Global decline of amphibian populations causes particular concern about their vulnerability to novel environmental pollutants, including engineering nanomaterials and pharmaceutical products. We evaluated the bioavailability of nanoform of zinc oxide (n-ZnO) in frog Pelophylax ridibundus and determined whether co-exposure to a common pharmaceutical, a calcium-channel blocker nifedipine (Nfd) can affect this bioavailability. Male frogs were exposed for 14 days to the tap water (Control) and n-ZnO (3.1 μM), Zn2+ (3.1 μM, as a positive control for n-ZnO exposures), Nfd (10 μM), and combination of n-ZnO and Nfd (n-ZnO + Nfd) in environmentally-relevant concentration. Exposure to Zn2+ or n-ZnO led to up-regulation of metal-binding proteins, metallothioneins (MTs) in the liver and Zn-carrying vitellogenin-like proteins in the blood plasma. Notably, upregulation of MTs by Zn2+ or n-ZnO exposures combined with increased binding of Zn and Cu to MTs. This was associated with the more reducing conditions in the liver tissue indicated by elevated lactate to pyruvate ratio. Nfd suppressed the binding of Zn and Cu to MTs and led to a decrease in Lactate/Pyruvate ratio and elevated protein carbonylation indicating pro-oxidant conditions. Redox status parameters were not directly related to DNA fragmentation, nuclear abnormalities or suppression of cholinesterase activity indicating that factors other than oxidative stress are involved in cytotoxicity of different pollutants and their combinations. Furthermore, activity of Phase I biotransformation enzyme (CYP450 oxidase measured as EROD) was elevated in Nfd-containing exposures and in Zn2+ exposed frogs. Tyrosinase-like activity in the frog liver was strongly stimulated by Zn2+ but suppressed by n-ZnO, Nfd and n-ZnO + Nfd. These findings show that Nfd modulates homeostasis of essential metals in amphibians and emphasize that physiological consequences of combined n-ZnO and Nfd exposures are difficult to predict based on the mechanisms of single stressors.

© 2017 The Authors. Production and hosting by Elsevier B.V. on behalf of King Saud University. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).
are often weaker than those of free Zn\(^{2+}\) indicating that metal from n-ZnO particles may not be readily bioavailable (Falfushynska et al., 2015b, Falfushynska et al., 2017).

Nanomaterials in freshwater environments commonly co-occur with other chemical stressors such as pharmaceuticals and personal care products (PPCPs) (Buzza et al., 2007, Daughton, 2016). Ecotoxicological effects of PPCPs are likewise poorly understood, although their high biological activity in target species (such as humans or domestic animals) creates potential for similar effects in aquatic organisms. In this study, we focused on a common calcium influx inhibitor nifdefpine (Nfd), which is widely used as an antiinflammatory and antihypertensive medicine and is on the World Health Organization (WHO) Model List of Essential Medicines (WHO, 2015). Despite its partial microbial and photocatalytic breakdown in the environment, Nfd and its degradation products (nitroso-, nitro-, azoxy- and N,N'-dioxide-derivatives) are common pollutants in municipal sewage effluents and in surface waters (Hayase et al., 1995). Our earlier study showed that n-ZnO toxicity in bivalve mussels is modulated by Nfd causing increase in the DNA fragmentation (Falfushynska et al., 2015a). In amphibians, Ndf exposure caused oxidative stress indexed by elevated production of reactive oxygen species, accumulation of lipid peroxidation products and oxidized glutathione (Falfushynska et al., 2017).

Understanding ecotoxicological effects of multiple pollutant exposures in amphibians has become a focus of attention due to global amphibian population declines (Johnson et al., 2016, Prokić et al., 2017). Frogs are sensitive to a range of environmental pollutants due to their semi-permeable skin especially during the aquatic life cycle stages, and exposures to sublethal concentrations of environmental pollutants may be complicit factors in deteriorating amphibian health (Kloas and Lutz, 2006). The ecotoxicity of nanoparticles and PPCPs in frogs has not been extensively studied (Nations et al., 2011). Our recent study in Pelophylax ridibundus, a common European marsh frog, showed that Nfd may act as a pro-oxidant and endocrine disruptor in this species (Falfushynska et al., 2017). Nfd also increased the cellular thiol content due to elevated levels of the mostinteractive thiol-containing proteins (putative MTs) and glutathione (Falfushynska et al., 2017). Based on these findings, we hypothesized that Nfd could affect homeostasis of essential metals with high affinity to thiol groups (such as Zn\(^{2+}\) and Cu\(^{2+}\)) increasing metal toxicity in frogs and inducing genotoxicity, which is a common consequence of disrupted metal homeostasis (Mulware, 2013). To test this hypothesis, we measured Zn\(^{2+}\) and Cu\(^{2+}\) binding to MTs and Zn-containing vitellogenin-like proteins in frogs exposed to n-ZnO, Nfd and their combinations, assessed parameters of genotoxicity (DNA fragmentation and nuclear abnormalities). We also determined the changes in cellular reduct status (by measuring the ratio of lactate to pyruvate reflective of the NADH/NAD\(^{+}\) ratio (Sun et al., 2012) and assessing the levels of carbonylated proteins) to determine whether changes in the Zn\(^{2+}\) and Cu\(^{2+}\) binding are associated with the cellular reduct shifts. Activity of CYP450 monoxygenase (measured as EROD activity) was determined to assess activation of detoxification pathways by n-ZnO and Nfd exposures, and activities of metal-sensitive enzymes (including tyrosinase and cholinesterase (Frasco et al., 2005, Han et al., 2007, Pohanka, 2014) was measured to test the potential effects of disrupted Zn\(^{2+}\) and Cu\(^{2+}\) metabolism for cellular processes.

