SUBLETHAL EFFECTS OF COPPER ON COHO SALMON: IMPACTS ON NONOVERLAPPING RECEPTOR PATHWAYS IN THE PERIPHERAL OLFACTORY NERVOUS SYSTEM

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Abstract—The sublethal effects of copper on the sensory physiology of juvenile coho salmon (*Oncorhynchus kisutch*) were evaluated. In vivo field potential recordings from the olfactory epithelium (electro-olfactograms) were used to measure the impacts of copper on the responses of olfactory receptor neurons to natural odorants (L-serine and taurocholic acid) and an odorant mixture (L-arginine, L-aspartic acid, L-leucine, and L-serine) over a range of stimulus concentrations. Increases in copper impaired the neurophysiological response to all odorants within 10 min of exposure. The inhibitory effects of copper (1.0–20.0 μg/L) were dose-dependent and they were not influenced by water hardness. Toxicity thresholds for the different receptor pathways were determined by using the benchmark dose method and found to be similar (a 2.3–3.0 μg/L increase in total dissolved copper over background). Collectively, examination of these data indicates that copper is broadly toxic to the salmon olfactory nervous system. Consequently, short-term influxes of copper to surface waters may interfere with olfactory-mediated behaviors that are critical for the survival and migratory success of wild salmonids.

Keywords—Salmon   *Oncorhynchus kisutch*   Copper   Olfaction   Biotic ligand model

INTRODUCTION

Water and sediment pollution are currently a concern for the management of declining Pacific salmon populations in the western United States. These declines have resulted, in part, from the deterioration or loss of critical freshwater and estuarine habitat [1]. Salmon and steelhead need cool, clean water in adequate supply to grow, migrate, and spawn in freshwater systems. In the past decade, numerous salmon populations have been listed for protection under the Endangered Species Act in California (USA) and the Pacific Northwest. The widespread contamination of surface waters in western river systems [2,3] may be a limiting factor in the recovery of some of these stocks listed under the Endangered Species Act.

For salmon, the olfactory nervous system provides a potential link between sublethal measures of neurotoxicity and biological consequences at the scale of individual animals or even natural populations. Salmon have a highly developed sense of smell, and previous studies have shown that direct measures of olfactory capacity are reliable indicators of sublethal neurotoxicity (reviewed by Klaprat et al. [4]). Use of the salmon olfactory system has several specific advantages for sublethal toxicity screening. First, primary olfactory receptor neurons are in direct contact with the surrounding environment and therefore are more vulnerable to the toxic impacts of dissolved chemicals. Second, the olfactory epithelium is easily accessible for the purpose of measuring odor-evoked voltage potentials [5]. Third, the olfactory nervous system is highly sensitive to dissolved chemicals in the animal's surrounding environment (detection thresholds of 10^{-7} to 10^{-11} M, depending on the odorant [6–8]). Finally, and perhaps most importantly, olfaction plays a key role in the recognition and avoidance of predators [9,10], the recognition of kin [11], and in the reproductive synchronization of prespawning animals [7]. Moreover, a salmon's sense of smell determines, in part, the long-term genetic integrity of wild and geographically distinct populations. Salmon rely on chemical cues to form olfactory memories [12] and return to their natal river system to spawn [13]. Critically, the migratory patterns of adult salmon are disrupted when olfactory function is lost [13]. Collectively then, environmental contaminants that damage the olfactory system could potentially impact salmonids at higher scales of biological organization.

An established technique for measuring peripheral olfactory function in fish is the use of field potential recordings, or electro-olfactograms (EOGs), to monitor the effects of pollutants on the active (or odor-evoked) properties of primary sensory neurons in the olfactory epithelium [14,15]. The EOG is a large, negative voltage transient and is measured with an electrode positioned near the surface of the sensory epithelium. The amplitude of the EOG reflects the summated electrical response of olfactory receptor neurons (ORNs) as they bind to odor molecules in the surrounding environment [16]. Therefore, the EOG provides a direct measure of receptor neuron function in the intact animal.

In the present study we evaluated the effects of copper on odor-evoked EOGs from the olfactory epithelium of coho salmon (*Oncorhynchus kisutch*) by using a computer-driven exposure and data acquisition system. Copper is a widespread source of water pollution in salmon habitat. Although the highest levels of copper pollution are generally found in river systems that have been impacted by mining activities, copper also can be transported to salmon habitat from several other anthropogenic sources, including nonpoint sources. Copper is an important pollutant in urban stormwater runoff [17], in part

because of the use of copper in vehicle brake pads. Copper leaches out of treated wood placed in rivers and estuaries [18] and copper compounds are widely used as algicides in waterways and as fungicides on agricultural crops. As an example of surface water contamination, copper was the most frequently detected trace element at agricultural and mixed-use sites in the Willamette River Basin (Oregon, USA) [19].

Copper has previously been shown to impair the olfactory nervous system and olfactory-mediated behaviors in salmonids [15,20–22] and other fish species [23]. The aim of the present investigation was to extend these earlier studies by quantifying the effects of copper on the sensitivity of the salmon olfactory system to odorants that activate different classes of receptor neurons in the sensory epithelium, mixtures of natural odorants, and odorants presented over a range of different stimulus concentrations. We also examined the influence of water hardness on sublethal copper toxicity. We used this approach in combination with the benchmark dose method [24] to calculate thresholds for sublethal neurotoxicity to different peripheral olfactory pathways.

