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AN EVALUATION OF SODIUM LOSS AND GILL METAL BINDING PROPERTIES IN RAINBOW TROUT AND YELLOW PERCH TO EXPLAIN SPECIES DIFFERENCES IN COPPER TOLERANCE

LISA N. TAYLOR,* CHRIS M. WOOD, and D. GORDON MCDONALD
McMaster University, Department of Biology, 1280 Main Street West, Hamilton, ON, Canada, L8S 4K1

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Abstract—The main objective of the study was to use a species comparison approach in order to understand sensitivity and tolerance differences to copper. We hypothesized that species differences in toxicity would be reflected by differences in copper binding to high-affinity sites on the gill. Specifically, the strength of copper binding (affinity, log*K*) and maximum number of binding sites (saturation, B_{max}) for copper at the gill surface would vary among different species of fish. Two species that are different in their copper sensitivity are the rainbow trout (*Oncorhynchus mykiss*) and yellow perch (*Perca flavescens*). We explicitly compared acute toxicity (median lethal concentrations via 96-h LC50s) and whole-body Na^+ loss in both organisms in two distinct water chemistries (i.e., hard and soft water). For both species, the copper binding sites at the gill surface were characterized for their affinity and saturability. The binding properties of the gill were quite similar between the two species in each water chemistry. Based on estimations of the free cupric ion concentration, the affinity, or log*K*, was 8.4 for both species in soft water, whereas in hard water, the affinity was higher (~9.7). The B_{max} value in soft water was 1.88 nmol/g for rainbow trout and yellow perch, while in hard water, saturation occurred at 3.63 nmol/g for rainbow trout and 9.01 nmol/g for yellow perch. More importantly, the amount of copper bound to the gills at 50% mortality (i.e., lethal accumulation; the LA50) was different between the two species (yellow perch LA50s were nine times higher than those of rainbow trout in soft water and hard water), indicating that the copper binding to the yellow perch gill must not have been 'biologically reactive.' According to 96-h LC50s, yellow perch were less sensitive to copper than were rainbow trout; however, the difference between the two species was similar in hard water (1.05 vs 4.16 μM) and soft water (~0.10 vs 0.44 μM). Perch were more tolerant because they lost less sodium upon exposure to copper; yet this mechanism of tolerance was not reflected by the amount of copper at the gill surface. The influence of water chemistry on the binding properties of the gill demonstrates the dynamic nature of the gill in maintaining ionoregulatory homeostasis, a key issue in the future development of the chronic biotic ligand model.

Keywords—Copper Gill binding Sodium loss Yellow perch Rainbow trout

INTRODUCTION

It has long been recognized that different fish species can vary in their tolerance to waterborne copper, and two such families that are different are Salmonidae and Percidae. According to Spear and Pierce [1], salmonids can be almost two orders of magnitude more sensitive than Perciformes to waterborne copper. In rainbow trout (RBT; a salmonid; *Oncorhynchus mykiss*), the mechanism of acute copper toxicity is clearly understood as an ionoregulatory disturbance of sodium levels that exceeds homeostatic control [2–4]. This mechanism is believed to result when copper causes a chemical disruption (i.e., an inhibition of Na^+/K^+-ATPase) and a physical disruption (i.e., weakening of cell junctions) of the gill surface. The yellow perch (YP; *Perca flavescens*), a member of the Percidae family but one not included in the review by Spear and Pierce [1], is endemic to metal-contaminated areas. In YP, the mechanism of toxicity may be qualitatively or quantitatively different from that of RBT.

Currently, only water hardness and the total dissolved metal concentration are used in the Canadian copper water quality standards, even though it is now well known that pH, dissolved organic matter, and alkalinity also play important roles in modifying the toxic effect of copper [e.g., 3,5–12]. In an attempt to influence existing water quality standards, the acute toxicity of copper has been characterized by the accumulation of copper on the gill surface in a limited number of fish species (fathead minnow [6,7]; rainbow and brook trout [13], and RBT [14]). This relationship, termed the Biotic Ligand Model [15], is the first to take into account the influence on toxicity of both the abiotic and biotic ligands, which are present in natural waters. The abiotic ligands [e.g., $-CO_3$, dissolved organic matter (-DOM), -OH] and the biotic ligands of the fish gill surface epithelium compete for copper in natural waters. Geochemical modeling programs (e.g., Windermere Humic Aqeous Model, or WHAM; [16]) can adequately model the state of the abiotic ligands; however, modeling the behavior of the biotic ligands is more difficult, since only a few fish species have been characterized under simplified conditions. Ideally, the binding properties of the gill surface may explain variances in copper tolerance.

