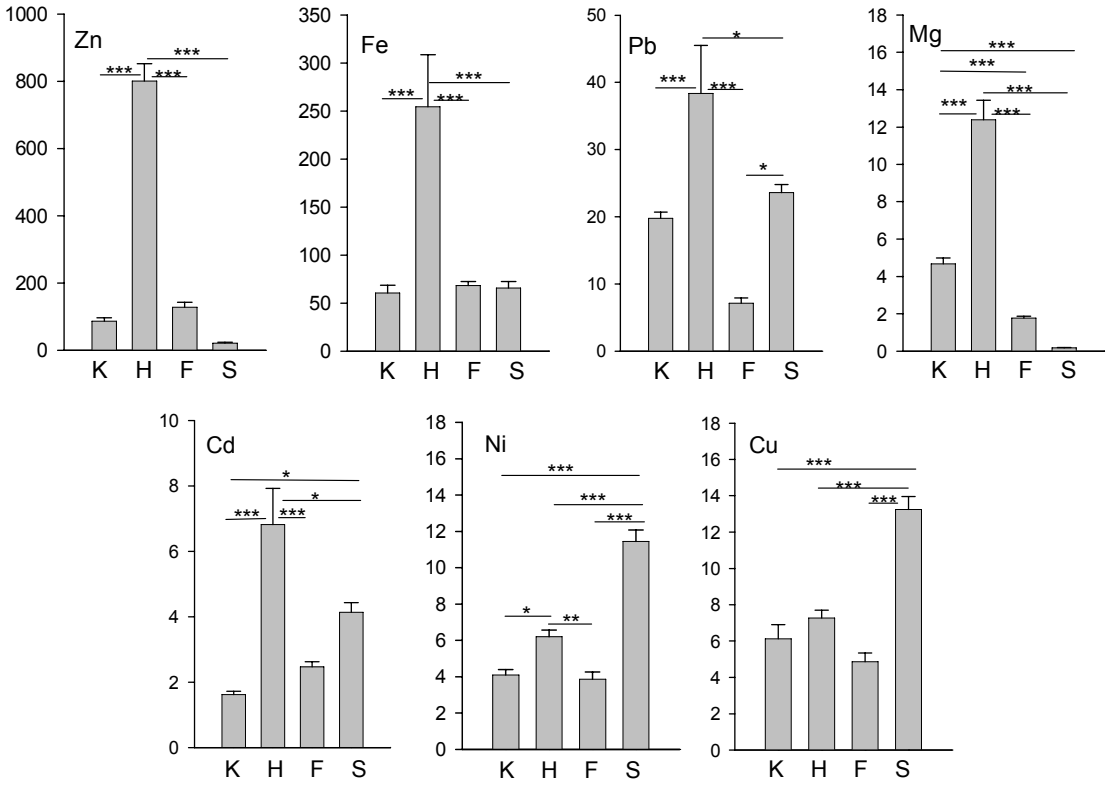

Fig. 1. Concentrations of metals in the kidney (K), hepatopancreas (H), foot (F), and shell (S) of *H. aspersa* snails. Values are presented as means ±SE. Differences between the organs *** P<0.001, ** P<0.01, * P<0.05. Concentrations of Zn, Fe, Pb, Cd, Ni, and Cu were expressed in µg per gram of wet mass, whereas Mg concentration was expressed in mg per gram of wet mass.

Data Analysis

Data were presented as mean values±standard error (SE). A one-way ANOVA was applied to analyze activity of the glutathione-related enzymes, activity of catalase and concentrations of reduced glutathione and TBARS as well. One-way ANOVA was also applied to analyze concentration of metals in organs and shell. In both analyses the Tukey-Kramer (HSD) test was used as a *post hoc* test. The threshold of statistical significance was $P<0.05$ for all tests. The data analyses were performed with the use of Statistica (StatSoft, Kraków, Poland).

Results

Concentrations of Metals

One-way ANOVA showed that concentrations of all metals (Fig. 1) was organ-dependent: Zn ($F_{3,20}=178.55$; $P<0.001$), Ni ($F_{3,20}=63.45$; $P<0.001$), Cu ($F_{3,20}=35.58$; $P<0.001$), Cd ($F_{3,20}=15.36$; $P<0.001$), Fe ($F_{3,20}=11.74$; $P<0.001$), Mg ($F_{3,20}=97.32$; $P<0.001$), and Pb ($F_{3,20}=12.22$; $P<0.001$). The metals e.g. Zn, Fe, Pb, Mg, and Cd were deposited mainly in the hepatopancreas, whereas, the highest accumulation of Ni and Cu was recorded in the shell.