MATERIALS AND METHODS

Experimental animals

Coho salmon eggs were obtained from the University of Washington hatchery (Seattle, WA, USA) and raised in the hatchery facility at the Northwest Fisheries Science Center. City water was passed through activated charcoal filters to provide dechlorinated source water for the hatchery. Fish were maintained in 2,400-L fiberglass tanks supplied by a filtered, recirculating water system (11–13°C, pH 7.1, buffered to 120 ppm total hardness as $CaCO_3$). The experiments reported here were performed on 77 juvenile animals with a weight of 143 $\pm$ 8 g (mean $\pm$ standard error of the mean [SEM]) and fork length of 22.7 $\pm$ 0.4 cm. Fish were anaesthetized with tricaine methane sulfonate (MS-222; 50 mg/L in hatchery water for 20 min) and then injected intramuscularly with the paralytic gallamine triethiodide (0.3 mg/kg body mass). The skin overlying the olfactory chamber (containing the olfactory rosette) was removed and the fish were placed in a Plexiglas holder on a vibration isolation table (Newport, Irvine, CA, USA). Chilled (12°C), oxygenated hatchery water containing MS-222 (50 mg/L) was delivered to the gills through a mouthpiece at a rate of 120 ml/min. A schematic diagram and photograph of the perfusion and electrophysiological recording systems are shown in Figure 1.

Stimulus solutions

Concentrated stock solutions of the bile salt taurocholic acid (TCA), the amino acid L-serine, and a mixture of the amino acids L-arginine, L-aspartic acid, L-leucine, and L-serine (Sigma, St. Louis, MO, USA) were prepared each week in distilled water and refrigerated at 4°C. These three solutions were used as odorants to stimulate the coho olfactory epithelium. Amino acids and TCA have previously been shown to evoke electrophysiological and behavioral responses in salmonids [16]. Previous studies have shown that TCA and each of the four amino acids used in this study act on nonoverlapping populations of ORNs in fish [25–29]. Within a mixture, the four amino acids were applied at the same concentration (i.e., a 10^{-8} M mixture contained 10^{-8} M L-arginine, 10^{-8} M L-aspartic acid, 10^{-8} M L-leucine, and 10^{-8} M L-serine). Odorant stimulus solutions were prepared daily by dilution of the stocks into filtered,
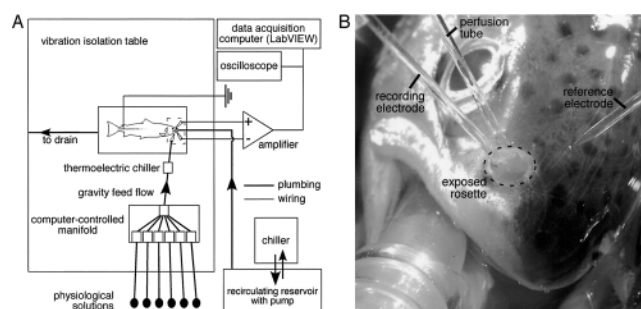

Fig. 1. Electophysiological recording system used to measure odor-evoked electro-olfactograms from the sensory epithelium of juvenile coho salmon. See the *Materials and Methods* section for more information. (**A**) Schematic diagram showing the major components of the apparatus. The dashed box denotes the area shown in more detail in (**B**). (**B**) Photograph showing the rostrum of a coho salmon and the positioning of the perfusion tube and glass microelectrodes. (LabVIEW, National Instruments, Austin, TX, USA).

dechlorinated city water (the source water for the Northwest Fisheries Science Center hatchery). The same water (without odorant) was used for baseline and wash perfusions. For experiments to test the influence of hardness on copper toxicity, the total hardness of the water was increased by the addition of $CaCl_2$ before adding the odorants. All solutions were adjusted to pH 7.5 to 7.6 with NaOH.

Copper solutions

A concentrated stock of copper chloride in distilled water (3.4 g/L) was prepared each week and refrigerated. The copper stock was adjusted to pH 3.0 with HCl to maintain the copper in ionic form. For exposures, copper solutions at different concentrations were prepared daily by diluting the copper stock into filtered, dechlorinated city water (source water) and adjusting each solution to a pH of 7.5 to 7.6 with NaOH. For some experiments, the hardness of the water was increased by the addition of $CaCl_2$ before adding the copper and adjusting the pH.

To determine the concentrations of total dissolved copper in exposure solutions, triplicate samples of the source water and each nominal copper exposure solution (1, 2, 5, 10, and 20 μg/L) were collected from the output of the perfusion system and submitted to an outside laboratory (Frontier Geosciences, Seattle, WA, USA) for analysis by inductively coupled plasma mass spectrometry (detection limit of 0.03 μg/L). Samples were passed through a precleaned 0.45-μm filter before analysis. The source water used in this study contained total dissolved copper at 3.0 μg/L. The measured concentrations of dissolved copper in the exposure solutions were 87% $\pm$ 5% (mean $\pm$ 1 SEM) of the expected concentrations (i.e., nominal plus a background of copper at 3.0 μg/L in the source water for the Northwest Fisheries Science Center hatchery). Exposures are reported as nominal copper concentrations, or an increase over background.

Copper and odorant delivery to the olfactory epithelium

A continuous flow of chilled source water (12°C) was delivered to the exposed rosette at a rate of 7 ml/min through a perfusion tube (Fig. 1B). Solutions containing source water only, source water plus odorant, source water plus copper, or source water plus odorant and copper were stored in amber bottles and gravity fed via separate, Teflon tubes to a series of computer-controlled manifolds (Neptune Research, West

Caldwell, NJ, USA). One input line to the manifolds was reserved for source water. The remaining input lines were varied as needed for each experiment. The manifolds allowed for automated, rapid (10-ms) switching between the different solutions. The single output from the manifolds passed through a custom-designed thermoelectric chiller just before delivery of the selected solution (at 12°C) to the rosette. With this approach, a custom computer program in LabVIEW (National Instruments, Austin, TX, USA) was used to select a perfusion source from as many as 10 different copper- and odorant-containing solutions.

