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LETTER TO EDITOR

# Avoidance of Copper and Zinc by Rainbow Trout *Oncorhynchus mykiss* Pre-exposed to Copper

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**Abstract** Laboratory tests were conducted on 1-year-old rainbow trout *Oncorhynchus mykiss* in a counter-current flow, steep-gradient chamber to evaluate their ability to detect and avoid copper and zinc at concentrations of 0.1 mg Cu/L and 1 mg Zn/L, respectively, after 10-day pre-exposure to five copper sublethal concentrations ranging from 0.1 to 0.5 mg Cu/L and after 10-day re-acclimation period in clean water. Avoidance response intensity in affected fish significantly decreased with increase in pre-exposure Cu concentration. The strength of avoidance response to Cu and Zn test solutions in pre-exposed fish after re-acclimation gradually increased in a concentration-dependent order.

**Keywords** Fish · Behavior · Avoidance · Toxicity · Cu and Zn · Pre-exposure

Avoidance of polluted water is one of the most significant sublethal responses of fish (Sprague and Drury 1969). Occurrence of this behavior in natural environments can result in disturbances of fish migration and distribution patterns (Saunders and Sprague 1967; Hansen et al. 1999a; Wells et al. 2004). The ability of aquatic animals to detect and avoid toxic substances is thought to be one of the forms of phenotypic adaptation allowing them to survive in altered environment (Flerov 1989).

Fishes spontaneously avoid many types of toxicants (see reviews: Beitinger and Freeman 1983; Brown et al. 1982; Giattina and Garton 1983; Atchison et al. 1987; Tierney et al. 2010). These responses occur when the choice is given to test fish to discriminate between clean and polluted water. However, insufficient information is compiled about how avoidance response of fish can be modified in the case when they are deprived of such opportunity and pre-exposed to toxicants for long periods of time. Several investigations have been devoted to this problem (Anestis and Neufeld 1986; Hartwell et al. 1987; Myllyvirta and Vuorinen 1989; McNicol and Scherer 1993; Farr et al. 1995; Hansen et al. 1999a, b; Svecevičius 1999a; 2003). However, these studies were conducted under different experimental conditions, and the results often are difficult to compare. Information is totally lacking about how fishes acclimated to one toxicant later will respond to other toxicants. However, it is clear that pre-exposure to a chemical can alter fish behavior to the same chemical at a later time. For example, Svecevičius (1999a) established that rainbow trout *O. mykiss* (Walbaum) pre-exposed for 3 months to sublethal concentrations of 0.1 and 0.2 mg Cu/L changed their behavioral response and started to demonstrate significant preference towards copper solutions to which they have been pre-exposed.

The objectives of the present study were (1) to evaluate the ability of rainbow trout to avoid copper and zinc solutions after 10-day pre-exposure to sublethal and nearly lethal copper concentrations ranging from 0.1 to 0.5 mg Cu/L and (2) to evaluate the same avoidance ability of rainbow trout after a 10-day re-acclimation period in clean water.

## Materials and Methods

Rainbow trout adults (1-year-old) were obtained from Žeimena Hatchery (Švenčionys District, Lithuania).

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Average total length of test fish was 100 ± 10 mm and the total weight was 10 ± 2 g (mean ± SEM). The fish were acclimated to laboratory conditions for 1 week prior to testing. They were kept in flow-through 1,000-L holding tanks supplied with aerated deep-well water (minimum flow rate 1 L per 1 g of their wet body mass per day), under natural illumination and were fed commercial trout feed daily in the morning; the total amount was no less than 1% of their wet body mass per day. During the tests the fish were also fed in the same manner.

Copper and zinc sulphates ($CuSO_4 \cdot 5H_2O$, $ZnSO_4 \cdot 7H_2O$, reagent grade 99%) (≪ REAKHIM ≫ Company, Russia) were used as the toxicants, and stock solutions were prepared by dissolving a necessary amount of salts in distilled water.

Deep-well water was used as the dilution water. Average hardness of the water was approximately 284 (271–296) mg/L as $CaCO_3$, alkalinity was 200 (190–210) mg/L as $CaCO_3$, pH ranged from 7.9 to 8.1, temperature was maintained at 10.5 to 11.5°C, dissolved oxygen concentration was maintained at 8–10 mg/L, and dissolved organic carbon (DOC) was less than detection limit (0.3 mg/L).

