Odor-evoked field potentials as indicators of sublethal neurotoxicity in juvenile coho salmon (*Oncorhynchus kisutch*) exposed to copper, chlorpyrifos, or esfenvalerate

Jason F. Sandahl, David H. Baldwin, Jeffrey J. Jenkins, and Nathaniel L. Scholz

Abstract: The sublethal effects of three different pesticides (a metal, organophosphate, and pyrethroid) on juvenile coho salmon (*Oncorhynchus kisutch*) were evaluated using paired electrophysiological recordings from the olfactory epithelium and the olfactory bulb. Animals were exposed to copper (5–20 $\mu g \cdot L^{-1}$), chlorpyrifos (0.625–2.5 $\mu g \cdot L^{-1}$), or esfenvalerate (0.05–0.20 $\mu g \cdot L^{-1}$) for 7 days. Sublethal neurotoxicity was examined by recording odor-evoked field potentials from the sensory epithelium and olfactory forebrain using two natural odorants (taurocholic acid or L-serine). Copper and chlorpyrifos decreased the amplitudes of the epithelial and bulbar responses to both odorants in a concentration-dependent manner. Benchmark concentrations for a 20% loss of sensory function were 4.4 $\mu g \cdot L^{-1}$ for copper and 0.72 $\mu g \cdot L^{-1}$ for chlorpyrifos. Esfenvalerate did not affect the amplitude of odor-evoked field potentials. However, in the olfactory bulbs of coho exposed to 0.2 μg esfenvalerate$\cdot L^{-1}$, L-serine evoked distinct and irregular bursts of postsynaptic activity in the olfactory bulb, possibly indicating sublethal excitotoxicity to central networks. Collectively, these data indicate that periodic, non-point source contamination of salmon habitats with current-use pesticides could interfere with olfactory function and, by extension, olfactory-mediated behaviors that are important for the survival and migration of salmonids.

Résumé : Des enregistrements électrophysiologiques appariés de l'épithélium olfactif et du bulbe olfactif nous ont permis d'évaluer les effets sublétaux de trois pesticides distincts, soit un métal, un organophosphate et un pyréthroïde, chez de jeunes saumons coho (*Oncorhynchus kisutch*). Les animaux ont été exposés pendant 7 jours à du cuivre (5–20 $\mu g \cdot L^{-1}$), du chlorpyrofos (0,625–2,5 $\mu g \cdot L^{-1}$) ou à de l'esfenvalérate (0,05–0,20 $\mu g \cdot L^{-1}$). L'enregistrement des potentiels de champ de l'épithélium sensoriel et du cerveau antérieur olfactif en réaction à deux substances odorantes naturelles (l'acide taurocholique ou la L-sérine) a servi à examiner la neurotoxicité sublétale. Le cuivre et le chlorpyrifos causent une baisse d'amplitude des réactions épithéliales et bulbaires aux deux substances odorantes en proportion des concentrations administrées. Les concentrations repères pour une perte de 20 % de la fonction sensorielle sont de 4,4 $\mu g \cdot L^{-1}$ pour le cuivre et de 0,72 $\mu g \cdot L^{-1}$ pour le chlorpyrifos. L'esfenvalérate n'affecte pas l'amplitude des potentiels de champ provoqués par les odeurs. Cependant, dans les bulbes olfactifs de saumons coho exposés à 0,2 $\mu g \cdot L^{-1}$ d'esfenvalérate, la L-sérine cause des explosions distinctes et irrégulières d'activité post-synaptique qui signalent peut-être une excitotoxicité aux réseaux centraux. Dans leur ensemble, ces données indiquent que la contamination diffuse non-périodique des habitats du saumon par les pesticides courants peut perturber la fonction olfactive et, par conséquent, les comportements reliés à l'olfaction qui sont d'importance pour la survie et la migration des salmonidés.

[Traduit par la Rédaction]

Introduction

Surface water monitoring studies in the Pacific Northwest of the United States (e.g., Wentz et al. 1998; Rinella et al. 1999; Voss and Embrey 2000) have found a wide range of current-use pesticides and trace elements in watersheds that provide freshwater habitat for various species of Pacific salmon (*Oncorhynchus* spp.) and steelhead (*Oncorhynchus mykiss*). In general, pesticides occur in salmon habitats at levels far below thresholds for acute mortality. However, pesticides are still a concern for salmon, as these chemicals can cause sublethal effects that could ultimately lead to the ecological death of exposed animals (Kruzynski and Birtwell 1994).

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J.F. Sandahl and J.J. Jenkins. Oregon State University, Department of Environmental and Molecular Toxicology, 1007 Ag and Life Sciences Bldg., Corvallis, OR 97331, U.S.A.
D.H. Baldwin and N.L. Scholz.[1] NOAA Fisheries, Northwest Fisheries Science Center, 2725 Montlake Boulevard E., Seattle, WA 98112, U.S.A.

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Major efforts are currently underway to recover wild salmonid populations in the Pacific Northwest, including coho salmon (*Oncorhynchus kisutch*), chinook salmon (*Oncorhynchus tshawytscha*), steelhead, and other anadromous species that have been listed for protection under the U.S. Endangered Species Act. Much of this effort is focused on restoring the physical and chemical quality of degraded river systems and estuaries. Pesticides represent a key uncertainty for the recovery planning process, in part because of a general lack of sublethal toxicity data that are specific to the biology and life histories of anadromous Pacific salmon. To date, very few studies have investigated the effects of pesticides over the range of concentrations that are actually detected in salmon habitats. In addition, few studies have focused on endpoints with clear significance for the viability of wild salmon populations, such as the survival, migration, or reproductive success of individual animals.

To address some of these data gaps, we have investigated the effects of copper, chlorpyrifos, and esfenvalerate on the sensory physiology of juvenile coho salmon. These pesticides are commonly associated with agricultural (and some urban) land-use activities in the Pacific Northwest. Copper, a metal, is used as a fungicide on various crops and as an algaecide in irrigation canals and other waterways. In urbanized watersheds, copper can also originate from other sources, such as wear from vehicle brake pads. Chlorpyrifos, an organophosphate insecticide, is widely used on fruit trees and other agricultural crops. For example, in the spring, chlorpyrifos is applied in pear and apple orchards for scale and leaf roller control. Esfenvalerate is a pyrethroid insecticide that is being used increasingly in the Pacific Northwest for residential and agricultural applications.

Copper, chlorpyrifos, and esfenvalerate are all known neurotoxicants in salmon and other vertebrates, and each has a different mechanism of toxic action. To assess the sublethal effects of these contaminants on the nervous system of juvenile coho, we used a paired neurophysiological recording method to simultaneously monitor odor-evoked field potentials from the olfactory epithelium and the olfactory bulb in the forebrain. Coho were exposed to each pesticide for 7 days over a range of concentrations that approximate nonpoint source contamination in salmon habitats. Sublethal neurotoxicity was measured as a change (relative to control fish) in the field potentials evoked by two natural odorants: taurocholic acid (TCA) and L-serine. These odorants stimulate different populations of receptor neurons in the sensory epithelium (Hara 1982; Sveinsson and Hara 1990). Collectively, this approach allows for two independent measures of nervous system function within a single fish. We specifically focused on the functional properties of the olfactory system because olfaction plays a critical role in imprinting, kin recognition, predator avoidance, homing, and other important aspects of the life histories of Pacific salmon.