The main objective of the present study was to use a species comparison approach in order to understand sensitivity differences to copper, which may explain the documented difference in tolerance between these two families. We explicitly compared (1) acute copper toxicity (lethal concentrations via 96-h LC50s) to document the difference in tolerance, (2) the pattern of whole-body ion loss (Na^+) to understand the mechanism of toxicity and mechanism of copper tolerance, (3) gill–copper (Cu) binding relationships, and (4) the accumulation of copper at the gill at 50% mortality (lethal accumulations;

* To whom correspondence may be addressed (ltaylor@esg.net). The current address of L. N. Taylor is ESG International, 361 Southgate Drive, Guelph, ON, Canada N1G 3M5.

LA50s). In order to assess copper bioavailability, the comparison was completed in two distinct water types, hard water (HW) and soft water (SW), thereby incorporating the influence of abiotic ligands. In both species, the biotic ligand was characterized (i.e., affinity and saturability of the gill), and we hypothesized that species differences in toxicity would be reflected by differences in copper binding to high-affinity sites on the gill, which are the target sites used in the biotic ligand model.

MATERIALS AND METHODS

Experimental animals

Juvenile RBT (*Oncorhynchus mykiss*, 1–2 g) and YP (*Perca flavescens*, 1–2 g) were obtained from Rainbow Springs Hatchery (Thamesford, ON, Canada) and Kinmount Fish Farm (Kinmount, ON, Canada), respectively. Fish were maintained for 8 to 12 weeks in dechlorinated Hamilton tap water (HW) of moderate hardness (18°C, pH 8, 0.6 mM Na^+, 0.7 mM Cl^-, 1.0 mM Ca^{2+}, 120 mg/L as $CaCO_3$ hardness, 95 mg/L alkalinity, 3 mg C/L DOM) prior to testing. For experiments using SW (16°C, pH 7.2, 0.13 mM Na^+, 0.10 mM Cl^-, 0.13 mM Ca^{2+}, 20 mg/L as $CaCO_3$ hardness, 15 mg/L alkalinity, 0.4 mg C/L DOM), fish were acclimated for a minimum of nine weeks. Soft water (SW) was synthesized by mixing one part HW to six parts ion-reduced water, the latter produced by reverse osmosis (Anderson Water Systems, Dundas, ON, Canada). The photoperiod was set to a light/dark cycle similar to the natural photoperiod for western Lake Ontario from January through June. Fish were fed (to satiation) a dry ration of commercial trout pellets (Martins Feed Mill, Elmira, ON, Canada) daily. The feed contained approximately 3 μg Cu/g dry weight.

Experimental protocol

Acute toxicity of copper in hard and soft water. The concentrations of copper, which were lethal to 50% of the animals (i.e., 96-h LC50s), were determined in HW and SW for both species. Rainbow trout [5.68 ± 0.28 g (n = 60) for HW and 10.58 ± 0.46 g (n = 99) for SW] and YP [2.73 ± 0.18 g (n = 60) for HW and 5.27 ± 0.27 g (n = 97) for SW] were exposed at the same time and in the same tanks to each concentration in order to ensure the exactness of environmental conditions for both species. Fish (n = 8–12 in SW and n = 9–16 in HW, per species and per concentration) were exposed in a flow-through system to a minimum of five Cu concentrations plus a control. In HW and SW, the Cu concentrations ranged from 0.38 to 15.35 μM and from 0.11 to 0.77 μM, respectively. Background Cu concentrations were 46.4 ± 16.5 (12) and 10.2 ± 3.8 (9) nM in HW and SW controls, respectively. Copper stocks ($CuSO_4 \cdot 5H_2O$, Fisher Scientific, Toronto, ON, Canada) were metered into diluent water in mixing head tanks. These tanks then fed 20-L polyethylene tanks at a rate of 300 ml/min each. Tanks were individually covered, aerated, and checked for mortality daily. Mortality checks were made approximately every 1 to 2 h during the first 12 h. Upon death, fish were removed and their whole bodies placed in 1 N trace metal–grade nitric acid (Fisher Scientific) for digestion. Whole bodies were analyzed for sodium content by flame atomic absorption spectrophotometry (AAS, Varian AA-1275, Walnut Creek, CA, USA). Water samples (15 ml) were acidified with 50 μl concentrated trace metal–grade nitric acid and analyzed for Cu by either flame or graphite furnace AAS (Varian AA-1275) against certified copper standards (Fisher Scientific).