Groups of 5 fish were pre-exposed to each concentration under semi-static conditions in 30-L tanks filled 2/3 with continuously aerated water. Test fish were exposed for 10 days in dilution water or to a series of 5 nominal concentrations ranging from 0.1 to 0.5 mg Cu/L. That pre-exposure concentration range was chosen based on a 96-h LC50 of 0.65 mg Cu/L derived from acute toxicity tests conducted previously in water from the same well (Svecevičius and Vosylienė 1996). Test solutions were renewed every day, test fish were transferred into the freshly prepared solutions, and mortality was recorded at 24-h intervals. The tests were replicated twice (5 + 5).

Avoidance tests were conducted in a counter-current flow, plastic steep-gradient chamber described in detail by Svecevičius (2007). The chamber was 127 × 15 × 15 cm, and total water flow rate was 6 L/min. Both sides of the chamber were fed by gravity water flows (hydraulic retention time in each side of the chamber was 2.5 min). Control tests with dye (methylene blue) showed that 10 min after the introduction of test solution into one end of the chamber, a stable, steep gradient with a sharp boundary between clean water and test solution was established.

Each trial used one fish and consisted of three periods; an acclimation period, a control period, and a test period. In order not to stress the fish, they were placed into copper solution at a concentration to which they were pre-exposed after water flow in a gradient chamber was stopped. Each fish was placed into the gradient chamber and acclimated until it swam freely exploring the chamber. After that, the next 10 min was the control period during which fish movements in the chamber were recorded with a digital PC video camera (GILE, WA-008, China) for further analysis. Immediately after the control period, water flow in the gradient chamber was restored and a test solution was introduced into one end of the chamber. The fish were allowed to choose between clean water and toxicant solution for 20 min and their movements were recorded during the last 10 min of this. Fish responses to 0.1 mg Cu/L and 1 mg Zn/L were examined. In a previous study using the same dilution water, these copper and zinc concentrations were avoided by rainbow trout with almost maximal intensity and the nominal threshold avoidance concentrations were 0.001 mg Cu/L and 0.0001 mg Zn/L of added metal (Svecevičius 1999b) (Lithuanian inland water standards are: 0.01 mg Cu/L and 0.1 mg Zn/L). In the present study avoidance of copper was tested first; then after 10 min was allowed for the gradient chamber to clean itself, avoidance of zinc was tested. Test solutions were added at one end or at the other of the chamber, in random order to avoid potential bias resulting from one end preference of the fish.

When the tests were over, fish were placed into the same pre-exposure tanks but now containing clean water for recovery during the next 10 days. After that re-acclimation period behavioral tests were performed with the same copper and zinc solutions.

The intensity of behavioral response was calculated as the following Response index:

$$\text{Response index} = 50(2 - N_T/N_C),$$

where $N_C$ is equal to the time (in s) spent by each test fish in the contaminated end during the control period (when that water was clean), and $N_T$ is the time (in s) spent by each test fish in the same end during the test period.

The value of index 100 denoted maximal avoidance, while 0 signified maximal preference and 50 stood for indifference. Preliminary tests in clean water showed that the test fish preferred both ends of the gradient chamber with the same probability. Because the experimental data were normally distributed and had homogeneous variance, the significance of behavioral responses was determined by comparing the mean Response index value to the theoretically neutral response, i.e. to a Response index value of 50 by use of Student's *t* test at $p \leq 0.05$. The threshold effect concentration (TEC) was estimated by calculating the geometric mean of the lowest observed effect concentration (LOEC) and the no observed effect concentration (NOEC) of pre-exposure copper (Van Leeuwen and Hermens 1995). The data were analyzed statistically through STATISTICA (Version 6.0) and GraphPad Instat$^{TM}$ (Version 2.04) softwares.

Dissolved oxygen in the tanks, temperature and pH were measured routinely with a hand held multi-meter (WTW

Multi 340i/SET, Germany). Nominal copper and zinc concentrations were checked during the tests with no fish in the chamber with an atomic absorption spectrophotometer (SHIMADZU AA-6800, Japan) by flame or graphite furnace techniques using proprietary software. Each sample was analyzed in triplicate. Mean measured concentrations were within 5%–10% of target.

## Results and Discussion

One of the ten fish died during pre-exposure to 0.4 mg Cu/L and two of the ten fish died during pre-exposure to 0.5 mg Cu/L. A sudden increase in locomotor activity (burst, erratic swimming) was followed by loss of equilibrium, tremor, breath interruption and death.

As pre-exposure copper concentration increased, the intensity of avoidance response to test concentrations of 0.1 mg Cu/L and 1 mg Zn/L significantly decreased and reached indifference at higher copper concentrations (Table 1).

