Copper Binding Dynamics and Olfactory Impairment in Fathead Minnows (*Pimephales promelas*)

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When fish are exposed to sublethal, environmentally relevant Cu concentrations, olfactory acuity is impaired. The goals of the present study were to investigate the binding dynamics of waterborne Cu in the olfactory epithelium (OE), to examine the influence of calcium (Ca^{2+}) on Cu binding, and to link Cu-OE binding to changes in olfactory acuity. Using short-term in vivo waterborne exposures to ^{64}Cu, we found that Cu accumulates rapidly in the OE, reaching a plateau by 3 h. The binding affinity (log K_{Cu-OE}) and binding capacity (B_{max}) of ^{64}Cu in the OE were 6.7 and 10.0 nmol Cu g^{-1}, respectively. As waterborne Ca^{2+} was increased from 50 to 1000 μM L^{-1}, the B_{max} of Cu decreased by ~50% while the log K_{Cu-OE} remained constant, indicative of noncompetitive inhibition. Using electro-olfactograms (EOG), short-term exposures to 160 and 240 nmol Cu L^{-1} were found to reduce olfactory responses to 10^{-5} M L-arginine by 72 and 79%, respectively. Short-term exposure to 160 nmol Cu L^{-1} also caused a 15-fold reduction in behavioral responses to a food stimulus. Interestingly, increasing waterborne Ca^{2+} did not reduce the effects of Cu on EOG or behavioral responses. These results demonstrate that short-term, environmentally realistic concentrations of Cu not only bind to the OE of fathead minnows but also impair their olfactory sensitivity and behavioral responses to olfactory stimuli. Waterborne Ca^{2+} reduces Cu−OE binding but does not protect against olfactory impairment.

Introduction

The biotic ligand model (BLM) (*1*−*3*) uses laboratory-derived gill-metal binding constants log *K* (binding affinity, an index of the strength of binding between the metal and the biotic ligand) and B_{max} (binding capacity, the maximum number of binding sites on the biotic ligand) as well as site-specific water chemistry in order to predict acute metal toxicity in fishes (i.e., 96 h LC50) (*4, 5*). The Cu gill−metal binding constants have been determined, as well as how a variety of different water quality variables (e.g., Ca^{2+}, Na^+, Mg^{2+}, H^+, DOC) influence gill−Cu accumulation (*6*−*9*) ultimately leading to the production of a Cu BLM for both fathead minnows (*Pimephales promelas*) and rainbow trout (*Oncorhynchus mykiss*, RBT) (*2, 3*).

Fish gills are appropriate as biotic ligands for predicting acute toxicity because they are physiologically sensitive to metals (*10*) and BLM predictions typically correspond to laboratory-derived acute toxicity data (*3*). However, predictions of Cu toxicity may be inappropriate using a gill-based BLM when fish are exposed to chronic sublethal waterborne and/or dietary metal concentrations because gill-metal binding characteristics (log K_{Cu} and B_{max}) may shift due to acclimation (*11*). Furthermore, when Cu concentrations are too low to cause mortality, they still may cause disruption of physiological processes in other tissues (*11*).

One such target tissue is the olfactory epithelium (OE). Olfaction is particularly important in aquatic environments for communication, especially at night or when the water is turbid (*12*). Olfactory chemical cues play a crucial role in locating food (*13*), assessing mating potential (*14*), recognizing kin (*15, 16*), and avoiding predators (*12, 17, 18*). The OE of fishes is in constant contact with the aquatic environment and does not possess a protective membrane, which can make it susceptible to toxicants (*19*). Waterborne concentrations of Cu can vary greatly and have been found in the range of 2−25 μg Cu L^{-1} in lakes along a metal contamination gradient (*20*). Exposure to dissolved Cu can cause degeneration of the OE via apoptosis, thereby reducing the number of ciliated and microvillar olfactory sensory neurons (OSN) (*21*−*23*). Furthermore, exposures to environmentally relevant concentrations of Cu (1−20 μg Cu L^{-1}; 15.6−312.5 nmol L^{-1}) impair peripheral olfactory responses (EOG−electro-olfactogram) and central olfactory responses (EEG−electroencephalogram) to a variety of olfactory stimuli (i.e., amino acids, bile salts, alarm cues) in juvenile coho salmon (*Oncorhynchus kisutch*) (*24*−*26*). Copper-impaired EOG responses to conspecific skin extracts (i.e., alarm substance) are correlated to decreases in behavioral responses to the same stimuli (*26*). Thresholds for sublethal olfactory Cu toxicity in juvenile coho salmon were determined to be ~3 μg Cu L^{-1} (~47 nmol L^{-1}) based on a 25% reduction in EOG response to standardized chemical stimuli (*24*).