Materials and methods

Animals

Coho salmon eggs were obtained from the University of Washington hatchery (Seattle, Wash.) and raised at the Northwest Fisheries Science Center's hatchery facility. Juveniles were maintained in tanks supplied with filtered, dechlorinated municipal water (hereafter referred to as "source water"; 120 $mg \cdot L^{-1}$ total hardness as $CaCO_3$, pH 7.1, 11–13 °C). A total of 120 fish were used for this study, with eight fish per pesticide exposure group. Fish were 10 to 12 months old with an average (±SD) length of 14 ± 0.8 cm and weight of 30 ± 5.4 g.

Pesticide exposures

Fish were exposed to pesticide solutions for 7 days in aerated glass aquaria (51 cm long by 26 cm wide with a water depth of 19 cm) using a static-renewal (12-h) dosing regimen. Nominal exposure solutions were prepared by diluting 0.5 mL of pesticide stock (or carrier alone for controls) into 25 L of source water. Three water samples from each exposure concentration were analyzed to compare nominal and measured values. Samples were collected in acid-washed amber glass bottles fitted with teflon-coated lids or acid-washed teflon bottles (for copper analysis) and refrigerated at 4 °C.

Copper chloride (99% purity, cupric chloride, dihydrate) was purchased from Sigma Chemical Co. (St. Louis, Mo.). Copper stocks were prepared in distilled water and adjusted to pH 3.0 with HCl to maintain copper in ionic form. Exposure solutions were prepared at nominal concentrations of 0, 5, 10, and 20 μg copper$\cdot L^{-1}$. The solutions were analyzed for total dissolved copper by an outside laboratory (Frontier Geosciences, Seattle, Wash.). Samples were filtered through a precleaned 0.45-μm filter unit and preserved with concentrated HNO_3. The filtrate was subsequently analyzed by inductively coupled plasma – mass spectrometry (Perkin-Elmer ELAN 6000, detection limit of 0.03 $\mu g \cdot L^{-1}$).

Chlorpyrifos (99.2% purity, *O*,*O*-diethyl-*O*-(3,5,6-trichloro-2-pyridinol)-phosphorothionate) and esfenvalerate (98% purity, (*S*)-alpha-cyano-3-phenoxybenzyl-(*S*)-2-(4-chloro-phenyl)-3-methylbutyrate) were purchased from Chem Service, Inc. (West Chester, Pa.). The two pesticide stocks were made in ethanol. Nominal exposure solutions of chlorpyrifos were prepared at 0, 0.625, 1.25, and 2.5 $\mu g \cdot L^{-1}$ and were analyzed by gas chromatography – mass spectrometry using established methods (Sandahl and Jenkins 2002).

Nominal exposure solutions of esfenvalerate were prepared at 0, 0.05, 0.1, and 0.2 $\mu g \cdot L^{-1}$ and exposure solutions were analyzed following the methods of Fairchild et al. (1992) with slight modification using a Hewlett-Packard 6890 gas chromatograph (Hewlett-Packard, Avondale, Pa.) coupled with a 5972A mass selective detector (gas chromatography – mass spectrometry). Initial oven temperature was held at 70 °C for 2 min, increased 15 $°C \cdot min^{-1}$ to a final temperature of 290 °C, and held for 7 min. The mass spectrometer operated under selective ion monitoring (*m*/*z* 125, 225, 419).

The source water for the hatchery facility contained 0.4 μg total dissolved copper$\cdot L^{-1}$. No chlorpyrifos or esfenvalerate was detected in the source water. Measured concentrations of copper, chlorpyrifos, and esfenvalerate in exposure tanks ranged from 70% to 72%, from 91% to 105%, and from 85% to 116% of nominal values, respectively (Table 1). For all three pesticides, sublethal effects on coho physiology are reported in terms of the nominal exposure concentrations.

Electrophysiological measurements

Before electrophysiological recordings, fish were anesthetized by immersion for 15 min in 50 mg tricaine methanesulfonate (MS-222)·L^{-1}) (Sigma Chemical Co.) and then injected intramuscularly with the paralytic gallamine triethiodide (0.3 mg·kg body weight^{-1}). In preliminary experiments, control fish anesthetized with MS-222 were found to have odor-evoked responses from the olfactory epithelium comparable to those of fish anesthetized using other methods (2-phenoxyethanol injections or chilling on ice) (t test, $p > 0.05$; data not shown). Upon immobilization, fish were placed in a Plexiglas holder with chilled (12 °C), oxygenated source water containing MS-222 (25 mg·L^{-1}) delivered to the gills at 120 mL·min^{-1} (Fig. 1*a*). Skin overlying the right olfactory chamber was removed and the rosette (the supporting structure for the olfactory epithelium) was gently rinsed with 12 °C source water, pH 7.6. A section of the skull was then removed and the mesenchymal tissue cleared, exposing the olfactory bulbs, posterior olfactory nerve bundles, and anterior telencephalon. The rosette was subsequently perfused with source water through a glass capillary tube at a rate of 4 mL·min^{-1}. Fish were allowed to acclimate for 20 min before the start of electrophysiological recordings.

Two classes of odorants were used to test the effects of pesticides on different populations of olfactory receptor neurons. TCA (a salmonid bile salt) and L-serine (an amino acid) were selected as the test odorants. Each odorant has previously been shown to activate separate populations of olfactory receptor neurons (Hara 1982; Sveinsson and Hara 1990). Stock solutions of TCA and L-serine (Sigma Chemical Co.) were prepared in 1-mL aliquots of distilled water and stored at –20 °C. Test solutions were prepared daily in acid-washed amber glass bottles by diluting thawed aliquot stocks into source water. Test solutions of TCA and L-serine were prepared at 10^{-5} and 10^{-4} mol·L^{-1}, respectively, as these concentrations elicit similar olfactory responses in juvenile coho (Baldwin et al. 2003).

Electroolfactograms (EOGs) were obtained using the experimental method of Evans and Hara (1985) as modified by Baldwin et al. (2003). A glass recording microelectrode was positioned along the midline of the rosette just above the base of the large, posterior-most lamella and was held stationary during the recording phase of each experiment (Fig. 1*b*). The amplitude of each EOG response was measured as the negative phasic displacement in millivolts from prestimulus baseline to evoked peak.

Electroencephalograms (EEGs) were recorded by pressing a blunt glass microelectrode against the surface of the right olfactory bulb. During the acclimation period, the location of the maximal EEG response to the two odorants was determined by positioning the microelectrode at different points across the olfactory bulb. Maximal responses to TCA were typically found at the central–medial region of the bulb, and responses to L-serine were maximal at the caudal–medial region (Fig. 1*c*). A third microelectrode was placed on the head midway between the olfactory bulb and rosette, serving as a reference for both the EOG and EEG recordings. Differential signals were acquired (10 000× amplification) and filtered (1- to 100-Hz band pass) with an AC amplifier (A-M Systems, Carlsborg, Wash.). Recorded responses were measured in microvolts, half-wave rectified, and integrated with a 3-s time constant. The magnitude of each EEG response was measured as the ratio of the integrated evoked peak over prestimulus basal activity. Bulbar responses from esfenvalerate-exposed fish were also analyzed using a shorter (1-s) time constant to improve the resolution of brief bursts of activity (relative to the odor-evoked activity; see Results).

Table 1. Nominal and measured pesticide concentrations from initial static-renewal (12-h) exposure solutions.