Electrophysiological recordings

Electro-olfactograms were obtained by using an experimental technique modified from established methods [5,14]. Odor-evoked EOGs were recorded by using a pair of glass microelectrodes filled with 2% agar-saline and bridged to Ag-AgCl electrodes by 3 M KCl. The recording electrode was placed along the midline of the rosette at the base of the large, posteriormost lamella [5] with the aid of a stereomicroscope (SMZ645, Nikon Instruments, Melville, NY, USA) mounted on a boom stand. A reference electrode was placed in the skin above the rosette (see Fig. 1B). A separate ground (a hypodermic needle) was placed in the muscle near the tail. The differential signal was amplified (500×) and filtered (100-Hz low-pass) with a direct current (DC) amplifier (A-M Systems, Carlsborg, WA, USA). The signal was then digitized at 240 samples/s by using a computerized data acquisition system (PowerMac G4, Apple Computer, Cupertino, CA, USA, and 6035E and DIO-96, National Instruments). As with the perfusion system, the collection of odor-evoked EOGs was controlled by using a custom data acquisition program in Lab-VIEW.

Testing procedure

After the placement of electrodes, fish were allowed to acclimate for 15 min before tests began. The rosette was continuously perfused with source water or test solutions throughout each experiment. The EOGs were elicited by briefly switching from source water (with or without added copper) to an odorant-containing solution. Blank (or control) responses were obtained by switching between source water delivered through the dedicated line and source water from a line normally used for odor-containing solutions. Blank pulses were used to monitor for responses to any residual odorants in the lines or manifolds.

A standard set of odor pulses was used for the copper exposure experiments. The pulse duration, pulse interval, and odor concentrations were determined from preliminary experiments (see the *Results* and Fig. 2). Eight odor pulses (each 10 s in duration and separated by 120 s) were presented in the following order: 10^{-5} M L-serine, 10^{-6} M TCA, 10^{-8} M amino acid mixture, 10^{-7} M amino acid mixture, 10^{-6} M amino acid mixture, 10^{-5} M amino acid mixture, and 10^{-5} M L-serine. The source water perfusing the rosette was then switched to source water plus copper for the duration of the exposure (30 or 60 min). During the copper exposure, 10-s pulses of 10^{-5} M L-serine (in copper plus source water) were presented every 5 min to track the time to effect. After the copper exposure, the perfusion was switched back to source water alone and the preexposure sequence of eight odor pulses was again presented to the rosette. Additional 10-s pulses of 10^{-5} M L-serine then were presented to track recovery. Each fish was tested once and were presented to track recovery. Each fish was tested once

at a single copper concentration, and *n* refers to the number of individual fish that were evaluated in each experiment.

Data analysis

Electro-olfactogram responses were quantified by measuring the peak negative amplitude relative to the preodorant baseline. In pilot experiments, the coefficient of variance for between-animal olfactory responses was found to be 25.1% (mean response to 10^{-5} M L-serine, *n* = 9 fish). This was slightly higher than the mean within-animal coefficient of variance for the same fish (21.4%, 4–15 responses per fish, *n* = 9 fish) and similar to the variability seen in the ensuing experiments (e.g., Fig. 2). The effects of copper were determined by comparing the pre- and postexposure responses of each fish to the various odorants. Thresholds for effect were determined by following the benchmark dose method [24]. The threshold values obtained are referred to as benchmark concentrations, because they reflect exposure concentrations and not administered doses. Statistical analyses were performed with StatView® (SAS, Cary, NC, USA). Kaleidagraph (Synergy Software, Reading, PA, USA) was used for plotting nonlinear regressions and the production of graphs.

RESULTS

Optimizing odor-evoked EOGs from the coho olfactory epithelium

To evaluate the effects of different pulse parameters on the amplitude of odor-evoked EOGs in juvenile coho, we varied several aspects of the stimulus delivery. These included the duration of a single pulse, the interval between two pulses, and the concentration of each odorant presented during a pulse. The aim was to empirically determine the appropriate conditions for delivering stimulus solutions before, during, and after copper exposures. The EOG amplitudes varied as each of these pulse parameters was varied (Fig. 2).

The influences of odor pulse duration on peak amplitude and the time to peak for EOGs evoked by 10^{-5} M L-serine are shown in Figure 2A and B. A 10-s pulse was necessary to evoke a maximal response for L-serine at 10^{-5} M. Shorter pulses produced smaller peaks or reduced the time to peak. Longer pulse durations did not increase peak amplitude, but did increase the duration of ORN activity (and thus the potential for adaptation). Based on these results, pulse durations of 10 s were used for all subsequent experiments.

The consequences of varying the interval between two pulses (10^{-5} M L-serine) on the amplitude of the second pulse are shown in Figure 2C and D. When odor pulses were presented in sequence, the amplitude of the second pulse increased with the duration of the interpulse interval (Fig. 2C and D). As indicated in Figure 2D, the responses of the olfactory epithelium to identical odorant pulses were equivalent if the pulses were separated by at least 120 s. Therefore, interpulse intervals of 120 s were used in the experiments that followed.

