doi: 10.17221/125/2016-VETMED

Effects of the pharmaceutical contaminants ibuprofen, diclofenac, and carbamazepine alone, and in combination, on oxidative stress parameters in early life stages of tench (*Tinca tinca*)

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ABSTRACT: In the present study, the effects of sub-lethal sub-chronic doses of ibuprofen, diclofenac, and carbamazepine alone, and in combination (concentration range 0.02–60 µg/l), on the early life stages of tench (*Tinca tinca*) were investigated. The lower concentrations of pharmaceuticals tested (0.02, 0.2, 2 µg/l) represent the concentration values of these substances commonly present in surface waters or effluents from wastewater treatment plants. Multiple biomarkers of biotransformation, antioxidant defence systems, and lipid peroxidation were determined in fish after 35 days of exposure. The evaluated pharmaceuticals induced oxidative stress in fish both alone and in combination with each other. Generally, 60 µg/l of each single pharmaceutical influenced the activity of antioxidant enzymes significantly ($P < 0.05$), whereas the same concentration of these pharmaceuticals in combination (1 : 1 : 1) did not have any impact on the activity of these enzymes. However, changes in biotransformation and antioxidant enzymes were apparent if lower concentrations of these pharmaceuticals were administered in the mixture. Significant changes ($P < 0.05$) in the activities of glutathione reductase, glutathione peroxidase, and glutathione-S-transferase were observed even at environmental concentration ranges. A significant effect ($P < 0.05$) on lipid peroxidation levels was found only in the experimental group exposed to carbamazepine.

Keywords: antioxidant defence system; lipid peroxidation

List of abbreviations

ANC = acid-neutralising capacity, **CAT** = catalase, **CBZ** = carbamazepine, **COD_{Mn}** = chemical oxygen demand, **COX** = cyclooxygenase, **DCF** = diclofenac, **DMSO** = dimethylsulfoxide, **GPx** = glutathione peroxidase, **GR** = glutathione reductase, **GSH** = reduced active form of glutathione, **GSSG** = oxidised inactive form of glutathione, **GST** = glutathione-S-transferase, **IBU** = ibuprofen, **LC** = liquid chromatography, **LC-MS/MS** = liquid chromatography with tandem mass spectrometry, **LPO** = lipid peroxidation level, **NSAIDs** = non-steroidal anti-inflammatory drugs, **ROS** = reactive oxygen species

Pharmaceutical mixtures that contaminate water sources are currently a problem worldwide. Ibuprofen (IBU), diclofenac (DCF), and carbamazepine (CBZ) are among the most frequently detected drugs in aquatic ecosystems. Their concentrations in surface water range from ng/l to tens of µg/l. Higher concentrations have been detected in developing countries due to the direct discharge of untreated wastewater from residences and hospitals into surface waters (Tran et al. 2014).

Supported by the Ministry of Education, Youth and Sports of the Czech Republic (Project No. OPVK CZ.1.07/2.3.00/30.0053).

doi: 10.17221/125/2016-VETMED

Water-borne pharmaceuticals may be absorbed into aquatic organisms. According to Brozinski et al. (2013), drugs detected in water could be found also in the bile of wild bream (*Abramis brama*) and roach (*Rutilus rutilus*) living in a lake in which DCF and IBU were present as pollutants. Despite the fact that many researchers around the world are actively investigating the possible impact of these substances on non-target organisms, there is still a lack of information on the sub-lethal effects of these pharmaceuticals on fish, especially regarding the combined effects of these substances at environmentally relevant concentrations.

Besse and Garric (2008) devised a ranking for pharmaceuticals according to the risk they pose to the environment, with Class IA including compounds posing the highest risk, and Class IV denoting those which pose only a very low risk. IBU, DCF, and CBZ are classified as Class IA, IIB and IB compounds, respectively. Similarly, environmental risk assessment of active pharmaceutical compounds in surface waters based on 'measured environmental concentration' and 'predicted no effect concentration', indicates that CBZ, DCF, and IBU pose a high risk to aquatic ecosystems (Tran et al. 2014).

The aim of this study was to assess the effects of both single pharmaceuticals (IBU, DCF, CBZ) and their combinations on embryo-larval stages of tench after sub-chronic exposure. As many pharmaceuticals found in the aquatic environment are thought or have been proven to induce oxidative stress in fish (Nava-Alvarez et al. 2014), changes in the activities of oxidative stress biomarkers and in biotransformation enzymes were investigated. Early life stages were used in the experiment as they are considered more susceptible to environmental contaminants than juvenile and adult fish.

