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The Effects of Salicylic Acid on Juvenile Zebrafish *Danio rerio* Under Flow-Through Conditions

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Abstract The aquatic environment is becoming increasingly contaminated with pharmaceuticals. Salicylic acid (SA), which can be used individually or appear as a degradation product of the widely used acetylsalicylic acid was chosen for testing. Juvenile zebrafish *Danio rerio* were subjected to OECD test No. 215 (fish, juvenile growth test) with salicylic acid concentrations of 0.004; 0.04; 0.4; 4 and 40 mg/L. Specific growth rate (SGR), histological changes, and parameters of oxidative stress were evaluated. SA had no effects on histological changes, SGR, glutathione reductase, and lipid peroxidation. Increased catalytic activity of GPx was found at 0.04 mg/L compared to control, increased catalytic activity of catalase was found at 0.04 and 4 mg/L compared to control, and increased catalytic activity of glutathione-*S*-transferase was found at 0.004 and 0.04 mg/L compared to control ($P < 0.05$). Juvenile zebrafish turned out to be relatively insensitive to both environmentally relevant (0.004 mg/L) and higher concentrations of salicylic acid.

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Increasing attention is being paid to the occurrence and effects of pharmaceutical residues in the water environment. Drugs are common contaminants of freshwater, seawater (Gaw et al. 2014), and unfortunately sometimes even groundwater (Heberer 2002). The main sources of pharmaceuticals are municipal, hospital (Santos et al. 2013), and stock farming waste waters (Gaw et al. 2014; Shore et al. 2014). The medicines themselves are, of course, sometimes inappropriately disposed of directly into such waste waters; however, the biggest contribution to environmental pollution with respect to pharmaceuticals comes from the active substances of these drugs or their metabolites excreted from the body of both human and animal patients via urine or faeces (Martin et al. 2012). Other, usually local sources of contamination are dumps and waste waters from the pharmaceutical or chemical industries (SanJuan-Reyes et al. 2013; Cardoso et al. 2014). Unfortunately, waste water/sewage treatment plants (STP) are missing in some areas and countries, and, if present, are often not equipped with efficient technologies that would lead to the complete degradation of pharmaceutical products. Residues of these substances then enter water sources via treated, but not completely clean water. The resulting cocktails of different drugs and their metabolites, whose contents change quantitatively and qualitatively over time, are presumed to influence the health of non-target aquatic organisms negatively, as such organisms are exposed to these substances over their whole lives.

Non-steroidal anti-inflammatory drugs (NSAIDs) are some of the most commonly found contaminants in the water environment, some of which (mainly diclofenac and

ibuprofen) are considered to be substances exhibiting an increased hazard coefficient for water environment (Orias and Perrodin 2014). Their concentrations in most natural or STP samples vary from hundreds of ng/L to units of μg/L, but, in some hot spots with a local source of pollution, may be up to units of mg/L. Salicylic acid (SA) is a drug used individually, e.g. in dermatology, and is also a metabolic and degradation product of acetylsalicylic acid (aspirin). Surprisingly, it is found in the water environment more often and in higher concentrations than the massively used drug acetylsalicylic acid (Heberer 2002; Metcalfe et al. 2003; Crouse et al. 2012; Lacina et al. 2013; Kotowska et al. 2014; Shanmugam et al. 2014). Despite the fact that for other NSAIDs many negative effects on aquatic biota are described, e.g. disturbances in hormonal regulation (Han et al. 2010; Fernandes et al. 2011; Ji et al. 2013) and osmotic regulation (Saravanan and Ramesh 2013), damage to DNA (Rocco et al. 2010; Parolini and Binelli 2012), or damage at the organ level (Schwaiger et al. 2004), almost no articles are available on the effects of salicylic acid on aquatic organisms. The aim of this study was to investigate the influence of sub-chronic exposure to salicylic acid on growth, histopathological changes, and oxidative stress in juvenile zebrafish *Danio rerio*.