Gill–copper binding characteristics in hard and soft water. In order to determine interspecific differences in copper tolerance, we characterized the high-affinity gill–Cu binding sites using the 3-h radiolabeled ^{64}Cu technique of Taylor et al. [14]. In HW, RBT and YP were exposed for 3 h to five different nominal Cu concentrations of 0.08, 0.16, 0.24, 0.32, and 0.48 μM radiolabeled with ^{64}Cu (specific activity 92.77 to 148.05 mBq/μmol). Mean weight was 4.49 ± 0.25 g (n = 50) and 3.73 ± 0.28 g (n = 50) for RBT and YP in HW, respectively. In SW, the six nominal exposure concentrations were 0.08, 0.16, 0.24, 0.32, 0.48, and 0.80 μM radiolabeled with ^{64}Cu (specific activity 65.45 to 120.73 mBq/μmol). The mean weight was 12.72 ± 0.75 g (n = 60) and 5.82 ± 0.29 g (n = 60) for RBT and YP in SW, respectively. The McMaster Nuclear Reactor (Hamilton, ON, Canada) supplied the ^{64}Cu (as $CuNO_3$). Exposures were conducted under static conditions in Ziploc® bags containing 5 L of water. Five RBT and five YP were placed in each bag, with two bags at each concentration. Bags were individually aerated and placed in black plastic boxes. Water samples (5-ml) were taken at the beginning and end of the exposure and acidified (50 μl concentrated trace metal–grade nitric acid). After 3 h, fish were sacrificed with a blow to the head. Gill arches were removed and rinsed for 10 s in control water, and the radioactivity was determined in both the tissue and water samples using a well-type gamma counter with a 7.62-cm NaI crystal (Packard Minaxi Auto-Gamma 5000 Series, Packard Instruments, Meridan, CT, USA). All samples were counted for a maximum of 5 min unless an acceptable counting error was achieved earlier. The appearance of radioactivity in the gills allowed for the calculation of newly accumulated gill copper (i.e., with no background). Water samples were further analyzed for total copper by flame or graphite furnace AAS, as appropriate (i.e., graphite furnace was used when lower detection limits were required). All water samples were analyzed against certified copper standards (Fisher Scientific). The geochemical modeling program WHAM [16] and our water chemistry constituents were used to convert total copper concentrations into the bioavailable free cupric ion concentrations (Cu^{2+}). Input variables included all major cations and anions (Ca^{2+}, Na^+, Cl^-, Mg^{2+}, HCO_3^-, CO_3^{-2}, K^+, Cu^{2+}, SO_4^{-2}), plus DOM and pH.

The 3-h gill binding technique was also used to calculate the lethal accumulation on the gill surface at the copper concentration that caused 50% mortality at 96 h in HW (i.e., the 3-h LA50). The measured exposure concentrations in HW were 1.17 μM for RBT and 4.31 μM for YP, both of which were radiolabeled with ^{64}Cu (specific activity was 43.21 and 47.66 mBq/μmol, respectively). In SW, the LC50s for both species fell within the range used to characterize the high-affinity binding sites; therefore, we were able to calculate the LA50s from the equation of the gill–Cu binding curve at the corresponding LC50 (~0.1 and 0.44 μM for RBT and YP, respectively).