	Concentration (μg·L^{-1})			
Copper				
Nominal	0	5.0	10	20
Measured	0.4	3.6	7.0	14.0
Chlorpyrifos				
Nominal	0	0.625	1.25	2.50
Measured	0.0	0.57	1.31	2.59
Esfenvalerate				
Nominal	0	0.05	0.1	0.2
Measured	0.0	0.06	0.09	0.23

Note: Each measured value is the average of three replicate samples.

The odorant delivery system has been previously described (Baldwin et al. 2003). Test EOG and EEG responses were measured simultaneously after brief (5 s) deliveries of each odorant to the rosette (Fig. 1*d*). Fish received three pulses of TCA followed by three pulses of L-serine with 3-min intervals between pulses. Triplicate responses to TCA and L-serine (Ser) were averaged to produce a single response value for each odorant. A combination of four neurophysiological responses (TCA–EOG, TCA–EEG, Ser–EOG, and Ser–EEG) was obtained from each fish. In a few cases (8% of fish), surgeries were unsuccessful, and we were unable to obtain the EEG component of the paired recording.

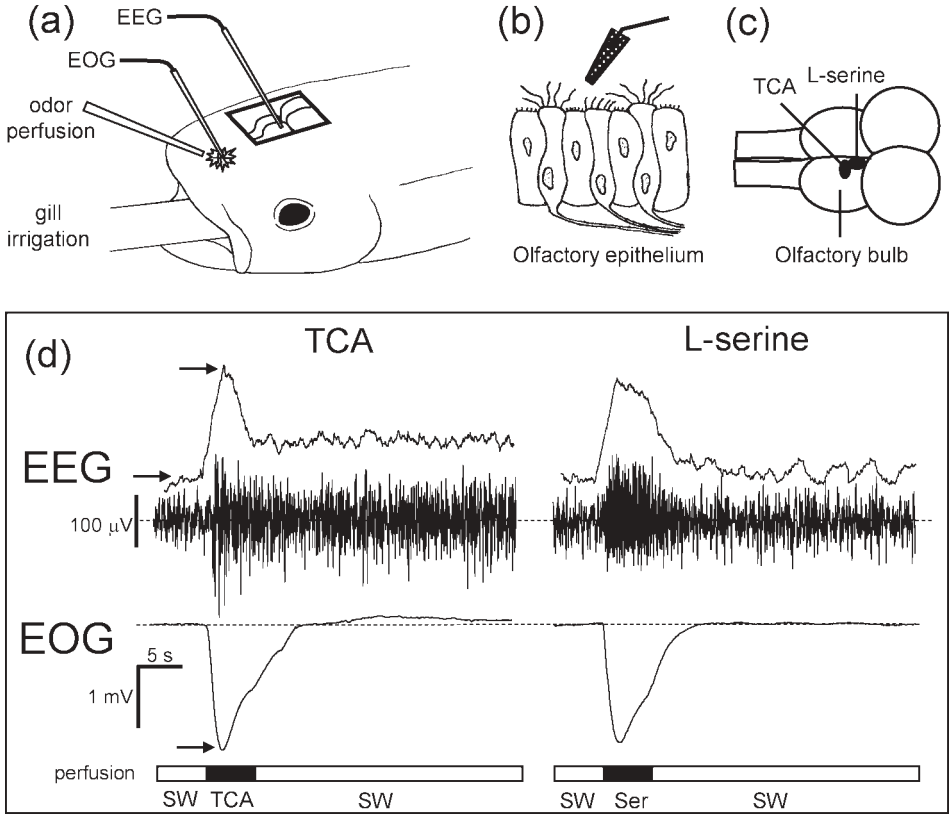
Acetylcholinesterase (AChE) assays

At the end of each experiment, the olfactory bulbs were removed and frozen at –20 °C. The rate of substrate hydrolysis by AChE was analyzed using the colorimetric method of Ellman et al. (1961), as modified by Sandahl and Jenkins (2002). Sample protein content was determined by the method of Bradford (1976). Measurements were performed on a SpectraMax Plus spectrophotometer (Molecular Devices, Sunnyvale, Calif.) and all reagents were purchased from Sigma Chemical Co. Samples were analyzed in triplicate wells, including tissue and substrate blanks. Acetylthiocholine iodide was added as the enzyme substrate, and 5,5′-dithiobis(2-nitrobenzoic acid) was used as a chromogen. The assay was run at 25 °C, and change in absorbance (412 nm) was measured at 12-s intervals for 10 min. AChE activities were normalized to total protein content and expressed as nanomoles of substrate hydrolyzed per minute per milligram protein.

Data analysis

For individual fish, the four odor-evoked field potential recordings (TCA–EOG, TCA–EEG, Ser–EOG, and Ser–

Fig. 1. Paired electrophysiological recordings from the olfactory epithelium and olfactory bulb of juvenile coho salmon. (*a*) Positions of the gill irrigation, odorant perfusion, microelectrode used to record odor-evoked field potentials from the sensory epithelium (EOG) in the olfactory chamber, and the microelectrode pressed against the surface of the olfactory bulb to monitor odor-evoked field potential oscillations (EEG). The reference microelectrode is not shown. (*b*) The EOG recording electrode is positioned over the apical surface of the ciliated and microvillar receptor neurons in the sensory epithelium (modified from Hara 1992). (*c*) Locations of the EEG recording electrode for TCA and L-serine-evoked EEGs from the olfactory bulb. (*d*) Examples of EOGs and EEGs elicited by switching from a perfusion of source water (SW) to TCA or L-serine (Ser) over the sensory epithelium. The EOG olfactory responses (lower traces) were measured as the negative phasic displacement from prestimulus baseline to evoked peak (arrow). The recorded EEG signals (middle traces) were half-wave rectified and integrated with a 3-s time constant (upper traces), and responses were measured as the ratio of the integrated evoked peak over prestimulus basal activity (arrows).



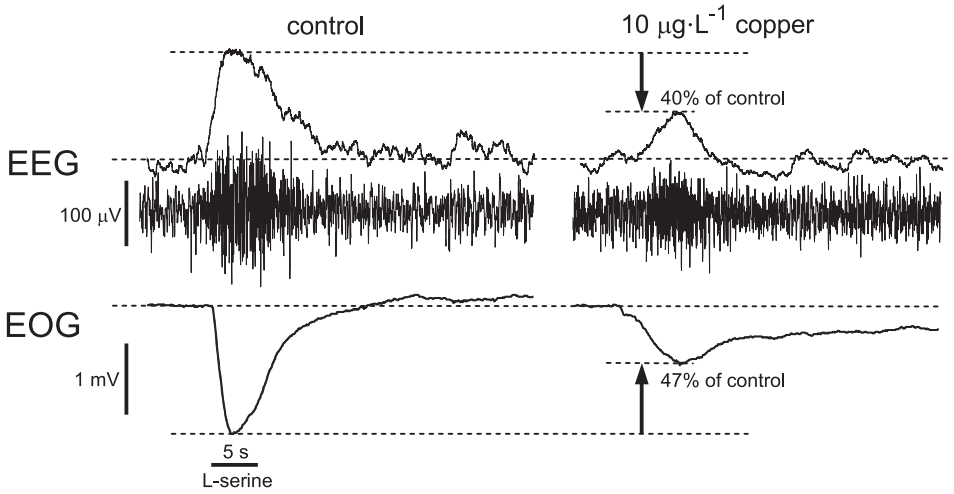
EEG) were each divided by the respective mean response of the control fish and expressed as a percentage of the control mean, which, in turn, was considered 100%. This allowed for comparisons between epithelial and brain recordings as a ratio of their respective control response and not in absolute units (millivolts or microvolts). For each pesticide, the effect of exposure (nominal concentrations) on the active properties of olfactory neurons in the sensory epithelium and the bulb was evaluated using a two-way ANOVA with pesticide concentration and neurophysiological response as the two variables tested. The pooled responses of each exposure group were analyzed using one-way ANOVA followed by a Bonferroni multiple comparison (GraphPad Prism 3.02) (San Diego, Calif.). Individual and pooled responses were fit by linear regression with the *y* intercept constrained to 100% (the control response in the absence of a pesticide exposure). The regression was used to calculate benchmark inhibitory concentrations for copper and chlorpyrifos (U.S. Environmental Protection Agency Benchmark Dose Software (http://cfpub.epa.gov/ncea/cfm/bmds.cfm)). Changes in activity patterns of bulbar neurons from esfenvalerate-exposed fish were evaluated using a Kruskal–Wallis test followed by Dunn's multiple comparison. Correlations between the two different odorants, and between EOG and EEG recordings, were evaluated by the Pearson correlation procedure. Relative AChE activity was compared using one-way ANOVA followed by Dunnett's multiple comparison.