Materials and Methods

A sub-chronic growth toxicity test was performed on juvenile zebrafish (30 days old, average weight 14.3 g) according to OECD (Organisation for Economic Cooperation and Development) guideline No. 215 (fish, juvenile growth test). All experiments were approved by the ethical committee of the University of Veterinary and Pharmaceutical Sciences Brno, Czech Republic and complied with national guidelines for animal care. The salicylic acid (p.a. purity, Sigma-Aldrich, St. Louis, MO) concentrations chosen for the testing were 0.004 mg/L (the reported environmental concentration, Ternes 1998) and 0.04, 0.4, 4 and 40 mg/L. Salicylic acid was dissolved in water using an ultrasound device. The control group was exposed to dilution water only. Each concentration was tested in duplicate (at the same time, same room, with the same solution; this arrangement was done only to avoid loss of all fish from a certain concentration in case of any technical difficulties); the number of fish in each aquarium was 50. The duration of the test was 28 days with the tested solution changed continuously (flow-through system). The temperature, pH, and oxygen saturation were recorded daily. Fish were fed with dried *Artemia salina* without nutshells in the amount of 8 % of their body weight per day; the food ration was based on initial fish weight and was recalculated after 14 days. All validation criteria for the test (that mortality in the control group was under 10 %, that oxygen saturation in the solution was above 60 %, that the concentration of the tested substance was more than 80 % of the nominal value, and that the weight gain in the control group was at least 50 % of the initial weight) were fulfilled. At the end of the test, fish were euthanized with an overdose of the anaesthetic MS 222 (250 mg/L) and specimens were collected for further analyses.

The values of the basic physical and chemical parameters of the tap water (dilution water) used in the tests were as follows: acid neutralization capacity ($ANC_{4.5}$) – 1.0–1.3 mmol/L; chemical oxygen demand (COD_{Mn}) – 1.2–1.5 mg/L; total ammonia – below the limit of determination (<0.04 mg/L); nitrates – 12.5–13.7 mg/L; nitrites – below the limit of determination (<0.01 mg/L); Cl^- – 15.5–17.0 mg/L; and Σ Ca + Mg – 3.1 mmol/L. The water temperature was 25 ± 1°C; and pH was between 7.5 and 8.2.

The tank-average specific growth rate (SGR) was calculated for each experimental group for the period from day 0 (prior to the beginning of the test, after acclimatization) to the completion of the test (day 28) according to the following equation:

$$\mathrm{SGR} = \frac{\overline{\log \mathrm{e}\ \mathrm{w}_2} - \overline{\log \mathrm{e}\ \mathrm{w}_1}}{\mathrm{t}_2 - \mathrm{t}_1} \times 100,$$

where w_1 and w_2 are the weights of a particular fish at time t_1 and t_2, respectively; t_1 is the first sampling time and t_2 is the final sampling time; and $\overline{\log \mathrm{e}\ \mathrm{w1/w2}}$ is the average of the logarithms of the values w1/w2 for the fish in the tank at the start/end of the study period.

At the end of the test, samples for histopathological examination were taken randomly from all tested groups and the control group (altogether 60 fish; 10 fish from each concentration, 5 fish per each duplicate aquarium). The fish were euthanized, prepared for histological examination, fixed in buffered 10 % neutral formalin, dehydrated, embedded in paraffin wax, sectioned (in cross sections) on a microtome at 4 μm, and stained with haematoxylin and eosin. The histopathology of skin, gill and liver was examined by light microscopy.

Samples for the evaluation of oxidative stress parameters were taken randomly from all tested groups and the control group (altogether 180 fish; 30 fish from each concentration, 15 fish per each duplicate aquarium) at the end of the test. Mixed samples (usually 3 fish from the same tested concentration per one sample, approximate total weight 300 mg) were prepared to obtain a sufficient amount of specimen for the analyses. Whole fish bodies were mixed with phosphate buffer (pH 7.2) and homogenized. Lipid peroxidation was assessed in the pure homogenate using a modified thiobarbituric acid reactive substances (TBARS) method (Lushchak et al. 2005). Then,

the rest of the homogenate was centrifuged (11,000 g, 4°C, 20 min) and the supernatant was used to determine enzymatic activities. Catalytic concentrations of glutathione-*S*-transferase (GST), glutathione peroxidase (GPx), glutathione reductase (GR), and catalase (CAT) were measured spectrophotometrically using Varioskan Flash Spectral Scanning Multimode Reader (Thermo Fisher Scientific Inc., USA) and Uvikon XS (Secomam, France) (Habig et al. 1974; Carlberg and Mannervik 1975; Aebi 1984; Flohe and Gunzler 1984).

