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CHINOOK SALMON (*ONCORHYNCHUS TSHAWYTSCHA*) AND RAINBOW TROUT (*ONCORHYNCHUS MYKISS*) EXPOSED TO COPPER: NEUROPHYSIOLOGICAL AND HISTOLOGICAL EFFECTS ON THE OLFACTORY SYSTEM

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Abstract—Olfactory epithelial structure and olfactory bulb neurophysiological responses were measured in chinook salmon and rainbow trout in response to 25 to 300 μg copper (Cu)/L. Using confocal laser scanning microscopy, the number of olfactory receptors was significantly reduced in chinook salmon exposed to ≥50 μg Cu/L and in rainbow trout exposed to ≥200 μg Cu/L for 1 h. The number of receptors was significantly reduced in both species following exposure to 25 μg Cu/L for 4 h. Transmission electron microscopy of olfactory epithelial tissue indicated that the loss of receptors was from cellular necrosis. Olfactory bulb electroencephalogram (EEG) responses to 10^{-3} M L-serine were initially reduced by all Cu concentrations but were virtually eliminated in chinook salmon exposed to ≥50 μg Cu/L and in rainbow trout exposed to ≥200 μg Cu/L within 1 h of exposure. Following Cu exposure, EEG response recovery rates were slower in fish exposed to higher Cu concentrations. The higher sensitivity of the chinook salmon olfactory system to Cu-induced histological damage and neurophysiological impairment parallels the relative species sensitivity observed in behavioral avoidance experiments. This difference in species sensitivity may reduce the survival and reproductive potential of chinook salmon compared with that of rainbow trout in Cu-contaminated waters.

Keywords—Copper Chinook salmon Rainbow trout Olfactory bulb electroencephalogram Olfactory histology

INTRODUCTION

Behavioral avoidance experiments have shown that fish avoid low copper (Cu) concentrations, but high concentrations are not avoided [1,2]. Failure to avoid a toxic concentration of a metal, particularly when low concentrations are avoided, suggests that some chemosensory system (e.g., olfaction, taste, etc.) that mediates the detection and avoidance of the metal is not functioning properly at higher metal exposure concentrations. Dysfunction of sensory systems is of special concern, because many behaviors are mediated by chemoreception [3,4], and many anthropogenic sources currently produce high Cu concentrations in natural waterways. Some of these sources of elevated Cu concentrations include precipitation runoff over and through mine tailings as well as municipal and industrial discharges [5].

Although olfaction has not been proven to mediate Cu avoidance in fish, Cu is known to impair olfactory system function and to damage olfactory epithelial structure. Olfactory system impairment has been shown by measuring the olfactory receptor potential (i.e., electro-olfactogram, or EOG) [6,7] and the olfactory bulb electroencephalogram (EEG) [8]. Both of these measures have demonstrated that neurophysiological responses to olfactory stimulants are reduced by Cu exposure, and the magnitude of impairment was dependent on exposure concentration and duration. Previous light microscopy studies of Cu-exposed fish have described histological changes in olfactory epithelial tissues without definition of damage to specific cell types or to fine cellular structure [9,10]. Transmission electron microscopy of Cu-exposed olfactory tissue has shown that olfactory receptors are eliminated by cellular necrosis [11], or they degenerate by apoptosis [12], depending on exposure duration, concentration, and possibly on other water-quality parameters. Although studies on Cu have shown a loss of behavioral avoidance [1,2], neurophysiological impairment [6–8], and disruption of receptors [11,12], to date no multidisciplinary studies have investigated how these three indicators of injury interrelate, especially under identical Cu-exposure and water-quality conditions.

In the research presented here, we investigated the effects of Cu on the olfactory epithelial structure and the electrophysiological function of the olfactory system in two representative salmonids: chinook salmon (CS, *Oncorhynchus tshawytscha*) and rainbow trout (RBT, *O. mykiss*). The objectives of this research were to determine if structural damage and functional impairment of the olfactory system was a probable cause of the loss of behavioral avoidance of Cu in each species [2] and to determine if the differences in sensitivity to Cu between CS and RBT that were observed in the companion (behavioral avoidance) study [2] were due to differential susceptibility to olfactory damage. A unique feature of these studies was the use of matched Cu concentrations and water-quality conditions across studies in order to permit the strongest possible causal inferences to be made between Cu effects on the olfactory system and Cu effects on behavioral avoidance. In addition, we report a new technique to quantify olfactory receptors and other cells in the olfactory epithelium.

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In order to investigate the effect of Cu on olfactory epithelial structure, we used a confocal laser scanning microscope (CLSM) to quantify the numbers of olfactory receptors on the surface of the rosette of both species following 1- and 4-h exposures to several Cu concentrations. We used transmission electron microscopy (TEM) to augment our observations from CLSM. In order to test the hypothesis that Cu causes olfactory dysfunction, we used a standard stimulus, L-serine, to elicit an olfactory EEG response, and we evaluated the effect of Cu exposure on that response. In order to further augment the functional inferences to be drawn between histological and electrophysiological components of the study, tissues for 1-h CLSM analysis were from the same fish used in electrophysiology measurements. The results indicated that Cu exposure reduced the number of olfactory receptors and disrupted the olfactory bulb response in a dose-dependent manner that closely paralleled the species-typical effects of Cu on behavioral avoidance [2] of the metal.

MATERIALS AND METHODS

Experimental fish

Chinook salmon were obtained as juveniles from McNenny State Fish Hatchery in Spearfish, South Dakota, USA, and were cultured to between 170 and 275 mm in length prior to use in experiments. Rainbow trout were obtained as eggs from Dubois State Fish Hatchery in Dubois, Wyoming, USA, and were cultured to between 197 and 280 mm in length before use. Both species were certified as disease-free prior to entering the laboratory, and no obvious signs of disease, injury, or distress were observed prior to or during experiments. Fish culture, Cu exposures, neurophysiological measurements, and tissue fixation were conducted at the Red Buttes Environmental Biology Laboratory at the University of Wyoming (Laramie, WY, USA). For histological investigations, the remainder of tissue preparation and imaging was completed in the microscopy laboratory of the Department of Zoology and Physiology at the University of Wyoming (Laramie, WY, USA).

Experimental water

All acclimation (14-d minimum) and experimental water was produced by mixing well water with deionized water to a nominal 25 mg/L $CaCO_3$ hardness, a pH of 7.5, a temperature of 12°C, and, except where noted, to a concentration of <0.7 μg Cu/L. The pH of all solutions was adjusted with dilute H_2SO_4 or KOH.

The same fish were used in electrophysiological measurements and in CLSM analysis from 1-h Cu exposures, so experimental and acclimation water were the same in these two phases of research. This experimental water was used for pretest acclimation, during and after the surgical preparation, and as rinse water over the olfactory rosette prior to removing tissues for CLSM analysis. Stimulus water contained 10^{-3} M L-serine in water the same as is described above. Copper-exposure water was produced with appropriate additions of $CuCl_2 \cdot 6H_2O$ to the water described above so that we could achieve nominal concentrations (treatments) of 0, 25, 50, 100, 200, and 300 μg Cu/L. These solutions were delivered to the olfactory rosette at constant temperature, flow rate, and pH. During Cu exposures, the L-serine stimulus solution did not contain any Cu.

Fish used for CLSM and TEM analysis after 4-h exposures were exposed to Cu in 10-L aquaria using the water-quality parameters described above. A concentrated stock solution of Cu was metered at 2.5 ± 0.05 ml/min into the 1 L/min aquaria water inflow using a metering pump (Fluid Metering QG-20, Syosset, NY, USA). Nominal Cu concentrations for CLSM studies of CS and RBT were 0, 25, 50, 100, and 200 μg Cu/L. In addition, RBT were also exposed to 300 μg Cu/L. For TEM studies with CS, nominal concentrations were 0 and 50 μg Cu/L.

Chemical analysis of water

Water used during acclimation and Cu exposures was analyzed for hardness by EDTA titration. Temperature and pH were measured using a standardized pH meter with a temperature compensation electrode. A 25-ml sample was collected from each exposure and was acidified with 25 μl analytical grade HNO_3 for Cu analysis by graphite furnace atomic absorption spectrophotometry (method detection limit was 0.7 μg Cu/L).

