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Low-level copper exposures increase visibility and vulnerability of juvenile coho salmon to cutthroat trout predators

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Abstract. Copper contamination in surface waters is common in watersheds with mining activities or agricultural, industrial, commercial, and residential human land uses. This widespread pollutant is neurotoxic to the chemosensory systems of fish and other aquatic species. Among Pacific salmonids (*Oncorhynchus* spp.), copper-induced olfactory impairment has previously been shown to disrupt behaviors reliant on a functioning sense of smell. For juvenile coho salmon (*O. kisutch*), this includes predator avoidance behaviors triggered by a chemical alarm cue (conspecific skin extract). However, the survival consequences of this sublethal neurobehavioral toxicity have not been explored. In the present study juvenile coho were exposed to low levels of dissolved copper (5–20 μg/L for 3 h) and then presented with cues signaling the proximity of a predator. Unexposed coho showed a sharp reduction in swimming activity in response to both conspecific skin extract and the upstream presence of a cutthroat trout predator (*O. clarki clarki*) previously fed juvenile coho. This alarm response was absent in prey fish that were exposed to copper. Moreover, cutthroat trout were more effective predators on copper-exposed coho during predation trials, as measured by attack latency, survival time, and capture success rate. The shift in predator–prey dynamics was similar when predators and prey were co-exposed to copper. Overall, we show that copper-exposed coho are unresponsive to their chemosensory environment, unprepared to evade nearby predators, and significantly less likely to survive an attack sequence. Our findings contribute to a growing understanding of how common environmental contaminants alter the chemical ecology of aquatic communities.

Key words: alarm behavior; coho salmon; copper; cutthroat trout; olfaction; predation; skin extract; sublethal; survival.

Introduction

Various forms of water pollution are known to interfere with chemical communication in aquatic habitats (Sutterlin 1974). There are senders and receivers of chemical signals both within and among species in aquatic communities, and certain contaminants are directly toxic to the olfactory, mechanosensory, or gustatory sensory neurons of receivers. This form of sublethal ecotoxicity has been termed info-disruption (Lurling and Scheffer 2007) because it diminishes or distorts the sensory inputs that convey important information about an animal's surrounding environment. Contaminant-exposed receivers thereby respond inappropriately (or not at all) to cues that signal the proximity and status of predators, mates, food, and other factors that can influence growth, survival, distribution, or reproduction.

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One of the most extensively studied examples of info-disruption is the neurotoxicity of dissolved copper to the peripheral olfactory system of fish (Tierney et al. 2010). Olfactory receptor neurons are located in the epithelium of the olfactory rosette, within the nasal cavity. Cilia containing odor receptors extend from the apical surfaces of olfactory neurons into the nasal cavity, separated from ambient waters by a thin layer of mucous. Olfactory receptor neurons are continuously exposed to ambient waters and are therefore highly vulnerable to dissolved toxicants in aquatic habitats.

Copper is a widely occurring pollutant in association with diverse human activities, including agricultural, industrial, commercial, and residential land uses. For example, copper is used in various agriculture and homeowner pesticide formulations, in building materials, as an antifoulant in hull paints for vessels, and in motor vehicle friction materials (i.e., brake pads). As a consequence, copper is commonly transported to aquatic systems in land-based stormwater runoff (Davis et al. 2001). Copper contamination is also associated with hard rock mining and municipal wastewater discharges.

Similar to fish mechanosensory receptor neurons (i.e., lateral line; Linbo et al. 2006), olfactory receptor

neurons undergo cell death in response to dissolved copper concentrations above approximately 20 μg/L (Julliard et al. 1996, Hansen et al. 1999). At lower concentrations in the 2–20 μg/L range, dissolved copper reversibly inhibits the physiological responsiveness of olfactory receptor neurons in a concentration-dependent manner (Baldwin et al. 2003, Sandahl et al. 2004). The loss of sensory function occurs rapidly, within the first few minutes of copper exposure (Baldwin et al. 2003). In most fish species that have been studied to date, peripheral sensory neurons do not acclimate to copper during exposures lasting days (Julliard et al. 1996, Linbo et al. 2006) or weeks (Saucier et al. 1991, Saucier and Astic 1995).

Chemical signals of predation risk are an ecologically important category of olfactory information for fish (Wisenden 2000, Ferrari et al. 2010). For many species (Chivers and Smith 1998), including juvenile salmonids, an olfactory alarm cue released via mechanical tearing of the skin (e.g., during a predation event) triggers predator avoidance behaviors by nearby conspecifics. Juvenile salmon and trout, for example, become motionless in response to the alarm cue (Brown and Smith 1997, Berejikian et al. 1999, Scholz et al. 2000). This reduces their visibility and corresponding vulnerability to attack by motion-sensitive predators such as piscivorous fishes and birds (Webb 1986, Martel and Dill 1995). Numerous studies have demonstrated a survival benefit for alarm-cue-responsive prey (Mirza and Chivers 2001, 2003, Chivers et al. 2002).

Previous studies have shown that peripheral olfactory toxicity and diminished sensory responsiveness correspond to a disruption in alarm behaviors in copper-exposed fish (Beyers and Farmer 2001, Sandahl et al. 2007). For individual juvenile coho salmon (*Oncorhynchus kisutch*), loss of alarm behavior triggered by an ecologically relevant olfactory alarm cue is directly correlated with loss of olfactory function at copper exposures ranging from 2 to 20 μg/L (Sandahl et al. 2007).

Copper's effect on chemical communication in aquatic systems has broad implications for the chemical ecology and conservation of aquatic species and communities. In the case of salmon, subtle but important impacts on sensory physiology and behavior at the juvenile life stage could increase predation mortality and thus increase losses from wild salmon populations, many of which remain at historic lows in large river basins throughout the western United States (Good et al. 2005). Conversely, improving water quality conditions (i.e., by reducing copper loading) could potentially improve juvenile survival and abundance, thereby enhancing ongoing efforts to recover depressed stocks. However, the cascading effects of copper across biological scales, from salmon physiology and behavior to predator–prey interactions and survival, have not been empirically determined.