2. Materials and methods

2.1. Materials

Suspensions of n-ZnO (mean particle size 35 nm), reduced glutathione (GSH), bovine serum albumin, phenylmethylsulfonyl fluoride (PMSF), 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB), nicotinamide adenine dinucleotides (NADH, NAD, NADPH), EDTA, dihydrodorhadamine, Hoescht 33342 dye, 2,4-Dinitrophenylhydrazine, tyrosin, hemoglobin, chymotrypsigen, cytochrome c, myoglobin, ubiquitin, Sephadex G-50, β-mercaptoethanol, ethoxyresorufin, certified reference material ERM-BB422 and Lactobacillus leichmanlii D-Lactate dehydrogenase were purchased from Sigma Chem. Co. (St. Louis, USA). All other chemicals were obtained from the Synbias (Kyiv, Ukraine), Bayer (Kyiv, Ukraine) and Balkanpharma-Dupnitsa (Dupnitsa, Bulgaria) commercial suppliers. All reagents were of the analytical grade or higher.

2.2. Animal maintenance and experimental exposures

A total of 60 replicates of adult males of Pelophylax ridibundus (Rana ridibunda) (8–10 cm long) were collected in September 2013, outside the breeding season of the local population of P. ridibundus, from a country site in the upstream portion of river Seret (near the village Ivachiv, 49°49′N, 25°23′E) where no industrial contamination could be detected. Frogs were transported to the laboratory in 60 L cages with aerated native water (dissolved oxygen concentration was 8.67 ± 0.51 mg L\(^{-1}\)). Experiments were carried out in accordance with the national and institutional guidelines for the animal protection and welfare with permission of the Ministry of Ecology and Natural Resources of Ukraine, No 466/17.04.2013 and approval of the Committee on the Bio-Ethics at Ternopil National Pedagogical University (No 2/10.06.2013).

The P. ridibundus specimens in the trial, exposure conditions and experimental design of the study were the same as described in our earlier published work (Falfushynska et al., 2017). Briefly, frogs were acclimated in aerated, softened tap water (total hardness was 1.1 ± 0.07 mmol L\(^{-1}\) and fed throughout the experiment with commercial “Turtle menu” (21% of protein, Aquarius, Ukraine). After seven days of preliminary acclimation, frogs were randomly distributed into five groups (10 individuals per group). One group was exposed to the fresh tap water only and was considered as a control (C). Four other experimental groups were exposed to the following compounds: (1) zinc nanooxide (n-ZnO), (2) dissolved zinc (Zn\(^{2+}\) added as ZnSO\(_4\) as a positive control), (3) a potent vasodilator agent, dihydropropyridine Calcium Channel Blocker nifdefpine (dimethyl-2,6-dimethyl-4-(2-nitrophe nyl)-1,4-dihydropropyridine-3,5-dicarboxylate) (Nfd, 10 µm), and (4) a combination of n-ZnO with 10 µm Nfd (n-ZnO + Nfd). The concentrations of n-ZnO and ZnSO\(_4\) were adjusted to correspond to the nominal concentration of 3.1 µm Zn in suspension and/or solution. The concentrations of the nanoparticles, free Zn\(^{2+}\) and Nfd chosen for this study were within the range of concentrations range found in the field (for Zn\(^{2+}\) and n-ZnO) and/or reflected the lowest observed effect concentrations (LOEC) (for Nfd). For n-ZnO approximately 95% of rivers in the EU would be predicted to amount 500 ng L\(^{-1}\) or less (<6 nM n-ZnO) (NanoFATE 2010–2014). Concentration of Zn in the water at the sampling site was 20.2 ± 2.7 µg L\(^{-1}\) (n = 3). The concentrations used in this study (3.1 µm Zn) were well below this level.

All exposures were carried out for 14 days at 18 °C in 60 L tanks with aerated, softened tap water (10 animals per tank). There was no mortality of frogs during the acclimation or experimental exposures. After 14 days of exposure, the frogs were anesthetized by clove oil, the heparinized blood was collected from the heart, and plasma was separated immediately by centrifugation at 2000g for 10 min. The frogs were killed by a blow to the head, the spinal cord severed, and liver was removed immediately for experiments. Isolated livers of 8 frogs per each experimental group were perfused on ice. The circulating blood was drained using a 24-gauge needle (Yuria-Farm, Ukraine) attached to a syringe was inserted.
into the lumen of the liver with 8 ml of the saline/Ringer’s solution (NaCl, 6.5 g, KCl, 0.14 g, CaCl₂, 0.03 g, NaHCO₃, 0.2 g, Glucose, 2.0 g, Distilled Water, 1000 ml), then livers were shock frozen and stored at −20 °C until further analyses. Plasma samples were obtained as the supernatants of heparinized (Biolik, Ukraine), anti-coagulated blood and immediately used for vitellogenin-like protein determination. For metal analysis in metallothioneins (MTs) samples were isolated from liver tissues and kept at −20 °C until metals assessed. Analysis of the results of studying water parameters and thermostable proteins was carried out in triplicate, and all other measurements were carried out in 8 frog specimens.