Our aim was to quantify the binding of waterborne Cu to the OE of fathead minnows using an environmentally relevant range of Cu concentrations and to examine how increasing waterborne Ca^{2+} influences this binding. In order to link Cu/Ca^{2+} binding dynamics in the OE with a biological effect we examined the influence of waterborne Cu and Ca^{2+} concentrations on EOG and behavioral responses to a suite of odorants. By understanding Cu−OE binding constants, the influence of water chemistry on ligand binding, and ethological or ecological end points such as the inability to detect chemical cues that facilitate predator avoidance, we may be able to develop a model to predict metal toxicity in the OE. Such a model may be useful in devising environmental regulations to protect against sublethal metal toxicity in fishes.

Experimental Procedures

Acclimation of Experimental Animals. Adult fathead minnows (3−5 g) were acclimated for 2+ weeks to either synthetic softwater (SSW- a mixture of dechlorinated Hamilton, ON municipal water and reverse osmosis water) (mean ± SEM; Ca^{2+} = 122.7 ± 4.4 μM, Na^+ = 106.6 ± 6.6 μM, Mg^{2+} = 35.7 ± 1.3 μM, dissolved organic carbon ~ 1 mg L^{-1}, temperature = 12 ± 1 °C) for ligand binding experiments or dechlorinated North Bay, ON municipal water (Ca^{2+} = 167.5 ± 2.0 μM, Na^+

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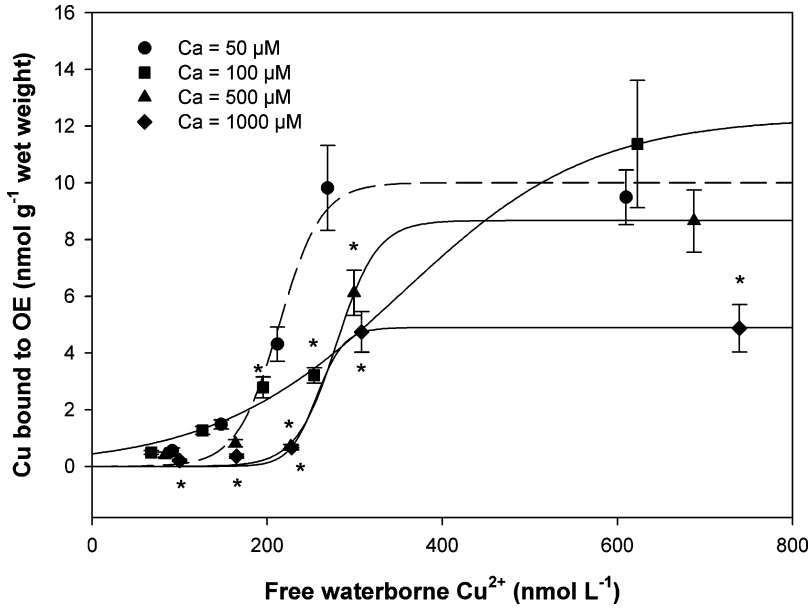

FIGURE 1. New copper binding to the olfactory rosettes of fathead minnows across a range of waterborne Cu concentrations in different waterborne Ca^{2+} concentrations after a short-term 3 h copper exposure. Means ± SEM (n = 6−7). Asterisks represent significant difference of Cu binding from Cu binding in baseline softwater (hatched line) within the respective Cu concentration.

= 560.9 ± 12.5 μM, Mg^{2+} = 63.3 ± 2.3 μM, temperature = 19 ± 1 °C) for electro-olfactogram and behavioral experiments. Fish were kept on a 16 h:8 h light:dark cycle. The pH of the experimental water was measured (PHM 82 standard pH meter with a GK2401C electrode, Radiometer Copenhagen) before all experiments, and was always in the range 6.72−6.93.

Effect of Ca on Cu−OE binding. Five 2 L Cu-exposure treatments were prepared in aerated SSW (mean ± SEM; Ca^{2+} = 41.7 ± 2.2 μM, Na^{+} = 51.6 ± 0.9 μM, Mg^{2+} = 11.1 ± 0.06 μM, dissolved organic carbon ~ 0.3 mg L^{-1}, temperature = 14 °C). Treatments were spiked with ^{64}Cu to yield concentrations of (nominal) 79, 160, 236, 315, and 787 nmol L^{-1} (5, 10, 15, 20, 50 μg L^{-1}) (measured: 100.5 ± 8.2 nmol L^{-1}, 176.7 ± 10.8 nmol L^{-1}, 252.9 ± 9.2 nmol L^{-1}, 331.7 ± 15.2 nmol L^{-1}, 780.9 ± 36.2 nmol Cu L^{-1}). Radioactive ^{64}Cu [as $Cu(NO_3)_2$] was created by irradiation in the McMaster University Nuclear Reactor (specific activity = 2 μCi μg^{-1}; half-life = 12.9 h). Exposure solutions were also prepared at three additional dissolved Ca^{2+} concentrations (nominal 100, 500, and 1000 μM Ca; measured 104.1 ± 0.9 μM, 474.7 ± 2.1 μM, 971.5 ± 5.8 μM Ca) in order to test the effect of Ca^{2+} on Cu−OE binding. Calcium concentrations were manipulated by adding measured quantities of $Ca(NO_3)_2$ (Fisher Scientific, Oakville, ON) to SSW to yield the desired concentrations.