Here we explored the influence of environmentally relevant copper exposures on juvenile coho salmon (see Plate 1) predator avoidance and survival during encounters with coastal cutthroat trout (*O. clarki clarki*). Cutthroat trout are visual foragers (Henderson and Northcote 1985, Mazur and Beauchamp 2003) that commonly prey on juvenile salmon in stream, lake, and nearshore marine habitats (Nowak et al. 2004, Duffy and Beauchamp 2008). We used a range of sublethal copper exposures (5–20 μg/L) and a duration (3 h) previously shown to impair both peripheral olfaction and alarm behavior in juvenile coho (Sandahl et al. 2007). In a subset of trials, predators were also exposed to dissolved copper (10 μg/L for 3 h).

Methods and Materials

Animals

Juvenile coho.—

1. *Behavior experiments.*—In 2007, wild juvenile coho salmon were collected as needed by seining a side channel of Big Beef Creek at the University of Washington's Big Beef Creek Research Station (Seabeck, Washington, USA). Coho were maintained on well water (Table 1) in indoor raceways under natural light regime and fed pellets daily (1–2 mm extruded; Silver Cup Fish Feed, Murray, Utah, USA). Coho grew slightly throughout the experimental period, from April–May (39–49 mm total length [TL], $\bar{x} = 42.8$, SD $= 3.3$, $n = 13$) to June–July (36–60 mm TL, $\bar{x} = 48.7$, SD $= 5.6$, $n = 79$).

2. *Predation experiments.*—In 2008, juvenile coho were produced from eggs fertilized at the Big Beef Creek Research Station. Hatchlings were maintained outdoors in 1-m^3 net pens suspended in a 5 m diameter circular tank continuously supplied with well water. One net pen of juveniles (approximately 1000 fish) provided the experimental prey. Coho were fed pellets daily. Coho grew slightly throughout the experimental period; random samples in April–May were 30–40 mm TL ($\bar{x} = 36.2$, SD $= 2.5$, $n = 24$) and in June–July were 35–46 mm TL ($\bar{x} = 41.3$, SD $= 2.7$, $n = 64$). During predation trials, there was a significantly higher attack rate on the larger coho in June–July compared to those used in April–May ($t_{29} = -2.136$, $P = 0.041$), likely related to the slightly larger size and therefore visibility of coho in the second set of predation trials. Other predator prey metrics were not affected ($P = 0.084$–0.625).

Cutthroat trout.—

1. *Behavior experiments (response to upstream predator).*—During April 2007, wild cutthroat trout (sizes 178–245 mm TL, $\bar{x} = 205$, SD $= 18$, $n = 16$) for use as predators were obtained from Big Beef Creek in smolt traps at a weir operated by Washington Department of Fish and Wildlife. Predators were maintained outdoors in flow-through circular holding tanks supplied with well water. On experimental days, predators were fed one juvenile coho each. Other days, predators were fed one fish each every other day. Predators were divided

TABLE 1. Conventional water chemistry characteristics, including total organic carbon (TOC), for source (well) water at the Big Beef Creek Research Station (Seabeck, Washington, USA).

Parameter	Units	D.L.	N	Mean	SE
pH			11	7.5	0.3
Alkalinity	mg/L $CaCO_3$	1.0	11	46.7	0.7
Hardness	mg/L $CaCO_3$	1.0	11	56.0	0.0
Bicarbonate	mg/L	1.0	11	46.7	0.7
Calcium	mg/L	0.05	11	18.00	0.00
Potassium	mg/L	0.10	11	0.50	0
Magnesium	mg/L	0.05	11	2.67	0.03
Sodium	mg/L	0.05	11	11.00	0.00
Chloride	mg/L	1.0	11	15.7	0.3
Sulfate	mg/L	1.0	11	2.0	0
TOC	mg/L	0.1	7	0.07†	0.01
0 Cu	μg/L	0.04	6	0.16	0.04
5 Cu	μg/L	0.04	2	4.54	0.07
10 Cu	μg/L	0.04	6	9.21	0.13
10 Cu‡	μg/L	0.04	8	8.94	0.54
10 Cu§	μg/L	0.04	4	8.06	0.34
20 Cu	μg/L	0.04	2	17.25	0.55

Notes: Also shown are measured copper concentrations for the different exposures; copper measurements are for exposure aquaria unless otherwise noted. D.L. stands for instrument detection limit.

† An eighth sample had anomalously high TOC (0.68 mg/L) and was excluded

‡ Experimental arenas for predator + prey trials.

§ Predator holding tanks for predator + prey trials.

randomly into four groups of four. On experimental days, predators within a group were randomly assigned to one of four arenas. Groups were rotated such that each predator was exposed to each treatment.

2. *Predation experiments.*—During April 2008, wild cutthroat trout for use as predators (sizes 150–215 mm TL, $\bar{x} = 183$, SD = 18, $n = 32$) were again obtained from Big Beef Creek and divided into three groups: groups 1 and 2 contained 8 predators each and were used in predation trials, while group 3, containing 16 predators, was held in reserve. Between the first set of predation trials (15–30 May) and the second set (25 June–3 July), predators in groups 1 and 2 were replaced with inexperienced fish from group 3. On experimental days, predators in Group 1 and Group 2 were fed one juvenile coho each during the predation trial. On other days, fish in all three groups were fed one fish each, every other day. For six days prior to collecting experimental data, predators were trained daily by simulating the experimental sequence. Trout were acclimated in the tank behind the divider for 1 h. The divider was then lifted, allowing the predators to locate, attack, and consume up to two prey fish.

Experimental arenas and alarm cue delivery

Behavior experiments with upstream predator.—Outdoor raceways (0.84 m width) were divided into segments (1.2 m long) with steel mesh barriers to create one experimental arena per raceway. A PVC sheet (1/16 inch [~0.16 cm]; Calsak Plastics, Kent, Washington, USA) subdivided by gridlines (5 cm^2) was placed at the bottom of each arena. Well water flowed into the raceway (2 L/s) from an underwater pipe upstream of the arena. A standpipe downstream of the arena maintained a water depth of 25 cm. Dividers partitioned each arena into an upstream predator-containing compartment (46 × 84 cm) and an adjacent downstream compartment containing prey (76 × 84 cm). Dividers were frames (13 cm wide) constructed from PVC sheets (1/16 inch) and covered with window screen.