2.3. Metal-binding proteins and metal content

Metallothioneins (MTs) were isolated from liver tissues of frogs as a thermostable protein fraction using size-exclusion chromatography on Sephadex G-50 as described elsewhere (Falfushynska and Stollar, 2009). Briefly, 5% homogenate (w/v) of the liver tissue was prepared in ice-cold isolation buffer (10 mM Tris–HCl, pH 8.0) containing 10 mM 2-mercaptopoethanol (to maintain the reducing conditions and avoid oxidation of MTs) and 0.1 mM PMSF (to inhibit proteolysis). Fractions of the chromatographic peak with high absorbance at 254 nm and a relatively high D254/D280 density ratios were identified as MTs-containing peaks (Kagi and Schaffer, 1988), pooled to obtain a total of 10 mL and applied to determine the content of metals (Cu and Zn).

Concentrations of metals (Cu and Zn) were determined in the liver tissue of the frogs and in the MT fraction of liver proteins. For this, fresh liver tissues (250 mg) or the pooled eluate of MTs fraction (10 mL) were digested in 5 mL metal-grade HNO₃ (LLC Stoliar, 2009). Briefly, 5% homogenate (w/v) of the liver tissue was prepared in ice-cold isolation buffer (10 mM Tris–HCl, pH 8.0) containing 10 mM 2-mercaptopoethanol (to maintain the reducing conditions and avoid oxidation of MTs) and 0.1 mM PMSF (to inhibit proteolysis). Fractions of the chromatographic peak with high absorbance at 254 nm and a relatively high D254/D280 density ratios were identified as MTs-containing peaks (Kagi and Schaffer, 1988), pooled to obtain a total of 10 mL and applied to determine the content of metals (Cu and Zn).

Concentrations of metals (Cu and Zn) were determined in the liver tissue of the frogs and in the MT fraction of liver proteins. For this, fresh liver tissues (250 mg) or the pooled eluate of MTs fraction (10 mL) were digested in 5 mL metal-grade HNO₃ (LLC Stoliar, 2009). Briefly, 5% homogenate (w/v) of the liver tissue was prepared in ice-cold isolation buffer (10 mM Tris–HCl, pH 8.0) containing 10 mM 2-mercaptopoethanol (to maintain the reducing conditions and avoid oxidation of MTs) and 0.1 mM PMSF (to inhibit proteolysis). Fractions of the chromatographic peak with high absorbance at 254 nm and a relatively high D254/D280 density ratios were identified as MTs-containing peaks (Kagi and Schaffer, 1988), pooled to obtain a total of 10 mL and applied to determine the content of metals (Cu and Zn).

Metal content in the MTs was presented as 10 g wet mass for the tissue and MT fraction. The reliability of the metals measurements was assessed by analyzing ERM-BB422 fish muscle matrix certified reference material with known mass fractions of eight elements, assessed by analyzing ERM-BB422 fish muscle matrix certified reference material with known mass fractions of eight elements, assessed by analyzing ERM-BB422 fish muscle matrix certified reference material with known mass fractions of eight elements.

2.4. Biomarkers of redox status and cellular stress

Liver tissue was homogenized (1:10 w:v) in ice-cold phosphate buffer (0.1 M, pH 7.4) containing 100 mM KCl, 1 mM EDTA and 0.1 mM PMSF with 12–15 strokes of a motor-driven Teflon Potter–Elvehjem homogenizer. The homogenate was centrifuged at 6000 g for 10 min at 4 °C, and supernatants used for biomarker analysis. Concentrations of metabolites and enzyme activities (described in Sections 2.4.1–2.4.3) were standardized to the protein content of the homogenates. Protein concentrations were measured by the method of Lowry et al. (1951) using bovine serum albumin as a standard. The absorbance values were measured on the UV/Vis spectrophotometer LOMO-S6 (LOMO, St. Petersburg, Russian Federation), and the extinction/emission values were measured on the f-max fluorescence microplate reader (Molecular Devices, Sunnyvale, CA, USA).

2.4.1. Oxidative lesions and redox status

Protein carbonyl (PC) content was measured in the supernatant using the reaction of carbonyls with 2,4-Dinitrophenylhydrazine (DNPH) or HCl as a negative control (Reznick and Packer, 1994). After incubation with DNPH, proteins were precipitated by 100% trichloroacetic acid and centrifuged at 12,000 g for 10 min. The pellet was collected, washed with ethanol ethylacetate and dissolved in 8 M urea. The differences in absorbance between the DNPH- and the HCl-treated samples were determined spectrophotometrically at 375 nm, and the amount of carbonyl was determined by using a molar extinction coefficient of 2.2·10⁴ M⁻¹ cm⁻¹. Data were expressed as μmole g⁻¹ wet mass.

Tissue redox status was assessed using the lactate/pyruvate ratio. Lactate and pyruvate were assayed with standard spectrophotometric methods using bacterial D-Lactate Dehydrogenase (EC 1.1.1.28) from Lactobacillus leichmannii (D-LDH) as a coupling enzyme. Lactate concentrations were measured following the NAD-dependent enzymatic oxidation of lactate to pyruvate by D-LDH (Gawehn, 1988). Pyruvate was measured following the D-LDH-dependent conversion of pyruvic acid to D-lactic acid in the presence of NADH. Differences in the absorbance at 340 nm (reflecting a change in NADH concentration) measured before the start and 60 min after the start of the reactions were used to calculate lactate or pyruvate concentrations of the samples using the millimolar extinction coefficient for NADH of ε₉₅ = 6.22·10⁴ M⁻¹ cm⁻¹ (Lamprecht and Heinz, 1988).