Dose–response data for different odorants (Fig. 2E and F) were collected to determine appropriate stimulus concentrations for the copper exposure experiments. Three different odorants were chosen for these experiments. As shown in Figure 2E, perfusion of L-serine over the olfactory epithelium evoked a dose-dependent increase in the amplitude of the measured EOGs. At 10^{-5} M, L-serine stimulated an EOG response approximately three times the amplitude of the blank control (Fig. 2F). Although larger EOG responses could be elicited

by a higher concentration of L-serine (e.g., 10^{-4} M), 10^{-5} M L-serine was chosen as a test odorant to minimize the possibility of receptor adaptation. The dose–response data for TCA, shown in Figure 2F, indicated that 10^{-6} M TCA produced an EOG response that was comparable, in terms of peak amplitude, to that of 10^{-5} M L-serine. Therefore, 10^{-6} M TCA was chosen as the second test odorant. The final set of test pulses consisted of the amino acid mixture (see the *Materials and Methods* section) presented at four concentrations. Figure 2F shows that mixture concentrations of 10^{-8} M to 10^{-5} M evoked EOG amplitudes comparable to the dose-response data from L-serine or TCA alone. The EOG responses to the mixture are presumed to reflect the summed activity of four nonoverlapping classes of ORNs [29].

In summary, 10^{-5} M L-serine, 10^{-6} M TCA, and an amino acid mixture presented at four concentrations (10^{-8} M, 10^{-7} M, 10^{-6} M, and 10^{-5} M of L-arginine, L-aspartic acid, L-leucine, and L-serine) were chosen as model odorants for the copper exposure experiments. In addition, odorant pulses lasting 10 s with interpulse intervals of 120 s were selected. Overall, the experimental sequence of odorant stimulus, copper exposure, and odorant stimulus required continuous recordings from the olfactory epithelium for a period of at least 1.5 h. To ensure that EOG amplitudes were stable and that the preparation was otherwise healthy for this interval, odor-evoked EOGs were recorded from unexposed fish for 3 h. The amplitudes of the EOGs evoked by either 10^{-5} M L-serine or a 10^{-6} M amino acid mixture were stable and did not decline within a three-hour interval ($n = 6$ fish, data not shown).

Copper inhibits multiple olfactory pathways in coho salmon

A short-term copper exposure at a nominal concentration of 10 µg/L reduced the responsiveness of the sensory epithelium to all three model odorants (Fig. 3A to C). For the fish shown in Figure 3, a 30-min exposure reduced the L-serine response by 57%, the TCA response by 67%, and the response to the amino acid mixture by 35% (all relative to pre-exposure EOG amplitudes). The effect of copper on the response to the amino acid mixture (Fig. 3C) was measured as a shift in the dose–response curve (Fig. 3D). To quantify the copper-induced shift across the range of mixture concentrations, the pre-exposure response amplitudes were plotted against the postexposure amplitudes (Fig. 3E). The effect of copper was expressed as the slope of the regression line, with a slope of one indicating no impact of copper on mixture-evoked EOGs. The L-serine and TCA responses are point estimates (i.e., a single olfactory pathway at a single stimulus concentration), whereas the mixture data capture the effects of copper on four olfactory pathways over three log units of stimulus intensity.

The time to effect for copper is shown in Figure 4A. L-Serine pulses were delivered every 5 min during 30-min exposures to copper at 1, 2, 5, 10, and 20 µg/L nominal concentrations ($n = 6$ individual fish for each exposure concentration). A reduction in EOG amplitude was evident within 10 min of exposure for all but the lowest copper concentration. Moreover, a 30-min exposure was sufficient to produce a maximal reduction in EOG peak amplitude at a given concentration of copper. For example, no additional reduction in the amplitude of the odor-evoked EOG was observed when the duration of the copper exposure (5 µg/L) was increased to 60 min (Fig. 4B; $n = 3$).

Repeated pulses of 10^{-5} M L-serine at 5-min intervals were used to follow the recovery of the olfactory epithelium after the rosette was returned to source water. At least some recovery was apparent after 30 min of wash (Fig. 4A). However, as is evident from the 10 µg/L trace in Figure 4A, ORNs did not recover completely within the time course of these experiments. Therefore, the inhibitory effects of a short-term copper exposure persist for hours and possibly longer.

To evaluate the potential role of hardness in copper's sublethal toxicity to the coho nervous system, the hardness of the source water delivered to the rosette was adjusted by adding $CaCl_2$. The water perfusing the gills (hatchery water) was not changed. The source water used in previous experiments (e.g., Fig. 4A) was soft, with a total hardness of 20 ppm. Amending the source water to an intermediate hardness of 120 ppm or a high hardness of 240 ppm did not significantly alter the inhibitory effects of 10 µg/L copper on L-serine–evoked EOGs (Fig. 4C). Therefore, water hardness does not seem to influence the toxicity of copper to coho sensory neurons.

Thresholds for sublethal copper neurotoxicity

One of the goals of this study was to determine thresholds for sublethal, copper-induced neurotoxicity in coho salmon. To this end, dose–response data were collected for the three odorants (L-serine, TCA, and the amino acid mixture) at six exposure concentrations for each. The exposure groups consisted of a blank or control group and 30-min copper exposures at nominal concentrations of 1, 2, 5, 10, and 20 µg/L ($n = 6$ fish per exposure group). For the control group, the source water perfusion was switched from the dedicated line to a line normally used to deliver copper-containing solutions. Notably, a slight reduction occurred in the amplitude of the evoked EOGs for the control animals (mean $\pm$ 1 SEM: L-serine, 0.84 $\pm$ 0.09; TCA, 0.83 $\pm$ 0.08; amino acid mixture, 0.97 $\pm$ 0.09). These reductions in evoked EOGs were not significantly different from each other (one-way analysis of variance, $df = 2$, $F = 1.186$, $p = 0.330$) and none were significantly different from 1 (one-group t test, hypothetical mean $= 1$, $p > 0.05$). The reductions were likely due to the presence of residual copper in the line used to deliver the copper exposures.