Results

Odor-evoked field potential recordings from control fish

A brief delivery of odorants to the olfactory rosette evoked negative, or downward, shifts in the local field potential of the sensory epithelium. The waveform of the evoked potential consisted of both phasic and tonic components (Fig. 1*d*). When the rosette was rinsed with source water, the field potential would return to the initial baseline, typically within 10–20 s. Baseline bulbar activity, as recorded by the EEG, consisted of irregular waves of oscillation in the local field potential. These waves typically had amplitudes between 20 and 100 μV (peak to peak). Odor-evoked stimulation of the rosette induced a pattern of regular oscillations with a fre-

Fig. 2. Paired EOG and EEG recordings from typical control and copper-exposed (10 μg·L^{-1}) fish. Responses were elicited by a 5-s presentation of L-serine to the sensory epithelium in the olfactory chamber. In this example, the EOG and EEG responses were reduced to 47% and 40% of the control responses, respectively.



quency typically between 8 and 12 Hz and amplitudes between 50 and 200 μV (Fig. 1*d*).

The paired recording configuration allowed for comparisons of odor-evoked activity at two separate points within the olfactory pathway of a single fish in response to the same odor pulse. The EOGs and EEGs evoked by L-serine were similar in both relative amplitude and shape (phasic and tonic components) and were highly correlated temporally. The paired recordings elicited by TCA, however, were similar in amplitude but not in shape. The EEG response did not return to baseline immediately. Instead, the TCA-evoked potential had a sustained component that lasted 1–2 min after the sensory epithelium was rinsed (Fig. 1*d*).

For control fish (n = 23 individual animals), the mean (±SD) EOG amplitudes elicited by TCA and L-serine were 2.1 ± 0.4 and 1.5 ± 0.6 mV, respectively, and the mean EEG response ratios were 1.9 ± 0.5 for TCA and 2.1 ± 0.5 for L-serine. Within fish, responses to the two odorants were significantly correlated, i.e., fish that were relatively sensitive to TCA were also sensitive to L-serine. This was true of recordings from the sensory epithelium (Pearson's correlation coefficient (r) = 0.51, $p < 0.02$) and also from the olfactory bulb (r = 0.73, $p < 0.001$). Moreover, the amplitudes of the epithelial responses were significantly correlated with bulbar responses for both odorants (r = 0.41, $p < 0.001$).

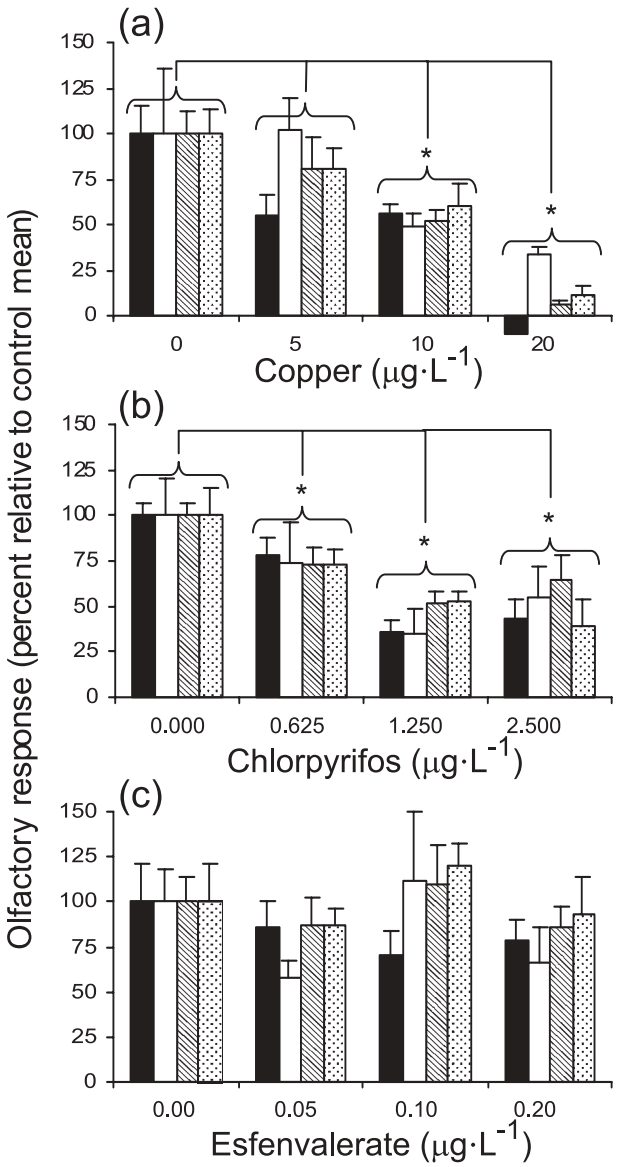
Copper and chlorpyrifos reduce the magnitude of the neurophysiological response to TCA and L-serine

Copper inhibited the olfactory responses to both TCA and L-serine. This included both the peripheral response and the bulbar response. An example of the inhibitory effects of copper (10 μg·L^{-1}) on the active properties of the sensory epithelium and olfactory bulb is shown (Fig. 2). The effects of copper on each of the four categories of field potential recording (i.e., EOGs and EEGs evoked by TCA and L-serine) are also shown (Fig. 3*a*). For a given odorant, the amplitude of the response from each fish was expressed as a percentage of the mean control response to that particular stimulus. The response ratios for individual fish were then averaged within a treatment group. A significant effect of copper concentration was observed (two-way ANOVA: df = 3, SS = 116 000, F = 28.1, $p < 0.0001$). There was no significant effect of electrophysiological recording category (df = 3, SS = 6316, F = 1.53, p = 0.21) and no significant interaction between copper exposure and recording category (df = 9, SS = 8929, F = 0.72, p = 0.69). The four categories of olfactory response were therefore combined for each treatment group. The pooled responses at 10 and 20 μg·L^{-1} were significantly different from controls (Fig. 3*a*) and were reduced by approximately 50% and 90%, respectively. Copper had no effect on AChE activity in the olfactory bulb (data not shown).