Well water or skin extract was delivered to the prey compartment through evenly spaced holes in a tube (Tygon tubing, 1/4 inch outer diameter [~0.63 cm]) crossing the upstream divider, approximately 5 cm below the surface. Even dispersion was confirmed visually by dye tests. A three-way valve connected to a syringe allowed for injection of water or water plus alarm odor from outside the visual field of the fish.

Predation experiments.—Circular fiberglass tanks (bottom diameter = 130 cm, height = 90 cm) were used as experimental arenas. Gridlines were drawn at 5-cm intervals on the tank bottom to track fish location via video. An external standpipe maintained water depth (30 cm, 400 L). A sheet of PVC (90 × 60 cm) suspended vertically was used to divide cutthroat trout predators into a small sub-area (34 L) of the arena during acclimation. Juvenile coho prey were introduced into the arena and allowed to acclimate within a clear acrylic cylinder (25 cm inner diameter, 38 cm tall; U.S. Plastic Corp, Lima, Ohio, USA). The acclimation chamber was placed in one of the quadrants opposite the predator divider, within 15 cm from the tank edge. Predator dividers and acclimation chambers were attached by rope to overhead pulleys so they could be gently raised without the observer coming into view of the fish.

Skin extract was introduced to the prey acclimation chamber via Tygon® tubing just below the water surface connected to a three-way valve fitted with two syringes outside the tank. The skin extract solution was immediately flushed from the line with well water (60 mL).

Skin extract alarm cue

An alarm cue-containing skin extract from juvenile coho was prepared as previously described (Sandahl et al. 2007).

Behavior experiments with upstream predator.—In each flow-through arena, 1 mL of concentrated skin extract (160 cm^2 juvenile coho skin/L) was diluted in 50 mL of well water to a final concentration of 2 cm^2/L. This solution was introduced over 60 s into an average flow of 2 L/s for an exposure of approximately 1×10^{-3} $cm^2 \cdot L^{-1} \cdot s^{-1}$. Pilot trials confirmed a behavioral reaction to the alarm cue at this diluted concentration ($\bar{x}$ activity reduction = 51%, SD = 15%, $n = 8$).

Predation experiments.—Initial range-finding tests indicated that 2×10^{-5} cm^2 of homogenized skin extract per liter of water was the minimum concentration to evoke an alarm response ($\bar{x}$ activity reduction = 77%, SD = 24%, $n = 4$). This agrees closely with previously published thresholds for conspecific skin extract evoking predator avoidance behavior in salmonids (1.85×10^{-5} cm^2/L in *O. mykiss* [Mirza and Chivers 2003]; 2×10^{-5} cm^2/L in *O. kisutch* [Sandahl et al. 2007]). In static arenas, diluted skin extract (1 cm^2/L) was prepared daily from a frozen aliquot of concentrated skin extract (22 cm^2/L). At the end of the 15-min prey acclimation, 257 μL of diluted skin extract in 50 mL of well water was injected into the prey acclimation chamber (12.9 L) for a final skin concentration of 2×10^{-5} cm^2/L. Dye tests indicated that injected water did not diffuse from the acclimation chamber prior to the chamber being lifted from the experimental arena.

Copper exposures

Juvenile coho were exposed to dissolved copper prior to experimental trials. Exposures took place in 30-L glass aquaria wrapped in black plastic and supplied with an airstone. Aquaria were filled with 15 L of well water (controls) or well water containing varying copper concentrations (conventional water quality parameters shown in Table 1). Copper was added to the aquaria just prior to the onset of the 3-h exposures. Copper chloride stock solution (0.15 g Cu/L) was diluted to achieve nominal concentrations of 0, 5, 10, or 20 μg/L.

Experimental sequence

Behavior experiments with upstream predator.—Individual predators were placed in the predator compartment of each arena, upstream of the prey compartment, the evening before a trial and allowed to acclimate (>13 h). The following morning, juvenile coho (1 prey/predator) were exposed to either well water or well water containing 20 μg/L copper for 3 h. They were then transferred to the prey compartment of the experimental arena (one prey per arena) and allowed to acclimate for 30 min prior to the injection of stimulus solutions (water or water plus skin extract).

Predation experiments.—The timeline for predation trials is delineated in Table 2. For trials in which only juvenile coho prey were exposed to copper, predators (two per arena) were acclimated behind the divider during the last hour of the 3-h prey exposure interval. Exposed prey were then transferred to the acrylic chamber (two fish per arena) for 15 min, an interval brief enough to minimize olfactory recovery in clean water and yet long enough to produce reliably robust control activity (swimming speed ~5 cm/s). Filming began at the time of prey transfer. Following prey acclimation, skin extract was administered and given 30 s to disperse (verified with dye tests) before the chamber was gently lifted and removed from the experimental arena. Thereafter, predators were released from their enclosure. Two consecutive sets of trials using a different group of predators were run each day, and the arenas were drained and filled between sets.

TABLE 2. Predation trial timeline.

Timeline	Duration	Event
−3 h 15min	3 h	prey exposure
−1 h 0 min	1 h	predator acclimation
−15 min	15 min	prey acclimation
0 min	10 s	skin extract injected
30 s	10 s	prey released
50 s	5 s	predators released

For trials in which both prey and predators were exposed to copper, both exposures were for 3 h, including acclimation time in the experimental arena. Predators were exposed to copper for 2 h in their holding tanks followed by a 1-h exposure in the experimental arena. Prey were exposed to copper in the exposure aquarium for 2.75 h. This was followed by 15 min in the acclimation chamber of the experimental arena.

Water chemistry analyses

Conventional water quality parameters and total organic carbon (TOC) were measured in water samples collected in 2008 between 20 May and 3 July. This interval spans most of the experimental period (16 May–3 July). Concurrently, dissolved copper (DCu) concentrations were measured in 28 samples that were representative of the different copper exposures. For conventional parameters, samples were stored at 4°C in polyethylene bottles until analysis by standard methods at an EPA-certified laboratory (AmTest Laboratories; Redmond, Washington, USA). Samples for TOC were stored in glass vials at −20°C until analysis by combustion catalytic oxidation/NIDR method with a Shimadzu TOC-VCSH (University of Washington, Oceanography Technical Services, Seattle, Washington, USA). Samples for dissolved copper were stored at 4°C for up to 72 h prior to analysis by inductively coupled plasma mass spectrometry (Frontier Global Sciences, Seattle, Washington, USA).