Calculations

Net sodium flux. The rate (Jnet) of sodium loss in nmol/g·h during Cu exposure was calculated based on the concentration of whole-body sodium for both species in SW and HW.

$$\text{Jnet} = \frac{\text{WB Na}_{\text{exposed}} - \text{WB Na}_{\text{control}}}{\text{time}}$$

where $\text{WB Na}_{\text{exposed}}$ is the whole-body concentration of sodium at the end of the Cu exposure in nmol/g, $\text{WB Na}_{\text{control}}$ is the mean whole-body sodium concentration of control (unex-

Table 1. Acute toxicity of copper to rainbow trout (RBT) and yellow perch (YP) in hard water (HW) and soft water (SW). Fish were acclimated to SW for at least 9 weeks prior to experimentation

Water	Toxicity test	Species	Median lethal concentration (μmol/L)	95% Confidence intervals	Mean wet wt (g)	Standard error	*n*
HW[a]	96-h-LC50	RBT	1.05	0.68–1.58	5.6	0.3	60
		YP	4.16	3.54–4.88	2.7	0.2	60
SW[b]	96-h-LC50	RBT	~0.10	—	10.6	0.5	99
		YP	0.44	0.37–0.53	5.3	0.3	97
	48-h-LC50	RBT	0.14	0.13–0.15	10.6	0.5	99
		YP	0.53	0.48–0.60	5.3	0.3	97

[a] 18°C, pH 8, 0.6 mM Na^+, 0.7 mM Cl^-, 1.0 mM Ca^{2+}, 120 mg/L as $CaCO_3$ hardness, 95 mg/L alkalinity, 3 mg C/L dissolved organic matter.
[b] 16°C, pH 7.2, 0.13 mM Na^+, 0.10 mM Cl^-, 0.13 mM Ca^{2+}, 20 mg/L as $CaCO_3$ hardness, 15 mg/L alkalinity, 0.4 mg C/L dissolved organic matter.

posed) RBT or YP in nmol/g, and time is the amount of time (in hours) over which the fish was exposed to copper (i.e., until death or until sacrificed at the end of 96 h).

Gill–Cu binding. Newly accumulated copper concentrations in the gill (nmol/g) were calculated based on the appearance of radioactivity in the gill.

$$Cu_{gill} = \frac{^{64}Cu_{gill}}{SA}$$

where $^{64}Cu_{gill}$ is the radioactivity of the gill arches in counts per minute per gram of wet gill tissue and SA is the mean measured specific activity of the water in cpm/nmol.

Statistics

All data presented are means ± 1 standard error (*n*), except for the LC50 data, in which means and 95% confidence limits are given. LC50 values were determined by linear functions relating the log concentration of copper to probit transformation of percent mortality (TOXSTAT, Version 3.5, Western EcoSystems Technology, Cheyenne, WY, USA). All other data were analyzed for statistical significance by a Student's *t* test (two-tailed, unpaired). Significance was set at $p < 0.05$ unless otherwise stated. Percent data was transformed (arcsin[$\sqrt{}$]) in order to attain a normal distribution, producing asymmetrical error bars. Gill binding saturation curves were fitted by nonlinear regressions using Sigma Plot 2000® (SPSS, Chicago, IL, USA).

RESULTS

Acute toxicity of copper

In comparing the 96-h LC50 values for both species, copper was approximately four times more toxic to juvenile RBT than to YP in HW (Table 1). We were unable to accurately calculate a 96-h LC50 in SW for RBT in the present study because of the high mortality at the low copper concentrations, but it was approximately 0.1 μM. We were, however, able to calculate 48-h LC50 values in SW for both species, which revealed under this circumstance that copper was four times more toxic to RBT.

Sodium loss due to copper exposure

Regardless of water chemistry, the control whole-body sodium concentrations of RBT were significantly higher than those of YP ($p < 0.05$). Whole-body sodium concentrations from control RBT and YP in HW were 61.56 μmol/g ± 7.63 (7) and 46.73 μmol/g ± 0.98 (9), respectively, and were 55.90 μmol/g ± 0.64 (10) and 40.10 μmol/g ± 0.73 (10) in SW, respectively. In both cases, whole-body sodium concentrations were significantly lower for YP ($p < 0.001$).