Electrophysiology test procedure

Five fish of each species were used to investigate responses to each treatment concentration. For each exposure, a single fish was anesthetized with 100 mg/L MS-222 (tricaine methane sulfonate) and was placed in a plastic trough that contained adjustable lateral supports and provided constant recirculation of chilled anesthetic over the gills. While the fish was under anesthesia, a partial craniotomy was performed in order to expose the olfactory bulb, anterior telencephalon, and posterior olfactory nerve. The septum and skin around the left-side naris was removed in order to expose the olfactory rosette. A Teflon®, stimulus-delivery tube was placed directly over the rosette to deliver rinse water, stimulus, and one treatment (control or Cu-concentration) of water, all at constant temperature and flow. Constant temperature and flow rate of the stimulus and other fluids was achieved by running the stimulus and other solutions through polypropylene tubing inside a well water–chilled PVC water jacket and then into a delivery funnel that had an overflow.

A pair of 125-μm diameter, Teflon-coated, stainless-steel wire electrodes was placed on the olfactory bulb in a standard position that yielded maximum response amplitude to L-serine for each fish species. The exact location of the recording electrode was determined empirically for each fish used. The maximum response was obtained in RBT by placement of one electrode on the anterolateral surface and of the other electrode on the medial surface of the olfactory bulb. The maximum response in CS was obtained from placement of one electrode on the posteriolateral surface and of the other on the posteriomedial surface of the olfactory bulb. Signals from the electrode pair were differentially amplified (1 to 100-Hz band pass) with a Grass P15 AC preamplifier (Grass Instrument, Quincy, MA, USA). This preamplified signal was displayed with a Grass model 7 chart recorder (Grass Instrument) and a Tektronix TDS 420 digital oscilloscope (Beaverton, OR, USA). The olfactory bulb activity was simultaneously displayed in two forms: as a raw EEG signal and also after half-wave rectification and integration with a time constant of 10 s.

Following preparation for recording, the fish was injected with Flaxedil (gallamine triethiodide 1 mg/100 g body weight) in order to induce paralysis by myoneural blockade, and the anesthetic flowing over the gills was replaced by clean water. A recovery period of 60 to 90 min was allowed, during which olfactory bulb EEG responses to L-serine (10^{-3} M) became

reliably elicitable and stable. The amino acid (L-serine) was used as a test stimulus because it is a well-studied olfactory stimulus of known functional significance [3]. Although bile salts, steroids, and prostaglandins are potentially useful stimuli, these chemicals have great potential for having a priori species differences in effectiveness that would have confounded our species comparisons [3].

Following the recovery period, the L-serine solution was delivered to the olfactory rosette for 15 s every 5 min, and responses were recorded with a digital oscilloscope, as explained below. Each fish was constantly exposed to stimuli at this rate for 3 h, with the first hour constituting a control period during which clean water rinsed the rosette between each L-serine stimulus. The second hour was the exposure period, where Cu-containing water at the desired concentration replaced the clean-water rinse between each L-serine stimulus. Finally, in the third hour, responses were recorded while clean water again rinsed the rosette between each L-serine stimulus.

Confocal laser scanning microscopy

For 1-h Cu exposures, the olfactory rosettes were collected from fish following olfactory bulb EEG recordings described above, where the fish were exposed to Cu for 1 h followed by 1 h of clean water flow over the rosette. For 4-h exposures, the fish were placed into an exposure aquarium containing the desired Cu concentration for 4 h and were then moved to another aquarium containing clean water for 16 h prior to tissue collection. The 16-h recovery period allowed more time for the pathological effects of the 4-h Cu exposure to take effect.

Immediately following each Cu-exposure and recovery period, each fish was anesthetized with 100 mg/L MS-222, and the olfactory rosette was flooded with phosphate-buffered fixative containing 4% formaldehyde, 0.1% glutaraldehyde, and 0.1% Triton X-100 (Sigma Chemical, St. Louis, MO, USA). The olfactory rosette was quickly removed and placed into the same fixative for 1 h. Several lamella were separated from each olfactory rosette. With intermediate rinses in phosphate-buffered saline, the tissue was labeled with rabbit anti-keyhole limpet hemocyanin (anti-KLH; Sigma Chemical) primary antibody and secondarily labeled with Fluorescein (FITC)-conjugated goat anti-rabbit antibody (Pierce, Rockford, IL, USA) mixed with Rhodamine (TRITC)-conjugated phalloidin (Sigma Chemical). The anti-KLH is a novel marker of salmonid olfactory receptors [13], and the phalloidin binds to actin in cells. In our study, the actin associated with adhesion belts just under cellular tight junctions [14] was labeled with the phalloidin to delineate cell borders. Each lamella was then mounted in a well-slide with Vectashield (fluorescence antifade mounting medium; Vector Laboratories Burlingame, CA, USA) and was stored at 4°C in the dark until analyzed. Microscopic analysis was completed within 8 h of mounting the tissue.

Topographical images of three 100-by-100–μm epithelial areas were collected from each of five fish exposed to each Cu concentration and from five control fish using a Leica TCS-4D CLSM. The selection of the location for each image was pseudo-random because of the topography of the epithelial surface. In salmonids, each olfactory lamella contains numerous folds, where the ridges are largely nonsensory, and the valleys contain the greatest density of receptors [15]. The peripheral one-half of the valleys was flatter and more conducive to obtaining topographical images of the epithelial surface. Toward the central and inner regions of each lamella, the valleys became steeper, and the topographical epithelial area of each image became much more variable. The different focal points required to view the epithelial surface of the valleys also limited image collection to certain orientations. Therefore, images were collected from the peripheral one-half of each lamella within the valley regions.

Transmission electron microscopy

The CS used in TEM studies were first acclimated to the soft water and were then placed in an exposure aquarium containing either clean soft water (controls) or soft water containing 50 μg Cu/L for 4 h. Because we wanted to observe the immediate cellular pathology resulting from Cu exposure, no recovery time was allowed. Immediately following the Cu exposure, each fish was pithed using a sharp probe, and the olfactory rosette was fixed in situ with 0.09 M cacodylate-buffered glutaraldehyde (2.5%). The olfactory rosette was quickly removed and placed into the primary fixative for one additional hour and was then rinsed in 0.09 M cacodylate buffer. The tissue was postfixed in 0.1 M cacodylate-buffered osmium tetroxide (1%), dehydrated in a graded ethanol series, and embedded in Araldite 502 resin (Ted Pella, Redding, CA, USA), with propylene oxide as the transition solvent. Thin sections were mounted on 200-mesh, formvar-coated grids and were stained with 2% uranyl acetate and 0.25% lead citrate. Olfactory epithelial tissue from three control fish and three fish exposed to 50 μg Cu/L were examined using either an RCA (Chicago, IL, USA) EMU-4 electron microscope or a Hitachi (Tokyo, Japan) H-7000 electron microscope.

Electrophysiology data analysis and statistics

Response parameters were derived from calculations performed by the digital oscilloscope. These included peak-to-peak amplitude of the oscillatory olfactory bulb EEG response, the root mean square (RMS) voltage of the oscillatory olfactory bulb EEG response, peak height of the integrated EEG signal, and area under the integrated signal. Because these measures proved to be highly correlated, only the RMS response is presented here. Exposure of the rosette to Cu greatly increased the amplitude of EEG activity in the olfactory bulb, so responses for each fish were standardized by subtracting the RMS measure of spontaneous EEG activity (during a 50-s sample of EEG activity 2 min after a stimulus) from each RMS measure of L-serine–evoked EEG response. The responses of treatment fish were standardized by dividing individual responses by the mean control response.

Fish olfactory responses were analyzed separately during the Cu-exposure and post–Cu recovery periods, as these periods represented distinct phenomena. During the Cu-exposure period, there was an initial (relatively immediate) drop in RMS followed by a gradual decline in RMS. Simple linear regression was used as a measurement tool to condense the individual data points for each fish into a slope and intercept term. The intercept from that regression was our choice for a measure of the initial drop in RMS for each fish, and the slope measured the subsequent rate of decline in RMS for each fish. These measurements were then each subjected to a formal regression analysis, using Cu concentration, species, and their interaction as predictor variables.

This regression approach was used to formally analyze the RMS measure of EEG responses from the Cu-exposure period and the post–Cu recovery period as well as the RMS measure of spontaneous EEG activity during these periods. With the

Table 1. Mean (SE, *n*) of analyzed copper (Cu) concentrations from electrophysiological and histological experiments. Samples were analyzed with graphite furnace atomic absorption spectrophotometry

Nominal Cu concentration (μg/L)	Analyzed Cu concentration (μg/L)	
	Rainbow trout	Chinook salmon
0	<0.7 (7)	<0.7 (8)
25	27.8 (0.79, 7)	26.2 (0.77, 9)
50	49.6 (0.66, 7)	50.0 (1.1, 9)
100	99.3 (1.8, 7)	100 (l.6, 8)
200	199 (2.4, 9)	200 (2.4, 9)
300	315 (7.6, 5)	Not tested

exception of the RMS measurements of EEG responses for the Cu-exposure period, this analysis incorporated the 12 data points from each fish that were collected from 5 to 60 min of each period. Because the RMS measurements of spontaneous EEG activity increased so abruptly following Cu exposure, the first three RMS measurements for each fish were eliminated from the analysis of L-serine–evoked EEG responses during the Cu-exposure period. Therefore, only nine data points from each fish were used in the analysis of responses from this period.

