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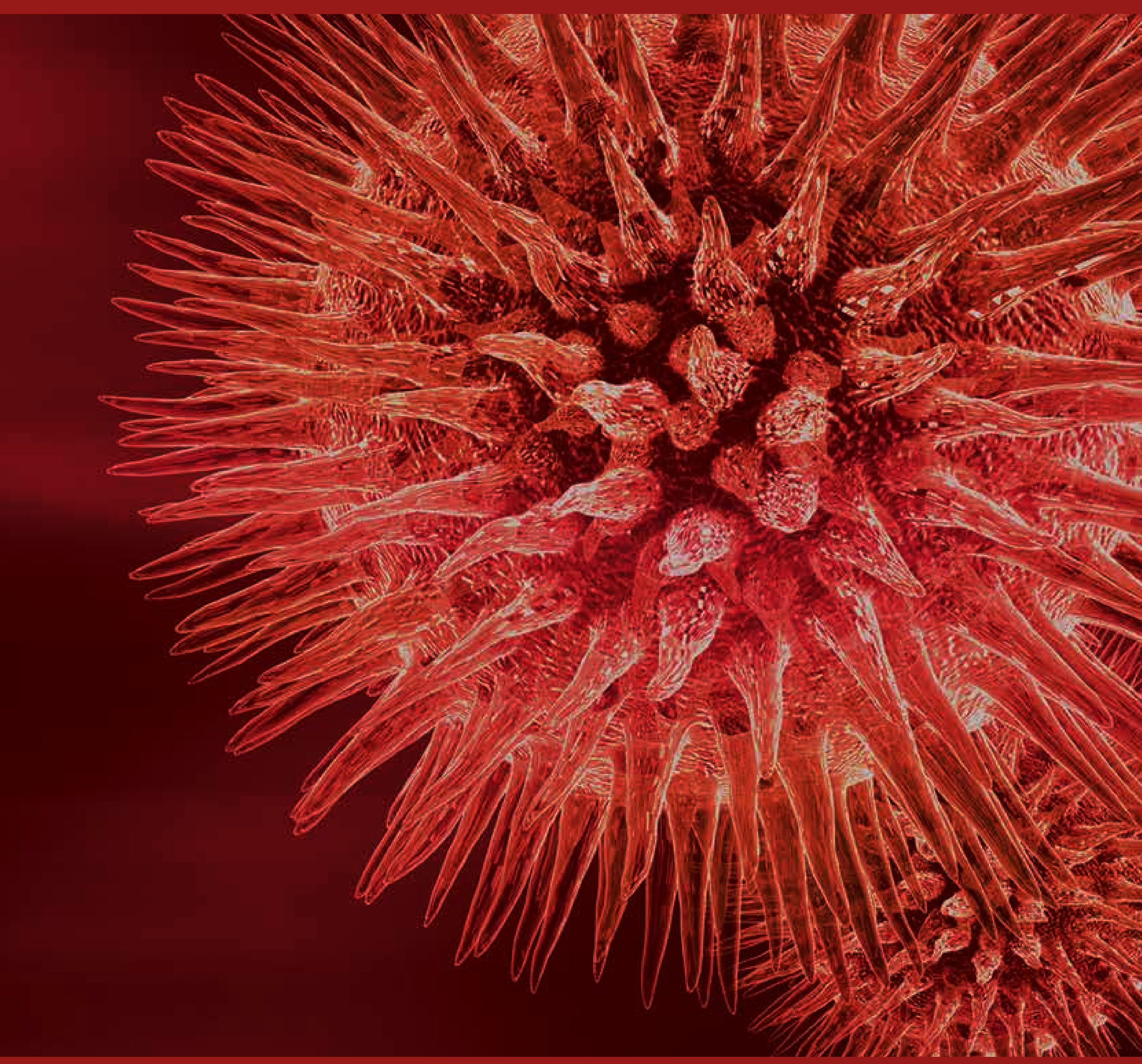
Fish and Crayfish Toxicology

Guest Editors: Josef Velíšek, Zdenka Svobodova, Antonín Kouba, and Zhi-Hua Li



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Editorial

Fish and Crayfish Toxicology

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Pollution of the environment and its protection have become increasingly to the forefront of mankind's concerns. Aquatic ecosystems are exposed to a continual inflow of pollutants of both natural and anthropogenic origin. These substances can in many cases result in negative changes in water quality. Water-inhabiting organisms constitute one of the essential components of the ecosystem. Fish and crayfish are a very important part of the aquatic ecosystem and simultaneously are also important economic organisms for human consumption. Over the last 50 years, there have been significant developments in the field of aquatic toxicology. The subject of aquatic toxicology is research and estimation of the effect of xenobiotic on aquatic ecosystem and organisms living there. This issue on fish and crayfish toxicology represents recent results of several studies, mostly carried out on fish. Laboratories from United States, China, and Czech Republic submitted 16 papers. Altogether, 7 original papers and 1 review were accepted after the reviewing process. The following briefly summarizes highlights of the published papers.

Phytoestrogens. Two papers with emphasis on effects of phytoestrogens on fish have been accepted. In the first paper, A. C. Brown et al. evaluated effects of phytoestrogens produced by plants on female Siamese fighting fish (*Betta splendens*). In this study, authors found no effects of phytoestrogens in natural concentration ($1\,\mu g\,L^{-1}$) of β-sitosterol and genistein on steroids and gonads. Effect of phytoestrogens on behavior was only detected in higher concentrations of $1000\,\mu g\,L^{-1}$. In the second study, E. D. Clotfelter and H. K. Gendelman evaluated effects of phytoestrogens on male reproductive function of Siamese fighting fish. Their observation suggests that acute exposure to waterborne phytoestrogens during activation does not reduce the motility of fish sperm.

Effects of Xenobiotics on Fish. Three papers with emphasis on effects of xenobiotic on fish have been accepted. DEET (N,N-diethyl-m-toluamide), the most common active ingredient in insect repellents, as described by A. Slaninova et al., was shown to affect the hematological, biochemical, and oxidative stress parameters in common carp (*Cyprinus carpio* L.) at a concentration of $1\,mg\,L^{-1}$. A study by M. Bartoskova et al. investigated the effects of fluoroquinolone norfloxacin (antibacterial agents) on selected oxidative stress parameters in zebrafish (*Danio rerio*). From their results, we can conclude that norfloxacin has a negative impact on specific biochemical processes connected with the production of reactive oxygen species in tested fish. The third study by V. Stancova et al. examined the long term effect of a mixture of ibuprofen, diclofenac, and carbamazepine on early life stages of tench (*Tinca tinca*). Exposure to the mixture of pharmaceuticals at a concentration of $60\,\mu g\,L^{-1}$ for each substance caused an increase in mortality as well as increase in growth, elevated incidence of malformations, and histopathological changes of liver, kidney, skin, and gill.

However, environmentally relevant concentrations (0.02 and $0.2\,\mu g\,L^{-1}$) used in this experiment did not result in toxic impairment of early life stage of tench.

Mycotoxins. One important paper with emphasis on effects of deoxynivalenol (DON), produced by the *Fusarium* genus, a major contaminant of cereal grains, on rainbow trout (*Oncorhynchus mykiss*), was accepted. DON in a dose of $2\,mg\,kg^{-1}$ feed, as described by I. Matejova et al., was shown to affect hematological, biochemical, and histopathological parameters in this fish.

Fish Nutrition. Y. Zhu et al. conducted experiments for the detection of effects of different diets on the growth performance and hypoxia adaptation capacity of Mississippi paddlefish (*Polyodon spathula*) larvae. The larval paddlefish fed with an appropriate proportion of live food and formulated diets exhibited improved adaptive capacity to hypoxia.

Fish as Model Organisms. One review by S. Dong et al. dealt with attempts to find the most suitable environmentally relevant fish model for toxicity testing. This review provides a literature survey highlighting the steady increase of ecotoxicological research on marine medaka (*Oryzias melastigma*), summarizes the advantages of using marine medaka as a tool for toxicological research, and promotes the utilization of this fish in future studies.

We hope that this issue will attract interest from many people, including scientists, university teachers, and students studying aquatic toxicology.

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Research Article

Growth Performance and Stress Responses of Larval Mississippi Paddlefish *Polyodon spathula* to Hypoxia under Different Diet Treatments

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A growth trial was conducted to detect the effects of different diets on the growth performance and hypoxia adaptation capacity of Mississippi Paddlefish (*Polyodon spathula*) larvae. The larvae were fed with live food, formulated diets, and 1/2 live food with 1/2 formulated diets. After a 15-d growth trial, final body weight and total body length were measured, and five larvae from each dietary group were subjected to 1 h of hypoxia treatment. Serum total antioxidant capacity (T-AOC), serum superoxide dismutase (SOD), and liver malondialdehyde (MDA) were measured. Final body weight and weight gain of the fish fed live food were significantly higher than the values for the other two groups. Total body length of the fish fed live food and 1/2 live food with 1/2 formulated diets exhibited no significant difference. After hypoxia treatment, serum T-AOC and SOD activities of the fish fed formulated diets were significantly lower than those of the other two groups. Liver MDA content of the fish fed with live food was significantly higher than that of the other two groups. In conclusion, larval paddlefish fed with an appropriate proportion of live food and formulated diets exhibit improved adaptive capacity to hypoxia.

1. Introduction

The paddlefish, *Polyodon spathula*, is a freshwater chondrostean fish that belongs to the suborder Acipenseroidei (order Acipenseriformes) [1]. This North American relict species is indigenous only to the waters of the Mississippi-Missouri River system and neighboring coastal drainages that flow into the Gulf of Mexico [2]. The paddlefish was first introduced to China in 1988. The high prices for paddlefish roe and flesh have resulted in the recent development of paddlefish aquaculture. Paddlefish primarily feed on zooplankton and occasionally consume small insects, insect larvae, and small fish [3]. Moreover, paddlefish are ram suspension filter-feeders [4]. These features make appropriate food resource a bottleneck in the paddlefish aquaculture, especially in the early life stage. Diet items of larval and juvenile paddlefish include zooplankton and all stages of aquatic insects [3]. Larval paddlefish reared under laboratory conditions readily accept natural prey items (live and frozen), as well as commercially prepared powdered diets [5]. However, according to Chinese farmers, larvae with a total body length of less than 110 mm are difficult to wean to formulated diets. Moreover, the survival and growth rates of such fish were significantly lower than those of fish fed with live zooplanktons.

A variety of environmental factors, such as temperature and oxygen availability, can significantly affect fish metabolism, which is extremely important during fish larvae transportation [6]. Two possible responses to ambient oxygen level reportedly exist. In one approach, oxygen regulators adjust their ventilation rates to compensate for changing oxygen levels and to maintain a constant respiration rate and, thus, aerobic metabolism [7]. In the other approach, oxygen conformers enable a decline in respiration rates as environmental oxygen decreases, with concomitant reductions in metabolic rate and aerobic metabolism [7]. Paddlefish are oxygen regulators and maintain constant respiration rates from 150 mm Hg to 90 mm Hg [8]. Tolerance to hypoxia can be regulated by certain diet components, such as n-3 HUFA [9] and vitamin E [10].

Reactive oxygen species (ROS) is as a natural byproduct of normal oxygen metabolism that includes superoxide anion radical, hydrogen peroxide, and highly potent hydroxyl radical [11, 12]. Free radicals, such as superoxide anion radical and hydroxyl radical, with unpaired electrons on an otherwise open shell configuration, such as superoxide anion radical and hydroxyl radical, are usually highly reactive because they are likely to participate in chemical reactions [13]. The cytotoxic effects of ROS include membrane lipid peroxidation, redox balance alteration, enzyme inactivation, and DNA damage [14, 15]. Consequently, aerobic organisms have evolved effective defense systems against oxidative damage [16]. Such defense systems consist of both hydrophilic and lipophilic antioxidant compounds or scavengers and specific antioxidant enzymes, including serum superoxide dismutase (SOD), catalase, and glutathione peroxidase [17].

Oxidative stress is a situation in which steady-state ROS concentration is transiently or chronically enhanced. This condition disturbs cellular metabolism and regulation, aside from damaging cellular constituents [18]. Similar to all aerobic organisms, fish are susceptible to ROS attacks and have developed antioxidant defenses, especially adapted enzymes, such as SOD, catalase, and glutathione peroxidase [19]. Changes in antioxidant enzymes and their protective mechanisms are early indicators of cellular susceptibility to oxidant injury caused by ROS [20].

This study used different diet combination to investigate the possibility of weaning larval paddlefish with body length of approximately 55 mm to commercial formulated diets. We hypothesized that larval paddlefish fed with different diets would show different responses to hypoxia.

2. Materials and Methods

2.1. Animals, Diets, and 15-Day Growth Trial. Mississippi Paddlefish (*P. spathula*) larvae were acquired from Sturgeon Culture Base of Huazhong Agricultural University, MaCheng city, Hubei province, China. When the larvae were hatched, they were transferred to laboratory in oxygenated plastic bags. *P. spathula* ($n = 270$), with a mean ± SE initial mass of 1.14 ± 0.03 g, were randomly distributed to nine tanks with 30 fish per tank, with no significant differences in mean mass among the groups. The larval paddlefish were maintained indoors in 397 L tanks (diameter: 80 cm, height: 70 cm) with a continuous supply of biofiltered freshwater at the Experimental Aquaculture Center in Huazhong Agricultural University from late April to early May. The larvae were exposed to a natural photoperiod of approximately 12 : 12 L : D. The temperature throughout the experimental period was between 24 and 24.8°C, whereas the water flow velocity was approximately 729 mL/min. Actually, the water flow velocity into each tank cannot be exactly the same every day. But we could make the water flow velocity close among the tanks by manually adjusting the water faucet. Total ammonia-nitrogen [(NH_4^+ + NH_3)-N] was always maintained below 0.5 mg/L, and the pH value was about 8.07. Residual chlorine was determined weekly, and levels were consistently below 0.05 mg/L. Dissolved oxygen during the growth trial was maintained at approximately 8.5 mg/L.

Two fish feed were used in the growth trial. One feed was live zooplanktons, mainly cladocera and rotifera, captured from South Lake every morning. The proportions of cladocera, rotifera, and others in our water samples were about 15%, 80%, and 5% by number during the period of our experiment. The other feed was commercially formulated diets for first-feeding fish larvae. The feed we used was brought from the most famous larvae formulated feeds company in China and it was proved to be a good live food replacement for sturgeon larvae (S1 number microparticles of Shengsuo Commercial Formulated Feed, Shandong, China; diameter: 150 μm to 250 μm). The diet composition provided by the feed company was crude protein = 51.6%, crude lipid = 10.2%, ash = 15.2%, calcium = 1.60%, total phosphorus = 1.55%, and moisture = 11.2%. Three feeding strategies were adopted in the 15-day growth trial. The first treatment group was fed completely with live zooplanktons, the second treatment group was fed completely with formulated diets, and the last treatment group was fed with a combination of nearly half live zooplanktons and half formulated diets by biomass. In order to make half live zooplanktons and half formulated diets, we calculated the numbers of cladocera and rotifer (dominated in water samples by biomass) in 10 L water samples first and then collected the zooplanktons from 10 L water samples by filtering the water samples with 45 μm membrane filter, weighted wet weight and dry weight of the filtered zooplanktons. Using the weight and number of zooplanktons of 10 L water samples, after we checked the numbers of cladocera and rotifer of every day's water samples, we then knew how many zooplanktons we should give to the fish comparable to half amount of formulated diets. Each treatment had three replicates, and the diets were fed to the larvae four times a day to satiation (8:00, 12:00, 15:00, and 18:00). Excess feed and feces were collected every morning before feeding (siphoning), and dead larvae were removed and counted twice a day.

Initial body weight and body length data were obtained at the beginning of the growth trial. After the growth trial, five fish were randomly selected from each tank and transferred to another set of tanks (with similar tank size used in growth trial) for future hypoxia treatment. All of the other remaining fish were starved for 24 h and anesthetized with MS-222 (100 mg L^{-1}). The fish were then counted, bulk-weighed, and measured (body length) to determine the growth performance parameters.

2.2. Hypoxia Treatment. After a 15-day of feeding, five fish from each diet treatment were subjected to low oxygen treatment. Sodium sulfite anhydrous was added into the three tanks with 100 L of experimental water using a dissolved oxygen meter (HQ40d; HACH, USA). After several hours of monitoring and adjustment, the dissolved oxygen concentration in each tank changed from 8.5 mg/L to 3.33 mg/L–3.46 mg/L. The fish were then placed into the tanks and left to stand for 1 h.

After hypoxia treatment, fish were anesthetized by placing them immediately in a concentration ($100\,mg\,L^{-1}$) of MS-222. The five fish were then weighed and measured (body length). For serum samples preparation, blood was withdrawn from the caudal vein into plastic Eppendorf tubes using sterilized syringes and mixed and kept at 4°C for 1 h and then centrifuged (4°C, 3000 g for 10 min). The supernatants were collected as the serum samples and frozen at −20°C for subsequent analysis. Liver samples were dissected from fish, the blood samples if which were obtained. These fish were frozen immediately in liquid nitrogen and stored at −80°C until use. Measurements of enzymatic activities of total antioxidant capacity (T-AOC), SOD, and malondialdehyde (MDA) contents were determined spectrophotometrically using the corresponding kits (Nanjin Jiancheng Bioengineering Institute, Nanjin, China) and following the manufacturer's protocol.

2.3. Statistical Analysis. Data are reported as means ± SE. All data means were compared using Duncan's multiple range test after the homogeneity of variances was tested (Statistical Package Social Science, SPSS, version 16.0). ANOVA was performed for the statistical analyses. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Growth Performance of Larval Paddlefish. Table 1 presents the growth performance of larval paddlefish fed with live food, formulated diets, and 1/2 live food with 1/2 formulated diets.

The final body weight and weight gain of the fish fed with live food were significantly higher than those of the other two groups, whereas no significant difference existed between fish fed with formulated diets and those fed with 1/2 live food with 1/2 formulated diets. The total body length and mortality of the fish fed with live food and 1/2 live food with 1/2 formulated diets exhibited no significant difference. However, these two groups exhibited significantly higher values than the group fed with formulated diets.

3.2. Biochemical Indicators in Serum and Liver. Table 2 presents the serum T-AOC and SOD activities, as well as liver MDA content, of larval paddlefish fed with live food, formulated diets, and 1/2 live food with 1/2 formulated diets.

After hypoxia treatment, serum T-AOC and SOD activities of the fish fed with formulated diets were significantly lower than those of the other two groups, whereas no significant difference was observed between fish fed with live food and those fed with 1/2 live food with 1/2 formulated diets. The liver MDA content of the fish fed live food was significantly higher than that of the other two groups. By contrast, no significant difference was found between fish fed with formulated diets and those fed with 1/2 live food with 1/2 formulated diets.

4. Discussion

First-feeding larvae generally depend on live food. However, live food is difficult to sustain and requires considerable space and expense; formulated diets are easier to maintain [21]. Thus, formulated diet substitution for live food is crucial for reducing production costs and sustaining production of high- and constant-quality juveniles.

Survival rates and growth performance in larvae were indicative of suitable rearing conditions [22]. In this study, the larvae fed formulated diets had the highest mortality, in agreement with previous work [23].

The larvae fed with formulated diets exhibited the lowest growth performance, which agrees well with other findings observed in *Acipenser persicus* [24]. The results are mainly attributed to an incomplete primitive larval digestive system [25]. Moreover, formulated diets are commonly composed of denatured insoluble proteins and carbohydrates [26]. In this study, the relative denseness and hardness of formulated diets may adversely affect the digestibility of formulated food compared with live food.

This study detected serum T-AOC, serum SOD, and liver MDA to determine hypoxia adaptation capacity after feeding different diets.

SOD is an antioxidant enzymatic defense system that is an important biochemical parameter for antioxidant effects; SOD converts the superoxide radical to hydrogen peroxide [27]. T-AOC reflects the overall cellular endogenous antioxidative capability for both enzymatic and nonenzymatic antioxidants [19]. The results indicate that paddlefish fed with live food and those fed with 1/2 live food with 1/2 formulated diets responded to environmental changes more quickly than those fed with formulated diets.

Liver MDA content of the fish fed with live food was significantly higher than that of the other two groups, whereas no significant difference existed between fish fed with formulated diets and those fed with 1/2 live food with 1/2 formulated diets. The analogous observation was also supported by previous studies [28]. The lipid peroxidation level of *Solea senegalensis* larvae fed with live food was higher than that of the larvae fed with inert food.

Lipid peroxidation is one of the main processes induced by oxidative stress. MDA formation is a widely used assay for lipid peroxidation, which represents the final product of lipid peroxidation [19]. MDA concentration provides direct evidence of the toxic processes caused by free radicals, and MDA level is considered a suitable indicator of the extent of lipid peroxidation [29]. In this study, lipid peroxidation was elevated in the liver of paddlefish after exposure to hypoxia, as evidenced by increased MDA production. This result suggests the participation of free radical-induced oxidative cell injury in mediating hypoxia. Consequently, lipid peroxidation cannot be prevented despite the induction of T-AOC and SOD activities in the group of fish fed with live food. However, the decrease in the MDA level in the group of fish fed with 1/2 live food with 1/2 formulated diets may be an indicator of an increase in the enzymatic and nonenzymatic antioxidants of defense mechanisms.

Table 1: Growth performance of Mississippi paddlefish (*Polyodon spathula*) under different dietary treatments (mean ± SE).

Treatment	IBW (g)	FBW (g)	BL (cm)	WG (%)[1]	Mortality (%)
Initial			5.54 ± 0.07^{a}		
Live food	1.14 ± 0.02	5.67 ± 0.11^{a}	11.84 ± 0.50^{b}	435.31 ± 11.14^{a}	15.56 ± 2.94^{a}
1/2 Live food + 1/2 formulated food	1.14 ± 0.03	4.33 ± 0.18^{b}	11.48 ± 0.33^{b}	303.05 ± 25.06^{b}	26.67 ± 3.85^{a}
Formulated food	1.15 ± 0.03	3.77 ± 0.35^{b}	10.34 ± 0.39^{c}	246.14 ± 29.06^{b}	64.44 ± 7.29^{b}

Note: IBW: initial body weight; FBW: final body weight; BL: body length.
[1]WG (%): weight gain (%) = (final weight − initial weight)/initial weight × 100.
Values with different letters within the same column are significantly different ($P < 0.05$).

Table 2: Adaptive responses of Mississippi paddlefish (*Polyodon spathula*) to hypoxia under different dietary treatments (mean ± SE).

Treatment	Serum T-AOC (U/mL serum)	Serum SOD (U/mL serum)	Liver MDA (nmol/mgprot)
Live food	19.12 ± 4.97^{a}	71.83 ± 7.91^{a}	7.61 ± 1.60^{a}
1/2 Live food + 1/2 formulated food	12.13 ± 1.93^{a}	63.84 ± 5.37^{a}	2.93 ± 0.65^{b}
Formulated food	6.99 ± 2.08^{b}	49.20 ± 3.59^{b}	2.98 ± 0.41^{b}

Note: T-AOC: total antioxidant capacity; SOD: superoxide dismutase; MDA: malondialdehyde.
Values with different letters within the same column are significantly different ($P < 0.05$).

Formulated diets, as nonliving material, may induce low-intensity oxidative stress in paddlefish. Preexposure to low-intensity oxidative stress, regardless of how the stress is induced, may enhance tolerance and result in higher oxidative stress intensity. This phenomenon is called preadaptation or cross-adaptation approach [30]. However, this adaptation has associated metabolic costs, which includes diverting energy from normal metabolic functions to the functions that are used to cope with stress [31]. Except for the difference in the trophic structure between formulated diets and live food, metabolic cost is another reason for the lower growth performance of paddlefish fed with formulated diets than those fed with live food.

In farm production, any diet that reduces the reliance on live food production is of technical and economic interest in rearing larval paddlefish. All these factors indicate that feeding the larvae with a combination of live food and formulated diets is optimal. Other studies also reported that a mixture of live food and formulated diets can be used at first feeding in *Acipenser fulvescens* [32] and *Acipenser persicus* [24]. However, future experiments are needed to determine the proportion of live food and formulated diets more precisely to reach the optimal growth performance and hypoxia adaptation capacity.

5. Conclusion

In conclusion, larval paddlefish fed with an appropriate proportion of live food and formulated diets exhibited improved hypoxia adaptation capacity without affecting growth performance.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Effects of Subchronic Exposure to *N,N*-Diethyl-*m*-toluamide on Selected Biomarkers in Common Carp (*Cyprinus carpio* L.)

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DEET (*N,N*-diethyl-*m*-toluamide) is the most common active ingredient in the insect repellents commonly detected in European groundwater. The aim of this study was to investigate the effect of subchronic DEET exposure on biochemical and haematological parameters, antioxidant enzymes, including catalase, glutathione peroxidase, glutathione reductase, and glutathione S-transferase, and the amount of thiobarbituric acid reactive substances (TBARS) in common carp (*Cyprinus carpio* L.). Two specific proinflammatory and anti-inflammatory cytokine genes were selected to assess an immunological status of the fish. Fish were exposed for 28 days to three concentrations of DEET (1.0 μg/L, 0.1 mg/L, and 1.0 mg/L) where 1 μg/L is corresponding to the concentration found in the environment. DEET had a significant ($P < 0.05$) effect on increased RBC, decreased mean corpuscular volume (MCV), and mean corpuscular haemoglobin value (MCH) compared to control groups in the concentration of 1 mg/L. A significant decline ($P < 0.05$) in triacylglycerols (TAG) in plasma was found in the concentration of 1 mg/L compared to the control groups. The parameters of oxidative stress in tissues of common carp were weekly affected and immunological parameters were not affected.

1. Introduction

DEET (*N,N*-diethyl-*m*-toluamide) is the most common active ingredient in insect repellents used around the world due to its high efficacy against insects and arthropods bites [1–3]. DEET was produced and patented for usage in American military by the US government and registered for the general population in the 1950s [4, 5].

WHO and subsequently the US Environmental Protection Agency decided that an application of DEET-containing repellents in compliance with the instruction guidelines does not pose a health risk [6].

Behavioral and electrophysiological studies have demonstrated DEET interactions with antennal olfactory as well as gustatory receptors in insect [7–9]. Ditzen et al. [10] described DEET-dependent blockade of electrophysiological responses of olfactory sensory neurons to attractive odors in *Anopheles gambiae* and *Drosophila melanogaster*.

In addition, DEET inhibits insect acetylcholinesterase (AChE) [11] resulting in the accumulation of AChE in the synaptic cleft, which leads to a continuous stimulation of the postsynaptic neuron, finally causing the disruption of the transmission of the nerve impulse [12]. Moreover, it is unknown if the inhibition of the AChE is related to the repellency potential of DEET [11, 13, 14].

DEET is a mobile and persistent chemical which is commonly detected in aquatic environment around the world. Presence of DEET has been studied and monitored in various

aquatic environments, such as drinking water, streams, open seawater, effluents from sewage plant, groundwater, treated effluent, and even drinking water treated with conventional water-treatment systems [5, 15–18]. Costanzo et al. [5] state that the concentrations of DEET in aqueous samples are ranging from 40 to 3000 ng/L worldwide, while the acute toxic concentrations for aquatic species vary between 4 and 388 mg/L [19].

The aim of this study was to assess the subchronic influence of DEET-containing formulation on common carp (*C. carpio*) through biometric, biochemical, and haematological parameters, oxidative stress markers, and selected immunological indices. The lowest tested concentrations of DEET responded to the environmental concentration.

2. Material and Methods

2.1. Experimental Design. The test was performed using two-year-old common carps (*C. carpio*) with average weight 277.1 ± 42.6 g. After one month of acclimatization to experimental conditions (light/dark: 12/12 h, a flow-through system), the fish were randomly distributed into ten tanks (volume 200 L). Three concentrations of DEET (1.0 μg/L, 0.1 mg/L, and 1.0 mg/L) and two control groups were tested: one control with dilution dechlorinated water only and the second control with dilution dechlorinated water and solvent dimethyl sulfoxide (DMSO) in concentration 5 μL/L). Ten fish in each group were divided into two replicates of five in each.

Concentrations of DEET were prepared from formulation Expedition 100+ (Lifemarque Ltd., UK). This formulation contains 95% of *N,N*-diethyl-*m*-toluamide and 5% of inert components. DMSO solvent was added to the formulation in the amount of 5 μL/L of final solution. The duration of this subchronic toxicity test was 28 days. During the test, the condition of fish was checked twice daily and the temperature, pH, and the oxygen saturation of water were daily recorded. Water temperature in the test was 21–22°C. The dissolved oxygen concentrations were above 80–90% and pH ranged from 7.74 to 8.22. Other water quality parameters were as follows: COD_{Mn} (chemical oxygen demand) 1.4–1.9 mg/L; total ammonia 0.25–0.6 mg/L; NO_3^- 40 mg/L; NO_2^- 0.75–1.25 mg/L; Cl^- 30 mg/L; $Cl^-/N\text{–}NO_2^-$ 78.9–130,4.