The well water at BBC used in all experiments had low ion and organic carbon content (Table 1), which is similar to Pacific Northwest streams west of the

Cascades (e.g., Fig. 2 in McIntyre et al. 2008). The background copper concentration was very low (mean of 0.16 μg/L) and samples from copper exposures were 81–91% of nominal concentrations.

Video data acquisition

The four experimental arenas were sheltered outdoors beneath a wooden scaffolding to which cameras and pulleys were attached. The stand was covered by blue tarps to prevent direct lighting. Prey acclimation and predation trials were filmed with digital video cameras (SONY Exwave HAD SSC-M383) fitted with auto-iris lenses (2M-2812A, F1.4 DC AutoIris, 1/3″ varifocal 28–12 mm, angle of view 95.6–22.1 degrees; Sony, Tokyo, Japan) mounted over each arena. Video footage for the four concurrent trials were recorded on a digital video recorder (Pro 8-CH DVR; SecurityCameraWorld.com, Cooper City, Florida, USA) at 30 frames per second (FPS).

Data analysis

Coho activity.—

1. *Behavior experiments*.—Following the 30-min acclimation, the activity of juvenile coho was quantified for 5 min by measuring swimming speed, approximated by the sum of vertical and horizontal line crossings on the 5-cm^2 grid of the prey compartment.

2. *Predation experiments*.—We quantified prey activity after coho were released from the acclimation chamber, during the 10 s prior to releasing the predators. Average swimming speed across the 10-s period was determined by tracking each prey fish in two-dimensional space with image analysis software. Using Quicktime Pro (version 7.6; Apple, Cupertino, California, USA), video was exported as an image sequence at 1 frame per second. In Image J, the position (x, y) of each prey fish was tracked between images, converting changes in position into swimming speed (cm/s) by standardizing the pixels to the bottom tank dimensions (software *available online*).[4] We assumed that movement between frames was linear.

For most prey pairs (69/76), the two fish were equally active, and we averaged the swimming speed of the two prey each second. In the remaining 10% of cases, one prey was significantly more active (Kolmogorov-Smirnov distribution test, $P < 0.05$), and the more active prey was attacked first in seven of the eight cases. For these pairings, we used only the activity record for the more active prey in calculating prey activity.

Predation trial metrics.—Predator–prey interactions were analyzed from video recordings of each predation trial. Only attacks and captures of the first prey of the prey pair were quantified. Metrics were time to first attack (δA), time to capture (δC), time between first attack and capture (δC − δA), number of attacks (A), and attack frequency (attacks per second during attack period; A/[δC − δA]). For each copper concentration and predator exposure combination, 16 predation trials were conducted for a total of 112 data trials. Not all metrics could be quantified for all trials.

Statistical analyses

Coho prey activity.—For the experiments in 2007, a two-factor ANOVA was used to explore whether copper exposure (0 vs. 20 μg/L) affected the behavioral response (activity level) to predation risk (no risk, upstream predator, upstream predator plus skin extract). Simple main effects analysis used a Bonferroni adjustment for multiple comparisons. For 2008, single-factor ANOVA was used to test the effect of the various copper treatments on prey activity in the combined presence of predators and skin extract. Dunnett's post-hoc was used to compare activity in the copper treatments to the control treatment. Statistical analyses were conducted in SPSS 16.0 for MacIntosh (IBM, Armonk, New York, USA).

Predator–prey interactions.—Data for predator–prey interactions were not normally distributed and were positively skewed, being bounded by zero. Log-transformation resulted in normally distributed δA, δC, and A, which were analyzed by ANOVA followed by Dunnett's post-hoc for comparing copper treatments to controls. Log-transformation did not normalize δC − δA and attack frequency. Differences in central tendency of δC − δA and attack frequency were tested by Kruskal-Wallis nonparametric multiple comparison. For the separate set of predation trials in which predators were also exposed to copper, Tukey's post-hoc test was used following the ANOVA to compare among the three treatments (controls, prey exposed to 10 μg/L copper, predator + prey exposed to 10 μg/L copper).

The relationship between capture success probability (capture on first attack) and copper treatment was tested by linear regression of the natural log of the odds ratio for capture success weighted by sample size. This method transforms curvilinear data in a probability distribution to a linear function of the independent variable. We transformed capture success probability at each copper concentration to the $\log_e$ odds ratio (OR) as follows:

$$\log_e(\mathrm{OR}) = \ln\left(\frac{\mathrm{CSR}}{1-\mathrm{CSR}}\right) \quad (1)$$

where CSR is the capture success ratio across trials within each copper concentration.

Survival curves.—Time to capture of the first prey fish for each trial was used to assess differences in the distribution of survival times (δC) among treatments. Within each treatment, survival time was ranked across trials and each trial was assigned a decreasing proportion of the total survival of the first prey as per Vilhunen (2006). For example, the first prey captured among control trials had a survival time of 6 seconds. Up to 6 s,

[4] http://rsbweb.nih.gov/ij/

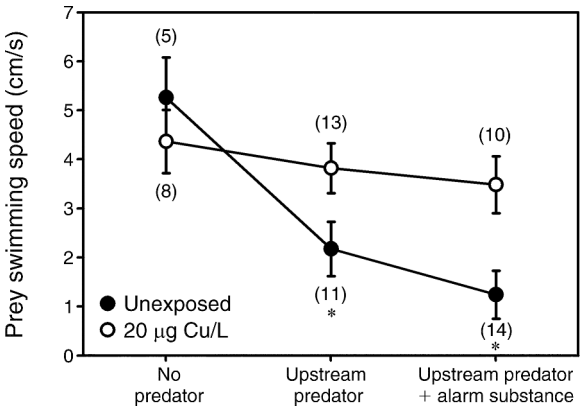

FIG. 1. Activity levels for control (unexposed) and copper-exposed (20 μg Cu/L for 3 h) juvenile coho downstream from one of three levels of predation risk; a compartment with a predator absent, a cutthroat trout predator present, and predator present plus the addition of juvenile coho skin extract. Swimming speed was recorded over 5 min at the end of the 30-min prey acclimation period. Significant differences ($P < 0.05$) from unexposed control are marked with an asterisk. Numbers by each symbol are the sample sizes. Error bars indicate ±SE.

prey survival was 100%. At 6 s, survival across control trials dropped to 15/16, or 93.75%.