Histology data analysis and statistics

Three classes of receptors were apparent in the tissues from both species: the microvillar receptors with 1-to-2–μm diameter apical knobs; the ciliated receptors (type I) with dendrites having 1-to-2–μm diameter apical knobs [16,17]; and a type II ciliated receptor having a 3.5-to-5–μm flat dendrite apex containing both cilia and microvilli [13,16,17]. The smaller classes of receptors could not be distinguished from one another, so these 1-to-2–μm diameter receptors were lumped together for quantification. The larger type II ciliated receptors, which have been a subject of debate (as to their identity as receptors) [11,16,18], were counted separately. For each Cu and control exposure, three CLSM images were collected and analyzed from five fish of each species. The numbers of receptors in the three images from each fish were averaged. Analysis of variance and Tukey's pairwise comparisons ($\alpha = 0.05$) were used to test for significant differences in the number of receptors between Cu and control treatments. The number of goblet cells were also counted from each fish. Differences between species at each concentration were determined by *t* tests ($\alpha = 0.05$).

RESULTS

Chemical analysis of exposure water

Mean analyzed water-quality parameters for pretest acclimation on both species were (SE, *n*) 23.6 (0.28, 38) mg/L $CaCO_3$ hardness, pH 7.30 (0.03, 38), and 12.2°C (0.03°C, 38). Mean analyzed water-quality parameters for experiments on both species were 24.5 (0.12, 57) mg/L $CaCO_3$ hardness and 12.3°C (0.03°C, 57). Acclimation pH, rinse pH, stimulus pH, and Cu-contaminated water pH were 7.64 (0.02, 55), 7.72 (0.02, 55), 7.70 (0.01, 55), and 7.67 (0.02, 55), respectively. Acclimation and control exposure water contained less than 0.7 μg Cu/L (method detection limit) as measured by GFAAS (Table 1). With the exception of the RBT 25 μg Cu/L exposures, all mean Cu-exposure concentrations were within 5% of nominal values. This exception was within 15% of nominal with low variance.

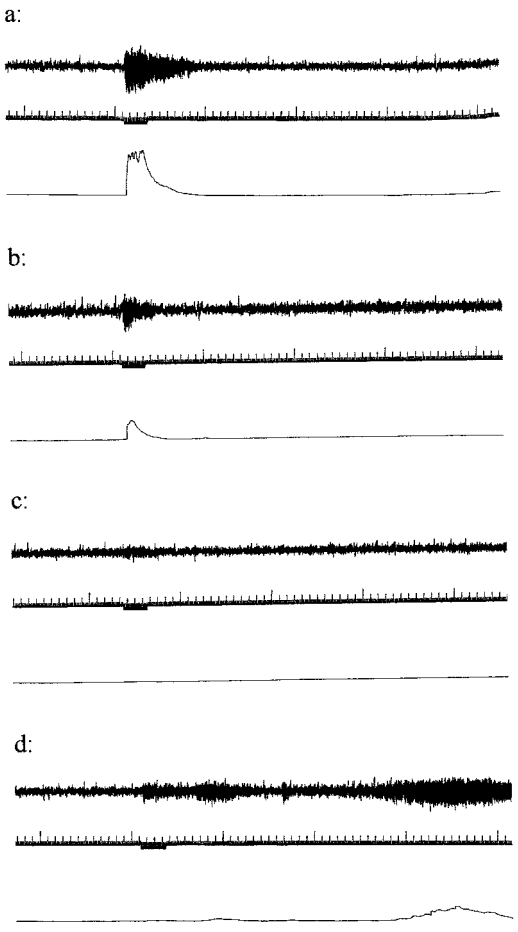

Fig. 1. Polygraph traces of typical olfactory bulb electroencephalogram (EEG) activity (top trace in each panel) and integrated signals of the EEG responses (bottom trace in each panel) from a rainbow trout exposed to 200 μg copper (Cu)/L. The center trace in each panel indicates the time in seconds, and the downward deflection in this center trace indicates the 15 s of 10^{-3} M L-serine stimulus (**a–c**) or the initiation of Cu exposure (**d**). Responses were collected before Cu exposure (**a**), following 30 min of exposure to 200 μg Cu/L (**b**), following 60 min of exposure to 200 μg Cu/L (**c**), and when the 200 μg Cu/L exposure was started (**d**).

Electrophysiology

Prior to Cu exposure, L-serine application caused an EEG amplitude increase that lasted until the clean water rinse (Fig. 1a). Following Cu application, both the amplitude and duration of L-serine–evoked EEG responses were reduced (Fig. 1b). The highest Cu concentrations caused complete or nearly complete elimination of EEG responses (Fig. 1c). The Cu application itself caused spontaneous EEG amplitude to increase (Fig. 1d), and in most fish, this amplitude remained elevated after clean water replaced Cu-contaminated water.

Throughout the pre-Cu control period, all RBT (Fig. 2a) and CS (Fig. 2b) responses were quite stable. The responses from RBT exposed to 25, 50, and 100 μg Cu/L immediately decreased to between 50 and 65% of control and remained at this level of depression throughout the 60-min Cu-exposure period (Fig. 2a). Responses from these fish began to recover during the subsequent 60-min post-Cu period. The decline of EEG responses from RBT exposed to 200 and 300 μg Cu/L were initially more dramatic than were responses to lower Cu concentrations, and they continued to decline until the response was virtually eliminated by the end of the Cu-exposure period.

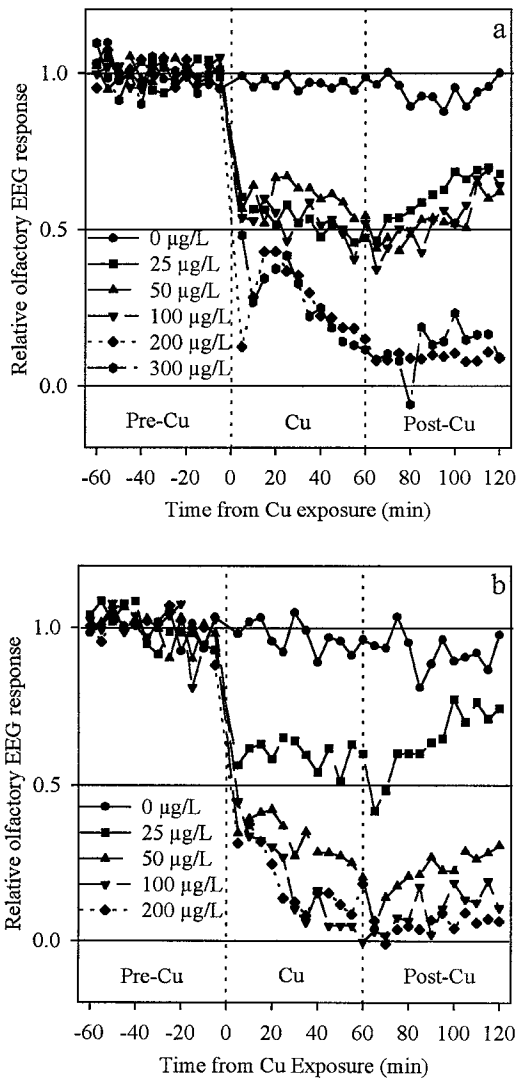

Fig. 2. Olfactory bulb electroencephalogram (EEG) responses to 10^{-3} M L-serine by rainbow trout (**a**) and chinook salmon (**b**). Each point is the mean from five fish exposed to each copper (Cu) concentration. Responses were standardized by subtracting the root mean square (RMS) measure of the spontaneous EEG activity from the RMS measure of the L-serine–evoked EEG response and then by dividing by mean control–period standardized RMS measure of the EEG response.

Furthermore, these fish showed a much slower recovery when no Cu was present. The EEG responses from CS exposed to 25 µg Cu/L were reduced by approximately 50%, with no further depressions in the response (Fig. 2b). After exposure to all higher Cu concentrations, responses to L-serine by CS were initially reduced by 55 to 70% and continued to decline until they were indistinguishable from the spontaneous EEG activity. Only after exposure to the 25 and 50 µg Cu/L concentrations did CS EEG responses show substantial recovery following the removal of the Cu solution.