The experiment was conducted in a flow-through system, and the test solutions were changed twice a day. The concentrations of DEET did not decrease 80% of original concentrations during the experiment. The fish were fed commercial pellets at total rate of 1.5% body weight twice a day.

At the end of the experiment, individual blood samples were taken by cardiac puncture and heparinized (50 IU per mL of blood). The carps were euthanized and their body weight and length (with/without tail) were recorded. Samples of tissues, such as kidney, gills, brain, and liver (hepatopancreas), were removed and stored at −85°C until analyses.

2.2. Biometric Parameters. Two biometric parameters were calculated: the condition factor (CF) and the hepatosomatic index (HSI). The condition factor of each fish was calculated as CF = (body weight (g)/standard length (cm)^3) × 100. The hepatosomatic index was calculated as HSI = liver weight (g)/body weight (g) × 100.

2.3. Haematological and Biochemical Profile. Haematological values, red blood cells count (RBC), white blood cells count (WBC), packed cell volume (PCV), haemoglobin (Hb), mean corpuscular volume (MCV), mean corpuscular haemoglobin value (MCH), and mean corpuscular haemoglobin concentration (MCHC), were determined according to Svobodová et al. [20]. Biochemical indices in plasma glucose, albumin, total protein, ammonium, lactate dehydrogenase (LDH), triacylglycerols (TAG), cholesterol, total calcium, inorganic phosphorus, lactate, alanine transaminase (ALT), aspartate transaminase (AST), alkaline phosphatase (ALP), and butyrylcholinesterase (ButChE) activities were determined using the biochemical analyzer Konelab 20i and commercial test kits (BioVendor, Czech Republic). To assess the ferric reducing ability of plasma samples (FRAP), the biochemical analyzer Konelab 20i was also used, according to Benzie and Strain [21] supplemented with slight modifications [22].

2.4. Immunological Profile. Samples of head kidney and spleen from 5 fish from 3 groups (control with DMSO and DEET in 1 μg/L and 1 mg/L) were immediately stabilized with RNAlater (Qiagen) and stored at −80°C. Tissue samples free of RNAlater were then lysed in 1 mL of TRI Reagent RT (Molecular Research Center) and homogenized on MagNA Lyser (Roche) with 2.3 mm zirconia/silica beads (BioSpec Products). Total RNA was obtained using combination of 4-bromoanisole and the RNeasy Kit (Qiagen) according to the manufacturer's instruction. Extracted RNA was reversely transcribed with M-MLV reverse transcriptase (200 U) (Invitrogen) and oligo-dT primers at 37°C for 1.5 h and then stored at −20°C. cDNA diluted 5 times (0.5 μL) was used in triplicate reactions in a final volume of 3 μL using the QuantiTect SYBR Green PCR Kit (Qiagen). Primers (10 pmol per reaction) [23] specific for proinflammatory (TNF-α and IL-1β) and anti-inflammatory cytokine genes (TGF-β and IL-10) and for two candidate reference genes (40S and β-actin) used are shown in Table 1. Each run included a control free of template to test the assay reagents for contamination. PCR was performed on the LightCycler 480 (Roche). To test the variation of mRNA expression in samples, RefFinder tool (http://www.leonxie.com/referencegene.php) was used and β-actin candidate reference gene was selected for normalization of expression data in our experiment. The relative expression of a gene of interest (GOI) was calculated according to formula $(1/2^{Ct}$ (GOI))/(1/2Ct (reference gene)) [24].

2.5. An Activity of Detoxifying Enzymes and Values of Oxidative Stress. An activity of detoxifying enzyme (glutathione S-transferase GST) and indices of oxidative stress (glutathione reductase GR, glutathione peroxidase GPx, catalase CAT, and the amount of thiobarbituric acid reactive substances TBARS) were measured in different fish tissues (liver, kidney,

Table 1: Primers used for gene expression by immunological examination of common carp.

Gene	Forward primer (5′-3′)	Reverse primer (5′-3′)
TNF-α	GCTGTCGCTTCACGCTCAA	CCTTGGAAGTGACATTTGCTTTT
IL-1β	AAGGAGGCCAGTGGCTCTGT	CCTGAAGAGGAGGCTGTCA
TGF-β	ACGCTTTATTCCCAACCAAA	GAAATCCTTGCTCTGCCTCA
IL-10	AAGGAGGCCAGTGGCTCTGT	CCTGAAGAAGAGGCTGTCA
β-actin	GCTATGTGGCTCTTGACTTCGA	CCGTCAGGCAGCTCATAGCT

gill, and brain). Tissue samples were weighed and homogenized using phosphate buffer (pH = 7.4). The homogenized samples were divided into two portions: the first one was for measuring of TBARS and the second one was centrifuged (11.000 g, 4°C, 20 min) to obtain supernatant fraction for measurement of GST, GR, GPx, and CAT activities and protein content. The enzyme activities were normalized and expressed per mg of protein content. Protein level was quantified by a spectrophotometric method using bicinchoninic acid [25]. All measurements were determined spectrophotometrically using Varioskan Flash Spectral Scanning Multimode Reader (Thermo Scientific). The GST activity was determined by measuring the conjugation of 1-chloro-2,4-dinitrobenzene with reduced glutathione at 340 nm and the activity was expressed as the nmol of the formed product per min per mg of protein [26]. The GR activity was determined by measuring of NADPH oxidation at 340 nm and expressed as the nmol of NADPH consumption per min per mg of protein [27]. The GPx activity was calculated from the rate of NADPH oxidation by the reaction with GR at 340 nm and expressed as the nmol of NADPH consumption per min per mg of protein [28]. The CAT activity was determined by measuring of H_2O_2 breakdown at 240 nm and it was expressed as the μmol of decomposed H_2O_2 per min per mg of protein [29]. To evaluate the level of lipid peroxidation, the amount of malondialdehyde was measured using the TBARS method at 535 nm and the concentration was expressed as nmol of TBARS per gram of tissue wet weight [30].

2.6. DEET Concentration in Water. The level of DEET in water was determined by gas chromatography with ion trap mass spectrometry. A sample was extracted in cyclohexane (4 mL samples: 4 mL cyclohexane). The separation, identification, and quantification of DEET were carried out using a Varian 450-GC gas chromatograph with 220-MS ion trap mass spectrometer and VF-5 ms (30 m × 0.25 mm) column (Varian, Inc., USA). A 1 μL of sample aliquot extract was injected in splitless mode. The injector temperature was 250°C. The initial oven temperature was set at 50°C for 1 min, increased in a rate of 30°C min^{-1} to 130°C for 1 min, increased in a rate of 16°C min^{-1} to 230°C, held for 1 min, increased in a rate of 60°C min^{-1} to 280°C, and held for 1 min. Total run time was 13.75 min. Certified standard DEET was purchased from (Sigma Aldrich, Co.). All solvents were GC/MS-grade purity (Chromservis s.r.o., Czech Republic).

2.7. Histopathological Examination. Samples of liver, gills, cranial, and caudal kidney were removed from 5 fish in each group. They were fixed in 10% neutral formalin solution and subsequently stained with haematoxylin and eosin. Histological changes in samples were examined by light microscopy.

2.8. Statistical Analysis. Statistical analysis was performed using Unistat 5.6 software. A Shapiro-Wilk test was done for the normal distribution. The differences among test groups were assessed with the Tukey-HSD test. Immunological parameters were evaluated by the unpaired nonparametric Mann-Whitney test.

3. Results

During the experiment, the mortality of fish was not recorded in both control groups as well as in the tested concentrations.

3.1. Biometric Parameters. There were no changes in HSI and CF in fish exposed to all DEET concentrations compared to both control groups after 28 days of exposure (Table 2).

3.2. Haematological Profile. The DEET exposure did not affect WBC, MCHC, values of Hb, and PCV of experimental fish. A significant increase ($P < 0.05$) in RBC was observed in the concentration of 1 mg/L compared to both control groups (Table 3). Further, a significant ($P < 0.05$) decrease in MCV and a decrease ($P < 0.05$) in MCH were found in the 1 mg/L concentration compared to both control groups (Table 3).

WBC and differential white blood cells count were not affected by treatment (data not shown).

3.3. Biochemical Profile. The only change in biochemical profile of the experimental fish was in the decrease ($P < 0.05$) of TAG in the DEET concentration of 1 mg/L compared to the control groups. The other parameters including activity of butyrylcholinesterase were not affected (Table 4).

3.4. Immunological Parameters. The exposure to DEET did not influence proinflammatory (TNF-α and IL-1β) and anti-inflammatory cytokine genes (TGF-β and IL-10) in any tested concentration of DEET (Figure 1).

3.5. Parameters of Oxidative Stress. Values of antioxidant enzymes activities (GR, GPx, GST, and CAT) and amount of TBARS are presented in tables for individual tissue (Tables 5, 6, 7, and 8). A significant ($P < 0.01$) increase in GPx was found in kidney in the exposure concentration of 1 mg/L compared to 1 μg/L and a significant ($P < 0.05$) decrease

Table 2: Biometric parameters in *C. carpio* for each tested group ($n = 10$).

Parameter	Control	Control with DMSO	DEET 1 μg/L	DEET 0.1 mg/L	DEET 1 mg/L
Body weight (g)	295.06 ± 40.76	267.33 ± 42.99	267.10 ± 44.88	273.17 ± 39.86	282.67 ± 46.26
CF	2.59 ± 0.19	2.59 ± 0.17	2.51 ± 0.21	2.59 ± 0.26	2.51 ± 0.14
HSI	1.87 ± 0.39	1.90 ± 0.26	1.79 ± 0.26	1.74 ± 0.27	1.81 ± 0.35

Table 3: Haematological values in *C. carpio* from control groups and groups exposed to DEET (mean ± SD, $n = 10$).

Indices	Control	Control with DMSO	DEET 1 μg/L	DEET 0.1 mg/L	DEET 1 mg/L
RBC (10^{12}/L)	1.69 ± 0.35^{a}	1.78 ± 0.41^{a}	2.21 ± 0.74^{a,b}	2.20 ± 0.62^{a,b}	2.49 ± 0.42^{b}
Hb (g/L)	74.05 ± 10.81	69.26 ± 13.93	73.56 ± 13.95	67.14 ± 8.31	71.70 ± 17.59
PCV (L/L)	0.26 ± 0.04	0.27 ± 0.03	0.26 ± 0.03	0.26 ± 0.02	0.26 ± 0.02
MCV (10^{15}/L)	162.11 ± 34.65^{a}	159.54 ± 34.23^{a}	134.76 ± 52.87^{a,b}	128.68 ± 41.65^{a,b}	107.27 ± 18.43^{b}
MCH (10^{12}/L)	44.12 ± 11.73^{a}	39.75 ± 8.36^{a,b}	36.35 ± 11.51^{a,b}	32.66 ± 10.30^{a,b}	29.88 ± 9.31^{b}
MCHC (g/L)	0.28 ± 0.04	0.25 ± 0.05	0.29 ± 0.11	0.25 ± 0.04	0.27 ± 0.07

Significant differences ($P < 0.05$) between groups are marked by different alphabetic superscripts.

Table 4: Biochemical indices in plasma of *C. carpio* from control groups andgroups exposed to DEET (mean ± SD, $n = 10$).

Indices	Control	Control with DMSO	DEET 1 μg/L	DEET 0.1 mg/L	DEET 1 mg/L
ALT (μkat/L)	0.70 ± 0.22	0.65 ± 0.11	0.88 ± 0.35	0.97 ± 0.36	0.67 ± 0.33
AST (μkat/L)	1.88 ± 0.69	1.69 ± 0.47	2.11 ± 0.39	1.95 ± 0.51	1.80 ± 0.52
ALP (μkat/L)	0.40 ± 0.21	0.54 ± 0.19	0.55 ± 0.22	0.52 ± 0.17	0.42 ± 0.17
Albumin (g/L)	10.94 ± 1.77	11.12 ± 1.57	10.89 ± 1.30	10.86 ± 1.22	10.92 ± 2.03
Total protein (g/L)	29.23 ± 2.12	27.67 ± 2.30	26.33 ± 3.02	27.26 ± 3.15	26.36 ± 2.52
Glucose (mmol/L)	3.46 ± 1.09	3.67 ± 1.18	3.24 ± 1.12	4.13 ± 1.39	2.93 ± 0.87
LDH (μkat/L)	5.67 ± 2.33	5.07 ± 0.73	4.45 ± 0.99	4.78 ± 1.61	4.15 ± 1.30
TAG (mmol/L)	2.37 ± 2.34^{a}	2.12 ± 0.73^{a,b}	2.21 ± 0.99^{a,b}	2.15 ± 1.60^{a,b}	1.88 ± 1.30^{b}
Ammonium (mmol/L)	196.12 ± 66.35	186.6 ± 104.77	164.39 ± 37.74	156.70 ± 50.76	172.33 ± 73.20
Calcium (mmol/L)	2.14 ± 0.09	2.06 ± 0.20	2.06 ± 0.06	2.03 ± 0.31	2.06 ± 0.12
Phosphorus (mmol/L)	1.85 ± 0.35	1.84 ± 0.33	1.88 ± 0.22	2.01 ± 0.58	1.80 ± 0.26
Lactate (mmol/L)	2.91 ± 1.29	2.64 ± 2.48	2.11 ± 0.95	3.29 ± 2.17	2.85 ± 1.77
Cholesterol (mmol /L)	3.33 ± 0.47	3.12 ± 0.54	2.97 ± 0.41	3.07 ± 0.52	3.06 ± 0.63
FRAP (Fe^{2+} equivalent μmol/L)	559.76 ± 91.74	448.57 ± 104.53	506.90 ± 60.87	483.81 ± 52.25	465.98 ± 116.96
ButChE (μkat/L)	1.55 ± 0.67	1.22 ± 0.85	1.54 ± 0.75	1.66 ± 0.59	1.69 ± 0.53

Significant differences ($P < 0.05$) between groups are marked by different alphabetic superscripts.

Table 5: Antioxidant enzymes activities and amount of TBARS in liver of *C. carpio* in each group (mean ± SD, $n = 10$).

Parameter	Units	Control	Control with DMSO	DEET 1 μg/L	DEET 0.1 mg/L	DEET 1 mg/L
GR	(nmol NADPH/min/mg protein)	5.69 ± 0.98	5.03 ± 1.98	5.43 ± 1.51	4.79 ± 1.14	5.86 ± 1.06
GPx	(nmol NADPH/min/mg protein)	182.3 ± 61.5	203.9 ± 70.7	169.1 ± 50.3	203.8 ± 47.2	194.7 ± 39.3
GST	(nmol /min/mg protein)	260.6 ± 82.9	344.9 ± 110.3	310.0 ± 91.4	284.1 ± 97.3	319.6 ± 100.3
CAT	(μmol H_2O_2/min/mg protein)	365.7 ± 70.4	351.3 ± 67.9	332.2 ± 62.7	344.7 ± 56.3	332.1 ± 73.0
TBARS	(nmol/g sample)	36.4 ± 10.4	30.6 ± 6.6	35.8 ± 11.2	36.1 ± 12.1	32.7 ± 14.4

Table 6: Antioxidant enzymes activity and amount of TBARS in kidney of *C. carpio* in each tested group (mean ± SD, $n = 10$).

Parameter	Units	Control	Control with DMSO	DEET 1 μg/L	DEET 0.1 mg/L	DEET 1 mg/L
GR	(nmol NADPH/min/mg protein)	5.02 ± 2.23	4.41 ± 1.62	5.67 ± 2.41	4.98 ± 1.68	4.76 ± 2.26
GPx	(nmol NADPH/min/mg protein)	193.4 ± 33.1^{a,b}	183.7 ± 45.7^{b}	164.9 ± 39.4^{a,b}	201.7 ± 29.8^{a,b}	221.8 ± 31.4^{a}
GST	(nmol/min/mg protein)	252.3 ± 43.7	275.4 ± 45.7	256.5 ± 55.9	265.2 ± 65.9	301.9 ± 65.1
CAT	(μmol H_2O_2/min/mg protein)	52.63 ± 8.60	42.90 ± 12.49	49.21 ± 15.98	42.06 ± 9.87	44.36 ± 17.40
TBARS	(nmol/g sample)	24.12 ± 4.53	25.06 ± 5.11	24.99 ± 4.66	26.27 ± 6.26	27.00 ± 8.85

Significant differences ($P < 0.05$) between groups are marked by different alphabetic superscripts.

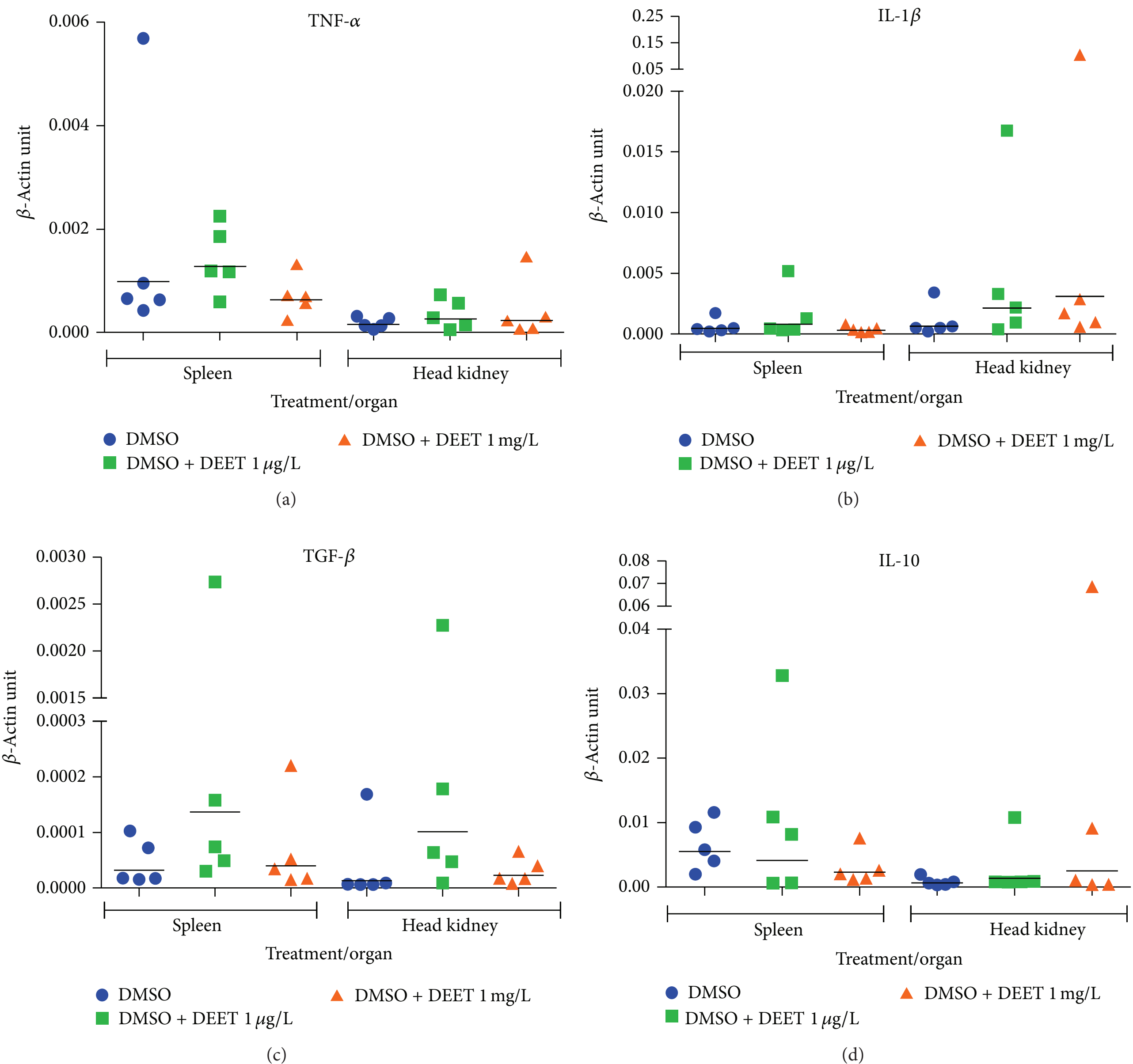

Figure 1: Graphs show individual values of the expression of cytokines versus housekeeping gene β-actin. Bars represent geometric mean values.

in GPx was found in gills in the exposure concentration of 1 mg/L compared to the control group with DMSO. The catalase activity could be not determined in brain due to very low activity of this enzyme.

3.6. Histological Examination. A subchronic exposure to DEET did not cause marked specific histopathological changes in the DEET-treated fish.

4. Discussion

The amount of data on mechanism of action and chronic toxicity for DEET to aquatic environment is still limited. Acute toxic studies have found DEET to be slightly toxic for fish: 96 h LC_{50} for tilapia mossambica (*Oreochromis mossambicus*) and rainbow trout (*Oncorhynchus mykiss*) is 120–150 mg/L and 71.3 mg/L, respectively [31, 32]. Nevertheless our study has shown that even low concentration of DEET can influence red blood parameters of fish after 28 days of exposure. The increase in red blood cells in DEET concentration 1 mg/L indicates rise of erythropoiesis. Although the total amount of haemoglobin and haematocrit in blood was not changed, erythrocytes (MCV and MCH) decreased. Two-third decrease in mean corpuscular volume (MCV) of erythrocyte indicates a breakdown of erythropoiesis and a development of nonadequate erythrocytes. Higher occurrence of erythroblast was not recorded. In the study of dogs, a weak reduction of haemoglobin and haematocrit

Table 7: Antioxidant enzymes activity and amount of TBARS in brain of *C. carpio* in each tested group (mean ± SD, $n = 10$).

Parameter	Units	Control	Control with DMSO	DEET 1 μg/L	DEET 0.1 mg/L	DEET 1 mg/L
GR	(nmol NADPH/min/mg protein)	3.64 ± 0.49	3.94 ± 0.35	3.88 ± 0.59	4.33 ± 0.81	4.01 ± 0.62
GPx	(nmol NADPH/min/mg protein)	56.20 ± 6.76	60.96 ± 6.87	56.86 ± 7.30	58.33 ± 6.16	56.93 ± 3.97
GST	(nmol/min/mg protein)	212.30 ± 50.11	220.28 ± 53.98	263.46 ± 62.05	222.51 ± 40.33	231.73 ± 78, 67
TBARS	(nmol/g sample)	9.09 ± 3.59	8.3 ± 2.23	8.92 ± 3.15	10.37 ± 4.50	10.02 ± 4.32

Table 8: Antioxidant enzymes activity and amount of TBARS in gills of *C. carpio* in each tested group (mean ± SD, $n = 10$).

Parameter	Units	Control	Control with DMSO	DEET 1 μg/L	DEET 0.1 mg/L	DEET 1 mg/L
GR	(nmol NADPH/min/mg protein)	6.38 ± 1.04	7.29 ± 1.37	6.93 ± 2.16	7.96 ± 0.96	6.35 ± 1.05
GPx	(nmol NADPH/min/mg protein)	43.67 ± 13.94^{a,b}	54.87 ± 18.11^{a}	43.09 ± 21.65^{a,b}	37.31 ± 6.94^{a,b}	34.28 ± 11.39^{b}
GST	(nmol /min/mg protein)	116.9 ± 24.2	112.6 ± 36.1	104.7 ± 30.3	116.4 ± 33.2	110.1 ± 22.7
CAT	(μmol H_2O_2/min/mg protein)	10.12 ± 3.55	9.20 ± 2.61	10.40 ± 2.73	11.07 ± 2.44	10.88 ± 2.27
TBARS	(nmol/g sample)	39.76 ± 17.62	29.23 ± 8.87	31.01 ± 9.66	36.66 ± 14.03	31.86 ± 8.61

Significant differences ($P < 0.05$) between groups are marked by different alphabetic superscripts.

was noticed after 6 and 12 months of oral intake of DEET in concentration 400 mg/kg/day [33], but other red blood parameters were not affected. In adult fish, a spleen, the head kidney (pronephros), and mesonephros have been found to be sites of erythropoiesis [34]; specific histopathological changes of these organs in the DEET-treated fish were not noticed in our study.

The decrease in triacylglycerides in DEET concentration 1 mg/L was recorded. TAG are the most important energy-storing lipids and belong to major energy sources for the fish [35]. In this study, TAG decrease can indicate exhaustion of energy sources due to long-term stress.

Because DEET is reported to act as a neurotoxin through inhibition of cholinesterase [11], we concentrated on butyrylcholinesterase activity. However, butyrylcholinesterase was not affected. This finding supports results of studies about elevation of cholinesterase inhibition in insect only after common impact of DEET and cholinesterase-inhibiting insecticides [36, 37].

The immunological toxicity of DEET has not been extensively studied in fish before. Our observation was focused on the expression of proinflammatory (TNF-α and IL-1β) and anti-inflammatory cytokine genes (TGF-β and IL-10). There were not changes of the cytokine expression in head kidney and spleen in tested fish. Cytokines are the key initiator of immune reaction. They arise at the sites of entry of pathogens into organism; they stimulate inflammatory signals and thus the ability of resident and newly recruited phagocytes to eliminate the invading pathogens is regulated [38]. In teleostean fishes, such as carp and rainbow trout, the expression of interleukin 1β (IL-1β) mRNA can be stimulated by lipopolysaccharide alone or in combination with cortisol [39–41]. On the contrary, some toxic compounds as cyanotoxin anatoxin-a, for example, significantly inhibited proinflammatory (IL-1β and TNF-α) cytokines and induced anti-inflammatory (IL-10 and TGF-β) cytokines in common carp [42].

The effect of DEET on formation of oxidative stress was studied especially in insect [43] and rats [44–46]. Antioxidant enzymes, that is, GPx, GR, CAT, and SOD, keep the oxidative status in the cell. They reduce either free or membrane-bound hydroperoxides [47]. Glutathione S-transferase catalyzes the conjugation of the reduced form of glutathione to xenobiotic substrates for the purpose of detoxification [48]. In our study, we observed alterations only in case of GPx activities. The activity of GPx in kidney tissues increased in experimental group exposed to 1 mg/L of DEET compared to the DMSO control group. This tissue-specific GPx increase might indicate the adaptive approach by the fish to defend the oxidative stress [49]. Moreover, we also found decline in GPx activity in gill tissues of experimental group exposed to 1 mg/L of DEET compared to the DMSO control group. This alteration in GPx in gills might be due to the depletion of the enzyme. In fact, the fish gills were the first organ exposed to the toxic effluent [50].

5. Conclusions

Fish are an appropriate model for a further investigation of the biological effect of DEET on vertebrates due to its high frequency of occurrence in aquatic environments around the world. Although acute toxicity levels of DEET are high, low concentration after subchronic exposition can cause adverse effects on haematological parameters. To assess the effect of diethyltoluamide on the fish immune system, more immunological parameters need to be included in the future studies.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Effects of Mixture of Pharmaceuticals on Early Life Stages of Tench (*Tinca tinca*)

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Ubiquitous occurrence of pharmaceuticals in aquatic environment results in concern about potential adverse the effects on nontarget organisms. In water, drugs are present in complex mixtures, in which complicated interactions affect toxicity of single components. The purpose of this study was to examine effect of 35-day-long exposure to mixture of ibuprofen, diclofenac, and carbamazepine on the mortality, growth, early ontogeny, and histopathological changes in tench (*Tinca tinca*). Early life stage toxicity test was carried out using a modified protocol according to OECD guideline 210. Exposure to mixture of pharmaceuticals at concentration of 60 $\mu g \cdot L^{-1}$ for each substance was associated with significant increase in mortality, as well as significant increase in growth and elevated incidence of malformations. Any of the tested concentrations resulted in histopathological changes of liver, kidney, skin, or gill. After fourteen days of exposure there was short-term delay of development related to increased concentrations of pharmaceuticals in the mixture (2, 20, and 60 $\mu g \cdot L^{-1}$). Environmentally relevant concentrations (0.02; and 0.2 $\mu g \cdot L^{-1}$) used in this experiment did not result in toxic impairment of tench.

1. Introduction

Human and veterinary pharmaceuticals and their metabolites belong due to their overproduction and indiscriminate usage to ubiquitous environmental contaminants present in the ground and surface waters at a magnitude of $ng \cdot L^{-1}$ to $\mu g \cdot L^{-1}$ [1]. In effluents of three Swiss wastewater treatment plants (WWTP), the concentrations of pharmaceuticals reached 1.3 $\mu g \cdot L^{-1}$ for ibuprofen, 0.99 $\mu g \cdot L^{-1}$ for diclofenac, and 0.95 $\mu g \cdot L^{-1}$ for carbamazepine [2]. The study of French scientists [3] demonstrates that concentration of pharmaceuticals in surface water is two or three times higher than in treated drinking water.