Fish exposed to the highest concentration of copper (20 μg·L^{-1}) showed an atypical EOG response to TCA. This was a positive (upward) shift in the voltage potential, shown as a negative EOG amplitude relative to controls (Fig. 3*a*). It is uncertain if the corresponding activity in the olfactory bulb reflected a response specific to TCA or a nonspecific response of damaged receptor neurons in the sensory epithelium.

Chlorpyrifos exposure also inhibited the olfactory response to both odorants in the sensory epithelium and in the bulb (Fig. 3*b*). The effect of chlorpyrifos concentration on olfactory response was significant (two-way ANOVA: df = 3, SS = 59300, F = 17.68, $p < 0.0001$). Similar to the results for copper, there was no significant effect of recording category (df = 3, SS = 1160, F = 0.346, p = 0.79) and no significant interaction between chlorpyrifos and the recording category (df = 9, SS = 3918, F = 0.389, p = 0.94). Accordingly, the different categories of measured olfactory response were combined within each chlorpyrifos treatment group. All pooled responses were significantly different from controls (see Fig. 3*b*). At the 0.625 μg·L^{-1} exposure level, the magnitude of the grouped response was reduced by approximately 25%. At the two highest concentrations tested (1.25 and 2.5 μg·L^{-1}), the responses were reduced by nearly 50%. Although this may represent an asymptotic level of reduction, there were too few exposure concentrations to make this determination. Although chlorpyrifos significantly inhibited bulbar AChE activity at the highest exposure level (a 25% reduction relative to controls, $p < 0.05$), there was no

Fig. 3. Exposures to (*a*) copper and (*b*) chlorpyrifos reduced the magnitude of EOG and EEG amplitudes to TCA and L-serine (two-way ANOVA). Because for both copper and chlorpyrifos, there was no significant difference between the four response measurements and no interaction between response measurement and exposure concentration, the measurements were grouped for comparisons between controls and exposure levels. Exposures to (*c*) esfenvalerate showed no significant differences within the measured responses or across exposure concentrations ($p > 0.05$). Olfactory responses are expressed as a percentage of the control response means. Asterisks indicate a statistically significant difference from controls (grouped response measures; one-way ANOVA, Bonferroni multiple comparison, $p < 0.05$); error bars represent 1 SE. Solid bars, EOG responses to TCA; open bars, EEG responses to TCA; hatched bars, EOG responses to L-serine; stippled bars, EEG responses to L-serine.



correlation between bulbar AChE activity and the magnitude of bulbar odor-evoked field potentials ($p > 0.8$).

Esfenvalerate treatment did not affect the magnitude of odor-evoked responses (n = 5–8) from the olfactory epithelium or olfactory bulb (Fig. 3*c*). A two-way ANOVA found no significant effect of esfenvalerate concentration (df = 3, SS = 13780, $F = 1.82$, $p = 0.15$), no significant differences between the four categories of neurophysiological response (df = 3, SS = 6200, $F = 0.821$, $p = 0.48$), and no interaction between esfenvalerate exposure and recording category (df = 9, SS = 12800, $F = 0.565$, $p = 0.82$). There was also no effect of esfenvalerate on AChE activity in the olfactory bulb (data not shown).

Threshold determination of copper and chlorpyrifos olfactory neurotoxicity

The four measures of olfactory response were separately fit by simple linear regression for both copper- and chlorpyrifos-exposed fish (Fig. 4). For each pesticide, the four individually derived slopes were not significantly different (copper: $F = 0.4098$, $p = 0.75$; chlorpyrifos: $F = 0.4611$, $p = 0.71$) and were thus combined to produce single dose–response curves for copper (Fig. 4*a*) (slope = –4.5, 95% confidence interval –3.6 to –5.3) and chlorpyrifos (Fig. 4*b*) (slope = –26.9, 95% confidence interval –20.6 to –33.2). Since the olfactory responses were expressed relative to the mean response values for control animals, or 100%, the y intercept of each dose–response curve was fixed at 100. Given the limitation of only three exposure concentrations, more complex, nonlinear models (e.g., a sigmoid logistic with three parameters) were not used. Benchmark concentration (BMC) estimates were calculated for 20% and 50% reductions in olfactory response (Table 2). The BMC_{20} values approximate reductions of 2 SE from copper (28%) and chlorpyrifos (15%) control means. The copper BMC_{20} and BMC_{50} estimates (and lower-bound 95% confidence limit) were determined to be 4.4 (4.3) and 11.1 (10.9) $\mu g \cdot L^{-1}$, respectively. The chlorpyrifos BMC_{20} and BMC_{50} estimates were determined to be 0.72 (0.56) and 1.81 (1.36) $\mu g \cdot L^{-1}$, respectively. These benchmark values are equivalent to effective concentrations (EC_{20} and EC_{50}) for olfactory inhibition.

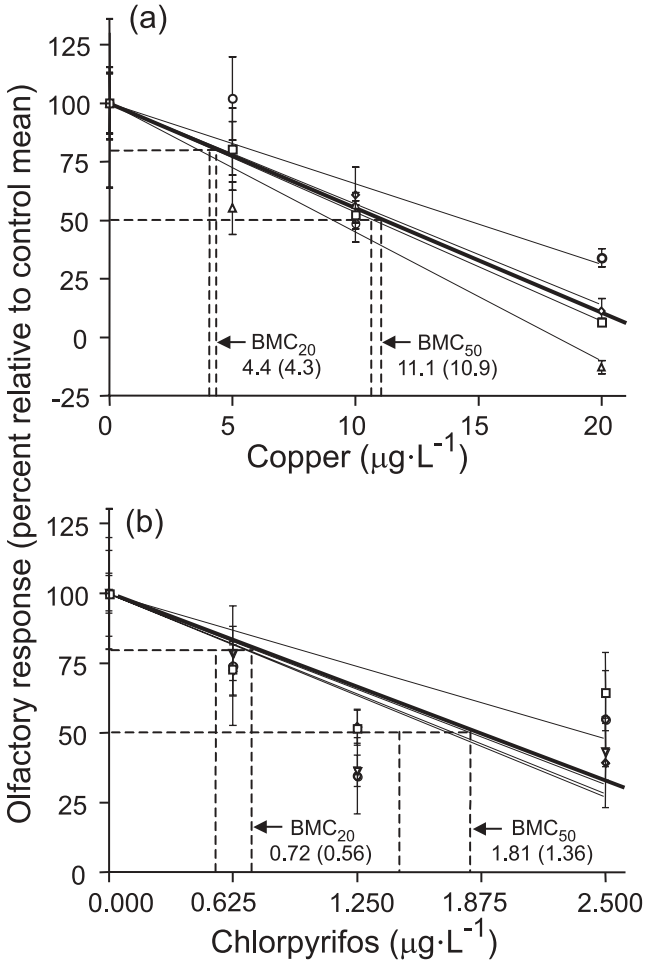
Esfenvalerate induces atypical postsynaptic burst activity in the olfactory bulb

Olfactory bulb recordings from esfenvalerate-treated fish showed an atypical pattern of activity after stimulation by L-serine. This consisted of a series of short (1–3 s), low-frequency (0–10 Hz) bursts of activity (Fig. 5). Comparable excitatory activity was not observed after TCA stimulation but may have been masked by the sustained component of TCA-evoked EEGs (Fig. 1*d*). To determine if the bursting activity observed in esfenvalerate-exposed fish was significantly different from that in control fish, the burst events in the bulb were quantified. A burst was defined as a 1- to 3-s event of low-frequency activity (0–10 Hz) that exceeded a threshold of 3 SD from mean prestimulus activity. Using these criteria, two of seven control fish showed postsynaptic burst activity that exceeded this threshold. Burst activity increased in esfenvalerate-exposed fish, with the 0.2 $\mu g \cdot L^{-1}$ exposure group significantly different from controls ($p < 0.05$) (Table 3).

