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DIFFERENCES IN NEUROBEHAVIORAL RESPONSES OF CHINOOK SALMON (*ONCORHYNCHUS TSHAWYTSCHA*) AND RAINBOW TROUT (*ONCORHYNCHUS MYKISS*) EXPOSED TO COPPER AND COBALT: BEHAVIORAL AVOIDANCE

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Abstract—Behavioral avoidance of copper (Cu), cobalt (Co), and a Cu and Co mixture in soft water differed greatly between rainbow trout (*Oncorhynchus mykiss*) and chinook salmon (*O. tshawytscha*). Chinook salmon avoided at least 0.7 μg Cu/L, 24 μg Co/L, and the mixture of 1.0 μg Cu/L and 0.9 μg Co/L, whereas rainbow trout avoided at least 1.6 μg Cu/L, 180 μg Co/L, and the mixture of 2.6 μg Cu/L and 2.4 μg Co/L. Chinook salmon were also more sensitive to the toxic effects of Cu in that they failed to avoid ≥44 μg Cu/L, whereas rainbow trout failed to avoid ≥180 μg Cu/L. Furthermore, following acclimation to 2 μg Cu/L, rainbow trout avoided 4 μg Cu/L and preferred clean water, but chinook salmon failed to avoid any Cu concentrations and did not prefer clean water. The failure to avoid high concentrations of metals by both species suggests that the sensory mechanism responsible for avoidance responses was impaired. Exposure to Cu concentrations that were not avoided could result in lethality from prolonged Cu exposure or in impairment of sensory-dependent behaviors that are essential for survival and reproduction.

Keywords—Behavioral avoidance Copper Cobalt Rainbow trout Chinook salmon

INTRODUCTION

Behavioral avoidance is often reported to be one of the most sensitive sublethal responses of fish to contaminants such as heavy metals [1]. Although the behavioral effects of many heavy metals have not been evaluated, most of those that elicit a consistent avoidance response, including copper (Cu), zinc (Zn), nickel, mercury, and others, are avoided at concentrations that are much lower than lethal [2]. For example, Sprague [3] compared behavioral avoidance of Cu and Zn in Atlantic salmon (*Salmo salar*) with time-independent lethal concentrations (incipient lethal level, ILL), and the lowest concentration to cause avoidance in 50% of fish tested was approximately 5 and 10% of the ILLs for Cu and Zn, respectively.

Of the heavy metals that have been evaluated for behavioral avoidance responses, Cu has been studied most often. Several studies have documented that anadromous and freshwater salmonids avoid low Cu concentrations ranging from 2.3 [3] to 7.3 μg/L [4]. This range of values suggests that many variables probably influence the lowest (threshold) concentrations of Cu that are avoided. For example, Giattina et al. [4] found that rainbow trout (RBT, *Oncorhynchus mykiss*) avoided 7.3 μg Cu/L in single-concentration, steep-gradient tests; 6.4 μg Cu/L in stepwise Cu concentration increases in steep-gradient tests; and 4.4 μg Cu/L in shallow-gradient tests. In these studies, the authors report that they first observed alterations in the behavior of these fish at concentrations of 1.4 to 3.2 μg Cu/L, but statistical significance could be inferred only at the much higher threshold concentrations identified above [4]. Thus, the experimental design, choice of avoidance chamber design, number of replicates, and exposure concentrations can greatly affect the results of a study. Additionally, other poorly understood variables that no doubt alter avoidance behaviors include the influence of metals acclimation [5,6], differences in species sensitivity [6], and water quality parameters of hardness, alkalinity, pH, and temperature, which can affect metal speciation and bioavailability [7].

Although behavioral avoidance of many heavy metals has been demonstrated, other metals, such as cobalt (Co), have not been investigated. In lethality studies with RBT, the 96-h LC50 for Co was 1,406 μg/L, and the ILL for Co was 346 μg/L [8]. Because fish avoid many heavy metals at less-than-lethal concentrations [2], we might expect fish to avoid Co at less than this ILL concentration. However, some metals, such as cadmium (Cd), which is lethal to salmonids at 1 to 1.8 μg/L [9], elicit inconsistent avoidance responses to concentrations ranging from 0.2 to 1 μg/L [10,11]. Therefore, we cannot assume that all metals will be avoided at less-than-lethal concentrations.

In most metals-affected waterways, rarely is only one metal present at elevated concentrations, but few studies have evaluated the avoidance of metals mixtures. Sprague [3] showed that, on average, Atlantic salmon avoided 5.2% of the Cu ILL and 9.2% of the Zn ILL when the fish were exposed to the single metal. However, when Cu and Zn were mixed, the fish avoided 2.1% of the ILL of each metal. So the fish were responding to the additive (or possibly synergistic) effects of the two metals. Although this study by Sprague [3] is convincing, the effect of other metals mixtures is poorly understood.