The inhibitory effects of copper for all animals were normalized to the mean reduction in odor-evoked responses of the control animals. The resulting dose–response data are shown in Figure 5. A two-way analysis of variance showed that copper had a significant, dose-dependent effect on the responsiveness of the sensory system to the odorants ($df = 4$, $F = 14.246$, $p < 0.001$), but no significant differences were found between the odorants ($df = 2$, $F = 0.890$, $p = 0.415$). Thresholds (benchmark concentrations) for copper toxicity were estimated by following the benchmark dose method [24]. The data for each odorant were fit with a sigmoid logistic model

$$y = 1/[1 + (x/k)^n]$$

where y is the relative EOG amplitude, x is the copper concentration, k is the copper concentration at half-maximum relative EOG amplitude (inhibition concentration of 50%), and n is the slope. Figure 5 shows that the model was a good fit for all three odorants. The benchmark concentration for each odorant was then determined based on the nominal concentration at which each curve crossed a criterion level. The benchmark criterion was set to 0.75, a level very close to the bottom of the 95% confidence interval for each odorant from the control group (L-serine $= 0.73$, TCA $= 0.77$, mixture $=$
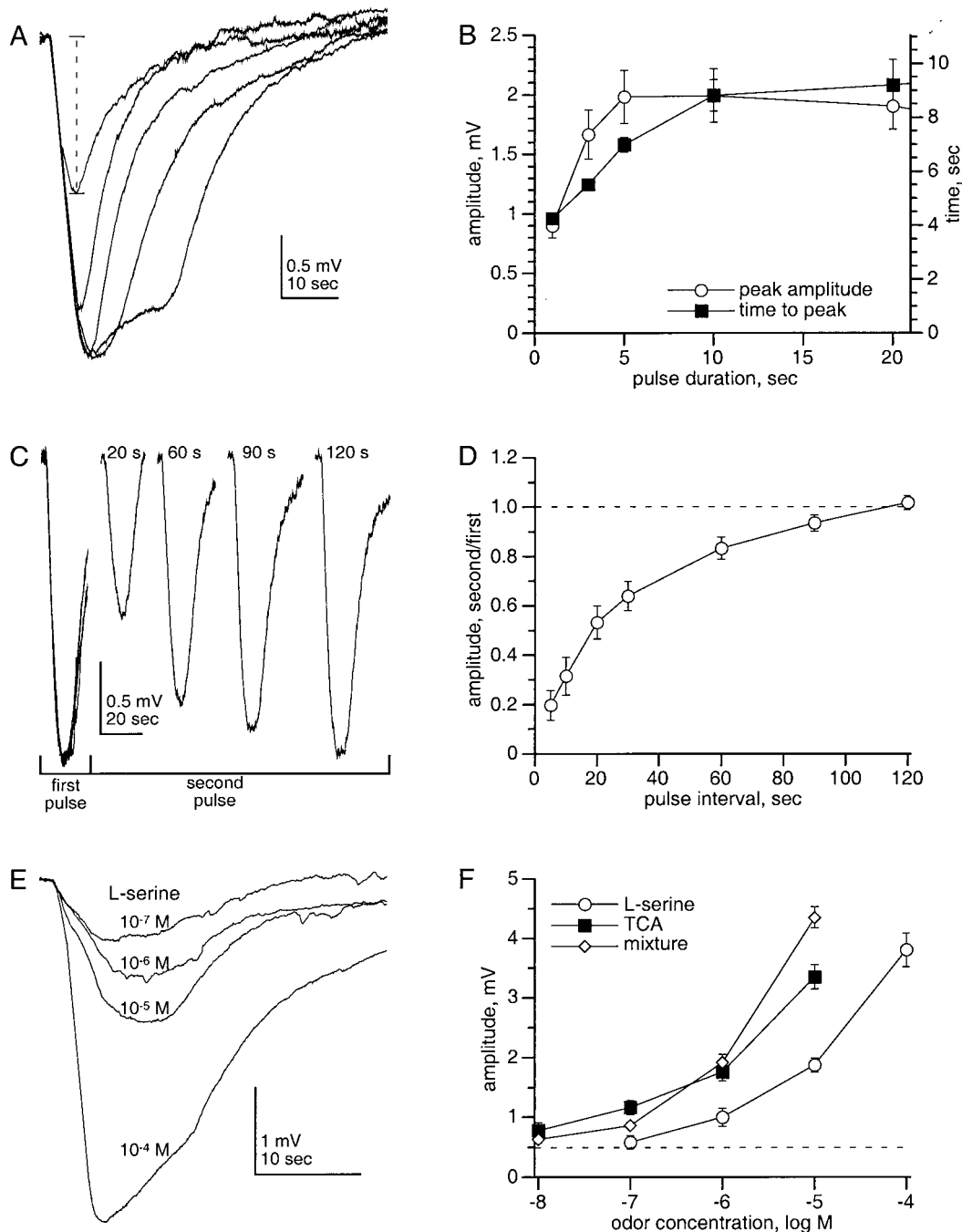
Fig. 2. Varying the duration, timing, and concentration of odorant pulses changes the waveform and amplitude of evoked electro-olfactograms (EOGs). The left column (**A, C,** and **E**) shows superimposed EOGs obtained from the same fish. The right column (**B, D,** and **F**) shows data averaged from multiple fish (mean ± 1 standard error). (**A**) The EOG responses to 10^{-5} M L-serine pulses delivered with durations of 1, 3, 5, 10, and 20 s. The dashed vertical line illustrates the peak amplitude (as measured from the baseline) for the EOG evoked by a 1-s pulse. (**B**) Double *y*-axis plot showing the effect of pulse duration on peak amplitude and time to peak ($n = 4$ fish). Time to peak was measured from the initial deflection from the baseline to the peak of the EOG. (**C**) Traces showing pairs of 10^{-5} M L-serine pulses (each 10 s long) presented with interpulse intervals of 20, 60, 90, and 120 s. For clarity, the intervening data have been deleted and only the evoked EOGs are shown. The first pulses (four total) are superimposed and the second pulses have been arbitrarily positioned horizontally and aligned vertically by their initial downward deflections. (**D**) Pooled data ($n = 4$ fish) showing the effect of pulse interval on the amplitude of the second pulse relative to the first pulse. (**E**) The amplitudes of EOGs evoked in response to 10-s L-serine pulses increase with increasing odorant concentration (10^{-7} M, 10^{-6} M, 10^{-5} M, and 10^{-4} M). (**F**) Dose–response data ($n = 6$ fish) for three odorants (L-serine, taurocholic acid [TCA], and an amino acid mixture). The dotted line shows the mean response to a blank control pulse (source water; $n = 4$ fish).