Seven fathead minnows were randomly assigned to each exposure treatment. We found that Cu reaches a plateau in OE after 3 h (Supporting Information (SI) Figure S1) so at this time, fish were euthanized in an overdose of buffered MS222 (500 mg L^{-1}; ethyl 3-aminobenzoate methanesulfonate salt, Sigma, Oakville, ON, Canada) and rinsed in SSW to remove any loosely bound ^{64}Cu. Both olfactory rosettes were removed, rinsed in SSW, blotted dry, weighed and counted for ^{64}Cu activity on a Canberra-Packard Minaxi Auto-Gamma 5000 series gamma counter with on-board decay correction for ^{64}Cu (Canberra-Packard Instruments, Meriden, CT). Duplicate 10 mL water samples were collected from each treatment at the beginning and end of the exposure period, passed through a 0.45 μm filter, and acidified to 1% with trace metal grade HNO_3 (Fisher Scientific, Oakville, ON). Dissolved Cu concentrations were determined using graphite furnace atomic absorption spectroscopy (GFAAS) (Varian GTA 110, Varian Scientific, Mulgrave, Australia). A certified reference material (SLRS-5) from the National Research Council of Canada was used to ensure accuracy. Ca^{2+}, Na^{+}, and Mg^{2+} concentrations in water samples were measured using flame atomic absorption spectroscopy (FAAS) (Varian SpectrAA-220 FS, Varian Scientific, Australia) against reference standards from Fisher Scientific, U.S.

Effect of Cu on EOG Response. The olfactory stimulus, 10^{-4} M L-arginine, was used to examine the effect of Cu on EOG response. The stimulus solution was prepared fresh daily from a stock solution (prepared fresh weekly) and SSW. Two experimental blank solutions were also prepared with SSW or SSW + Cu, where the Cu concentration corresponded to the exposure treatment (see below). All solutions were stored at 4 °C and brought to room temperature when needed.

Fathead minnows were anaesthetized in buffered MS222 (185 mg L^{-1}), immobilized by an epaxial intramuscular injection of Flaxedil (gallamine triethiodide, 3 mg kg^{-1} body mass, Sigma, Oakville, ON, Canada) (*27*), and wrapped in wet tissue to prevent desiccation, leaving the head and tail regions exposed. Fish were secured and electrically grounded in an acrylic glass perfusion chamber where the gills were perfused via a tube inserted into the mouth with a constant supply of aerated holding tank water containing 50 mg L^{-1} MS222 (*27*). The right olfactory rosette was exposed by removing the nasal septum, and the exposed olfactory chamber was irrigated with SSW prior to EOG testing. After this 1 h stabilization period, an EOG signal was continuously monitored throughout the course of the experiment. See SI for details of the EOG recording setup.

Experimental stimuli were delivered directly to the olfactory chamber in three phases: (i) a 30 min premetal exposure, (ii) a 60 min metal exposure, and (iii) a 30 min postmetal exposure. During the premetal exposure phase, experimental stimuli were delivered sequentially in 3 s pulses once every 10 min via a gravity-fed delivery system. Immediately following a 3 s stimulus pulse, the irrigation stream was switched back to SSW, thus maintaining a continuous flow to the olfactory chamber. A minimum of 2 min of continuous SSW irrigation was maintained between successive stimulus deliveries to minimize olfactory attenuation.

During the 60 min metal exposure phase, the irrigation stream was switched from SSW to SSW + Cu, where the nominal dissolved Cu concentration was one of 80, 160, or 240 nmol L^{-1} (5, 10, 15 μg L^{-1}) (measured: 74.5 ± 2.2, 134.6 ± 4.4, 163.1 ± 5.5 nmol L^{-1}). Successive olfactory stimulus delivery of 10^{-4} M L-arginine continued in 10 min intervals

as described above, and EOG signals continued to be recorded. During the 30 min postmetal exposure period, Cu was removed from the irrigation stream leaving only SSW perfusing the olfactory chambers. Again, experimental stimuli were delivered in 10 min intervals and EOG responses were recorded. Gill perfusion water, irrigation water, and all experimental stimuli were at room temperature (18−22 °C).