For each treatment, the proportion surviving was analyzed as a function of survival time by non-linear regression using the following sigmoid equation:

$$P(T) = \frac{1}{1 + e^{k(T - \mathrm{ST50})}} \quad (2)$$

where k was the slope of the linear portion of the curve, indicating how quickly survival declined with time, T was time in $\log_{10}$(number of seconds), and ST50 was the midpoint of the curve, the $\log_{10}$ survival time for 50% of trials—analogous to the median survival time. For significantly different distributions, a t test assessed differences in the slope and midpoint among treatments. The benefit of using this method over simply comparing the central tendency of survival time among treatments was that we could compare not only the median survival time, but also the shape of the relationship between survival and time.

To calculate survival probabilities for copper treatments relative to the control treatment, we solved Eq. 2 for survival time, T, using the control slope (k) and midpoint (ST50) from Table 4:

$$T = k^{-1} \times \ln\left(\frac{1}{P} - 1\right) + \mathrm{ST50}. \quad (3)$$

For given control survival probabilities (0.95 and 0.5), we used Eq. 3 to calculate the associated prey survival time. These times were then used in Eq. 2 with the respective slopes and midpoints for various copper exposures to estimate the related survival probability at that time for coho in each copper exposure.

RESULTS

Copper-exposed coho prey are behaviorally unresponsive to alarm cues.—We found a significant interaction between copper exposure and upstream predator cues with respect to their effect on coho activity ($F_{2,55} = 6.083$, $P = 0.054$; Fig. 1). In the absence of proximal predator cues, i.e., no upstream predator or conspecific skin extract, coho swam at an average speed of 5.2 cm/s (control condition; Fig. 1). A significant alarm response (tendency toward motionlessness) was elicited by the presence of a predator (2.1 cm/s; $F_{1,55} = 4.813$, $P = 0.032$) and a predator together with an upstream introduction of skin extract (1.2 cm/s; $F_{1,55} = 8.738$, $P = 0.005$). When the prey was exposed to copper, upstream predator cues had no effect on activity (combined 3.9 cm/s; $F_{2,55} = 0.518$, $P = 0.599$). Exposure to copper (20 μg/L) alone did not significantly affect baseline swimming activity (predator absent; 4.3 cm/s, $F_{1,55} = 0.734$, $P = 0.395$). Based on previous work (Baldwin et al. 2003), juvenile coho would be expected to recover ~20% of lost olfactory function during the 30 min acclimation interval in clean water used in these behavioral experiments. Nevertheless, copper-exposed fish were still unresponsive to chemical predator cues.

Similar to flow-through trials, control coho in static trials showed a strong alarm response to skin extract, as indicated by a reduction in swimming speed to 1.0 cm/s (Fig. 2). The magnitude of this alarm response decreased with increasing copper exposure. The average swimming speed of coho exposed to copper at 20 μg Cu/L was 4.9 cm/s and comparable to the baseline swimming speed of unexposed control fish in the flow-through trials (5.2 cm/s; Fig. 1). The loss of the alarm response was

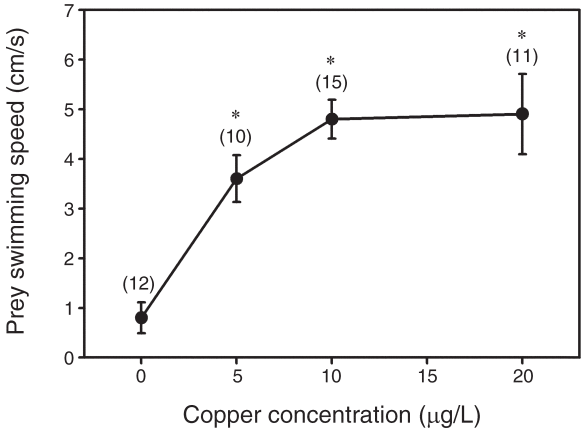

FIG. 2. Alarm behavior in juvenile coho prey at the outset of predation trials. Predators were located within the trial arena behind an opaque divider. Prey swimming speed was recorded at the end of the 15-min prey acclimation, after the presentation of conspecific skin extract. An asterisk indicates that juvenile coho unexposed to copper (0 μg/L) were significantly less active (i.e., were alarmed) relative to copper-exposed coho at all copper exposure concentrations ($P < 0.05$). Error bars indicate ±SE.

TABLE 3. Median values (min, max) for time to first attack (δA), time to first capture (δC), time between δA and δC, number of attacks to δC (*A*), and frequency of attacks.

[Cu]‡ (μg/L)	δA (s)	δC (s)	δC − δA (s)	*A*	Attack frequency (s^{-1}) §
May					
0	29.4 (4.2, 218.4)	41.7 (6, 256.8)	3.3 (0, 106.2)	2 (1, 5)	0.75 (0.029, 16.67)¶
5	8.4 (0, 102)*	13.2 (3, 175.8)†	3 (0, 73.8)	3 (1, 7)	1.11 (0.054, 16.67)
10	6 (1.8, 97.2)†	9.3 (3, 422.4)*	2.7 (0, 422.47)	2 (1, 6)	1.25 (0.007, 16.67)
20	4.5 (0.6, 426.6)*	9.6 (1.2, 426.6)*	3 (0, 6)	3 (1, 6)	1.15 (0.667, 16.67)
June					
0	22.2 (4.2, 156)	23.4 (5.4, 159)	1.8 (0, 7.2)	3 (1, 6)	1.67 (0.555, 16.67)
10	3 (0, 114)*	6.9 (0.6, 124.8)*	3 (0, 12)	3 (1, 6)	1.5 (0.222, 16.67)
10#	5.4 (1.2, 27)*	9 (1.2, 34.8)*	2.1 (0, 28.8)	3 (1, 10)	1.57 (0.347, 16.67)

* $P < 0.05$; † $P < 0.1$.
‡ Copper exposures for 3 h prior to predation trial.
§ A/(δC − δA).
¶ To calculate attack frequency for δC − δA = 0, number of attacks was divided by 0.06 s.
# Predators also exposed to copper.

significant among copper-exposed coho relative to controls ($F_{3,44} = 14.27$, $P < 0.001$; Dunnett's post hoc test, $P \leq 0.001$).