Regression analyses showed that the differences in species sensitivity to Cu stem from the initial affect of Cu on the EEG response to L-serine. The initial decline of EEG responses was significantly different between the two species, in that increasing Cu concentrations had more of an effect on CS than on RBT (Table 2). Following the initial decline of EEG responses, the rate of response decline was statistically similar for both species, but the rate of decline significantly increased with increasing Cu concentrations. During the post–Cu recovery period, the rate of EEG response recovery was statistically similar between the two species, but higher Cu concentrations caused significantly lower recovery rates. At the end of the recovery period, EEG responses in CS had not recovered as much as had responses by RBT, and each Cu concentration had depressed EEG responses more in CS than in RBT (Table 2).

The spontaneous EEG activity in the olfactory bulb of both species substantially increased following the application of Cu to the olfactory rosette (Fig. 1d). For RBT, all Cu concentrations (≥25 µg/L) caused the spontaneous EEG activity, measured by RMS, to increase by 1.5 to three times and to remain elevated throughout the recovery period (Fig. 3a). The spontaneous EEG activity in CS also increased from exposure to all Cu concentrations and remained elevated throughout the recovery period, but not to the same degree as in RBT (Fig. 3b).

Regression analysis of the spontaneous EEG activity revealed significant differences between species and Cu-exposure concentrations. The initial increase in spontaneous EEG activity during Cu exposure was significantly greater in RBT than in CS, but it was not Cu-concentration dependent (Table 3). Following the initial increase (Fig. 1d), the amplitude of spontaneous EEG activity declined much faster in RBT than in CS, and the EEG activity from fish exposed to higher Cu concentrations declined more quickly than did the activity of

Table 2. Regression analysis of the standardized root mean square measurements of electroencephalogram responses to L-serine by rainbow trout and chinook salmon. These responses to L-serine during the copper (Cu)-exposure and post–Cu recovery periods were analyzed by simple regression to condense these individual observations to a slope and intercept term for each fish. These measurements were then subjected to a formal regression analysis (results presented below), using Cu concentration, species, and their interaction as predictor variables. In the equations presented below, the term (Cu) is the Cu concentration in µg/L

	Chinook salmon		Rainbow trout	
Exposure period parameter	Equation	*p* value	Equation	*p* value
Cu exposure period				
Initial decline	0.783 − 0.00334(Cu)	0.003[a]	0.763 − 0.000932 (Cu)	0.003[a]
Rate of decline (×10³)	−1.33 − 0.0193 (Cu)	0.001[b]	−1.33 − 0.0193 (Cu)	0.001[b]
Post–Cu recovery period				
Rate of recovery (×10³)	4.65 − 0.0154 (Cu)	<0.001[b]	4.65 − 0.0154 (Cu)	<0.001[b]
Recovery at 1 h	0.756 − 0.00406 (Cu)	0.035[a]	0.828 − 0.00261 (Cu)	0.035[a]

[a] *p* value is for interaction term; its significance dictates different regression equations for each species.
[b] Significance of the common slope; no significant interaction and no difference in level (i.e., intercept) between species.

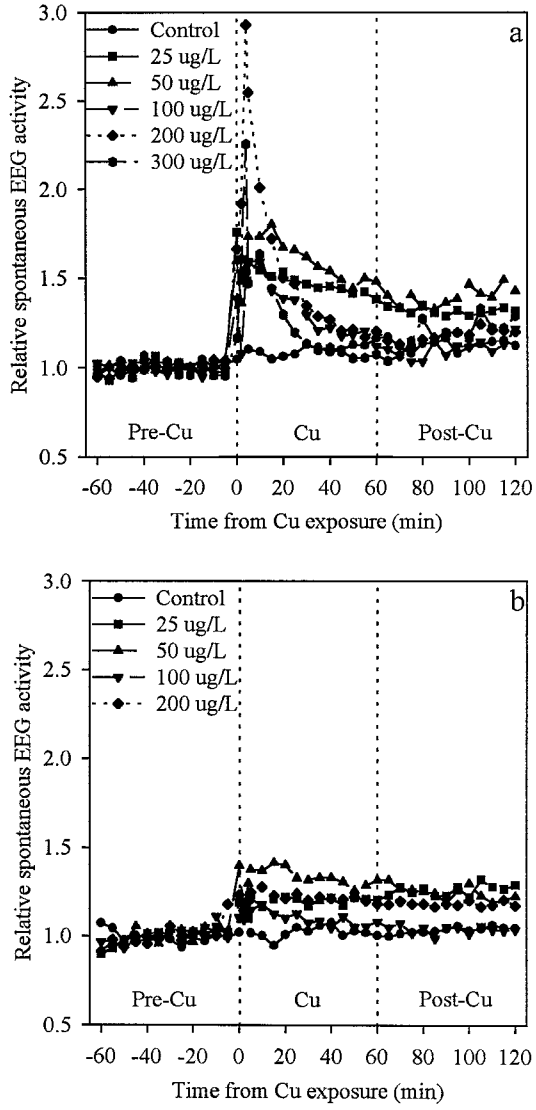

Fig. 3. Spontaneous electroencephalogram (EEG) activity from rainbow trout (**a**) and chinook salmon (**b**). Each point is the mean response from five fish collected 2 min after each L-serine stimulus. Responses were standardized by dividing the root mean square (RMS) measure of the spontaneous EEG activity by the mean control–period RMS measure of the spontaneous EEG activity.

fish exposed to lower Cu concentrations (Table 3). During the post–Cu recovery period, the amplitude of spontaneous EEG activity increased similarly between the two species, and higher Cu concentrations caused a higher rate of activity increase (Table 3).

Confocal laser scanning microscopy

The small-dendrite receptors (i.e., microvillar and type I ciliated) were much less intensely labeled with anti-KLH than were the larger type II ciliated receptors. The phalloidin intensely labeled the dense actin of the adhesion belts just under the cellular tight junctions, thus effectively outlining the borders of each cell at the epithelial surface. The small-dendrite receptors (1-to-2–μm diameter dendrites) could, therefore, be more reliably identified by their phalloidin-labeled cell borders using criteria similar to those of Miragall et al. [19]. The anti-KLH labeling colocalized within the receptors identified by using phalloidin, thus confirming the phalloidin technique. Only some emerging goblet cells had a size and shape similar to the phalloidin-identified small-dendrite receptors, but these goblet cells were distinguished by much less intensely labeled adhesion belts, and the interior of these cells appeared to have far fewer actin filaments, as seen in the almost complete absence of fluorescently labeled phalloidin inside the cytoplasm of the apex of these cells. The type II cells were easily identified with the phalloidin labeling, because the entire apex of the dendrite was intensely labeled, possibly because of the labeling of the actin in the short microvilli between the cilia on these cells. Both types of receptors were counted only from phalloidin-labeled images after the reliability of counting receptors from this technique was verified against the anti-KLH labeling.

Control tissues contained numerous small-dendrite receptors (Fig. 4a), whereas tissues from fish exposed to higher Cu concentrations had far fewer receptors (Fig. 4b). The control CS averaged 335 small-dendrite receptors per 10,000 μm^2 image area, but exposure to 50 μg Cu/L and greater significantly reduced the number of these receptors (Fig. 5a). Rainbow trout controls had a similar number of small-dendrite receptors when compared with those observed in CS, but the number of small-dendrite receptors was reduced only following exposure to 200 μg Cu/L and higher (Fig. 5a).

After a 4-h exposure to all Cu concentrations, including the lowest concentration tested (25 μg Cu/L), the numbers of small-dendrite receptors were significantly reduced in both

Table 3. Regression analysis of the standardized root mean square measurements of spontaneous electroencephalogram (EEG) activity by rainbow trout and chinook salmon. This spontaneous EEG activity during the copper (CU)-exposure and post—Cu recovery periods were analyzed by simple regression to condense these individual observations to a slope and intercept term for each fish. These measurements were then subjected to a formal regression analysis (results presented below), using Cu concentration, species, and their interaction as predictor variables. In the equations presented below, the term (Cu) is the Cu concentration in μg/L

	Chinook salmon		Rainbow trout	
Exposure period parameter	Equation	*p* value	Equation	*p* value
Cu exposure period				
Initial increase	1.20	0.001[a]	1.61	0.001[a]
Rate of decline ($\times 10^3$)	−0.775 − 0.000970 (Cu)	0.032[b]	−2.96 − 0.00421 (Cu)	0.032[b]
Post–Cu recovery period				
Rate of increase	−0.0733 + 0.00860 (Cu)	0.037[c]	−0.0733 + 0.00860 (Cu)	0.037[c]

[a] Significance of species; no significant interaction and no difference in rate of change (i.e., slope) between Cu concentrations.
[b] *p* value is for interaction term; its significance dictates different regression equations for each species.
[c] Significance of the common slope; no significant interaction and no difference in level (i.e., intercept) between species.