As far as the particular organ-specific differences are concerned hepatopancreatic concentrations of Zn, Fe, Pb, Mg, and Cd were significantly higher ($P<0.001$) that those in the kidney, foot and shell. Concentration of Ni and Cu in the shells was significantly higher than those recorded in the soft tissues ($P<0.001$). Moreover, hepatopancreatic concentration of Ni was higher than that recorded in the kidney ($P<0.05$) and in the foot ($P<0.01$).

Antioxidant Defence System and Oxidative Stress

Catalase activity (Fig. 2A) and total glutathione peroxidase (Fig. 2B) were organ-dependent ($F_{2,10}=19.66$; $P<0.001$; $F_{2,90}=6.64$; $P<0.01$). The highest levels of the enzymes activity were recorded in the hepatopancreas, where they were significantly higher ($P<0.001$ and $P<0.01$, respectively) than those recorded in the foot, where they were the lowest. Activities of CAT and total GPX, recorded in the kidney, were also higher ($P<0.01$) than those recorded in the foot.

Activity of Se-GPX (Fig. 2C) was also affected by type of organ ($F_{2,10}=4.54$; $P<0.05$) but the highest activity of the enzyme was recorded in the kidney and it was significantly higher than that in the hepatopancreas.

Activity of glutathione reductase (Fig. 2D) was organ-specific ($F_{2,14}=21.09$; $P<0.001$). The *post hoc* analysis showed that the activity of GR (in contrast to activities of CAT and total GPX) in the kidney and hepatopancreas of *H. aspersa* snails were significantly lower ($P<0.001$) than that recorded in the foot.

Activity of GST (Fig. 2E) was organ-dependent ($F_{2,10}=5.47$; $P<0.01$) and foot activity of the enzyme was significantly lower than that recorded in the kidney ($P<0.05$).

One-way ANOVA showed that concentration of glutathione was strongly affected by type of organ ($F_{2,23}=29.71$; $P<0.001$) but *post hoc* analysis showed that there were no significant organ differences in GSH concentration between the hepatopancreas and foot as well as between the kidney and foot. The lowest concentration of GSH was recorded in the hepatopancreas and was significantly lower ($P<0.001$) that that in the kidney (Fig. 2F).

TBARS concentration (Fig. 2G) was unaffected by type of organ ($F_{2,80}=1.13$; ns). Its concentration tended to be the highest in the foot. Surprisingly, this was associated with relatively low accumulation of metals in the foot (Fig. 1).

Discussion

Many papers dealing separately with mechanisms of defence against oxidative stress [5, 6, 7,9] and with toxicity of metals [2, 12, 21] in land snails have been published so far.

In the present investigation concentrations of zinc, nickel, copper, cadmium, iron, magnesium and lead were measured and we tried to find the relationship between metals content in selected organs of the *H. aspersa* snail and the status of antioxidant defence system in the organs. It should be mentioned that we measured accumulation of metals in snails living in a suburb area and untreated with metals. Therefore, accumulation of the metals in the snails' body may be regarded as a consequence of their limited ability to excrete the metals [1].

The present results indicate that all antioxidant enzymes activities were organ-specific. Activities of CAT, total GPX, and GST in the foot were significantly lower than those in the remaining organs. In contrast, activity of GR in the foot was significantly higher than those in the hepatopancreas and kidney. Łaszczyca et al. [22] have shown that high activity of GR, accompanied by low activity of GPX and GST, may be regarded as a compensatory mechanism. This mechanism may relay on intensification of turnover between reduced and oxidized glutathione under conditions, which cause increased consumption of the peptide for the synthesis of metal-binding proteins, such as metallothioneins. As far as glutathione transferase is concerned the highest activity of the enzyme was recorded in the kidney and hepatopancreas, which reflects central metabolic functions of the organs in detoxification and elimination of toxic and undesirable foreign compounds. GST activity appears to be altered also by exposure to xenobiotics and to be elevated in some field studies [23]. It should be mentioned again that *H. aspersa* used in this investigation were taken from the experimental farm, where they had no contact with xenobiotics.

In the present investigation we were able to show that Cd was accumulated mainly in the hepatopancreas. This

supports the statement that the hepatopancreas of snails is one of the major target tissues accumulating Cd [21]. Most of the Cd accumulated in this organ is bound to a Cd-specific metallothionein isoform [24] that is inducible by the metal itself.