Samples of the tested solutions were taken once a week directly from the fish tanks and the concentrations of salicylic acid were determined. The analysis was performed using high-performance liquid chromatography (HPLC, Alliance 2695 chromatographic system, Waters, Milford, MA) with fluorescence detection (FD 2475 fluorescence detector, Waters, Milford, MA) at excitation and emission wavelengths of 290 nm and 400 nm, respectively. The water samples were filtered through a 0.45 μm nylon filter (Millipore, Billerica, MA) before HPLC analysis. The sample volume injected into the HPLC system was 10 μL. Isocratic elution on a 150 × 4.6 mm, 5 μm Zorbax Eclipse XBD-C18 column (Agilent Technologies, Santa Clara, CA) with water/acetonitrile 30/70 (v/v) at a flow rate of 1 mL/min and at a temperature of 35°C was employed. Salicylic acid p.a. was purchased from Sigma-Aldrich (St. Louis, MO). All solvents were HPLC-grade purity (Chromservis, s.r.o., CZ). The detection limit for salicylic acid was 0.9 ng/mL, the limit of quantification was 3.0 ng/mL, and the coefficient of variation was 2.8 %.

Statistical analysis was performed using Unistat 5.6 for Excel software (Unistat Ltd., UK). Testing for the normality of all parameters of oxidative stress was done using the Shapiro–Wilk test and the homogeneity of variances across groups was assessed by Levene's test. Then, one-way ANOVA was performed and individual differences between the means were tested using Dunnett's test with $P < 0.01$ and $P < 0.05$ chosen as the levels of significance. Data on morphometric parameters did not exhibit a normal distribution and were thus subjected to Kruskal–Wallis ANOVA. Individual differences between experimental groups and control were tested using Dunn's test.

Results and Discussion

Salicylic acid is a common contaminant of the water environment. Its solubility (and thus toxicity) is temperature dependent, it dissolves better at higher water temperature (Delgado 2007). At the inlets to STPs in the Czech Republic, concentrations of SA ranging from 20.2 to 51.9 ng/L were detected (Lacina et al. 2013); in Germany, concentrations of up to 54 μg/L were detected. At the outlets from STPs in Greece and Spain, concentrations of SA of up to 13 μg/L were measured, demonstrating inefficiency of STP processes in eliminating this pharmaceutical from waste water (Heberer 2002). An older study by Ternes (1998) found concentrations of SA in rivers and streams of up to 4 μg/L; more recent studies show that environmental concentrations of SA vary in European streams: only around 10 ng/L in Spanish water sources (Gros et al. 2010), 0–143 ng/L in the Czech Republic (Lacina et al. 2013), and from 200 to 500 ng/L in Poland at locations near bigger cities (Baranowska and Kowalski 2012). Despite the fact that SA often appears in water sources, studies on the effects of salicylic acid on aquatic organisms are lacking.

In the presented study, the effects of SA on juvenile zebrafish were investigated. It was revealed that salicylic acid did not influence the growth of juvenile zebrafish at any of the tested concentrations and no statistically significant differences were found in specific growth rate between the control and tested groups. SGR ranged from 5.203 to 5.638. No histological changes in juvenile zebrafish tissues were found at any of the tested SA concentrations.