In both species upon exposure to copper, whole-body sodium decreased with increasing copper concentrations. In those circumstances in which there was 100% mortality, YP and RBT lost approximately 60% of their initial whole-body sodium concentration. However, the Cu concentration at which this level was reached was different between the two species (Fig. 1). In SW, this occurred at water Cu concentrations of 0.11 and 0.91 μM for RBT and YP, respectively. In contrast, the Cu concentrations were 8.39 μM for RBT and 15.34 μM for YP in HW. Rainbow trout had higher mortality and hence greater sodium loss at lower Cu concentrations than did YP. The sodium loss threshold for toxicity was defined as the percentage of loss occurring at the concentrations that produced 50% mortality (i.e., the LC50). In SW, based on the 48-h LC50 values for RBT, the sodium loss threshold was approximately 30%. For YP in SW at their 96-h LC50, the threshold was approximately 40%. These thresholds were the same in HW, where RBT and YP lost approximately 30 and approximately 40% of their whole-body sodium levels at their respective 96-h LC50 concentrations.

Overall, the rate of sodium loss was much greater in SW than in HW and was dependent on Cu concentration in both species (i.e., larger rates of loss at higher Cu concentrations; Fig. 2). In soft and HW, over the full range of Cu concentrations tested, YP had significantly lower rates of sodium loss than did RBT. For example, in SW, the maximum rate of loss in YP was 3,000 nmol Na^+/g·h, whereas it was 5,000 nmol Na^+/g·h for RBT. In HW this maximum was 1,500 and 2,300 nmol Na^+/g·h for perch and trout, respectively. At the threshold for toxicity (i.e., at their LC50), the rate of sodium loss in SW was lower for YP than for RBT; however, this was not the case in HW. The toxicity threshold rate of sodium loss in SW was approximately 250 and approximately 750 nmol/g·h for YP and RBT, respectively. In HW, the rate for perch was approximately 400 Na^+/g·h and only approximately 250 nmol Na^+/g·h in RBT.

Gill–copper binding characteristics

In the gill binding experiment, the two replicate bags were compared to each other before combining the data (i.e., all were not significantly different from each other, $p > 0.05$). The binding of copper to the gills increased with increasing copper exposure concentrations in both HW and SW (Fig. 3).