Cadmium is stored in soft tissues and induces lipid peroxidation in the liver, kidneys, brain, lungs, and heart of rats [11]. Contrary to other metals, Cd has a long (10-30 years) biological half-life, which means that it is excreted extremely slowly from the body [11]. Metals catalyse production of a large amount of reactive oxygen species, which should affect various cellular processes (mostly concerning the membrane system [12]) but our results do not show enhanced lipid peroxidation in tested organs. Lipid peroxidation has been found to be increased by lead [25], and there was a significant correlation between lead concentration and lipid peroxidaton [26]. Also, zinc [12] and copper [23] may cause a significant increase in TBARS. Our results show that despite the highest accumulation of Pb, Zn, and Cd in the hepatopancreas of *H. aspersa* snails, the organ concentration of TBARS was unchanged compared to other organs. In contrast, the lowest accumulation of the metals in the foot of our snails was associated with a surprisingly high TBARS concentration in the organ. This might be due to the relatively low activity of the antioxidant enzymes in the foot. Among them, only the activity of GR was enhanced, which might be responsible for a relatively high concentration of GSH in the organ.

There is some evidence that several enzymes of the antioxidant defence system are inactivated due to direct binding of metals to the enzymes' active sites, if they contain sulfhydryl groups [11]. On the other hand, there were no correlations between Fe, Zn, and Pb concentrations and enzyme activities [27]. Nevertheless, decreased activities of