No changes in the activity of glutathione reductase were found at any of the tested salicylic acid concentrations (data not presented). On the other hand, GPx activity exhibited a slight tendency to increase up to the tested concentration of 0.04 mg/L; then it returned to its basal value. A statistically significant difference ($P < 0.05$) was found only between the group exposed to 0.04 mg/L of salicylic acid and control (Fig. 1). Catalase activity exhibited a slight tendency to increase up to the tested concentration of 4 mg/L; then, at the highest tested concentration, it returned to its basal value. A statistically significant difference ($P < 0.05$) was found between the group exposed to 0.04 mg/L of salicylic acid and control, and a highly statistically significant difference ($P < 0.01$) was found between the group exposed to 4 mg/L of salicylic acid and control (Fig. 2). Similarly to GPx, activity of the detoxification enzyme GST exhibited a slight tendency to increase up to the tested concentration of 0.04 mg/L and then it returned to its basal value. A statistically significant difference ($P < 0.05$) was found between the group exposed to 0.004 mg/L of salicylic acid and control, and a highly statistically significant difference ($P < 0.01$) was found between the group exposed to 0.04 mg/L of salicylic acid and control (Fig. 3).

Tendencies to increase and then drop at the highest concentration were found also in TBARS, but a highly statistically significant increase ($P < 0.01$) was found only in the group exposed to 4 mg/L of salicylic acid compared to control (Fig. 4). As the above mentioned tendencies were not statistically significant, and the value of a significantly different concentration is extremely far from the

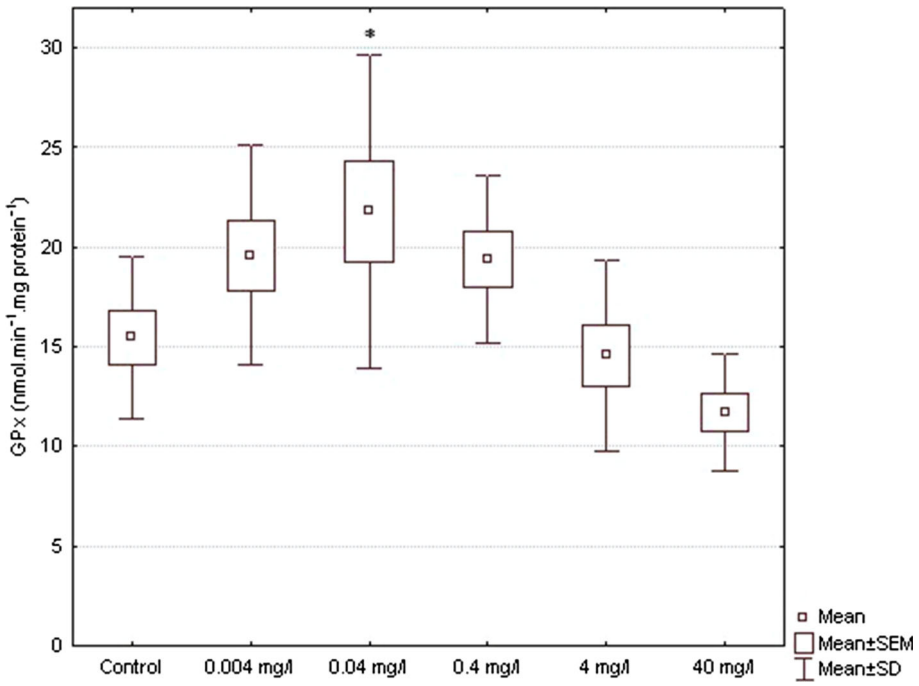

Fig. 1 GPx – glutathione peroxidase in juvenile zebrafish after 28 days of exposure to salicylic acid. Significant differences between tested groups and control are indicated by *($P < 0.05$) and **($P < 0.01$)