MATERIAL AND METHODS

Experimental protocol. Experiments were carried out using solutions of individual drugs - ibuprofen, diclofenac sodium salt, and carbamazepine and also mixtures of all three in a ratio of 1 : 1 : 1. All tested compounds were purchased from Sigma-Aldrich (Brno, Czech Republic). The embryo-larval toxicity test was performed according to OECD guideline No. 210 (OECD 1992). Fertilised eggs of tench were obtained from a commercial fish farm.

All experimental procedures were approved by the relevant institutional ethical committee and performed in compliance with institutional guidelines and national legislation (Act No. 246/1992 Coll., on the Protection of Animals Against Cruelty, as amended).

Experiments were conducted in 500 ml crystallisation dishes, each containing 100 randomly chosen embryos (24 h after fertilisation). Each experiment was conducted in triplicate (a total of 300 fertilised eggs for each concentration and for the control). The embryos and larvae of tench were exposed for 35 days to: (A) individual pharmaceuticals at a nominal concentration of 60 µg/l; (B) mixtures of pharmaceuticals at nominal concentrations of 0.02, 0.2, 2, 20, and 60 µg/l of each pharmaceutical.

Control fish (Group C) were kept in pure water used for dilution. As the tested substances required the use of dimethylsulphoxide (DMSO) as a solvent, additional control fish (Group C-S) were exposed to 0.01% DMSO in water. The concentration of DMSO in the control-solvent group was the same as the DMSO concentration in the tested solutions containing the highest drug concentration (60 µg/l). The basic physical and chemical parameters of water used in the experiment were as follows: acid neutralising capacity ($ANC_{4.5}$) 3.5–3.7 mmol/l; chemical oxygen demand (COD_{Mn}) 1.4–1.9 mg/l; total ammonia < 0.04 mg/l; nitrates 12.1–13.6 mg/l; nitrites < 0.01 mg/l; Cl^- 17.5–18.5 mg/l; and Σ Ca + Mg 3.06 mmol/l.

A semi-static method was used, in which solutions of drugs were exchanged for fresh ones in 12 h intervals. The concentrations of pharmaceuticals in tested solutions were determined once a week using LC; samples were taken both before and after the solution exchange. The dilution water was also regularly tested for the presence of the pharmaceuticals used in the experiment; however, no traces of these were detected in the dilution water during the experimental period.

Hatching, survival, temperature, pH, and oxygen saturation were recorded daily. The water temperature and pH ranged from 21 to 23 °C and from 8.4 to 8.8, respectively. The photoperiod chosen was a 12 h light/12 h dark regimen. The concentration of dissolved oxygen did not fall below 80%. Feeding was initiated on Day 7 of the experiment, when all the embryos progressed from the sac-fry stage to larvae. The larvae were fed with freshly hatched *Artemia salina* twice a day *ad libitum*. During the test, larvae were randomly sampled several times in

doi: 10.17221/125/2016-VETMED

order to record developmental changes, morphological abnormalities, total length, body weight, and Fulton's condition factor. The results of those observations and influence of the used pharmaceuticals on the growth and development of the tench are described in detail in our earlier study (Stancova et al. 2014).

The experiment was terminated on Day 35, when 10 fish from each control and test group were euthanised with carbon dioxide. Fish were frozen and stored at –85 °C pending further biochemical analyses.

Homogenisation and determination of the activity of antioxidant enzymes. Whole body samples were thawed, weighed, and after the addition of phosphate buffer (1 ml of buffer per 0.1 g of sample), homogenised on ice.

The catalytic activities of the enzymes glutathione reductase (GR), glutathione peroxidase (GPx), glutathione-S-transferase (GST), and catalase (CAT) were measured spectrophotometrically using the methods described by Habig et al. (1974), Carlberg and Mannervik (1975), Aebi (1984), and Flohe and Gunzler (1984). To determine lipid peroxidation levels (LPO) in fish samples, the modified thiobarbituric acid reactive substances (TBARS) method described by Lushchak et al. (2005) was used. The activities of enzymes were normalised to protein content. The concentration of proteins was determined using a Bicinchoninic Acid Protein Assay Kit (Sigma-Aldrich, St Louis, USA), in which bovine serum albumin was used as a standard (Smith et al. 1985). All spectrophotometric measurements were performed using the Varioskan Flash Spectral Scanning Multimode Reader (Thermo Fisher Scientific Inc., USA).