Effect of Ca on Cu-Impaired EOG Responses. To test whether dissolved Ca^{2+} could protect against Cu-impaired EOG responses, fathead minnows ($n = 4$) were randomly assigned to one of three pre-exposure treatments, where Cu concentrations were held constant at 160 nmol L^{-1} (10 μg L^{-1}) but Ca^{2+} concentrations increased (100, 500, or 1000 μM Ca^{2+}), and one control treatment (no Cu + 100 μM Ca^{2+}). Fish were pre-exposed to these solutions for 3 h prior to EOG testing. Olfactory stimuli were delivered successively in 10 min intervals as described above, and included 10^{-4} M L-arginine as well as two additional stimuli, 10^{-4} M L-alanine and the bile acid 10^{-5} M TCA. Irrigation water was SSW and did not include Cu. Each stimulus was delivered three times to each fish.

Behavioral Experiments. Fathead minnows ($n = 15$) were randomly assigned to one of four pre-exposure treatments corresponding to those of the previous section (i.e., constant 160 nmol L^{-1} Cu in 100, 500, and 1000 μM Ca^{2+}, and a control of no Cu in 100 μM Ca^{2+}). Pre-exposures were for 3 h.

Behavioral trials were conducted in 36 × 80 cm Y-mazes. Each Y-maze consisted of a large plastic container with an acclimation chamber located at one end and a corrugated plastic barrier separating each arm to prevent any mixing of chemicals. A removable barrier was placed between the acclimation zone and both arms (SI Figure S2). Arm preference trials found there was no significant difference in the amount of time spent in either arm ($t = 0.861$, df = 11, $p = 0.408$). A food stimulus to induce attraction was prepared from 500 mg of frozen brine shrimp in 250 mL of SSW, and filtered to remove large particles. For each trial one arm of the maze (SI Figure S2) was randomly assigned as experimental (food stimulus) and the other as control (SSW). Stimuli were injected into the distal end of each maze arm at the same time that a single fish from one of the four pre-exposures was introduced to the acclimation area at the proximal end. During a 20 min acclimation period, fish were prevented from accessing either arm of the maze. At the end of the acclimation period, the barrier was lifted and the fish's location was recorded every 10 s. Each trial ran for 10 min and was conducted in SSW (18−20 °C).

Calculations and Statistical Analysis. The amount of newly bound Cu (nmol g^{-1}) to the OE was calculated using

$$a(bc^{-1})^{-1}$$

where a = radioactivity counts in the OE (cpm g^{-1} wet tissue weight); b = radioactivity counts in the water (cpm L^{-1}); c = dissolved metal concentration in the water (nmol L^{-1}).

Nonlinear regressions (Sigmaplot 8.0 for Windows) were employed for the analysis of metal binding to the OE using a sigmoid 3-parameter curve fit:

$$y = \frac{a}{1 + \exp^{-}\left(\frac{x - x_0}{b}\right)}$$

B_{max} (binding capacity) and log K (binding affinity) were determined from these regressions. B_{max} was the value on the y-axis where the maximum amount of metal binding occurred (represented by "a" in the above equation). Log K was determined as the negative logarithm of the free Cu^{2+} ion concentration in the water ("x_0" in the above equation, in molar units) that provides metal binding equivalent to half the B_{max}. Free Cu^{2+} ion concentrations were estimated using measured water chemistry values and the aquatic chemistry program MINEQL+.

TABLE 1. Log K and B_{max} Values for Cu Binding to the Olfactory Epithelium (OE) and Gill Epithelium of Fathead Minnows in Artificial Softwater with Low Concentrations of Competitive Ions and Other Complexing Agents (e.g., DOC)[a]

metal	tissue	$[Ca^{2+}]$ (μM)	B_{max} (nmol g^{-1})	Log K
Cu	OE	50	10.0	6.7
		100	12.3	6.5
		500	8.7	6.6
		1000	4.9	6.6
Cu	Gill		10.0*	7.4*

[a] Values for the OE are after a 3 h exposure to waterborne Cu and are shown as a function of increasing waterborne $[Ca^{2+}]$. Asterisks indicate values from ref *7* and *8*.

The magnitude of an EOG response to an odor stimulus was measured as the difference between baseline and peak depolarization for each odor delivery. Relative responses to a particular odorant (e.g., 10^{-5} M L-arginine) were calculated by dividing each response value for a given fish by the corresponding premetal exposure (control) response average for that odorant.

All data are presented as means ± SEM (n) where n = number of fish. The effects of increasing waterborne [Cu] and $[Ca^{2+}]$ on metal−OE accumulation were examined by two-way ANOVA. Subsequent ANOVA's with a Tukey−Kramer HSD post hoc tests were used to determine if increasing waterborne $[Ca^{2+}]$ reduced Cu−OE accumulation at each of the Cu concentrations tested. To determine the effect of Cu exposure on EOG response to 10^{-5} M L-arginine throughout the entire testing period, repeated measures two-way ANOVA were utilized. The responses from the Cu/Ca exposure EOG data were analyzed by conducting three one-way ANOVA among Cu/Ca concentrations (one for each chemical stimulus) followed by post hoc Tukey analysis. In the behavioral trials, the difference in time spent between the two arms was calculated (experimental−control) for each Cu/Ca concentration. The differences among Cu/Ca concentrations were analyzed with one-way ANOVA followed by post hoc Tukey HSD tests. All statistical analyses employed a significance level of 0.05, and were performed using JMP statistical software (v.5.0; SAS, Cary, NC).