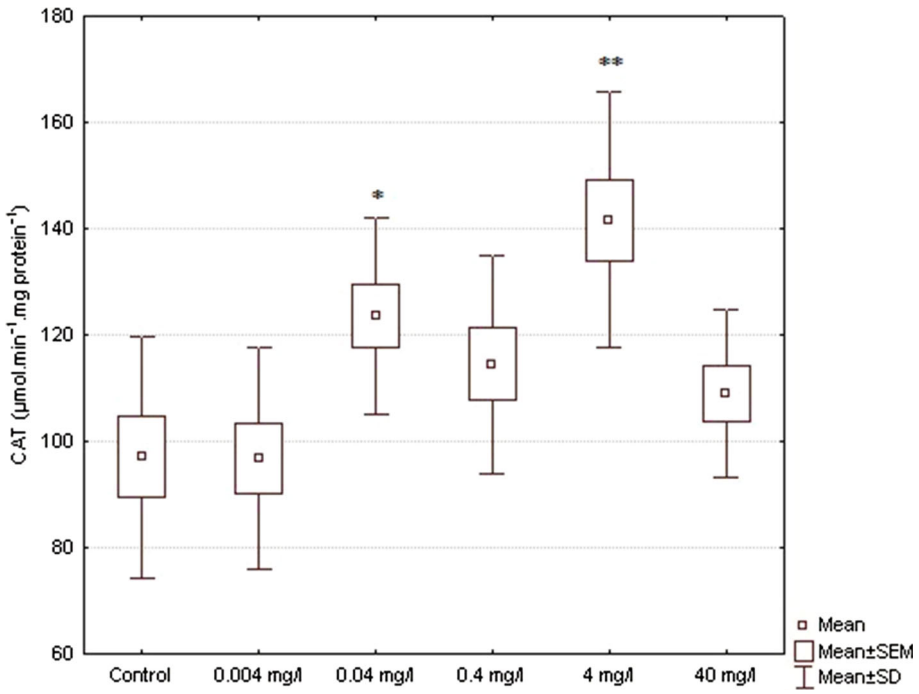

Fig. 2 CAT – catalase in juvenile zebrafish after 28 days of exposure to salicylic acid. Significant differences between tested groups and control are indicated by *($P < 0.05$) and **($P < 0.01$)

others and with high SD and SEM, we suppose this isolated significant change to be only an accidental finding. This may be supported also by the fact that in this concentration enzyme activities except CAT were similar to those in 40 mg/L concentration group, so we did not find any real basis for that accidental hike in lipid peroxidation at 4 mg/L. In our opinion, SA has no effect on lipid peroxidation in juvenile zebrafish.

The increase in the activities of antioxidant enzymes GPx and CAT responsible for peroxide degradation and in the activity of the detoxification enzyme GST in low tested concentrations indicates an activation of a defence system against xenobiotic presence and xenobiotic induced peroxide production in juvenile zebrafish. Their increased activity may be connected also with a slight, even though non-significant decrease in TBARS. No oxidative damage

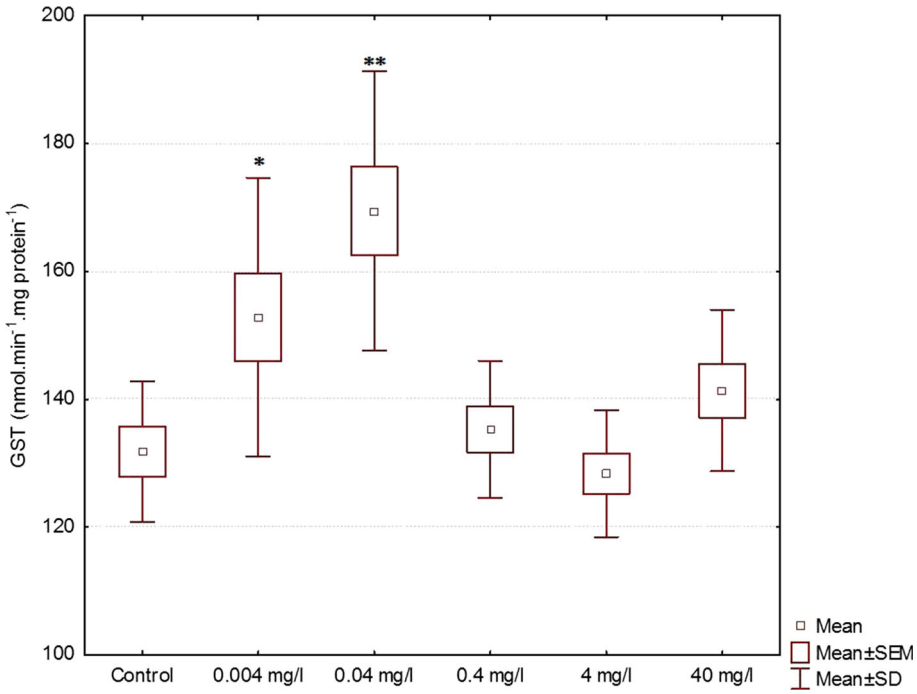

Fig. 3 GST – glutathione-S-transferase in juvenile zebrafish after 28 days of exposure to salicylic acid. Significant differences between tested groups and control are indicated by *($P < 0.05$) and **($P < 0.01$)