Determination of pharmaceuticals in tested solutions. Levels of IBU, DCF, and CBZ were measured using high-performance liquid chromatography coupled with triple quadrupole tandem mass spectrometry (LC-MS/MS). Samples were prepared using solid phase extraction. SPEC C18 AR cartridges (3 ml, 30 mg, Varian, Inc., Palo Alto, USA) were used. One millilitre of the sample was passed through a preconditioned cartridge (500 µl methanol and 500 µl water). The analyte was eluted with 1 ml acetonitrile and used for LC-MS/MS analysis. A Thermo Scientific UHPLC Accela 1250 system was connected to a Thermo Scientific TSQ Quantum Access MAX triple quadrupole instrument (Thermo, San Jose, USA) equipped with a heated electrospray ionisation (HESI-II) probe. A Thermo Scientific Hypersil C18 (2.1 mm × 50 mm, 1.9 µm) column was used at a constant flow rate of 300 µl/min in an isocratic elution method with acetonitrile/water 70/30 (v/v). The full loop injection volume of the sample was set at 20 µl. The heated electrospray ionisation was operated in the positive-ion mode for carbamazepine and in the negative-ion mode for ibuprofen and diclofenac under the following conditions: capillary temperature: 325 °C; vaporiser temperature 300 °C; sheath gas pressure 35.0 psi; auxiliary (drying) gas 10 a.u.; spray voltage 3300V (–3300V for ibuprofen and diclofenac). Standards were purchased from Sigma-Aldrich (St Louis, USA). The limits of detection were determined as 3 : 1 signal versus noise value (S/N) and were 9 ng/l for ibuprofen, 7 ng/l for diclofenac, and 5 ng/l for carbamazepine. Since a good agreement was found between the nominal and measured concentrations in the tested solutions, and the concentration of pharmaceuticals did not drop below 80% of the original concentrations, subsequent biological effects were evaluated on the basis of nominal concentrations.

Statistical analysis. Data are presented as mean ± SEM, $n = 10$. Oxidative stress parameters were tested using the statistical software Unistat 5.6 for Excel. Data were tested for normality using the Shapiro-Wilk test. If data were normally distributed, the effects of pharmaceuticals on the activities of antioxidant enzymes were evaluated using parametric ANOVA and the differences among tested groups were assessed with Tukey's test. For non-normally distributed data, the Kruskal-Wallis one way ANOVA test and the multi-sample median test were used. In our experiments, differences between groups were assessed to be significant at $P < 0.05$ and highly significant at $P < 0.01$.

RESULTS

Effects of ibuprofen and diclofenac on oxidative stress parameters

In the present study, no impairment of GR (Figure 1A), either by IBU or DCF, was found. GR is known for its particularly active role in preventing the onset of oxidative stress; it is involved in the recycling of glutathione by reducing the inactive oxidised (GSSG) form to the active reduced (GSH) form. On the other hand, significant ($P < 0.05$) and highly significant ($P < 0.01$) decreases in GPx activity were

doi: 10.17221/125/2016-VETMED

found in the IBU60 and DCF60 groups, respectively (Figure 1B). The main biological role of GPx is to reduce both lipid hydroperoxides and free hydrogen peroxide. GST activity was highly significantly ($P < 0.01$) inhibited as a consequence of exposure to NSAIDs (Figure 1C). GST is a Phase II detoxifying enzyme, which catalyses the conjugation of the reduced form of glutathione to xenobiotics for the purpose of detoxification. In our study, CAT was not affected to the same extent as GPx or GST by NSAIDs, but DCF still decreased ($P < 0.05$) its activity significantly and IBU non-significantly (Figure 1D).

Effects of carbamazepine on oxidative stress parameters

The results of our test show that CBZ did not have any impact on most of the investigated antioxidant enzymes (Figures 1A–D); only the activity of GR was increased ($P < 0.05$), which may be indicative of weak activation of the defence mechanism against oxidative stress. Surprising results with respect to lipid peroxidation were obtained in our study. A highly significant ($P < 0.01$) decrease in LPO occurred in tench after 35 days of exposure to CBZ (Figure 1E).