Discussion

It has been recognized for many years that the fish olfactory system is particularly vulnerable to the toxic effects of

Fig. 4. Benchmark concentration (BMC) response curves for the inhibitory effects of (*a*) copper and (*b*) chlorpyrifos on olfactory responsiveness to TCA and L-serine. Data from Figs. 3*a* and 3*b* are fit individually (thin lines), and as pooled responses (thick lines), by linear regression. Horizontal broken lines represent the 20% and 50% effect levels, and the vertical broken lines represent central BMC and 95% lower-bound estimates (in parentheses). Because esfenvalerate did not affect either the EOG or EEG responses, dose–response curves are not presented. Olfactory responses are expressed as a percentage of the control response means. Triangles, EOG responses to TCA; circles, EEG responses to TCA; squares, EOG responses to L-serine; diamonds, EEG responses to L-serine.



metals and other environmental contaminants (Sutterlin 1974; Klaprat et al. 1992). This is due, in part, to the direct physical contact between primary sensory neurons and dissolved toxicants in surface waters. Contaminants can also be transported to the central nervous system, where they have the potential to interfere with the processing of sensory information by various regions of the animal's brain (Scott et al. 2003). These two points in the olfactory pathway, signal transduction and transmission, can be monitored using simultaneous electrophysiological recordings from the sensory epithelium and the olfactory bulb, respectively. This approach provides for direct, functional measures of sublethal neurotoxicity in salmon and other fish species. For copper and chlorpyrifos, these recording techniques are largely complimentary. However, as our current results for esfenvalerate show, peripheral recordings from the olfactory epithelium alone may fail to reflect pesticide-induced changes in the firing patterns of central neurons.

Table 2. Copper and chlorpyrifos benchmark concentration (BMC) estimates were determined for olfactory neurotoxicity in juvenile coho salmon.

		BMC_{20} ($\mu g \cdot L^{-1}$)	BMC_{50} ($\mu g \cdot L^{-1}$)
Copper	TCA–EOG	3.6	8.9
	TCA–EEG	6.1	15.1
	Ser–EOG	4.3	10.7
	Ser–EEG	4.5	11.3
	Pooled	4.4	11.1
Chlorpyrifos	TCA–EOG	0.6	1.51
	TCA–EEG	0.78	1.96
	Ser–EOG	0.71	1.77
	Ser–EEG	0.69	1.71
	Pooled	0.74	1.81

Note: Responses to TCA and L-serine (Ser) were each measured by simultaneous EOG and EEG recordings. Data from the four measured responses (TCA–EOG, TCA–EEG, Ser–EOG, and Ser–EEG) were fit individually, and as a pooled response, by linear regression. BMCs were determined for 20% and 50% reductions in the magnitude of the olfactory response to the two odorants.

In the present study, a bile salt (10^{-5} mol TCA·L^{-1}) and an amino acid (10^{-4} mol L-serine·L^{-1}) stimulated field potentials in the sensory epithelium of control fish that were similar in terms of waveform and amplitude. The bulbar responses to these two odorants, however, were different. Whereas the response of forebrain networks to L-serine coincided with peripheral activation and termination, the bulbar response to TCA was sustained for a minute or more after the sensory epithelium was no longer activated. The significance of the sustained component is not known, but it does indicate a difference in the processing of the two odorants by the olfactory bulb.

Copper and chlorpyrifos exposures each reduced the responsiveness of the coho olfactory system to TCA and L-serine. The amplitudes of the evoked EOGs and EEGs (phasic peak) were diminished in a similar, concentration-dependent manner. Thus, like copper, chlorpyrifos appears to be a nonselective inhibitor of different classes of receptor neurons in the sensory epithelium. Seven-day exposures to 5, 10, and 20 μg copper·L^{-1} reduced the response to TCA and L-serine by approximately 20%, 50%, and 90%, respectively. Baldwin et al. (2003) reported similar results after 30-min perfusions of copper directly to the olfactory rosette of juvenile coho. Thus, the olfactory epithelium does not appear to recover function after several days of continuous copper exposure. Similar copper exposures lasting more than a few hours are known to trigger cell death among primary receptor neurons (Hara et al. 1976; Winberg et al. 1992; Hansen et al. 1999*a*). It is therefore likely that the diminished EOG and paired EEG responses of copper-exposed animals in this study were a consequence of peripheral degeneration or loss of receptor neurons.

Similarly, the diminished bulbar activity of chlorpyrifos-exposed fish is probably due to a smaller peripheral response

Fig. 5. Example EEG recordings from control and esfenvalerate-exposed (0.2 μg·L^{-1}) fish. Exposed fish showed bursts of low-frequency bulbar activity (0–10 Hz) after stimulation by L-serine (asterisks). Bursts were arbitrarily defined as 1- to 3-s events that exceeded a threshold integrated response (broken line) of 3 SD above prestimulus activity (solid line). The integration time constant used to generate the analyzed EEG signal was reduced to a 1-s window for better resolution of the burst events.

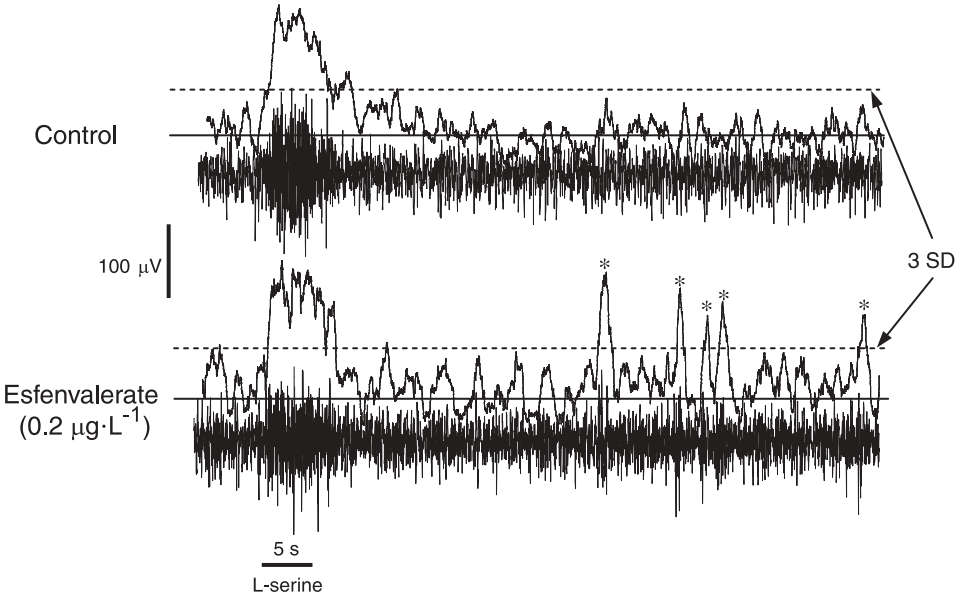


Table 3. Exposure to esfenvalerate increased spontaneous bursting in the coho salmon olfactory bulb.