2.4.2. Genotoxicity markers

To assay the genotoxicity of exposures, levels of protein-free DNA strand breaks in liver tissue cells were determined by the alkaline DNA precipitation assay (Olive, 1988) based on using Hoescht 33342 dye in the presence of 0.4 M NaCl, 4 mM sodium cholate, and 0.1 M Tris (pH 9) (Bester et al., 1994). The amount of protein-free DNA strand breaks was related to the level of the protein in sample.

Genotoxic effect of model exposures was assessed in the erythrocytes by the frequency of the micronucleated erythrocytes (MN) (Baršienė et al., 2006), as well as erythrocytes with lobed nuclei (L), dumbbell-shaped or segmented nuclei (S), and kidney-shaped nuclei (K). Frequency of nuclear lesions were assessed totally for MN and all other lesions (L + S + K) and expressed per 1000 cells. At least 2000 cells were assessed for MN and nuclear lesions in each sample.

2.4.3. Enzyme activities

Microsomal ethoxyresorufin-O-deethylase (EROD) activity was measured as a common and reliable indicator of the activity of the cytochrome P450 family 1 (CYP450 I) enzymes involved in Phase I biotransformation of xenobiotics in frog liver (Iwamoto et al., 2012). EROD was assayed in the microsomal fraction of the liver tissues. To obtain the microsomal fraction, postmitochondrial supernatant was obtained by centrifugation of the liver homogenates for 20 min at 12,000 g, and the microsomal fraction was obtained by calcium (80 mM CaCl₂) precipitation of the postmitochondrial supernatant in 10 mM Tris–HCl buffer, pH 7.4 as described elsewhere (Cinti et al., 1972). EROD activity was
detected by measuring the change in absorbance of resorufin at 572 nm (Klotz et al., 1984) and calculated using a molar extinction coefficient of 73.2 mM \text{-} 1 \text{ cm}^{-1} \text{ cm}^{-1} \text{ M}^{-1} and standardized to the microsomal protein content.

The phenoloxidase-like activity of tyrosinase (EC 1.14.18.1) was determined by recording the formation of o-quinones (Luna-Acosta et al., 2011). p-Phenylene diamine were used as substrate. Phenoloxidase-like activity was monitored during 2 h at 420 nm. The amount of o-quinones was determined by using a molar extinction coefficient of 43 160 M \text{-} 1 \text{ cm}^{-1} \text{ cm}^{-1} \text{ M}^{-1}. Data were expressed as nmol min \text{-} 1 mg \text{-} 1 protein.

Cholinesterase (ChE, EC 3.1.1.7) activity was determined in the brain as the acetylthiocholine-cleaving ChE activity at 25 °C according to the colorimetric method of Ellman et al. (1961). Cholinesterase activity in the frog brain measured in this study mostly represents activity of acetylcholine esterase. Butyrylcholinesterase which can also non-specifically cleave choline-based esters, is found in blood plasma and would not contribute significantly to the cholesterase activity in the perfused brain samples. Enzyme activity was calculated using a molar extinction coefficient of 13.6 \text{ M}^{-1} \text{ cm}^{-1} and standardized to the soluble protein content.

2.5. Statistical analysis

The results are presented as means ± standard deviation (S.D.). Data were tested for normality and homogeneity of variance by using Kolmogorov-Smirnoff and Levene’s tests, respectively. Whenever possible, data were normalized by Box-Cox common transforming method. One-way ANOVA was used to test the effect of experimental exposures, followed by post hoc procedures. Since data were not normally distributed (Lilliefors’ test), non-parametric tests (Kruskal–Wallis ANOVA and Mann–Whitney U-test) were performed (significant at p < .05). For detection of correlation, the Pearson’s correlation test was also used at 0.05 level of significance.

The classification tree was built using Classification and Regression Tree (CART) software on the basis of all determined biological characteristics. A CART tree is a binary decision tree that is built by splitting a node into two subsidiary nodes repetitively, beginning with the root node that contains the whole learning sample and predicts the value of a target based on the values of independent variables. All statistical calculations were performed with Statistica v 10.0 and Excel programs for Windows-2000.

3. Results

3.1. Metal content and binding

MTs level in the liver tissue and their Zn and Cu-binding ability were affected by all exposures with the most prominent changes in response to Zn2+ exposures (with up to 2.5 increase compared to the control levels) (Fig. 1A, C, D). All Zn-containing treatments (Zn2+, n-ZnO and n-ZnO + Nfd) induced an increase in the tissue MTs content, while exposure to Nfd alone suppressed the MT levels (Fig. 1A). Notably, metal exposures (Zn2+ and n-ZnO) led to an increased binding of Zn and Cu to MTs (Fig. 1C and D). Nfd treatment alone or in combination with n-ZnO caused the decrease of Zn and Cu binding to MTs (Fig. 1C and D).

All Zn-containing treatments (Zn2+, n-ZnO and n-ZnO + Nfd) led to an increase in the levels of Zn-binding Vtg-LP by 23.5–54.1% in the blood plasma of male frogs (Fig. 1B). Nfd alone had no effect on plasma levels of Vtg-LP (Fig. 3).