Results

Metal−ligand Binding Experiments. The concentration-dependence of ^{64}Cu binding was measured using a 3 h assay to establish the affinity (log K) and binding capacity (B_{max}) of Cu to the OE in baseline SSW. The curve representing Cu binding to the OE was S-shaped over the [Cu] range of 0−800 nmol L^{-1} (0 − 50 μg L^{-1}). Cu−OE binding increased significantly above [Cu] of ~177 nmol L^{-1} (11 μg L^{-1}) and reached a plateau at ~330 nmol L^{-1} (21 μg L^{-1}) ($F_{4,30} = 26.70$; $p < 0.0001$) with Cu−OE binding increasing by ~1600% over the [Cu] range tested. Log K_{Cu-OE} was calculated to be 6.7, representing a free Cu^{2+} concentration of 214 nmol L^{-1} (13.5 μg L^{-1}), and B_{max} was 10.0 nmol g^{-1} (Figure 1, Table 1).

Increasing waterborne $[Ca^{2+}]$ from 50 μM (baseline SSW) to 100, 500, or 1000 μM (2, 4, 20, 40 mg L^{-1}) reduced Cu−OE binding between 200 and 600% relative to Cu−OE binding in baseline SSW at each of the waterborne [Cu] tested (whole model $F_{19,119} = 22.28$; $p < 0.0001$) (Figure 1). However, at Cu concentrations ≤160 nmol L^{-1} (≤10 μg L^{-1}), Cu−OE binding was significantly reduced only at waterborne Ca^{2+} concentrations of 1000 μM L^{-1} (40 mg L^{-1}). Notably, as waterborne $[Ca^{2+}]$ increased, the B_{max} of Cu decreased by up to 50% while the log K_{Cu-OE} remained the same (Table 1), indicative of noncompetitive inhibition.

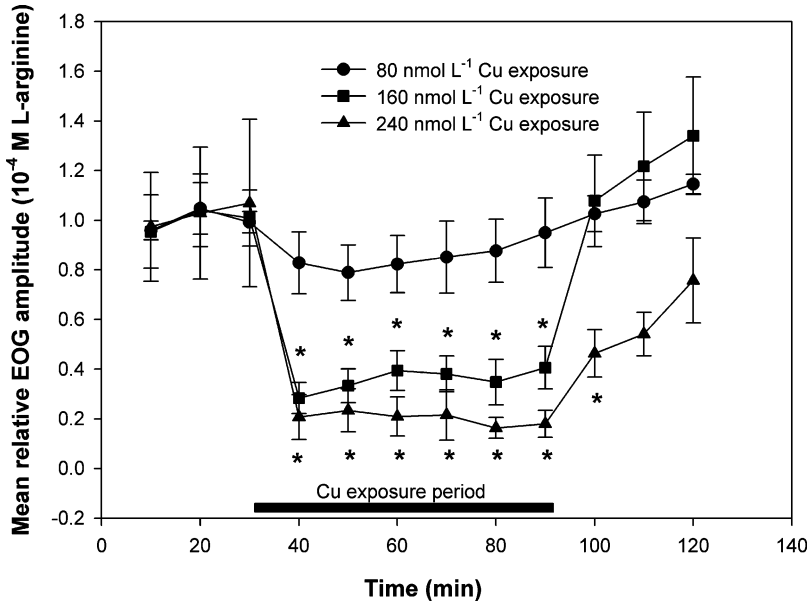

FIGURE 2. Mean ± SEM (n = 5) of the relative EOG response of fathead minnows to 10^{-4} M L-arginine for the precopper, copper exposure, and postcopper exposure periods at concentrations of 80, 160, and 240 nmol Cu L^{-1}. Relative responses were determined by dividing each response value for a given fish by the respective premetal exposure response average. Plotted means were determined by averaging the response to 10^{-4} M L-arginine of all five fish for a given time period. Repeated measures two-way ANOVA was conducted for each Cu concentration and asterisks indicate significant differences from the respective premetal exposure responses ($p \leq 0.05$). The solid bar indicates the copper exposure period.