Copper-exposed coho are more vulnerable to predation.—Prior copper exposure significantly affected time to first attack (ANOVA, $F_{3,58} = 3.550$, $P = 0.020$) and time to first capture ($F_{3,58} = 4.33$, $P = 0.008$) of juvenile coho by predators (Table 3). Time to attack (δA) and time to capture (δC) were reduced for all copper treatments compared to controls (Dunnett's post hoc test (0 vs. 5, 10, 20 μg/L): $P_{\delta A} = 0.031, 0.069, 0.014$; $P_{\delta C} = 0.062, 0.020, 0.004$). Other predator–prey interactions were unaffected by copper exposure (Table 3), including time between first attack and capture (Kruskall-Wallis $\chi^2_{3,63} = 2.43$, $P = 0.488$), number of attacks ($F_{3,58} = 0.624$, $P = 0.602$), and attack frequency ($\chi^2_{3,63} = 6.00$, $P = 0.111$).

Time to attack and time to capture were positively correlated because time to capture includes time to first attack (δC = δA + [δC − δA]). The correlation between time to attack and time to capture was very strong ($r_S = 0.959$, $n = 63$, $P < 0.001$). When log-transformed to allow calculation of a coefficient of determination, time to attack explained nearly all the variation in time to capture ($r^2 = 0.912$). Capture–attack interval (δC − δA) was not significantly different among treatments ($\chi^2_{3,63} = 2.43$, $P = 0.488$, median = 3 s), and was not correlated with δA ($r_S = 0.094$, $n = 63$, $P = 0.470$), suggesting that the primary component of the predation sequence affected by copper was prey detection leading to attack (δA).

Although the number of attacks to capture (*A*) was not different among treatments (Table 3), the capture success rate (probability of capturing prey on the first attack) increased with copper concentration (Fig. 3). Capture success rate was significantly correlated with increasing copper exposure concentration ($F_{1,3} = 60.060$, $P = 0.016$, $r^2 = 0.968$) following the equation $\log_e(\text{OR}) = 0.062[\text{Cu}] - 2.039$, where [Cu] is dissolved copper concentration in μg/L. Standard error for the slope was 0.008 and was 0.092 for the intercept.

Exposing predators to copper does not improve the evasion success of prey.—In a separate set of predation trials, we determined the effect of co-exposing predators and prey to copper at 10 μg/L (Table 3). Similar to the first set of predation trials, copper exposure affected time to attack ($F_{2,42} = 8.639$, $P = 0.001$) and time to capture ($F_{2,42} = 6.368$, $P = 0.004$). However, these metrics were not significantly different from experiments in which prey alone were exposed (Tukey's post hoc, δA, $P = 0.340$; δC, $P = 0.715$). Number of attacks ($F_{2,42} = 1.429$, $P = 0.251$), time between first attack and capture ($\chi^2_{2,45} = 0.732$, $P = 0.693$), and attack frequency ($\chi^2_{2,45} = 0.318$, $P = 0.853$) were not affected by copper exposure (prey exposed and predators plus prey exposed were similar to controls). In addition, exposing predators to copper did not change the likelihood of capturing prey on the first attack (25% for exposed prey only vs. 31% for co-exposed predators and prey; $\chi^2_1 = 0.643$, $P = 0.423$).

Copper exposure reduces prey survival.—Survival curves for each treatment were constructed from the

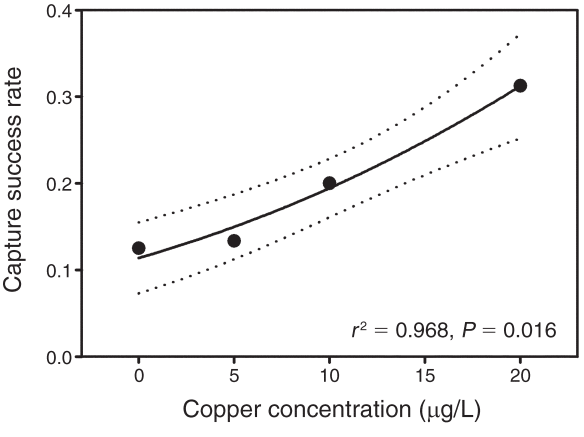

FIG. 3. Proportion of trials for which prey were captured on the first attack (capture success rate). Dashed lines are 95% confidence bands for the logistic regression. Capture success rate is described by the equation $e^F/(1 + e^F)$, where $F = 0.062[\text{Cu}] - 2.039$ (see *Results* for associated statistics).

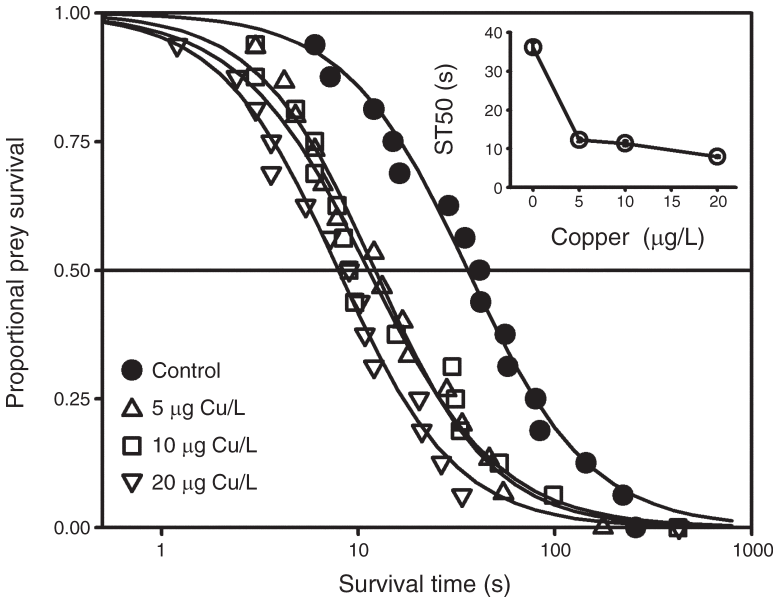

FIG. 4. Survival curves for control and copper-exposed coho in predation trials. Each point represents one predation trial, and survival times are based on the first prey fish consumed. The inset shows the midpoints of each curve, representing median survival time (ST50) for each treatment as a function of copper exposure.

time to first capture among trials (Figs. 4 and 5). Slopes, midpoints, and coefficients of determination for these curves are presented in Table 4.