In the study presented here, we conducted-behavioral avoidance experiments on chinook salmon (CS, *Oncorhynchus*

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tshawytscha) and RBT under water-quality conditions and metals concentrations that simulated a mine-affected stream, Panther Creek in Idaho, USA, where both species once resided. We designed this study to determine the avoidance response of CS and RBT to Cu, Co, and Cu+Co mixtures over a wide range of realistic concentrations; to determine the fundamental differences in avoidance responses between the two fish species; and to determine the effects of Cu acclimation on the avoidance of Cu by both fish species.

MATERIALS AND METHODS

All experiments were conducted at the Red Buttes Environmental Biology Laboratory, University of Wyoming, in Laramie, WY, USA.

Experimental fish

Juvenile CS were obtained from McNenny State Fish Hatchery (Spearfish, SD, USA) and ranged in total length from 65 to 160 mm during tests. Rainbow trout eggs were obtained from Dubois State Fish Hatchery (Dubois, WY, USA) and, after hatching, were reared to between 34 and 110 mm in total length prior to use in tests.

All fish were held in the laboratory for a minimum of 30 d and were then acclimated to appropriate hardness, alkalinity, pH, and temperature conditions for at least 72 h prior to testing. During tests to determine the effects of Cu acclimation on behavioral avoidance responses, the fish were acclimated to a nominal 2 μg Cu/L for 25 to 30 d prior to testing. All CS used in tests were free from obvious disease, injury, or distress. Experiments on CS were completed within 12 months of hatch, and, therefore, these fish were pre-smolt. However, following the first RBT experiment, the RBT became infected with an external parasite (*Gyrodactalus* spp.). The infected trout were successfully treated with a 20-s dip into a 1:500 v/v dilution of glacial acetic acid. Following a 2-week recovery from the treatment, pilot studies revealed that avoidance responses were statistically not different from preinfection responses. No further infection was observed in these fish, and no other disease, injury, or distress was noted.

Water quality

Desired water hardness and other water-quality characteristics for acclimation and testing were achieved by continuously mixing deionized water with well water. The pH of this water was continuously monitored and adjusted with dilute H_2SO_4 or KOH using a Leeds and Northrup (North Wales, PA, USA) model 7084 pH controller/analyzer. Nominal water-quality parameters during acclimation and testing were as follows: hardness and alkalinity, 25 mg/L as $CaCO_3$; conductivity, 50 μS/cm; pH, 7.5; and temperature, 10°C.

During each test series, stock solutions for each nominal metal-exposure concentration were prepared by serial dilutions of concentrated $CuCl_2 \cdot 6H_2O$ and/or $CoCl_2 \cdot 2H_2O$ solutions and were stored in 8-L glass bottles. Stock solutions were metered at 2.5 ± 0.05 ml/min into avoidance chamber inflow water using Fluid Metering QG-20 (Syosset, NY, USA) metering pumps.

Chemical analysis of water

Water used during acclimation and avoidance tests was analyzed daily for hardness, alkalinity, pH, conductivity, dissolved oxygen, and temperature using standard methods [12]. Copper and Co samples were collected from 50% of all replicate Cu and/or Co exposures and were collected once every 3 d during Cu acclimation. Samples were collected in acid-washed, 25-ml scintillation vials and acidified with 25 μl of Optima™ nitric acid (HNO_3) (Fisher Scientific, Pittsburg, PA, USA). Samples were analyzed by graphite-furnace atomic absorption spectrophotometry (GFAAS), with method detection limits of 0.7 μg Cu/L and 1.2 μg Co/L.

Behavioral avoidance chambers

Three countercurrent avoidance chambers (PVC, 15 cm diameter by 98 cm long) were used for behavioral avoidance tests. The chamber design was similar to that used by Sprague [5] and essentially the same as that previously described and used by Woodward et al. [13] and Hansen et al. [6]. Water entered each end of the chamber at 1,000 ml/min (±20 ml/min maximum tolerance) and flowed toward nine center drain holes in order to establish a stable, steep gradient between the waters entering each end of the chamber. Mixing boxes allowed for adequate mixing of water and metals stock solutions prior to the time at which the water entered the avoidance chamber. The three chambers were isolated within a black plywood enclosure in order to reduce external disturbances. A video camera was mounted above the chambers in order to record fish movements for later analysis.

Trials with colored dyes demonstrated that a steep gradient between the waters flowing from each end of the chamber was established within 10 min of dye introduction, and this gradient was maintained within 3 cm of the drain holes at the chamber center. Metals analysis with 100 μg Cu/L test water similarly indicated a stable, steep gradient at the chamber center that cleared from the chamber within 15 min after halting the input of Cu-contaminated water.