Electro-Olfactogram Experiments. Exposure to 160 and 240 nmol Cu L^{-1} (10 and 15 μg L^{-1}) for 10 min reduced the EOG responses to 10^{-4} M L-arginine by approximately 72 and 79%, respectively, relative to controls ($F_{11,121} = 3.47$, $p < 0.0001$) (Figure 2). This inhibition continued throughout the Cu exposure period. Interestingly, even after a 1 h exposure to 80 nmol Cu L^{-1} (5 μg L^{-1}), EOG responses to 10^{-4} M L-arginine were not affected (Figure 2). During the post-Cu exposure period, the EOG response to 10^{-4} M L-arginine returned to pre-Cu exposure levels within 10 min after a 160 nmol Cu L^{-1} (10 μg L^{-1}) exposure and 20 min after a 240 nmol Cu L^{-1} (15 μg L^{-1}) exposure, indicating relatively rapid recovery. Further electrophysiological experiments using additional odorants revealed that increasing Ca^{2+} concentrations from 100 to 1000 μM had no protective effect against a 160 nmol Cu L^{-1} (10 μg L^{-1}) exposure (Figure 3a).

Behavioral Experiments. Fathead minnows not exposed to Cu spent 15-fold more time in the experimental arm with the food stimulus compared to fathead minnows exposed to any of the Cu/Ca combinations ($F_{3,56} = 6.03$, $p = 0.001$). The behavioral responses paralleled the EOG results in that increasing Ca^{2+} concentrations from 100 to 1000 μM did not have a protective effect against a 3 h, 160 nmol Cu L^{-1} (10 μg L^{-1}) exposure (Figure 3b) regardless of the fact that increasing waterborne Ca reduced B_{max} by up to 50%.

Discussion

It is well established that waterborne Cu concentrations above 80 nmol L^{-1} (~5 μg L^{-1}) can be toxic to the olfactory system of fishes causing reduced olfactory sensitivity and impaired behavioral responses (*26, 28, 29*). A recent study by the authors (*30*) demonstrated that waterborne Cu also alters EOG and behavioral responses in wild yellow perch chronically exposed to metals. The results shown here are in general agreement with previous studies and build upon our previous research in order to characterize Cu−OE binding and provide insight into the potential mechanism of Cu toxicity in the OE. These results are the first to demonstrate that increasing water Ca^{2+} noncompetitively inhibits Cu−OE binding. In addition, our results show that although increases in waterborne Ca^{2+} reduce Cu-OE binding they do not protect against Cu induced impairment of olfactory sensitivity or behavioral responses.

Copper binding in the OE is thought to occur on cellular surface proteins, membrane structures, and internal organelles. At high concentrations Cu accumulation is known to cause cell death via apoptosis (*21, 22, 31*). Moreover, Cu may disrupt odor responses by blocking ligand-gated and (or) voltage-gated ion channels (*32*). Our results demonstrate that both EOG and behavioral responses of FHM were significantly reduced at an exposure of 160 nmol Cu L^{-1} (~10 μg Cu L^{-1}). Previous studies in salmon have also found that Cu (as low as ~31−47 nmol L^{-1} (2−3 μg Cu L^{-1})) reduces EOG responses and impairs behavioral responses (at ~80 nmol L^{-1} (≥5 μg Cu L^{-1})) of fishes to a variety of odorants (*24−26, 28*). In addition, our previous research has shown that wild yellow perch chronically exposed to sublethal metal concentrations, including Cu (~140 − 390 nmol L^{-1}), actually exhibited increased EOG responses, presumably in compensation for prolonged exposure, but behavioral responses to a variety of odorants were still inhibited (*30*). In the current study, increasing waterborne Ca^{2+} concentrations up to 1000 μM L^{-1} did not restore EOG responses during a 160 nmol L^{-1} (10 μg L^{-1}) Cu exposure. Similar results in salmon have shown that increasing water hardness up to ~6000 μM L^{-1} (240 mg Ca L^{-1}) does not restore EOG responses to a variety of odorants during exposures of 10−20 μg Cu L^{-1} (~160−320 nmol L^{-1}) (*24, 33*). Similarly, our current study shows that increasing waterborne Ca^{2+} up to 1000 μM L^{-1} does not restore behavioral responses to a food stimulus during a low, sublethal Cu exposure (160 nmol L^{-1} (10 μg L^{-1}). Overall, our findings demonstrate that the effects of Cu on the olfactory system occur at approximately 160 nmol L^{-1}. Interestingly, the binding curve data show that at 160 nmol Cu L^{-1}, calcium does not reduce OE Cu binding and therefore does not provide a protective benefit. In fact, calcium only provides a protective benefit against Cu-binding at much higher Cu concentrations. This suggests that Cu and Ca could be binding at two different sets of binding sites in the OE. However, another possible explanation for the effect of Ca on Cu