3.2. Tissue redox status

Lactate concentrations in the liver tissues were slightly but significantly elevated by exposures to n-ZnO (alone or in the presence of Zn2+ or n-ZnO).

Fig. 1. Metallothioneins traits (A–D) and Zn-binding protein vitellogenin level (B) in frogs exposed to nano-zinc oxide (ZnO), zinc ions (Zn), nifedipine (Nfd) and combination of ZnO and Nfd. A: metallothioneins level, B: vitellogenin-like protein level, C: copper concentration in metallothioneins, D: zinc concentration in metallothioneins in the liver of frog P. ridibundus. Data are presented as means ± SD. N = 8 for MTs level and Vtg-LP and N = 3 (for joined samples from 5 specimens each) for Zn-MT, Cu-MT. Here and on the Figs. 2 and 3, the columns that share the same letters indicate the values that are not significantly different (P > .05).
of Nfd) (Fig. 2A). Other treatments had no effect on tissue lactate levels. Pyruvate concentrations were suppressed by Zn²⁺ and n-ZnO treatments and elevated in the presence of Nfd (Fig. 2B). As a result, lactate/pyruvate ratio increased in Zn²⁺ and n-ZnO treat-ments and declined in Nfd-containing exposures (Fig. 2C).

PC levels in the liver tissues slightly but significantly increased after exposure to Zn²⁺, and were considerably elevated after exposure to Nfd alone (Fig. 2D). In contrast, joint exposure to n-ZnO and Nfd led to a decrease in the protein carbonyl content in the frog liver (Fig. 2D).

3.3. Cellular stress and toxicity biomarkers

All experimental exposures resulted in elevated levels of nuclear abnormalities indicating genotoxicity (Fig. 3A). No neuro-cytotoxicity (assessed as ChE activity in the brain) was induced by the experimental exposures to Zn²⁺ and n-ZnO + Nfd, while ChE activity was suppressed in n-ZnO exposed group (Fig. 3B). Notably, exposure to Nfd alone led to an increased activity of activation of ChE.

All experimental treatments (except n-ZnO) led to elevated activity of a Phase I biotransformation enzyme (EROD) in the liver (Fig. 3C). The tyrosinase-like activity was suppressed in Nfd- and n-ZnO- exposed groups but not in those co-exposed to n-ZnO and Nfg (Fig. 3D). Notably, Zn²⁺ exposure caused a twofold increase in tyrosinase activity compared to the control (Fig. 3).

3.4. Data integration

Pearson correlation analysis showed that all parameters related to metal homeostasis (MT levels, Cu- and Zn-MTs, Vtg-LP) are strongly and positively correlated with each other and with the lactate/pyruvate ratio (Table 1). CART analysis generated a tree with 4 splits and 5 terminal nodes, and the overall classification accuracy of 95% (Fig. 4). Nfd-treated groups were distinguished from the rest primarily by the low Cu-MT content. Within the Nfd-containing treatment groups, n-ZnO + Nfd treated group differed from those treated with Nfd alone by higher MT content. Control group and n-ZnO-exposed groups were segregated by the lactate/pyruvate ratio (lower in n-ZnO group), and Zn²⁺-exposed group was distinguished by elevated tyrosinase activity.

4. Discussion

Ca-channel blockers such as Nfd are expected to affect Zn²⁺ metabolism due to the tight linkages between the metabolism and transport of Ca²⁺ and Zn²⁺ in the cell (Hogstrand et al., 1996, Nolte et al., 2004, Inoue et al., 2015). Thus, Zn²⁺ can replace Ca²⁺ in the binding sites of wide range of transport proteins such as the mitochondrial Ca²⁺ transporter and the Ca²⁺ channels located in excitable membranes (Csermely et al., 1989), and Zn²⁺ blocks Ca²⁺ entry through the same channels (Kerchner et al., 2000, Rogers and Wood, 2004). On the other hand, Zn uptake can be inhibited by Ca channel blockers Nfd, verapamil and lanthanum in some organisms, among them an euryhaline black sea bream (Acanthopagrus schlegeli) (Zhang and Wang, 2007) and a mangrove crab Ucides cordatus (Sá and Zanotto, 2013). Our present study is in line with these earlier findings showing that n-ZnO-induced elevation of Zn-MT levels is prevented by exposure to Nfd. Interestingly, Nfd exposure also suppressed levels of Cu-MT indicating that this Ca channel blocker may affect metabolism of several bivalent metals. One possible mechanism of Zn-MTs and Cu-MTs suppression may be related to Nfd-induced inhibition of the L-type voltage-gated Ca channel. Last one is responsible for the influx of zinc and copper ions and paracellular uptake route of these metals in P. ridibundus (Glover and Hogstrand, 2002).