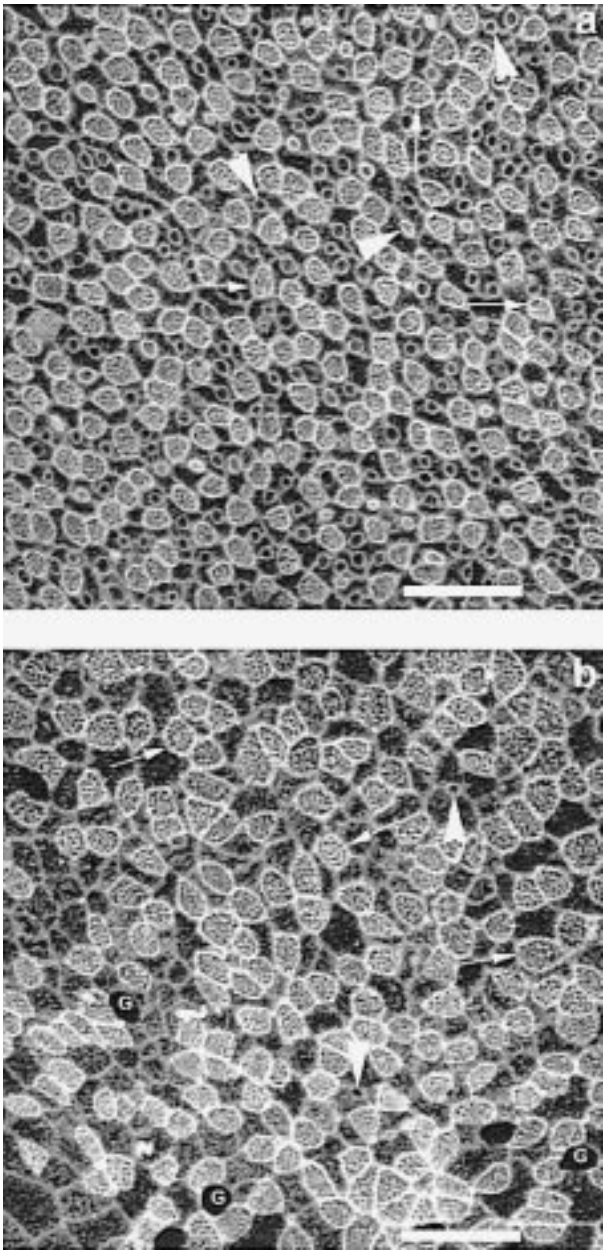

Fig. 4. Confocal laser scanning microscope images of control (**a**) and 100 μg copper (Cu)-L exposed (**b**) olfactory epithelial tissue from a chinook salmon. Copper exposure times were 1 h followed by 1 h in clean water. The tissue was labeled with Rhodamine (TRITC)-conjugated phalloidin that was bound to the actin of the adhesion belts of cells. These adhesion belts lie just beneath the tight junctions of epithelial cells. The wide arrowheads indicate either ciliated type I or microvillar receptors (i.e., small-dendrite receptors). The arrows represent ciliated type II receptors. G indicates a goblet cell. Bar indicates 20 μm.

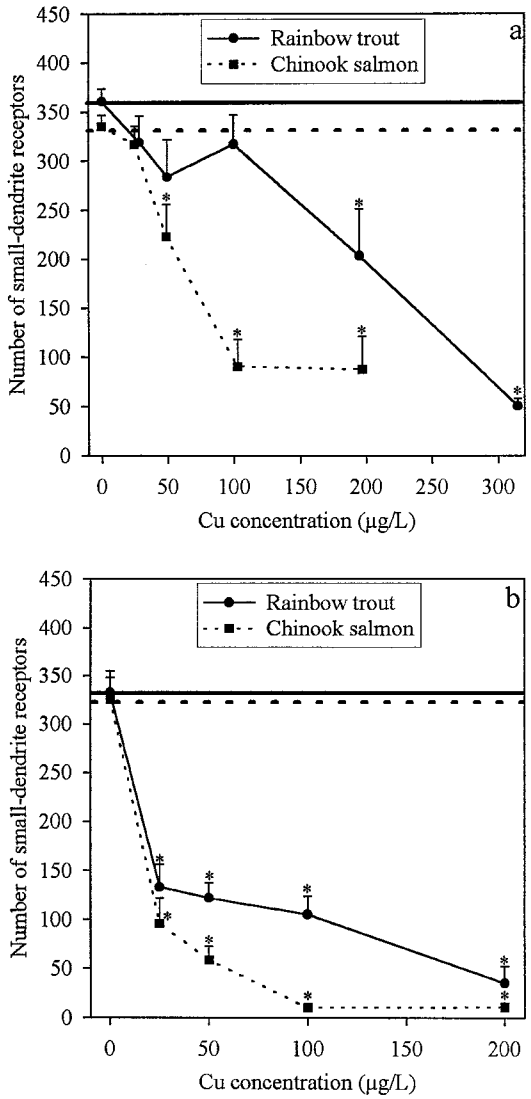

Fig. 5. Mean number of small-dendrite receptors (sum of ciliated type I and microvillar) counted from lamellae of chinook salmon and rainbow trout olfactory rosettes after 1-h (**a**) and 4-h (**b**) exposures to copper (Cu). Three 100-by-100–μm confocal laser scanning microscope images from each of five fish were counted for each Cu concentration. Horizontal lines indicate the mean number of receptors from control fish. Vertical bars indicate the SE. Asterisks indicate significant differences from control numbers using analysis of variance ($\alpha = 0.05$).

species (Fig. 5b). However, CS had fewer small-dendrite receptors at each Cu concentration than did RBT. In both species, the number of these receptors in control exposures was similar to that seen in 1-h–exposed control fish (Fig. 5a).

The numbers of type II ciliated receptors were unaffected by exposure for 4 h to any of the Cu concentrations tested (Fig. 6). The rosette of both species contained approximately 175 type II ciliated receptors per 10,000 μm^2. Receptor numbers in fish exposed to Cu for 1 h were essentially the same as those seen in the 4-h exposed fish.

The number of goblet cells was also counted from fish exposed to Cu for 1 h. In all treatments, including controls, RBT olfactory tissue contained more discharged goblet cells than did CS olfactory tissue, and at the control Cu concentration and at 50 μg Cu/L, RBT had significantly more goblet cells than did CS (Fig. 7).

Transmission electron microscopy

Transmission electron microscopy was used to investigate the cellular pathology associated with the receptor loss that was documented by CLSM. The structure of healthy olfactory tissue has been described elsewhere [11,16], and our control olfactory tissue was similar to this description (Fig. 8a). As in these previous studies, the olfactory epithelium from our control fish contained numerous microvillar and ciliated type I receptors with the classic 1-to-2–μm dendritic knobs (Fig.

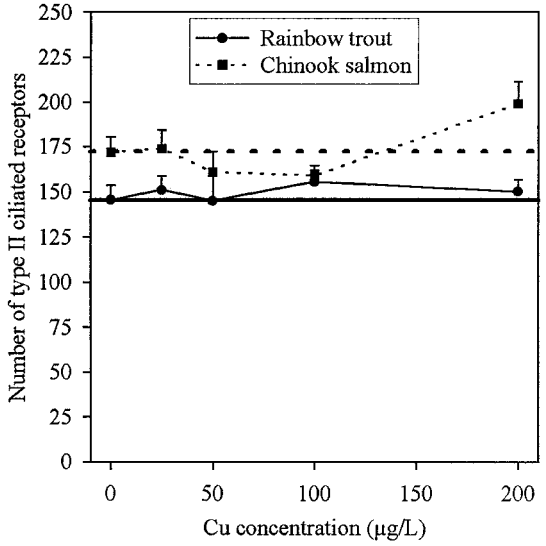

Fig. 6. Mean number of ciliated type II receptors counted from lamellae of chinook salmon and rainbow trout olfactory rosettes after a 4-h exposure to copper (Cu). Three 100-by-100–μm confocal laser scanning microscope images from each of five fish were counted for each Cu concentration. Horizontal lines indicate the mean number of receptors from control fish. Vertical bars indicate the SE. Asterisks indicate significant differences from control numbers using analysis of variance ($\alpha = 0.05$).