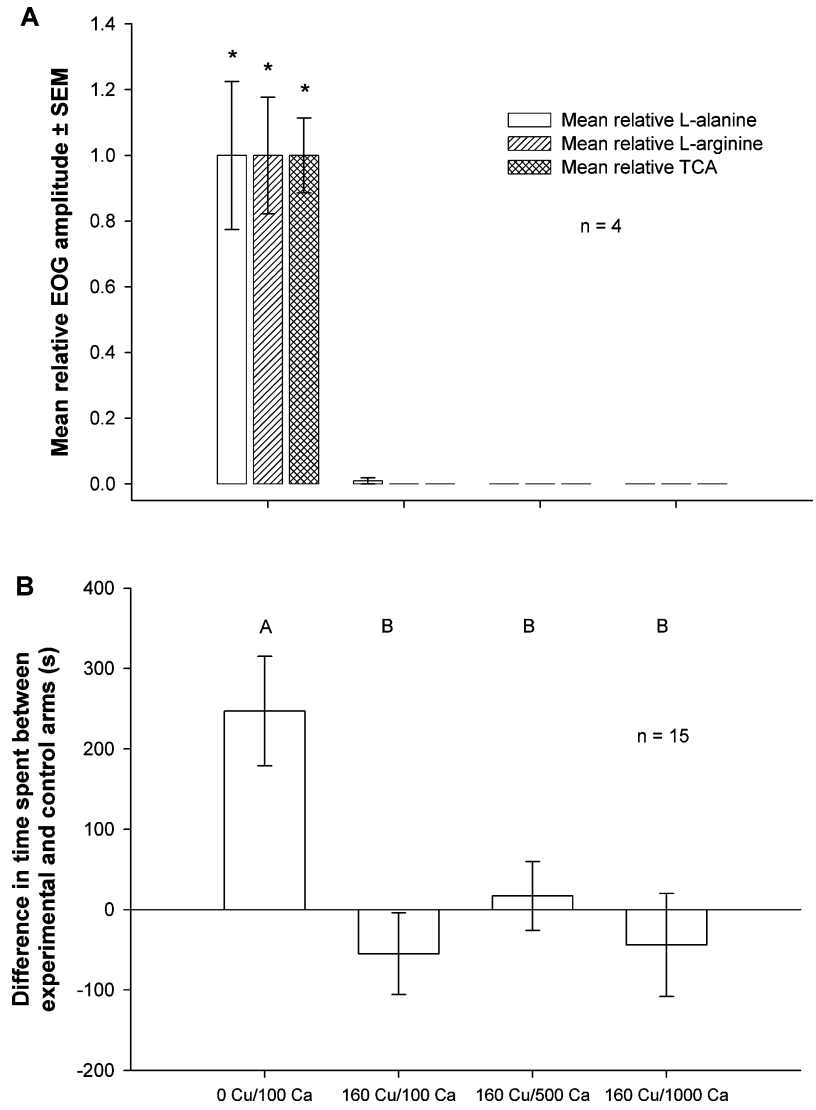

FIGURE 3. Mean ± SEM of (A) relative EOG response of adult fathead minnows to 10^{-4} M L-alanine, 10^{-4} M L-arginine and 10^{-5} M taurocholic acid (TCA) and (B) difference in time spent between experimental (brine shrimp stimulus) and control (blank water) arms of a Y-maze after 3 h exposure to waterborne copper/calcium solutions of 0 nmol Cu L^{-1} /100 μM Ca L^{-1}, 160 nmol Cu L^{-1} /100 μM Ca L^{-1}, 160 nmol Cu L^{-1} /500 μM Ca L^{-1}, and 160 nmol Cu L^{-1} /1000 μM Ca L^{-1}. $n = 15$/treatment. * indicates a significant difference at $p < 0.001$. Separate comparisons were conducted between Cu/Ca combinations for each stimulus. Different letters above bars indicate a significant difference ($p \leq 0.05$).

binding at the OE is the change in plasma membrane surface potential following increases of ionic strength in the exposure medium (*34*).

Recently, it has been shown that the current gill-based BLM does not adequately predict and protect against Cu induced toxicity in the olfactory epithelium (*33*). Specifically, gill BLM LC50 and OE IC50 predictions for Cu exposure were compared across a range of calcium, bicarbonate, and dissolved organic matter concentrations with calculated gill BLM LC50 values in each instance being higher than OE IC50 values (*33*). Increases in calcium over the range of ~0−1500 μM had little effect on predicted OE IC50 values, which were always <10 μg Cu L^{-1} (~160 nmol L^{-1}), whereas calculated gill LC50 values were 3 times higher (*33*). The results of the current experiments also demonstrate that Cu toxicity in the OE occurs at ~160 nmol L^{-1} despite the fact that the binding capacity for Cu in the OE was decreased by up to 50% with increasing calcium. In addition, BLM predicted gill LC50 values using our reported water chemistry data for SSW in the EOG and behavioral experiments are ~416 nmol Cu L^{-1} and increase up to ~800 nmol Cu L^{-1} as Ca concentrations increase from ~50 to 1000 μM. Therefore, a distinct set of binding constants and end points for the olfactory system are required in order to produce a model that adequately predicts metal binding at the OE and protects against toxicity.