Survival curves for copper treatments (Fig. 4) were significantly different from the control curve (F test, all $P < 0.001$). This was due to differences in midpoint (t test, all $P < 0.001$), as slope between survival and time for each copper treatment was similar to the slope of the control curve (t test, all $P > 0.480$). Among copper treatments, 5 μg/L and 10 μg/L produced similar survival curves ($F_{2,27} = 2.222$, $P = 0.128$), with similar slopes (t_{27}, $P = 0.314$) and midpoints (t_{27}, $P = 0.274$),

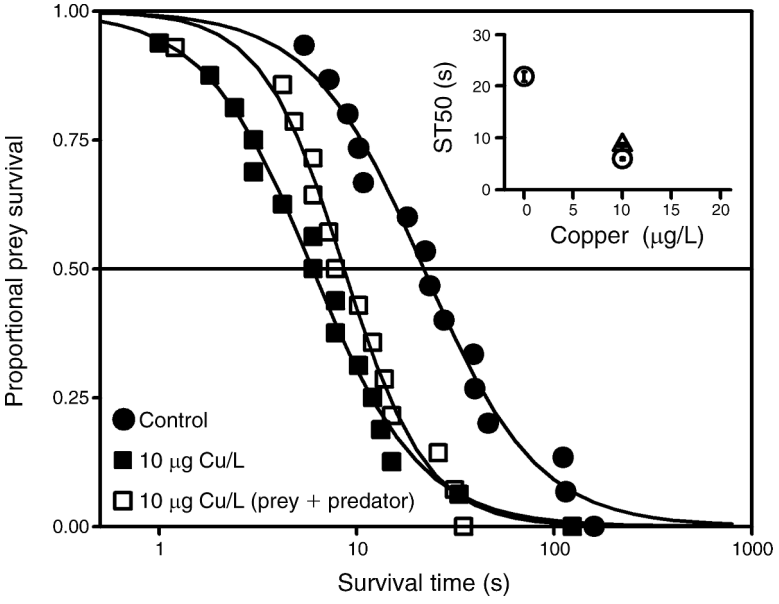

FIG. 5. Survival curves for predation trials in which prey alone or predators and prey were both exposed to copper (10 μg/L). Each point represents one trial, and survival times are based on the first prey fish consumed. Insets show the midpoints of each curve, representing median survival time (ST50) for each treatment as a function of copper exposure. The triangle symbol in the inset represents the ST50 for trials in which both predator and prey were exposed to copper.

TABLE 4. Sigmoid regression parameters for the survival curves.

[Cu] (μg/L)	r^2	ST50†	SE	K‡	SE	N
May						
0	0.984	1.557	0.016	3.219	0.186	16
5	0.988	1.085	0.014	3.36	0.166	15
10	0.965	1.052	0.026	3.042	0.262	16
20	0.987	0.898	0.014	3.333	0.17	16
June						
0	0.983	1.338	0.016	3.493	0.213	15
10	0.985	0.774	0.014	3.659	0.203	16
10§	0.985	0.935	0.012	4.768	0.302	14

Note: All $P < 0.001$.
† Log of time to 50% survival across trials, midpoint of curve, measured in seconds.
‡ Slope of the sigmoid regression curve.
§ Predators and prey both exposed to copper.

whereas these curves had significantly different midpoints (Table 4) than the curve for 20 μg/L (both $P < 0.004$).

For the predation trials in which both predators and prey were exposed (Fig. 4), survival curves for copper treatments (10 μg/L) were again different from the control curve (F test, both $P < 0.001$). Prey alone exposed to 10 μg/L resulted in a survival curve that had a similar slope (t_{27}, $P = 0.577$), but different midpoint (t_{27}, $P < 0.001$) than the control curve. Exposing predators and coho to 10 μg/L affected both the slope (t_{25}, $P = 0.002$) and the midpoint (t_{25}, $P < 0.001$) of the survival curve compared to the control curve. The predator + prey copper curve also had a different slope (t_{26}, $P = 0.005$) and midpoint (t_{26}, $P < 0.001$) compared to the prey-only copper exposures. Therefore, exposing predators to copper resulted in a subtle change in the shape of the survival curve, although it was not strong enough to alter predator–prey metrics (see *Exposing predators to copper does not improve the evasion success of prey*).

We calculated survival probabilities for copper exposures relative to controls using Eqs. 1 and 2. At 4.4 s, 95% of control coho were alive. Relative survival probabilities for copper-exposed coho were 82% for 5 μg/L, 78% for 10 μg/L, and 70% for 20 μg/L. The median survival time for controls was 36.1 s (50% survival; Table 4). Corresponding survival probabilities for copper exposures were 17%, 18%, and 10% for 5 μg/L, 10 μg/L, and 20 μg/L treatments, respectively.

DISCUSSION

We have evaluated the effects of copper exposure on juvenile coho predator avoidance behaviors and the related consequences for coho survival during encounters with predatory wild cutthroat trout. We find that relatively brief (3 h) exposures to copper at 5–20 μg/L eliminated the behavioral alarm response in coho prey, leading in turn to increased detection, reduced evasion, and reduced survival during predation trials.

The magnitude of the coho alarm response was greatest when the presence of an upstream predator was paired with skin extract, consistent with previous studies (e.g., Lautala and Hirvonen 2008). Our results showing a copper-induced loss of antipredator behavior reinforces and extends previous observations for juvenile coho. Sandahl et al. (2007) found that hatchery-raised coho become motionless (freeze) following presentation of a conspecific skin extract, and that this alarm response is reduced or abolished by copper exposure (3h; 2–20 μg/L). We have extended this behavioral toxicity to wild coho, and shown that copper also renders coho unresponsive to possibly distinct chemical cues emanating from a proximal upstream predator. This is consistent with copper's broad neurotoxicity across non-overlapping olfactory receptor neuron populations in the salmon olfactory epithelium (Baldwin et al. 2003).

