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Vulnerability of marsh frog *Pelophylax ridibundus* to the typical wastewater effluents ibuprofen, triclosan and estrone, detected by multi-biomarker approach

Halina I. Falfushynska[a], Lesya L. Gnatyshyna[a,b], Oksana Horyn[b], Oksana B. Stoliar[a,*]

[a] Research Laboratory of Comparative Biochemistry and Molecular Biology, Ternopil National Pedagogical University, 2, M. Kryvonosa Str., Ternopil 46027, Ukraine
[b] I.Ya. Horbachevsky Ternopil State Medical University, 1, Maidan Voli, Ternopil 46001, Ukraine

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ABSTRACT

Pharmaceutical and personal care products (PPCPs) are the environmental pollutants of growing concern. The aim of this study was to indicate the effects of typical PPCPs on the marsh frog *Pelophylax ridibundus*. We treated male frogs with waterborne ibuprofen (IBU, 250 ng·L^{-1}), triclosan (TCS, 500 ng·L^{-1}), or estrone (E1, 100 ng·L^{-1}) for 14 days. Common vulnerability of the frogs was detected from dramatic decrease of Zn, total and metalated metallothionein (MT) concentrations, Zn/Cu ratio, the elevation of activity of glutathione-*S*-transferase, cathepsin D and DNA instability in the liver, the depletion of cholinesterase in the brain and cortisol in the blood plasma in all exposures. Nevertheless, lipofuscin concentration in the liver was always decreased. The groups were best distinguished by cytochrome P450 (CYP450) activity determined by ELISA. The exposure to IBU caused lesser damage, but elevated the levels of oxyradicals and glutathione (GSH and GSSG) and lysosomal membrane instability. Exposures to TCS and E1 provoked the endocrine disturbance (increased levels of vitellogenin and thyrotropin in blood plasma), decreased lactate dehydrogenase activity and increased level of pyruvate in the liver. TCS caused the increase of GSSG by 7.3 times and lactate levels. Only E1 lead to decrease of deiodinase activity in the liver, activation of CYP450 and caspase-3 and efflux of cathepsin D from lysosomes. *Spectrophotometric and ELISA assays* of MTs and CYP450 gave distinct results in E1-group. Broad disruption of the hormonal pathways caused by E1 could be of concern for the health status of frogs in their habitats.

1. Introduction

Pharmaceutical and personal care products (PPCPs) are the environmental pollutants of growing concern (Schröder et al., 2016). The increased usage of these synthetic substances in the casual life and the shortcoming or deprivation of the communal service makes the mixture of PPCPs the most common kind of the waters pollution (Directive 2013/39/EU; Ellis, 2006; Falconer et al., 2006; Erturk et al., 2007). Main expected toxic effect of the water pollution by PPCPs is their activity as endocrine disrupters (EDs) leading to modulation of fundamental biological functions in aquatic animals (Jung et al., 2012; Boberg et al., 2013). Amphibians belong to the most targeted wild animals in the contemporary world (Sodhi et al., 2008; Warne et al., 2016). The effects of EDs, particularly on thyroid activity during metamorphosis, are among the major contributors to the suspected reasons of amphibian declines (Venturino et al., 2003; Hayes et al., 2006; Kloas et al., 2009; Mortensen et al., 2006; Tompsett et al., 2013; Garmshausen et al., 2015). However, the common molecular basis of the particular susceptibility of amphibian in comparison with other aquatic species to the contemporary environmental challenges is unclear.

Substances selected for this study are PPCPs that have been detected in surface waters worldwide. The anti-inflammatory agent ibuprofen (IBU) was found in different sewage samples (sewage treatment plants, hospital effluents) and in seawater and has reached concentrations up to 85 μg·L^{-1} (Weigel et al., 2004; Blaise et al., 2006). Its accumulation in the bile of two wild fish species was in the range of 15 to 34 μg·L^{-1} (Brozinski et al., 2013). The synthetic antimicrobial agent, triclosan (TCS), has been incorporated into > 700 different personal care products including deodorants, soaps, toothpastes, and various plastic products, but also in textile fibers used in a range of other consumer products (e.g., toys, undergarments and cutting boards) (Dann and Hontela, 2011; Bedoux et al., 2012). It is among the top 10 most commonly detected organic wastewater compounds. Its concentration in the effluents of the treatments plants reaches hundreds of ng·L^{-1} in different parts of world (Brausch and Rand, 2011; Kookana et al., 2011; Kumar et al., 2015). It has been shown to produce cytotoxic, genotoxic,

* Corresponding author.
E-mail address: Oksana.Stolyar@tnpu.edu.ua (O.B. Stoliar).

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and ED effects in the aquatic animals.

(Bedoux et al., 2012). Native estrogen, estrone (E1) is the priority ED (ranged from 10^{-1} to 10^{-3} ng·L^{-1}) in municipal sewage plants (Sun et al., 2013; Schröder et al., 2016) and had been reported at concentration of 74.2 ng·L^{-1} even after the wastewater treatment (Zhou et al., 2012).

Our recent study of the effects of the selected substances (IBU, TCS and E1) on bivalve mollusk demonstrated some particular responses in each exposure (Falfushynska et al., 2014b, 2015b; Fal'fushinskaya et al., 2015). The lesser toxicity and activation of glutathione-related responses was shown for IBU. On the other hand, TCS and E1 caused elevated genotoxicity, apoptotic activity and a decrease in lysosomal stability. The changes in the microsomal monooxygenation and vitellogenesys were also detected but the application of these markers in mussels is questionable (Viarengo et al., 2007). Therefore the aim of the current study was to extend the expertise of the toxicity of selected PPCPs with utilizing of highly vulnerable vertebrate model marsh frog *Pelophylax ridibundus* basing on the approach elaborated in the previous study of mollusk. The scopes of investigation were extended by the evaluation of different axes of endocrine activities, specific for the vertebrate animals and particularly sensitive in amphibians (Kloas, 2002).

To evaluate the endocrine activity, the thyroid axis (using concentration of thyrotropin (TSH) and hepatic deiodinase), the stress axis (the cortisol concentration) and reproductive axis (assessed by the level of vitellogenin (Vtg) in males as a biomarker for xenooestrogen exposure) were characterized. The hepatic cytochrome P450 (CYP450)-relative activity and Glutathione *S*-transferase (GST) to evaluate the hepatic transformation were detected. The characteristics of zinc (Zn) and copper (Cu) homeostasis and oxidative stress indices were included in the set of parameters taking into consideration the particular susceptibility of metal-buffering and stress- responsive proteins metallothioneins (MTs) of frog to the environmental pollution and experimental contaminants (Falfushynska et al., 2008, 2015a). We utilized two or three alternative assays for the detection of the levels of MTs, vitellogenin and CYP450-related activities to strengthen the validity of expertise. Geno-, cyto- and neurotoxicity markers and the activities of the apoptotic proteases caspase-3 and cathepsin D were detected to estimate the severity of lesions.

2. Materials and methods

2.1. Chemicals

All chemicals were purchased from Sigma Aldrich (St. Louis, USA) or Merck (Synbias, Kyiv, Ukraine), and were of the analytical grade or higher (Suppl. 1).

2.2. Experimental exposures

The experiments were carried out in mid-September of 2013. Adult males of marsh frog *Pelophylax ridibundus* (8–10 cm long) were collected from a pristine site in the upstream portion of river Seret (49°49′ N, 25°23′ E). The animals were out of the breeding season. 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 performed in accordance with the national and institutional guidelines for the protection of animal 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).

Frogs were acclimated in aerated, softened tap water and fed throughout the experiment with commercial sticks “Turtle menu” (21% of protein, Aquarius, Ukraine) placed in a tray inside the container. After seven days of preliminary acclimation, frogs were randomly distributed into four groups (15 individuals per group). One group was exposed to the aquarium water only and was considered control (C). Other groups were exposed to nonsteroidal anti-inflammatory drug ibuprofen ((*RS*)-2-(4-(2-methylpropyl)phenyl)propanoic acid, IBU, 250 ng·L^{-1}); the antimicrobial chlorinated biphenyl ether triclosan (5-chloro-2-(2,4-dichlorophenoxy)-phenol, TCS, 500 ng·L^{-1}), or to estrone, a typical residual steroid estrogen (E1, 100 ng·L^{-1}). The selected conditions of exposure were corresponding to the design applied in study of bivalve mollusks (Falfushynska et al., 2014b).