Test procedure

Test procedures were similar to those reported previously by Woodward et al. [13] and by Hansen et al. [6]. During tests, reference water was used for pretest acclimation, avoidance chamber habituation (acclimation to surroundings), and as an alternative choice to the test water. Test water was the same as reference water, but it contained Cu and/or Co, which was added as the experimental variable.

Each avoidance test consisted of four periods: a 20-min rinse period before and between tests with reference water entering both ends of each chamber to ensure uniform water quality prior to the introduction of the fish; a 20-min habituation period that commenced when one fish was added to each chamber; a 10-min latency period when metals were introduced into one randomly selected end of each chamber and allowed to establish a stable, steep gradient between the test water and reference water; and a 20-min observation period when all avoidance data were collected with reference water in one end and test water in the other end of each chamber.

Each test was videotaped from the beginning of the habituation period to the end of the observation period. After completing each test series, an observer reviewed each videotape and recorded into a computer file the time each fish crossed into and out of the test-water end of the chamber. From these data, the number of trips, average trip time, and total time in the test water were calculated. Because the total-time parameter was a robust indicator of avoidance and because the other parameters were redundant, only the data for total time in test water are presented here.

A valid test was declared when no external disturbances or

Table 1. Means (SE, *n*) of analyzed Cu concentrations during the Cu-avoidance tests. Samples were analyzed from 50% of the avoidance tests performed

Nominal Cu concentration	Analyzed Cu concentration (μg/L)	
	Chinook salmon tests	Rainbow trout tests
REF[a] (0)	0.2 (0.20, 15)[b]	0.4 (0.29, 14)[b]
Test waters		
0	0.2 (0.08, 15)[b]	0.2 (0.13, 15)[b]
0.25	NT[c]	0.0 (0.10, 5)[b]
0.5	NT[c]	0.1 (0.18, 4)[b]
0.8	0.7 (0.16, 5)	0.6 (0.09, 5)[b]
1.6	1.6 (0.10, 5)	1.6 (0.15, 10)
3.1	2.8 (0.21, 5)	2.9 (0.11, 10)
6.3	6.0 (0.26, 10)	5.2 (0.36, 4)
12.5	11 (0.32, 10)	11 (0.20, 5)
25	22 (0.57, 10)	23 (0.25, 10)
50	44 (1.0, 10)	46 (0.90, 5)
100	92 (1.8, 10)	88 (3.1, 5)
200	180 (9.1, 4)	180 (5.7, 5)
400	340 (12.0, 5)	360 (21.0, 5)

[a] REF = reference water that was used in the chamber as an alternative choice to the test water in the behavioral avoidance chamber.

[b] Mean concentration less than the detection limit.

[c] NT = nominal concentration was not tested for this species.

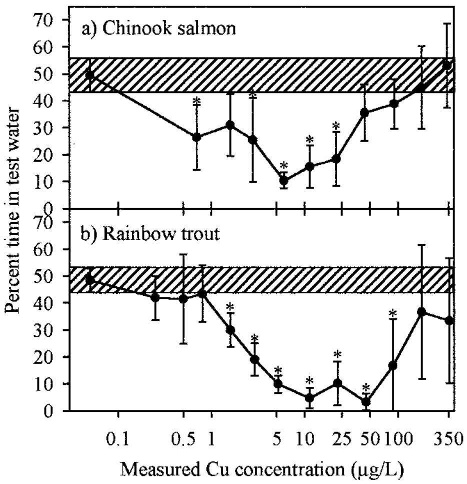

Fig. 1. Chinook salmon (**a**) and rainbow trout (**b**) avoidance of copper. Each vertical bar indicates the 95% confidence interval for the fish tested at that concentration. The shaded region indicates the 95% confidence interval for the control response. An asterisk indicates a response that differed significantly from the control response ($p < 0.05$), as determined by Dunnett's multiple comparison procedure.

inconsistent water chemistries were identified. Additionally, in order to be included in the final data compilation and analysis, each fish was required to make at least one trip into the test water during either the latency period or the observation period. This criterion eliminated four tests from the 960 tests that were conducted. Eliminated tests were not replaced, so some data sets were analyzed without equal replication.

Experimental design

Four experimental series were completed with both CS and RBT, including the avoidance of Cu, the avoidance of Co, the avoidance of Cu+Co mixtures, and the avoidance of Cu following acclimation to low concentrations of Cu. With the exception of the Cu-acclimation experiments, all fish were naive to metals exposure. For each series of experiments, the nominal and analyzed metals concentrations are presented in Tables 1 through 4.