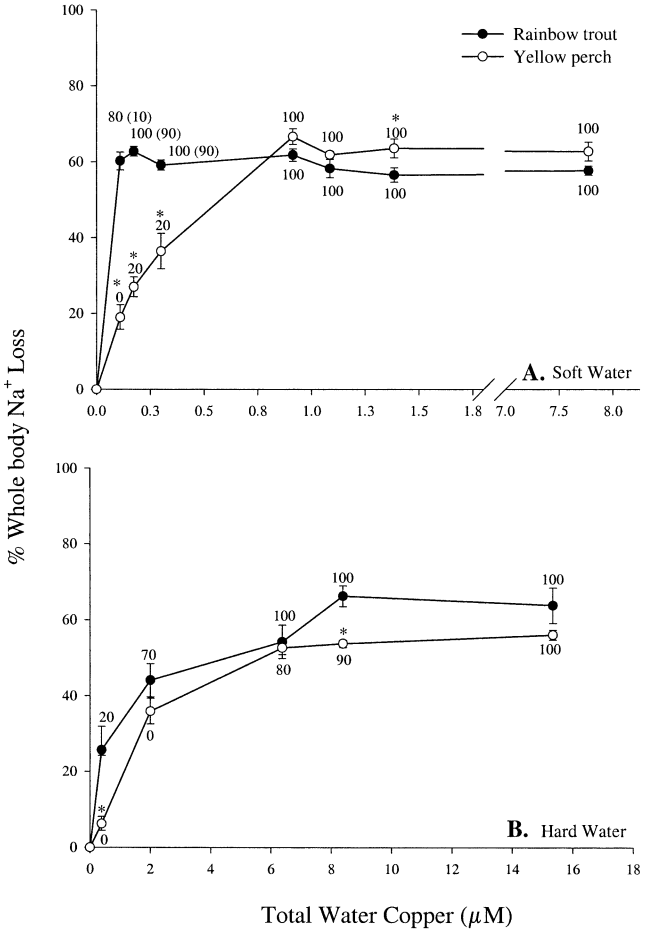

Fig. 1. Whole-body sodium loss (expressed as a percent of control levels) in rainbow trout (RBT) and yellow perch (YP) exposed to various copper (Cu) concentrations during an acute toxicity challenge in (**A**) soft water (SW) and (**B**) hard water (HW). Fish were sampled upon death or at 96 h. In HW, $n = 8$ to 12, and in SW, $n = 9$ to 16. The asterisk indicates significant difference between RBT and YP at that Cu concentration ($p < 0.05$). Numbers at each data point represent the percent mortality at 96 h (or 48 h).

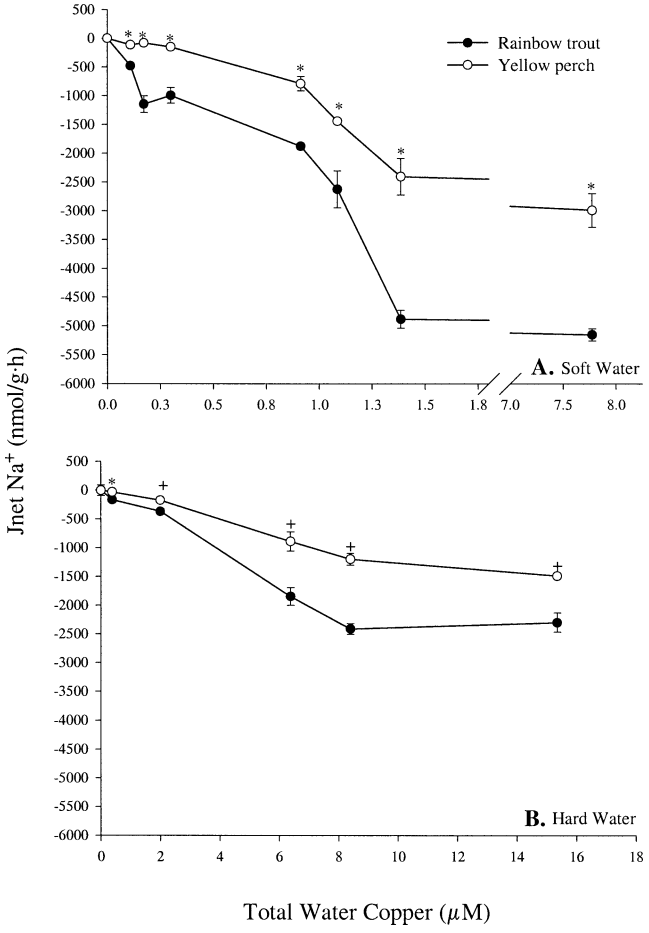

Fig. 2. The net rate of sodium loss (Jnet) in rainbow trout (RBT) and yellow perch (YP) exposed to various copper (Cu) concentrations during an acute toxicity challenge in (**A**) soft water (SW) and (**B**) hard water (HW). Fish were sampled upon death or at 96 h. In HW, $n = 7$ to 12, and in SW, $n = 9$ to 16. The asterisk and plus signs indicate significant differences between RBT and YP at that Cu concentration (* = $p < 0.001$ in SW, $p < 0.05$ in HW; + = $p < 0.01$).