8a). Although not quantified using TEM, very few of these small-dendrite receptors could be found in tissues from CS exposed to 50 μg Cu/L. We examined numerous thin sections from multiple salmon and observed cellular damage in all of the identified receptors from CS exposed to 50 μg Cu/L for 4 h. Frequently, these receptors had ruptured plasma membranes on the exposed dendrite, swollen mitochondria, and no cilia or microvilli projecting from the dendrite (Fig. 8b). Some receptors showed structural features that suggested that cilia had been resorbed into the receptor (Fig. 8c). Copper-induced damage was evident only in ciliated type I and microvillar receptors. The ciliated type II receptors and other cells in the epithelium were not visibly damaged by the Cu exposure.

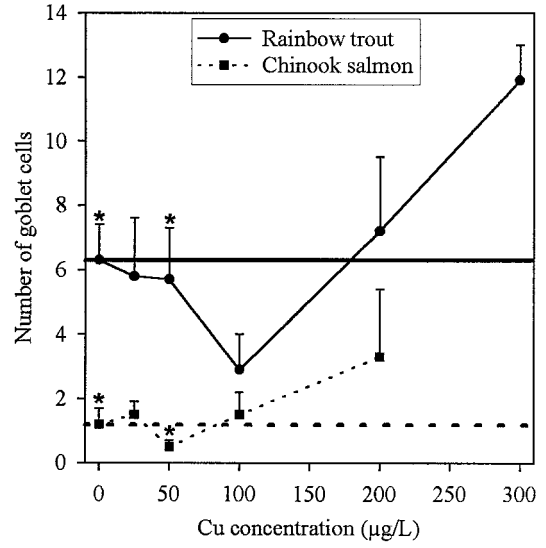

Fig. 7. Mean number of goblet cells counted from lamellae of chinook salmon and rainbow trout olfactory rosettes after a 1-h exposure to Cu. Three 100-by-100–μm confocal laser scanning microscope images from each of five fish were counted for each Cu concentration. Horizontal lines indicate the mean number of goblet cells in control fish. Vertical bars indicate the SE. Asterisks indicate significant differences between species at that Cu concentration ($\alpha = 0.05$).

DISCUSSION

L-serine–evoked olfactory bulb EEG responses

Exposure of both RBT (Fig. 2a) and CS (Fig. 2b) to all Cu concentrations in this study immediately reduced the RMS measure of the EEG response to less than 60% of controls. We expected that even the lowest concentration of 25 μg Cu/L would have some physiological effect, because this concentration was similar to that previously shown to be lethal to both species within 96 h [20]. This immediate reduction in EEG response was similar to those effects reported by Thompson and Hara [21], who observed that arctic char EEG responses were immediately depressed to 25% of the original value following exposure to 100 μg Cu/L. However, in other studies, slower depressions in EEG response were observed with RBT exposed to 50 μg Cu/L, where the maximal depression of approximately 50% occurred after 1 h of exposure [8].

The rate and degree of olfactory bulb EEG response decline following the initial exposure to Cu probably depends on hardness, alkalinity, and other water parameters that influence Cu speciation and, thus, the bioavailable Cu concentration. Increased hardness, alkalinity, pH, and dissolved organic carbon concentrations have all been shown to reduce toxicity in studies of lethality [22,23] and metal binding on fish gills [24]. In EOG studies of olfactory epithelium, increases in calcium (Ca) concentration, ionic strength [7], or alkalinity [6] have reduced the effects of Cu on the olfactory system. The slower depressions in EEG responses due to Cu exposure reported by Hara et al. [8] may have stemmed from lower bioavailable Cu concentrations in their water, which had a higher degree of hardness (90 mg/L $CaCO_3$), compared with our experiments using approximately 25 mg/L $CaCO_3$–hardness water. The likelihood that the bioavailable Cu concentration is important in olfactory neuroelectrical measurements was further shown by Thompson and Hara [21]; in their study, full-strength water from a heavy metals–contaminated lake depressed olfactory bulb EEG responses immediately, whereas slower, more gradual depressions in EEG responses were observed from 1% solutions of the same water.

In the present study, exposure to lower Cu concentrations produced maximum depression of EEG responses rapidly, and further Cu exposure did not produce any greater depression. The EEG responses from CS exposed to 25 μg Cu/L and from RBT exposed to 25 to 100 μg Cu/L did not substantially decrease beyond the initial depression after the onset of the Cu exposure. As in our study, Thompson and Hara [21] observed that Cu caused a rapid initial decline in olfactory bulb EEG response to L-serine, with little change from this initial depression until the Cu exposure was stopped. Furthermore, although Hara et al. [8] had previously shown a more gradual response depression with Cu exposure, the depression reached an asymptote at approximately 50% of the original EEG response amplitude. Thus, low bioavailable Cu concentrations likely result in slower depressions, whereas relatively higher concentrations have a more rapid effect.

Following Cu exposure, EEG activity recovered more quickly and to a greater degree in fish exposed to lower concentrations. In CS (Fig. 2b) exposed to 25 μg Cu/L and in

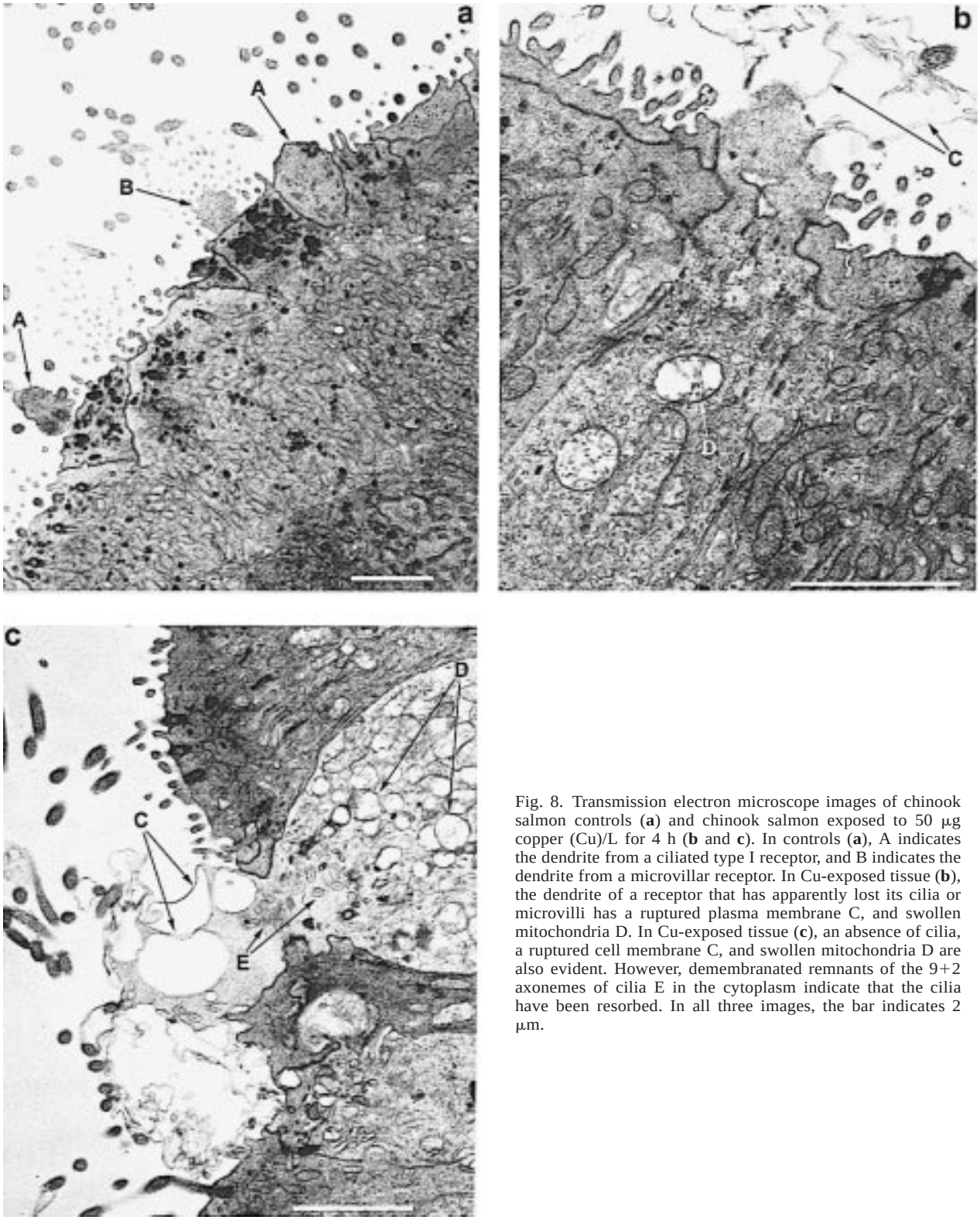

Fig. 8. Transmission electron microscope images of chinook salmon controls (**a**) and chinook salmon exposed to 50 μg copper (Cu)/L for 4 h (**b** and **c**). In controls (**a**), A indicates the dendrite from a ciliated type I receptor, and B indicates the dendrite from a microvillar receptor. In Cu-exposed tissue (**b**), the dendrite of a receptor that has apparently lost its cilia or microvilli has a ruptured plasma membrane C, and swollen mitochondria D. In Cu-exposed tissue (**c**), an absence of cilia, a ruptured cell membrane C, and swollen mitochondria D are also evident. However, demembranated remnants of the 9+2 axonemes of cilia E in the cytoplasm indicate that the cilia have been resorbed. In all three images, the bar indicates 2 μm.