MTs are the unique small cysteine-rich (up to 30%) cytosolic proteins which play a critical role in Zn and Cu buffering, transition
metals, preferentially cadmium and mercury, detoxification, scavenging of reactive oxygen species (Kagi and Schaffer, 1988, Isani and Carpenè, 2014, Kimura and Kambe, 2016). It has been proposed that MTs play an important role in Zn homeostasis by controlling cellular Zn uptake, distribution, storage, and release (Maret, 2011, Kimura and Kambe, 2016). Moreover, Zn can stimulate MTs synthesis (Suzuki et al., 1990). Despite Zn is a weaker inductor of MTs than cadmium (Onosaka et al., 1984), numerous investigations have reported that Zn should affect MTs concentration and their mRNA synthesis in various species, among them freshwater vertebrate. It has been shown that the muscle MT-A and MT-B genes were up-regulated in mature rainbow trout (Oncorhynchus mykiss) under Zn exposure (1 mg L\(^{-1}\) ZnSO\(_4\).7H\(_2\)O) during 6, 12, 24, and 48 h of treatment (Ceyhun et al., 2001). Moreover, Zn\(^{2+}\) in concentration of 100 \(\mu\)g caused elevation of the Zn-MTs in the frog (P. ridibundus) liver, but no changes were observer for total MTs concentration (Falfushynska et al., 2015b). However, the ability of nanosized Zn either in n-ZnO or synthetic polymeric complex to affect MTs induction has not been well understood yet. Some results proposed different assertions related to wide range of animals (different fila), which vary from MTs induction to their oppression. A rapid up-regulation of MTs mRNA by n-ZnO has been reported in the liver of Arbor Acres chickens (Xiao-bo et al., 2009), in the kidney of Wistar rats (Hejazy et al., 2014). In present work both Zn and n-ZnO provoked prominent changes in MTs-related traits in frog liver either concentration or metal-binding ability refer to Zn or Cu. Obviously the up-regulated expression of MTs should occur due to releasing free metal ions from the surface of the nanoparticles (Shaw and Handy, 2011). In agreement with abovementioned, Zn-containing exposures alone or in combination had slight or no effect on antioxidant system of frog liver, independently of the effect on DNA fragmentation. In contrast, products of oxidative damage, PC, and genotoxicity, fragmented DNA and frequency of nuclear abnormalities, were strongly increased after Nfd treatment. This event was happened with a simultaneously deep oppression of putative radical scavenger, MTs. Of interest, the scavenging capacity of MT2A towards free \(\bullet OH\) and peroxyl radicals is found to be 100-fold higher than that of GSH (Lian et al., 2013).

It has been expected that Zn- and Nfd-containing exposures provoked opposite changes of lactate/pyruvate ratio in frog liver. Several studies give the evidence of Zn linkage to the glucose homeostasis (Jansen et al., 2009), but generally it was obtained with using mammals as a model. For instance, the addition of ZnCl\(_2\)
cytes from fed rats (Brand and Kleineke, 1996). This effect is believed to be caused by elevation of intracellular Zn levels (Rofe et al., 2000). Treatment of cells with ZnO, but not TiO2, depressed mitochondrial membrane potential, leading to a dose-dependent increase in glycogen breakdown by up to 430%, with an increase of both glycolysis and glucose release (Filippi et al., 2015). We have shown that increases of anaerobic processes and acidosis under Zn and n-ZnO were consistent with MTs level (r = 0.35, p < .05) and Zn-MT (r = 0.88, p < .001) in frog liver tissue. Indeed, stimulation of MTs synthesis may sufficiently reduce labile Zn in hepatocytes with subsequent stimulation of glycolysis via alteration of glycogen metabolism steps catalyzed by pyruvate kinase and by phosphofructokinase-1 (Brand and Kleineke, 1996). Thereby, Zn and its partitioning with MTs participation plays an important role in regulation of carbohydrate metabolism in frog organism.

The calcium channel blockers, when given either in vivo or in vitro, usually have marked decreased energy demands and both oppressed lactate dehydrogenase activity and lactate level. Several evidences of this phenomenon have been obtained mainly with using guinea pig hearts and rat hearts and muscles as a model (de Jong and Huizer, 1985, Becker and Möbert, 1999, Sato et al., 1999). However, our previous study with using bivalve mollusk Unio tumidus has shown significant lactate accumulation and a decrease in pyruvate levels after Nfd treatment, indicating transitory decrease in pyruvate levels after Nfd treatment, indicating transitory.
dependent on Zn, both endo- and exogenous. We've shown that Vtg-LP level correlated with Zn-MTs (r = 0.76, p < 0.001), providing probably the sufficient supply of Zn in frog liver whenever Vtg synthesis occurs. On the other hand, moderate but significant activation of vitellogenesis was found in response to Zn, n-ZnO and n-ZnO + Nfd co-exposure. Moreover, Vtg-LP level in blood plasma was the primer marker for distinguishing of groups subjected to Zn-containing and Zn-free exposures according to CART analysis (Fig. 4). These data confirm the results obtained by alternative methods in the same model exposure (Falfushynska et al., 2017).

There are several notes that Vtg level and/or expression is up-regulated as a response to estrogen-mimicking chemicals in male fish and amphibians (Palmer et al., 1998, Jones et al., 2000, Hutchinson et al., 2006). The up-regulation of Vtg-LP by exposures to Zn, Zn-containing nanomaterial and corresponding polymer substance based on vinylpyrrolidone was also found in frog P. ridibundus and fish Carassius sp. (Falfushynska et al., 2014, 2015b). Besides, in these studies Vtg-LP elevation was congruent in most cases with responses of tyrosinase-like and EROD activities.