The water quality parameters were: temperature 17 ± 1 °C, pH 7.3 ± 0.2, CaCO3 86.8 ± 1.0 mg·L^{-1}, dissolved oxygen concentration 8.67 ± 0.51 mg·L^{-1}, ammonia (NH_3/$NH4^+$) and nitrite concentrations below 0.1 mg·L^{-1}. There was no mortality of frogs during the performed experiments.

After the exposure, frogs were anesthetized by clove oil (Goulet et al., 2010), the heparinized blood was collected from the heart, and plasma was immediately separated by centrifugation at 10,000 × *g* for 10 min. For serum preparation, whole blood was allowed to clot and centrifuged for 10 min at 1500 × *g* for 10 min. The frogs were killed by a blow to the head, the spinal cord severed, and liver was immediately removed for experiments. For each biochemical parameter, 8 samples were used. Vitellogenin was determined in blood plasma, TSH and cortisol − in the blood serum, cholinesterase (ChE) activity − in the brain while all other biomarkers – in the liver. Samples of perfused hepatic and brain tissues, blood plasma or serum from 8 specimens in each group were prepared individually and kept at − 40 °C (for three days or less) until analyses except EROD activity and lysosomal stability which were determined immediately. Hepatic tissue was homogenized (1:10 w:v) in 0.1 M pH 7.4 phosphate buffer containing 100 mM KCl, 1 mM EDTA and 0.1 mM PMSF to inhibit proteolysis. Microsomal pellet obtained by calcium (80 mM CaCl2) precipitation of the post-mitochondrial supernatant of liver homogenates was centrifuged for 20 min at 12,000 × *g* in 10 mM Tris-HCl buffer, pH 7.4 (Cinti et al., 1972). Homogenization was carried out at 4 °C using 12–15 strokes of a motor driven Teflon Potter-Elvehjem homogenizer and centrifuged at 6000 × *g* for 10 min at 4 °C. PMSF (0.1 mM) was also added to plasma/serum to inhibit proteolysis. Protein concentration in the supernatant, microsomal pellet and blood plasma/serum was measured by the method of Lowry et al. (1951) with using bovine serum albumin as a standard. The absorbance values were measured on the UV/Vis spectrophotometer “LOMO-56” (LOMO, Russian Federation), and the fluorescence was measured on the *f*-max fluorescence microplate reader (Molecular Device, USA).

For all ELISA assays, the absorbance was measured in an ELISA reader (Awareness Technology Stat-Fax-303 + Microstrip Reader, USA); linearity of the assay was tested using the serial dilutions of the samples and compared to the dilutions of the respective standards provided with the kits. Samples showed acceptable linearity with an average R2 of 0.97 ± 0.02 and the lines that were parallel to the standard curve.

2.3. Endocrine activities and biotransformation enzymes

The 96-well-plate *semi-quantitative* Biomarker *ELISA Kit* (Biosense, Norway) was used to determine of vitellogenin (Vtg) concentration in the blood plasma according to the manufacturer's protocol. Also, vitellogenin-like proteins (Vtg-LP) were evaluated as the alkali-labile phosphate level, according to the method described by Nagler et al. (1987). It is based on the principle that trichloroacetic acid-precipitated phosphoproteins are subjected to an alkali treatment in order to release labile phosphates. The content of free phosphates was defined by the phosphomolybdenum assay.

The blood serum thyrotropin (TSH) concentration was measured with diagnostic ELISA kit employing biotin-streptavidin-ELISA detection system (Sigma-Aldrich, USA) according to the manufacturer's instructions.

Hepatic deiodinase (EC 1.97.1.10) activity was detected using

Tetraiodothyronine (T4) as a substrate (Cinti et al., 1972). The deiodinase assay contained 100 μL of the microsomal pellet and 2 μM T4 in a final volume of 125 μL, and was incubated at 37 °C for 60 min. The reaction was stopped by the addition of 250 μL ice-cold 96% ethanol. The amount of formed triiodothyronine was determined in the ethanol extracts using an ELISA kit (Sigma Aldrich, USA). The deiodinase activity was expressed as nmol $T_3 \cdot g^{-1}$ FW.

Cortisol concentration in frog blood serum was measured using the 96-well-plate solid phase competitive ELISA *Kit* (Sigma-Aldrich, USA) according to the manufacturer's protocol.

Cytochrome P450 (CYP450) concentration in the microsomal pellet was measured using the 96-well-plate *semi-quantitative* Biomarker *ELISA Kit* (Biosense, Norway) according to the manufacturer's protocol. The activity of the microsomal ethoxyresorufin *O*-deethylase (EROD) is a common and reliable indicator of the activity of the cytochrome P450 family I (CYP450 I) enzymes involved in Phase I biotransformation of xenobiotics in frog liver (Iwamoto et al., 2012). EROD activity was detected by measuring the absorbance of resorufin at 572 nm (Klotz et al., 1984) in the microsomal pellet. The reaction at 30 °C was initiated by the addition of 0.5 mM NADPH. EROD activity was calculated using a molar extinction coefficient of 73.2 $mM^{-1} \cdot cm^{-1}$ and standardized to the microsomal protein content.

Activity of glutathione-*S*-transferase (GST, EC 2.5.1.18) in liver extracts was measured according to Habig et al. (1974) using CDNB as a substrate. The enzymatic activity was determined by monitoring changes in absorbance at 340 nm for 2 min at constant temperature. The GST activity was presented as nmol $min^{-1} \cdot mg^{-1}$ soluble protein.

2.4. Quantification of metallothioneins and metals

Metallothioneins (MTs) were determined in liver by different methods. The level of MT- related thiols (MT-SH) was measured after ethanol/chloroform extraction with DTNB as described by Viarengo et al. (1997) and calculated by assuming the relationship: 1 mol MT-SH = 20 mol GSH and expressed as μg of MTs per gram of fresh weighted (FW) tissues.

To assess metal concentration in the MTs (MT-Me), they were isolated as thermostable proteins by size-exclusion chromatography on Sephadex G-50 with necessary adjustments needed to avoid their oxidation (Suzuki, 1991), as described in (Falfushynska et al., 2011). A 5% homogenate (w/v) was prepared in ice-cold 10 mM Tris-HCl buffer, pH 8.0, containing 10 mM 2-mercaptoethanol for maintaining of the reducing conditions and 0.1 mM PMSF for the inhibition of proteolysis. Fractions of the chromatographic peak with high absorbance at 254 nm and comparative high density ratio D254/D280 identified as MT-containing peak (Kagi and Schaffer, 1988), were pooled (total 10 mL) and applied to metal determination. MT-Me was calculated from the concentrations of metals in the MTs, considering that one molecule of MTs binds seven Zn^{2+} ions or twelve Cu + ions (Nielson and Winge, 1985). These values were expressed as $\mu g \cdot g^{-1}$ FW.

The concentration of MTs from their immunoreactivity (MTi) was detected by 96-well-plate *Semi-quantitative* Biomarker *ELISA Kit* (M04406201-100, Biosence, Sweden). A dilution series of thermostable hepatic tissue extract were incubated in the microtiter plates overnight at 4 °C. Samples were incubated successively with primary rabbit polyclonal antibody against cod MT and secondary antibody conjugated with Horseradish Peroxidase. Thermostable extract of liver tissue of *Cyprinus carpio* was used as positive control.

To determine metal (Zn, Cu) concentration, fresh tissues (250 mg) and pooled eluate of MTs fraction after the size-exclusion chromatography (10 mL) were digested in 5 mL HNO3 (Merck) for 3 h at 105 °C for metal analysis using hermetic acid-cleaned Teflon bomb. Concentration of metals was analyzed by the atomic absorption spectrometer with flame detector (C-115, "LOMO", Russian Federation). The detection limits for metals were 0.1 $\mu g \cdot g^{-1}$ FW for tissue. The reliability of the metals measurements was assessed by analyzing *ERM-BB422* certified reference material; metal recovery was between 90% and 110%. Quality control was assessed by method of Standard Addition (Beukelman and Lord, 1960). Metal concentration in the tissue and MTs was presented as $\mu g \cdot g^{-1}$ FW and $nmol \cdot g^{-1}$ FW.

2.5. General stress assay

Superoxide dismutase (SOD, EC 1.15.1.1) activity was measured by the method of Beauchamp and Fridovich (1971) based on the aerobic reduction of NBT at 535 nm by superoxide radicals and expressed as relative $units \cdot mg^{-1}$ soluble protein; 1 unit of SOD activity being defined as the amount of protein causing 50% inhibition of the rate of NBT reduction.