The relationship between pre-exposure copper concentration and the intensity of avoidance response was nonlinear. Regression analysis was conducted and Harris model (Harris 2001) for all four behavioral test variants was applied (Table 1).

The TEC for pre-exposure copper was 0.25 mg Cu mg/L for both the Cu and Zn challenges. Although re-acclimated fish summarily avoided the Cu and Zn challenges better than they did immediately after pre-exposure to copper, the intensity of avoidance response in fish pre-exposed to higher Cu concentrations (0.3–0.5 mg Cu/L) was still significantly lower than in control fish (Table 1).

In a previous study (Svecevičius 1999a), rainbow trout were pre-exposed for 3 months to sublethal concentrations of 2 different model mixtures consisting of five heavy metals (Cu – 0.11, Zn – 0.25, Ni – 0.12, Cr – 0.15, Fe – 2.9 and Cu – 0.13, Zn – 0.38, Ni – 0.13, Cr – 0.23, Fe – 2.4 mg/L, respectively) and 2 copper concentrations of 0.1 and 0.2 mg/L. During the 3-month test period the behavior of fish was investigated monthly. The control fish avoided test solutions with almost maximal intensity. However, pre-exposed fish demonstrated significant preference to all concentrations of test solutions.

In this study using the same well-water source, we did not obtain preference at copper concentrations of 0.1 and 0.2 mg/L. Only avoidance response intensity was affected. Consequently, 10-day pre-exposure period to these solutions is quite insufficient to induce significant changes in fish organism responsible for behavioral orientation. Apparently, this phenomenon is related to evidence of phenotypic adaptation. This kind of adaptation has two forms: long-term (biochemical-physiological) and short-term behavioral (avoidance) (Flerov 1989). Perhaps, 1-month was quite enough for biochemical-physiological adaptation to occur, which caused copper-polluted water preference in test fish.

Overall, the data on behavioral responses of fish pre-exposed to the toxicants seem to be species-specific, concentration and exposure-duration dependent, and often contradictory and difficult to compare. For example, rainbow trout pre-exposed to sublethal concentrations of chromium ranging from 0.01 to 3 mg Cr/L for 7–20 weeks demonstrated much higher avoidance thresholds of chromium than control fish (Anestis and Neufeld 1986). Hartwell et al. (1987) pre-exposed fathead minnows *Pimephales promelas* from 3 to 9 months to a single concentration of fly ash slurry (Cu—1.3, Cr—0.7, Cd—0.03, As—2.4, Se—0.5 mg/L, respectively). Pre-exposed fish demonstrated only mild avoidance at concentrations that control fish strongly avoided, while showing strong attraction to high concentrations of the slurry. Similarly, vendace *Coregonus albula* avoided bleached kraft mill effluent (BKME), although fish pre-exposed to BKME

**Table 1** Avoidance response of rainbow trout (response indices ± SEM, N = 8–10) to test concentrations of copper and zinc

| Pre-exposure Cu concentration (mg/L) | Fish after 10-day pre-exposure to copper | | Fish after 10-day re-acclimation in clean water | |
|---|---|---|---|---|
| | 0.1 mg Cu/L | 1 mg Zn/L | 0.1 mg Cu/L | 1 mg Zn/L |
| 0 (control) | 90 ± 4.0* | 98 ± 0.7* | 89 ± 4.2* | 97 ± 0.9* |
| 0.1 | 70 ± 3.8*# | 62 ± 5.1*# | 88 ± 4.2* | 95 ± 1.7* |
| 0.2 | 65 ± 5.5*# | 58 ± 3.5*# | 82 ± 4.0* | 89 ± 3.8* |
| 0.3 | 54 ± 4.2# | 50 ± 4.0# | 82 ± 3.8* | 88 ± 5.1*# |
| 0.4 | 54 ± 4.4# | 52 ± 3.9# | 67 ± 3.6*# | 72 ± 4.3*# |
| 0.5 | 48 ± 5.0# | 47 ± 4.5# | 61 ± 4.4*# | 60 ± 3.9*# |
| Regression equation | $Y = 1/(0.0111 + 0.0155 \cdot X^{0.7174})$ | $Y = 1/(0.0102 + 0.0140 \cdot X^{0.3810})$ | $Y = 1/(0.0113 + 0.0298 \cdot X^{2.4884})$ | $Y = 1/(0.0105 + 0.0523 \cdot X^{3.0623})$ |
| $R^2$ | 0.98 | 0.98 | 0.96 | 0.97 |

* Values significantly different from 50 ($p \leq 0.05$); # denote values significantly different from control