The log *K* values for the OE are ~1 log unit lower than that estimated for the gill of fathead minnows, but the B_{max} values are very similar (Table 1). This indicates that although OE Cu affinity is approximately 10-fold lower than gill Cu affinity, the OE has a relatively large binding capacity for Cu, probably due to the fact that its normal function is to bind odor molecules from the water column. Interestingly, as waterborne [Ca^{2+}] increased from ~50 to 1000 μM, the Cu B_{max} decreased but log *K* remained the same (i.e., unchanged binding affinity), indicative of noncompetitive inhibition. The binding and consequent toxic effects of Cu in fish gills are mainly reduced with increasing waterborne [Ca^{2+}] (*6, 7, 35*) and partly by increasing waterborne [Na^{+}] (*3, 35, 36*). This may be because Ca^{2+} controls the permeability of the membrane, thereby reducing Na^{+} and Cl^{-} loss through paracellular pathways (*36*). In the BLM approach, this effect at the gills is implicitly assumed to occur by competitive inhibition (*1*−*5*), but the original experiments (*3, 6*−*9, 35, 37*)

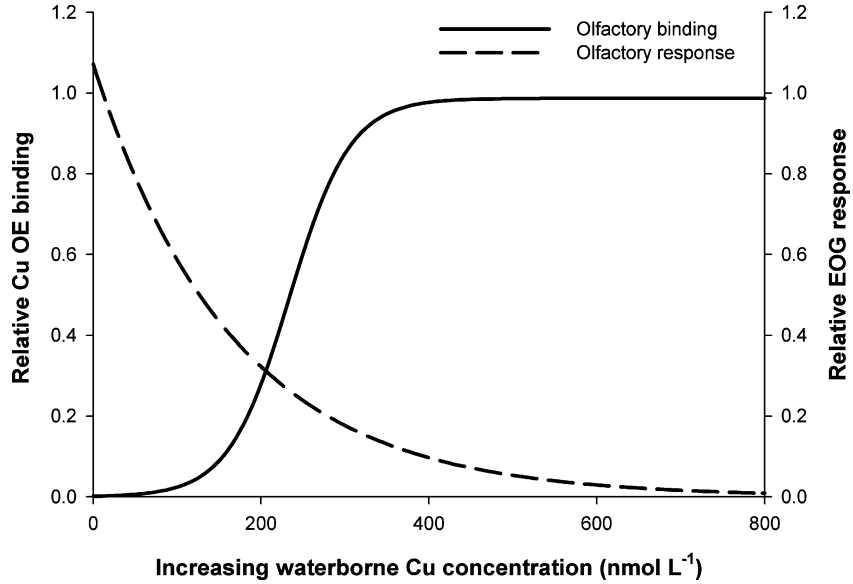

FIGURE 4. Diagram of the relationship between increasing copper binding to the olfactory epithelium and decreasing electro-olfactogram responses in fathead minnows exposed to increasing waterborne copper concentrations. Diagram based on data presented in Figures 1 and 2 in baseline synthetic softwater. All data on *y*-axes are relative to their respective no-copper control responses.

were not done in such a manner as to distinguish competitive from noncompetitive inhibition. Predictions of the gill BLM do not protect the OE from Cu toxicity, despite the fact that the log *K* values for the OE are 10-fold lower than that of the gill epithelium because changes in water chemistry do not reduce toxicity at the OE and ligand binding at the OE does not follow competitive binding principles of the BLM. In addition, toxicity at the OE occurs at lower waterborne Cu concentrations than at the gill epithelium (*33*). Combined, these findings indicate that the OE is more sensitive than the gill to Cu toxicity. The inhibition of peripheral olfactory function by sublethal Cu exposure has implications for the survival and reproductive success of fishes in metal-contaminated aquatic habitats (*12, 18, 28*).

When examining the binding dynamics of Cu in the OE and the inhibition of EOG responses across the same range of environmentally relevant Cu concentrations, it is clear that as little as a 15% increase in Cu accumulation in the OE can lead to more than a 50% decrease in EOG response (Figure 4). Furthermore, as our results show, a 50% or greater loss of olfactory response leads to a loss of behavioral responses to ecologically important odorants (*12, 18*). By understanding the relationship between metal binding dynamics in the OE and inhibition of peripheral olfactory function, it may be possible to develop a model to predict sublethal metal toxicity to the olfactory system of freshwater fishes. Such a model may be used to relate metal accumulation in the olfactory rosettes and site-specific water chemistry to predict end points such as a 50% inhibition of olfactory response leading to impaired behavioral responses. This may ultimately lead to improved ecological risk assessment.

Acknowledgments

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Supporting Information Available

Additional information regarding the experimental methods and results of the metal ligand binding and electro-olfactogram experiments. Figure S1 illustrates the time course of Cu binding to the OE of fathead minnows over a 24-h period. Figure S2 is a diagram of the Y-maze utilized for behavioral experiments. This material is available free of charge via the Internet at http://pubs.acs.org.

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