Data analysis and statistics

Because all treatments could not be tested simultaneously, a balanced incomplete block design was used to investigate and eliminate time as an experimental variable [14]. Six treatments were tested within a data set (five different metal concentrations plus a no-metal control), with only three treatments completed at any one time. Each treatment was tested simultaneously with each other treatment four times. Therefore, each treatment was compared with every other treatment equally often and with the same precision. With the exception of Cu-acclimation tests, multiple data sets were completed throughout a wide range of metals concentrations. Because the effect of time was insignificant ($p > 0.05$), the data were analyzed using a simplified general linear model procedure in SAS version 6.02 [15]. Differences between control and metals treatments were determined using Dunnett's multiple comparison procedure ($\alpha = 0.05$). The data were normally distributed and had homogeneous variance. No transformations were performed, and the total time in the test water was used in analysis. For graphic presentation, analyzed metals concentrations below the GFAAS method detection limits are presented using nominal concentrations.

RESULTS

General water-quality parameters

Mean hardness (SE) values were maintained at 25.3 (0.24) mg/L as $CaCO_3$. Mean values for alkalinity, as analyzed by Gran titration, were 28.0 (0.80) mg/L as $CaCO_3$. Mean water temperature was 10.2°C (0.01°), with a maximum variation within each test of 0.4°C. Measured pH was 7.5 (0.02), and conductivity was 53.8 (2.00) μS/cm. Dissolved oxygen was 8.2 (0.01) mg/L.

Avoidance of Cu

For both CS and RBT tests, measured Cu concentrations were within 20% of the nominal concentration. However, all measured Cu concentrations above detection limits were within 10% of the mean value, which indicated low variance among samples (Table 1).

Chinook salmon significantly avoided 0.8 μg Cu/L and from 2.8 to 22.5 μg Cu/L, but 1.6 μg Cu/L and 44 to 340 μg Cu/L were not significantly avoided (Fig. 1a). The maximum mean avoidance response was at 6 μg Cu/L, with fish averaging approximately 10% of their time in the contaminated water.

Rainbow trout avoided Cu concentrations from 1.6 to 88 μg Cu/L (Fig. 1b). Like CS, RBT did not avoid 180 or 360 μg Cu/L. The most intense avoidance responses were between 5 and 50 μg/L, where the fish averaged less than 10% of their time in the contaminated end of the chamber.

Avoidance of Co

Measured Co concentrations were consistently less than the nominal concentrations, but variability between samples at each nominal concentration was low (Table 2).

Chinook salmon displayed a bimodal response to Co, where low concentrations (24 and 46 μg Co/L) were avoided but 89 μg Co/L was not (Fig. 2a). All concentrations at or higher than 180 μg Co/L were avoided, with increasing avoidance as

Table 2. Means (SE, *n*) of analyzed Co concentrations during the Co-avoidance tests. Samples were analyzed from 50% of the avoidance tests performed

Nominal Co concentration	Analyzed Co concentration (μg/L)	
	Chinook salmon tests	Rainbow trout tests
REF[a] (0)	1.6 (0.53, 10)	0.4 (0.05, 10)[b]
Test waters		
0	0.6 (0.15, 10)[b]	0.5 (0.17, 10)[b]
25	24 (1.3, 5)	22 (0.56, 5)
50	46 (1.5, 5)	44 (0.60, 5)
100	89 (2.1, 10)	91 (0.67, 9)
200	190 (2.6, 10)	180 (3.0, 10)
400	360 (7.0, 10)	360 (2.4, 10)
800	720 (4.9, 5)	710 (7.3, 5)
1,600	1,480 (12.0, 5)	1,450 (12.0, 5)

[a] REF = reference water that was used in the chamber as an alternative choice to the test water in the behavioral avoidance chamber.

[b] Mean concentration less than the detection limit.

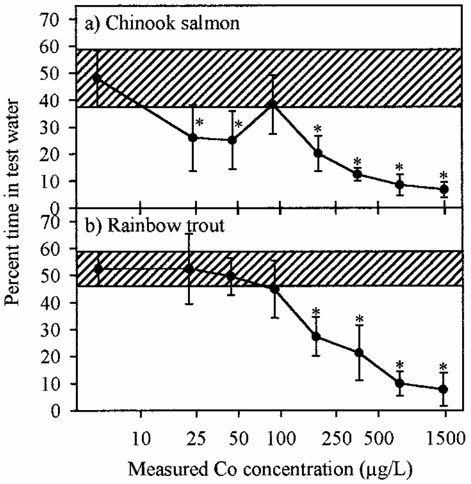

Fig. 2. Chinook salmon (**a**) and rainbow trout (**b**) avoidance of cobalt. Each vertical bar indicates the 95% confidence interval for the fish tested at that concentration. The shaded region indicates the 95% confidence interval for the control response. An asterisk indicates a response that differed significantly from the control response ($p < 0.05$), as determined by Dunnett's multiple comparison procedure.

the concentration increased. We did not identify an upper Co concentration that these fish failed to avoid.