RBT (Fig. 2a) exposed to 25 to 100 μg Cu/L, responses showed fairly rapid recovery and approached pre–exposure response magnitudes. The rates of recovery were dependent on the Cu-exposure concentration, and no recovery was evident in fish exposed to the highest Cu concentrations for either species. Similarly, in Hara's previous work [25], no recovery was evident in fish exposed to 320 μg Cu/L, whereas recovery was observed in fish exposed to lower Cu concentrations [8,21]. Furthermore, slower recovery rates were observed with longer exposure times [8].

Spontaneous EEG activity

The amplitude of spontaneous EEG activity greatly increased immediately in both species following exposure of the

rosette to Cu (Fig. 1d). This immediate effect was typically short-lived and was followed by a general prolonged, gradual increase in amplitude. Hara [25] observed similar changes in the olfactory bulb EEG following exposure of the rosette to at least 6 mg/L. However, the control exposure for these studies [25] also produced an elevated EEG amplitude. The elevated EEG amplitude was used as a basis of comparison for responses to Cu, and with this comparison, the EEG amplitude appeared to decrease with increasing Cu concentrations. Differences in responses to control exposures and in methods of measuring responses between Hara's study [25] and the present study lead to dramatically different conclusions about the mechanisms of avoidance behavior. Hara [25] suggested that olfactory impairment caused avoidance behavior and that olfactory stimulation led to preference for Cu. Our results indicate that the olfactory system is stimulated by Cu exposure and that this stimulation may lead to avoidance responses. Higher Cu concentrations may overload, overstimulate, or damage the olfactory system, leading to olfactory impairment and to a loss of avoidance behavior. We cannot determine in our studies whether there are specific Cu receptors or if Cu is eliciting a response through receptor depolarization caused by Cu toxicity. Regardless, the specific or nonspecific detection of Cu by the olfactory system may be controlling Cu avoidance behaviors.

In our studies, RBT (Fig. 3a) showed a more pronounced increase in spontaneous EEG amplitude than did CS (Fig. 3b). We cannot determine if RBT are more sensitive or if differences in responses to Cu by the two species are a result of the different electrode placement on the olfactory bulb. However, because the spontaneous activity was subtracted from the L-serine–evoked EEG activity, the very high increase in spontaneous EEG activity in RBT during the first 10 to 15 min of Cu exposure (Fig. 3a) has greatly influenced the RBT EEG responses toL-serine that were reported for the first 10 to 15 min of Cu exposure (Fig. 2a).

Mechanisms of Cu toxicity related to olfactory function

Although our study and studies by others [21,26] show dose–response relationships in the initial magnitude of response decline, rate of decline, and rate of recovery from extended Cu exposure, our results suggest that two distinct mechanisms seem to be controlling the impairment of the olfactory system. The fast initial response depression was largely reversible following exposure to low Cu concentrations, indicating that receptor function was not irreversibly impaired. In our histological evaluations, we found no significant reductions in the number of receptors on the olfactory epithelium following exposure to the low Cu concentrations that reduced EEG activity by only 50%. These lower concentrations of Cu may be impairing ion pumps or blocking ion channels, as has been shown in other fish epithelial tissue [27]. Because signal transduction depends on Ca^{2+} in the external water contacting the cilia and microvilli of receptors [28], Cu may be blocking Ca channels and, therefore, reducing the olfactory response, as indicated by the EEG. However, the sustained increase in spontaneous EEG activity suggests that Cu was acting to depolarize receptors and, thus, to increase the discharge of action potentials to the olfactory bulb.

Initially, higher Cu concentrations ($\geq$50 μg Cu/L for CS and $\geq$200 μg Cu/L for RBT) decreased EEG activity in the same way as did lower concentrations, but prolonged exposure appeared to cause irreversible effects on the peripheral olfactory system. Histological examination of receptors from fish treated with these high Cu concentrations revealed significant reductions of receptors on the epithelial surface of the rosette. Furthermore, with TEM, we observed that the external plasma membranes of many receptors in the olfactory rosette were ruptured. These higher Cu concentrations apparently damage and rupture receptor membranes, possibly through lipid peroxidation [29,30]. The further, gradual reduction in EEG activity, with reduced or no recovery, is likely to have resulted from cell destruction caused by the high Cu concentrations.

Receptor cell types

The olfactory epithelium of most teleost fish contains both microvillar and ciliated (type I) receptors that are easily identifiable as having 1-to-2–μm diameter dendritic knobs projecting from the epithelial surface [11,16,17]. However, the existence of a ciliated type II receptor has been disputed by some investigators. In channel catfish (*Ictalurus punctatus*) and goldfish (*Cerassius auratus*), Muller and Marc [16] described the ciliated type II receptor as having a 3.5-to-5–μm diameter dendritic apex that was flush with the epithelial surface. These cells were identified as receptors after they were retrogradely labeled with horseradish peroxidase (HRP). Similarly, Riddle and Oakley [17] demonstrated retrograde labeling of ciliated type II receptors in RBT with fluorescent microspheres. Moran et al. [11] also conducted retrograde transport studies on brown trout (*Salmo trutta*) and observed labeling of the microvillar and type I ciliated receptors but not of the disputed type II receptor. They described the type II cell as a ciliated, nonsensory epithelial cell and argued that Muller and Marc's [16] results were an artifact of using 3 atm pressure to inject HRP into the olfactory bulb. Eisthen [18] reviewed several published studies and concluded that cells morphologically resembling the type II ciliated cells are present in the olfactory epithelium of many Osteichthian fishes. That review concluded that these cells could be receptors in some fish but did state that, in most fish, these cells do not appear to have axons and are therefore assumed to be nonsensory cells.

During the course of our research with CS and RBT, we observed that a cell resembling the type II ciliated cell was consistently labeled with our anti-KLH antibody (Fig. 9). These cells were similar to those described as type II ciliated receptors [16], each with a broad, flat dendritic apex and an apparent axon that extends at least to the basal lamina of the epithelium. The anti-KLH antibody has previously been shown to be a novel marker of small-dendrite and ciliated type II receptors in salmonids [13,31]. Furthermore, other antibody-labeling methods, including anti-olfactory marker protein, have labeled the same cells as anti-KLH, thus strengthening the argument that this ciliated cell is a ciliated type II receptor [13,31]. Our observations concur with the notion that this cell is a receptor.

We were not able to distinguish between microvillar and ciliated type I receptors. Therefore, we counted them as one receptor type and referred to them as small-dendrite receptors. In control tissues of both RBT and CS (Fig 5a and b), the number of small-dendrite receptors averaged 330 to 360 per 10,000 μm^2. In a comparable study, in which microvillar and ciliated type I receptors were quantified using scanning electron microscopy, Thommesen [15] found that the density of small-dendrite receptors in several salmonid species ranged from approximately 400 receptors per 10,000 μm^2 near the

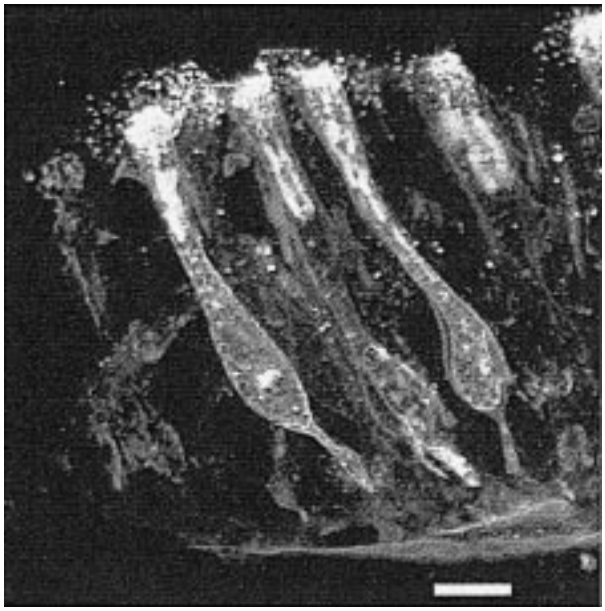

Fig. 9. Confocal laser scanning microscope image of ciliated type II receptors from a longitudinal tear in the olfactory epithelium. This tissue was labeled with anti-keyhole limpet hemocyanin. Note the apparent axon leading from the cell body to the basal lamina. The bar indicates 10 μm.

periphery of the lamella to approximately 200 receptors per 10,000 μm^2 in the central regions of the lamella. Apparently, because we counted receptors from the periphery of each lamella, the densities of receptors in our study were similar to those observed by Thommesen [15].