0.76). When using this approach, the benchmark concentrations for copper were found to be similar for all three odorants. The estimates (± 1 SE) for the benchmark concentrations were 2.7 ± 0.4 μg/L for L-serine, 2.3 ± 0.6 μg/L for TCA, and 3.0 ± 0.7 μg/L for the mixture.

DISCUSSION

Identification of the functional or mechanistic relationships between chemical habitat quality and the viability of at-risk species is one of the major conceptual and empirical challenges
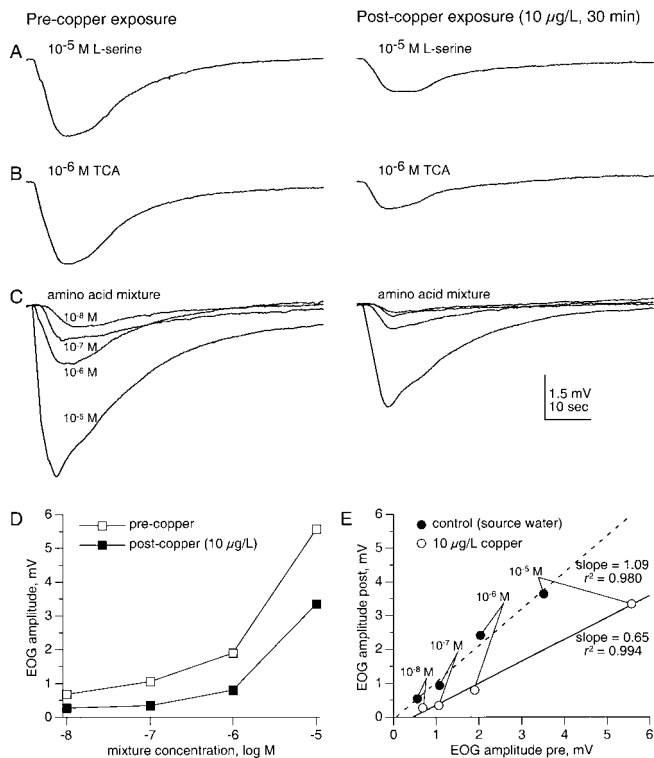
Fig. 3. Short-term copper exposures diminish the responsiveness of the olfactory epithelium to natural odorants. A 30-min exposure to copper at 10 µg/L reduced the electro-olfactogram (EOG) evoked by 10^{-5} M L-serine by 57% (**A**) and the response to 10^{-6} M taurocholic acid (TCA) by 67% (**B**). Similarly, the EOG responses to all four concentrations of the amino acid mixture (**C**) were reduced. (**D**) The odorant dose–response curves before and after copper exposure show a consistent decrease in EOG amplitude. (**E**) Scatter plot comparing EOG amplitudes in response to the amino acid mixture before and after copper exposure. Filled circles are data from a single control fish and open circles are the data shown in C from a single exposed fish. Regression lines indicate a change in evoked EOGs after a 30-min perfusion of source water (dashed line) or source water containing copper at 10 µg/L (solid line).

that currently confronts ecotoxicologists, conservation biologists, and natural resource managers [30]. The consequences of habitat degradation must first be measured in terms of the health or performance of exposed animals, and then extrapolated to natural populations. However, this is not a trivial task [31]. It requires that toxicological endpoints be measured at scales below the level of the individual animal and, at the same time, have clear significance for processes at higher scales of biological complexity.