Pharmaceuticals can be excreted, primarily via urine and faeces, either as an unchanged parent compound or in the form of metabolites or as conjugates of glucuronic and sulphuric acid, and they can then enter aquatic ecosystems via different ways [4]. Moreover, conventional technology used in wastewater treatment plants appears as insufficient to remove these specific compounds [5]. Some substances (e.g., carbamazepine) are not removed during wastewater treatment. Carbamazepine passes the plants without any reduction and effluent concentrations are in the range of influent concentrations [6]. However, removal efficiencies for ibuprofen and diclofenac are high 65–100 and 30–100%, respectively; they are still ubiquitous and are present at considerable concentrations in river waters [7–10]. This could be due to the fact that their concentrations in the inlets are so high that the remains in the effluents are still significant [11]. Carbamazepine is relatively lipophilic, with an octanol/water partition coefficient of 2.2, and is consistently found at relatively high concentrations in aquatic environment. This compound was proposed as a suitable anthropogenic marker of urban effluents [12]. Information about concentrations of

Table 1: Concentrations of ibuprofen (IBU), diclofenac (DCF), and carbamazepine (CBZ) in surface waters.

	Surface water (dams, lakes, and rivers)					Location	Source
	Frequency (%)	Median ($ng{\cdot}L^{-1}$)	Mean ($ng{\cdot}L^{-1}$)	Min ($ng{\cdot}L^{-1}$)	Max ($ng{\cdot}L^{-1}$)		
IBU		20	20		100	Rhine river	[13]
	62	6	31.3		395	EU rivers	[14]
	96		93			Ebro river, Spain	[11]
	100		790	160	2710	Llobregat river, Spain	[15]
DCF		46	50		900	Rhine river	[13]
	83	5	17		247	EU rivers	[14]
	93		58			Ebro river, Spain	[11]
	100		2200	80	18740	Llobregat river, Spain	[15]
CBZ		110	103		640	Rhine river	[13]
	95	75	11.6		248	EU rivers	[14]
	100		55			Ebro river, Spain	[11]
	90		1070	80	3090	Llobregat river, Spain	[15]
	100	455.5				Madrid	[16]

ibuprofen, diclofenac, and carbamazepine in surface water is summarized in Table 1. Detection of pharmaceuticals in the environment has resulted in concern for potential adverse effects on nontarget species.

Experiment by Loos et al. [14] showed that the polar pharmaceuticals (ibuprofen and diclofenac) are slowly degraded in water (by a factor of around 20% after 3 weeks). Photodegradation half-life time of diclofenac is much faster than carbamazepine and humic acids (concentration of $5.0\,mg{\cdot}L^{-1}$) act as inner filters during the photodegradation of carbamazepine and diclofenac [17].

The wide spectrum of substances detected in receiving river waters indicates that WWTP outlets are major contributors to pharmaceuticals in the aquatic environment [11]. Although most of these compounds are present at low concentrations, many of them raise considerable toxicological and ecotoxicological concerns, particularly when present as components of complex mixtures. It is very difficult to assess the effect on the aquatic environment of the thousands of synthetic and natural trace contaminants that may be present in water at low concentrations [18].

Pharmaceuticals are designed to have specific mode of action and many of them for persistence in the organism. According to Láng and Köhidai [19], toxicity of diclofenac based on the proliferation inhibition of ciliate *Tetrahymena pyriformis* is higher than toxicity of ibuprofen. The reported order of toxicity diclofenac > ibuprofen was confirmed by Cleuvers [20] too. Although detected environmental concentrations of pharmaceuticals are often low, these compounds are present in mixtures [21]. Substances applied at no effect concentration can contribute to the total mixture effect which in turn can become significant. Concentration addition was observed, for example, for NSAIDs (nonsteroidal anti-inflammatory drugs) in Daphnia and algal bioassays [20]. For pharmaceuticals chronic exposure is much more relevant than acute exposure.

The aim of this study was to assess subchronic toxic effects of mixture of drugs (ibuprofen, diclofenac, and carbamazepine) on tench (*Tinca tinca*). Our work mainly focused on the growth parameters, histopathology, early ontogeny, incidence of malformations and mortality of embryos, and larval stages in tench affected by mixture of pharmaceuticals.

2. Materials and Methods

2.1. Experimental Protocol. Our laboratory experiment was carried out using test solutions containing mixture of ibuprofen (IBU), diclofenac sodium salt (DCF), and carbamazepine (CBZ) as test substances (Table 2). All test substances were purchased from Sigma-Aldrich Co.

Table 3 summarizes various physical properties of IBU, DCF, and CBZ, including the dissociation constant (pK_a), octanol-water partition coefficient ($\log K_{ow}$) [21], and the percentage of parent compound excreted from the human body [22].

Embryo-larval toxicity test was carried out using a modified protocol according to OECD guideline 210 (fish, early life stage toxicity test) [23]. Fertilized eggs of tench were obtained from a commercial fish farm.

Experiment took place in 500 mL crystallization dishes, each containing 100 randomly distributed embryos (24 hours after fertilization). The experiment was conducted in triplicate (a total of 300 fertilized eggs for each experimental group and each control). The embryos and larvae of tench were exposed to the mixtures of pharmaceuticals on nominal concentrations of 0.02, 0.2, 2, 20, and $60\,\mu g{\cdot}L^{-1}$ (for each pharmaceutical), and experimental groups were named M1, M2, M3, M4, and M5, respectively. Duration of the test was 35 days. The two lowest used concentrations simulated environmental conditions, the third concentration approaches concentration of pharmaceuticals in waste water, and the highest concentration is up to 3000 higher than environmental concentration. Control fish were kept in tap water. Due to use of ibuprofen, diclofenac, and carbamazepine, as test substances, which require the use of dimethylsulfoxide (DMSO) as a solvent, additional control fish were exposed to 0.01% DMSO as a solvent control. The concentration of DMSO in the solvent

Table 2: List of pharmaceuticals used in the experiment.

Pharmaceutical	Molecular formula	Molecular structure	CAS number	Use of drug
Ibuprofen (IBU)	$C_{13}H_{18}O_2$		15687-27-1	Anti-inflammatory, analgesic
Diclofenac sodium salt (DCF)	$C_{14}H_{10}Cl_2NNaO_2$		15307-79-6	Anti-inflammatory, analgesic
Carbamazepine (CBZ)	$C_{15}H_{12}N_2O$		298-46-4	Antiepileptic, mood modulating

Table 3: Physical properties of ibuprofen (IBU), diclofenac (DCF), and carbamazepine (CBZ).

Compound	pK_a	$\log K_{ow}$	Percentage of parent compound excreted
IBU	4.5–5.2	4.0	≤5
DCF	4.15	4.5	6–39
CBZ	13.9	2.24	≤5

control corresponded to the DMSO concentration present in the test water containing the highest drug concentration (M5 group). A concentration of 0.01 $mL \cdot L^{-1}$ of DMSO did not result in lethal effects, abnormalities, or changes in growth parameters during an embryo-larval toxicity test on carp [24].

A semistatic method was used, in which solutions of drugs were replaced twice a day. Concentrations of pharmaceuticals in test waters were determined once a week by HPLC. None of the pharmaceuticals used in this study were detected in the dilution water during the study period. During the test, concentrations of pharmaceuticals did not fall below 80% of the nominal concentration. Dead embryos and larvae were removed from crystallization dishes twice a day. Feeding was initiated on day 7. Larvae were fed with freshly hatched *Artemia salina* twice a day *ad libitum* after the bath exchange. Hatching, survival, temperature, pH, and oxygen saturation were recorded daily. The water temperature and pH ranged from 21 to 23°C and 8.4 to 8.8, respectively. A photoperiod consisted of 12 h light/12 h dark segments for each day. Concentration of dissolved oxygen did not fall below 80%.

During the test, larvae were sampled to investigate developmental changes, morphological abnormalities, length, weight, and Fulton's condition factor (FCF). Samples from each concentration and each control were collected on day 7 (after feeding initiated), and on days 14, 21, 29, and 35. All collected fish were fixed in 4% formalin. Ending of the experiment was at the 35th day, when fish were euthanized by carbon dioxide.

2.2. Determination of Developmental Stages. The developmental stages of tench were determined according to Peňáz et al. [25–27] who described ontogenesis in tench and common carp, both belong to the same cyprinid family (Cyprinidae). Peňáz distinguished nine embryonic (E1–E9), six larval (L1–L6), and two juvenile (J1-J2) stages.

2.3. Length, Weight, and Growth Evaluation. Total length (TL) from the mouth to caudal peduncle was measured stereomicroscopically using a micrometer to be 0.01 mm. Whole body wet weight (W) was measured to be 0.1 mg. Fulton's condition factor (FCF) is widely used in fisheries and general fish biology studies. This factor is calculated from the relationship between the weight of a fish in grams and its length in millimeters, with the intention of describing the condition of that individual [28]. In our study FCF was

calculated for each sampling time and for each experimental group. Consider

$$\mathrm{FCF} = \frac{W \times 10^5}{\mathrm{TL}^3}. \quad (1)$$

The specific growth rate (SGR) is a measure of the percentage body weight increase per day. The mean SGR was calculated for each experimental group in the beginning on day 7 (the first sampling time) and on day 35 (the last sampling time). The SGR was calculated in the following formula:

$$\mathrm{SGR}\,(\%) = \frac{\overline{\ln W_2} - \overline{\ln W_1}}{T_2 - T_1} \times 100, \quad (2)$$

where ln is natural logarithm. W_1 is weight of one fish at time T_1 in grams; W_2 is weight of one fish at time T_2 in grams. T_1 is first sampling time; T_2 is last sampling time.

The inhibition of specific growth rate (I) for each experimental group was calculated as follows:

$$I\,(\%) = \frac{\mathrm{SGR}\,(\mathrm{control}) - \mathrm{SGR}\,(\mathrm{group})}{\mathrm{SGR}\,(\mathrm{control})} \times 100. \quad (3)$$

2.4. Histopathological Examination. The fish were prepared for histopathological examination, fixed in buffered 10% neutral formalin, dehydrated, embedded in paraffin wax, sectioned (cross-section) on a microtome at 4 μm, and stained with hematoxylin and eosin (H&E). The histology of skin, gill, kidney, and liver was examined by light microscopy.

2.5. Determination of Ibuprofen, Diclofenac, and Carbamazepine. Measurement of diclofenac, ibuprofen, and carbamazepine in water samples was performed by high-performance liquid chromatography coupled with triple quadrupole tandem mass spectrometry (LC-MS/MS). Sample preparation was based on solid phase extraction. SPEC C_{18} AR cartridges (3 mL, 30 mg, Varian, Inc., Palo Alto, CA, USA) were used. One milliliter 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, CA, USA) equipped with heated electrospray ionization (HESI-II) probe. A Thermo Scientific Hypersil C_{18} (2.1 mm × 50 mm, 1.9 μm) column was used at a constant flow rate of 300 μL·min^{-1} by 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 ionization 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: 325.0°C; vaporizer temperature 300.0°C; sheath gas pressure 35.0 psi; auxiliary (drying) gas 10 a.u.; spray voltage 3300 V (−3300 V for ibuprofen and diclofenac). Standards were purchased from Sigma-Aldrich (St. Louis, MO, USA). All solvents were residual analysis purity (Chromservis s.r.o., CZ). For our QA/QC program, the instrument was calibrated daily with multilevel calibration curves. Procedural blank and solvent blank were analyzed for every set of 20 samples. The spiked recoveries were 97% for ibuprofen, 99% for diclofenac, and 99% for carbamazepine. Reported concentrations are after corrections based on the recoveries. Coefficients of variation for between-series were 4.1% for ibuprofen, 3.5% for diclofenac, and 2.9% for carbamazepine. The limits of detection were determined as 3 : 1 signal versus noise value (S/N) and were 9 ng·L^{-1} for ibuprofen, 7 ng·L^{-1} for diclofenac, and 5 ng·L^{-1} for carbamazepine.

2.6. Statistical Analysis. Growth parameters were tested using statistical analysis performed using software Unistat 5.6 for Excel. Data were evaluated for normal distribution by Shapiro-Wilk test. Normal distributed data were compared using parametric ANOVA. Since data were not normally distributed, significance of differences between control, control-solvent group, and experimental groups was tested by non-parametric Kruskal-Wallis followed by multiple comparisons. Mortality was evaluated using contingent tables. Levels of significance were set to $P < 0.001$ highly significant (**) and $P < 0.01$ significant (*).

3. Results

3.1. Exposure Concentrations. See Table 4.

3.2. Hatching. There was not any observed negative effect of any concentration of mixture of drugs on the hatching. The hatching began in both control groups and in all experimental groups on the second day of the experiment and was completely finished on the fourth day. Hatching success was at least 95% for both control groups and all experimental groups.

3.3. Mortality. Mortality at the end of the experiment was 19.3, 19.3, 18.5, 16.0, 16.7, 22.3, and 36.3% in C, CS, M1, M2, M3, M4, and M5, respectively. Significant ($P < 0.001$) increase in mortality between control and M5 groups has been found. Differences between mortality in CS, M1, M2, M3, and M4 in comparison with C group were not significant. Cumulative mortality is depicted in Figure 1.

3.4. Length and Weight Parameters, Condition, and Growth Rate. Growth parameters did not differ between control and control-solvent groups. Statistically significant ($P < 0.01$) effect of the highest tested concentration of drugs mixture (M5) on weight of fish on the 7th and 14th days and high significant ($P < 0.001$) effect on weight on the 35th day of the test have been found. Effect on total length of fish of groups M4 ($P < 0.01$) and M5 ($P < 0.001$) after seven-day-long exposure to pharmaceuticals has been found. After fourteen days, statistical significance on decrease of length ($P < 0.01$) of fish in experimental group M5 has been found. Highly significant increase of length ($P < 0.001$) of fish of M5 group occurred after 35-day-long exposure. Length and weight parameters are depicted in Table 5.

Table 4: Average (A) exposure concentrations of ibuprofen (IBU), diclofenac (DCF), and carbamazepine (CBZ) ± standard error of mean (SEM) in control (C), control-solvent (CS), and experimental groups (M1–M5).

	C	CS	M1	M2	M3	M4	M5
	A ± SEM	A ± SEM	A ± SEM	A ± SEM	A ± SEM	A ± SEM	A ± SEM
IBU	N.d.	N.d.	0.02 ± 0.01	0.20 ± 0.05	1.66 ± 0.20	18.10 ± 0.52	56.20 ± 8.56
DCF	N.d.	N.d.	0.02 ± 0.00	0.19 ± 0.05	1.67 ± 0.17	18.30 ± 2.20	59.45 ± 9.10
CBZ	N.d.	N.d.	0.02 ± 0.01	0.18 ± 0.04	1.58 ± 0.15	18.00 ± 1.50	58.10 ± 4.51

N.d.: not detected.

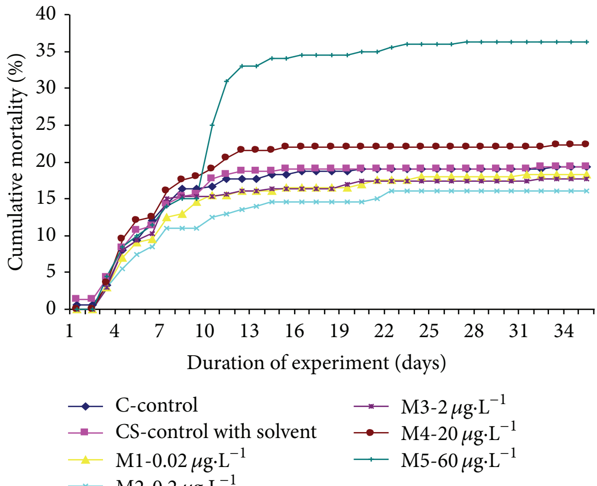

Figure 1: Cumulative mortality in controls and experimental groups affected by mixture of ibuprofen, diclofenac, and carbamazepine, during 35-day-long test.

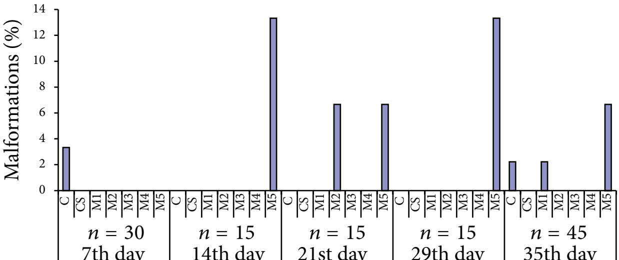

Figure 2: Percentage of malformations found in C-control, CS-control with solvent, M1, M2, M3, M4, and M5—mixture of ibuprofen, diclofenac, and carbamazepine on concentration of 0.02, 0.2, 2, 20, and 60 $\mu g{\cdot}L^{-1}$, respectively, after 7, 14, 21, 29, and 35 days of exposure.

After 35 days of experiment, significantly higher ($P < 0.001$) FCF has been found in tench exposed to M5 than in tench from control group (Table 6).

3.5. The Occurrence of Malformations. At first sampling on the seventh day of the experiment, shortened body and curved tail have been found in control group. The same kind of malformations was found in group M5 ($c = 60\,\mu g{\cdot}L^{-1}$) at second sampling on the fourteenth day. Deformations of eyes and lower jaws in groups M2 ($c = 0.2\,\mu g{\cdot}L^{-1}$) and M5 were discovered at the third sampling. Only in M5 group there were uncovered deformations of eyes and lower jaws at the fourth sampling. In samples from the 35th day, malformations in control and M1 ($0.02\,\mu g{\cdot}L^{-1}$) and M5 group were found. Most common were ocular malformations (lack of eye and pigment-deficient eye) and lower jaws defects. Alimentary canal defects and scoliosis occurred rarely. The percentage of malformations is depicted in Figure 2.

3.6. Histopathology. Histopathological examination of liver, kidney, gill, and skin of tench in both control groups and experimental groups (C, CS, M1, M2, M3, M4, and M5) showed no pathomorphological changes.

3.7. Early Ontogeny. At first sampling on the seventh day, all fish in all experimental groups were L1 larval stage. After 14 days of exposure, all fish from C, CS, M1, and M2 groups belonged to L3 stage. In M3 and M4, almost 7% of fish were determined as L1 and L2 stages, respectively. M5 group consisted of L3 (majority) and more than 13% were L1 and L2 stages. After 21 days of exposure, most fish were in L5 stage. In C, M4, and M5, L6, L4, and L3 also occurred, respectively. Fish from both control groups and all experimental groups belong to L6 and L5 stages after 29-days-long exposure. At the end of the test (the 35th day of exposure) most fish were determined in larval L6 stage and some fish were determined in stage L5 and some already achieved to be in first juvenile stage-J1 (Figure 3).

3.8. Behavior. All fish in both control groups and experimental groups (M1, M2, M3, M4, and M5) showed normal behavior.

4. Discussion

The aquatic environment is increasingly exposed to complex mixtures of pollutants. A chemical can be more toxic when it is mixed with other chemicals, because of chemical interactions commonly referred to as cocktail effects. Mixed exposure can result in interactions, which means that one chemical affects absorption, distribution, metabolism, or excretion of another chemical [29]. The predominant interaction type observed in mixtures of pharmaceuticals (IBU, DCF, propranolol, and metoprolol) was antagonism, and the

Table 5: Average (A), median (M), and standard error of mean (SEM) of weight (W) and length (TL) parameters of tench exposed to C-control, CS-control with solvent, M1, M2, M3, M4, and M5—mixture of ibuprofen, diclofenac, and carbamazepine on concentration of 0.02, 0.2, 2, 20, and 60 μg·L^{-1}, respectively.

	$W_{7th\ day}$ A ± SEM (M)	$W_{14th\ day}$ A ± SEM (M)	$W_{21st\ day}$ A ± SEM (M)	$W_{29th\ day}$ A ± SEM (M)	$W_{35th\ day}$ A ± SEM (M)
C	0.95 ± 0.02 (0.90)	5.13 ± 0.12 (5.00)	13.66 ± 0.69 (12.20)	25.96 ± 1.19 (25.10)	41.98 ± 1.42 (42.10)
CS	1.00 ± 0.03 (1.00)	5.00 ± 0.10 (5.10)	16.33 ± 0.69 (15.85)	25.94 ± 1.69 (23.40)	46.35 ± 1.81 (47.00)
M1	0.99 ± 0.03 (0.95)	4.94 ± 0.18 (4.80)	16.33 ± 0.68 (15.85)	28.15 ± 2.32 (28.20)	50.18 ± 1.93 (48.10)
M2	0.99 ± 0.03 (0.95)	4.56 ± 0.19 (4.70)	15.52 ± 0.73 (14.19)	27.79 ± 1.39 (27.10)	45.74 ± 1.92 (44.40)
M3	1.02 ± 0.02 (1.00)	5.03 ± 0.15 (4.95)	16.22 ± 0.86 (14.65)	25.52 ± 1.55 (24.90)	45.95 ± 2.22 (47.50)
M4	1.03 ± 0.04 (1.00)	4.90 ± 0.30 (5.20)	13.69 ± 1.42 (15.10)	30.95 ± 2.41 (30.60)	46.72 ± 2.29 (47.60)
M5	1.09 ± 0.03 (1.10)*	3.82 ± 0.42 (4.35)*	10.12 ± 1.65 (9.60)	25.71 ± 2.87 (24.30)	62.93 ± 2.81 (68.20)**
	$TL_{7th\ day}$ A ± SEM (M)	$TL_{14th\ day}$ A ± SEM (M)	$TL_{21st\ day}$ A ± SEM (M)	$TL_{29th\ day}$ A ± SEM (M)	$TL_{35th\ day}$ A ± SEM (M)
C	5.14 ± 0.03 (5.12)	8.50 ± 0.06 (8.52)	11.33 ± 0.14 (11.30)	13.58 ± 0.21 (13.32)	15.70 ± 0.16 (15.89)
CS	5.18 ± 0.02 (5.18)	8.49 ± 0.07 (8.51)	11.43 ± 0.14 (11.42)	13.73 ± 0.22 (13.37)	16.13 ± 0.20 (16.30)
M1	5.09 ± 0.03 (5.13)	8.26 ± 0.14 (8.36)	11.78 ± 0.14 (11.67)	13.84 ± 0.35 (13.86)	16.40 ± 0.20 (16.55)
M2	5.26 ± 0.03 (5.27)	8.35 ± 0.12 (8.41)	11.48 ± 0.20 (11.24)	13.80 ± 0.21 (13.81)	15.95 ± 0.20 (16.08)
M3	5.18 ± 0.03 (5.16)	8.57 ± 0.07 (8.58)	11.92 ± 0.14 (11.96)	13.66 ± 0.20 (13.75)	15.97 ± 0.22 (16.10)
M4	5.27 ± 0.04 (5.32)*	8.06 ± 0.17 (8.23)	11.18 ± 0.32 (11.50)	14.22 ± 0.34 (14.22)	16.26 ± 0.23 (16.52)
M5	5.31 ± 0.02 (5.32)**	7.70 ± 0.22 (8.00)*	10.22 ± 0.42 (10.24)	13.57 ± 0.35 (13.20)	17.46 ± 0.24 (17.94)**

The asterisks indicate significant difference ($^{*}P < 0.01$ and $^{**}P < 0.001$).

Table 6: Fulton's condition factor (FCF), specific growth rate (SGR), inhibition factor (I), average (A), and standard error of mean (SEM) of C-control, CS-control with solvent, M1, M2, M3, M4, and M5—mixture of ibuprofen, diclofenac, and carbamazepine on concentration of 0.02, 0.2, 2, 20, and 60 μg·L^{-1}, respectively.

	C A ± SEM	CS A ± SEM	M1 A ± SEM	M2 A ± SEM	M3 A ± SEM	M4 A ± SEM	M5 A ± SEM
FCF_{35}	1.07 ± 0.01	1.07 ± 0.01	1.11 ± 0.01	1.12 ± 0.02	1.07 ± 0.02	1.06 ± 0.01	1.15 ± 0.02**
SGR	13.52	13.70	14.01	13.68	13.59	13.62	14.49
I (%)	1.31		−2.26	0.15	0.80	0.58	−5.77

The asterisks indicate significant difference ($^{*}P < 0.01$ and $^{**}P < 0.001$).

frequency of its detection increased in general with the mixture concentration. The predominance of antagonism in the higher concentration range might be explained by a potential competitive inhibition between two pharmaceuticals acting in the same way. Additivity was observed only in the 37% of the mixtures and synergism was the rarest type of interaction that was obtained only in 4% of the cases [19]. Mixture toxicity of the compounds could be accurately predicted using the concept of concentration addition. Evaluation of the ecotoxicity of DCF, IBU, naproxen, and acetylsalicylic acid using acute *Daphnia* and algal tests showed that toxicity of the mixture was considerable, even at concentrations at which the single substances showed no or only very slight effects, with some deviations in the *Daphnia* test [30].

In our experiment, hatching success was not disrupted by mixture of IBU, DCF, and CBZ up to concentration of 60 μg·L^{-1}. Similarly, embryonic exposure to mixture of CBZ, acetaminophen, gemfibrozil, and venlafaxine at concentrations of 0.5 and 10 μg·L^{-1} or diluted wastewater treatment effluent (5% and 25%) did not affect hatching success of zebrafish (*Danio rerio*) [31]. In hatching, there were some differences between vulnerability of fish species. While there was recorded delay in hatching time after exposure of zebrafish embryos to DCF at 1, 1.5, and 2 mg·L^{-1} [32, 33], no negative effects of DCF up to concentration of 3 mg·L^{-1} on the hatching and viability of carp embryos were found [34]. Consequences of long-term exposure to DCF up to 3 months were evaluated in a freshwater medaka fish (*Oryzias latipes).* Exposure to 0.001–10 mg·L^{-1} resulted in significant decreasing trend in hatching success and delay in hatch. The hatching of the eggs produced from the fish exposed to 10 mg·L^{-1} was completely interfered [35]. Parental exposure to as low as 0.1 μg·L^{-1} IBU in *Oryzias latipes* delayed hatching of eggs even when they were transferred to and cultured in clean water. Delayed hatching is environmentally relevant because this may increase the risk of being predated [36]. Nanoinjection of DCF and CBZ into medaka embryos clearly decreased their hatchability and some doses delayed the

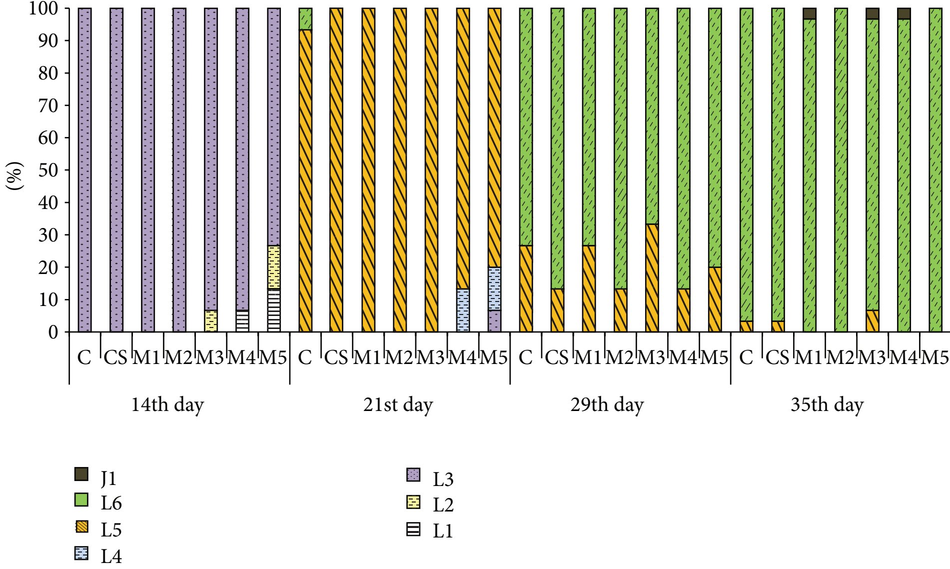

Figure 3: Representation of development stages in experimental groups C-control, CS-control with solvent, M1, M2, M3, M4, and M5—mixture of ibuprofen, diclofenac, and carbamazepine concentration of 0.02, 0.2, 2, 20, and 60 μg·L^{-1}, respectively, on the 14th, 21st, 29th, and 35th day of experiment.

hatching time [37]. Memmert et al. [38] conducted early life stage test with rainbow trout and zebrafish. Fish were affected by DCF in concentration range: 3.2–1000 μg·L^{-1}. Hatching rate at all tested concentrations did not differ significantly from the control in both fish species.