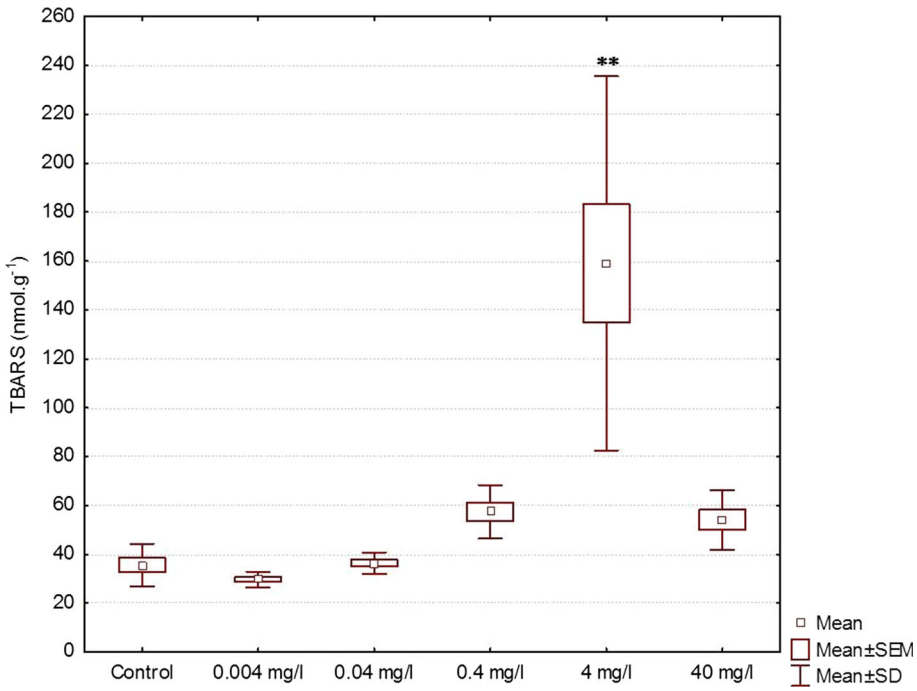

Fig. 4 TBARS – thiobarbituric acid reactive substances in juvenile zebrafish after 28 days of exposure to salicylic acid. Significant differences between tested groups and control are indicated by *($P < 0.05$) and **($P < 0.01$)

to lipids in low SA concentrations reveals the efficiency of the antioxidant and degradation system activation. Return to the basal values found in enzyme activities in the highest or higher concentrations of SA groups may indicate a down-regulation/inhibition of the antioxidant defense system known e.g. from high dose metal or pesticide exposure (Qu et al. 2014; Blahova et al. 2013 respectively). Due to the fact that TBARS were not increased in the highest concentration and thus lipids were not damaged by reactive oxygen species (ROS), decrease in ROS production and no need for the increased activity of antioxidant enzymes may play a role. Some studies show that salicylic acid inhibits the mitochondrial enzyme ferrochelatase, which is important for haem synthesis. Haem is a prosthetic group in many haemoproteins, including cytochromoxidases, which are responsible for cellular breathing and are connected to

reactive oxygen species production. Ferrochelatase inhibition is considered to participate in the decrease of ROS production and anti-inflammatory effect of SA (Gupta et al. 2013). Thus, the exposure of fish with a sufficiently well-developed enzymatic system to SA may not cause a significant increase in ROS production. As the system is otherwise balanced, minor changes are efficiently compensated and the activities of the enzymes of antioxidant defence do not change much, meaning that there is no gross damage and no changes to the health status of such fish.