Effects of pharmaceutical mixtures

As mentioned above, several significant changes in the antioxidant system were found for the 60 µg/l concentration of IBU and DCF, and some minor changes were also detected for CBZ. By contrast, the same concentrations of pharmaceuticals acting together in a mixture (1 : 1 : 1) did not have any significant effect on any antioxidant enzyme inves-

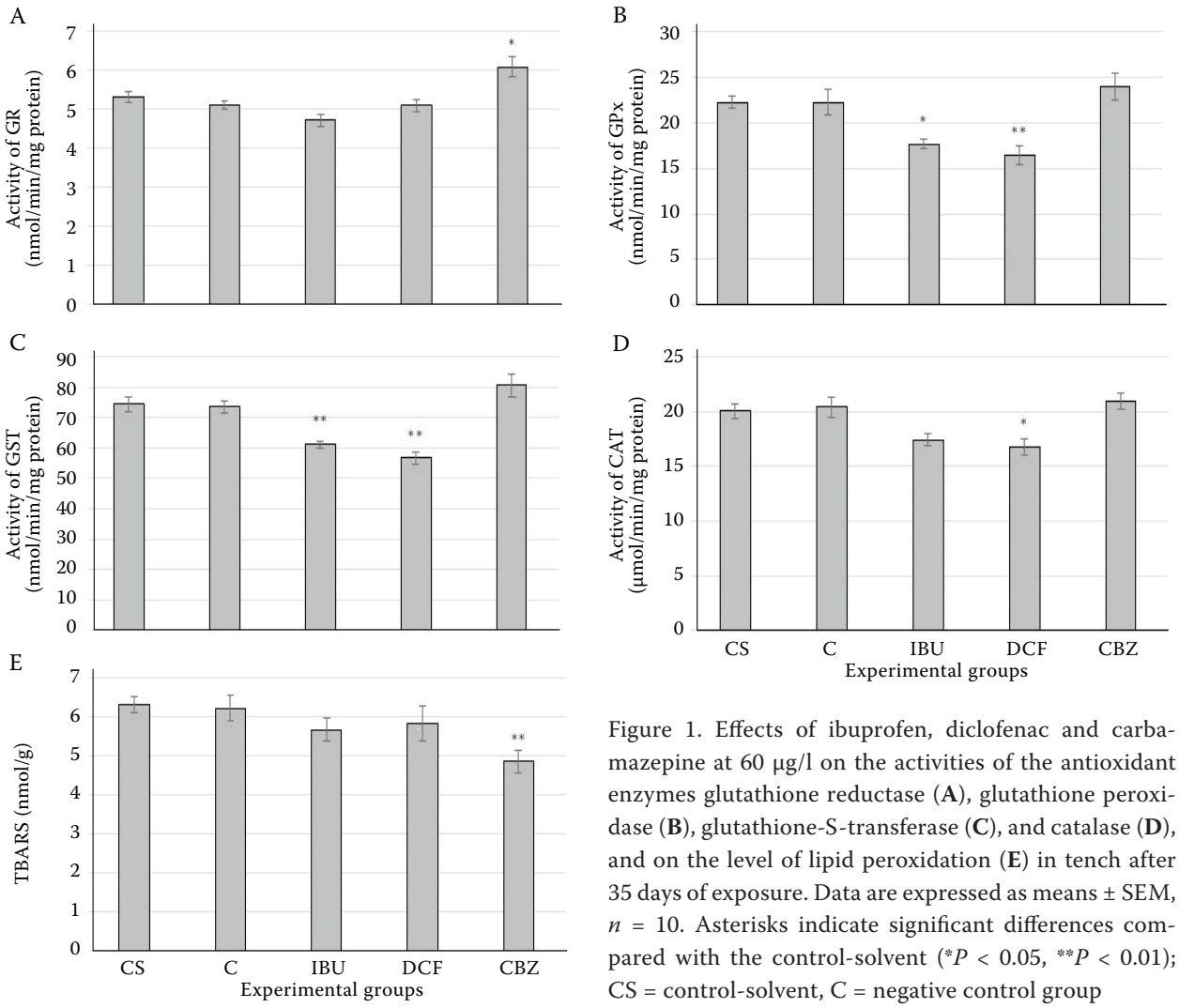

Figure 1. Effects of ibuprofen, diclofenac and carbamazepine at 60 µg/l on the activities of the antioxidant enzymes glutathione reductase (**A**), glutathione peroxidase (**B**), glutathione-S-transferase (**C**), and catalase (**D**), and on the level of lipid peroxidation (**E**) in tench after 35 days of exposure. Data are expressed as means ± SEM, $n = 10$. Asterisks indicate significant differences compared with the control-solvent (*$P < 0.05$, **$P < 0.01$); CS = control-solvent, C = negative control group