For many years, the olfactory system of fish has been recognized to be particularly vulnerable to the neurotoxic effects of copper and other dissolved pollutants in the aquatic environment. This includes the olfactory nervous system [4,32] as well as olfactory-mediated behaviors [23]. However, the extent of this vulnerability is not well understood. Are all olfactory pathways and, by extension, all olfactory-mediated behaviors, impaired by copper? This question has been difficult to answer for salmonids, in part because previous copper studies have generally used a single odorant, delivered at a single concentration, as a standard stimulus in toxicity evaluations [15,22,33]. In the present study, we show that copper has similar inhibitory effects on ORNs that respond to different classes of olfactory stimuli (bile salts vs amino acids). Moreover, for mixtures of amino acids that stimulate nonoverlapping
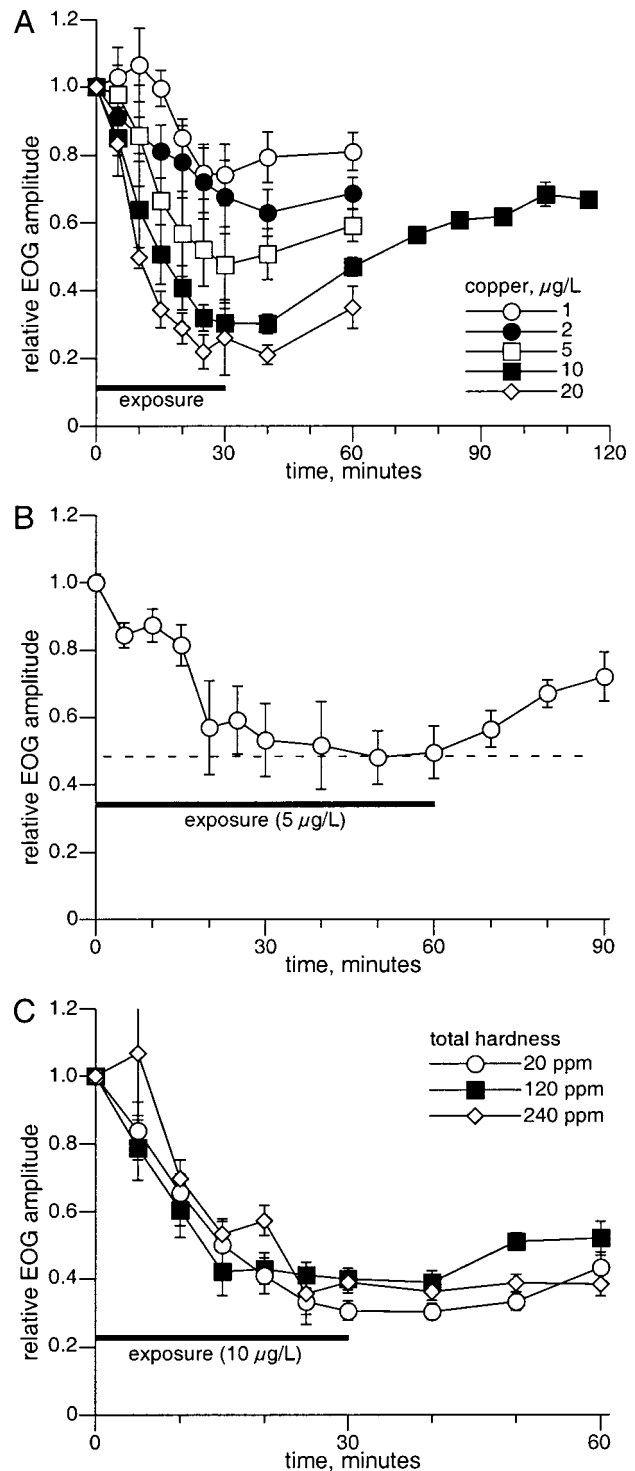

Fig. 4. The inhibitory effects of copper as a function of concentration, exposure duration, and water hardness. For all three panels, the post-exposure electro-olfactogram (EOG) of an individual fish is normalized to the pre-exposure response for that animal before averaging (mean $\pm$ 1 standard error) within a treatment group. (**A**) Responses to 10^{-5} M L-serine pulses were obtained at intervals during and after copper exposures at concentrations ranging from 1 to 20 µg/L ($n = 6$ animals for each copper concentration). The horizontal bar denotes the 30-min exposure. (**B**) The effects of perfusing the olfactory epithelium with copper at 5 µg/L for 60 min ($n = 3$ fish). The horizontal bar denotes the 60-min exposure and the dashed line indicates the maximal EOG reduction after the shorter (30-min) exposure in (**A**). (**C**) The influence of water hardness (as $CaCl_2$) on the inhibitory effects of copper ($n = 4$–6 fish per hardness category). The horizontal bar denotes a 30-min exposure to copper at 10 µg/L.
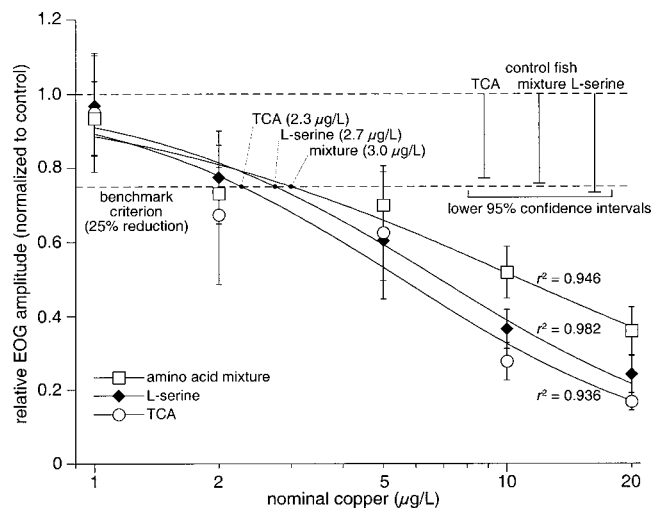
Fig. 5. Dose–response curves and threshold determinations for sublethal copper neurotoxicity. Data were obtained from six treatment groups (control and five copper exposures; *n* = 6 fish per group). The evoked electro-olfactogram (EOG) amplitudes for all copper exposures were normalized to the mean response of the controls and expressed as a group (mean ± 1 standard error). The dashed line indicates a benchmark criterion of 0.75, or a 25% reduction in evoked response (relative to controls). Three vertical lines in the upper right show the lower limits of the 95% confidence interval for the control response to the three different odorants. Filled circles indicate the benchmark concentrations for the different olfactory pathways (L-serine, taurocholic acid [TCA], and the amino acid mixture). Note that the benchmark values are nominal concentrations, or a change (increase) from the copper present at approximately 3 µg/L in the source water for the Northwest Fisheries Science Center hatchery (Seattle, WA, USA).

populations of ORNs, the inhibitory effects of copper are evident across three log units of stimulus intensity. Consequently, all olfactory-mediated behaviors in Pacific salmon could be affected by sublethal copper exposures if, as examination of the present data suggests, the toxicological impacts of copper are similar for all ORNs (irrespective of their affinities for different natural odorants). Although the peripheral olfactory pathways involved in imprinting, predator avoidance, and homing behaviors have not been identified in salmon, a general copper-induced loss of olfactory capacity could be expected to disrupt these and other higher-order processes.