Tyrosinase is a widespread indispensable binuclear Cu-enzyme that presents in the tissues of wide range of vertebrates, among them frog skin and liver pigment cells (Cicero et al., 1989). It takes part in melanogenesis and is able to protect cells in vitro against ROS-mediated toxicity (Rolf et al., 2011, Marino et al., 2011) and plays a crucial role in the innate immune (Pang et al., 2013). CART analysis has revealed tyrosinase-like activity as criterion of partitioning n-ZnO-treated animals (Fig. 4). There are several studies reported Zn-containing proteins or substrates' caused inhibition either tyrosinase protein level or activity in a mixed-type inhibition manner (Hale, 2002, Han et al., 2007). However for n-ZnO such information has been unknown. Meanwhile, the same regularities were observed in our studies Vtg-LP elevation was congruent in most cases with responses of tyrosinase-like and EROD activities.

Tyrosinase is a widespread indispensable binuclear Cu-enzyme that presents in the tissues of wide range of vertebrates, among them frog skin and liver pigment cells (Cicero et al., 1989). It takes part in melanogenesis and is able to protect cells in vitro against ROS-mediated toxicity (Rolf et al., 2011, Marino et al., 2011) and plays a crucial role in the innate immune (Pang et al., 2013). CART analysis has revealed tyrosinase-like activity as criterion of partitioning n-ZnO-treated animals (Fig. 4). There are several studies reported Zn-containing proteins or substrates' caused inhibition either tyrosinase protein level or activity in a mixed-type inhibition manner (Hale, 2002, Han et al., 2007). However for n-ZnO such information has been unknown. Meanwhile, the same regularities were observed in our studies Vtg-LP elevation was congruent in most cases with responses of tyrosinase-like and EROD activities.

Tyrosinase is a widespread indispensable binuclear Cu-enzyme that presents in the tissues of wide range of vertebrates, among them frog skin and liver pigment cells (Cicero et al., 1989). It takes part in melanogenesis and is able to protect cells in vitro against ROS-mediated toxicity (Rolf et al., 2011, Marino et al., 2011) and plays a crucial role in the innate immune (Pang et al., 2013). CART analysis has revealed tyrosinase-like activity as criterion of partitioning n-ZnO-treated animals (Fig. 4). There are several studies reported Zn-containing proteins or substrates' caused inhibition either tyrosinase protein level or activity in a mixed-type inhibition manner (Hale, 2002, Han et al., 2007). However for n-ZnO such information has been unknown. Meanwhile, the same regularities were observed in our studies Vtg-LP elevation was congruent in most cases with responses of tyrosinase-like and EROD activities.