While mortality in M1, M2, M3, and M4 groups was comparable with control, situation in group M5 at the end of our experiment was different and mortality increase was significant ($P < 0.001$). Early life stage parameters such as egg and embryo mortality did not show significant differences in comparison with control group after exposure to diclofenac (1–2000 μg·L^{-1}) and its solvent DMSO [32]. Embryo mortality was elevated with exposure to 10 μg·L^{-1} of mixture of CBZ, acetaminophen, gemfibrozil, and venlafaxine [38]. The effect of IBU on medaka increased with duration of exposure. Survival of adult fish (120 dph—days after hatching) exposed to IBU as little as 1 μg·L^{-1} was significantly less than in the controls, while survival of fry (7 dph) was not affected even at the maximum test concentration of 1000 μg·L^{-1} [36]. There were no significant changes in survival for CBZ and IBU (up to 1000 μg·L^{-1}) in fathead minnow at 28 days after hatching [39].

The rate at which fish grow depends on a number of factors including species, age, genetic potential, water temperature, health, and quantity and quality of food. Mixture of drugs used in this experiment at concentrations of 0.02, 0.2, 2, and 20 μg·L^{-1} did not have any effect on the growth parameters of tench after 35 days of exposure, but the concentration of 60 μg·L^{-1} affected weight and length of tench significantly. Surprisingly, fish growth was not retarded but boosted. Furthermore, FCF of tench exposed to 60 μg·L^{-1} mixture of IBU, DCF, and CBZ increased more significantly than control fish after 35 days. However, there was the highest ($P < 0.001$) mortality in experimental group M5 and amount of fish in aquaria could also affect fish growth. Exposure to amiodarone and clozapine in early life stage toxicity test on fathead minnow (*Pimephales promelas*) also resulted in a significant increase in growth at concentrations of 1020 and 30.8 μg·L^{-1}, respectively [39]. Generally, stress conditions such as polluted aquatic environment result in fish growth decrease. Stepanova et al. [34] studied impact of DCF in early life stages of common carp after 30 days of exposure. They did not find any effect on the growth up to concentration of 3 mg·L^{-1}. After 144 days of exposure to IBU (0.01–1000 μg·L^{-1}), the length, weight, and condition factors of surviving adults of medaka were not affected [36]. Generally, stress conditions such as polluted aquatic environment result in fish growth decrease. Retarded growth of zebrafish in concentrations of DCF above 1.5 mg·L^{-1} [33] was recorded. Neither length nor weight of rainbow trout was affected by DCF up to 1000 μg·L^{-1} [38]. CBZ and IBU did not result in any significant changes in fathead minnow growth at concentrations of 62.5, 125, 250, 500, and 1000 μg·L^{-1} [39]. Chronic toxic effects of CBZ on rainbow trout were investigated. Fish were exposed to sublethal concentrations for 42 days. Compared with the control, there was a significantly lower ($P < 0.05$) FCF in fish exposed to the highest concentration of 2.0 mg·L^{-1}, but FCF in fish exposed to 1.0 μg·L^{-1} and 0.2 mg·L^{-1} were not changed significantly [40].

The early stages of embryonic development in fish generally exhibit a high incidence of malformations. These commonly include deformities of head and spinal column, ocular deformities, or yolk-sac resorption abnormalities. The natural background level of embryonic malformations is generally expected to be less than 10% [41]. In our

experiment we recorded almost 14% of malformations in M5 group at the 14th and 29th days of exposure. We suppose that such occurrence of malformations is consequence of exposure to high concentration ($60\,\mu g{\cdot}L^{-1}$) of pharmaceuticals. Most common were ocular malformations and lower jaws defects. Alimentary canal defects and scoliosis occurred rarely. Exposure to DCF (more than $1.5\,mg{\cdot}L^{-1}$) resulted in yolk-sac and tail deformations of zebrafish [33]. Exposure of embryos to mixture of CBZ, acetaminophen, gemfibrozil, venlafaxine ($10\,\mu g{\cdot}L^{-1}$), or diluted wastewater treatment effluent (25%) significantly increased the incidence of developmental abnormalities. This increase was primarily caused by an increase in the occurrence of yolk-sac edema [38]. *In ovo* nanoinjection of diclofenac (12 ng DCF egg^{-1}) and carbamazepine (12 ng CBZ egg^{-1}) caused significant impairments of embryonic development [37]. Early developmental stages of fathead minnow fish exposed to mixture of drugs (DCF, triclosan, naproxen, gemfibrozil, IBU, salicylic acid, and acetaminophen) at concentrations of 100 and $300\,ng{\cdot}L^{-1}$ showed a significant increase in yolk-sac abnormalities, eye deformities and hemorrhaged embryos, and spinal deformities in comparison with control [42]. It seems that early life stages of fish are particularly vulnerable to damage caused by pharmaceuticals.

Study of chronic histopathological effects of DCF on rainbow trout (*Oncorhynchus mykiss*) revealed that 28 days of exposure resulted in renal lesions and alterations to the gills at a concentration of $5\,\mu g{\cdot}L^{-1}$ [43]. The lowest observed effect concentration (LOEC) of $1\,\mu g{\cdot}L^{-1}$ for induction of cytological alterations in liver, kidney, and gills in rainbow trout [44] was reported. Similar results have been found in brown trout (*Salmo trutta* f. *fario*). Exposure to DCF in concentration ranges commonly found in the environment can result in adverse effects in various organs, especially kidney or gill [45]. Adverse effects of DCF on kidney and villi in the intestine from concentration of $1\,\mu g{\cdot}L^{-1}$ after 21 days of exposure in rainbow trout were recorded [46]. CBZ at 0.5 and $10\,\mu g{\cdot}L^{-1}$ caused altered ovarian histology in female zebrafish. Six-week -long exposure to CBZ also significantly altered kidney proximal tubule morphology but did not change liver histology [47]. Upper described impacts of single pharmaceutical to histopathology of fish organs are in contrast with our findings, where either concentration of $60\,\mu g{\cdot}L^{-1}$ of mixture of IBU, DCF, and CBZ did not cause any histopathological changes in liver, kidney, skin, or gill. Our results are in compliance with many other studies [34, 36, 38], which focused on the effect of different drugs (DCF, IBU) on different fish species (carp, medaka, and rainbow trout) and their organs (kidney, liver, and gonad) and did not find any tissues alterations.

The development of carp appeared to be delayed due to DCF exposure at the beginning of the early life stages test, but there was no difference after 30 days of exposure [34]. Similar trends occurred also in our experiment. After 14 days of exposure to mixture of drugs, there were all larvae in L3 stage up to concentration of $0.2\,\mu g{\cdot}L^{-1}$. Along with increase of mixture concentration (2, 20, and $60\,\mu g{\cdot}L^{-1}$) count of larvae of L1 and L2 stages was increased. Similarly, slightly delayed development occurred after 21 days of exposure. However, fish after 29 and 35 days of exposure had similar development.

DCF and CBZ affect fish behavior through different mechanisms. Feeding behavior of adult Japanese medaka fish was affected by exposure to CBZ and DCF, while swimming speed was altered only by exposure to CBZ [48]. The ability of hatched larvae to swim upward was affected after embryonic injection with DCF and CBZ. The number of larvae of medaka fish failing to swim upward significantly increased with the higher doses [37]. Rainbow trout affected by DCF up to $1000\,\mu g{\cdot}L^{-1}$ showed normal behavior [38], as well as in our experiment.

5. Conclusion

In summary, this paper assesses subchronic toxicity of human pharmaceuticals mixture in embryos and larvae of tench by analysing mortality, hatching success, growth, early ontogeny, histopathological changes, and incidence of malformations. Our results lead us to conclude that environmental concentrations (0.02 and $0.2\,\mu g{\cdot}L^{-1}$) of mixture of ibuprofen, diclofenac, and carbamazepine do not have an adverse effect on tench. The highest used concentration ($60\,\mu g{\cdot}L^{-1}$ for each substance) of that mixture significantly elevated mortality, delayed development of larvae, and increased occurrence of malformations. The parameters analyzed provide useful information about the effect of commonly occurred human pharmaceuticals in water environment on fish.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgment

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Research Article

Norfloxacin—Toxicity for Zebrafish (*Danio rerio*) Focused on Oxidative Stress Parameters

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The aim of the study was to investigate the effects of subchronic exposure of zebrafish (*Danio rerio*) to a fluoroquinolone norfloxacin, using selected oxidative stress parameters as a target. Toxicity tests were performed on zebrafish according to the OECD Guidelines number 203 and number 215. In the Subchronic Toxicity Test, a significant ($P < 0.01$) increase in the activity of glutathione peroxidase, glutathione S-transferase, and catalase was found. In the test, norfloxacin did not affect lipid peroxidation and catalytic activity of glutathione reductase. From the results, we can conclude that norfloxacin has a negative impact on specific biochemical processes connected with the production of reactive oxygen species in fish tested.

1. Introduction

Fluoroquinolone antibacterial agents are widely used for the treatment of various infections, especially against gram-negative bacteria [1]. Although this type of use leads to an entry of these compounds into the environment through the excretion of unmetabolised quinolones and the disposal of unused drugs, the main source of aquatic compartments pollution with these drugs is their use in aquaculture [2].

Fluoroquinolones are among the antimicrobial chemotherapeutics frequently detected in the aquatic environment in relatively high concentrations ranging from ng L^{-1} to $\mu g\ L^{-1}$. Their ubiquitous presence has been reported, for example, in waste water treatment plant influents [3], as well as groundwater [4], surface waters [5], and even in drinking water [6].

These drugs are rather resistant to microbial degradation [7] and may persist in water bodies because of their strong sorption properties. Photodegradation is expected to play an important role in fluoroquinolone fate in some sunlit surface waters [8]. Also chemical oxidation may be significant for their degradation [9].

Norfloxacin is a synthetic chemotherapeutic agent usually used to treat urinary tract infections [10, 11]. Norfloxacin belongs to the third generation of quinolones. The mechanism of norfloxacin action is the inhibition of DNA gyrase (a type II topoisomerase), which is an essential bacterial enzyme [12]. NOEC (no observed effect concentration) for norfloxacin was determined to be 10.38 $\mu g\ L^{-1}$ by long-term bioluminescence inhibition assay with *V. fischeri* [2]. The EC_{50} and NOEC of this compound for *Chlorella vulgaris* are 10.4 and 4.1 $mg\ L^{-1}$, respectively [13].

The aim of this study was to investigate the subchronic effect of norfloxacin on zebrafish (*Danio rerio*). For the determination of norfloxacin effects, selected oxidative stress parameters such as glutathione peroxidase, glutathione reductase, glutathione S-transferase, catalase, and lipid peroxidation were used as a target.

2. Materials and Methods

In the study, two tests of norfloxacin were performed. As the model organism, zebrafish (*Danio rerio*) was used. The first test was performed according to the OECD Guideline number 203 (Fish, Acute Toxicity Test) [14] and the second one was performed according to the OECD Guideline number 215 (Fish, Juvenile Growth Test) [15].

2.1. Acute Toxicity Test. Acute Toxicity Test was performed according to OECD number 203. Norfloxacin of ≥98.0% chemical purity (Sigma-Aldrich, Czech Republic) was dissolved in water with the addition of solvent, dimethyl sulfoxide (DMSO). The final concentration of DMSO was 0.1% in each test aquarium. For the test, we used five ascending concentrations of norfloxacin (0.05, 1, 5, 10, and 30 mg L^{-1}), all in a duplicate and two control groups (only water and water with a solvent). After 7 days of acclimatization, ten juvenile fish (aged 30 days) were placed in each 4 liter glass aquarium. A total of 120 zebrafish were used in the test. Food had been withheld 72 hours before the test; during the test fish were not fed. The test was performed using a semistatic method with the solutions renewed every 24 hours. Fish conditions and a number of dead fish were checked every 24 hours. Duration of the test was 96 hours. Water temperature, pH, and oxygen saturation were monitored every 24 hours and were as follows: temperature 23 ± 0.5°C, oxygen concentrations above 60% (ranged from 85% to 96%), and pH ranged from 8.28 to 8.61.

Water samples were collected for the measurement of the real concentration of norfloxacin in water every 48 hours. The concentrations of the test substance were measured using HPLC with photometric detection. Norfloxacin concentration did not fall under 90% of the nominal value.

The aim of the acute toxicity test was to determine the range of concentrations for a subsequent Subchronic Toxicity Test.

2.2. Subchronic Toxicity Test. Subchronic Toxicity Test of norfloxacin was performed according to OECD Guideline number 215. In the test, juvenile (30 days old) zebrafish (*Danio rerio)* were used. Fish had been acclimatized for 14 days before the test started.

Norfloxacin was dissolved in water using DMSO in volume of 2 μL L^{-1} as a solvent. Fish were exposed to five ascendant sublethal concentrations, that is, 0.0001 (an environmental concentration), 0.1, 1, 10, and 30 mg L^{-1} (all in a duplicate). In the test, two control groups (the first one with only water, the second one with water and solvent, DMSO) were used. Fish were randomly distributed into 30 liter glass aquaria, 50 specimens in each aquarium. A total of 600 fish were used in the test.

The experiment was conducted in a flow-through system with a test solution renewal every twelve hours. During the test, water condition parameters were monitored at 24-hour intervals. Water quality values were measured as follows: temperature 23.7–24.6°C, oxygen saturation above 60% (ranged between 80% and 94%), and pH 8.19–8.60. In the course of the test, fish were fed with dried *Artemia salina* without shells at 8% of body weight per day. The food ration was based on an initial fish weight and recalculated after 14 days of the test. Duration of the test was 28 days.

Water samples were collected every 7th day to measure real concentrations of norfloxacin in water. Analyzed concentrations of norfloxacin in water were found to be above 92% of the nominal concentrations in the course of subchronic test, which is in accordance with the test validation criteria.

At the end of the test, fish were euthanased and then weighed. Their tank-average specific growth rates were determined. Tank-average specific growth rates were calculated using the following formula according to OECD method number 215:

$$r = \frac{\overline{\log_e W_2} - \overline{\log_e W_1}}{t_2 - t_1} * 100, \quad (1)$$

where r is the tank-average-specific growth rate; W_1, W_2 are the weights of a particular fish at times t_1 and t_2, respectively; $\overline{\log_e W_1}$ is the average of the logarithms of the values W_1 for the fish in the tank at the start of the study period; $\overline{\log_e W_2}$ is the average of the logarithms of the values W_2 for the fish in the tank at the end of the study period; t_1, t_2 is the time (days) at the start and end of the study period.

2.3. Fish Sampling and Homogenization. At the end of the Subchronic Toxicity Test, fish were euthanased by approved anesthetic (MS222). Body weight and length of each fish were recorded. Fish were frozen and stored at −85°C until homogenization. Whole body samples were weighed and homogenized (1 : 10 w/v) using a phosphate buffer (pH 7.2). The homogenate was then divided into two parts. The first one was used for the measurement of thiobarbituric acid reactive substances (TBARS), the second was centrifuged (10,500 g at 4°C for 20 min), and the obtained supernatant fraction was then used for the determination of catalytic activities glutathione peroxidase (GPx), glutathione reductase (GR), glutathione S-transferase(GST), and catalase (CAT).

2.4. Measurement of Oxidative Stress Parameters. To determine a lipid peroxidation in fish samples, the TBARS (thiobarbituric acid reactive substances) method described by Lushchak et al. (2005) was used. TBARS were measured at 535 nm and the concentration was expressed in nmol of TBARS per gram of tissue wet weight [16].

The catalytic activity of glutathione peroxidase and glutathione reductase was determined spectrophotometrically at 340 nm by catalysis conversion of oxidized glutathione (GSSG) to reduced glutathione (GSH) for the consumption of NADPH. The specific activities were expressed as the nmol of NADPH consumption per min per mg of protein [17].

Total catalytic activity of glutathione S-transferase was determined by the measurement of the conjugation of 1-chloro-2,4-dinitrobenzene with reduced glutathione (GSH) at 340 nm [18]. The specific activity was expressed in the units of nmol of formed product per min per mg of protein.

For the assessment of GST and GR catalytic activities, the concentration of proteins was determined by Bicinchoninic Acid Protein Assay Kit (Sigma-Aldrich, St. Louis, MO), in which bovine serum albumin was used as a standard [19].

The catalytic activity of catalase was determined by a spectrophotometrical measurement of H_2O_2 breakdown at 240 nm. The specific activity of the enzyme was expressed as μmol of decomposed H_2O_2 per min per mg of protein [20].

All spectrophotometric measurements were performed using the Varioskan Flash Spectral Scanning Multimode Reader (Thermo Fisher Scientific Inc., USA).

2.5. Determination of Norfloxacin Concentration in Water. Measurement of norfloxacin was based on high-performance liquid chromatography coupled with triple quadrupole tandem mass spectrometry (LC-ESI-MS/MS). Samples were filtered and used for LC-ESI-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, CA, USA) equipped with heated electrospray ionization (HESI-II) probe. A Thermo Scientific Hypersil C_{18} (2.1 mm × 50 mm, 1.9 μm) column was used at a constant flow rate of 300 $\mu L\,min^{-1}$. Mobile phase consisted of water containing 0.1% formic acid (v/v) (solvent A) and acetonitrile containing 0.1% formic acid (solvent B). The full loop injection volume of the tissue extract was set at 20 μL. The heated electrospray ionization was operated in the positive-ion mode under specific conditions: standard of norfloxacin was purchased from Sigma-Aldrich (St. Louis, MO). All solvents were residual analysis purity (Chromservis, Ltd., CZ). Coefficient of variation for between-series was 3.1%. The limit of detection was 91 ng L^{-1}.

2.6. Statistical Analysis. Oxidative stress biomarkers were tested for a normal distribution using the Shapiro-Wilk test. After testing for homogeneity of variance across groups, an analysis of variance (one-way ANOVA) was used. The differences among test groups were assessed with the Tukey-HSD test with $P < 0.05$ as the level of significance.

2.7. Ethical Statement. All experimental procedures were approved by the institutional committee and performed in a compliance with institutional guidelines and national legislation (Act number 246/1992 Coll., on the Protection of Animals Against Cruelty, as amended).

3. Results

No significant difference was found between the results of control group with only water and the control one with water and the addition of a solvent (DMSO). Therefore, we used only one average value for the control group.

3.1. Mortality of Fish in Subchronic Test. Mortality of juvenile fish was found to be less than 8% in all experiment groups. In the control group, mortality did not exceed 4%, which is in an agreement with the validation criteria of the juvenile growth test.

3.2. Growth Rate. At the end of the Subchronic Toxicity Test, all fish were weighed and their length was measured. Statistical analysis of the somatic parameter was then performed.

The specific growth rate r was calculated for all tested concentrations and control. No significant differences were found among the groups tested (Figure 1).

3.3. Effect of Norfloxacin on Biotransformation and Antioxidant Enzymes. An increase in glutathione peroxidase (GPx) activity was found in fish exposed to all concentrations of

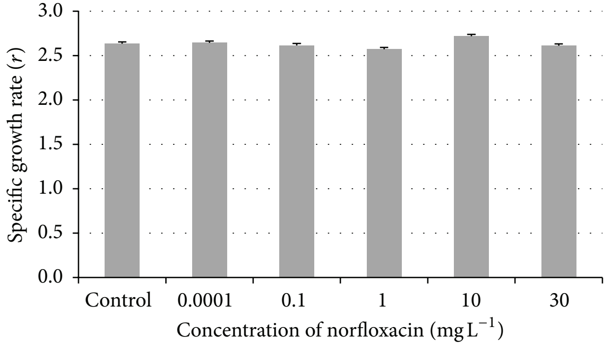

Figure 1: Zebrafish specific growth rate (r) in subchronic toxicity test (values in mean ± SEM); SEM = standard error of mean.

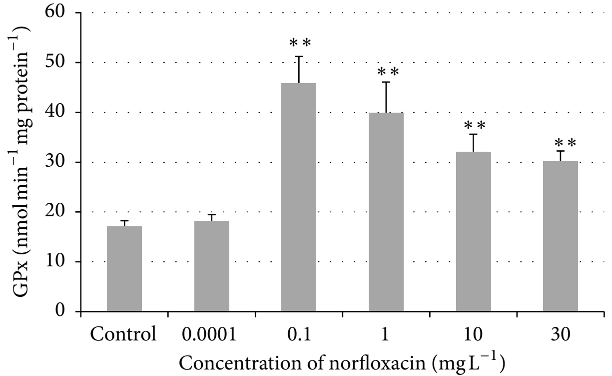

Figure 2: Glutathione peroxidase (GPx) activity in zebrafish exposed to norfloxacin (values in mean ± SEM); SEM = standard error of mean; ∗∗ = a significant increase in GPx activity ($P < 0.01$).

norfloxacin (0.0001, 0.1, 1, 10, and 30 mg L^{-1}) compared to the control group (Figure 2). But only in the groups exposed to norfloxacin concentrations of 0.1, 1, 10, and 30 mg L^{-1} (45.86, 39.92, 32.13, and 30.24 nmol min^{-1} mg $protein^{-1}$) GPx activity was significantly higher ($P < 0.01$) compared to the control group (17.16 nmol min^{-1} mg $protein^{-1}$). The increase in the activity of glutathione reductase (GR) was found in norfloxacin concentrations of 0.0001, 0.1, 1, and 30 mg L^{-1} (11.33, 12.96, 13.41, and 12.18 nmol min^{-1} mg $protein^{-1}$) compared to the control group (10.59 nmol min^{-1} mg $protein^{-1}$), but the increase did not reach significance (Figure 3).

The activity of glutathione S-transferase (GST) increased in all tested groups exposed to 0.0001, 0.1, 1, 10, and 30 mg L^{-1} (163.16, 137.49, 147.64, 152.06, and 145.03 nmol min^{-1} mg $protein^{-1}$) of norfloxacin compared to the control group (131.86 nmol min^{-1} mg $protein^{-1}$). The highest and significantly different ($P < 0.01$) value of GST activity was found in the environmental concentration 0.0001 mg L^{-1} compared to the control group (Figure 4).

A significant ($P < 0.01$) increase in catalase (CAT) activity was found in all tested concentrations compared to the control group (Figure 5). In the concentrations of 0.0001, 0.1, 1, 10, and 30 mg L^{-1}, catalase activity was found to be 128.29, 128.37, 149.35, 127.53, and 145.96 μmol min^{-1} mg

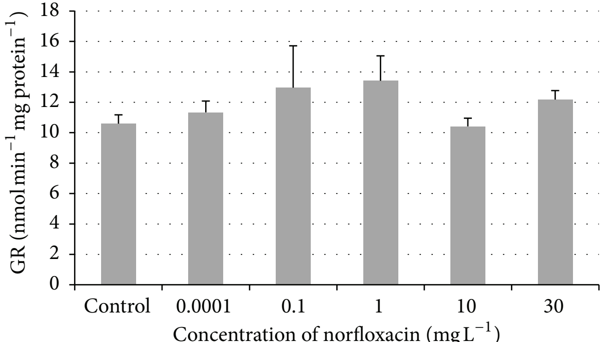

Figure 3: Glutathione reductase (GR) activity in zebrafish exposed to norfloxacin (values in mean ± SEM); SEM = standard error of mean.

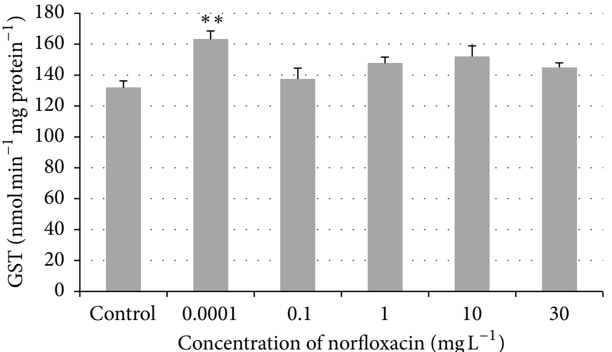

Figure 4: Glutathione S-transferase (GST) activity in zebrafish exposed to norfloxacin (values in mean ± SEM); SEM = standard error of mean; ∗∗ = a significant increase in GST activity ($P < 0.01$).

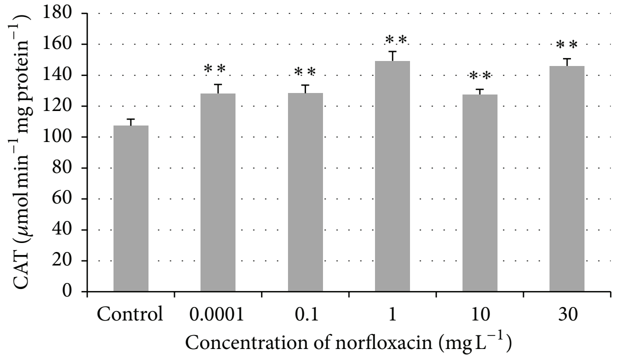

Figure 5: Catalase (CAT) activity in zebrafish exposed to norfloxacin (values in mean ± SEM); SEM = standard error of mean; ∗∗ = a significant increase in CAT activity ($P < 0.01$).

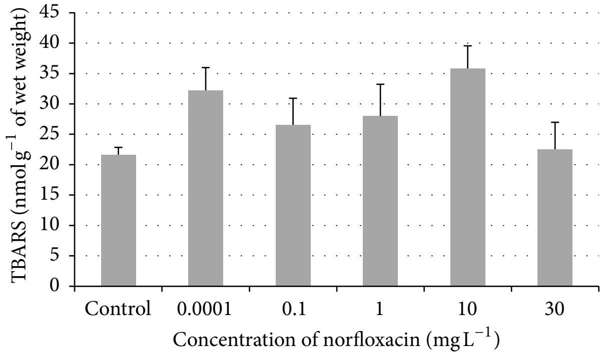

Figure 6: TBARS concentrations in zebrafish in Subchronic Toxicity Test (values in mean ± SEM); SEM = standard error of mean.

protein^{-1}. In the control group, its activity was found to be 107.36 μmol min^{-1} mg protein^{-1}.

3.4. Effect of Norfloxacin on Lipid Peroxidation (TBARS). No significant difference was found in all tested concentrations –0.0001, 0.1, 1, 10, and 30 mg L^{-1} (32.22, 26.56, 28.01, 35.86, and 22.54 nmol g^{-1} of wet weight) of norfloxacin when compared to the control group (21.63 nmol g^{-1} of wet weight). In our study, norfloxacin did not affect the lipid peroxidation (Figure 6).

4. Discussion

In the literature, there is little information on the effect of norfloxacin (or other fluoroquinolones) on fish and other water organisms; therefore, we had to compare our results with the effect of other pharmaceuticals on fish.

In our study, we did not find any significant differences in growth rates in zebrafish exposed to all norfloxacin concentrations when compared to the control group. These results disagree with the findings of Nie et al.'s study [21], in which the effect of norfloxacin on the freshwater microalga (*Scenedesmus obliquus*) was evaluated. Nie et al. proved that the concentrations used in the test (0.0–60.0 mg L^{-1}) inhibited growth rates of tested alga, which can be probably explained as a direct toxic effect of norfloxacin, an antimicrobial agent, to unicellular alga. The results of his study demonstrated that the concentration resulting in 50% inhibition of algal growth rate after 48 hours of exposition (48 h IC_{50}) was found to be 38.49 mg L^{-1} [21]. In the study on the subchronic effect of diclofenac (a nonsteroidal anti-inflammatory drug, NSAID) on early stages of common carp (*Cyprinus carpio*), no effect on body weight and growth was found in experimental fish compared to control [22]. This result is in an agreement with our results. In the study of Zivna et al. [23], toxic effect of acetylsalicylic acid (NSAID) in the concentration range from 0.004 to 250 mg L^{-1} on zebrafish was tested and the increase in body weight and specific growth rate was found in all experimental groups compared to the control group.

Glutathione peroxidase (GPx) is an enzyme which transforms hydroperoxides to hydroxyl compounds using a reduced glutathione as a substrate. In our study, a significant increase ($P < 0.01$) was found in the activity of glutathione peroxidase in zebrafish exposed to norfloxacin at the concentrations of 0.1, 1, 10, and 30 mg L^{-1}. It can be explained by the presence of oxidative substances in cells, which may

cause an increase in antioxidant enzymes activities as a defense mechanism [24]. Environmental concentration of norfloxacin (0.0001 mg L^{-1}) did not cause an increase in the activity of GPx in our experiment. The increase in GPx activity was also found in the study performed on zebrafish exposed to ibuprofen (NSAIDs) in the concentrations of 0.05, 1, 8, and 25 mg L^{-1} [25].

Glutathione reductase (GR) is an enzyme catalyzing the conversion of glutathione disulfide to reduced glutathione. The main function of reduced glutathione is the protection of cells from chemical insult [26, 27]. In our study, we did not find significant differences in glutathione reductase activity in all concentrations tested. On the contrary, in the study of Zivna et al. [23], the exposure of zebrafish to acetylsalicylic acid in the concentrations of 0.004, 0.4, 40, 120, and 250 mg L^{-1} caused a significant increase in the activity of GR when compared to the control group.