Rainbow trout did not express the bimodal response seen with CS (Fig. 2b). Concentrations ≤91 μg Co/L were not avoided, and concentrations ≥180 μg Co/L were avoided with increasing intensity.

Avoidance of Cu+Co mixtures

Measured Cu+Co concentrations were generally at a 1:1 ratio, with low variability within each nominal concentration (Table 3).

As with the avoidance of Cu (Fig. 1a) and Co (Fig. 2a) tested individually, the CS response to low concentrations of the Cu+Co mixture was bimodal (Fig. 3a). The lowest concentration tested (nominal 0.8 μg Cu+Co/L) was significantly avoided, whereas the next higher concentration (nominal 1.6 μg Cu+Co/L) was not avoided. All concentrations between the nominal 3.1 μg Cu+Co/L and the nominal 50 μg Cu+Co/L were avoided. With the exception of the nominal 50 μg Cu+Co/L exposure, the intensities of the avoidance responses to the mixture were similar to those seen in the Cu-only exposures (Fig. 1a). All higher concentrations were neither avoided nor preferred.

Rainbow trout avoided the mixtures from the nominal 3.1 to the nominal 50 μg Cu+Co/L (Fig. 3b). Whereas the nominal 1.6 and 100 μg Cu/L were avoided in Cu-only exposures (Fig. 1b), the nominal 1.6 and 100 μg Cu+Co/L exposures in the mixture experiments were not (Fig. 3b).

Influence of Cu acclimation on avoidance of Cu

Both CS and RBT were acclimated to a nominal 2 μg Cu/L concentration for 25 to 30 d prior to Cu-avoidance testing. During the habituation period, both chamber ends contained the nominal 2 μg Cu/L, and during the latency and observation periods, the reference-water end still contained 2 μg Cu/L, but the test end contained from 0 to 24 μg Cu/L concentrations.

Table 3. Means (SE, *n*) of analyzed Cu + Co concentrations during the Cu + Co–avoidance tests. Samples were analyzed from 50% of the avoidance tests performed

Nominal Cu + Co concentrations	Analyzed Cu + Co concentrations (μg/L)			
	Chinook salmon tests		Rainbow trout tests	
	Cu	Co	Cu	Co
REF[a] (0)	0.3 (0.11, 9)[b]	0.4 (0.15, 9)[b]	0.7 (0.32, 9)[b]	0.5 (0.16, 10)[b]
Test waters				
0	0.0 (0.09, 10)[b]	0.0 (0.30, 10)[b]	0.7 (0.21, 10)[b]	0.3 (0.12, 10)[b]
0.8	1.0 (0.06, 5)	0.9 (0.24, 5)[b]	1.0 (0.38, 5)	0.9 (0.39, 5)[b]
1.6	1.7 (0.07, 5)	1.5 (0.12, 5)	1.7 (0.39, 5)	1.2 (0.15, 5)
3.1	3.5 (0.24, 5)	2.9 (0.14, 5)	2.6 (0.12, 5)	2.4 (0.13, 5)
6.3	7.1 (0.12, 5)	5.4 (0.10, 5)	5.5 (0.35, 5)	5.1 (0.27, 5)
12.5	14 (0.62, 5)	11 (0.52, 5)	11 (0.30, 5)	10 (0.36, 5)
25	21 (0.46, 4)	23 (0.64, 4)	25 (4.0, 5)	21 (1.0, 4)
50	43 (0.71, 5)	46 (1.3, 5)	43 (2.2, 5)	44 (1.7, 5)
100	88 (1.5, 5)	90 (2.2, 5)	92 (1.7, 5)	86 (3.2, 5)
200	180 (3.0, 5)	190 (5.2, 5)	179 (13.0, 5)	170 (12.0, 5)
400	370 (5.7, 5)	370 (5.1, 5)	380 (6.0, 5)	370 (3.3, 5)

[a] REF = reference water that was used in the chamber as an alternative choice to the test water in the behavioral avoidance chamber.

[b] Mean concentration less than the detection limit.

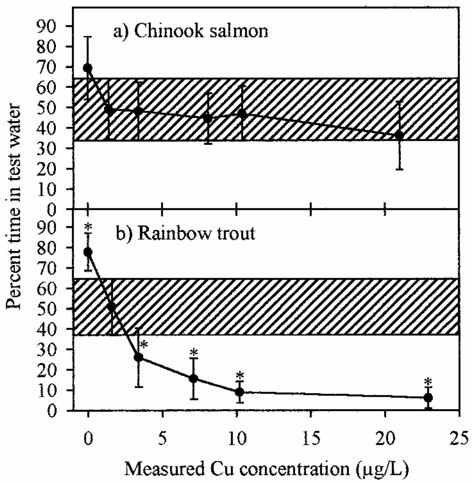

Fig. 3. Chinook salmon (**a**) and rainbow trout (**b**) avoidance of a 1:1 ratio mixture of copper and cobalt. Each vertical bar indicates the 95% confidence interval for the fish tested at that concentration. The shaded region indicates the 95% confidence interval for the control response. An asterisk indicates a response that differed significantly from the control response ($p < 0.05$), as determined by Dunnett's multiple comparison procedure.