Total glutathione (GSH) concentration in frog liver was quantified by the glutathione reductase recycling assay (Anderson, 1985). Standards were prepared from the reduced glutathione (GSH), and concentrations were presented as μmol per g FW. To estimate the oxidized glutathione (GSSG) level, the protein free sample was treated with 2-vinylpyridine prior to the assay (60 min) at final concentration 2% (Griffith, 1980). The rate of 5-thionitrobenzoic acid formation was monitored spectrometrically at 412 nm. The redox index of glutathione as the ratio of concentrations ([Total glutathione]–[GSSG])/[Total glutathione] was also calculated. Standards were prepared from reduced glutathione (GSH), and concentrations were expressed as μmol per g wet weight.

Oxyradical formation in tissue 1/10 w/v homogenates was determined by a signal of non-fluorescent dye dihydrorhodamine which is converted to the fluorescent derivative rhodamine-123 in a reaction with the reactive oxygen species (Viarengo et al., 1999). Probe fluorescence signal was detected by using fluorescence plate-reader [excitation (ex.) = 485 nm, emission (em.) = 538 nm] immediately, and in 20 min.

Lipofuscin concentration in the hepatic tissue was determined using chloroform: methanol (2:1, v/v) extraction of the homogenate. The lower clear chloroform phase obtained after the centrifugation was dried and redissolved in chloroform. The fluorescence intensity of extractable lipofuscin was calibrated using quinine sulphate (1 $\mu g \cdot mL^{-1}$ of 0.1 N H_2SO_4) and was detected by using an *f*-max fluorescence plate-reader [excitation = 350 nm, emission = 450 nm] (Manibabu and Patnaik, 1997).

Lactate was assayed by the spectrophotometric measurement of enzymatic oxidation of lactate to pyruvate in the presence of bacterial D-Lactate Dehydrogenase (EC 1.1.1.28) from *Lactobacillus leichmannii* and NAD. Absorbance at 340 nm was measured immediately, and within 60 min. The difference between two absorbance readings was used in the calculations ($\varepsilon_M = 6.22 \cdot 10^6$) (Gawehn, 1988).

Pyruvate was measured by a similar method based on using D-LDH which converts pyruvic acid to D-lactic acid in the presence of NADH. NADH consumption was measured by a decrease in absorbance at 340 nm ($\varepsilon_M = 6.22 \cdot 10^6$) (Lamprecht and Heinz, 1988).

The activity of lactate dehydrogenase (LDH, EC 1.1.1.27) was measured in the hepatic tissue using a standard UV assay with pyruvate and NADH as substrates and following oxidation of NADH at 340 nm (Bergmeyer and Bernt, 1974). A molar extinction coefficient of $6.22 \cdot 10^6\ M^{-1} \cdot cm^{-1}$ for NADH was used. For all enzyme activities, assay conditions were selected to achieve linear reactions non-limiting concentrations of the substrates over the assay time, and initial reaction velocity was used to estimate Vmax. Enzymatic assays were conducted at 37 °C to achieve the maximum LDH activities; pilot studies indicated that the enzyme is not damaged by the temperatures below 40 °C.

2.6. Assays of cytotoxicity and apoptotic activities

DNA damage was evaluated by the levels of protein-free DNA strand breaks in the liver by the alkaline DNA precipitation assay (Olive, 1988) using Hoescht 33,342 dye. To reduce the possible interference with

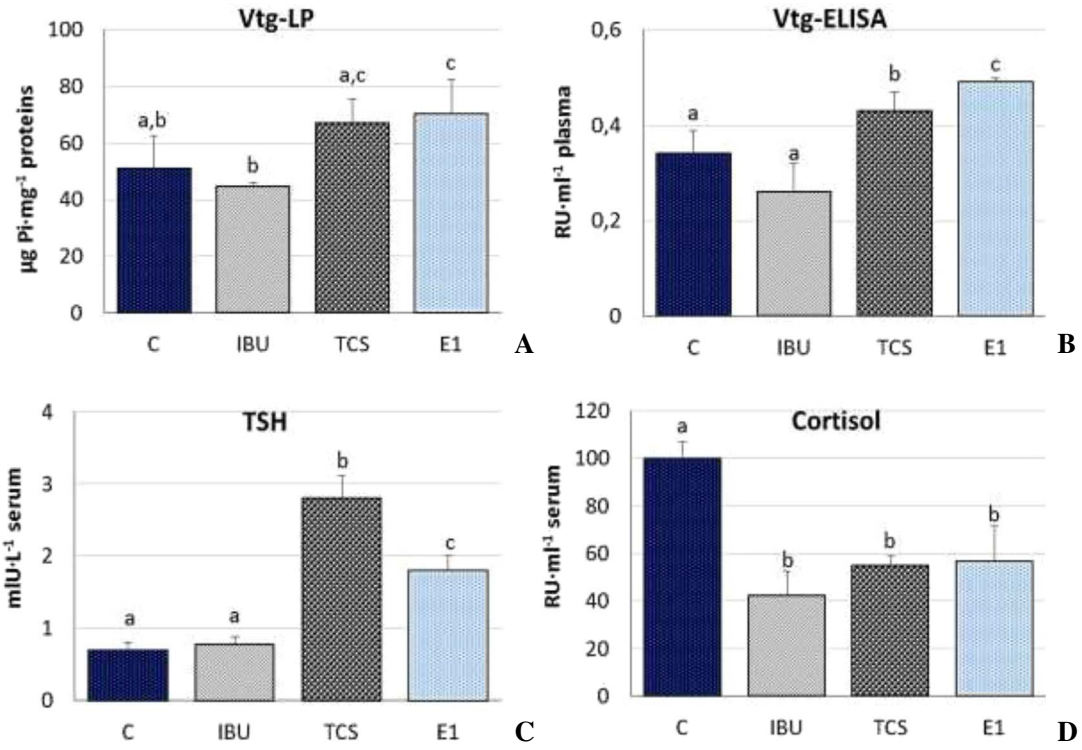

Fig. 1. Concentrations of endocrine-related indices in the blood plasma (A, B) and serum (C, D) of frogs exposed to selected PPCPs (IBU, TCS, E1). A, vitellogenin-like proteins; B, vitellogenin; C, thyrotropin; D, cortisol. Data are presented as means ± SD ($N = 8$). Here and on the Figs. 2–5, the columns that share the same letters indicate the values that are not significantly different ($P > 0.05$).

traces of sodium dodecyl sulphate (SDS), the assay was carried out in the presence of 0.4 M NaCl, 4 mM sodium cholate, and 0.1 M Tris (pH 9) (Bester et al., 1994). Probe fluorescence signal was detected by using *f*-max fluorescence plate-reader (excitation = 360 nm, emission = 450 nm).

Nuclear lesions were determined by the frequency of the erythrocytes with micronuclei (MN). The suspension of erythrocytes, obtained from each frog, was spread on a slide, transferred to a lightproof humidity chamber for 15 min to allow cells to attach. Cells were then fixed in methanol/acetic acid (3/1), stained with 5% Giemsa and mount in Canada balsam. The stained slides were analyzed under the light microscope (Olympus BX40) at a final 1000 × magnification. In total, 2000 cells were scored in each specimen studied (Baršienė et al., 2006). Erythrocytes with MN as well as with lobed nuclei (L), dumbbell-shaped or segmented nuclei (S), and kidney-shaped nuclei (K) were registered. Frequency of nuclear lesions was assessed separately for MN and all other lesions (L + S + K) and expressed per 1000 cells.

For the characteristic of apoptosys, the cytosolic and lysosomal proteases activities were detected. Activity of an executor caspase-3 was assayed colorimetrically based on the hydrolysis of peptide acetyl-Asp-Glu-Val-Asp *p*-nitroanilide (Ac-DEVD-pNA) by caspase-3 that produces a colored product *p*-nitroaniline (pNA). *p*-Nitroaniline was detected at 405 nm (εmM = 10.5 $mM^{-1}{\cdot}cm^{-1}$) (Bonomini et al., 2004; Falfushynska et al., 2015a). Cathepsin D (EC 3.4.23.5) activity was determined with 1% hemoglobin as substrate as described by Dingle et al. (1971). Cathepsin D acts on acid denatured hemoglobin resulting in a soluble colored complex which can be read spectrophotometrically at 280 nm. Free cathepsin D activity was assessed in tissue homogenate without detergent addition, whereas the total cathepsin D activity was measured after the enzyme release by Triton X100 treatment. Lysosomal cathepsin D activity was calculated as a difference of total and free activities. Activities were determined using a standard curve with thyrosine, and expressed as nmol tyrosine $min^{-1}{\cdot}mg^{-1}$ of soluble extracted protein.

