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The effects of early chronic exposure to sublethal copper on the olfactory discrimination of rainbow trout, *Oncorhynchus mykiss*

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Synopsis

The impact of a long-term sublethal copper exposure on the olfactory discrimination performance was examined in rainbow trout, *Oncorhynchus mykiss*. Two fish groups, one from the 14th day post-fertilization and the other from hatching, were exposed continuously to 22 $\mu g\ Cu^{+2} \cdot l^{-1}$ for 41 and 37 weeks, respectively. Preference/avoidance responses to different odor conditions (own rearing water/well water; own rearing water/heterospecific water) were evaluated during the 8th month after hatching. The 2 odor conditions appear reliable criteria to evaluate the fish discrimination ability. When controls were given a choice between own rearing water against either well water or heterospecifc water, they significantly preferred their own rearing water, whereas both copper-exposed groups showed no preference. The behavioral response of exposed fish indicates impairment of their olfactory discrimination ability; however, it cannot be determined if this reflects a loss of olfactory sensitivity or an olfactory hyposensitivity. Behavioral tests performed 2 and 10 weeks after removal of copper, showed some functional recovery of the olfactory discrimination ability which could be related to the renewal property of olfactory receptor cells. Results demonstrate that a long-term sublethal exposure to copper, as it commonly occurs in the 'natural' condition, may result in olfactory dysfunction with potential impacts on fish survival and reproduction.

Introduction

Copper is widely distributed in aquatic ecosystems and may exceed the 5–15 $\mu g \cdot l^{-1}$ non-effect value (Birge & Black 1979). Many investigations of copper toxicity have dealt with the impact of this heavy metal on sensory modalities, and the olfactory system has received particular attention because feeding, defense, schooling, spawning and migration are significantly influenced by olfactory cues (see Hara 1986). Many behavioral studies have evaluated the response of fish to copper and their ability to mitigate the effects of toxicant. Tests have revealed the capability of fish to detect and generally avoid copper at low concentrations (see Rand 1985). But no data are available regarding impairment of fish olfactory discrimination ability as a result of copper exposure. This question is of obvious importance for aquatic animals since olfaction, like the other chemical channels, plays an important role in mediating physiological and behavioral responses to the environment. Sparse histopathological studies have shown that short-term exposures to high copper concentration ($\geq 150\ \mu g\ Cu^{+2} \cdot l^{-1}$) induce

lesions in the fish olfactory organ with degeneration of receptor cells (Gardner & LaRoche 1973, Hara et al. 1983), the severity of injury being dependent upon the metal concentration and the exposure duration. In such exposure conditions, little or no electro-olfactogram (EOG) response to food extract and L-serine was noted in rainbow trout (Hara et al. 1983), suggesting some impairment of the olfactory function. On the other hand, no behavioral or histopathological study has been carried out to evaluate the olfactory dysfunction following a copper exposure comparable, to some extent, to that occuring in the 'natural' environment as a result of man's industrial activities. It is obviously important to understand how a long-term sublethal copper exposure may alter chemosensory function and modify odor detection. Behavioral modifications resulting from such exposure conditions may not lead directly to death but may cause disturbance of major ecological significance.

Materials and methods

The present study evaluated whether chronic exposure to sublethal copper impairs fish olfactory discrimination. Emphasis was given to early life interval which appears, during the fish life cycle, as one of the most sensitive to toxicants (McKim 1985). Rainbow trout, *Oncorhynchus mykiss,* were continuously exposed to copper from the 14th day after fertilization or from hatching. Fish olfactory discrimination ability was evaluated during the 8th month after hatching by recording preference/avoidance reactions in 2 different odor conditions. The effect of copper removal upon the discrimination performance was also measured 2 and 10 weeks after the end of exposure.

Experimental fish and exposure conditions

Rainbow trout eggs came from the hatchery of the Aquarium & Centre Marin of Shippagan where the experiments were conducted. Eggs were collected from one female and were inseminated by sperm from 3 males. Fertilized egg masses were incubated in well water until they were divided into 3 experimental groups: (1) 700 embryos (embryo group) were placed in well water to which copper was added from the 14th day after fertilization, stage 21 according to Vernier (1969); (2) at hatching, 300 embryos (alevin group) were placed in well water containing copper and (3) the 1000 remaining newly hatched fish (control group) were left in plain well water. Each experimental group was reared in a flow-through circular 125 l fiber-glass tank.