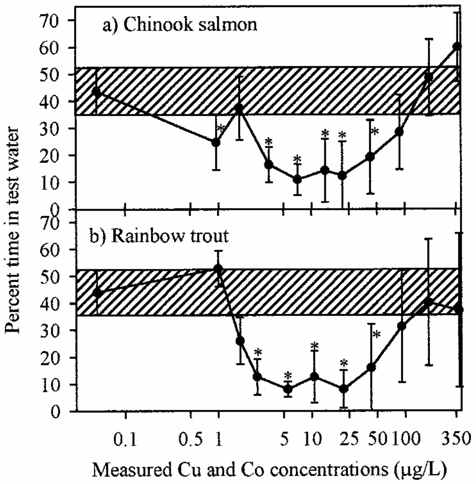

Fig. 4. Chinook salmon (**a**) and rainbow trout (**b**) avoidance of copper (Cu) following acclimation to a nominal 2 μg Cu/L for 25 to 30 d. Each vertical bar indicates the 95% confidence interval for the fish tested at that concentration. The shaded region indicates the 95% confidence interval for the control response. An asterisk indicates a response that differed significantly from the control response ($p <$ 0.05), as determined by Dunnett's multiple comparison procedure.

Between-sample variance of Cu concentrations was low (Table 4).

Once acclimated to Cu, CS did not avoid any of the Cu concentrations tested (3.4 to 21 μg/L) when the alternative to this higher Cu concentration was 1.6 μg Cu/L reference water (Fig. 4a). Moreover, these fish did not significantly prefer 0 μg/L over the reference water with 1.6 μg Cu/L, although a trend toward preference was noted.

Copper-acclimated RBT significantly preferred clean water and avoided all Cu concentrations higher than the control 1.6 μg/L (Fig. 4b). These avoidance responses to Cu were similar in intensity to those of non–Cu-acclimated RBT (Fig. 1b).

DISCUSSION

As with other Cu-avoidance studies, we have shown that salmonids avoid extremely low concentrations of Cu. In our study, RBT avoided a minimum of 1.6 μg Cu/L (Fig. 1b) and CS avoided a minimum of 0.7 μg Cu/L (Fig. 1a). From behavioral avoidance responses of fish exposed to a stepwise increase in Cu concentrations, Sprague [3] calculated the avoidance threshold concentration for Atlantic salmon to be 2.3 μg Cu/L. Similarly, Giattina et al. [4] calculated Cu-avoidance threshold concentrations for RBT to be between 4.4 and 7.3 μg Cu/L, depending on the chamber design and the exposure method.

Table 4. Means (SE, *n*) of analyzed Cu concentrations during the Cu-avoidance tests following 25- to 30-d acclimation to 2 μg Cu/L (nominal). Samples were analyzed from 50% of the avoidance tests performed

	Analyzed Cu concentrations (μg/L)	
Nominal Cu concentrations	Chinook salmon tests	Rainbow trout tests
Acclimation (2)	2.2 (0.40, 9)	1.5 (0.17, 9)
REF[a] (2)	1.6 (0.79, 5)	1.4 (0.10, 5)
Test waters		
0	0.0 (0.29, 5)[b]	0.0 (0.36, 5)[b]
2	1.4 (0.16, 5)	1.6 (0.29, 5)
4	3.4 (0.38, 5)	3.4 (0.10, 5)
8	8.1 (0.92, 5)	7.1 (0.33, 5)
12	10 (0.32, 5)	10 (0.25, 5)
24	21 (0.28, 5)	23 (0.44, 5)

[a] REF = reference water (2 μg Cu/L nominal) that was used in the chamber as an alternative choice to the test water in the behavioral avoidance chamber.

[b] Mean concentration less than the detection limit.

The lowest concentrations that were avoided in our study were lower than those previously reported, possibly because of our high replication at each concentration. Sprague [3] used a total of six Atlantic salmon in his Cu studies, and Giattina et al. [4] used between four and eight fish per test concentration, depending on the chamber design and the exposure method. For our Cu-avoidance experiments, we conducted 30 control exposure tests and either 10 or 20 replicate exposures for each Cu concentration. From Figure 1a and b, the results from more highly replicated concentrations (i.e., 20 replicates) are apparent because of their smaller confidence intervals. Responses from CS exposed to the nominal 0.8 to 3.1 μg Cu/L concentrations probably would not have been significant if we had conducted fewer than the 10 replicates we did complete. With lower replication at these concentrations, it is conceivable that the lowest concentration avoided by CS would have been 6.0 μg Cu/L. Similarly, the significant avoidance seen in RBT at 1.6 μg Cu/L was influenced by the completion of 20 replicate exposures at this concentration. Thus, in avoidance experiments, the calculation of a threshold is greatly influenced by the replication as well as by the exposure design, and comparisons of thresholds between studies is not always entirely appropriate.