Stability of the lysosomal membranes was determined by the Neutral Red Retention (NRR) assay. Hepatocytes were isolated according to Nazar et al. (2008) and monitored under oil immersion at 1000 × magnification as described in Moore et al. (2004). Observations for NRR were recorded at 5-min intervals. The NRR time was estimated as time at which 50% of lysosomes released the accumulated neutral red dye (ET50).

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 13.6·103 $M^{-1}{\cdot}cm^{-1}$ and standardized to the soluble protein content.

2.7. Statistical analysis

For analysis of MT-Me, sample size was three per group, each biological replicate consisting of pooled tissues from five frogs. For all other traits and all experimental treatment groups, sample size was eight. The data are presented as means ± standard deviation (SD) unless indicated otherwise. 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. For the data that were not normally distributed, non-parametric tests (Kruskall–Wallis ANOVA and Mann–Whitney *U* test) were performed. Mahalanobis distance was calculated as a measure of the distance between the groups based on the values of multiple traits. The classification tree based on all studied traits was built using Classification and Regression Tree (CART) software using raw (non-transformed) data. All statistical calculations were

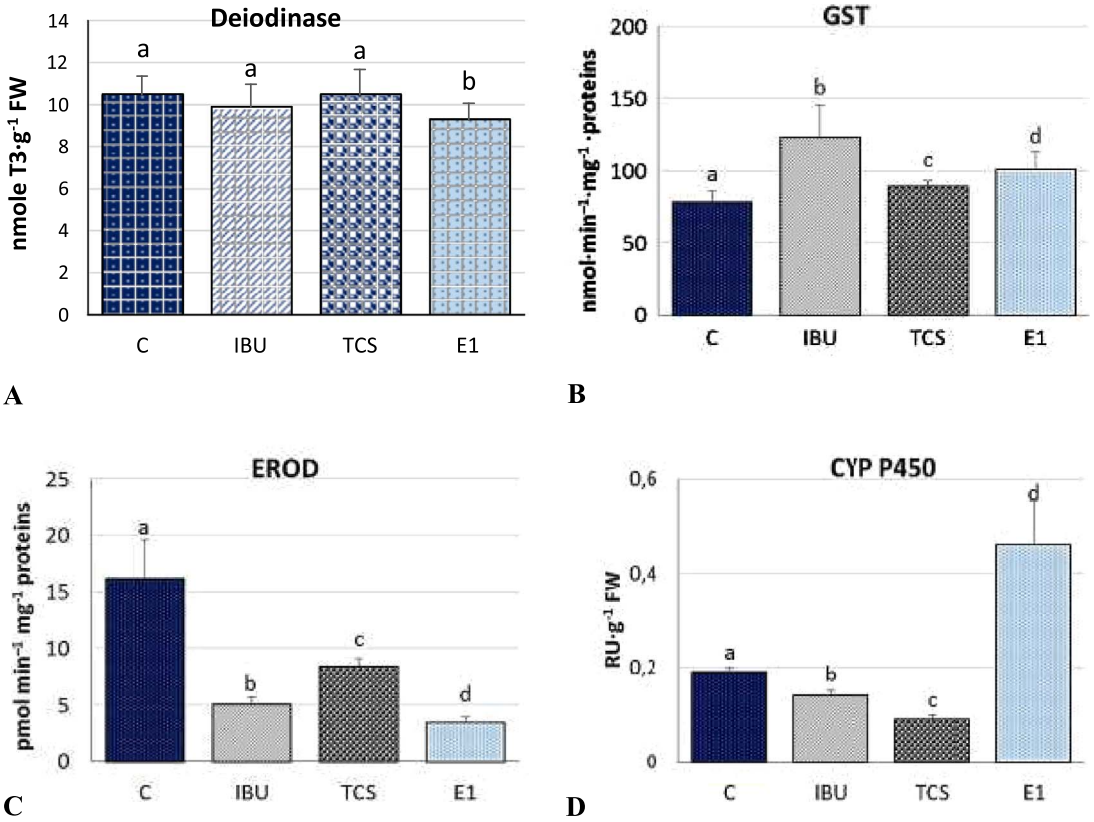

Fig. 2. Detoxification-related activities in the liver of frogs exposed to selected PPCPs (IBU, TCS, E1). A, deiodinase; B, glutathione-*S*-transferase; C, EROD; D, CYP450. Data are presented as means ± SD ($N = 8$).

performed with Statistica v. 10.0 and Excel for Windows-2010. Differences were considered significant if the probability of Type I error was < 0.05.

3. Results

3.1. Endocrine activity and enzymes of detoxification

The results have shown the increasing of the level of Vtg-LP and Vtg in the blood plasma in the exposures to TCS and E1 (by 1.3 and 1.4 times respectively) with similar range of changes obtained by two assays (Fig. 1A,B). The exposures to TCS and E1 elevated also prominently the level of thyrotropin (by 4.0 and 2.6 times respectively) (Fig. 1C). The level of cortisol in blood serum decreased dramatically by all exposures (by 1.8–2.4 times) (Fig. 1D).

Deiodinase activity was decreased only by the exposure to E1 (by 11.4%) (Fig. 2A). All exposures led to the up-regulation of GST (by 14.4–57.8%) (Fig. 2B). The exposures to IBU and TCS caused the decrease of the activity of CYP450-dependent transformation detected from EROD activity and ELISA (by 3.2–1.4 and 1.9–2.1 times for IBU and TCS respectively), whereas the changes evaluated by two methods for the effect of E1 were opposite (decrease by 4.6 times and increase by 2.4 times) (Fig. 2C,D).

3.2. Metal distribution and metallothionein characteristics

The analysis of metal uptake in the liver of frog has demonstrated similar changes in all exposures (Fig. 3A,B). The concentration of Zn decreased and concentration of Cu increased, leading to remarkable decrease in Zn/Cu concentration ratio (from 29.2 ± 3.2 in control to 13.1 ± 1.4, 12.7 ± 1.5, 18.4 ± 2.2 in IBU, TCS and E1 groups respectively). Fig. 3.

The concentrations of MT-SH and MT-Me decreased in all groups (by 23–24% for MT-SH and by 42–61% for MT-Me) (Fig. 3C,D). The evaluation of the immunoreactive form of MTs (MTi) (Fig. 3E) has shown the same decrease in all cases except for the E1-exposed group (increase). MT-SH/MT-Me concentration ratio increased from 1.1 ± 0.1 in control to 2.1 ± 0.2, 1.9 ± 0.2 and 1.5 ± 0.2 in IBU, TCS and E1 groups respectively (Fig. 3F).

3.3. Stress-relating and metabolic responses

The evaluation of the oxidative stress indices (Fig. 4A,B,C) in the liver detected the decrease of SOD activity in the E1-treated group. In the IBU- and TCS-groups, the SOD activity was corresponding to the control value (Fig. 4A). Elevation of oxyradical level was detected in the IBU- and E1-treated groups. However, the concentration of lipofuscin was decreased in the each exposure, particularly by IBU (by 39.7%). The glutathione system was targeted by all exposures (Fig. 4D,E) with the increasing of GSH level only by IBU but increasing of GSSG level in all exposures (by 7.3 times in TCS-group). Resulting redox index of GSH decreased in TCS-group from 0.99 ± 0.003 to 0.96 ± 0.005 ($p < 0.05$).

Exposures caused the changing in the metabolic activities (Fig. 4F,G,H). The LDH activity was increased by IBU but decreased by TCS and E1. Decreased pyruvate concentration and Lactate/Pyruvate balance (from 7.6 ± 0.4 in control group to 6.2 ± 0.7 and 2.4 ± 0.3 ($p < 0.05$)) was detected in the TCS and E1 groups respectively. The level of lactate was elevated only by TCS.