doi: 10.17221/125/2016-VETMED

tigated; interestingly however, mixtures with lower concentrations of the tested drugs affected GR, GPx and GST activity (Figures 2A–C). The most pronounced impact of the tested mixtures was on GR (Figure 2A); at concentrations of 0.2, 2, and 20 µg/l activity increased significantly ($P < 0.01$). GST activity (Figure 2C) increased significantly ($P < 0.01$) at the concentration of 2 µg/l, while GPx activity was inhibited in the M2 group (Figure 2B). LPO and CAT activity were not altered in response to any of the mixtures investigated (data not shown).

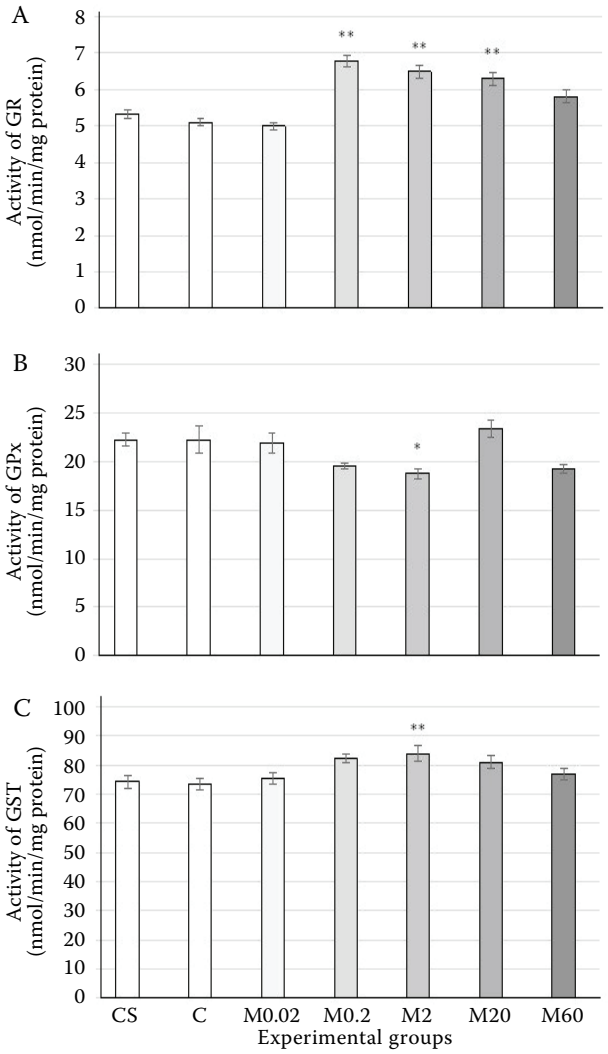

Figure 2. Effects of pharmaceutical ibuprophen (IBU), diclofenac (DCF) and carbamazepine (CBZ) mixtures containing 0.02, 0.2, 2, 20, and 60 µg/l of each substance on the activity of glutathione reductase (**A**), glutathione peroxidase (**B**), and glutathione-S-transferase (**C**) in tench after 35 days of exposure. Data are expressed as means ± SEM, $n = 10$. Asterisks indicate significant differences compared with control-solvent (*$P < 0.05$, **$P < 0.01$); CS = control-solvent, C = negative control group

DISCUSSION

Although most pharmaceuticals are present in the aquatic environment at only very low concentrations, many of them raise considerable toxicological and ecotoxicological concern, particularly when present as components of complex mixtures. Despite the use of many approaches, it is still very difficult to evaluate the effects of what are probably hundreds of such substances on aquatic biota, natural processes in water as well the environment in general, as their concentrations and ratios change constantly.

The effects of IBU and DCF (which belong to the same pharmacological group of non-steroidal anti-inflammatory drugs – NSAIDs) on antioxidant defence enzymes are shown in Figures 1A–E. It is obvious that both substances influenced the evaluated parameters in a similar manner, whereas the antiepileptic drug CBZ affects antioxidant enzymes in a different way.