The observation that CS did not significantly avoid 1.6 μg Cu/L is probably due to the higher variability and the weaker responses near the threshold (Fig. 1a). As in the responses to the 0.7 and 2.8 μg Cu/L, each individual fish was not avoiding the Cu as strongly as were fish in higher concentrations. Therefore, the nonsignificant response at 1.6 μg Cu/L is likely a consequence of lower replication in the experimental design, but this concentration still elicits a biologically important response. With this interpretation, the CS apparently are slightly more sensitive than the RBT, in that CS avoided the nominal 0.8 μg Cu/L (Fig. 1a) and RBT did not (Fig. 1b).

As Cu concentrations increased beyond acutely lethal concentrations, avoidance responses to Cu deteriorated until the fish failed to avoid. Chinook salmon failed to avoid 44 μg Cu/L and higher (Fig. 1a), and RBT failed to avoid 180 μg Cu/L and higher (Fig. 1b). In other studies in which responses to low Cu concentrations were not examined, RBT failed to avoid 500 μg Cu/L and higher [16], the 10-spined stickleback (*Pygosteus pungitius*) failed to avoid 32 mg Cu/L and higher [17], and green sunfish (*Lepomis cyanellus*) failed to avoid 20 to 100 mg Cu/L [18]. Using more reasonable concentrations, Giattina et al. [4] reported that RBT did not avoid or may even prefer 334 μg Cu/L and higher in shallow-gradient tests. In their study, the next lowest concentration, which was strongly avoided, was 191 μg Cu/L. Given the differences in study design, potential influences of metal-binding constituents in the water that may influence Cu speciation (e.g., dissolved organic carbon, carbonates, sulfates, chlorides, etc.), and potential differences in the RBT used in experiments, the results of their study and of ours are similar.

The Cu concentrations that fish fail to avoid may be related to physiological differences between fish species and may not be related only to the amount of aqueous Cu and/or specific Cu species in the water. In our study, using identical water-quality conditions, RBT failed to avoid 180 μg Cu/L and higher (Fig. 1b), whereas CS failed to avoid 44 μg Cu/L and higher (Fig. 1a). Moreover, the most intense avoidance response by RBT occurred at 46 μg Cu/L, whereas the 44 μg Cu/L concentration was not avoided by CS. Unlike the lowest concentrations that were avoided, the replication of exposures does not appear to have influenced the results. Twenty replicate exposures were completed for each of the Cu concentrations with which differences occurred between CS and RBT. Therefore, compared to RBT, CS were more likely to fail to avoid Cu concentrations above the 96-h LC50 reported by Chapman [9] for CS in soft water.

As with many other metals, Co has not been extensively studied either in relation to behavior or mortality. Our results indicate that, as with other metals, the avoidance thresholds for Co are lower than acutely lethal concentrations, but not by much. The avoidance thresholds for both CS and RBT were, at most, 180 μg Co/L (Fig. 2a and b). In a companion study using the same water quality in the same laboratory, Marr et al. [8] reported the 96-h LC50 for Co to be 1,406 μg/L and the ILL for Co to be 346 μg/L. This large discrepancy between these two lethality endpoints (LC50 and ILL) was due to the greatly delayed mortality associated with this metal [8].

Although both species avoided 180 μg Co/L and higher, CS demonstrated a bimodal response to Co by significantly avoiding 24 and 46 μg Co/L and by not avoiding 89 μg Co/L (Fig. 2a). Aside from this possible anomaly, CS are likely more sensitive in their ability to detect aqueous Co than are RBT. Although not convincing in Cu-only experiments (Fig. 1a), the bimodality that is apparent in Co-only (Fig. 2a), and Cu+Co mixtures (Fig. 3a) suggests that the avoidance response may be controlled by two different sensory mechanisms, each with a different sensitivity.