3.4. Toxicity of exposures

The applying of the micronuclear test has shown (Fig. 5A,B) the increase in the frequency of the erythrocytes with micronuclei or nuclear abnormalities in the IBU-and TCS-exposed frogs. The increased fragmentation of hepatic DNA was detected in all exposures (by

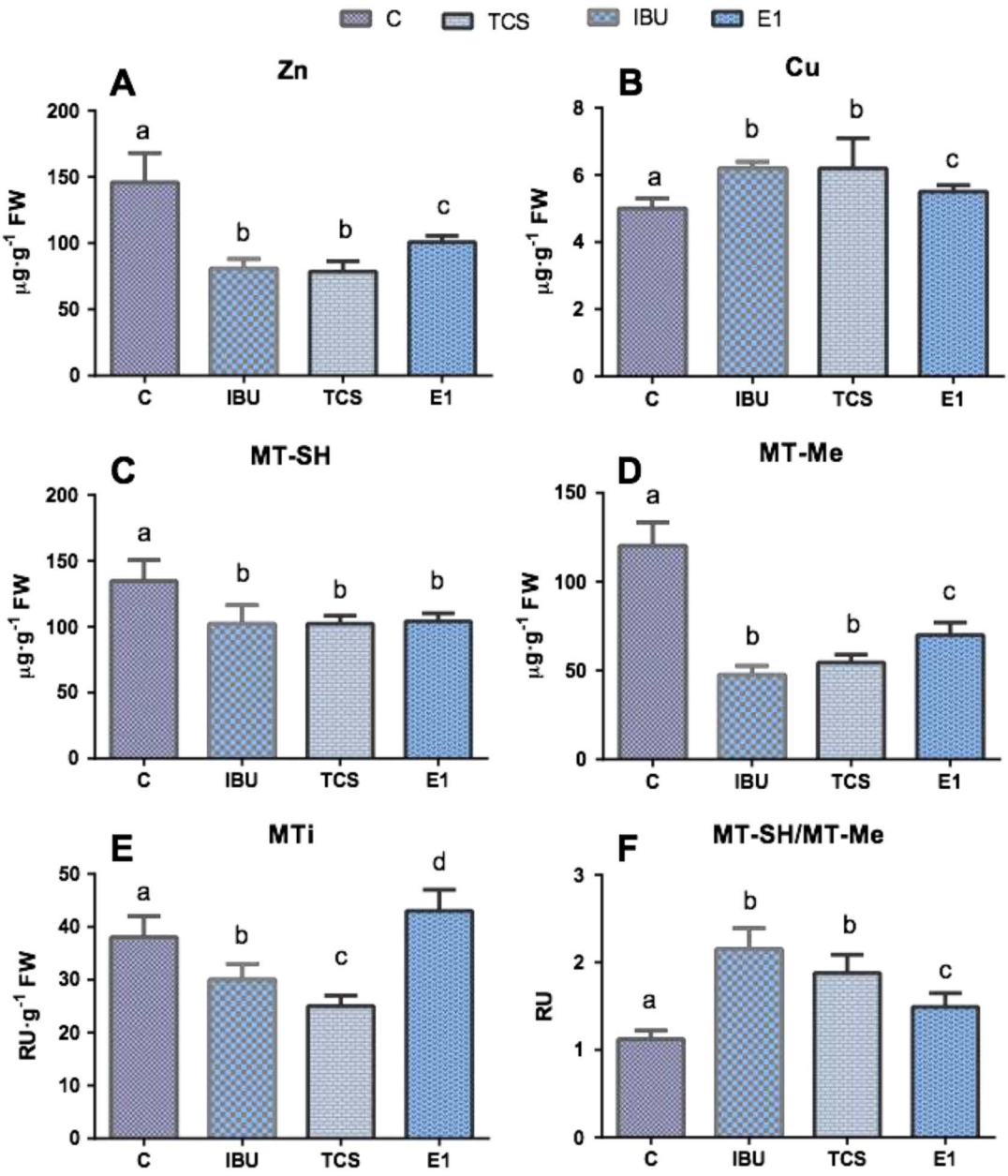

Fig. 3. Effects of experimental exposures to selected PPCPs (IBU, TCS, E1) on the tissue levels of Zn (A), Cu (B), metallothionein total (MT-SH) (C), metalated (MT-Me) (D) and immunoreactive (MTi) (E), and the ratio of the concentrations of MT-SH/MT-Me (F) in the liver of frog. Data are presented as means ± SD. $N = 8$ with the exception for MT-Me, where $N = 3$ (for joined samples from 5 specimens each).

30.1–112.0%) (Fig. 5C).

Caspase-3 activity in the liver was targeted only by E1 (increase by 43.0%) (Fig. 5D). The activity of lysosomal protease cathepsin D was strongly increased in all exposures (particularly, by 3.8 fold, under the effect of E1). However, it was accompanied by the elevated efflux of this protease from the lysosomes only in the case of E1. In opposite, the effect of IBU and TCS led to strong decrease of cathepsin D free activity (by 4.6 fold in the case of TCS) (Fig. 5E,F).

The lysosomal membrane stability was affected by IBU (decreased by 33.3%) and did not change significantly in other exposures (Fig. 5G). All exposures led to strong decrease of ChE activity, particularly TCS (by 4.1 fold) (Fig. 5H).

3.5. Data integration

Pearson correlation analysis revealed multiple strong correlations between the studied traits of frog across all experimental groups (Suppl. 2). Cathepsin D (total activity) and CYP-450 activity correlated with the largest number of the studied traits (9 and 8 correspondingly). When the characteristic was evaluated by different approaches, the correlation between the indices was positive for Vtg/Vtg-LP ($p < 0.001$). Comparison of CYP450/EROD and MT-SH/MT-Me has shown negative correlations ($p < 0.05$). The lesser number of correlations possessed TSH: only three positive correlations with Vtg, Vtg-LP and MT-Me and negative correlation with MT-SH ($p < 0.02$). The squared Mahalanobis distance based on all the measured traits have shown that exposures induced shift from control group in the position within the multiparameter space. The group exposed to IBU had lesser distance from control group, whereas distance of TCS- and, particularly, E1-groups from control and IBU-groups were notably bigger (Fig. 6A).

CART analysis used to identify the main distinguishing biomarker for each group resulted in a tree with three splits and four terminal