It is important to note that salmonids will actively avoid copper [21,34]. Where distinct copper gradients are present in salmon habitat (e.g., near a point-source discharge), salmon may use their sense of smell to detect and avoid contaminated surface waters. As a consequence, low levels of copper pollution could serve as a barrier to migration or exclude salmon from habitats that are otherwise productive [34]. However, behavioral avoidance may not be an adequate defense against stormwater runoff, agricultural runoff, or other nonpoint sources of copper. In the absence of defined spatial gradients, it is unlikely that salmon and steelhead will be able to avoid non–point-source inputs of copper to lakes, rivers, and estuaries.

The copper exposures used in this study were environmentally realistic. They were designed to emulate non–point-source copper pollution in salmon habitat arising from surface water runoff. In the Willamette River study, for example, copper was detected in 19 of 23 filtered water samples at concentrations ranging up to 21 µg/L [19]. Thus, during storm events, juvenile salmonids in urban and agricultural watersheds are likely to be exposed to short-term increases in copper

levels above the background concentrations in base flows. In the present study, the source water for the hatchery contained a small amount of copper (3 µg/L), and this may have contributed to the observed toxicity in the nominal exposures. When juvenile coho salmon were transiently exposed to copper at concentrations above this background, short-term increases of 3 µg/L or more significantly impaired the sensory physiology of the exposed animals. It should be noted that the U.S. Environmental Protection Agency's water-quality criterion for dissolved copper (at a hardness of 100 mg/L) in freshwater systems is 13 µg/L for a 1-h average maximum concentration. This is approximately equivalent to our 30-min nominal exposures at 10 µg/L. Thus, based on our present results, a stormwater pulse containing copper at 13 µg/L could be expected to cause a >50% loss of sensory capacity among resident coho in freshwater habitats.

Copper's toxicity to the salmonid olfactory system manifests on a time scale of minutes. Although the precise mode of action in sensory neurons is not known [4], the inhibitory effects of a brief (30-min) copper exposure are at least partially reversible. However, previous studies on other salmonid species have shown that exposures lasting 4 h or longer cause ORNs to undergo cell death [22,35,36]. In chinook salmon, a loss of ORNs is accompanied by a loss of sensitivity to amino acids [22] and an inability to detect and avoid copper-containing water [21]. Therefore, olfactory function will be impaired if salmon are unable to avoid copper pollution within the first few minutes of exposure. If copper levels subsequently exceed a threshold for sensory cell death, it may be weeks before the functional properties of the olfactory system recover [37].

For salmon and steelhead, olfactory cues convey important information about habitat quality (e.g., pollution), predators, conspecifics, mates, and the animal's natal stream. Therefore, a substantial copper-induced loss of olfactory capacity is likely to impair behaviors that might be considered essential for survival or reproductive success. For example, copper exposures will shift the detection thresholds for natural odorants to higher concentrations (Fig. 3D), and juvenile salmonids may fail to respond to pheromones [8] and other cues that naturally occur at levels near the lower detection limits for unexposed animals. If the pheromone signals the proximity of a predator [9,38], a failure to respond could result in ecological death. Of course, in the natural environment, the magnitude of behavioral impairment will depend on the olfactory cue and the site-specific ecological context. We cannot infer specific behavioral impacts from our neurophysiological results because, to our knowledge, neurophysiological and behavioral thresholds for sublethal copper toxicity have never been directly compared in fish. Clearly, salmon and steelhead cannot detect and respond to environmental cues in the absence of a functional sensory system. However, evaluating the effects of a partial loss of sensory capacity on behavioral function is considerably more complex, and this is an important area for future research.

Water hardness, expressed as the sum of Ca^{2+} and other divalent cations in solution, is known to affect the acute toxicity of copper and other metals to fish [39]. Dissolved copper is more acutely lethal to salmonids as water hardness decreases. According to the biotic ligand model, the bioavailability of copper can be decreased by increasing the concentrations of cations that compete for metal binding sites on gills and other tissues [40]. The biotic ligand model is generally applied

to data from copper exposures at higher concentrations and longer durations than those used in the present study [40]. However, our results indicate that water hardness may not influence the sublethal impacts of copper on coho sensory neurons. For example, a 10-fold increase in the Ca^{2+} content of the perfusion solution did not alter the inhibitory effects of copper on ORNs (Fig. 4C). Presumably, copper and Ca^{2+} do not compete for binding sites in the olfactory epithelium. Based on these preliminary data, it should not be assumed that the neurotoxicity of copper is proportionally less in harder surface waters.

The present study used a benchmark dose method [24] to calculate sublethal thresholds for functional neurophysiological impairment in coho. Compared with the no-observable-effect concentration, the benchmark concentration is a more precise determination of threshold, because it is not specifically constrained by a single exposure concentration. Although the sample sizes for the dose–response experiments were relatively small ($n = 6$ fish per exposure concentration), the measurements obtained were sufficient to calculate inhibitory effects thresholds for copper on different olfactory pathways. Thus, the in vivo electrophysiological recording methods used in this study, in combination with benchmark concentration statistics, provide an empirical and reproducible approach to determining thresholds for sublethal neurotoxicity in salmonids.

In summary, we have shown that transient exposures to copper significantly impair the sensory physiology of juvenile coho salmon. These exposures are typical of copper concentrations that have been measured in surface waters from urban and agricultural watersheds. The sublethal thresholds for copper toxicity are very similar for different olfactory pathways. Therefore, copper may interfere with many (or all) olfactory-mediated behaviors in coho that cannot avoid storm water and other non–point-source inputs to salmon habitat. More work is needed to define the behavioral consequences of this sensory neurotoxicity.

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