(at 0.13–4.5 v/v %) for 1 week significantly preferred polluted water (Myllyvirta and Vuorinen 1989). Lake whitefish *Coregonus clupeaformis* pre-exposed to cadmium ranging from 0.0002 to 0.005 mg Cd/L for 3 weeks preferred water containing sequentially increasing sublethal cadmium concentrations (McNicol and Scherer 1993). Farr et al. (1995) studied avoidance response of fathead minnows surviving acute exposure to fluoranthrene. Affected and control fish avoided concentrations ranging from 0.0086 to 0.0438 mg/L with the same intensity. Rainbow trout and chinook salmon *Oncorhynchus tshawytscha* were pre-exposed to a nominal 0.002 mg Cu/L concentration for 25 to 30 days prior to Cu-avoidance testing. Copper-acclimated chinook salmon did not avoid any of the Cu concentrations tested (0.0034–0.021 mg/L). Moreover, these fish significantly preferred water with 0.0016 mg Cu/L. Meanwhile, rainbow trout avoided all Cu concentrations higher than 0.0016 mg/L. Avoidance responses to Cu were close in intensity to those of non-Cu-acclimated fish (Hansen et al. 1999b). Similarly, rainbow trout acclimated to a metal mixture (Cu – 0.012, Zn – 0.05, Pb – 0.0032, Cd – 0.0011 mg/L) for 45 days period significantly avoided higher metal concentrations (Hansen et al. 1999a). Rainbow trout underwent 70-day acute and chronic toxicity test to a model heavy metal mixture (Cu – 0.0075, Zn – 0.64, Ni – 0.0021, Cr – 0.0028, Pb – 0.0142, Cd – 0.00018, Mn – 0.0099 mg/L, respectively) in early development (eggs, larvae). After 1 year when fish became adult they avoided lower and higher concentrations of the mixture in the same manner as control fish (Svecevičius 2003).

The mechanisms underlying such large differences in fish behavior are not clear. However, fish responses are probably directly connected with changes in fish chemoreceptor sensitivity to pollutants. It has been established that avoidance response to copper in lake whitefish *C. clupeaformis* (Brown et al. 1982) and to copper and zinc in vimba *Vimba vimba* (Svecevičius 1991) are olfactory-mediated since experimentally anosmated fish failed to avoid these metals.

Tierney et al. (2010) summarized the data on olfactory toxicity in fishes and concluded that copper like other heavy metals is a general-purpose toxicant for all freshwater fish. Moreover, copper can affect the fish olfactory system, directly inducing structural and functional alterations (histopathological destruction of olfactory epithelium and nerve, disturbance of behavioral responses to natural odors: alarm substances, food extracts, L-serine, etc.). Recovery from copper in several days is also possible. Changes in ambient water chemistry have an influence on copper olfactory toxicity. Increases in hardness, alkalinity and dissolved organic carbon (DOC) are protective against copper. However, the authors emphasized that in some cases, it can be difficult to separate olfactory toxicity from other forms of toxicity.

Recovery of the fish pre-exposed to pollutants was demonstrated as well. Thus, Anestis and Neufeld (1986) have found that a rainbow trout population pre-exposed to a critical chromium concentration of 0.8 mg/L for 7–20 weeks followed by acclimation for 7 days in clean water demonstrated a functional recovery within that period of time, since their respective avoidance curves were similar to the control fish populations. Hara et al. (1983) suggested a 12-week rehabilitation period for biologically affected fish after pre-exposure to a pollutant. The data obtained in the present study suggests a much shorter period for functional recovery from copper exposure.

Without discussing possible mechanisms, it is evident that the influence of pre-exposure to pollutants in fish is ecologically significant. Anestis and Neufeld (1986) suggested that pollutant preference in a natural environment might be a “physiological trap” and might affect their behavioral patterns if fish previously were pre-exposed to specific toxic substances. McNicol and Scherer (1993) suggested that pre-exposed fish populations can be attracted to “familiar” polluted water for chemosensory impairment, sensitization, desensitization, or familiarization although preference for pollutants has not been reported in-field.

The data obtained in the present and earlier (Svecevičius 2003) studies partially negate these suggestions and confirm that fish acclimation to metals is of temporary character and test fish recover functionally after a period of time independently of whether they were pre-exposed to these substances in early development or in considerably later-life periods. Moreover, fish acclimated to one kind of metal later respond to the same and other metals very similarly. The question is whether this pattern can be extended to other chemicals or it can be applied only to metals. Therefore, in further studies the number and concentration range of toxicants should be expanded, and more exhaustive investigation into the mechanisms by which fish can adapt to pollutants is needed.

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