Avoidance experiments using mixtures of Cu and Co in a 1:1 ratio suggest that the fish were avoiding Cu and that Co had a minor influence. Although the RBT significantly avoided 88 μg Cu/L (Fig. 1b) but did not significantly avoid the mixture of 92 μg Cu/L + 86 μg Co/L (Fig. 3b), the difference between responses was not large and may be attributable to random chance coupled with the lower mean control responses in the Cu+Co experiments. Alternatively, CS avoided the mixture of 43 μg Cu/L + 46 μg Co/L (Fig. 3a) but did not avoid the comparable Cu-only exposure (Fig. 1a). The 95% confidence limits for this mixture ranged from 9 to 30% of the total time in the test water, whereas the Cu-only response ranged from 28 to 44% of the total time. Because CS avoided 46 μg Co/L, this difference in responses between the avoidance of the mixture and the nonavoidance of the comparable Cu concentration may stem from the added effect of Co in the mixture.

Perhaps the most dramatic difference between CS and RBT was seen in the effect of Cu acclimation on the avoidance of Cu. Low background Cu concentrations (<4 μg/L) are commonly observed in natural waterways, yet CS failed to avoid any higher Cu concentrations following an acclimation to a nominal 2 μg Cu/L (Fig. 4a). This response was so unexpected that we ran a second set of exposures (J.A. Hansen, unpublished data) that yielded the same results. If CS will not avoid any Cu concentrations following acclimation to low Cu concentrations, the behavioral defense against chronic and acute exposures to Cu is lost, and high mortality or chronic physiological effects are probable if subsequent Cu exposure occurs. Unlike CS, Cu-acclimated RBT preferred clean water and avoided higher Cu concentrations (Fig. 4b). Similarly, RBT avoidance responses were unaffected by acclimation to 12 μg Cu/L in a mixture with 50 μg Zn/L, 3.2 μg lead/L, and 1.1 μg Cd/L [6].

This study demonstrated dramatic differences in overall behavioral avoidance responses between CS and RBT, differences that could have ecological consequences for the distribution and survival of the two species. Chinook salmon appear to be much more susceptible to the physiological effects of Cu on the subsequent ability to detect and avoid Cu. In related studies using similar water-quality parameters and exposure concentrations, the Cu concentrations that each species failed to avoid also caused a significant loss of olfactory receptors in the olfactory rosette [19] and a significant loss of olfactory function, as measured by electroencephalogram activity in the olfactory bulb, in response to stimulation of the rosette with L-serine [19]. Similar pathological [20,21] and electrophysiological [22] effects have been observed following short-term exposure to Cu. However, the parallel effects between loss of avoidance, loss of olfactory receptors [19], and loss of olfactory function [19] suggest that the olfactory reception of Cu is the mechanism, or that it is at least as sensitive as other mechanisms, that controls the behavioral avoidance of Cu. Therefore, when CS are exposed to at least 50 μg Cu/L and when RBT are exposed to at least 200 μg Cu/L, these fish probably will fail to avoid the Cu-contaminated water and will thus have impaired olfaction for extended periods of time. If these impaired fish do not die from short-term exposure to these lethal concentrations, as might occur during exposure to a pulse of Cu-contaminated water or as a result of swimming through a mixing zone below a discharge containing Cu, many olfactory-dependent survival and reproductive behaviors will likely be affected. The behaviors that would be impaired or eliminated by the loss of olfaction include the imprinting behaviors by smolts on home streams, migration behaviors, spawning behaviors, feeding behaviors, predator avoidance behaviors, and contaminant avoidance behaviors [23].

These neurobehavioral effects, including the failure to avoid metals-contaminated waters and dysfunction of the olfactory system, may be a contributing cause of the demise of anadromous salmonids. Nihlsen et al. [24] concluded that 214 native stocks of anadromous trout and salmon from the northwest United States face a moderate to high risk of extinction, or are stocks of special concern. Furthermore, they estimate that at least 106 stocks are already extinct. Panther Creek, a mine-affected tributary of the Salmon River in USA, once supported 2,000 to 3,000 spawning CS adults and even more steelhead (i.e., anadromous RBT) [25]. Following a large-scale mining operation, Cu and Co concentrations in Panther Creek greatly increased. Average dissolved Cu concentrations range from 36 to 82 μg/L and have recently been observed to be as high as 620 μg/L. Cobalt concentrations were similar to Cu concentrations in roughly a 1:1 ratio [26]. Presently, no CS spawn in Panther Creek, whereas reduced numbers of steelhead continue to spawn in lower tributary sections [27]. Although hydroelectric dams, forestry practices, and overfishing are all important causes of declining anadromous salmon and trout populations in western North America [24], adult CS still return to spawn in tributaries above Panther Creek [25]. Extensive efforts to reestablish spawning runs of CS and RBT have been completely unsuccessful [25]. The neurobehavioral effects of Cu could greatly impair the success rate for seaward migration of smolts, imprinting of smolts on home-stream water and conspecifics, and spawning migrations of adult fish. Moreover, this study has shown that RBT are less vulnerable than CS to these neurobehavioral effects, and while steelhead (RBT) populations have been greatly reduced in Panther Creek, CS populations have been eliminated.

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