The water circulating in the rearing tanks of the embryo and alevin groups was kept in a 520001 container which was filled weekly with well water to which a calculated quantity of $CuSO_4$ was added to maintain a copper concentration between 20 and 25 $\mu g \cdot l^{-1}$. This copper concentration is between the non-effect dose of 5–15 $\mu g \cdot l^{-1}$ (Birge & Black 1979) and the 37 $\mu g \cdot l^{-1}$ which induces a 100% embryo mortality (McKim et al. 1978). An average copper concentration of $22 \pm 8.85\,\mu g \cdot l^{-1}$ was maintained during the exposure period whereas that of the control tank contained less than 5 μg $Cu^{+2} \cdot l^{-1}$. The other water characteristics were similar in the 3 rearing tanks and were as follows: pH 6.50 to 6.64; total hardness 61.8 to 64 ppm as $CaCO_3$; conductance 200 μmhos; dissolved oxygen 8.68 to 8.90 mg $\cdot l^{-1}$; temperature 7.6 to 10.1°C, and the ion concentration (ppm): $Al^{+3} <$ 0.10; Ca^{+2} 20.2; $Cd^{+2} <$ 0.0004; $Fe^{+3} <$ 0.003; $Mn^{+2} <$ 0.004; $Pb^{+2} <$ 0.034; $Zn^{+2} <$ 0.0043. Temperature was checked daily and the other parameters weekly. The ion concentration was measured twice during the experiments except for copper which was checked weekly. Fish were kept under illumination from 8 h until 18 h and were fed daily with salmonid starter and then with trout grower dry pellets (Corey Feed Mills Ltd., Fredericton, NB).

As a minimum duration exposure of 60 days is suggested for estimating chronic copper toxicity upon survival and growth rates (McKim et al. 1978), we chose an exposure of 41 and 37 weeks for the embryo and alevin groups, respectively. The treatment which started on 29 April 1987 for the embryo group and on 28 May 1987 for the alevin group, was stopped on 3 February 1988. Fish were then placed in plain well water for 10 weeks.

Testing apparatus

The rectangular two-choice apparatus (Quinn & Tolson 1986) consisted of 2 arms, each measuring 69 × 9.5 cm and a downstream central section of 22.5 × 28.5 cm which was separated from the 2 arms by a removable screen. For tests performed during the post-exposure period, a 1.5 × larger two-choice apparatus was used. To avoid disturbing the test fish, the apparatus was covered with a plexiglass shade panel. Each arm received flow from one of two 200 l plastic tanks containing the stimulus water sources. One plastic tank was filled with the fish' own rearing water which was directly pumped from its rearing tank. The other one was filled, depending upon the test, either with well water or with stimulus water siphoned directly from the rearing tank fo heterospecifics, which were adult largemouth bass, *Micropterus salmoides*. The stock density of fish in this rearing tank was 9 kg · m^3; they were fed with smelts and maintained in conditions similar to those of experimental fish. The same temperature was maintained in the two stimulus water tanks. Stimulus water flowed into each arm at a rate of 1 l · min^{-1} and the water level in the testing apparatus was maintained at 7.5 cm by an outflow located in the downstream central section. Methylene blue dye placed at the inflow of one arm of the testing apparatus revealed a flow-through time of 2 min. No intermixing within the arms was seen at the end of the 13 min test period.

Testing procedure

During the exposure period, behavioral testing was performed between the 29th and the 33th week after hatching. Tests were repeated 2 weeks and 10 weeks after the end of the copper exposure.

Two odor conditions were tested during the exposure period. Fish (n = 30) of each experimental group were individually given a choice between their own rearing water and another water which could be, as mentioned above, either well water or heterospecific water. In addition, a test was made to determine if there was any bias for one arm of the testing apparatus; 18 fish of the control group were individually tested with well water flowing in both stimulus arms. For the 2 post-exposure tests, 18 fish of each experimental group (control, alevin and embryo) were given a choice between their own rearing water and well water.