IBU and DCF act as inhibitors of cyclooxygenase isoenzymes, which are responsible for the formation of prostanoids. Among these enzymes, COX-1 is considered to be the constitutive form and catalyses the formation of molecules that, e.g. protect the mucosa of the stomach and intestine from erosion by hydrochloric acid and xenobiotic substances. COX-2 is believed to be mainly an inducible form responsible for causing inflammation and pain (Hong et al. 2007). IBU and DCF exhibit no selectivity between these isoenzymes; they inhibit both COX-1 and COX-2. Both COX isoforms as well as the synthesis of prostaglandins share a high similarity between humans and fish (David and Pancharatna 2009). The side-effects and toxicity of non-selective NSAIDs are well documented in mammals (Lascelles et al. 2007; Khan and McLean 2012) and the negative influence of aquatic contaminants of this type are expected, and in some cases, have already been proven in non-target aquatic organisms (Bhandari and Venables 2011; Deeti et al. 2014).

Inhibition of the functions of multiple antioxidant enzymes was observed in our experiments after the exposure of tench to NSAIDs, manifesting as a decrease in GPx activity (Figure 1B) and CAT

doi: 10.17221/125/2016-VETMED

activity (Figure 1D), which may lead to increased oxidative stress and lipid peroxidation, as well as decreased activity of the detoxification enzyme GST (Figure 1C). Interestingly, however, no oxidative damage to lipids was found as a consequence of either IBU or DCF exposure (Figure 1E). Reactive oxygen species (ROS) can oxidise different cellular macromolecules such as lipids, proteins, and DNA, as well as xenobiotics present within organisms. Thus, in our study, if there had been any significant oxidative damage, it is possible that there would also have been some changes in LPO. Unfortunately, the activity of antioxidant enzymes and level of LPO were not assessed during the experiment, so we do not know how these parameters changed with time, but it can by hypothesised that the organisms may have adapted to this low level of oxidative stress during the long exposure period. The fact that long-term exposure to NSAIDs does not induce lipid peroxidation in fish can be supported by the findings of Bartoskova et al. (2013) and Zivna et al. (2013), who revealed a mild decrease in LPO in zebrafish after exposure to IBU and acetylsalicylic acid, respectively. The fact that lipids remain intact or that LPO mildly decreases can also be attributed to the mechanism of action of NSAIDs which inhibit COX, so the membrane lipid peroxidation connected with the physiological function of cyclooxygenase and prostanoid formation is decreased.

Carbamazepine is an anticonvulsant drug, indicated for epilepsy, mood stabilisation in bipolar disorder, and it is also effective in the treatment of neuropathic pain. It acts in the central nervous system by blocking voltage-dependent sodium channels of excitatory neurons (Brandao et al. 2013). Our results show that CBZ did not have any impact on most of the antioxidant enzymes which were investigated, except for an increased activity of GR (Figures 1A–D). The consequence of increased GR activity is more intensive reduction of the oxidised form of glutathione. Our findings are in concordance with Brandao et al. (2013), who found that exposure to CBZ caused an increase in GST and GR activity in pumpkinseed sunfish (*Lepomis gibbosus*), as well as with the findings of Li et al. (2011), who reported a significant increase in GR activity in rainbow trout (*Oncorhynchus mykiss*) exposed to CBZ for 96 h.

Lipid peroxidation is one of the most biologically important reactions involving free radicals. Peroxidative injury to plasma phospholipids may lead to severe cell damage (Ercegovac et al. 2013). Surprising results with respect to lipid peroxidation were obtained in our study. The highly significant ($P < 0.01$) decrease in LPO observed after CBZ exposure (Figure 1E) is not consistent with the literature. In fact, we expected CBZ to exhibit pro-oxidant behaviour on the basis of the results of Tsiaka et al. (2013) and Li et al. (2011), who found that exposure to CBZ resulted in increased lipid peroxidation in the unicellular marine algal species *Dunaliella tertiolecta,* haemocytes of the mussel *Mytilus galloprovincialis*, and rainbow trout. Brandao et al. (2013) found no changes in lipid peroxidation levels in pumpkinseed sunfish after CBZ exposure. An important factor which should be taken into account is the tested concentration used. Li et al. (2011) used a range of concentrations in mg/l, while in our study 60 µg/l was used. The peroxidation of lipids by CBZ may also be species-specific and be influenced by the length of exposure. The study of Almeida et al. (2014) supports these ideas. While in the clam *Venerupis philippinrum* exposed to 9 µg/l of CBZ, LPO increased, in *Venerupis decussate* the same dose of CBZ decreased LPO significantly. To the best of our knowledge, no other positive effect of CBZ on lipids has been reported in fish to date. On the other hand, a positive effect of long-term treatment with CBZ on the antioxidant balance was described in humans (Ercegovac et al. 2013).