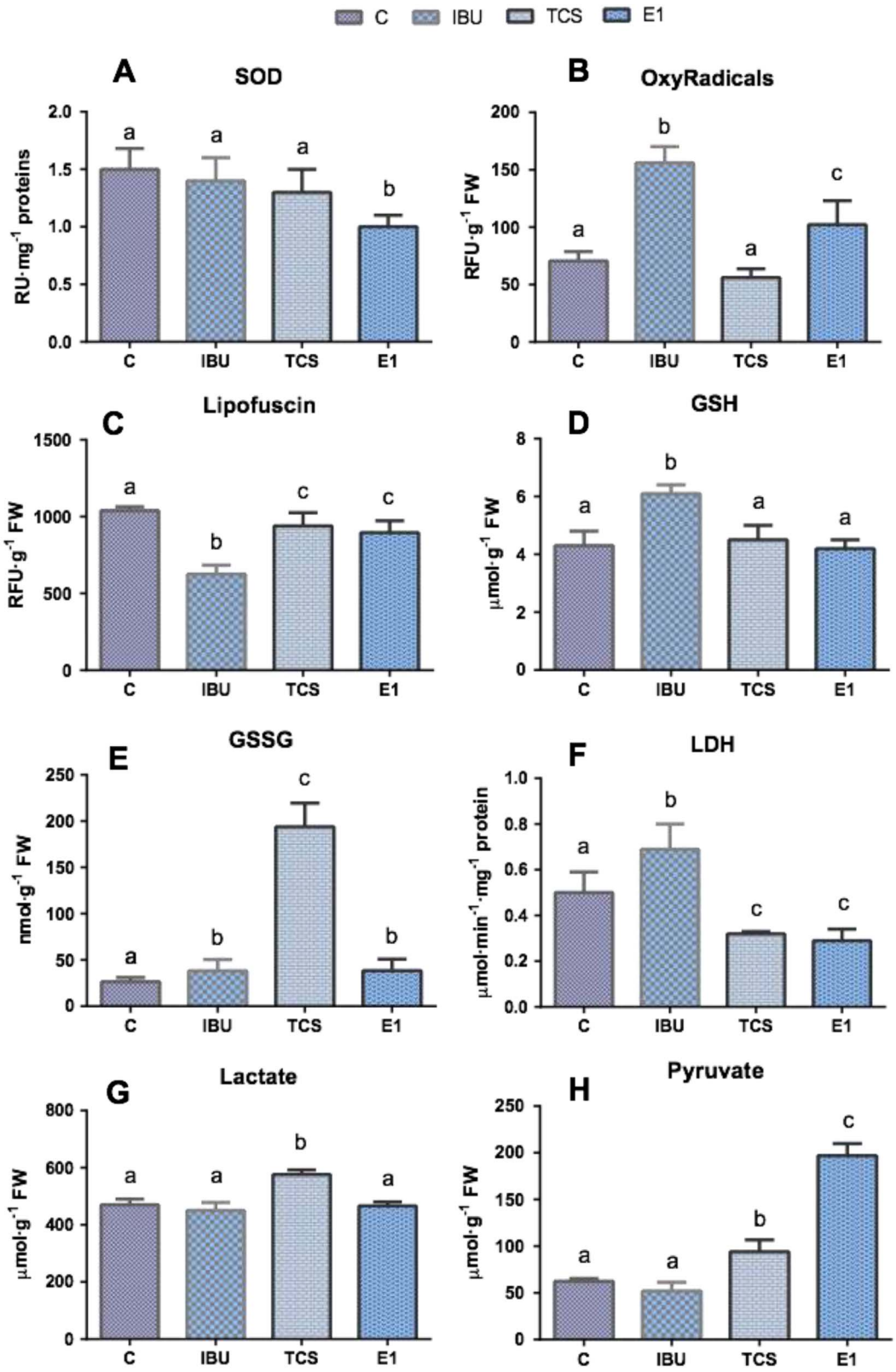

Fig. 4. Traits of oxidative stress and metabolic activity in the liver of frog *P. ridibundus* exposed to selected PPCPs (IBU, TCS, E1). A, superoxide dismutase (SOD) activity; B, rate of oxyradicals formation; C, lipofuscin concentration; D, reduced glutathione (GSH), E, oxidized glutathione (GSSG); F, Lactate dehydrogenase activity; J, Lactate concentration; I, Pyruvate concentration. Data are presented as means ± SD ($N = 8$).

nodes (Fig. 6B). The first branch on the tree separated E1-treated group from all other groups basing on the highest level of the CYP450 in this group. The distinguishing characteristic for IBU- and TCS-treated groups was also the level of CYP450 with the lowest level in the TCS-group.

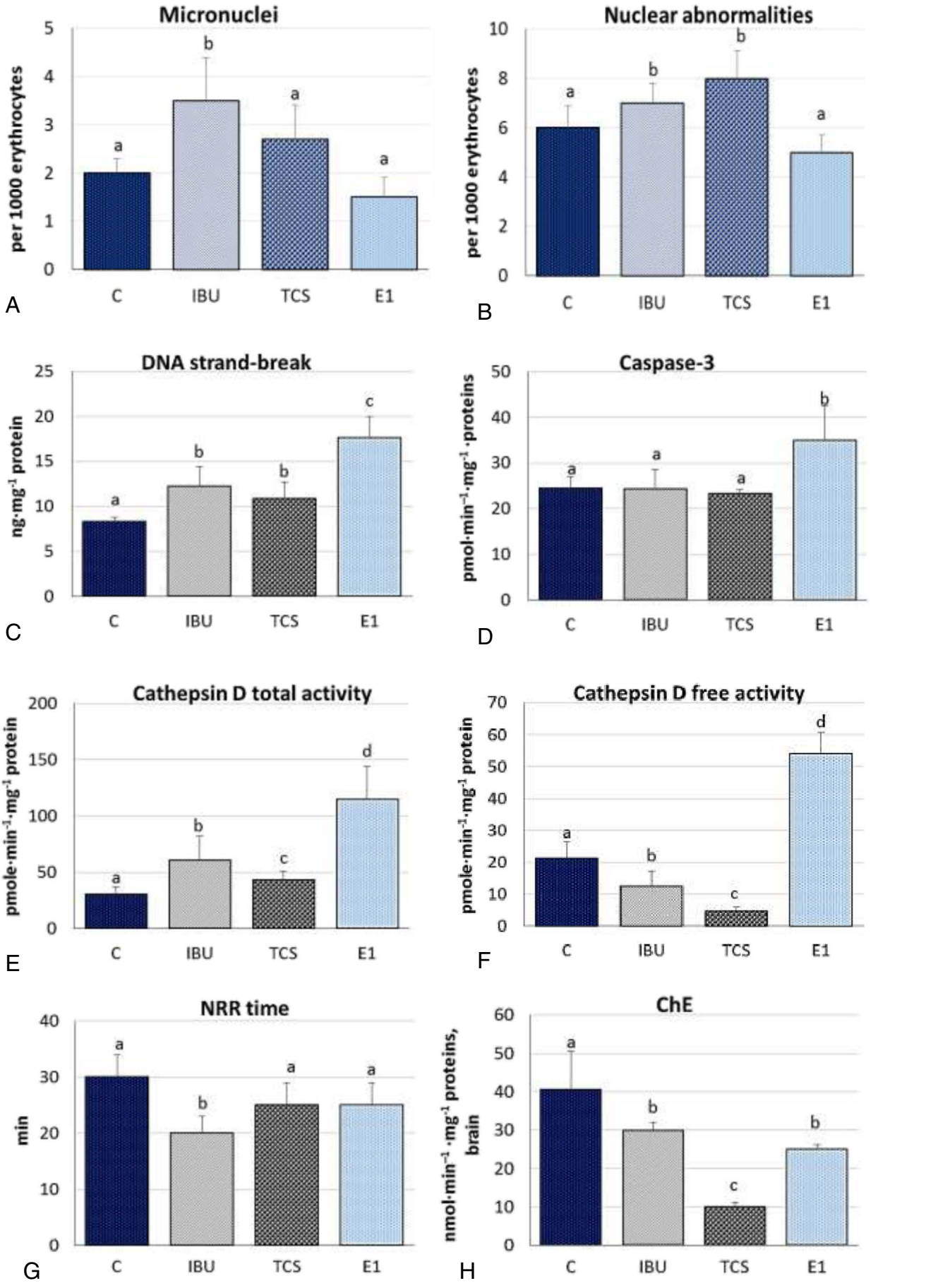

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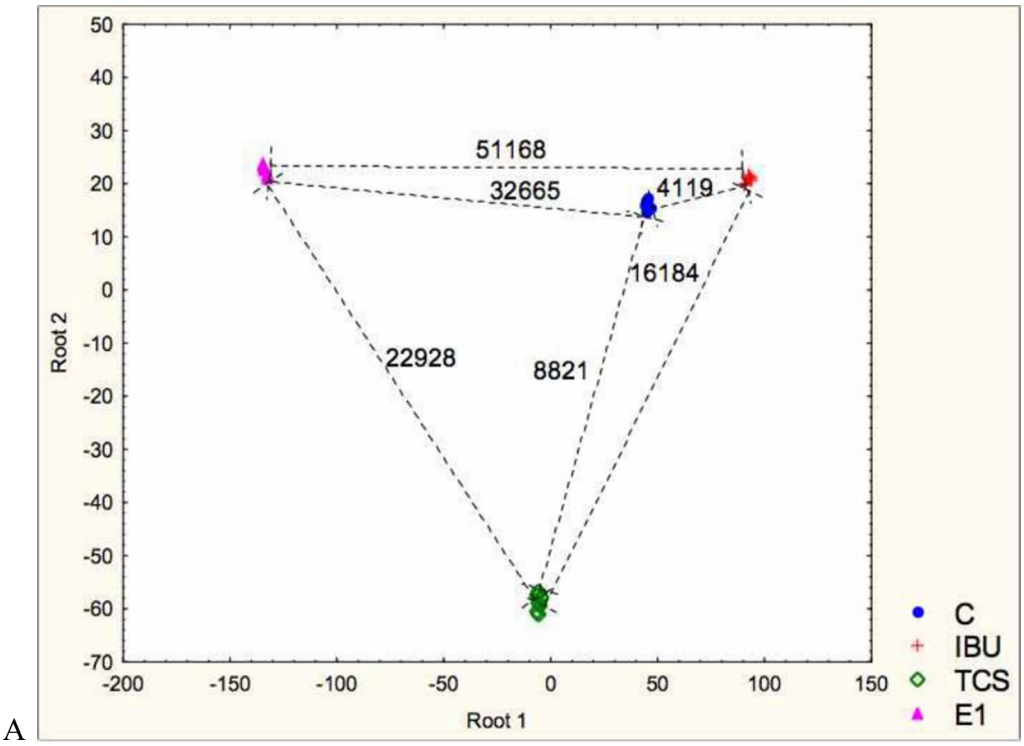
Fig. 5. Toxicity indices in the erhytrocytes (A,B), liver (C, D, E, F, G) and brain (H) of frogs exposed to selected PPCPs (IBU, TCS, E1): the frequency of the cells with micronuclei (A), nuclear abnormalities (B), level of the DNA strand breaks (C); caspase-3 activity (D); total activity of cathepsin D (E); activity of free cathepsin D (F); lysosomal membrane stability (neutral red retention time) (G); cholinesterase activity (H), means ± SD (N = 8).