For the test, an individual fish was placed in the screened central section. After 3 min of acclimation, the screen was raised and the time spent by the fish in each compartment, the central section and the two odor arms, was recorded for 10 min. A test fish was considered to be in a given compartment only if its entire body was in that compartment. After 10 min, the fish was removed, the water sources were switched to the other arms, and the testing apparatus was drained, cleaned and refilled for the next test. Fish were not tested more than once.

Data analysis

The total amount of time spent by individual fish in each compartment of the testing apparatus was computed for every test and the mean time was calculated for each experimental group and odor condition. A preference score for each subject was determined as the difference in time it spent orienting toward the different compartments, studied two by two. The null hypothesis that the median of these scores equaled zero was tested by the nonparametric Wilcoxon matched-pairs signed-ranks test (Runyon & Haber 1971). A two-tailed region of rejection was chosen for this analysis. Results were regarded to be within a significant level of preference when for 2 given compartments, the ratio of time spent significantly differed from random ($p < 0.05$).

Results

Embryo survival in the embryo group represented 69% of the control group value. From hatching to the end of the experiment, survival and growth of the embryo and alevin groups did not differ from the controls.

Control fish exposed to only well water flowing in each arm of the testing apparatus showed no consistent preference for any compartment; they spent comparable lengths of time in each of the 3

compartments (mean times 183, 211 and 206 sec, respectively).

During the exposure period, 2 odor conditions were tested. In the own rearing/well water odor condition (Fig. 1), only the control group significantly preferred their own odor arm over the 2 other compartments. The 2 exposed groups did not display preference for any of the 3 compartments. In the own rearing/heterospecific odor condition, the control group significantly preferred their own odor arm over the foreign odor one. Conversely, the 2 copper-exposed groups did not show any preference between the 2 odorized arms of the testing apparatus. Moreover, the embryo group presented no preference for any of the 3 compartments whereas the alevin group stayed longer in the central section than in the foreign odor arm.

Behavioral tests were performed 2 weeks and 10 weeks after the copper exposure was stopped. Only the clear-cut own rearing/well water odor condition was tested. Two weeks after copper removal, the control group preferred both their own odor arm and the central section over the foreign odor arm (Fig. 2). For the alevin group, the time spent in each compartment differed significantly, fish staying longer in the central section than in their own odor arm which was significantly preferred over the foreign odor arm. Lastly, the embryo group significantly preferred the central section over their own odor arm whereas no significant difference could be noted between the 2 stimulus arms. Ten weeks after the end of the exposure, the nonparametric Wilcoxon test showed similar results in control and embryo groups, fish preferring their own odor arm and the central section over the foreign odor arm. The behavioral response of the alevin group was the same as that recorded 2 weeks after the end of the exposure.

Discussion

The 31% mortality observed during embryo life stages in the copper-exposed embryo group does not differ substantially from that of 25% reported in rainbow trout exposed to a 25 μg $Cu^{+2} \cdot l^{-1}$ from fertilization (Birge & Black 1979). Otherwise, the 9 months of chronic exposure to copper had no significant effects upon fish survival and growth after hatching. Even during the crucial period of resorption and assimilation of the egg yolk mass, no increased mortality was recorded in the embryo group. This result does not confirm the suggestion of McKim et al. (1978) that sublethal copper exposure during embryo stages produce subtle changes which probably influence survival and growth of newly hatched fish and early juveniles. The lower copper concentration used in the present study may have induced less toxicant accumulation in the egg yolk mass, but it is also possible that by some mechanisms of adaptation, only the more tolerant or the less sensitive embryos may have survived.

Results in the control group demonstrate that the 2 odor conditions tested, own rearing odor against either well water or heterospecific odor, could be regarded as reliable odor conditions to evaluate fish olfactory discrimination ability. In both odor conditions, controls significantly preferred their own rearing odor over the other odor. Preference for their own odor over well water was also reported in adult coho salmon (Quinn et al. 1983, Quinn & Tolson 1986), in adult sockeye salmon (Groot et al. 1986) and in young cichlid fish (Barnett 1982). In the own rearing/heterospecific odor condition which requires greater acuity, control fish were able to distinguish between the 2 odors, even though they had no prior experience with heterospecific chemical cues.