With respect to exposure to single pharmaceuticals, higher concentrations generally elicit more profound responses as a consequence of dose-dependency. This was confirmed in our study where IBU, DCF and CBZ exposure resulted in multiple significant changes to antioxidant systems. On the other hand, the same concentrations of pharmaceuticals acting together in a mixture (1 : 1 : 1) did not have any significant effects on any of the antioxidant enzymes investigated (Figures 2A–C). This is in sharp contrast with our previously published results (Stancova et al. 2014), which indicate that a mixture of IBU, DCF and CBZ, and also each alone at a concentration of 60 µg/l, causes temporary slowdown of ontogeny, higher incidence of malformations and higher mortality in tench. Taken together, our results indicate that the damage to the early life stages of this fish is not necessarily caused by the oxidative injury of cells.

Although we did not find any impairment of tench ontogeny or any increased mortality at environmen-

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tally relevant concentrations (0.02 and 0.2 μg/l) in our previous study (Stancova et al. 2014), our present results revealed that mixtures with lower concentrations of the tested drugs affected GR, GPx and GST activity (Figures 2A–C). According to Lang and Kohidai (2012), the additive effect of DCF and IBU occurred only in the mixture containing the lowest concentrations of both compounds, while in all other concentrations, antagonism was the observed type of interaction. In general, antagonistic interactions increased with the higher concentrations of the mixture. Similarly, Gomez-Olivan et al. (2014) found that interactions between two different NSAIDs are fundamentally antagonistic in type.

In our study, the most pronounced impact of the tested mixtures was on the GR (Figure 2A), which increased. From our tests with single pharmaceuticals, we found that CBZ acts as a GR enhancer. Similarly, mixtures with lower concentrations of the pharmaceuticals acted as slight enhancers of GST activity. This is in contrast with individual substances, where a decrease in GST was found for NSAIDs and no change was reported for CBZ. The boosted activity of both enzymes indicates the promotion of antioxidant defence and biotransformation processes and, therefore, a protective effect against environmental contaminants. GPx activity was inhibited in the M2 group (Figure 2B), which may be caused by NSAIDs, which inhibit this enzyme as individual substances. Although a reduction in GPx activity might enhance the toxic effects of the tested substances on fish (Li et al. 2012), no differences in LPO levels between the control and experimental groups exposed to the mixtures were found in our study.

Pharmaceuticals, as well as other contaminants, are present in complex and variable mixtures in the aquatic environment. The testing of all these different mixtures at all the possible environmentally relevant concentrations is very complicated. Moreover, any single pharmaceutical in the water environment can also be considered a mixture consisting of the active compound, its metabolites, and its transformation products, which can act in different ways. Pomati et al. (2008), who studied the interactions of 13 environmentally relevant drugs (such as ibuprofen, carbamazepine, etc.), found complex concentration-dependent interaction profiles which were difficult to predict or model. Thus, although real environmental conditions were mimicked as much as possible in our experiment, it would be difficult to predict the effects of the same tested substances if they were used in different ratios or in combination with other pollutants.

Aquatic wildlife organisms are chronically exposed to low doses of cocktails of dozens of pharmaceuticals. In our study, ibuprofen and diclofenac affected the antioxidant defence system in tench more profoundly than carbamazepine. From the results obtained, it is obvious that both IBU and DCF have similar effects on the activities of antioxidant enzymes. Mixtures of pharmaceuticals induce oxidative stress already at environmentally relevant concentrations. However, neither single drugs nor their mixture caused any significant damage to lipids. Further evaluation of the toxicity of pharmaceuticals and especially their mixtures for non-target aquatic organisms is necessary, as they are biologically active, are produced in huge amounts, occur worldwide, and are often not removed by water treatment processes.

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Received: August 23, 2016
Accepted after corrections: December 14, 2016