Copper-exposed prey were easier for predators to identify, attack, and capture. This was due primarily to higher activity than alarmed controls, leading to a more rapid detection by cutthroat trout. For juvenile salmon, activity critically determines the likelihood of detection by visually guided predators such as larger salmonids, piscivorous birds, and river otters. For example, in predation trials with Mergansers, attacks on active juvenile coho were 15 times more frequent than attacks on inactive coho (Martel and Dill 1995). In the current study, copper also negatively influenced evasion of a predator once an attack was initiated, i.e., it became increasingly likely that prey would be captured on the first attack at higher copper exposure concentrations. Evasion success depends in part on whether the prey fish is aware of proximal danger (Lima and Dill 1990). In the current study the threat awareness of unexposed controls was heightened via the introduction of conspecific skin extract prior to the onset of the trial. By comparison, copper-exposed coho were unresponsive to the chemical alarm cue, thus unaware of the impending threat, and less prepared to evade once an attack sequence was initiated.

Copper toxicity to the coho lateral line mechanosensory system may have contributed to the observed reduction in evasion success. As with olfactory receptor neurons, copper is toxic to lateral line neurons that are directly exposed to contaminated waters (Linbo et al.

PLATE 1. Juvenile coho salmon are sensitive to olfactory alarm cues. Photo credit: Morgan Bond.

2006). The lateral line system in salmon and other fish responds to water displaced by an approaching predator and triggers a well-studied sequence of evasive behaviors (the C-type startle reflex: reviewed by Bleckmann 1993). Conversely, predators can capture prey without a functioning lateral line system. For predatory bass (*Micropterus salmoides*) and muskellunge (*Esox masquinongy*), prey capture success rate was unaffected by cobalt exposures at concentrations toxic to the lateral line (New 2002). Despite similar prey capture success, some aspects of the attack sequence were altered in cobalt-exposed predators relative to controls, including shorter distance to strike (both predators) and mean angular approach (muskellunge). We found a subtle shift in the midpoint and slope of the prey survival curve when predators were co-exposed to copper, possibly due to copper neurotoxic effects on the lateral line of cutthroat trout predators. Additional behavioral studies with a focus on lateral line function are warranted, particularly for predator–prey encounters under low visibility conditions.

Prey may make compensatory behavioral changes to improve their likelihood of surviving an attack (Lima and Dill 1990, Lind and Cresswell 2005); however, we saw no evidence of this among copper-exposed coho. Also, co-exposing predators and prey to copper did not eliminate the reduced survival time of prey relative to exposing prey alone. This indicates that sublethal copper toxicity will have a disproportionate impact on prey in predator–prey dynamics, irrespective of whether the visually guided predators occupy the same contaminated surface waters (e.g., cutthroat trout and other piscivorous fish) or attack from the air above (e.g., Kingfishers and other birds).

The arena used for the predation trials lacked substrate, making it easier for cutthroat trout to detect and successfully capture alarmed coho relative to an encounter under natural conditions. Substrate complexity improves juvenile coho crypsis (Donnelly and Dill 1984) and provides refuge. Turbidity in streams can further constrain visual detection (Mazur and Beauchamp 2003). Thus, our observed differences in predation vulnerability between copper-exposed and unexposed prey would likely be magnified in natural stream habitats where survival rates for alarmed (predator aware) coho are higher.

Our findings likely extend to other fish species. For example, Baldwin et al. (2011) recently showed that the olfactory toxicity of copper is comparable in coho and steelhead, and also comparable among fish raised in hatchery and natural environments. Numerous other studies have demonstrated the olfactory-mediated neurobehavioral toxicity of copper for alarm behavior (reviewed by Tierney et al. 2010) in both controlled laboratory settings (e.g., Beyers and Farmer 2001, Jaensson and Olsen 2010) and in situ in copper-contaminated habitats (McPherson et al. 2004, Mirza et al. 2009). Copper impacts on chemosensory function also extends to other taxa; for example, disruption of the kairomone-mediated morphological predation defense of zooplankton (*Daphnia pulex*) and altered olfactory-based feeding behaviors of leeches (*Nephelopsis obscura*; Pyle and Mirza 2007) have similar toxicity thresholds (~5 μg/L).

The toxic effects of copper have been remarkably consistent in coho salmon across biological scales, from the functional responsiveness of receptor neurons in the olfactory epithelium (Baldwin et al. 2003, Sandahl et al. 2004, 2007, McIntyre et al. 2008, Baldwin et al. 2011) to the olfactory-mediated behavior of individual animals (Sandahl et al. 2007; this study) to coho survival in predator–prey interactions (this study). Across these studies, the thresholds for neurobehavioral toxicity have been in the range of 2–5 μg/L (although this will shift upward in waters with relatively high dissolved organic carbon content: McIntyre et al. 2008). Notably, this is very close to the toxicity threshold reported for rainbow trout olfaction more than 35 years ago (7 μg/L: Hara et al. 1976). Olfactory disruption as measured at the olfactory epithelium is therefore a reliable proxy for behavioral impairment and reduced survival.

In conclusion, our findings are an example of how chemical habitat degradation in the form of water pollution can have nuanced but important impacts on the behavioral ecology of salmon. The effects of copper on coho survival are context-dependent and likely to go unnoticed in conventional field surveys of juvenile salmon abundance, habitat use patterns, and physical habitat quality. New biological indicators of copper toxicity, including diagnostic changes in gene expression within the salmon olfactory epithelium (e.g., Tilton et al. 2008), may eventually reveal the extent of sensory isolation in wild salmon under natural exposure regimes. In the interim, copper control strategies will likely improve juvenile salmon survival and minimize the disruption of a range of chemosensory-dependent behaviors. This includes, for example, legislation recently enacted in Washington State (SB6557) and California (SB346) to phase out the use of copper and other metals in motor vehicle brake pads.

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