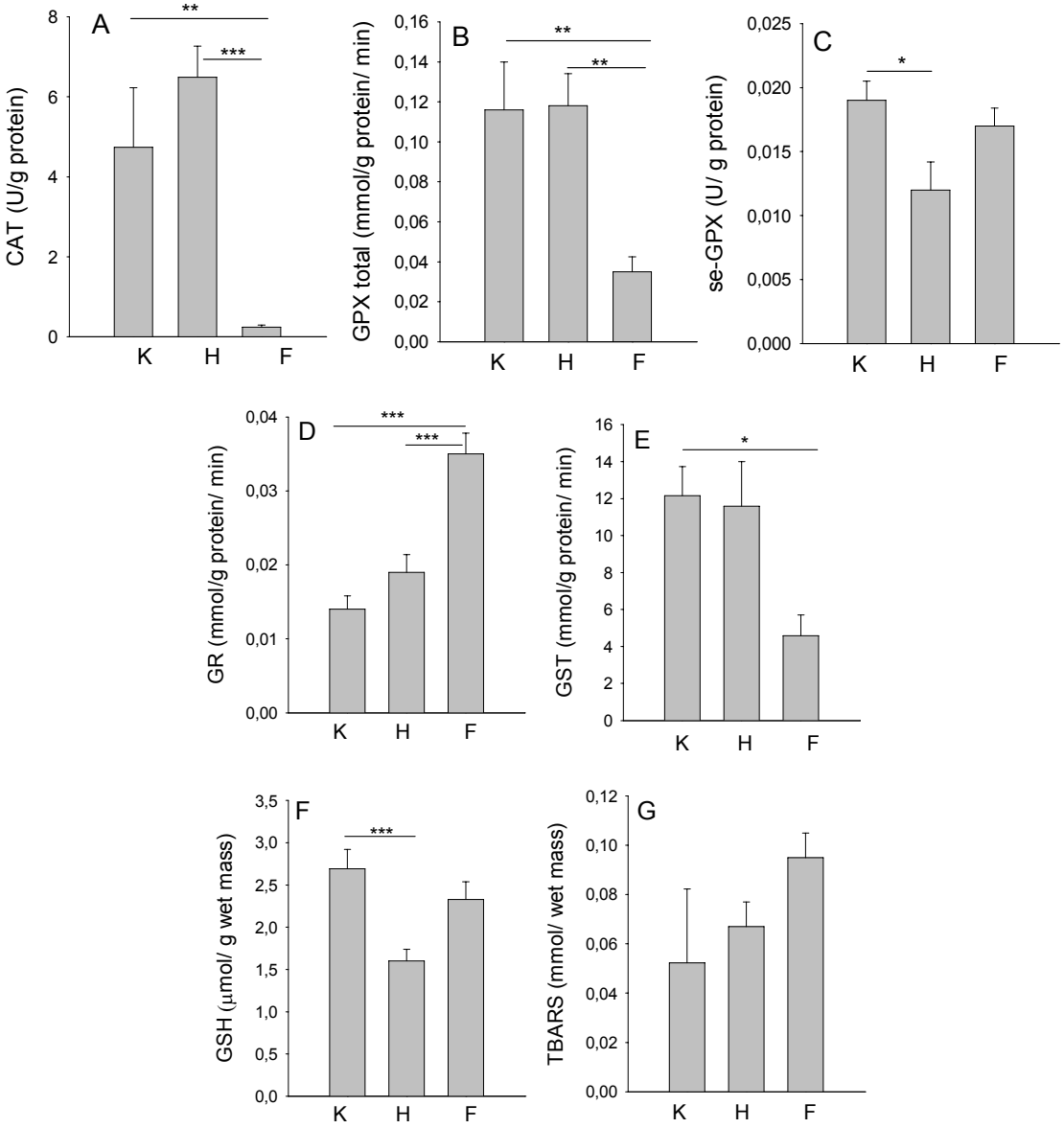

Fig. 2. Activity of catalase (panel A) and glutathione-related enzymes – total glutathione peroxidase (B), selenium-dependent glutathione peroxidase (C), glutathione reductase (D), and glutathione s-transferase (E), as well as concentrations of reduced glutathione (F), and TBARS (G) in the kidney (K), hepatopancreas (H) and foot (F) of *H. aspersa* snails. Values are presented as means ±SE. Differences between the organs ***P<0.001, **P<0.01 *P<0.05.

SOD, CAT, and GPX, recorded in *Achatina fulica* snails, may be due to over-production of reactive oxygen species by the metals [12]. *H. aspersa* snails from polluted areas exhibited higher CAT activity compared with control snails [28]. Because the enzymatic activities are regarded as reliable indices of individual responses to environmental stress, they can be used to estimate pollution at population and ecosystem levels [22].

Metals have electron-sharing affinities that can result in the formation of covalent attachments, which are mainly formed between metals and sulfhydryl groups of proteins [11]. Because GSH has carboxylic acid groups, an amino group, a sulfhydryl group, and two peptide linkages as sites for the reaction with metals, an interaction of many metals with GSH metabolism is an essential part of their toxicity [11]. Therefore, the functional groups play an important role in metals binding. It has been demonstrated that Cu may cause a significant decrease in GSH concentration while Cd and Zn do not [23]. Surprisingly again, in the present study we were able to show that concentrations of GSH were inversely proportional to accumulations of Cd and Zn in the hepatopancreas of *H. aspersa* snails. An association of Cd accumulation with GSH concentration is not clear. Cadmium is very similar to Zn in terms of easy absorption by animals and in terms of storage in the form of complexes with metallothioneins containing numerous –SH groups [29]. Nevertheless, some authors conclude that Cd can alter antioxidant defence systems in various tissues of many animals, causing depletion of the reduced glutathione [11]. In contrast, Cd caused an increase of GSH levels in the frog *Rana ridibunda* [29]. The present investigation proves that Cd can affect antioxidant defence systems, which confirms the previous findings [12], although its effect appears to be equivocal.

Lead is commonly regarded as the least toxic metal for the majority of invertebrates [30] and it is 5-6 times less toxic than Cu, Cd, and Zn for *Cantareus aspersus* [31, 32] and *Helix engaddennsis* snails [33]. In our *H. aspersa* snails, concentrations of Cd and Pb were the highest in the hepatopancreas and both metals exhibited the same profile of organ accumulation.

As far as the shell's metal accumulation is concerned accumulation of Zn and Mg was extremely low, whereas concentrations of Ni and Cu were extremely high. Accumulation of metals in the shell should be discussed in the wider context of calcification processes. There is an association between accumulation of Pb and Ca, but the mechanism of Pb assimilation is not fully understood. On the other hand, Ca-rich tissues have little detectable Pb. Accumulation of Pb in the shells of our snails was higher than that in soft tissue such as the foot. Lead reduces Ca concentration and enhances Mg concentration in fish plasma [34]. In mammals plasma Mg is buffered by bone Ca, but according to Burton [35] calcium does not affect Mg assimilation in shells of snails. Also in *Cantareus aspersus* snails Mg does not play any role in limiting Pb assimilation [30]. Nickel is regarded as a metal preferentially accumulated in soft tissues of *Cerithium vulgatum* [36] and *Patella aspersa* snails [37]. Surprisingly again, in the present investigation the highest accumulation of Ni was recorded in shells of our *H. aspersa* snails.

Interaction between metals and the components of the antioxidant defence system is involved in ecotoxological responses of an organism to environmental pollution [28]. The pollution enhanced accumulation of Cd and Zn in the shell of *Cepaea nemoralis* snails and concentrations of these metals were higher in shells from polluted than from unpolluted soils [38]. Both of the mentioned metals were not accumulated in the shells of our *H. aspersa* snails, which proves that the metals did not pollute their environment.

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