A long-term sublethal exposure to copper results in an absence of odor preference which indicates impairment of fish olfactory discrimination performance. Whatever the odor condition tested, a similar response was recorded in both the embryo and alevin groups. It could not be determined, however, if the olfactory impairment is identical and if it appeared at the same time in both exposed groups. Nevertheless, this behavioral response resembles those reported in preference/avoidance tests when no odor stimulus is presented as for controls in the above well water/well water condition or when animals which have been made anosmic are tested (Brown et al. 1982, Rehnberg et al. 1985, Waldman 1985, Royce-Malmgren & Watson 1987). Thus, exposed fish behaved as if they had

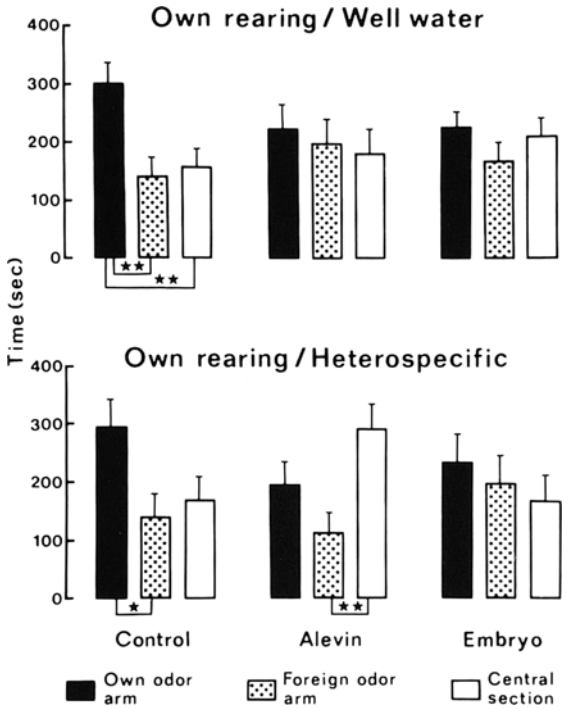

Fig. 1. Comparison of mean time (± SEM) spent in each compartment (own odor arm, foreign odor arm and central section) of the testing apparatus by the 3 experimental groups (control, alevin and embryo). For each odor condition tested (own rearing/well water and own rearing/heterospecific), 30 fish of each group were recorded individually over 10 min periods. Time spent orienting toward the different compartments, studied two by two, were compared for the different experimental groups and odor conditions by the Wilcoxon matched-pairs signed-ranks test; asterisks refer to significant differences for two-tailed probabilities at $p < 0.05^{*}$ and $p < 0.01^{**}$.

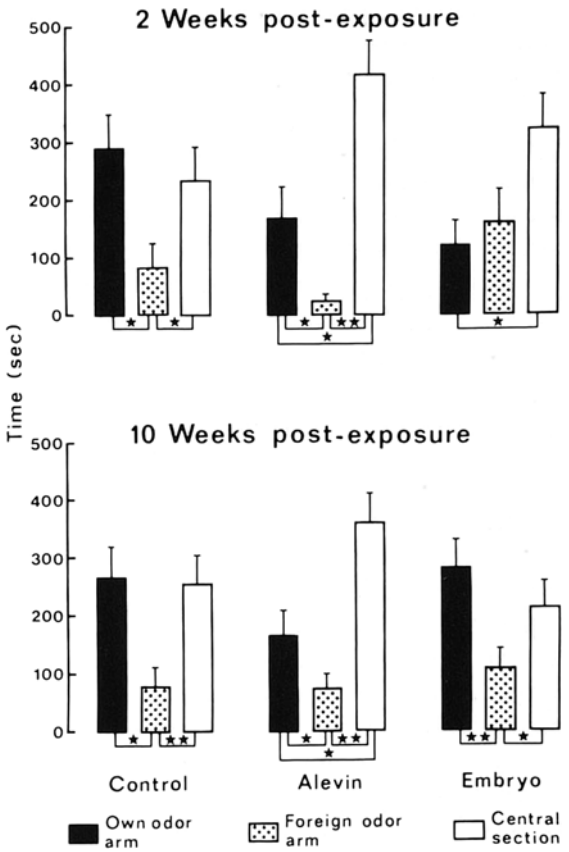

Fig. 2. Comparison of mean time (± SEM) spent in each compartment of the testing apparatus by the 3 experimental groups in response to own rearing/well water odor condition. Testing was performed 2 weeks and 10 weeks after the end of copper exposure. See Figure 1 for explanation of the statistical analysis.