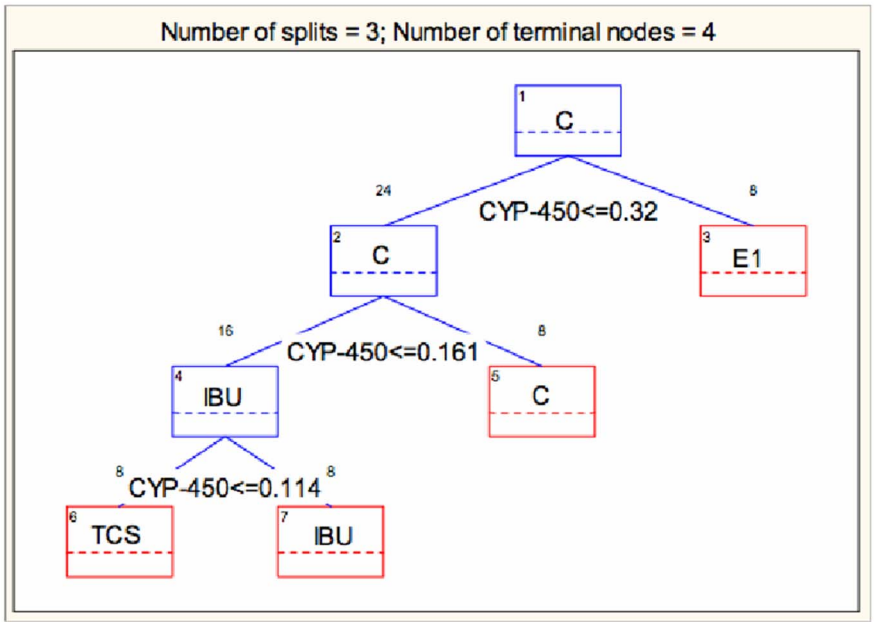
4. Discussion

4.1. Common signs of the vulnerability of frog to chemical impact

In the present study the evaluation of a wide spectrum of indices allowed us to prove high similarity in the responses of frogs to three different substances. Most common response was the distortion of Zn metabolism in the liver: reduction in the concentrations of Zn and MTs (particularly MT-Me) and Zn/Cu concentration ratio in the liver. The participation of essential metals in the responses to PPCPs did not attract investigators' attention, despite Zn being involved in supplying health status via molecular stabilization, catalysis, and cell signaling (Maret, 2011). The syndrome of Zn deficiency is reported as a non-specific manifestation of different inappropriate effects, mainly in humans (Fukada et al., 2011; Prasad, 2012; Falfushynska et al., 2015c; Yasuda and Tsutsui, 2016). Moreover, when the concentrations of Zn and Cu in the tissues are studied simultaneously, the Zn/Cu imbalance is reported as a typical sign of adverse effects (Rutherford and Bird,



A



B

Fig. 6. Results of discriminant (A) and classification and regression tree (B) analyses of the studied biological traits of *P. ridibundus* from different treatment groups (C, IBU, TCS and E1 – frogs exposed to ibuprofen, triclosan and estrone, respectively). A - the layout of control and exposed frogs for discriminant analysis based on Mahalanobis square distance. C –control group, IBU, TCS, E1 - exposed groups. Numbers above lines indicate Mahalanobis square distance between two groups; B - a classification tree based on the studied biological traits. The stepwise classification procedure identifies, at each step, a treatment group that is most different from all others based on the values of a certain biomarker. The distinguishing biomarker is shown near the branch split, and the group differentiated by that biomarker is shown in the corresponding terminal node. The lesser (<) sign next to the biomarker indicates that the value of the biomarker is greater in the terminal node group compared to all other groups. The number above each box indicates the number of animals in the respective group. Note that there was no misclassification of animals from any of the treatment groups, as N = 8 in each of the terminal nodes.

2004). For examples, high serum Cu to Zn ratio in humans is associated with mortality in elderly, oxidative stress, inflammation and hormonal impact (Malavolta et al., 2015). In frogs, Zn depletion was described for specimens exposed to coal combustion waste (Ward et al., 2009), Zn-contained nanomaterials and Zn ions (Naab et al., 2001; Falfushynska et al., 2015a). Decreased Zn/Cu ratio was detected in the livers of frogs from the municipal site (Stolyar et al., 2008) confirming the vulnerability of Zn homeostasis in this species.

The induction of metal-buffering proteins MTs is a typical response to toxic metals in different phyla, including frogs (Papadimitriou and Loumbourdis, 2003; Zocche et al., 2014; Carvalho et al., 2017). However, MTs are also sensitive to other than metals stressors (Viarengo et al., 1999, 2007). At that, MTs of frog are characterized by particular oxidative vulnerability, which manifests as an imbalance of metal saturation (Formigari et al., 2007; Isani and Carpenè, 2014). The simultaneous determining of MT-SH and MT-Me has given the opportunity to detected their imbalance with negative correlation ($p = 0.004$) in the present study. The same imbalance was shown in frogs inhabiting municipal site (Falfushinska et al., 2008a) and after the exposure to oxidants in vitro (Falfushynska et al., 2010). The opposite relation of metal accumulation and tissue thiol was found in the frogs from the polluted environment (Borković-Mitić et al., 2016). Relating the studied substances, similar phenomenon of Zn release from intra-cellular store by sublethal concentrations of TCS (1–3 μM) was detected for the rat thymocytes (Tamura et al., 2012). The utilizing of ELISA assay confirmed the decrease of the level of MTs with corresponding antigenic properties in IBU- and TCS-groups. We cannot explain the discrepancies between the MTi and MT-Me & MT-SH changes in E1-group. However this phenomenon attracts the attention to intercalibration in the evaluation of biomarkers (Falfushynska et al., 2014a).

The decrease of MT-SH correlates with the level of cortisol in the serum ($p < 0.001$) that also shown nonspecific dramatic decrease witnessing common depression of stress response. At the first glance, this manifestation seems opposite to the typical stress response in vertebrates (Gagnon et al., 2006; Martínez-Porchas et al., 2009; Lee et al., 2015). However, the suppression of cortisol secretion was detected in yellow perch *Perca flavescens* as a result of a polluted environment and under the acute capture stress (Dorval et al., 2003; Gravel et al., 2005). High positive correlation between cortisol and EROD level ($p < 0.001$) was found, probably reflecting the dependence of amphibian neurosteroid metabolism on the microsomal transformation (Martínez-Porchas et al., 2009; Takase et al., 2011).

Another highly vulnerable characteristic detected in the present study was the CYP450-related biotransformation in the liver. Its induction by persistent organic xenobiotics (polycyclic aromatic hydrocarbons and their chlorinated derivates) is utilized as a biomarker of their toxicity to vertebrate animals, including amphibians (Denslow et al., 2004; Katagi and Ose, 2014; Falfushynska et al., 2017). On the other hand, instances of decreased activity of P450 monooxigenase in the liver of frogs were reported after exposures to the ions of chromium or its mix with cadmium (Kostaropoulos et al., 2005), Zn and metal-contained nanoscale organic polymers (Falfushynska et al., 2015a). In the current study the applying of two assays confirms the same responses to IBU and TCS (Fig. 2C,D). The phenomenon of distinct responses of EROD and CYP450 in the case of E1 could be explained by the differences in the substrate specificity and/or induction of specific forms (Cribb et al., 2006; Havelková et al., 2008; Zamaratskaia and Zlabek, 2009; Ku et al., 2014). For example, the effect of TCS on the yellow catfish *Pelteobagrus fulvidraco* caused the decrease of EROD activity as the most consistent response, whereas CYP1A and CYP3A expression levels were dependent on the exposure period and concentration of TCS (Ku et al., 2014). In any case, the application of ELISA for MTs and CYP450 revealed in both cases the particular responses to E1 that were not evident in the traditional assays. Moreover, the groups were best distinguished by CYP450 activity.

Next common response was GST activation. The positive correlation of GST and GSH levels ($p < 0.001$) attests the involvement of GST in the oxidative stress response in accordance with the GSH function. Elevated GST activity was reported for frogs of different life stages after exposure to pesticides (see for review Katagi and Ose, 2014). The studied PPCPs also activated GST in freshwater mussel *Unio tumidus* (Falfushynska et al., 2014b). The exposure to IBU (1 $\mu g \cdot L^{-1}$) caused the up-regulation of GST in oysters *Crassostrea gigas* (Serrano et al., 2015).