In SW, there was no significant difference in gill–copper binding between the two species at any concentration (Fig. 3A). However, in HW, there was a trend toward lower copper binding in RBT than in YP; this trend was significant at 0.33 μM (Fig. 3B). In SW, gill–copper binding reached an apparent plateau between 0.28 and 0.42 μM total Cu (since gill–Cu concentrations returned to those found at lower water Cu concentrations), but then sharply increased with gill–Cu concentrations reaching three times the plateau value at approximately 0.61 μM total Cu. For RBT in HW, the pattern was similar, with the plateau around 0.24 to 0.33 μM (i.e., no significant difference in newly accumulated gill Cu over these water Cu concentrations, $p > 0.05$), and the increase at 0.47 μM total water Cu ($p < 0.01$). This was not the case for YP in HW, where the gills bound Cu in a linear fashion (Fig. 3B, $y = 14.17x$, $r^2 = 0.96$, $p < 0.01$). The calculated LA50 values for the two species in SW were 0.2 and 1.7 nmol/g for RBT and YP, respectively. These values were calculated from the equation of the saturation curve, since the 96-h LC50 values were within the range of copper concentrations used in the 3-h gill binding assays. In striking contrast, the measured LA50 concentrations in HW were 3.1 and 27.8 nmol/g for RBT and YP at their 96-h LC50 concentrations, respectively.

DISCUSSION

The present study explicitly tested and answered three important questions. First, is the mechanism of copper toxicity different between RBT and YP? Second, what is the basis for the difference in tolerance between the two species? Third, does gill–copper binding predict the difference in tolerance? Our results show that the mechanism of copper toxicity is apparently not different between RBT and YP. Specifically, the threshold for toxicity was reached when both species lost 30 to 40% of their whole-body Na^+, and at 60% loss there was complete mortality. As for the mechanism of tolerance, clearly YP possess an ability to resist Na^+ loss; however, this resistance was not a result of binding less copper at their gills. Interestingly, we found that the gills of YP were able to bind the same amount of Cu as the gills of RBT in SW, and they were able to bind slightly more copper than RBT in HW. In summary, there was only a very small difference in gill–copper binding despite a very large difference in toxicity.

Species differences in acute copper toxicity

We have demonstrated that YP are more tolerant to waterborne copper; however, they are not nearly as tolerant as predicted from the 96-h LC50 equation developed by Spear and

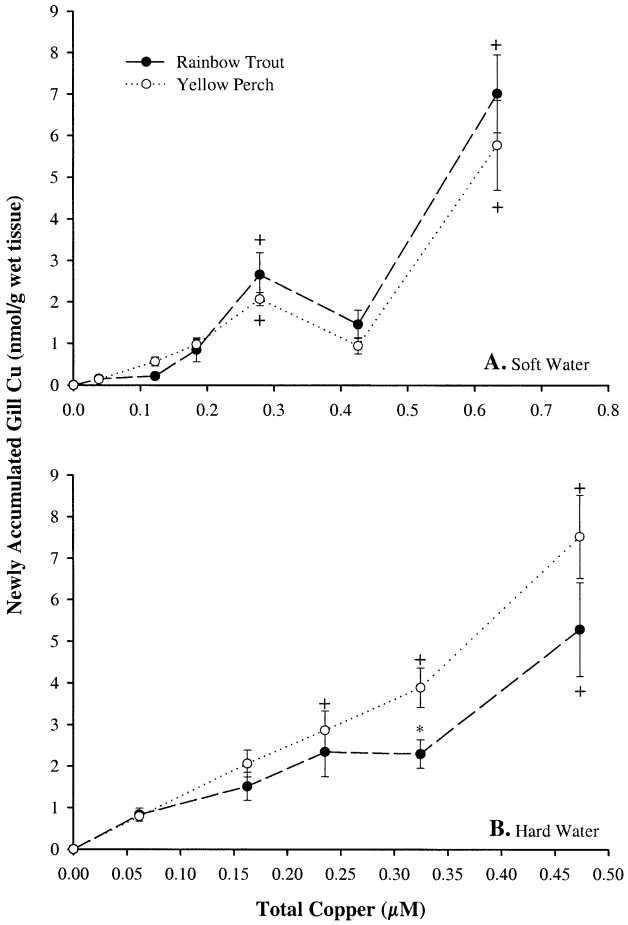

Fig. 3. The short-term (3-h) binding of copper (Cu) to the gill in juvenile rainbow trout (RBT) and yellow perch (YP) in (**A**) soft water and (**B**) hard water. Data represents means ± 1 standard error (n = 10). The asterisk indicates a significant difference between RBT and YP at that copper concentration, whereas a plus sign indicates a significant increase in newly accumulated gill Cu from the first measurement, within the species type ($p < 0.05$).