	Esfenvalerate (μg·L^{-1})			
Burst events	0 (n = 7)	0.05 (n = 6)	0.1 (n = 8)	0.2 (n = 8)
Mean	0.4	1.7	1.4	3.7*
Median	0	1.5	1	3
Range	0–2	0–4	0–4	0–8

Note: Burst events were counted over a 2-min period beginning 30 s after stimulation of the rosette by L-serine. A burst was defined as a 1- to 3-s event of low-frequency activity (0–10 Hz) that exceeded a threshold integrated response (1-s time constant) of 3 SDs above prestimulus activity. The asterisk denotes a significant difference from control (Kruskal–Wallis test followed by Dunn's multiple comparison, $p < 0.05$).

to TCA and L-serine. A significant reduction in AChE activity in the olfactory bulbs from these animals indicates uptake and transport of chlorpyrifos to the forebrain. However, there was no significant correlation between reduced AChE activity in the bulb and reductions in odor-evoked EEGs. Rather, chlorpyrifos appears to be inhibiting the responsiveness of olfactory receptor neurons in the sensory epithelium, leading in turn to smaller EEGs. The mechanism of action for chlorpyrifos in the olfactory rosette has yet to be determined. Chlorpyrifos inhibits AChE, an enzyme that regulates neurotransmitter levels at cholinergic synapses in the vertebrate nervous system (Massoulié et al. 1993). Other anticholinesterase insecticides, including diazinon and carbofuran, also reduce odor-evoked EOGs in Atlantic salmon (*Salmo salar*) (Moore and Waring 1996; Waring and Moore 1997). Although AChE is expressed in the olfactory rosette of Pacific salmon (chinook; N.L. Scholz, unpublished data), there are no known synapses in the sensory epithelium. AChE may serve a nonsynaptic function in the olfactory rosette. Alternatively, chlorpyrifos may target other cellular proteins, such as adenylate cyclase (Song et al. 1997), that are important for olfactory signal transduction.

In contrast with the inhibitory actions of copper and chlorpyrifos, esfenvalerate is an excitatory neurotoxicant. More specifically, esfenvalerate binds to voltage-activated sodium channels and slows or blocks channel inactivation (Narahashi 1996). As a consequence, neurons are more likely to fire in response to synaptic input, and neurons that are actively firing trains or bursts of action potentials may be unable to effectively repolarize and thus terminate activity. Positive ions passing into receptor neurons through cyclic nucleotide-gated cation channels, and not voltage-activated sodium channels, are the basis for odor-evoked, negative extracellular field potentials recorded from the olfactory epithelium (Scott and Scott-Johnson 2002). Therefore, it is not surprising that esfenvalerate did not affect the amplitude of TCA- and L-serine-evoked EOGs. However, the effects of esfenvalerate on postsynaptic activity in the olfactory bulb are consistent with sublethal excitotoxicity. Esfenvalerate-exposed fish showed unusual bursts of activity for a minute or more after a brief 5-s stimulation with L-serine. Similarly, atypical burst patterns have been observed in auditory-evoked EEGs from rats exposed to the pyrethroid decamethrin (Ray 1980). The implication of this delayed burst

activity for processing odorant signals from the periphery in the olfactory bulb is not known. However, these data show that esfenvalerate can trigger atypical bursting activity in the coho forebrain at concentrations in the low parts per trillion.

The ranges of pesticide exposures in this study were chosen to reflect actual environmental conditions in river systems from the Pacific Northwest of the United States. Copper is a common non-point source contaminant in urban and agricultural watersheds where copper compounds are used as fungicides and algicides. For example, in the Willamette River Basin, Oregon, copper was frequently detected in surface waters at concentrations ranging up to 21 $\mu g \cdot L^{-1}$ (Anderson et al. 1996). In the Sacramento – San Joaquin River Basin, California, chlorpyrifos is applied intensively as a broad-spectrum insecticide, with detectable residues present in surface waters. Concentrations of chlorpyrifos in streams from this drainage have reached 0.5 $\mu g \cdot L^{-1}$ (Werner et al. 2000). In other geographical regions, including the Willamette, Yakima, and Puget Sound basins, chlorpyrifos co-occurs in streams with other anticholinesterase insecticides, including azinphos-methyl, diazinon, malathion, and carbaryl (Wentz et al. 1998; U.S. Geological Survey 1999). This raises the possibility of cumulative effects on the sensory physiology of juvenile salmonids. These cumulative effects could occur within a contaminant class (e.g., between chlorpyrifos and other organophosphate insecticides) or across classes (e.g., between chlorpyrifos and copper). To our knowledge, environmental monitoring of esfenvalerate has not been conducted in Pacific Northwest streams, and therefore, the concentrations of this insecticide in surface waters are not known (although they are unlikely to exceed 1 $\mu g \cdot L^{-1}$ under typical exposure conditions).

Olfaction is an important sensory modality for salmonids. Accordingly, a loss of olfactory function could interfere with physiological processes or behaviors that are essential for survival, migration, or reproductive success. It is well established that Pacific salmon are unable to navigate back to their natal streams to spawn when olfactory function is lost (Wisby and Hasler 1954). Sublethal exposures to neurotoxic pesticides that inhibit olfaction could potentially increase rates of straying in wild salmon and hatchery fish (Scholz et al. 2000). Salmon rely on olfaction to avoid water pollution, and their risk of contaminant exposure is higher when their olfactory system is impaired (Hansen et al. 1999*b*). Olfactory neurotoxicity has been shown to disrupt electrophysiological responses to natural odorants, as well as pheromone-mediated reproductive priming in Atlantic salmon (Moore and Waring 1996; Waring and Moore 1997). Moreover, juvenile chinook salmon exposed to the organophosphate insecticide diazinon are significantly less responsive to chemical cues that signal a predation threat (Scholz et al. 2000). Similar results have been observed in Colorado pikeminnow (*Ptychocheilus lucius*) exposed to copper (Beyers and Farmer 2001). In some species, such as goldfish (*Carassius auratus*), the relative thresholds for odor-evoked neurophysiological responses and behavioral responses are very similar (Sorensen et al. 1988). Presumably, for salmonids, a reduction or loss of olfactory sensitivity could interfere with imprinting, kin recognition, predator avoidance, homing, spawning, and other aspects of Pacific salmon biology that are reliant on olfaction. To explicitly address linkages between physiology and behavior, it will be necessary to relate sublethal thresholds for neurotoxicity, as measured with the electrophysiological recording techniques used in the present study, to individual behaviors that have clear significance at the scale of natural populations. This is an important focus for future research.

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References

Anderson, C.W., Rinella, F.A., and Rounds, S.A. 1996. Occurrence of selected trace elements and organic compounds and their relation to land use in the Willamette River basin, Oregon, 1992–94. U.S. Geol. Surv. Water-Resour. Invest. Rep. 96-4234.

Baldwin, D.H., Sandahl, J.F., Labenia, J.S., and Scholz, N.L. 2003. Sublethal effects of copper on coho salmon: impacts on nonoverlapping receptor pathways in the peripheral olfactory nervous system. Environ. Toxicol. Chem. **22**: 2266–2274.

Beyers, D.W., and Farmer, M.S. 2001. Effects of copper on olfaction of Colorado pikeminnow. Environ. Toxicol. Chem. **20**: 907–912.

Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. Anal. Biochem. **72**: 248–254.

Ellman, G.L., Courtney, K.D., Valentino, A., Jr., and Featherstone, R.M. 1961. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem. Pharmacol. **7**: 88–95.