The main function of glutathione S-transferase (GST) in the endogenous metabolism is the detoxification of xenobiotics and products of oxidative stress. GST provides cellular protection against toxic effects of a variety of environmental and endogenous chemicals [27]. The exposure of zebrafish to norfloxacin caused a nonsignificant increase in GST activity in all tested groups compared to the control group. A significant ($P < 0.01$) increase was found only in the fish exposed to environmental concentration (0.0001 mg L^{-1}). In freshwater microalga (*Scenedesmus obliquus*) exposed to norfloxacin in the concentration range between 0.0 and 60 mg L^{-1}, GST activity was found to be significantly increased at higher norfloxacin concentrations, reaching a peak value at 15 mg L^{-1} [21]. Oliveira et al. [28] investigated the effect of two antibiotics (oxytetracycline and amoxicillin) on zebrafish enzymes. GST activity was induced in muscle and gill samples. On the contrary, in head samples an inhibition trend was observed. In zebrafish exposed to oxytetracycline, GST activities were increased at almost all concentrations above 1 mg L^{-1} in muscle and liver samples. GST activity in zebrafish exposed to 40, 120, and 250 mg L^{-1} of acetylsalicylic acid in the study of Zivna et al. [23] was also found to be significantly higher. Stepanova et al. [22] studied the effects of diclofenac on early stages of common carp and found a significant increase in the GST activity at the highest tested concentration of diclofenac (3 mg L^{-1}).

Catalase is an enzyme that catalyzes the conversion of a potentially oxidative molecule, H_2O_2 into H_2O and O_2 [29]. In our study, significantly higher catalase activity was found in all tested concentrations (0.0001, 0.1, 1, 10, and 30 mg L^{-1}) compared to control. In the study of Wang et al., the toxicity of fluoroquinolone enrofloxacin associated with environmental stress in Tra catfish (*Pangasianodon hypophthalmus*) was tested. Fish were fed with pellets containing 1 g kg^{-1} enrofloxacin for 7 days. A 1.7 times increase in CAT activity in gills of the fish exposed to enrofloxacin was found in a low-density group (40 fish m^{-3}) contrary to one and three days after the end of exposure (day 8 and 10); CAT activity was significantly lower than in the control group [29]. In the study of Oliveira et al., amoxicillin at the highest concentration (221 mg L^{-1}) inhibited the activity of CAT in gills and head samples of zebrafish. Zebrafish exposed to oxytetracycline in the concentration of 25.0–100.0 mg L^{-1} exhibited a dose-dependent inhibition of CAT activity in head samples, whereas no alteration was observed in the other tissues analyzed [28].

The level of TBARS is used to measure the extent of lipid peroxidation. One of the major terminal products of lipid peroxidation is malondialdehyde [30, 31]. Lipid peroxidation has been a major contributor to the loss of cell function under oxidative stress [32]. In our study, we did not find any significant effect of norfloxacin on lipid peroxidation. In the study of Wang et al., in which the effect of enrofloxacin on Tra catfish (*Pangasianodon hypophthalmus*) in the relation to density stress was tested, lipid peroxidation in gills of enrofloxacin-fish reared at low (40 fish m^{-3}) or high (120 fish m^{-3}) density was significantly (more than 5-fold) higher than their respective control at day 7. On the contrary, lipid peroxidation in gills of enrofloxacin-fish reared at medium density (80 fish m^{-3}) was significantly, 3-fold lower than in the control fish [29]. Juvenile rainbow trout (*Oncorhynchus mykiss*) exposed to six ascending concentrations (5, 10, 15, 20, 25, and 30 mg L^{-1}) of carbamazepine (anticonvulsant drug) for 96 hrs showed an increase in lipid peroxidation ($P < 0.05$) in gill and brain when compared to the control group [33].

5. Conclusions

The results of our study indicate that norfloxacin does not affect the growth rate in zebrafish. Norfloxacin in the tested concentrations affects some biomarkers of oxidative stress (GPx, GST, and CAT). The subchronic exposure of zebrafish to norfloxacin causes the increase in the activities of some antioxidant and biotransformation enzymes. Based on these results, we can conclude that norfloxacin (even in environmental concentrations) may have a negative impact on some biochemical processes connected with the production of ROS (reactive oxygen species) in aquatic organisms.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

The Effect of Mycotoxin Deoxynivalenol on Haematological and Biochemical Indicators and Histopathological Changes in Rainbow Trout (*Oncorhynchus mykiss*)

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Deoxynivalenol (DON), produced by the *Fusarium* genus, is a major contaminant of cereal grains used in the production of fish feed. The effect of mycotoxin deoxynivalenol on rainbow trout (*Oncorhynchus mykiss*) was studied using a commercial feed with the addition of DON in a dose of 2 mg/kg feed. The fish ($n = 40$) were exposed to the mycotoxin for 23 days. The trout were divided into two groups, control and experimental groups. Control groups were fed a commercial feed naturally contaminated with a low concentration of DON (225 μg/kg feed); experimental groups were fed a commercial feed with the addition of DON (1964 μg/kg feed). Plasma biochemical and haematological indices, biometric parameters, and histopathological changes were assessed at the end of the experiment. The experimental groups showed significantly lower values in MCH ($P < 0.05$). In biochemical indices, after 23-day exposure, a significant decrease in glucose, cholesterol ($P < 0.05$), and ammonia ($P < 0.01$) was recorded in the experimental group compared to the control group. Our assessment showed no significant changes in biometric parameters. The histopathological examination revealed disorders in the caudal kidney of the exposed fish. The obtained data show the sensitivity of rainbow trout (*O. mykiss*) to deoxynivalenol.

1. Introduction

Mycotoxins are toxic secondary metabolites produced by different types of filamentous fungi. The most relevant mycotoxins to animal health and production are produced by *Aspergillus, Fusarium,* and *Penicillium* genera. Mycotoxins are thermally and chemically stable and this renders them resistant to feed manufacturing techniques [1].

Deoxynivalenol (DON), also known as vomitoxin, is a trichothecene mycotoxin produced by the *Fusarium* genus. Deoxynivalenol and zearalenone belong to the most prevalent mycotoxins produced by the *Fusarium* species [2]. Although DON is the least toxic type of trichothecene, it can cause significant harm to animals and humans [3].

Animal feed components and finished feedstuffs normally contain this mycotoxin, it is one of the most frequently found mycotoxin in cereal grains, such as wheat, barley, and corn [4].

Despite the fact that rainbow trout belongs to a carnivorous species, commercial salmonoid feeds contain plant components. Due to a decrease in the availability of fish meal for the production of aquaculture feeds, alternative protein sources need to be used in fish feed production. When we use more plant source ingredients in commercial feeds for farmed

fish, we increase the possibility that mycotoxins contaminate those feeds [5].

Deoxynivalenol causes a broad variety of toxic effects in animals, the toxicity is well recognized in mammals. The main effect at the cellular level is the inhibition of protein synthesis through it being bound to the ribosomal subunit [6]. Chronic oral exposure induces anorexia, decreased weight gain, reduction in feed conversion, gastrointestinal hemorrhaging, inflammation, and immune system alterations. The effects of deoxynivalenol depend on dose and duration of exposure, age, species, health, and nutritional status. There are differences in sensitivity to DON contaminated feed in fish. Rainbow trout (*O. mykiss*) is extremely sensitive [7].

2. Materials and Methods

2.1. Animals. The experiment was carried out on one-year-old rainbow trout (*O. mykiss*) obtained from a commercial fish farm. Groups of ten fish were randomly distributed into four tanks of 200 L volume with dechlorinated tap water. The test was performed using a flow-through system with the bath exchanged every 12 h and individually aerated. A photoperiod regime of 12 h light : 12 h dark was used. Trout were acclimated for two weeks and during this acclimation were supplied twice a day with commercial pellets (BioMar, Denmark) at a total rate of 1% of body weight.

2.2. Experimental Diet. The control fish were fed a commercial diet (BioMar, Denmark) containing rapeseed oil, blood meal, fish meal, soya cake, sunflower cake, rapeseed meal, horse beans, wheat, soya concentrate, fish oil, pea proteins, vitamins, and minerals.

The experimental diet was prepared by adding DON to commercial pellets in several separate steps. The amount of 32.50 g of Eudragit E (Basic Butylated Methacrylate Copolymer) was dissolved in 227.50 g of acetone on the electromagnetic stirrer for a period of 60 minutes (solution A). 60 mL of this solution was put aside (solution B). Into each of the three vials containing 5 mg of DON, 10 mL from the solution B was injected for the reconstitution of DON. Next, the dissolved content of these vials was added to the original solution A. The vials were then rinsed with the rest of solution B (10 mL for each vial) and solutions (A and B) were mixed together. The resultant common solution was divided into the two equal parts with the weight of 130 g that is equivalent to 7.5 mg of DON.

2470.75 g of pellets and 13.00 g of AEROSIL were added to a cubic blender and mixed for 5 minutes at 40 rpm ("Blend A"). 130 g of solution with a content of 7.5 mg of DON was uniformly and carefully poured onto the surface of mixed excipients and this moistened mixture was kneaded for 5 minutes at 40 rpm. The same procedure was performed with the "Blend B". The final mixtures were placed in a hot air dryer and dried at 50°C for 4 hours.

The polymer forms a specific layer on the surface of the pellets, which is formulated from the solid dispersion of the active ingredient fixed in a polymer. As a result, it is assumed that there is a highly uniform content of active substance in each of the individual pellets.

Table 1: Mycotoxin concentration in control and experimental feed.

Mycotoxin contamination μg/kg	Control feed	Experimental feed
Deoxynivalenol	225	1964
3-Acetyldeoxynivalenol	ND[1]	ND
15-Acetyldeoxynivalenol	ND	ND
Diacetoxyscirpenol	ND	ND
Fumonisin B_1	ND	ND
Fumonisin B_2	ND	ND
HT-2 toxin	ND	ND
T-2 toxin	ND	ND
Nivalenol	ND	ND
Ochratoxin A	ND	ND
Zearalenone	ND	1

[1]ND: not detectable.

2.3. Analysis of Mycotoxin. The contents of deoxynivalenol, 3-acetyldeoxynivalenol, 15-acetyldeoxynivalenol, diacetoxyscirpenol, fumonisin B_1 and B_2, HT-2 toxin, T-2 toxin, nivalenol, ochratoxin A, and zearalenone in control and experimental feed were analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) by Metrology and Testing Laboratory (Institute of Chemical Technology, Prague). The analyzed mycotoxin contents are described in Table 1.

2.4. Experimental Protocol. Fourty fish were divided into control ($n = 20$) and experimental ($n = 20$) groups. Control groups were twice a day fed the commercial diet used during the initial acclimation period at a total rate of 1% of body weight. Experimental groups were fed with mycotoxin-contaminated feed at a dose of 2 mg/kg feed. The samples were taken after 23 days from the beginning of the experiment. Water temperature during the test ranged from 14.0 to 15.1°C, pH ranged from 7.9 to 8.2, and oxygen saturation of the water ranged from 80.5 to 95.2%. The physicochemical parameters of the water used in the test during the experiment were total ammonia 0.1–0.5 mg/L, NO_3^- 20–30 mg/L, NO_2^- 0.1–0.4 mg/L, and Cl^- 20–25 mg/L.

2.5. Haematology Profile. Blood samples were taken from each fish by puncturing the caudal vessel and stabilized with sodium heparin (50 IU per 1 mL of blood). Heparinized blood samples were used for the evaluation of haematological indicators including erythrocyte count (RBC), haemoglobin concentration (Hb), hematocrit (PCV), mean erythrocyte volume (MCV), mean erythrocyte haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), and leukocyte count (WBC). Samples were determined according to Svobodova et al. [8].

2.6. Biochemical Profile. For biochemical analysis, a part of heparinized blood, after centrifugation at 855 g for 10 min

Table 2: Values of haematological indices 23 days after the beginning of the experiment.

Indices	Control mean ± SD (n = 20)	Experimental mean ± SD (n = 20)
RBC ($T \cdot L^{-1}$)	1.41 ± 0.36	1.58 ± 0.41
Hb ($g \cdot L^{-1}$)	76.94 ± 12.78	69.49 ± 12.92
PCV ($L \cdot L^{-1}$)	0.30 ± 0.06	0.29 ± 0.05
MCV (fL)	228.14 ± 66.62	198.26 ± 53.05
MCH (pg)	57.86 ± 16.26	46.93 ± 14.12*
MCHC ($g \cdot L^{-1}$)	250.31 ± 27.58	235.67 ± 22.97
WBC ($G \cdot L^{-1}$)	12.13 ± 4.97	14.23 ± 3.58

Significant difference between test groups ($^{*}P < 0.05$).
RBC: erythrocyte count, Hb: haemoglobin concentration, PCV: haematocrit, MCV: mean erythrocyte volume, MCH: mean erythrocyte haemoglobin, MCHC: mean corpuscular haemoglobin concentration, WBC: leukocyte count.

Table 3: Values of biochemical parameters 23 days after the beginning of the experiment.

Indices	Control mean ± SD (n = 20)	Experimental mean ± SD (n = 20)
ALB ($g \cdot L^{-1}$)	15.69 ± 2.90	15.25 ± 2.74
TP ($g \cdot L^{-1}$)	37.45 ± 5.77	36.38 ± 3.98
GLU ($mmol \cdot L^{-1}$)	4.84 ± 0.79	4.36 ± 0.48*
NH_3 ($\mu mol \cdot L^{-1}$)	398.14 ± 75.85	280.79 ± 57.99**
TRIG ($mmol \cdot L^{-1}$)	1.90 ± 0.40	1.63 ± 0.68
LACT ($mmol \cdot L^{-1}$)	2.62 ± 0.98	2.10 ± 0.69
CHOL ($mmol \cdot L^{-1}$)	6.50 ± 1.43	5.54 ± 1.12*
ALP ($\mu kat \cdot L^{-1}$)	1.64 ± 0.78	1.40 ± 0.69
ALT ($\mu kat \cdot L^{-1}$)	0.33 ± 0.12	0.42 ± 0.28
AST ($\mu kat \cdot L^{-1}$)	7.66 ± 2.35	7.71 ± 1.85
LDH-L ($\mu kat \cdot L^{-1}$)	16.57 ± 6.50	16.77 ± 6.81
Ca^{2+} ($mmol \cdot L^{-1}$)	2.32 ± 0.15	2.32 ± 0.16
PHOS ($mmol \cdot L^{-1}$)	3.49 ± 0.41	3.67 ± 0.42

Significant difference between test groups ($^{*}P < 0.05$; $^{**}P < 0.01$).
ALB: albumins, TP: total proteins, GLU: glucose concentration, NH_3: ammonia, TRIG: triacylglycerols, LACT: lactate, CHOL: cholesterol, ALP: alkaline phosphatase, ALT: alanine aminotransferase, AST: aspartate aminotransferase, LDH: lactate dehydrogenase, Ca^{2+}: calcium, PHOS: inorganic phosphate.

at cooled centrifuge (4°C), was used. Biochemical parameters including albumins (ALB), total proteins (TP), glucose (GLU), ammonia (NH_3), triacylglycerols (TRIG), lactate (LACT), cholesterol (CHOL), alkaline phosphatase (ALP), alanine aminotransferase (ALT), aspartate aminotransferase (AST), lactate dehydrogenase (LDH), calcium (Ca^{2+}), and inorganic phosphate (PHOS). Plasma biochemical indicators were measured using a biochemical automatic analyzer Konelab 20i (ThermoScientific, Czech Republic) and commercial test kits (BioVendor, Czech Republic).

2.7. Biometric Parameters. After blood sampling, the fish were stunned with a blow to the head and killed by spinal transection. Then, the biometrical indices were defined (total and standard length, body and liver weight) from which there were derived and calculated somatic parameters such as the Fultons condition factor and the hepatosomatic index. The Fultons condition factor (CF) was calculated using the formula CF = (body weight [g]/standard length [cm^3]) × 100. The hepatosomatic index (HSI) was calculated using the following formula: HSI = liver weight/body weight × 100.

2.8. Histological Examination. The samples of gills, skin, liver, cranial, and caudal kidney and spleen of ten fish from each group were immediately fixed in buffered 10% neutral formalin. The samples were later dehydrated, embedded in paraffin wax, sectioned on a microtome at a thickness of 4 μm, and stained with haematoxylin and eosin (H+E). The sections were examined by light microscopy and photographed using a digital camera.

2.9. Statistical Analysis. The results of the haematological and biochemical examinations and biometric parameters were carried out with UNISTAT statistica 5.6 software. Data were first tested for normality (Shapiro-Wilk test). When necessary, logarithmic transformations were used for the analysis of variance. A one-way analysis of variance (ANOVA) and a Tukey-HSD testwere applied. If the normal distribution

Table 4: Biometrical indices 23 days after the beginning of the experiment.

Indice	Control mean ± SD ($n = 20$)	Experimental mean ± SD ($n = 20$)
Total length	26.10 ± 1.37	25.89 ± 0.85
Standard length	23.44 ± 1.25	23.67 ± 0.85
Body weight	199.33 ± 33.22	185.48 ± 29.95
Liver weight	2.98 ± 0.69	2.92 ± 0.66
Fultons condition factor	1.54 ± 0.17	1.39 ± 0.17
Hepatosomatic index	1.49 ± 0.26	1.58 ± 0.33

was not satisfied, a nonparametric Kruskal-Wallis test was applied. Significance was accepted at $P < 0.05$.

3. Results

3.1. Haematology Profile. The results of the analyses of the haematological indices of both control and experimental groups after 23 days from the beginning of the experiment are presented in Table 2. The exposure caused a significant decrease of MCH ($P < 0.05$) in the experimental fish. The other measured haematological indices showed no statistically significant differences between the experimental and control groups.

3.2. Biochemical Profile. The results of plasma biochemical indicators are presented in Table 3. The experimental rainbow trout exposed to the feed with deoxynivalenol showed statistically significant lower values of glucose, cholesterol ($P < 0.01$), and ammonia ($P < 0.05$) in comparison with negative control.

3.3. Biometric Parameters. The mean values of fish total and standard body length, body and liver weight, Fultons condition factor, and hepatosomatic index did not show significant differences (Table 4).

3.4. Histopathological Examination. The histopathological examination revealed severe hyaline droplet degeneration in the tubular epithelial cells (tubulonephrosis) of the caudal kidney in 9 out of 10 fish fed the diet containing DON (Figure 1). No substantial histopathological changes were demonstrated in the other tissues (gills, skin, liver, cranial kidney, and spleen).

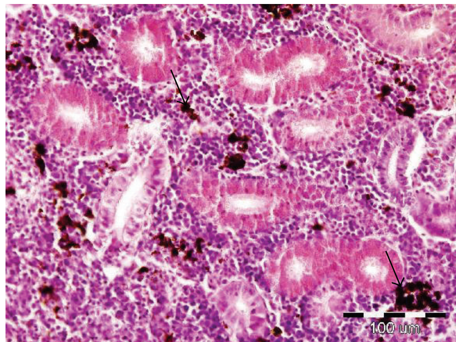

Figure 1: Effect of deoxynivalenol exposure on caudal kidney histology. Hyaline droplet degeneration of tubular epithelial cells (arrows). HE, 400x.

4. Discussion

The study showed posttreatment changes in the haematological and biochemical profiles and histopathological changes in rainbow trout fed a commercial feed with the addition of the mycotoxin deoxynivalenol. No fish mortality was observed in the control or experimental groups during the test.

The main haematological response of rainbow trout after 23 days exposure to DON was a statistically significant decrease in MCH ($P < 0.05$). The lower values of MCH, MCHC, MCV, and Hb suggested that the concentration of haemoglobin in red blood cells is lower due to an anaemic condition [9]. The common clinical symptoms of DON toxicity include among others gastrointestinal hemorrhaging which we demonstrated in our study (Figure 2) and might be the reason for the anaemic condition of the trout. A moderate increase in the erythrocyte count might be caused by the higher percentage of immature red blood cells in the circulation and might be the other reason for the MCH, MCHC, and MCV decrease in the present study [10].

The main biochemical response of rainbow trout to the effect of DON showed a decrease in glucose, cholesterol ($P < 0.05$), and ammonia ($P < 0.01$) in comparison with the control groups. The lower values of these parameters might be caused by a lower intensity of metabolism. Here the effect of feeding a diet containing DON on feed intake and fish weight was observed. The fish weight was nonsignificantly lower in the DON-treated group. The decrease in feed intake could subsequently lead to a decreased intensity in nitrogen metabolism [11]. The decrease in blood glucose during the 23 days exposure can be attributed to the high utilization of glucose for hypoxic conditions and oxidation [12]. It is well established that DON consumption inhibits protein synthesis [6]. In the current study, feed with the addition of toxin caused a nonsignificant decrease in the total protein in the plasma when compared with the control fish.

We observed severe hyaline droplet degeneration in the tubular epithelial cells of the renal tubules of the caudal kidney in the experimental group. The kidney is a target organ

(a)

(b)

Figure 2: Experimental rainbow trout after exposure of DON-haemorrhages in the liver and gastrointestinal tract.

of certain toxicants; it is a major route for the excretion of foreign chemicals. On the other hand, Hooft et al. reported that deoxynivalenol caused considerable morphological changes in the liver, including subcapsular haemorrhages, subcapsular edema, altered hepatocytes, and fatty infiltration [7]. In the current study, we have demonstrated subcapsular haemorrhages in the liver in some of the fish fed diets containing the DON (Figure 2).

In conclusion, the results of the present study indicate that exposure of deoxynivalenol in a dose of 2 mg/kg feed induces significant changes in the haematological and biochemical parameters and in the histopathological examination of rainbow trout. The alterations of these parameters may provide a better understanding of the toxicological effect of mycotoxin deoxynivalenol on aquaculture fish, such as rainbow trout.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

Development of a Promising Fish Model (*Oryzias melastigma*) for Assessing Multiple Responses to Stresses in the Marine Environment

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With the increasing number of contaminants in the marine environment, various experimental organisms have been "taken into labs" by investigators to find the most suitable environmentally relevant models for toxicity testing. The marine medaka, *Oryzias melastigma*, has a number of advantages that make it a prime candidate for these tests. Recently, many studies have been conducted on marine medaka, especially in terms of their physiological, biochemical, and molecular responses after exposure to contaminants and other environmental stressors. This review provides a literature survey highlighting the steady increase of ecotoxicological research on marine medaka, summarizes the advantages of using *O. melastigma* as a tool for toxicological research, and promotes the utilization of this organism in future studies.

1. Introduction

Estuaries and coastal waters are contaminated by high levels of anthropogenic pollutants [1], creating an urgent need for ecotoxicological studies of marine pollution. The ecotoxicological characteristics of pollutants in saltwater and freshwater environments are different. The parameters of seawater are significantly different from those of freshwater (i.e., salinity, density, buoyancy, pH, ionic strength, and dissolved oxygen (DO)), and these differences impact the ecotoxicological characteristics of the pollutants, such as the packing fraction and size, the distribution of the contaminants in liquid and solid phases, and the bioaccumulation of the contaminants [2–4].

In addition, the studies of the organisms living in the two different environments have also presented different results. Although *Oryzias latipes* (freshwater fish) and *Oryzias melastigma* (seawater fish) are closely related, their branchial FXYD domain-containing ion transport regulator (FXYD) proteins exhibit divergent expression patterns [5]. *Kif7* is not expressed in *O. melastigma* but is highly expressed in the brain of zebrafish, which is a freshwater fish [6]. There is an inverse correlation between the muscle water contents (MWC) and salinity in *O. latipes*; however, the two parameters are not related in *O. melastigma* [7]. Exposure to perfluorooctane sulfonates (PFOS) shortened the hatching time and increased the hatching rate of *O. melastigma* but had the opposite effects in zebrafish [8–10]. These differences illustrate that ecotoxicological results from freshwater environments cannot be directly applied to the marine environment. At present, aquatic toxicological research is largely carried out under freshwater environmental conditions, and research in the marine environment is urgently needed.

The biologic impact of toxic pollutants on fish is an important area of study in ecotoxicology. Fish models, such as zebrafish (*Danio rerio*), tilapia (*Oreochromis niloticus*), and rainbow trout (*Oncorhynchus mykiss*), have been widely used for ecotoxicological studies in the freshwater environment. Although some estuarine species, for example, *Corophium acherusicum*, *Enteromorpha linza*, and *Ctenogobius giurinus*, can be used for the study of ecotoxicology in marine environments, the research still lags well behind that in freshwater environments, and problems such as species specificity and the lack of genetic information in these species do exist.

O. melastigma, also named *O. dancena* or Indian medaka, has many advantages as a fish model in marine toxicological research. This review summarizes the advantages and research findings of marine toxicological studies using *O. melastigma* and encourages further investigation of ecotoxicology in the marine environment using this fish model.

2. Advantages of *O. melastigma* as a Research Model in Toxicological Studies

O. melastigma originates from the coastal waters and fresh waters of Pakistan, India, Myanmar, and Thailand. In classification, *O. melastigma* and *O. latipes* belong to the order Beloniformes, family Adrianichthyidae and genera *Oryzias*. The embryo of this species has been identified as an important tool for toxicology investigations by the regime of ILSI Health and Environmental Sciences Institute (HESI). As a fish model, it shares many advantages as follows.

(1) *O. melastigma* is small in size (4.5 to 23 mm) and has a short generation time (2-3 months). These characteristics make it available to culture on a large scale under laboratory conditions (30‰ artificial seawater, 28 ± 1°C, and in a 14 h light: 10 h dark cycle). The relatively large eggs and transparent color simplify experimental observations and operations, such as observing developmental changes during each stage of growth [11].

(2) *O. melastigma* has distinct sexual dimorphism, and the morphology of the anal fin is very prominent approximately 1 month after hatching, rendering it highly desirable for gender studies [12]. Researchers have recommended that future risk evaluation of immune-modulatory chemicals must include parallel assessment of both genders. This makes *O. melastigma*, owing to its characteristics of distinct gender dimorphism and the presence of sex-determining *Dmy* gene of its homologous species *O. latipes*, suitable for toxicity evaluation [13].

(3) *O. melastigma* possesses strong environmental tolerance. This organism is capable of adapting to a wide range of temperatures; thus, mutants can be derived that are conveniently temperature sensitive [14]. *O. melastigma* has the ability to survive in aquatic environments with a wide range of salinity. Although *O. latipes* can adapt to varying salinity environments to some degree, the adaptive capacity of *O. latipes* is lower than that of *O. melastigma*, which can thrive in water of varying salinity ranging from 0 to 35 ppt [1].

(4) The eggs and larvae of *O. melastigma* are sensitive to many environmental pollutants. If the specific sensitive gene responding to pollutants or other environmental stresses can be identified at the molecular level, then environmental pollution can be quickly identified. The molecular staging of *O. melastigma* embryos, focusing on the heart, pectoral fin, brain, eye, pancreas, muscle, liver, and neuron system, has been fully described [15].

(5) Studies of *O. latipes* in anatomy, physiology, and other aspects have been increasingly extensive and systematic, and the genome sequences of *O. latipes* have been completed. Many common characteristics exist between *O. latipes* and *O. melastigma* in phylogeny; thus, the brackish *O. melastigma* can serve as a good marine fish model for developmental studies by utilizing the resources developed from *O. latipes*. The corresponding genetic chip information of *O. melastigma* has been acquired which makes it convenient for the study of *O. melastigma* [1, 14, 16]. Additionally, homologous species could be fully used for comparative biology, in a similar manner to *Drosophila*, for which the genome analysis of multiple species has greatly promoted the study of comparative biology [14, 17].

All of these advantages enhance the potential of *O. melastigma* to be a competent model organism in marine ecotoxicology.

3. The Research Background of *O. melastigma* in Molecular Biology

Sharing a high degree of similarity, most of the research findings of the congeneric species of *O. melastigma*, such as *O. latipes*, could be applied to *O. melastigma* mostly. Notably, even though *O. melastigma* is similar to the other medaka species, some differences still exist. For example, *omChgh* is characterized by eight exons and seven introns, while the second isoform of the *Chgh* gene has only seven exons in the *O. latipes* genome [6, 18, 19]. *Dlx2* is expressed only in the telencephalon and diencephalon of *O. melastigma*, while it is also expressed in the rhombencephalon of *O. latipes* [1]. *O. latipes* and *O. melastigma* share completely identical peptide sequences but bear very different glycan structures [19]. This phenomenon suggests that further exploration of the marine medaka genome and proteome is needed [20].

3.1. The Research Background of O. melastigma Genes. A substantial number of molecular biological studies for *O. melastigma* are being conducted. The complete mitochondrial genome of *O. melastigma* has been obtained from the genome data sequenced by next-generation sequencers [21]. A batch of organ-specific molecular markers have also been identified, such as the makers for brain, eyes, heart, liver, and muscle [15]. These markers can be used to indicate the developmental status of specific organs, and their abnormal expression can be used to indicate the toxicity of pollutants on organ development. Chen et al. [1] analyzed the expression of 11 organ-specific expression genes during each period of embryonic development by *in situ* hybridization (ISH) and determined that 8 of the 11 genes are similar to those expressed during the embryonic development of zebrafish and *O. latipes*.