The depletion of ChE in the brain is a typical sign of neurotoxicity. ChE decrease in the liver was detected in the frogs from a polluted site under exposure to thiocarbamate fungicide (Falfushinska et al., 2008a, 2008b). The same simultaneous depletion of EROD, cortisol and ChE was detected in the exposures of frog to metal-contained organic nanocomplexes and Zn (Falfushynska et al., 2016), confirming general vulnerability of these indices.

The common sign for all exposures was also the up-regulation of lysosomal aspartatic protease cathepsin D. In amphibian this protease plays particular role during the metamorphosis and regeneration because it participates in degradation or modification of intra- and extracellular matrix molecules (Ju and Kim, 2000; Sung et al., 2001; Benesa et al., 2008; Mahapatra and Mahapatra, 2011). Its activation in the adult frog seems to be sensitive response to different xenobiotics (Falfushynska et al., 2016, 2017) indicating the development of necrosis (Benesa et al., 2008). All mentioned above indices were strongly coordinated ($p < 0.01$). The conclusion concerning the common high vulnerability to PPCPs even in low concentrations is confirmed by the signs of genotoxicity (DNA instability and nuclear abnormalities) detected in each exposure.

4.2. Distinct manifestations of the effects of selected PPCPs

Despite the multiple common signs of vulnerability, the application of Mahalonobis distance (Fig. 6A) detected the specificity of response to each exposure. The activity of CYP450 was selected as a main distinguished criterion for all groups by CART analysis with highest similarity between control and IBU-group and highest distinction of E1-group (Fig. 6B). Indeed, the lesser impact was shown in the case of IBU. It did not cause the same disruptive endocrine effect as was reported for male fish, *Oryzias latipes*, in chronic exposure to 100 $ng \cdot L^{-1}$ (Han et al., 2010). Most remarkable effect of IBU was strong oxidative stress that was not accompanied by destructive consequences. LDH up-regulation could add to this prominent and specific response. The ability of non-steroid anti-inflammatory drugs to induce oxidative stress was previously confirmed in the mollusks. For example, the early activation of antioxidant enzyme expression and lipid peroxidation was reported for bivalve mollusks *Dreissena polymorpha* (Contardo-Jara et al., 2011). Similarly to present results, the lesser signs of toxicity among three PPCPs and the increase of GSH & GSSG were detected in the exposure to IBU in the mussel *Unio tumidus* (Falfushynska et al., 2014b). Gagné et al. (2006) established the enhanced respiration rates and oxidative stress in the isolated mitochondrion of freshwater mussel *Elliptio complanata* under the effects of pharmaceutical products including IBU.

Unlike IBU, both TCS and E1 caused prominent endocrine disturbance. In the case of vitellogenin, it was confirmed by two assays. The increase in TSH level (by several times) could be of particular concern due to the role of thyroid activity in the development of anurans (Manzon and Denver, 2004; Opitz et al., 2006; Kloas, 2002; Kloas et al., 2009; Chai et al., 2016). All known information concerning the endocrine effects of TCS relates mostly to mammalian species and the effects of high doses (several orders of magnitude greater than the estimated exposure levels of TCS in humans) (Stoker et al., 2010; Witorsch, 2014). However, there is strong evidence that aquatic species such as algae, invertebrates and certain types of fish are much more sensitive to TCS than mammals (Dann and Hontela, 2011; Witorsch, 2014). Distortion of thyroid function was observed in the early stages of frog development; however, the concentrations of TCS in most of these studies were in the range of $\mu g \cdot L^{-1}$. The exposure of *Bufo gargarizans* to

60 and 150 μg·L^{-1} of TCS during embryogenesis resulted in delayed growth and development that was associated with the disruption of thyroid homeostasis (Chai et al., 2016). In the premetamorphic *Rana catesbeiana* tadpoles, exposure to TCS promoted the distortion of the gene expression programs for tail fin and back skin induced by triiodothyronine (Veldhoen et al., 2006; Hammond et al., 2015). In Pacific tree frog (*Pseudacris regilla*), TCS concentrations of 0.3, 3 and 30 μg·L^{-1} disrupted coordination of postembryonic tadpole development (Marlatt et al., 2013). The altering of steroid activities by TCS was reported for male and female rats but in dozen more higher concentration than could be fixed in the human plasma (Stoker et al., 2010). The endocrine activity of TCS was explained by similar chemical structures to 17β-estradiol (Brausch and Rand, 2011; Kumar et al., 2015; Yuan et al., 2015).

Treatment by TCS also caused prominent oxidation of GSH (by 7.3 times). The sensitivity of cellular thiol to sublethal concentrations of TCS (1–3 μM) was detected in rat thymocytes (Tamura et al., 2012). The signs of metabolic depression by TCS were shown in the present study and for various aquatic animal models (Canesi et al., 2007; Cherednichenko et al., 2012; Falfushynska et al., 2014b). Notably, TCS caused almost complete depletion of ChE in frog. These manifestations add to the conclusion about the high toxicity of TCS for different phyla of aquatic animals, leading to violation of population growth (Han et al., 2016) and genotoxicity (Binelli et al., 2009; Riva et al., 2012; Matozzo et al., 2012).

A particular imbalance of endocrine activities was provoked in frogs by E1 (highest level of vitellogenin, detected by two assays, elevated thyrotropin level in the blood serum and decrease of deiodinase activity) and was distinguished from all other groups by high Cyp450 activity (Fig. 6C). The induction of vitellogenesis by estrogen E1 in frog was expected. The dependence of the serum vitellogenin level on physiological doses of estradiol in *Rana esculenta* was described earlier (Gobbetti et al., 1985). The chronic exposure of wood frog *Lithobates sylvaticus* to 17α-ethynylestradiol, the synthetic estrogen used in oral contraceptives, during the larval period led to the elevated abundances of transcripts of vitellogenin A2 in the liver (1.8–280-fold greater compared to controls). A significant effect was provoked even by about 2-fold increase in equivalent concentration of estrogen as compared to the environment (Tompsett et al., 2013). Interestingly, in the previous study of the mussels, E1 decreased the level of Vtg-LP in honads (Falfushynska et al., 2014b). The inability of mollusks to transform cholesterol into vertebrate- type steroids could explain this opposite response to typical vertebrate steroid and must be taken into account with regard to utilization of mussels in the bioindication of EDs.

Additionally, the exposure of frogs to E1 provoked specific oxidative change (superoxide dismutase down-regulation accompanied by increasing in the levels of oxyradical and GSSG). It also caused prominent and particular metabolic shift (a 3.2-fold increase in pyruvate concentration), which can attest to its important effect on the glycolytic pathway (Klinge, 2008; Cai et al., 2013). Endocrine and metabolic effects of E1 were accompanied by particular changes in the apoptotic activities: up-regulation of cytosolic executive apoptotic enzyme caspase-3 and cathepsin D efflux from the lysosomes (Fig. 5). High dependence of apoptotic activities in frogs on endocrine regulation is well described for the stage of metamorphosis triggered by thyroid hormone (for review see Ishizuya-Oka et al., 2010). Caspase-3 up-regulation was involved in this activity. However, the relation between estrogens and apoptotic activities is studied mostly in human models (Urata et al., 2006). Estrogen partially prevented the activation of caspase-3 in neurons (Sawada et al., 2000; Park et al., 2007) but stimulated apoptosis, including caspase-3 activation in the renal carcinoma cells (Yu et al., 2013). The transcriptional activation of cathepsin D gene expression by estrogens was shown in human breast cancer cells (Wang et al., 2000). The apoptotic activation in frogs by environmental estrogens was described for *Xenopus laevis* embryos (Bevan et al., 2003).

5. Conclusion and perspectives

To summarize, the comparison of results obtained in the same experimental conditions for mussels (Falfushynska et al., 2014b) and frogs (present study) confirms the similarity in the comparative severity of impact and several responses caused by three substances in both species. Nevertheless, only the experimental model of frog has given the opportunity to detect the endocrine disruption caused by TCZ and E1 that confirms it as a sentinel organism for endocrine disruption in vertebrate animals. Deep common depression of stress response is also worthy of particular concern for the amphibian in their native environment. The further investigations will be concentrated on the selection of biomarkers of frogs for the assessment of environmentally relevant combined impact of PPCPs.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.cbpc.2017.07.004.

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