Mechanisms of cellular damage

With TEM, we observed several pathological changes in small-dendrite receptors of the CS olfactory epithelium following a 4-h exposure to 50 μg Cu/L. Although few receptors could be found in this Cu-exposed tissue, those that were identified as receptors were clearly damaged. The most common signs of damage included a loss of cilia and microvilli, ruptured cell membranes, and swollen mitochondria (Fig. 8b). Similar pathology following Cu exposure has been reported by others [11,12,32,33]. Although more rare and previously not described, demembranated remnants of the 9+2 axonemes of cilia were observed inside the cytoplasm of some receptors (Fig. 8c). The cilia may have been resorbed as a consequence of Cu toxicity.

Julliard et al. [12] described the loss of small-dendrite receptors by apoptosis. They described the histological indicators of death by apoptosis to be condensed chromatin, increased electron density of the cytoplasm, and swollen mitochondria. In the Julliard et al. [12] study, this process of apoptosis required up to 10 d at 20 μg Cu/L before most of the receptors showed signs of advanced stages of cell death. As shown by our CLSM analysis, 1- and 4-h exposures to higher Cu concentrations significantly reduced the number of small-dendrite receptors (Fig. 5a and b). Julliard et al. [12] proposed that marginally toxic heavy metal concentrations (e.g., the 20 μg Cu/L in their study) can induce apoptosis, whereas higher concentrations can initiate cell death by necrosis. In the present study, significant reductions in small-dendrite receptors occurred in RBT after a 1-h exposure to at least 200 μg Cu/L. In addition to our higher Cu concentrations, our water was less than one-half the degree of hardness of that used by Julliard et al. [12] (25 vs 63 mg/L $CaCO_3$), and Cu has more toxic effects on epithelial tissue in water with lower hardness and alkalinity [6,7,34]. Therefore, the damage to receptors in our study was likely due to necrosis. However, receptors that were not initially eliminated by necrosis might have been eventually lost through apoptotic mechanisms.

The apparent necrosis of receptors in our study was probably mediated by lipid peroxidation and loss of cellular ionoregulation. Several researchers have shown that Cu oxidizes plasma membranes by lipid peroxidation, causing them to rupture [29,30]. Additionally, Cu inhibits cellular ionoregulation by blocking several ion pumps and channels [27]. These mechanisms depend on the binding of Cu to an epithelial binding site (i.e., a ligand [35]) [24], and saturation of these membrane-bound ligands can occur within 2 to 3 h of Cu exposure [36]. Because the epithelial membrane of olfactory receptors has a high number of membrane-bound receptor proteins [37], Cu may be able to bind to receptors on these ligands with high affinity and accumulate to high concentrations on the membrane. Thus, Cu could quickly affect receptors by binding to ligands on the plasma membranes. The high number and variety of ligands on receptors compared with other cells in the olfactory epithelium may explain the initial selectivity of Cu for olfactory receptors rather than for other cells.

The possibility remains that exposure to Cu caused the small receptors to recede under the epithelial surface, as demonstrated for chloride cells in the gill following exposure of fish to altered pH [38]. However, the remnants of dendrites with ruptured plasma membranes protruding from the epithelial surface in TEM micrographs (Fig. 8b and c) strongly suggests that reduced cell counts were due to loss by necrosis and not by receptor retreat into the epithelium.

Quantification of cell types

We observed significant losses in small-dendrite olfactory receptors in CS exposed to ≥50 μg Cu/L and in RBT exposed to ≥200 μg Cu/L for as short a period as 1 h (Fig. 5a). Furthermore, the number of these receptors was significantly reduced in both species after a 4-h exposure to ≥25 μg Cu/L (Fig. 5b). To our knowledge, this is the first study to look at tissues after exposure to environmentally relevant Cu concentrations for such short exposure times. Julliard et al. [12] observed significant increases of apoptotic receptors in RBT exposed for 1 d to 20 μg Cu/L, and Moran et al. [11] observed significant degeneration of receptors in brown trout exposed for 1 d to 18 μg Cu/L. Another study demonstrated that exposures to extremely high Cu concentrations (7.6 g/L) for a few seconds significantly reduced the number of receptors in channel catfish [32].

No changes were observed in the numbers of ciliated type II cells following 1- or 4-h exposures to any of the Cu concentrations tested (Fig. 6). This lack of injury suggests that these cells are more resistant to Cu exposure than are the small-dendrite receptors. In order to be toxic, Cu must often outcompete other cations (e.g., Ca^{2+}, Na^{+}, etc.) to bind to a ligand on the epithelial surface [24]. Because Cu reduces L-serine binding to the olfactory rosette [39], Cu is likely binding to olfactory receptor proteins (e.g., ligands), thus allowing the Cu to damage receptor cells and to disrupt their function. In the gill, membrane-bound ligands have a specific binding affinity and capacity for Cu [24]. The type II ciliated receptors and the small-dendrite receptors may have receptor proteins

that are selective for very different chemicals, and the receptor proteins in type II ciliated receptors may have a much lower binding affinity for Cu than do the small-dendrite receptors.

Goblet cells were also counted from the olfactory epithelium of fish exposed to Cu for 1 h. In control exposures and at all Cu concentrations tested, olfactory tissues from RBT contained many more discharged goblet cells per image than did CS olfactory tissues (Fig. 7). Within each fish species, exposure to Cu did not significantly increase the number of goblet cells at the epithelial surface. Julliard et al. [33] and Saucier and Astic [40] observed a significant increase in the number of goblet cells related to Cu exposure. Since the mucus released by goblet cells may bind metals and slow access of the metal to epithelial tissues [41], the greater sensitivity to Cu exposures by CS may be caused by lower mucus production in the olfactory epithelium.

Functional and ecological implications

The close dose-effect relationship between behavioral dysfunction [2], neuroelectrical impairment, and neurohistological effects of Cu exposure support a strong argument that olfactory dysfunction underlies impaired behavioral avoidance of the metal. The possibility remains that other mechanisms, including taste and irritation of the gills, could be involved in the detection and avoidance of Cu, but this awaits further study. Our behavioral data, however, indicate that any role of these other afferent nervous systems in detection and avoidance of Cu must also be disabled at Cu concentrations equal to or less than the concentrations reported here for effects on the olfactory system. Otherwise, avoidance behavior would remain at Cu concentrations that cause damage to the olfactory system.

The large differences in sensitivity between CS and RBT are likely to have important ecological implications. The greater sensitivity of CS results in the loss of significant numbers of receptors, dysfunction of the olfactory system, and behavioral abnormalities at much more environmentally prevalent Cu concentrations. Because the histological measurements reported here show the loss of significant numbers of olfactory receptors, an exposed fish is likely to be impaired for extended periods of time until the receptors are replaced. Moran et al. [11] found that many receptors were replaced within 8 d of clean-water recovery following exposure to Cu, but Zielinski and Hara [42] report that as many as 42 d were required to replace receptors following olfactory nerve transection. Because olfactory receptors are constantly replaced, immature receptors that have not protruded from the epithelial surface could quickly replace the lost receptors [33]. Thus, the lost receptors in our study would likely be replaced after several days to a few weeks. The time required to reestablish the functional, preexposure sensitivity is unknown.

Depending on when Cu exposure occurs during the life cycle of the fish, these fish may not be able to imprint on home streams or conspecifics, locate home streams as spawning adults, find food normally, avoid predators, or avoid other harmful environmental conditions [4,37,43]. Because olfactory imprinting occurs during the smolt stage [44,45] and because fish use olfaction to find home streams for spawning [44], CS are more likely to have impaired olfactory systems during these sensitive periods. These fish would likely have more difficulty finding home streams if olfactory function was disrupted by Cu exposure either during the smolt stage or during spawning migrations. Therefore, compared to RBT, CS are more susceptible to olfactory injury, and CS populations are potentially more threatened by elevated Cu concentrations.

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