In addition to the above specified genes of organ development, some functional genes in different tissues have been analyzed as well (Table 1). Some immune-related genes,

Table 1: Expression of the cloned genes of *O. melastigma* in different tissues under various environmental stresses.

Functions	Genes	Exposed tissues	Exposed to	References
Reference genes	*18S*, *Rpl7*, and *β-actin*			
Hypoxia-responsive	*Telomerase reverse transcriptase (Tert)*	Ovary, liver, testis, kidney, gill, brain, spleen, intestine, eye, muscle, and skin	Hypoxia	[16, 22]
	Hypoxia-inducible factor-1α (Hif 1α)	Liver, testis	Hypoxia	[22]
	Erythropoietin (Epo)	Liver, testis, and embryos	Hypoxia, PFOS	[22, 23]
	Leptin receptor (Lepr)	Liver, gill, heart, kidney, gill, brain, spleen, intestine, eye, muscle, ovary, and testis	Hypoxia	[24]
	Hemoxygenase-1 (Ho)	Liver, gill, and heart	WAFs, Hypoxia	[24, 25]
Immune toxicity	*Glutathione peroxidase (Gpx)*	Embryos	PFOS	[26]
	Catalase (Cat)	Embryos	PFOS	[26]
	Uncoupling protein 2 (Ucp2)	Embryos	PFOS	[26, 27]
	Cyclooxygenase-2 (Cox2)	Embryos	PFOS	[26]
	Peroxisome proliferator-activated receptors(Ppars): Pparα, Pparβ, and *Pparγ*	Embryos, whole fish	PFOS, WAFs	[25, 26, 28]
Complement-related genes	*Lectin, mannose-binding 2 (Mbl2)*	Liver	PBDE-47	[29]
	Cyan fluorescent protein (Cfp)	Liver	PBDE-47	[29]
	Complement component: C1r/s, C3, C9, C3-2, C4, C1q, C5, C8, C1 inhibitor	Liver	PBDE-47, *Vibrio parahaemolyticus*	[29, 30]
	Prothrombin (F2)	Liver		[30]
	Complement factor: Hf, Bf	Liver	*Vibrio parahaemolyticus*	[30]
	Hepcidin (Hep): Hep1, Hep2	Liver, spleen, gill, intestine, ovary, testis, brain, and embryos	*Vibrio parahaemolyticus*	[30]
	Mannose-binding lectin-associated serine protease (Masp)	Liver	*Vibrio parahaemolyticus*	[30]
Inflammation-related genes	*Tumor necrosis factor-α (Tnfα)*	Embryos	PFOS, BPA	[26, 31]
	Interleukin (Il): Il1β, Il8	Embryos	PFOS, BPA	[26, 31]
	CC chemokine eotaxin-1 (Ccl11)	Embryos	BPA	[31]
	Superoxide dismutase (Sod)	Embryos	PFOS, BPA	[26, 31]
Osmoregulatory mechanism	Na^+/K^+*-ATPase (Nka)*	Gill, embryos	BPA, SW (35‰), BW (15‰), and FW (0)	[7, 31, 32]
	Na^+, K^+, $2Cl^-$ *cotransporter (Nkcc): Nkcc1a, Nkcc1b,* and *Nkcc2*	Gill, liver, testis, intestine, ovary, brain, muscle, kidney, heart, Fin, and eye	SW (35‰), BW (15‰), and FW (0)	[32]
	FXYD domain-containing ion transport regulator (Fxyd): Fxyd5, Fxyd6, Fxyd7, Fxyd8, Fxyd9, Fxyd11, and *Fxyd12*	Gill, intestine, kidney, brain, eye, liver, and caudal fin	SW (35‰), BW (15‰), FW (0)	[5]
Cardiac development-related genes	*NK2 transcription factor related 5 (Nkx2.5)*	Embryos	PFOS, BPA	[23, 31]
	Cyclooxygenase (Cox): Cox1, Cox2	Embryos	PFOS, BPA	[23, 31]
	ATP synthase	Embryos	PFOS	[23, 27]
	Bone morphogenetic protein (Bmp4)	Embryos	PFOS, BPA	[23, 31]
	Fibroblast growth factor 8 (Fgf8)	Embryos	PFOS, BPA	[23, 31]
	GATA-binding protein 4 (Gata4)	Embryos	PFOS, BPA	[23, 31]
	Leptin receptor (Lerp)	Embryos	BPA	[31]
	SET and MYND domain containing 1 (Smyd1)	Embryos	PFOS	[23]

Table 1: Continued.

Functions	Genes	Exposed tissues	Exposed to	References
Metabolisms	*Cytochrome P450 (Cyps)*	Liver, gill, embryos, intestine, and ovary	PFOS, WAFs	[25, 28, 33]
	Aldehyde dehydrogenase (Aldh)		WAFs	[25]
	Glutathione S-transferase (Gst): Gsta, Gstk, Gsto, Gstt, and *Gstz*		WAFs	[25]
	Sulfotransferase (Sult): Sult1, Sult2 cytosolic, Sult2b1, Sult2b2, Sult3 cytosolic, Sult3-like, Sult4a1, and *Sult6b1*		WAFs	[25]
	UDP-glucuronyltransferases (Ugts): Ugt1b, Ugt2a, Ugt2a2, Ugt2a3, Ugt2b33, Ugt2b3-like, Ugt5a1, and *Ugt5g1*		WAFs	[25]
	Hydroxysteroid dehydrogenase (Hsd): 3β-Hsd, 11 β-Hsd, and *17β-Hsd*		WAFs	[25]
	Aryl Hydrocarbon Receptor (Ahr): Ahr1, Ahr2	Embryos, whole fish	PFOS, WAFs	[25, 28]
	5 α-reductase (Srd5a)		WAFs	[25]
	Steroidogenic acute regulatory protein (Star)		WAFs	[25]
	ATP-binding cassette (Abc): Abcb1, Abcc2, Abcc3, Abcc4, and *Abcg2*		WAFs	[25]
	Heat shock protein (Hsp): Hsp10, Hsp22, Hsp27, Hsp30, Hsp60, Hsp70, Hsp75, Hsp90a, Hsp90β, Hspβ7, and *Hspβ11*		WAFs	[25]
	Choriogenin H and L (Chgh and Chgl)	Liver, embryos, and larvae	PFOS, E2, EE2, BPA, and NP	[28, 34]
	Kinesin superfamily7 (Kif7)	Brain, kidney, liver, muscle, ovary, and testicle		[6]
	Aryl hydrocarbon receptor nuclear translocator (Arnt)	Embryos	PFOS	[28]
	Vitellogenin (Vtg)	Embryos, liver, gill, intestine	PFOS	[28]
	Estrogen receptor (Er)	Embryos	PFOS	[28]
	Horiolysin H and L (Hce and Lce)	Embryos	PFOS	[10]

Notes: 2,2′,4,4′-tetrabromodiphenyl ether (PBDE-47), bisphenol A (BPA), polycyclic aromatic hydrocarbons (PAHs), sea water (SW), fresh water (FW), brackish water (BW), 17β-Estradiol (E2), 17α-ethinylestradiol (EE2), 4-nonylphenol (NP).

including complement-related genes and inflammation-related genes, have been analyzed. Bo et al. used suppression subtractive hybridization (SSH) to identify differentially expressed immune genes in the liver of *O. melastigma* infected with *Vibrio parahaemolyticus* [30]. Based on an NCBI BLAST search of the 1279 sequenced clones in the SSH libraries, 396 genes were identified, and 38 were involved in the immune process. Additionally, genes involved in cellular metabolism, biological regulations, general response to stimuli, transport processes, signal transduction, and cellular component organization were obtained [30]. Some genes related to metabolism, osmoregulatory and cardiac development in *O. melastigma* have also been submitted. Whole *omCyp* genes were registered at the GenBank database. To date, various *Cyp* gene families have been identified. The transcript profiling of whole *omCyp* genes has been finished for *O. melastigma* exposed to water accommodated fractions (WAFs) of Iranian crude oil [25, 33].

Second generation high-throughput sequencing technology has greatly enhanced the ability to obtain genetic information. Huang et al. extracted RNA from *O. melastigma* following exposure to pollutants during various developmental periods and used Illumina high-throughput sequencing to obtain 6 GB data. They performed bioinformatics analyses and identified a large number of toxicology-related genes, thus providing a broad molecular basis for further toxicological investigations [27].

Differentially expressed genes can be largely obtained in fish after exposure to pollutants using gene chip technology. Chinese scholars have constructed a dedicated gene chip for *O. melastigma*, which contains 180 genes related to cell division, detoxification reactions, hypoxia response, oxidative stress, apoptosis, growth, sex determination, gonadal differentiation, and reproductive hormone secretion [35]. This chip includes the most common marker genes for toxicological studies and can be used effectively for gene screening with

differential expression. Using newly developed sequencing technology (Illumina RNA-Seq) and digital gene expression (DGE) technology, a total of approximately 145 thousand unigenes were obtained with 565 bp of unigene N50 [27], which were further enriched in various molecular pathways involved in the response to PFOS exposure and related to neurobehavioral defects, mitochondrial dysfunction, and the metabolism of proteins and fats.

3.2. The Research Foundation of O. melastigma Proteins. The detection of protein expression levels requires corresponding antibodies. Because of the conservation of homologous proteins, antibodies have certain commonalities in allied species. The antibody library of zebrafish has been relatively completed; thus we can use them to directly screen for the specific antibody that reacts with the homologous protein in *O. melastigma*, avoiding the tedious processes of antibody preparation. Through immunohistochemical assay (IHCA) screening of whole embryos, 17 types of zebrafish antibodies can cause specific immune reactions with *O. melastigma*. These antibodies have a close relationship with the development of nerve, heart, and brain, providing a basis for toxicological research on protein levels [15, 16]. In addition, mouse anti-human TERT monoclonal antibody mAb476 can specifically combine with the TERT protein of *O. melastigma* [16].

The tissue distribution of the protein expression in *O. melastigma* under various environment stresses has been partly finished intuitively by WB, IHCA, and matrix-assisted laser desorption/ionization tandem time-of-flight mass spectrometry (MALDI-TOF/TOF MS) (Table 2). The expression of the TERT protein in the cytoplasm and nucleus of *O. melastigma* can be quantified by Western blotting (WB) [41]. Kong et al. observed the TERT protein expression of *O. melastigma* in the testis, ovary, muscle, brain, gill, intestine, kidney, and liver of adult fish using IHCA [16]. Proliferating Cell Nuclear Antigen (PCNA) is the protein marker reflecting cell proliferation, which can be detected by means of IHCA in *O. melastigma*. Experimental results showed a significant correlation between PCNA and TERT in transcriptional and translational expression levels [11]. PCNA detection can also reflect the spatial and temporal characteristics of *O. melastigma* embryonic development [15].

Proteomics refers to the research method of identifying protein characteristics on the large-scale level, and it has become one of the hot spots of aquatic toxicology [39]. Quantitative proteomic analysis demonstrated that hepatotoxicity caused by Hg might involve oxidative stress, cytoskeleton impairment, and energy metabolism alteration, highlighting that the fish liver might be an important target for Hg attack. And proteins such as cathepsin D, GST, and peroxiredoxin-1 responding to Hg treatment in a dose-dependent manner could be used as potential biomarkers of aquatic Hg monitoring [38]. Exposure to PbTx-1 resulted in the alteration of the protein expression involved in cell structure, macromolecule metabolism, neurotransmitter release, and the distribution of signal transduction which may help explain the damage mechanisms of aquatic toxins in fish [36].

4. Utilization of *O. melastigma* in Toxicological Studies

O. melastigma has been used as a research model for assessing multiple responses to stresses of organic chemicals, inorganic chemicals, detrimental organisms, and environmental stress (Table 3). The toxicity responses of *O. melastigma* are different from some species under environmental stresses, which may even have a totally opposite effect (Table 4).

4.1. Toxicological Studies for Organic Chemicals. The choriogenin of teleost fish is considered to be part of the structural interlayer of chorionic precursor cells, which are sensitive to estrogenic contaminants. It increased the expression of the egg-shell precursor protein gene in the liver when exposed to a high concentration of 17β-Glycol and 17α-ethinyl estradiol [34]. The *Chgh* and *Chgl* of *O. melastigma* are sensitive to exposure to estradiol and nonylphenol, and the response of the male fish is more sensitive compared to the female. This indicates that the two genes can be used as sensitive biomarkers to detect pollution levels of estrogen contaminants in the marine environment [34].

The WAF exposure induced CYP-involved detoxification effects but reduced CYP-involved steroidogenic metabolism in the marine medaka. As well-characterized biomarkers of toxicants exposures, *omCyp1a* and *omCyp1b* were highly induced following WAF exposure [25, 43]. Some previous studies have shown potentially synergistic effects after coexposure of *O. melastigma* embryos to CYP1A inhibitors and PAH-type CYP1A inducers [43]. The acute aquatic toxicity of some seawater organisms exposed to polycyclic aromatic hydrocarbons (PAHs) in the laboratory is summarized in Table 5. Distinctly, *O. melastigma* showed high tolerance to PAHs compared to other species. The heart elongation (heart tube) of *O. melastigma* embryos and heart deformities of these juvenile fishes have been recommended as potential biomarkers of the existence of PAH pollution by Mu et al. [43].

Studies quantified the endogenous expression of all six complement system genes including *C1r/s*, *Mbl2*, *CfpF2*, *C3*, and *C9*, in the liver of marine medaka and found that the expression levels were higher in males than in females. BDE-47 exposure downregulates the expression of all six genes in males, while in females the expression of *Mbl2*, *Cfp*, and *F2* mRNAs was upregulated and *C3* and *C9* remained stable with exposure time and dose. These results indicate that the future direction for fish immunotoxicology should include parallel assessment for both genders [29]. Two hepcidins in *O. melastigma* play a complementary role in the innate defense system. Gender specificity should be taken into consideration in immunotoxicological studies in time and extent of induction of the two hepcidin genes in infected *O. melastigma* [48].

PFOS has estrogenic activity and endocrine-disruptive properties that elicit transcriptional responses on POPs-related pathways in a stage-specific manner [61–63]. The marine biological toxicity of PFOS was systematically studied by Dong et al. using *O. melastigma* [10, 23, 26–28]. Their

Table 2: Expression of proteins in different tissues of *O. melastigma* under various environmental stresses.

Related functions	Proteins	Expression tissues and exposure condition	References
Cell structure	Histone-binding protein RBBP4	Gill (Br)	[36]
	Gelsolin	Gill, brain (Br)	[36]
	Krt4 protein	Gill (Br)	[36]
Oxidative stress response	Hemoglobin beta chain	Gill (Br)	[36]
	Histone H3	Gill (Br)	[36]
	Glial fibrillary acidic protein	Brain (Br)	[36]
	Keratin 15 [KRT15]	Brain (Br), liver (Hg)	[36, 37]
	Zgc: 65851	Brain (Br)	[36]
	Type I cytokeratin, enveloping layer [CYT1]	Brain (Br), liver (Hg)	[36, 37]
	Myosin light chain 2	Brain (Br)	[36]
	Tropomyosin alpha-3 chain	Brain (Br)	[36]
	α-Tubulin 1	Liver (Hg)	[37]
	Keratin 8	Liver (Hg)	[37]
	α-Actin	Liver (Hg)	[37]
	Keratin 18	Liver, brain (Hg)	[37]
	β-Actin	Liver, brain (Hg)	[37]
	Type I keratin-like protein	Liver (Hg)	[37]
	Lamin type B	Liver (Hg)	[37]
	Krt5 protein	Brain (Hg)	[37]
	Type II basic cytokeratin	Brain (Hg)	[37]
	Keratin K10 [KRT10]	Liver (Hg)	[38]
	Novel protein similar to vertebrate plectin 1 [PLEC]	Liver (Hg)	[38]
	Peroxiredoxin 4	Liver (Hg)	[38]
	Peroxiredoxin 6	Liver (Hg)	[38]
	Glutathione S-transferase [GSTR]	Liver (Hg)	[38]
	SOD [Cu-Zn]	Liver (Hg)	[38]
	Aldehyde dehydrogenase 1 family, member A2	Brain (Hg)	[38]
	Aldehyde dehydrogenase, mitochondrial	Brain (Hg)	[38]
	Peroxiredoxin-2 [PRDX2]	Liver (Hg)	[38]
	Natural killer enhancing factor	Liver (Hg)	[37]
	Peroxiredoxin-1 [PRDX1]	Liver (Hg)	[38]
	DJ-1 protein [DJ-1]	Liver (Hg)	[38]
	Cathepsin D [CTSD]	Liver (Hg)	[38]
	proliferating cell nuclear antigen [PCNA]	Testis, muscle, kidney, liver Cheek, brain, intestine, and ovary embryo during each development period (H)	[16, 39]
	Telomerase Reverse Transcriptase [TERT]	Testis, brain, muscle, gill, intestine, kidney (N), and liver (H)	[16]
	superoxide dismutase [SOD]	Whole fish (Z)	[40]
	Metallothionein [MT]	Whole fish (Z)	[40]
	heat shock protein 70 [HSP70]	Whole fish (Z)	[40]

Table 2: Continued.

Related functions	Proteins	Expression tissues and exposure condition	References
Metabolism	ApoA-IV4	Gill (Br)	[36]
	Aldose reductase	Gill, brain (Br)	[36]
	Pyruvate carboxylase	Brain (Br)	[36]
	Dpysl5a protein	Brain (Br)	[36]
	Triosephosphate isomerase	Brain (Br)	[36]
	Enolase	Brain (Br)	[36]
	Glutamine synthetase	Brain (Br, Hg)	[36, 37]
	Isovaleryl coenzyme A dehydrogenase	Brain (Br)	[36]
	Glyceraldehyde 3-phosphate dehydrogenase	Brain (Br)	[36]
	Homogentisate 1,2-dioxygenase	Liver (Hg)	[37]
	Alanyl-tRNA synthetase, cytoplasmic	Liver (Hg)	[37]
	Dihydrolipoamide S-acetyltransferase	Liver (Hg)	[37]
	Adenosylhomocysteinase	Liver (Hg)	[37]
	Pyruvate dehydrogenase E1 component subunit alpha, somatic form, mitochondrial	Liver (Hg)	[37]
	Brain-type fatty acid binding protein	Liver (Hg)	[37]
	Methionine adenosyltransferase-like	Liver (Hg)	[37]
	S-formylglutathione hydrolase	Liver (Hg)	[37]
	Apolipoprotein A1	Brain (Hg)	[37]
	Pyruvate kinase	Brain (Hg)	[37]
	Dihydropyrimidinase-related protein 5	Brain (Hg)	[37]
	Dihydropyrimidinase-like 2	Brain (Hg)	[37]
	Enolase 1, (alpha)	Brain (Hg)	[37]
	Creatine kinase, brain b	Brain (Hg)	[37]
	Total glutathione [GSH]	Whole fish (W)	[25]
	Glutathione *S*-transferase [GST]	Whole fish (W)	[25]
	Sulfotransferase [SULT]	Whole fish (W)	[25]
	Superoxide dismutase [SOD]	Whole fish (W)	[25]
	Glutathione reductase [GR]	Whole fish (W)	[25]
	Glutathione peroxidase [GPx]	Whole fish (W)	[25]
	Catalase, CAT	Whole fish (W)	[25]
	ATP synthase subunit d, mitochondrial [ATP5H]	Liver (Hg)	[38]
	Electron-transferring-flavoprotein dehydrogenase [ETFDH]	Liver (Hg)	[38]
	Electron transferring flavoprotein subunit alpha, mitochondrial [ETFA]	Liver (Hg)	[38]
	Pyruvate dehydrogenase (lipoamide) beta [PDHB]	Liver (Hg)	[38]
	Phytanoyl-CoA dioxygenase domain-containing protein 1 [PHYD1]	Liver (Hg)	[38]
	Delta3,5-delta2,4-dienoyl-CoA isomerase, mitochondrial [ECH1]	Liver (Hg)	[38]
	Phosphorylase [PYGB]	Liver (Hg)	[38]
	Formimidoyltransferase-cyclodeaminase [FTCD]	Liver (Hg)	[38]

Table 2: Continued.

Related functions	Proteins	Expression tissues and exposure condition	References
Signal transduction	Putative transient receptor protein 2	Gill (Br)	[36]
	Myosin regulatory light chain 2	Gill (Br)	[36]
	FXYD domain-containing ion transport regulator	Gill (S)	[5]
	NKCC1a-like protein	Gill (S)	[32]
	NKA α-subunit	Gill (S)	[7, 32]
	Grancalcin	Gill (Br)	[36]
Protein modification	Myosin light chain 2	Gill (Br)	[36]
	Calreticulin, like 2	Gill (Br)	[36]
	Transforming protein RhoA	Brain (Br, Hg)	[36, 37]
	Calmodulin	Brain (Br)	[36]
	Annexin 4	Liver (Hg)	[37]
	14-3-3E1 protein	Liver (Hg)	[37]
	14-3-3 protein	Liver (Hg)	[37]
	Annexin A13	Brain (Hg)	[37]
	Cytosolic nonspecific dipeptidase	Liver (Hg)	[37]
	Proteasome alpha 1 subunit	Liver (Hg)	[37]
	HSP-90	Brain (Hg)	[37]
Other function related	Chaperonin containing TCP1, subunit 8 (theta)	Brain (Hg)	[37]
	Beta-synuclein	Brain (Br)	[36]
	SH3-domain GRB2-like endophilin B2	Brain (Br)	[36]
	Complement component C3-1	Liver (Hg)	[37]
	Carbonic anhydrase 1	Brain (Hg)	[37]
	ATPase, H+ transporting, V0 subunit D isoform 1	Brain (Hg)	[37]
	Transferrin	Brain (Hg)	[37]
	Eukaryotic translation initiation factor 3, subunit 2 beta [EIF3S2]	Liver (Hg)	[38]
	Histone H4	Liver (Hg)	[38]
	Ependymin [EPD]	Liver (Hg)	[38]
	GammaN1 crystallin [CRYGN1]	Liver (Hg)	[38]

Notes: the abbreviations in parentheses mean the protein expression in the environment of exposure to normal (N), hypoxia (H), brevetoxins (Br), $HgCl_2$ (Hg), salinity (S), nZnO (Z), and WAFs of Iranian crude oil (W).

results showed that exposure to PFOS could induce the hatching enzyme both at transcriptional and enzymatic activity levels and further lead to decreases of average hatching time and increases of the average hatchability of *O. melastigma* embryos, which in turn induced the mortality of the larvae hatched from exposed embryos. All of these effects were dose dependent [10]. They also found that PFOS is toxic to the development of the cardiovascular system of *O. melastigma*, affecting the expression of cardiac development-related genes, morphological development, and function of the heart in the marine medaka [23].

Some research has also been conducted in their laboratory with embryos exposed to low concentrations of bisphenol A (BPA). The result showed that the expression of heart development-related genes and inflammation-related genes in *O. melastigma* was altered, the body length and width decreased, and the larvae exhibited inflammation foci in the heart ventricles [31].

4.2. Toxicological Studies for Inorganic Chemicals. Subacute toxicity experiments with ambient concentrations of pollutants are often closer to environmental value and thus have great significance in toxicological evaluation. In evaluating the toxicity of ZnO, researchers evaluated the subacute toxicity of two zinc oxides on the expression of SOD, MT, and HSP70 in *O. melastigma* and found that the two zinc

Table 3: Utilization of *O. melastigma* as a research model for toxicological studies.

Responsive to	Toxicological research about	Age of fish	Exposure concentration and time	Main works	Main conclusions	References
Organic chemicals						
WAFs	CYP1A-involved detoxification mechanism	3-week-old fish and adults	2.5, 5, 10, 20, 40, 60, 80, and 100% WAF for 24 h; 5% for 6, 12, 24, 48, 72, and 96 h	Transcript profiling of whole *omCyp* genes, enzyme activity and steroid hormones assay, *omCyp1a* mRNA expression in different tissues during different developmental stages, and effects of β-NF, BaP, and WAF on expression of *omCyp1a*	WAF induced CYP-involved detoxification mechanism but reduced steroidogenic metabolism; *omCyp1a* would be associated with the initiation of the cellular defense systems.	[25, 33]
PBDE-47	Immune-modulatory effects	Three-month-old	290 and 580 ng/day from 2 dpf to hatching	Correlation between BDE-47 body burden and complement gene expression (RT-PCR) in different genders	Genes studied were gender dependent (males > females); BDE-47 is not biotransformed in marine medaka.	[29]
	Maternal transfer	2- and 3-month-old	1.3 ± 0.2 μg/day for 21 days	Accumulation of PBDE 47 in 2-month-old fish and maternal transfer of PBDE 47 from adult female medaka to eggs	PBDE 47 transfer is associated with lipid mobilization during egg production.	[42]
PFOS	Mitochondrial dysfunction	Embryos	0.25 and 1 mg/L from 2 dpf to 6 dpf	Sequence the RNA mixtures using Solexa/Illumina RNA-Seq at various developmental stages and after various types of exposure, and DGE and qRT-PCR analysis for relative gene expression	The mitochondrial dysfunction appears to be involved in multiple toxicological effects of PFOS on *O. melastigma* embryos.	[27]
	Precocious hatching	Embryos	1, 4, and 16 mg/L from 2 dpf to hatching	Record the time for hatching, hatching rate and mortality of fry hatched within a week, and hatching enzymatic activity and RT-PCR analysis for gene expression	PFOS induced the hatching enzyme, leading to the precocious hatching of embryos and the decrease of larvae survival.	[10]
	Endocrine-disruptive effect	Embryos	1, 4, and 16 mg/L for 2 dpf, 4 dpf, and 10 dpf, respectively	The mortality and malformation rates, the transcriptional responses of the ER, AHR, and PPAR pathways to PFOS by RT-PCR, and quantification of PFOS in exposure solutions and medaka embryos	PFOS has estrogenic activity and endocrine-disruptive properties and could elicit gene responses in a stage-specific manner.	[28]
	Cardiac toxicity	Embryos	1, 4, and 16 mg/L for from 2 dpf to hatching	Cardiac morphology, heart rates and the SV-BA distance of the heart was measured; RT-PCR analysis of gene expression profiles was conducted.	PFOS affected the development and function of the heart in the marine medaka embryos.	[23]
	Immunotoxicity	Embryos	0, 1, 4, and 16 mg/L from 2 dpf to hatching	PFOS body burden, survival rates, and growth parameters of fish larvae during 17 dph, liver histological examination, and gene expression in fish larvae after LPS exposure for 12 h at 27 dph	The immunosuppression effects caused by PFOS could lead to functional dysfunction or weakness of the immune system in the fish larvae.	[26]

Table 3: Continued.

Responsive to	Toxicological research about	Age of fish	Exposure concentration and time	Main works	Main conclusions	References
BPA	Cardiac toxicity	Embryos	200 μg/L for 2 dpf-incubation	Heart beat rate, SV-BA distance of embryos, body length and width, histology, and BPA-induced inflammation-related genes and heart-related genes	BPA induced cardiac toxicity of the *O. melastigma* embryos.	[31]
PAHs (ANF, Pyr, Phe, and BaP)	Developmental malformations	Embryos	Different PAHs for 18 days	Deformity assessment, heart rate, heart elongation, hatch rate, and EROD and Caspase-3/7 activity assays of embryos exposed to PAHs with or without 100 μg/L ANF	Inhibition of CYP1A, EROD, and Caspase-3/7 activities can be used as indicator in the ecological early warning and PAHs detection.	[43, 44]
Estrogen (E2, EE2, NP, and BPA)	Estrogenic pollutants	Sexually mature	E2, EE2 (1, 10, 100, and 500 ng/L); NP, BPA (1, 10, 100, and 200 μg/L) for 7 days	E2-inducible choriogenins expression in embryos and yolk-sac larvae by end-point PCR; effects of EE2, BPA, and NP, respectively, on *omChgh* and *omChgl* expression by RT-PCR	The rapid inducibility (within 24 h) of *omChgh* by E2 during early developmental stages was found to be more estrogen sensitive than *omChgl*.	[34]
Benzotriazole	Reproductive effect	3-month-old	0.01, 0.1, and 1 mg/L for 4 and 35 days	Benzotriazole can induce *Vtg* and *Cyp19a* gene expression but inhibits the *Cy1a1* gene expression (qPCR analysis).	Benzotriazole had adverse potential on the endocrine system.	[45]
Inorganic chemicals						
DWNTs	Ecotoxicity data of DWNTs	48 h posthatching	10, 50, and 100 mg/L for 14 days	Mortality and total length of medaka fish larvae over 14 days exposed to different concentrations of stirred and sonicated double-walled carbon nanotubes.	So-DWNTs are more toxic than st-DWNTs; the dispersion method and size of aggregations should be considered in DWNT toxicity testing.	[46]
nZnO	Sublethal toxicities	<24 h	4 and 40 mg/L ZnO for 96 h	Stress responses in fish after acute exposure (SDS-PAGE)	nZnO did not display the same toxicity as ZnO towards the fish.	[40]
$HgCl_2$	Hepatotoxicity and neurotoxicity	Weighing 0.5 ± 0.05 g	1000 μg/L for 8 h; 1 or 10 μg/L for 60 d	Protein expression profile in liver and brain exposed to HgCl2 (MALDI-TOF/TOF MS) and mercury accumulation and damaged liver ultrastructure in medaka	Hg hepatotoxicity might involve oxidative stress, cytoskeleton impairment, and a dysfunction in metabolism.	[37, 38]
Cd^{2+}, Hg^{2+}, Cr^{6+}, and Pb^{2+}	Toxic effects of heavy metals	Embryos and larvae	96 h and 14 d	The mortality, heart beat rate, and malformation rates	The fish species has relatively high sensitivity to heavy metal stress.	[47]
Detrimental organisms						
Vibrio parahaemolyticus	Immunotoxicity	5-month old	6 × 105 cfu/fish for 6 h, 24 h and 48 h	qPCR analysis of the complement genes in liver; age-, tissue-, and gender-differences in the expression of *hepcidin*; *hepcidin* expression in hepatocyte by ISH	*O. melastigma* can serve as a model to understand the basic biological processes related to immune function.	[30, 48]

TABLE 3: Continued.