lost their discrimination capability. It could be asked, however, if this behavioral response reflects a loss of olfactory sensitivity or an olfactory hyposensitivity. Further studies dealing with sensitivity thresholds in response to biological and pure odors are needed to clarify this point and bring out eventual differences in olfactory impairment between the 2 exposed groups.

Impaired behavioral responses observed in copper-exposed fish may probably reflect some anatomical changes occurring at the level of the olfactory neuroepithelium since this structure is directly in contact with the toxicant. Injury to the olfactory organ has been reported by Brown et al. (1982) and Hara et al. (1983) in adult rainbow trout and whitefish exposed to 150 μg $Cu^{+2} \cdot l^{-1}$ for 2 weeks. Such copper exposure induced degeneration and elimination of olfactory receptor cells stained for phospholipids. Following exposure to 500 μg $Cu^{+2} \cdot l^{-1}$ or more, necrosis of neuroreceptors and hyperplasia of sustentacular cells in the olfactory organ of *Fundulus heteroclitus* and *Menidia menidia* have been observed within 6 and 24 hours, respectively (Gardner & LaRoche 1973). The mode of action of copper at the level of receptor cell membranes is not fully understood (Rehnberg & Schreck 1986). Brown et al. (1982) and Hara et al. (1983) who observed accumulated copper in the olfactory rosettes of rainbow trout exposed for 2 weeks to

150 μg $Cu^{+2} \cdot l^{-1}$ have suggested that such accumulation might have resulted in membrane or organelle dysfunction.

Behavioral tests performed during the post-exposure period bring into evidence some recovery of fish discrimination ability, but delays for recovery are quite different for the 2 exposed groups. Thus, already 2 weeks after the end of copper exposure the alevin group distinguished their own rearing odor from well water whereas this behavioral response was observed only 10 weeks after copper removal in the embryo group. The functional recovery might be the result of some underlying anatomical recovery in the olfactory organ which has the unique property of renewal of its neuroreceptors (Graziadei & Monti Graziadei 1978). Olfactory cell regeneration has been observed in rainbow trout and in whitefish after the end of a 2 week exposure to 150 μg $Cu^{+2} \cdot l^{-1}$ (Hara et al. 1983). Twelve weeks seem sufficient for return to near normal quantity of receptor cells stained for phospholipids. However, anatomical and functional recoveries are probably not temporally superimposed. It has been demonstrated that after olfactory nerve section in rainbow trout, receptor cells reach the normal density within 84 days whereas the electro-olfactogram response to L-serine was only 50% of the control values at 230 days (Evans & Hara 1985). On the other hand, as the delay for functional recovery is quite different in the 2 copper-exposed groups, it could be hypothesized that patterns of injury at the peripheral level could also differ in extension and/or location in the lamellae of the olfactory rosette in both groups. This is all the more possible since the embryo group was exposed to toxicant earlier and longer than the alevin group. In our experiments in progress, we verify this hypothesis by studying the histopathological effects of a long-term sublethal copper exposure.

From this study, we conclude that a long-term exposure to sublethal copper impairs the olfactory discrimination ability of developing fish. The artificiality of laboratory studies precludes generalization to 'natural' behavior especially since the impact of such copper exposure on the whole chemosensory modalities is not fully established. Nevertheless, one can assume that the sensory dysfunction shown here could make fish more vulnerable to predators and unable to select favorable conditions for survival and spawning. Our investigations also demonstrate that functional recovery of olfactory discrimination may take place after the removel of pollutant. However, as the olfactory cell regeneration capability appears to decrease with age in mammals (Dodson & Bannister 1980, Breipohl et al. 1986), we do not know to what extent olfactory impairment induced by pollution may become irreversible as fish mature. Only studies on long-term sublethal pollution will allow to ascertain this point.

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