Evans, R., and Hara, T.J. 1985. The characteristics of the electro-olfactogram (EOG): its loss and recovery following olfactory nerve section in rainbow trout (*Salmo gairdneri*). Brain Res. **330**: 65–75.

Fairchild, J.F., La Point, T.W., Zajicek, J.L., Nelson, M.K., Dwyer, F.J., and Lovely, P.A. 1992. Population-, community- and ecosystem-level responses of aquatic mesocosms to pulsed doses of a pyrethroid insecticide. Environ. Toxicol. Chem. **11**: 115–119.

Hansen, J.A., Rose, J.D., Jenkins, R.A., Gerow, K.G., and Bergman, J.L. 1999*a*. Chinook salmon (*Oncorhynchus tshawytscha*) and rainbow trout (*Oncorhynchus mykiss*) exposed to copper: neurophysiological and histological effects on the olfactory system. Environ. Toxicol. Chem. **18**: 1979–1991.

Hansen, J.A., Marr, J.C.A., Lipton, J., Cacela, D., and Bergman, H.L. 1999*b*. Differences in neurobehavioral responses of chinook salmon (*Oncorhynchus tshawytscha*) exposed to copper and cobalt: behavioural avoidance. Environ. Toxicol. Chem. **18**: 1972–1978.

Hara, T.J. 1982. Structure–activity relationships of amino acids as olfactory stimuli. *In* Chemoreception in fishes. *Edited by* T.J. Hara. Elsevier, Amsterdam, Netherlands. pp. 135–157.

Hara, T.J. 1992. Mechanisms of Olfaction. *In* Fish chemoreception. *Edited by* T.J. Hara. Chapman and Hall, New York. pp. 150–170.

Hara, T.J., Law, Y.M.C., and Macdonald, S. 1976. Effects of mercury and copper on the olfactory response in rainbow trout. J. Fish. Res. Board Can. **33**: 1568–1573.

Klaprat, D.A., Evans, R.E., and Hara, T.J. 1992. Environmental contaminants and chemoreception in fishes. *In* Fish chemoreception. *Edited by* T.J. Hara. Chapman and Hall, New York. pp. 321–341.

Kruzynski, G.M., and Birtwell, I.K. 1994. A predation bioassay to quantify the ecological significance of sublethal responses to juvenile chinook salmon (*Oncorhynchus tshawytscha*) to the antisapstain fungicide TCMTB. Can. J. Fish. Aquat. Sci. **51**: 1780–1790.

Massoulié, J., Pezzementi, S.B., Krejci, E., and Vallette, F-M. 1993. Molecular and cellular biology of cholinesterases. Prog. Neurobiol. **41**: 31–91.

Moore, A., and Waring, C.P. 1996. Sublethal effects of the pesticide Diazinon on olfactory function in mature male Atlantic salmon parr. J. Fish Biol. **48**: 758–775.

Narahashi, T. 1996. Neuronal ion channels as the target sites of insecticides. Pharmacol. Toxicol. **78**: 1–14.

Ray, D.E. 1980. An EEG investigation of decamethrin-induced choreoathetosis in the rat. Exp. Brain Res. **38**: 221–227.

Rinella, J.F., McKenzie, S.W., Crawford, J.K., Foreman, W.T., Fuhrer, G.J., and Morace, J.L. 1999. Surface water quality assessment of the Yakima River Basin, Washington — distribution of pesticides and other organic compounds in water, sediment, and aquatic biota, 1987–91. U.S. Geol. Surv. Water Supply Pap. 2354-B.

Sandahl, J.F., and Jenkins, J.J. 2002. Pacific steelhead (*Oncorhynchus mykiss*) exposed to chlorpyrifos: benchmark concentration estimates for acetylcholinesterase inhibition. Environ. Toxicol. Chem. **21**: 2452–2458.

Scholz, N.L., Truelove, N.K., French, B.L., Berejikian, B.A., Quinn, T.P., Casillas, E., and Collier, T.K. 2000. Diazinon disrupts antipredator and homing behaviors in chinook salmon (*Oncorhynchus tshawytscha*). Can. J. Fish. Aquat. Sci. **57**: 1911–1918.

Scott, G.R., Sloman, K.A., Rouleau, C., and Wood, C.M. 2003. Cadmium disrupts behavioural and physiological responses to alarm substance in juvenile rainbow trout (*Oncorhynchus mykiss*). J. Exp. Biol. **206**: 1779–1790.

Scott, J.W., and Scott-Johnson, P.E. 2002. The electroolfactogram: a review of its history and uses. Microsc. Res. Tech. **58**: 152–160.

Song, X., Seidler, F.J., Saleh, J.L., Zhang, J., Padilla, S., and Slotkin, T.A. 1997. Cellular mechanisms for developmental toxicity of chlorpyrifos: targeting the adenylyl cyclase signaling cascade. Toxicol. Appl. Pharmacol. **145**: 158–174.

Sorensen, P.W., Hara, T.J., Stacey, N.E., and Goetz, F.W. 1988. F prostaglandins function as potent olfactory stimulants that comprise the postovulatory female sex pheromone in goldfish. Biol. Reprod. **39**: 1039–1050.

Sutterlin, A.M. 1974. Pollutants and the chemical senses of aquatic animals — perspective and review. Chem. Senses Flavor, **1**: 167–178.

Sveinsson, T., and Hara, T.J. 1990. Multiple olfactory receptors for amino acids in Arctic char (*Salvelinus alpinus*) evidenced by cross-adaptation experiments. Comp. Biochem. Physiol. A Comp. Physiol. **97**: 289–293.

U.S. Geological Survey. 1999. Pesticides detected in urban streams during rainstorms and relations to retail sales of pesticides in King County, Washington. U.S. Geol. Surv. Fact Sheet 097-99.

Voss, F.D., and Embrey, S.S. 2000. Pesticides detected in urban streams during rainstorms in King and Snohomish counties, Washington, 1998. U.S. Geol. Surv. Water-Resour. Invest. Rep. 00-4098.

Waring, C.P., and Moore, A.P. 1997. Sublethal effects of a carbamate pesticide on pheromonal mediated endocrine function in mature male Atlantic salmon (*Salmo salar*) parr. Fish Physiol. Biochem. **17**: 203–211.

Wentz, D.A., Bonn, B.A., Carpenter, K.D., Hinkle, S.R., Janet, M.L., Rinella, F.A., Uhrich, M.A., Waite, I.R., Laenen, A., and Bencala, K. 1998. Water quality in the Willamette Basin, Oregon, 1991–95. U.S. Geol. Surv. Circ. 1161.

Werner, I., Deanovic, L.A., Connor, V., de Vlaming, V., Bailey, H.C., and Hinton, D.E. 2000. Insecticide-caused toxicity to *Ceriodaphnia dubia* (Cladocera) in the Sacramento – San Joaquin river delta, California, USA. Environ. Toxicol. Chem. **19**: 215–227.

Winberg, S., Bjerselius, R., Baatrup, E., and Døving, K.B. 1992. The effect of Cu(II) on the electro-olfactogram (EOG) of the Atlantic salmon (*Salmo salar* L.) in artificial freshwater of varying inorganic carbon concentrations. Ecotoxicol. Environ. Saf. **24**: 167–178.

Wisby, W.J., and Hasler, A.D. 1954. Effect of occlusion on migrating silver salmon (*Oncorhynchus kisutch*). J. Fish. Res. Board Can. **11**: 472–478.