Responsive to	Toxicological research about	Age of fish	Exposure concentration and time	Main works	Main conclusions	References
K. brevis: PbTx-1	Neurotoxicity	Adult	0, 6, 8, 10, 12, 16 and 18 μg/L for 24 h; 6 μg/L for 2 days	Algal toxicity (toxic symptoms, 24 hour mortality, 1/LT50) and its supernatant, MeOH and TCM extracts of *O. melastigma*; changes in protein profiles in medaka gill and brain exposed to PbTx-1	*K. Brevis*-induced hypoventilation response in medaka; the down-regulation of several proteins involved in cell protection.	[36, 49]
C. marina	Ichthyotoxins of *C. marina*	4–8 months-old	10,000 cells/mL for 0, 24, 48 and 60 h	Algal cell density, growth rate, their toxicity (toxic symptoms, 24-hour mortality, 1/LT50) and its supernatant, MeOH and TCM extracts to *O. melastigma*	Fish susceptibility to *C. marina* is related to its growth rate, but not to cell density; *C. marina* developed the hyperventilation response of the fish.	[49, 50]
Environmental stress						
Hypoxia	Hypoxia-responsive	4-week old adult	1.8 ± 0.2 mg O_2/L for 3 months; 12 weeks 1.8 mg O_2/L for 24, 48 and 96 h	Adult male fish were processed for ISH and IHC; volume density indices of omTERT mRNA and protein, PCNA and TUNEL signals in liver hepatocytes after chronic exposure to hypoxia; expression of *Tert*, *Hif 1α*, *Epo*, *Lepr*, and *Ho* in tissues by RT-PCR	Hypoxia upregulates omTERT expression via omHIFhif-1 in liver and testis and the omLepR omLEPR expression demonstrated its independent control in endocrine and peripheral tissues.	[16, 22, 24]
	Ichthyotoxins of *C. marina*	4–8 months old	7 mg/L, 6.0 mg/L and 1 mg/L DO for 60 h	Oxygen consumption rate, threshold lethal DO and correlation between body weight and survival time of marine medaka inside the sealed syringe	Fish susceptibility to *C. marina* is related to the susceptibility of the fish to hypoxia.	[50]
Salinity	Osmoregulatory mechanism	2.50 ± 0.30 cm	SW (35‰), BW (15‰), FW (0) for Three weeks or 1 month	Plasma osmolality, MWC, Na^+/Cl^- concentration, time course, NKCC1a-like protein expression, NKA activity, NKA-IR cell activity, NKA α-subunit mRNA and protein expression in gills in response to hypoosmotic challenge; salinity effects on multiple *Fxyd* mRNA and FXYD11 protein abundance; co-immunoprecipitation of NKA with FXYD11 and the localization of *Fxyd11* mRNA in gill sections in freshwater-acclimated marine medaka	The expression pattern of branchial *Fxyd11* was similar to that of *Nkaα* in the *O. latipes*, but non-correlated expression patterns were observed in the *O. melastigma* at both the mRNA and protein levels; the lowest NKA activities were found in the environments with salinities similar to their natural habitats.	[5, 7, 32]
	Ichthyotoxins of *C. marina*	4–8 months old	70‰ seawater and natural seawater.	The LT50 of marine medaka at different ages (4–8 months-old) exposed to 70‰ hypersalinity (70‰-SW)	Fish susceptibility to *C. marina* is not related to its tolerance to hypersalinity stress.	[50]

Notes: days postfertilization (dpf); days posthatching (dph); sinus venosus-bulbus arteriosus (SV-BA); lipopolysaccharides (LPS); β-naphthoflavone (β-NF); benzo[a]pyrene (BaP); phenanthrene (Phe); pyrene (Pyr); methanol (MeOH); chloroform (TCM); quantitative polymerase chain reaction (qPCR).

Table 4: Comparative toxicity of *O. melastigma* and other species under various stresses.

Exposing to	*O. melastigma*	Other species	References
PFOS	Hatched in advance and hatching rate increased.	Hatch was delayed and hatching rate was not affected or decreased in zebrafish.	[8–10]
	Ke in the larvae ranged from 0.04/d to 0.07/d.	Ke ranged from 0.053 to 1.700 L/kg/d in blood, kidney, liver and gall bladder and from 0.02 to 0.23/d in carcass and liver concentrations in rainbow trout (*Oncorhynchus mykiss*).	[51–53]
	Did not alter *Epo*	Led to high mortality in zebrafish	[23]
Phe, Pyr, and BaP	NOEC values were 50, 25, and 10 μg/L, respectively.	NOEC values were 10, 50, and 1.8 μg/L, respectively, in the water flea (*Tigriopus japonicus*).	[54]
E2	The mRNA level of *3Bhsd* (steroidogenic enzymes) was increased.	Decreased the production of 11-KT and mRNA levels of steroidogenic enzymes in zebrafish and decreased the production of testosterone in human	[55–57]
DWNTs	Growth inhibition was observed at 10 mg/L for so-DWNTs but not for st-DWNTs.	Population growth was reduced to 0.1 mg/L for so-DWNTs and 10 mg/L for st-DWNTs in the water flea.	[23]
nZnO	Lack of change was observed in the SOD activities.	SOD activities were decreased for the first few days but recovered soon in *O. latipes* and were also significantly depleted in mouse embryo fibroblast cells, more toxic in Skeletonema costatum and Thalassiosira pseudonana, and less toxic in Elasmopus rapax and the water flea.	[40]
Cercariae	Did not infect	Infected in liver and kidneys of *Channa punctatus*, infected in the muscles of *Cliona orientalis*, and did not infect in *Puntius sophore* and *Gambusia affinis*	[58]
Salinity	Prefers hypoosmotic conditions	Prefers hyperosmotic conditions in Javanese medaka (*Oryzias Javanicus*)	[11, 59]
	MWC was constant with the increase of salinity in *O. melastigma*.	MWC was decreased with the increase of salinity in *O. latipes*.	[7]
Hypoxia	HAS was not present.	HAS was identified in zebrafish and *Fugu*.	[60]
	3Bhsd and *Cyp19a* mRNA expression upregulation	*3Bhsd* and *Cyp19a* mRNA expression was reduced in zebrafish.	[55]

Notes: the elimination rate constant (Ke); No Observed Effect Concentration (NOEC); 11-ketotestostrone (11-KT); HIF-1 ancillary sequence (HAS).

oxides show differences in the induction of three proteins [40]. In the toxicity assessing, double-wall carbon nanotubes (DWNTs) (10 mg/L) following ultrasonic treatment inhibited the growth of *O. melastigma* larvae [46].

O. melastigma is also used in the evaluation of heavy metal toxicity. The 96 h LC50s of this fish following exposure to Cu^{2+}, Cd^{2+}, Hg^{2+}, Cr^{6+}, Pb^{2+}, and Zn^{2+} are shown in Table 6, from which we could determine that *O. melastigma* has a strong sensitivity to metal stress compared to other marine species. Toxicity detection of *O. melastigma* for copper, tributyltin (TBT), and five commonly used antifouling fungicides, including s-triazine, diuron, pyrithione zinc, copper pyrithione, and chlorothalonil, indicate that the 96 h LC50 of this fish's tolerance for copper, s-triazine, and diuron is at the level of mg/L, while others are μg/L [91]. Exposing fertilized eggs and newly hatched *O. melastigma* juveniles to Cd^{2+}, Hg^{2+}, Cr^{6+}, and Pb^{2+} significantly reduced the hatching ability of the embryos and the heart rates above a certain concentration [47]. Metal accumulation of inorganic mercury in the liver and brain of *O. melastigma* induced oxidative stress, cytoskeletal reorganization and/or disruption, dysfunction in metabolism, protein modification, signal transduction, and other related functions [37, 38].

4.3. Toxicological Researches for Detrimental Organisms. The median lethal time (LT50) of *O. melastigma* is treated as an indicator of pollutant toxicity for toxicological comparison and correlation analysis. In addition, the acute toxicity test can provide the appropriate dose for the study of molecular toxicological mechanisms, such as the determination of the 24 h LC50 value in *O. melastigma* exposed to brevetoxins (PbTxs). These concentrations of PbTxs can be determined in follow-up proteomics studies [36].

ISH showed that *Vibrio parahaemolyticus* would induce the expression of hepcidin genes in the nuclei and cytoplasm of liver cells of *O. melastigma* [48]. It is also used to characterize the toxicity of the toxins generated by *Chattonella marina* and *Karenia brevis*. Test fishes exposed to the toxins generated by *C. marina* exhibit hyperventilation, while those exposed to the toxins generated by *K. brevis* exhibit hypoventilation [49]. With the assistance of the proteomic approach combined with other methods, the toxicological mechanism of aquatic toxins in marine organisms will be elucidated easily and conveniently [36].

4.4. Toxicological Studies for Environmental Stress. O. melastigma can serve as a marine fish model for assessing

Table 5: Acute toxicity data (96 h LC50/EC50) of seawater organisms exposed to PAHs.

Scientific name	LC50/EC50	PAHs (μg/L)			References
		Phe	Pyr	BaP	
Corophium acherusicum	LC50	310	49	—	[54]
Neomysis awatschensis	LC50	130	—	—	[54]
Tigriopus japonicus	LC50	546	174	3.46	[54]
Neomysis awatschensis	LC50	—	15.2	—	[54]
Nitzschia closterium	EC50	71.5	56.8	51	[54]
Enteromorpha linza	EC50	2070	209	286	[54]
Oncorhynchus mykiss	LC50	3200	2000	—	[64]
Acanthogobius lactipes	LC50	295	—	—	[54]
Ctenogobius giurinus	LC50	—	13.1	—	[54]
O. melastigma	LC50	6399	3127	5705	[54]
Sparus macrocephalus	LC50	800	—	—	[54]
Strongylocentrotus intermedius	LC50	520	—	—	[54]
Hemicentrotus pulcherrimus	LC50	—	1.056	1.56	[54]

Notes: median lethal concentration (LC50); median effective concentration (EC50).

Table 6: Acute toxicity data (LC50, mg/L) of metals in various species.

Species	Exposure ages	LC50 (mg/L)						References
		Cu^{2+}	Cd^{2+}	Pb^{2+}	Cr^{6+}	Hg^{2+}	Zn^{2+}	
Argopecten ventricosus	Juvenile	—	0.396	0.830	3.430	—	—	[65]
Chironomus furens	Larvae	52.8	0.3	0.3	0.3	0.03	4.5	[66]
Chironomus plumosus	Larvae	42.6	0.4	8.2	1	0.3	9.5	[66]
Chironomus riparius	Adult	0.043	0.021	—	—	—	—	[67]
Cynoglossus semilaevis	Postlarvae	0.025	0.178	1.026	—	0.045	1.18	[68]
Duttaphrynus melanostictus	Larvae	0.03	0.3	4.2	—	—	4.2	[69]
Echinogammars olivii	Adult	0.25	—	0.62	—	—	1.30	[70]
Farfantepenaeus paulensis	Postlarvae	—	0.83	—	—	—	3.31	[71]
Fundulus heteroclitus	Postlarvae	1.7	18.2	188	—	0.068	129.5	[72–74]
Hyalella azteca	Adult	0.21	0.013	—	—	—	—	[67]
Hexagenia spp.	Adult	0.073	7.82	—	—	—	—	[67]
Liza vaigiensis	Postlarvae	—	3.7	138	—	0.0835	—	[72]
Lutjanus argentimaculatus	Juvenile	—	—	98	20.1	0.38	—	[75]
Menidia menidia	Juvenile	—	6.3	—	91	0.112	—	[75, 76]
Oreochromis niloticus	Juvenile	0.80	—	—	—	0.82	—	[77]
O. latipes	Postlarvae	—	5.6	—	12.4	—	—	[72]
O. melastigma	Postlarvae	7.3	1.12	>20	1.456	0.097	43	[78]
Pagrosomus major	Postlarvae	0.31	5.6	—	—	—	3.6	[79, 80]
Palaemon elegans	Adult	2.52	—	5.88	—	—	12.3	[70]
Penaeus indicus	Postlarvae	0.8204	—	7.22	—	—	—	[81, 82]
Penaeus monodon	Postlarvae	—	2.28	5.77–7.28	—	—	3.02	[83, 84]
Penaeus penicillatus	Larvae	—	3.025	—	—	—	4.267	[85]
Poecilia reticula	Juvenile	2.36	17.71	—	43.4	—	—	[86]
Penaeus setiferus	Postlarvae	0.0308	—	—	—	0.017	—	[87, 88]
Priopidichthys marianus	Juvenile	—	—	140	31	0. 35	—	[72]
Rivulus marmoratus	Postlarvae	1.4	21.1	85.3	14.3	—	147.9	[72, 74]
Sphaeroma serratum	Adult	1.98	—	4.61	—	—	6.12	[70]
Sparus macrocephalus	Larvae	0.2	0.3	—	—	—	1.8	[80]
Stenocypris major	Adult	0.0252	0.0131	0.5262	—	—	1.1898	[89]
Tubifex tubifex	Adult	0.16	0.87	—	—	—	—	[67]
Zebrafish	Adult	0.174	6.497	116.432	181.09	0.14	44.48	[86, 90]

molecular responses to stresses in the marine environment. Hypoxia upregulates *omTert* expression via omHIF-1 in the liver and testis of nonneoplastic fish [22]. Significant changes were observed in the transcription, translation, cell proliferation, and apoptosis level of TERT in the liver of *O. melastigma* exposed to hypoxic conditions for 3 months [16]. Anoxic conditions can increase the expression of *Tert* in the liver and testicular tissue of *O. melastigma*, which is mediated by anoxia-induced factor-1 [24]. The expression of leptin receptor gene exhibits tissue specificity when exposed to hypoxia, and this gene was identified as a sensitive marker gene for a hypoxic environment [22].

The experimental animal, marine medaka, is suitable for studying the mechanism of hypoosmoregulatory. Studies show that branchial om*Nkcc1a* mRNA levels are induced significantly with an increase in environmental salinities. Salinity-dependent expression of *Nkcc1a* is in the branchial mitochondria-rich (MR) cells of *O. melastigma*, which suggests a critical role in hypoosmoregulatory endurance of this fish [32]. Studies have also indicated that *O. latipes* exhibited better hypoosmoregulatory ability, while *O. melastigma* exhibited better hyperosmoregulatory ability. These results support the hypothesis that the lowest branchial NKA activities of these two species were found in the environments that have similar salinities to their natural habitats [7].

5. Conclusion

O. melastigma have biological characteristics such as small size, high fecundity, short life cycle, sexual reproduction, and distinctive life stages that would allow their use as a marine fish model. Additionally, their ease of cultivation facilitates the use of *O. melastigma* in independent laboratories. The availability of knowledge on their sensitivity towards inorganic and organic compounds and the increasingly complete knowledge on their genes and proteins will also enhance the potential of *O. melastigma* as suitable models in marine aquatic ecotoxicology and toxicogenomics. Researchers have demonstrated the potential application of *O. melastigma* as an ideal marine test fish for marine pollution assessments and ecotoxicological studies of organic chemicals, inorganic chemicals, microorganism, and environmental stress in relation to cardiac toxicity, hepatotoxicity, neurotoxicity, ecotoxicity, immunotoxicity, and so forth.

O. melastigma can also serve as a model marine fish for assessing multiple *in vivo* molecular responses to stresses in the marine environment. *O. melastigma* showed high tolerance to PAHs and strong sensitivity to metal stress compared to other species. The heart elongation of *O. melastigma* embryo and *omChgh* and *omLepr* expression are used as potential biomarkers to indicate PAH mixtures contamination or an oil spill, estrogenic chemicals in the marine environment, and growth and/or endocrine disruption in this marine fish, respectively. The expression of the leptin receptor gene, which was identified as a sensitive marker gene for hypoxia environment, exhibits tissue specificity in *O. melastigma*. We may be able to develop biomarkers for more specific adverse effects that can be used for both ecotoxicology and human health risk assessment because of the high degree of evolutionary conservation among vertebrates [92].

Although some toxicological research has been conducted using this small fish species as a model, there is still much to be studied. Fortunately, transcriptome analyses and proteomic approaches, along with new methodologies in *O. melastigma*, such as gene knockdown, gene overexpression, gene chip technology, second-generation high-throughput sequencing technology, RNA-Seq, and DGE technology, can be expected to further accelerate the knowledge of the toxicological mechanisms of aquatic toxins in marine animals in the future. Demonstrating and understanding toxicity mechanisms in *O. melastigma* that are common between humans and fish and wildlife are necessary if we are to integrate findings from laboratory and ecotoxicology studies with human health risk assessment.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Phytoestrogens β-Sitosterol and Genistein Have Limited Effects on Reproductive Endpoints in a Female Fish, *Betta splendens*

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Phytoestrogens are produced by plants and may cause endocrine disruption in vertebrates. The present study hypothesizes that phytoestrogen exposure of female Siamese fighting fish (*Betta splendens*) may disrupt endogenous steroid levels, change agonistic behavior expression, and potentially also disrupt oocyte development. However, only the pharmacologic dose of β-sitosterol had a significant effect on opercular flaring behavior, while we did not find significant effects of β-sitosterol or genistein on steroids or gonads. These findings are in direct contrast with previous studies on the effects of phytoestrogens in female fish. Results of the current study support previous work showing that the effects of phytoestrogen exposure may be less acute in mature female *B. splendens* than in other fish.

1. Introduction

Phytoestrogens are structurally similar to endogenous estrogens such as 17β-estradiol but are produced by plants. The most well-understood phytoestrogen action on animal physiology, due to ingestion or exposure to contaminated water, involves competitive binding to estrogen receptors. Because of this ability, some phytoestrogens have documented medicinal potential [1], but in uncontrolled conditions they may adversely affect reproduction [2–5]. Furthermore, phytoestrogens may also interfere directly with steroid biosynthesis, intracellular signaling, cell proliferation, and gene expression [6], which has raised concerns in the medical community about their safety [7].

Consequences of exposure to these compounds are still unclear, as phytoestrogens have been reported to have estrogenic as well as antiestrogenic effects on vertebrates [8]. Estrogen receptor signaling and its importance in influencing development, behavior, and reproduction is only partially understood [9], so the far-reaching impacts of phytoestrogen exposure, including the alternative modes of action, are difficult to predict. Generally, phytoestrogens are considered safe for humans at common exposure levels, such as those found in soy products [10], but the large-scale anthropogenic production of phytoestrogens in runoff from agricultural areas [11], wood pulp mill discharge [12], and sewage treatment plant effluent [13, 14] may still pose a threat to aquatic ecosystems.

Even subtle reproductive impairments due to phytoestrogens can have detrimental impacts on wild fish populations [15]. Reproductive problems caused by phytoestrogens include delayed oocyte maturation in female medaka (*Oryzias latipes*) at 750 and 30,000 ng genistein per fish [3, 16], increased egg mortality and larval deformation of brown trout (*Salmo trutta*) at 10 and 20 $\mu g\,L^{-1}$ mixed phytosterols found in pulp mill effluent [17], and confused sexual differentiation in mosquitofish (*Gambusia affinis holbrooki*) [18]. Furthermore, the effects of phytoestrogen exposure in fish are not limited to the direct effects of endocrine disruption; zebrafish (*Danio rerio*) embryos injected with

genistein experience neural cell apoptosis in the hindbrain without binding to estrogen receptors [19]. Environmental concentrations of phytoestrogens have been reported to range from 1 to 41 μg L^{-1} in some areas, with increasing concentrations correlated to the physiological effects mentioned above [20].

Abnormal behavior can be the first and most readily observable manifestation of endocrine disruption [21]. Phytoestrogens cause decreased sex and social behavior [4, 22, 23] and increased aggression and anxiety [22–24] in mammalian studies, while fish responses range from depressed agonistic behavior in Siamese fighting fish (*Betta splendens* Regan, 1910) [25] to male-typical courtship behaviors in female mosquitofish [18]. To fully characterize the effects of phytoestrogen exposure on this species, the current investigation considers the potential effects of short-term exposure to both environmentally relevant and pharmacological concentrations of genistein and β-sitosterol on mature female reproductive behavior and ovary development.

Previous research from our lab posited that Siamese fighting fish are an ideal system in which to investigate the effects of endocrine disrupting chemicals on behavior, as they exhibit well-characterized and easily quantifiable agonistic behaviors [25–27] and have been used previously in studies of *in vitro* toxicology [28, 29]. An early study from our lab supported male fighting fish as good candidates for demonstrating the ecological impact of phytoestrogens, as they showed a significant decrease in agonistic behaviors as a result of exposures [26]. However, in a subsequent physiological study, the only effect detected was decreased sperm count, with no observable decline in fertilization [30].

The present study hypothesizes that phytoestrogens will reduce endogenous sex steroid hormone levels, resulting in behavioral changes and delayed ovulation and oocyte development in female Siamese fighting fish. We use two common phytoestrogens, genistein and β-sitosterol, which can produce disruptive effects in animals via competitive binding of estrogen receptors [22, 31–33] and are often present in the same plants [34]. We predict that circulating hormone levels will decrease, resulting in reduced gonad mass and oocyte maturation delay. Behavior towards conspecific males may be suppressed. If the present study does not observe these changes, we may conclude that Siamese fighting fish are relatively tolerant of endocrine disrupting chemicals in their environment.

2. Materials and Methods

2.1. Ethics Statement. All animal protocols used in these experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of Amherst College (Clotfelter).

2.2. Study Subjects and Phytoestrogen Exposure. Female *B. splendens* (n = 173) used in the following experiments were raised in captivity from domestic stock and obtained from a commercial supplier. Fish ranged in standard length from 24.33 to 38.85 mm and ranged in mass from 0.37 to 1.46 g. All fish were at least one year of age and sexually mature at the time of exposure. Each fish was individually housed in 800 mL of water in a 1 L glass beaker and fed to satiety with freeze-dried mosquito larvae once daily. Uneaten food was removed immediately with a bulbed syringe.

All the water used for housing and exposure tests was filtered using reverse osmosis and reconstituted to pH 6.5 and conductivity between 100 and 200 S cm^{-1}. Water temperature was maintained at 19 degrees C in a climate controlled room. Dissolved oxygen was 10 ± 1.5 mg L^{-1}. Phytoestrogens were dissolved in ethanol and resuspended in reconstituted reverse-osmosis water at the appropriate concentration for use in a semistatic exposure. Although phytoestrogens are relatively stable in the environment [35, 36], treatment and control water was changed and replenished with the appropriate concentration of phytoestrogens every day for 21 days to maintain exposure levels throughout the experiment [30]. The exposure duration of 21 days was selected because similar studies using other endocrine disrupting chemicals have shown significant negative effects on a range of fish species after 3-4 weeks of exposure [37–40].

Fish used to collect behavioral or hormonal data were placed in one of five treatment groups: negative control (ethanol vehicle only), 1 μg L^{-1} genistein or β-sitosterol, and 1000 μg L^{-1} genistein or β-sitosterol. For histological analyses, female fish (n = 73) were placed in the following groups: negative control (ethanol vehicle only), 100 μg L^{-1} 17β-estradiol, 1 μg L^{-1} genistein, 1 μg L^{-1} β-sitosterol, and a mixture of 1 μg L^{-1} genistein and 1 μg L^{-1} β-sitosterol. The low doses (1 μg L^{-1}) are nominal concentrations within the range of those reported in nature [26]. The high doses (1000 μg L^{-1}) used in the behavioral and hormonal experiments are pharmacological concentrations. 17β-estradiol was used in the histology experiment because it is known to depress oocyte maturation and yolk deposition in female guppies (*Poecilia reticulata*) [41]. 17β-estradiol was omitted from the behavioral and hormonal work because it cross-reacts with the detection assay probe used here. Exposure levels were confirmed using high performance liquid chromatography as described previously [42].

2.3. Behavioral Studies. After the 21-day exposure period, 100 treated and control females were transferred to 2 L cubical aquaria containing treatment water and then visually presented with one of 14 live, untreated males in an adjacent aquarium. Previous studies have shown that transportation to a novel tank has no measurable effect on stress response in domesticated *B. splendens* [43]. The female fish were videotaped for 10 minutes and these recordings were scored for duration of three behavioral responses: latency to respond to the presence of the male, duration of opercular displays (tonic movement of the operculum and branchiostegal membrane), and duration of fin displays (erection of the dorsal and caudal fins). These displays are important parts of the agonistic and courtship repertoires of both female and male *B. splendens* [21, 25, 27]. Where $n < 100$, subjects were removed from the experiment because they died or showed signs of illness (n = 12 total, across all treatment groups).

2.4. Hormonal Studies. In order to measure how phytoestrogen exposure affected levels of endogenous hormones testosterone and 17β-estradiol excretion, we used a noninvasive technique commonly used in *B. splendens* and other fish to provide a proxy for plasma hormone levels [44]. After the 21-day exposure period outlined in the previous section, fish were placed in 400 mL of reconstituted reverse osmosis (RO) water (not treated with phytoestrogens) and visually presented with a male stimulus fish for 12 hours. Following this treatment, the subject fish were then isolated for 2 hours. Water (100–150 mL) was collected from each fish. Fish excrete hormones through their urine, feces, and gills, which can be extracted from the surrounding water. In brief, a sample of 50 mL water was drawn through SPE extraction columns, eluted in methanol, dried under nitrogen gas, and resuspended in enzyme immunoassay (EIA) buffer. Concentrations of the steroid hormones testosterone and 17β-estradiol in the water of exposed and control fish were measured using EIA assay kits (Cayman Chemical Co.). The antiserums used in these assays, while prone to interference under certain circumstances, are highly specific to the endogenous steroid they are designed to test and therefore do not detect excreted phytoestrogens. Steroid hormone amounts are reported as pg of detected hormone per mL sample water per gram of fish mass (pg/mL/g).

2.5. Histological Analyses. Semistatic exposures were conducted as described previously, and female fish were euthanized after the 21-day exposure period. Fish mass and ovary mass were used to calculate gonadosomatic index (GSI; measured as (gonad weight/(total body weight))∗100). After weighing, ovaries were fixed in Bouin's solution (Ricca Chemical Co.) for 24 hours. Following fixation, ovaries were dehydrated with ethanol, cleared with Hemo-D xylene substitute, and infiltrated with paraffin. The specimens were then embedded in paraffin and trimmed. Ovaries were cut into 6 μm sections with a microtome and mounted on gelatin-coated slides. Sections were cleared with Hemo-D xylene substitute and stained with hematoxylin and eosin. Each 10th section was photographed under 400x magnification and scored using Image J software, version 1.43 [45]. Four stages of oocyte maturation were defined as described in Figure 1 [2] and counts of each cell type were performed. Final counts were converted to proportions in order to accommodate unequal numbers of histological slices.

2.6. Statistical Analyses. Data analysis for all experiments was performed using R (R Development Core Team, 2010). For variables that were not normally distributed, we used a log transformation to achieve normality. Steroid data were analyzed with ANOVA and Dunnett's post hoc tests. For analysis of behavioral data, we were unable to achieve normality of residuals through transformation, so treatment effects were estimated with robust regression using M-estimation from the MASS library [46]. Histological data met the assumptions of a multivariate analysis of variance (MANOVA), which we then used to analyze the data. Post hoc statistical power was calculated based on small, medium, and large effect sizes (f_2) of 0.02, 0.15, and 0.35, respectively.