The results of the present study indicate that the concentrations of SA used in the test induced no changes in histopathological or growth parameters, and only slight changes in some of the oxidative stress biomarkers in juvenile zebrafish. Nunes et al. (2015), who tested SA (concentrations 25–100 μg/L, 30 days) in the juvenile brown trout *Salmo trutta fario*, found only a mild increase in GPx and GR, while the activities of CAT and GST, and the concentration of TBARS were not influenced by the tested substance. These results are similar to those obtained from a growth test on zebrafish performed with acetylsalicylic acid (ASA) (Zivna et al. 2013). However, in contrast to the above mentioned test with ASA, glutathione reductase was not influenced in the present study, and GST activity increased mainly at the lower tested concentrations of SA. Similarly, while ASA decreases TBARS significantly (Zivna et al. 2013), no effect on lipid peroxidation was found in our study. Compared to ASA, salicylic acid appears to have a lower ability to influence oxidative stress parameters in the juvenile stages of fish of different species.

In contrast, another study that tested the effects of SA on the embryos and larvae of common carp *Cyprinus carpio* (Zivna et al. 2015) provided completely different results regarding the toxicity of SA to aquatic animals. Salicylic acid caused a slight decrease in GPx and significant decreases in GR and CAT. The concentration of TBARS was increased. GST, meanwhile, remained unchanged. Changes in most of the parameters were not concentration dependent; thus, simply the presence of salicylic acid itself in the environment had a negative effect on the tested fish. The mechanism underlying the GR decrease is unknown, but CAT, as a haemoprotein, can be decreased by the action of SA on ferrochelatase (Gupta et al. 2013). Thus, these results indicate that early life stages of common carp do not have a sufficient compensatory mechanism to defend themselves against the effects of SA. In particular, their inefficiency in increasing the conjugation with glutathione, which is the main detoxification pathway in fish, may lead to the decreased elimination and increased toxicity of SA in these fish. Moreover, the inability to convert inefficient oxidized glutathione to efficient reduced glutathione because of GR inhibition causes lipid peroxidation (increased TBARS) and membrane damage to these organisms.

Differences in the toxicity of SA between the developmental stages of fish can be explained by the lower enzymatic capacity in early life stages compared to juveniles, and also by the differences in absorption capacity between these stages – the presence of intestines and gills in quite developed juveniles compared to the chorion sheath of embryos, and the very vulnerable skin and gills of sac fry and larval stages (VanLeeuwen et al. 1985; Gellert and Heinrichsdorff 2001). In early life stages, the keratolytic effect of SA is probably pronounced. While chorion is impermeable to many substances, fish just after hatching are very sensitive to external damage. Zivna et al. (2015) described changes in growth around the 6th day of life and histology confirmed skin erosions in SA-exposed early life stages of fish. This may indicate increased absorption of SA into the bodies of sac-fry and larvae through the damaged skin barrier, and the increased influence of SA on the health of such early life stages of fish.

According to the available literature, salicylic acid has not been tested on other aquatic animals either, such as crustaceans, despite the fact that acetylsalicylic acid influences oxidative stress in *Daphnia magna* (Gomez-Olivan et al. 2014a) and *Hyalella azteca* (Gomez-Olivan et al. 2014b) significantly.

In contrast to early life stages of fish, juveniles (zebrafish and brown trout) turned out to be less sensitive to both environmentally relevant and higher concentrations of salicylic acid. Thus, to evaluate the real threat to aquatic organisms represented by SA residues in water, the use of other, preferably wild species, early developmental stages, zooplankton, and water molluscs should be considered in further testing. It is also important to mention that in the real environment, SA does not occur in water only as a result of a direct contamination with pure SA, but it is a metabolic and degradation product of the massively used acetylsalicylic acid as well (Needs and Brooks 1985; Heberer 2002). Thus, both drugs are often present in water in different ratios (Heberer 2002; Gros et al. 2010; Baranowska and Kowalski 2012; Lacina et al. 2013) and as both of them may influence aquatic organisms (Zivna et al. 2013; Gomez-Olivan et al. 2014a, b, Zivna et al. 2015), their possible combined effect should be taken into consideration and evaluated.

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

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