Tyrosinase is a widespread indispensable binuclear Cu-enzyme that presents in the tissues of wide range of vertebrates, among them frog skin and liver pigment cells (Cicero et al., 1989). It takes part in melanogenesis and is able to protect cells in vitro against ROS-mediated toxicity (Rolf et al., 2011, Marino et al., 2011) and plays a crucial role in the innate immune (Pang et al., 2013). CART analysis has revealed tyrosinase-like activity as criterion of partitioning n-ZnO-treated animals (Fig. 4). There are several studies reported Zn-containing proteins or substrates' caused inhibition either tyrosinase protein level or activity in a mixed-type inhibition manner (Hale, 2002, Han et al., 2007). However for n-ZnO such information has been unknown. Meanwhile, the same regularities were observed in our studies Vtg-LP elevation was congruent in most cases with responses of tyrosinase-like and EROD activities.
Jansen, J., Karges, W., Rink, L., 2009. Zinc and diabetes–clinical links and molecular
Gawehn, K., 1988. D-(-)-Lactate. In: Bergmeyer, H.U. (Ed.), Methods of Enzymatic
Luna-Acosta, A., Thomas-Guyon, H., Amari, M., Rosenfeld, E., Bustamante, P.,
Liu, J., Feng, X., Wei, L., Chen, L., Song, B., Shao, L., 2016. The toxicology of ion-
Lian, Y., Zhao, J., Xu, P., Wang, Y., Zhao, J., Jia, L., Fu, Z., Jing, L., Liu, G., Peng, S., 2013.
Klotz, A.V., Stegeman, J.J., Walsh, C., 1984. An alternative 7-ethoxyresorufin O-
Kim, R.O., Choi, J.S., Kim, B.C., Kim, W.K., 2017. Comparative analysis of
Jones, P.D., De Coen, W.M., Tremblay, L., Glesy, J.P., 2000. Vitellogenin as a
Hutchinson, T.H., Ankley, G.T., Segner, H., Tyler, C.R., 2006. Screening and testing for
Hogstrand, C., Verbost, P.M., Bonga, S.E., Wood, C.M., 1996. Mechanisms of zinc
Hejazy, M., Koohi, M.K., Asadi, F., Behrouz, H.J., 2014. Induction of renal
Han, H.Y., Zou, H.C., Jeon, J.Y., Wang, Y.J., Xu, W.A., Yang, J.M., Park, Y.D., 2007. The
Fremont, L., Riazi, A., 1988. Biochemical analysis of vitellogenin from rainbow trout
Crassostrea gigas
phenoloxidases from the Pacific oyster
Frost, C.M., Jones, P.D., Chester, A.D., 2003. Oxidative stress and antioxidant mechanisms
Freire, J. P., Mc Laughlin, P., Steinberg, H., Tyler, C. R., 1995. Screening and testing of
D-FABP3: an endogenous zinc transporter. Nature 373, 378–380.
Dawson, P., Sprecher, J., Vyas, D., Kinyanjui, S., Rautaharju, P., 2006. Oxidative stress and antioxidative defense in rainbow trout
Dawson, P., Vyas, D., Sprecher, J., Kinyanjui, S., Rautaharju, P., 2006. Oxidative stress and antioxidative defense in rainbow trout.
David, S., Milon, S., Millan, F., Hocquard, C., 1999. Cytosolic and mitochondrial zinc uptake in gills of freshwater rainbow trout: interplay with calcium transport.
Dawson, P., Vyas, D., Sprecher, J.,今年. 氧化应激与抗氧防御在彩虹 trout。
Dawson, P., Vyas, D., Sprecher, J., Kinyanjui, S., Rautaharju, P., 2006. Oxidative stress and antioxidative defense in rainbow trout.
Dawson, P., Vyas, D., Sprecher, J., Kinyanjui, S., Rautaharju, P., 2006. Oxidative stress and antioxidative defense in rainbow trout.
Dawson, P., Vyas, D., Sprecher, J., Kinyanjui, S., Rautaharju, P., 2006. Oxidative stress and antioxidative defense in rainbow trout.
Dawson, P., Vyas, D., Sprecher, J., Kinyanjui, S., Rautaharju, P., 2006. Oxidative stress and antioxidative defense in rainbow trout.
Dawson, P., Vyas, D., Sprecher, J., Kinyanjui, S., Rautaharju, P., 2006. Oxidative stress and antioxidative defense in rainbow trout.
Dawson, P., Vyas, D., Sprecher, J., Kinyanjui, S., Rautaharju, P., 2006. Oxidative stress and antioxidative defense in rainbow trout.
Dawson, P., Vyas, D., Sprecher, J., Kinyanjui, S., Rautaharju, P., 2006. Oxidative stress and antioxidative defense in rainbow trout.
Dawson, P., Vyas, D., Sprecher, J., Kinyanjui, S., Rautaharju, P., 2006. Oxidative stress and antioxidative defense in rainbow trout.
Dawson, P., Vyas, D., Sprecher, J., Kinyanjui, S., Rautaharju, P., 2006. Oxidative stress and antioxidative defense in rainbow trout.
Dawson, P., Vyas, D., Sprecher, J., Kinyanjui, S., Rautaharju, P., 2006. Oxidative stress and antioxidative defense in rainbow trout.
Dawson, P., Vyas, D., Sprecher, J., Kinyanjui, S., Rautaharju, P., 2006. Oxidative stress and antioxidative defense in rainbow trout.
Dawson, P., Vyas, D., Sprecher, J., Kinyanjui, S., Rautaharju, P., 2006. Oxidative stress and antioxidative defense in rainbow trout.
Dawson, P., Vyas, D., Sprecher, J., Kinyanjui, S., Rautaharju, P., 2006. Oxidative stress and antioxidative defense in rainbow trout.
Dawson, P., Vyas, D., Sprecher, J., Kinyanjui, S., Rautaharju, P., 2006. Oxidative stress and antioxidative defense in rainbow trout.
Dawson, P., Vyas, D., Sprecher, J., Kinyanjui, S., Rautaharju, P., 2006. Oxidative stress and antioxidative defense in rainbow trout.
Dawson, P., Vyas, D., Sprecher, J., Kinyanjui, S., Rautaharju, P., 2006. Oxidative stress and antioxidative defense in rainbow trout.
Dawson, P., Vyas, D., Sprecher, J., Kinyanjui, S., Rautaharju, P., 2006. Oxidative stress and antioxidative defense in rainbow trout.
Dawson, P., Vyas, D., Sprecher, J., Kinyanjui, S., Rautaharju, P., 2006. Oxidative stress and antioxidative defense in rainbow trout.
Dawson, P., Vyas, D., Sprecher, J., Kinyanjui, S., Rautaharju, P., 2006. Oxidative stress and antioxidative defense in rainbow trout.
Dawson, P., Vyas, D., Sprecher, J., Kinyanjui, S., Rautaharju, P., 2006. Oxidative stress and antioxidative defense in rainbow trout.
Dawson, P., Vyas, D., Sprecher, J., Kinyanjui, S., Rautaharju, P., 2006. Oxidative stress and antioxidative defense in rainbow trout.
Dawson, P., Vyas, D., Sprecher, J., Kinyanjui, S., Rautaharju, P., 2006. Oxidative stress and antioxidative defense in rainbow trout.
Dawson, P., Vyas, D., Sprecher, J., Kinyanjui, S., Rautaharju, P., 2006. Oxidative stress and antioxidative defense in rainbow trout.
Dawson, P., Vyas, D., Sprecher, J., Kinyanjui, S., Rautaharju, P., 2006. Oxidative stress and antioxidative defense in rainbow trout.
Dawson, P., Vyas, D., Sprecher, J., Kinyanjui, S., Rautaharju, P., 2006. Oxidative stress and antioxidative defense in rainbow trout.
Dawson, P., Vyas, D., Sprecher, J., Kinyanjui, S., Rautaharju, P., 2006. Oxidative stress and antioxidative defense in rainbow trout.
Dawson, P., Vyas, D., Sprecher, J., Kinyanjui, S., Rautaharju, P., 2006. Oxidative stress and antioxidative defense in rainbow trout.
Dawson, P., Vyas, D., Sprecher, J., Kinyanjui, S., Rautaharju, P., 2006. Oxidative stress and antioxidative defense in rainbow trout.