3. Results

Raw behavioral data is presented in Table 1. Females treated with the pharmacologic dose (1000 μg L^{-1}) of β-sitosterol spent less time performing opercular displays than fish in the negative control group ($t_{(88)} = -3.03$, $P = 0.002$; Figure 2). Opercular flare duration was not affected in the environmentally relevant β-sitosterol treatment group ($t_{(88)} = -0.86$, $P = 0.20$) or either genistein treatments (1 μg L^{-1}: $t_{(88)} = -1.61$, $P = 0.06$; 100 μg L^{-1}: $t_{(88)} = -0.34$, $P = 0.37$). The latency of females to respond to conspecific males was not significantly affected by genistein (1 μg L^{-1}: $t_{(78)} = 1.08$, $P = 0.85$; 100 μg L^{-1}: $t_{(78)} = 1.63$, $P = 0.95$) or by β-sitosterol (1 μg L^{-1}: $t_{(78)}= 1.72$, $P = 0.95$; 100 μg L^{-1}: $t_{(78)}= 0.79$, $P = 0.78$). Similarly, duration of female dorsal and caudal fin displays was not significantly affected by genistein (1 μg L^{-1}: $t_{(84)} = -0.39$, $P = 0.35$; 100 μg L^{-1}: $t_{(84)} = 0.66$, $P = 0.74$) or β-sitosterol (1 μg L^{-1}: $t_{(84)} = -0.31$, $P = 0.38$; 100 μg L^{-1}: $t_{(84)} = -0.68$, $P = 0.25$).

Circulating levels of testosterone in female *B. splendens* were not significantly affected by exposure to genistein or β-sitosterol ($F_{4,85} = 0.50$, $P = 0.73$). Neither were levels of endogenous 17β-estradiol in female fish significantly affected by genistein or β-sitosterol ($F_{4,90} = 0.41$, $P = 0.80$). Our statistical power to detect small (0.02), medium (0.15), or large (0.35) effect sizes of phytoestrogen exposure on endogenous testosterone was 0.05, 0.16, and 0.71, respectively, while our power to detect effect sizes for estrogen were 0.05, 0.17, and 0.74.

For details on GSI and oocyte histology results, see Table 2. There was no effect of the phytoestrogen treatment or positive control on female GSI ($F_{4,73} = 1.91$, $P = 0.12$). Analysis of ovary development indicated that there was no effect of genistein, β-sitosterol, or the positive control 17β-estradiol on oocyte maturation (MANOVA; df = 60, maturation categories: (i) $P = 0.23$, (ii) $P = 0.11$, (iii) $P = 0.18$, and (iv) $P = 0.71$). Our statistical power to detect small (0.02), medium (0.15), or large (0.35) effect sizes of phytoestrogen exposure on ovary development was 0.05, 0.13, and 0.58, respectively.

4. Discussion

The current study predicted that waterborne phytoestrogens would decrease endogenous steroid levels in female *B. splendens*, because male *B. splendens* exposed to environmentally relavent concentrations of estrogens show decreased reproductive behavior and altered monoamine neurotransmitter activity in the brain [26, 42, 47]. But we found no effects of phytoestrogens on circulating levels of testosterone and 17β-estradiol in females. One effect of phytoestrogens on female behavior towards males was detected, but only at the pharmacologic dosage, and no effects were detected on gonadosomatic index (GSI) or oocyte development.

Table 1: Behavioral results from fish exposed to phytoestrogens or control. Means and standard errors are expressed in seconds.

	Vehicle only	1 μg/L β-sitosterol	1 μg/L genistein	1000 μg/L β-sitosterol	1000 μg/L genistein
Fin display	552.8 ± 74.1	495.0 ± 189.9	530.4 ± 124.6	513.1 ± 127.6	563.0 ± 79.6
Opercular flare	215.4 ± 169.3	155.5 ± 163.8	125.6 ± 139.7	159.5 ± 201.7	195.3 ± 151.0
Response latency	10.9 ± 15.2	50.6 ± 64.8	69.6 ± 117.9	13.69 ± 8.77	55.2 ± 89.0

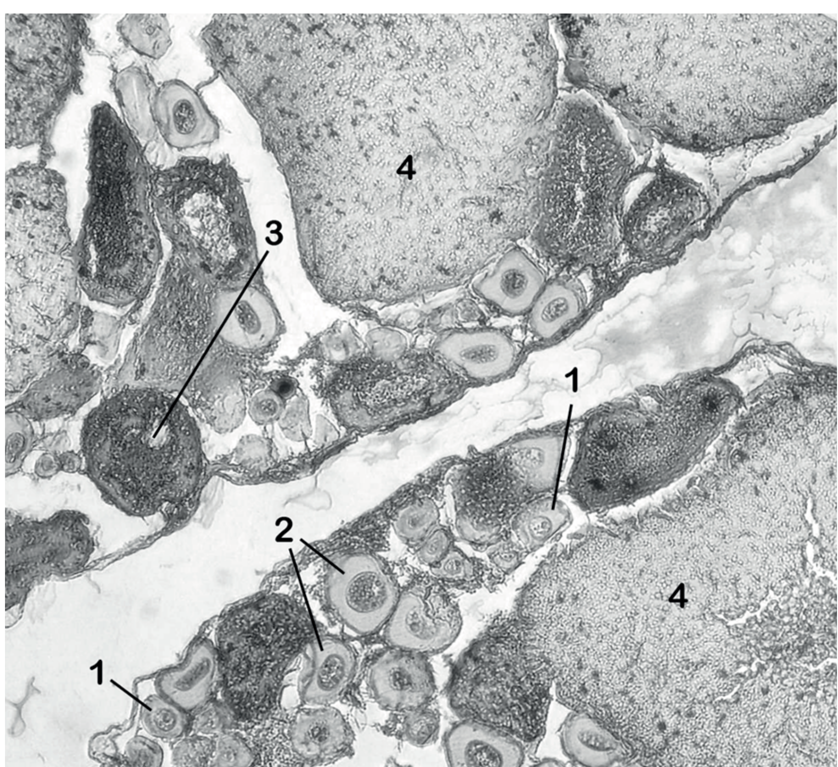

Figure 1: Female *B. splendens* gonad histological section under 400x magnification. Four distinct categories of oocyte maturation were identified: (1) one nucleolus present in the nucleus; (2) multiple nucleoli present and lipid droplets in the cytoplasm; (3) VTG globules in the periphery of the cytoplasm or layered in the zona radiata; (4) oocyte is large, full of VTG, with a nucleus at the oocyte periphery (or nucleus not visible due to slicing).

The biological significance of the present study's observation that a high dose of β-sitosterol depressed female opercular displays is difficult to interpret. While the pharmacological dose that produced the depression in flaring behavior was twice as concentrated as the highest levels of mixed phytoestrogens found in the environment [26], it is comparable to levels used in previous fish physiology studies [3, 16]. Opercular displays in male *B. splendens* are correlated with paternal care, and may be indicative of a male's physiological tolerance of hypoxia [48, 49], but the role of this behavioral display in determining the outcome of social interactions and fitness among female fighting fish is unknown. Nor did the present study detect an effect of phytoestrogens on endogenous hormones, even at the highest exposure levels. A few studies also suggest that different phytoestrogens may affect behavior through multiple pathways; for example, β-sitosterol may mimic cholesterol in addition to estrogen [50, 51]. These pose interesting questions for further research, but the present study cannot draw any conclusions about the mechanism that caused the depression in opercular display unaccompanied by hormonal disruption.

Histological examination revealed that ovaries contained a mix of oocytes at varying stages of maturation, but there was no difference in ovule maturation with respect to treatment group. Similarly, phytoestrogens did not cause a change in GSI. The nonsignificant results of the present study support the nonsignificant effects of genistein and β-sitosterol steroids and GSI data that we found previously in male *B. splendens* [30]; however, estrogenic compounds inhibit oocyte development [17, 52] and ovulation [2] as well as cause reductions in GSI [52] and gonad size [53] in other fish. It is possible that if we had included the pharmacologic doses (1000 μg L^{-1}) in the histological testing, we may have detected an effect of phytoestrogens on the gonads. However, as with the significant behavioral change, its biological significance would have been difficult to interpret.

The authors do not interpret this finding as an indication that anthropogenic phytoestrogen concentration in waterways is safe for fish. In addition to fish as important indicators of ecosystem health [54], the health of waterways and wild populations of fish are inextricably linked to human health through the consumption of fish [55] and fish products, such

Table 2: Histology and GSI measurements of adult female *B. splendens* across steroid and phytoestrogen exposures.

	Vehicle only	E2 (100 μg/L)	Genistein (1 μg/L)	β-Sitosterol (1 μg/L)	Gen (1 μg/L) β-sit (1 μg/L)
GSI $F_{73} = 1.90$, $P = 0.12$	0.08 ± 0.03	0.07 ± 0.03	0.06 ± 0.03	0.08 ± 0.03	0.10 ± 0.034
Oocyte type (i) $F_{60} = 1.48$, $P = 0.22$	0.31 ± 0.13	0.28 ± 0.14	0.29 ± 0.14	0.25 ± 0.11	0.37 ± 0.12
Oocyte type (ii) $F_{60} = 1.73$, $P = 0.16$	0.37 ± 0.14	0.38 ± 0.11	0.41 ± 0.14	0.42 ± 0.15	0.37 ± 0.14
Oocyte type (iii) $F_{60} = 1.17$, $P = 0.17$	0.15 ± 0.05	0.19 ± 0.04	0.17 ± 0.04	0.17 ± 0.03	0.16 ± 0.03
Oocyte type (iv) $F_{60} = 0.45$, $P = 0.77$	0.16 ± 0.11	0.15 ± 0.08	0.13 ± 0.09	0.16 ± 0.03	0.17 ± 0.07

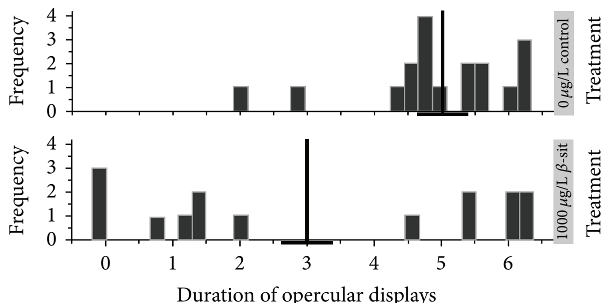

Figure 2: Female *B. splendens* exposed to 1000 μg L^{-1} of β-sitosterol spent significantly less time engaged in opercular displays than fish in the control treatment group. Mean and standard error of the residuals of the log transformed data for each group are indicated by the vertical and horizontal crossbars, respectively. This figure also illustrates the presence of many extreme values that could not be resolved by transformation.

as fish oil capsules [56]. The results of the present study are consistent with previous findings that endocrine disrupting compounds may have a reduced impact on steroid levels when exposure occurs in adulthood or over a short duration [57]. Greater exposure duration than 21 days or exposure to combinations of other chemicals in wood pulp mill effluent might produce an effect, as suggested by other researchers [58]. Although previous work found decreased sperm quality [42] and aggression [26] in male *B. splendens* exposed to phytoestrogens, the results of the present study reflect the assertion made previously [30] that *B. splendens* may be more resistant to phytoestrogens than previously thought.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Exposure to Environmentally Relevant Concentrations of Genistein during Activation Does Not Affect Sperm Motility in the Fighting Fish *Betta splendens*

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Sperm collected from male fighting fish *Betta splendens* were activated in control water, water containing the ion-channel blocker gadolinium (a putative positive control), or water containing the isoflavone phytoestrogen genistein to determine the effects of acute genistein exposure on male reproductive function. Computer-assisted sperm analysis was used to quantify the proportion of sperm that were motile and the swimming velocity of those sperm. The highest concentration of gadolinium (100 μM) tested was effective at reducing sperm motility and velocity, but neither concentration of genistein tested (3.7 nM or 3.7 μM) significantly affected these sperm parameters. Our findings suggest that acute exposure to waterborne phytoestrogens during activation does not reduce the motility of fish sperm.

1. Introduction

Fish sperm are immotile in the gonads and are activated upon release into hypotonic (freshwater fishes) or hypertonic (saltwater fishes) solutions. Particularly among freshwater species, motility may last less than two minutes following activation [1, 2]. Lacking an acrosome, fish sperm must enter the egg mechanically by swimming through the micropyle. In many species, however, the brief duration of sperm motility is insufficient for the sperm to traverse more than half of the egg's circumference [3, 4]. Thus, sperm swimming velocity is one of the most important predictors of fertilization success in fishes with both internal and external fertilization [5–7].

Fish sperm have limited ability to adjust to physicochemical changes in their external environment [1, 8]. As a result, fish sperm are vulnerable to disturbance by numerous environmental contaminants. Direct exposure of sperm to contaminants, via activation of sperm in contaminated water, has been shown to reduce sperm motility, velocity, and fertilization ability [9, 10]. The mechanisms for such disruptions are largely unknown but may include damage to the sperm plasma membrane or axoneme, or consumption of adenosine triphosphate (ATP) [10]. In some cases, however, reductions in sperm motility and velocity are only observed at concentrations lethal to the fish themselves [10, 11].

Deficits in sperm motility due to environmental contamination can also occur indirectly when long-term exposure in maturing or adult fish perturbs sexual differentiation, gonadal development, or spermatogenesis. Lahnsteiner et al. [12] reported declines in trout sperm motility and swimming velocity when adult males were exposed to environmentally relevant levels of the endocrine disruptor bisphenol A. Similarly, Montgomery et al. [13] found that long-term exposure to the synthetic estrogen 17α-ethinylestradiol reduced sperm swimming velocity in the fighting fish, *Betta splendens*, most likely through a depletion of ATP reserves.

Phytoestrogens are a broad class of estrogenic compounds found in plants that have the potential to disrupt fish sexual development and gamete quality when they are concentrated and released into the environment due to human activity. Numerous studies have reported biologically active levels of phytoestrogens (particularly genistein, equol, and β-sitosterol) in effluent from wood pulp mills or sewage treatment plants or in agricultural soils [14–16]. At these concentrations,

phytoestrogens can disrupt sexual differentiation and induce production of egg yolk protein in males [17–20]. The potential for fish populations to experience widespread and acute contamination from phytoestrogens is significant; undiluted plumes of pulp and paper mill effluent have been reported dozens of kilometers downstream from point sources [21].

Relatively little is known about the effects of phytoestrogen exposure on fish sperm quality. Sperm motility and concentration decreased in a dose-dependent manner in rainbow trout, *Oncorhynchus mykiss*, fed diets experimentally enriched with genistein, an isoflavone [18]. Stevenson et al. [22] exposed male fighting fish to waterborne genistein and β-sitosterol (a phytosterol) at environmentally relevant and pharmacological concentrations for four weeks but found that neither dose of either phytoestrogen had an effect on sperm quality. Sharpe et al. [23] found that β-sitosterol disrupted transcription of steroidogenic acute regulatory (StAR) protein in goldfish, *Carassius auratus*, which could interfere with steroidogenesis. Green and Kelly [24] incubated testes of channel catfish, *Ictalurus punctatus*, and walleye, *Sander vitreus*, in genistein and found a significant negative effect on sperm motility, ATP content, and *in vitro* fertilization rates in both species. The aforementioned studies all focus on phytoestrogen disruption of sperm maturation, but little work has been done on acute sperm exposure to phytoestrogens.

The purpose of the current study is to test the direct effects of environmentally relevant concentrations of genistein in activation water on the motility and velocity of sperm in the fighting fish, *Betta splendens*. By exposing sperm via activation water, the experimental design mimics the acute exposure that spawning fish might experience when phytoestrogen contamination is spatially or temporally variable. An ancillary goal of this research is to test the efficacy of gadolinium as a positive control in studies of fish sperm motility. Gadolinium blocks stretch-activated calcium ion channels, which are involved in sperm activation [25] and was deemed an appropriate positive control because genistein has been shown to affect motility of mammalian sperm by inhibiting spermatic calcium channels [26].

2. Materials and Methods

Sexually mature males from a domesticated strain of *Betta splendens* were purchased from a commercial supplier and acclimated for one week in the laboratory prior to each experiment. Fish were housed in individual, visually isolated 1-L beakers containing 800 mL of reverse-osmosis (RO) water reconstituted to a conductivity of 110–140 μS using R/O Right (Kent Marine). Fish were fed freeze-dried chironomid larvae five times per week, water was maintained at 27°C, and the light cycle was kept at 14 : 10 L : D. Animal care protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of Amherst College.

The first experiment was designed to test the effects of the ion channel blocker gadolinium on sperm activation and motility. Twenty-four *B. splendens* were anesthetized with buffered tricaine methanesulfonate (MS-222; Western Chemical) and sacrificed. The testes were removed and suspended in a quiescent state in 100 μL of "catfish" sperm extender [27], which has been used successfully in a variety of species [3]. The sperm extender contained 5.52 g/L NaCl, 2 g/L KCl, 2.42 g/L Trizma HCl, and 3.75 g/L glycine, dissolved in distilled water at pH 7.5. The testes were then punctured 40 times with a needle to release the sperm. This method has been used successfully in several previous studies on sperm motility in *B. splendens* [13, 22].

Sperm samples were assigned to one of four treatments that differed in the composition of the activation water: control (no gadolinium), 25 μM gadolinium, 50 μM gadolinium, and 100 μM gadolinium ($n = 6$ fish in each group). Gadolinium (Sigma-Aldrich Co.) was dissolved in trace quantities of ethanol; the control treatment contained only the ethanol vehicle. Four μL of activation water (taken from aquaria) was mixed with 12 μL of the sperm solution described above. Sperm were activated within 4 min after being extracted from testes. Five μL of activated sperm sample was immediately mounted onto Leja 20-micron slides and viewed under a Nikon Eclipse E400 microscope. Using a SPOT Insight QE, Model 4.1 camera, three videos (approximately 6 sec in duration, 70 frames/sec) from different parts of each slide were recorded within 60 sec of sperm activation. The rapid assessment of motility ensured that our results were not affected by the relatively high osmolality [28]. Videos were analyzed with computer-assisted sperm analysis (CASA) using the Java plug-in for ImageJ [29]. Sperm number was counted in each slide view and standardized by multiplying the number of detected sperm by the dilution factor and dividing by the testes mass (g) of each fish. Parameters obtained for each fish were known indicators of fertilization success in a range of fish species [8]. These included percent motility, as well as the average curvilinear velocity (VCL; point-to-point velocity per sec), straight line velocity (VSL; velocity measured along a straight line from the first point of movement to the point furthest from the origin), and smooth path velocity (VAP; point-to-point velocity based upon the average path) for each video. For each sperm sample, the values of the above parameters were averaged for the three recorded video sequences to obtain a single parameter for each fish. The three velocity measures (VCL, VSL and VAP) were strongly positively correlated ($r > 0.76$, $P < 0.001$ for all correlations) and showed the same differences among treatment groups. Therefore, for simplicity we report results from VCL only.

To test the effects of genistein in activation water on sperm motility, we used 41 *B. splendens* from the same supplier and housed these fish in an identical manner. These fish were divided among four treatments: a negative control (no genistein in activation water), a positive control (100 μM gadolinium), an environmentally relevant dose of 3.7 nM genistein, or a pharmacologic dose of 3.7 μM genistein [14, 22, 30]. Each treatment group had 10 fish, except the 3.7 nM genistein group, which had 11 fish. Genistein (number G6649; Sigma-Aldrich Co.) and gadolinium were dissolved in ethanol prior to addition to activation water; the negative control treatment contained similar concentrations of ethanol alone. Testes were prepared and sperm motility recorded following the procedures described above.

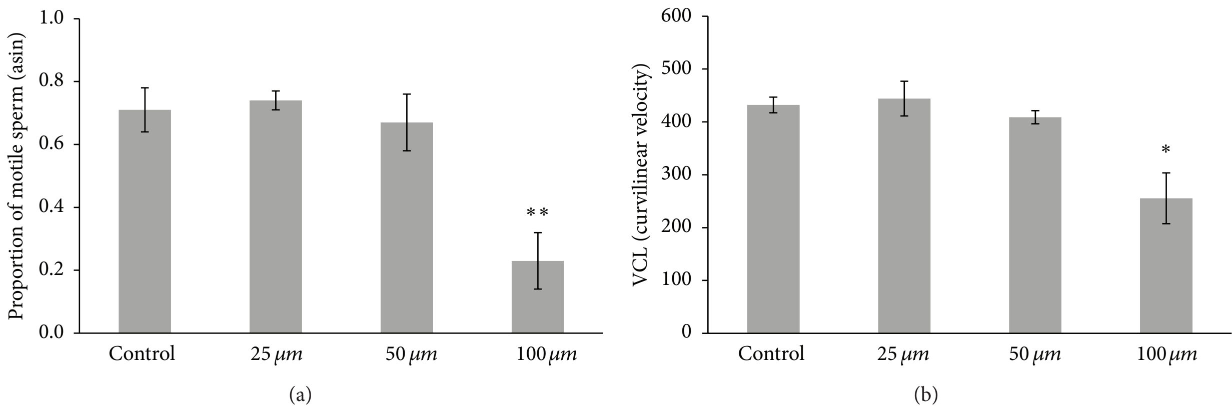

Figure 1: Motility (a) and curvilinear velocity or VCL (b) of *B. splendens* sperm exposed to negative control conditions or one of three doses of gadolinium (25, 50, or 100 μM). Sperm motility data were arcsin transformed prior to analysis. $^{*}P = 0.001$ and $^{**}P < 0.001$. VSL and VAP (not shown here; see text for descriptions) were similar in statistical significance to VCL ($P = 0.001$ for both).

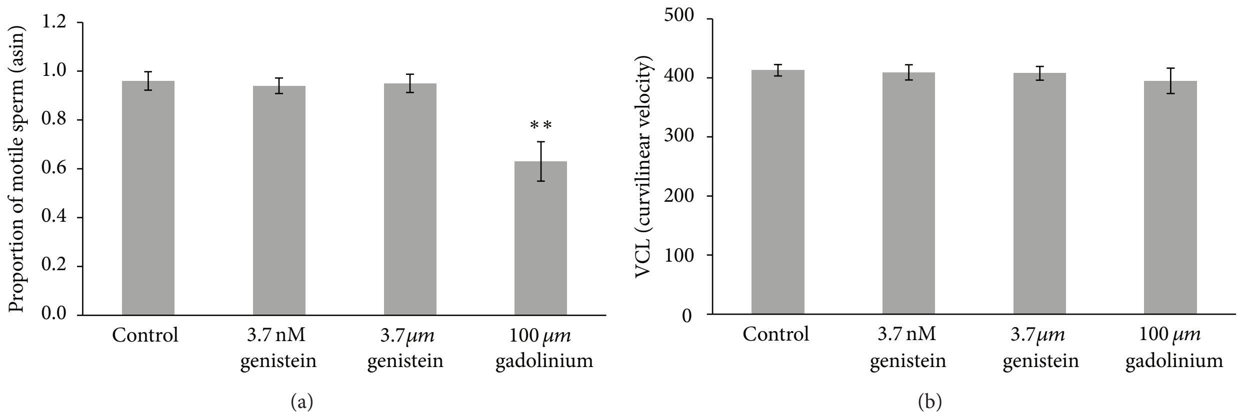

Figure 2: Motility (a) and curvilinear velocity or VCL (b) of *B. splendens* sperm exposed to negative control conditions, one of two doses of the phytoestrogen genistein (3.7 nM or 3.7 μM), or the positive control gadolinium (100 μM). Sperm motility data were arcsin transformed prior to analysis. $^{**}P < 0.001$. Differences among treatments in VSL and VAP (not shown here; see text for descriptions) were similarly nonsignificant ($P = 0.70$ and 0.79, resp.).

SPSS version 15.0 was used for statistical analysis. Data were checked for normality prior to analysis and arcsin transformations were used when necessary. Differences were considered significant if $P < 0.05$ and means are presented ± SE. The statistical power of the analysis was estimated using the effects size conventions of 0.1, 0.25, and 0.40 for small, medium, and large effects, respectively, using G*Power version 3 [31].

3. Results

In the first experiment, activation water containing the highest dose of gadolinium (100 μM) was found to have significant effects on *B. splendens* sperm. Gadolinium exposure resulted in fewer motile sperm ($F_{3,20} = 12.77$, $P < 0.001$; Figure 1(a)) and sperm with reduced curvilinear velocity or VCL ($F_{3,20} = 8.18$, $P = 0.001$; Figure 1(b)). Straight-line (VSL) and average path (VAP) velocity measures yielded similar results. A Scheffe's *post hoc* test revealed that motility and VCL were significantly reduced at 100 μM compared to 0, 25, and 50 μM ($P < 0.018$ for all). None of the other pairwise comparisons was significant ($P > 0.88$ for all). Thus, the 100 μM dose of gadolinium was selected for use as our positive control in the subsequent experiment.

In the second experiment, *B. splendens* sperm were activated in one of four treatments: negative control (ethanol vehicle only), positive control (100 μM gadolinium), or one of two concentrations of genistein (3.7 nM or 3.7 μM). Gadolinium exposure reduced the proportion of sperm that were motile ($F_{3,37} = 9.1$, $P < 0.001$; Figure 2(a)), but neither dose of genistein significantly affected the proportion of sperm that were motile (Figure 2(a)) or sperm VCL (Figure 2(b))

when compared to the negative control conditions ($P > 0.78$ for all Scheffe's *post hoc* comparisons). The statistical power of this analysis for small, medium, and large effect sizes was 0.07, 0.22, and 0.51, respectively.

4. Discussion and Conclusion

Fighting fish *B. splendens* sperm activated in water containing 100 μM gadolinium were less likely to be motile and swam more slowly than sperm activated in control water or water with lower (25 or 50 μM) concentrations of gadolinium. Sperm exposed to the highest dose of gadolinium showed similarly significant declines in all three measures of velocity (VCL, VSL, and VAP). This reduction in VCL, VSL, and VAP indicates asymmetric flagellar waveforms, perhaps due to the effect of gadolinium on intracellular Ca^{2+} concentrations [10].

Morisawa and Suzuki [2] were the first to demonstrate the particular role of potassium and calcium ions in the activation of fish sperm. Gadolinium ions (Gd^{3+}) are known to block stretch-activated ion channels [32] and it has been proposed that their effect on intracellular Ca^{2+} concentration can initiate sperm motility [33]. Consistent with this, gadolinium reduced sperm motility in both puffer fish, *Takifugu niphobles*, and carp, *Cyprinus carpio*, sperm [34, 35]. These effects were reversible, suggesting that gadolinium blocked the stretch-activated channels, and were dependent on both dose and incubation time. The difference between our finding and those of Krasznai et al. [34, 35], namely, that they found significant effects of gadolinium at lower concentrations (e.g. 10 μM), can be attributed to the longer incubation times used in their research. Thus, our gadolinium results are generally consistent with previous studies and support the conclusion that gadolinium is a suitable positive control for sperm motility studies in *B. splendens* and other fish species. Other potential positive controls include calcium ion chelating agent such as ethylene glycol tetra-acetic acid (EGTA) [25].

Activation water containing two environmentally relevant concentrations of the phytoestrogen genistein (3.7 nM and 3.7 μM) caused no reduction in *B. splendens* sperm motility or velocity. Some studies report significant reductions in sperm motility and swimming velocity following phytoestrogen exposure in rainbow trout, channel catfish, and walleye [18, 24]. However, these studies employed different experimental approaches than the ones we used in the current study. Bennetau-Pelissero et al. [18] fed trout genistein-enriched diets for a full year prior to testing, and Green and Kelly [24] incubated catfish and walleye testes for 14 h in genistein solutions that were much more concentrated (up to 0.01 M) than the solutions we used. The genistein exposure that *B. splendens* sperm experienced in activation water may have been too brief to reduce ATP levels or increase oxidative damage, which are some of the proposed mechanisms by which phytoestrogens disrupt sperm motility in fishes [18, 24]. Future work should focus on the temporal changes in sperm motility following phytoestrogen exposure, particularly in fishes with long-lived sperm.

Our results were generally consistent with a previous study on the long-term effects of phytoestrogens on reproductive performance in this same species. Stevenson et al. [22] exposed adult male *B. splendens* to environmentally relevant levels of two waterborne phytoestrogens (genistein and β-sitosterol, and their mixture) for 4 weeks and found no detrimental effects on a battery of reproductive parameters including sperm motility and velocity, as well as sex steroid hormone levels, gonad size, and fertilization success. Thus, there was no evidence in that study that phytoestrogens accelerated spermatogenesis or disrupted steroid hormone production in *B. splendens* [22]. Coupled with those results, the current study suggests that acute exposure to environmentally relevant concentrations of genistein has few deleterious consequences for fish sperm. Future work could verify this conclusion by incubating testes in genistein solutions or by subjecting the sperm of species more sensitive than *B. splendens* to the acute effects of phytoestrogen exposure. Reproductive performance may still be compromised by phytoestrogens in aquaculture, however, where soy-based protein is a common ingredient in fish feed [18].

Conflict on Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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