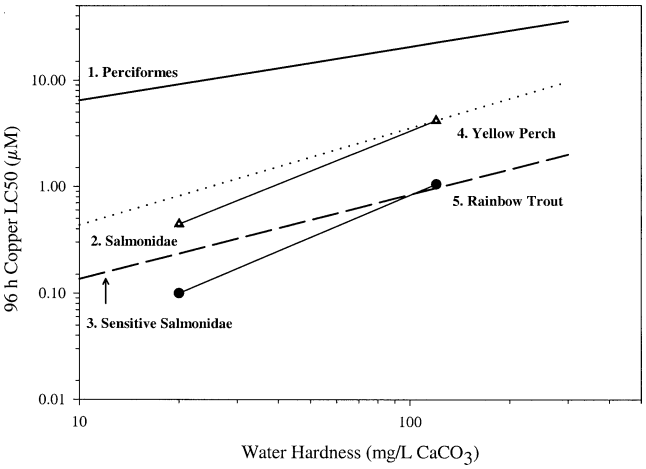

Fig. 4. The effect of water hardness on acute toxicity (96-h lethal concentration; LC50) of copper (Cu) to Salmonids and Perciformes. Perciformes data are representative of six bluegill, one striped bass, and four pumpkinseed tests (line 1 [1]). Salmonidae (line 2 [1]) and sensitive Salmonidae (line 3, *Onchorhynchus kisutch* and *Onchorhynchus tshawytscha*, [1]) are shown in relation to yellow perch and rainbow trout (lines 4 and 5, respectively) from the present study.

Pierce [1] for perchlike fishes belonging to the order Perciformes (which is represented by line 1 in Fig. 4). This equation was based on 11 studies using bluegills (*Lepomis marchochirus*), striped bass (*Roccus saxatilis*), and pumpkinseeds (*Lepomis gibbosus*) over a range of water hardnesses (i.e., 10 to 300 mg/L as $CaCO_3$; line 1 in Fig. 4), but these studies did not include data for YP. Also inconsistent with the general Perciformes data was the reported difference in toxicity between HW and SW. In our study, YP were approximately 9 times more sensitive in SW than in HW, whereas the Perciformes equation predicts only a threefold difference (as represented by our steeper slope, line 1 vs line 4 in Fig. 4). In fact, our YP toxicity data were more comparable to the Salmonidae values summarized in the same report (line 2 in Fig. 4; [1]).

The toxicity of copper to RBT in the present study confirms previous findings in our laboratory [14], which found our RBT to be comparable to the Spear and Pierce [1] sensitive Salmonidae data derived from two species of Pacific salmon (see lines 3 and 5 in Fig. 4). The toxicity equations of Spear and Pierce [1] predict a larger species difference in SW than in HW (Perciformes 11 times more tolerant in SW and 5 times more tolerant in HW). In contrast, we found a similar difference in HW and SW (YP were ~4 times more tolerant than RBT). A possible reason for this difference may be the historical SW data upon which the toxicity equations were based, in which it was generally accepted that two weeks was sufficient acclimation time (e.g., [17,18]). Previously we have argued against this concept, noting that two weeks was too short a period of time to fully acclimate [14]. We defined fully acclimated as that point at which whole-body sodium concentrations in SW were equal to the HW values [14]. In the present study, this definition applied to RBT; however, YP sodium concentrations were still different after a minimum of nine weeks of SW acclimation. This may explain why the difference in toxicity was smaller in SW than in HW between the two species. The importance of SW acclimation becomes extremely relevant when evaluating a toxicant, like copper, which disrupts ionoregulation.

Mechanism of copper toxicity

The mechanism of acute copper toxicity in RBT involves a concentration-dependent stimulation of Na^+ efflux and inhibition of Na^+ uptake at the gill [2]. The present study demonstrates that the same mechanism applies for YP. The biomarker used in this study to reflect this toxic action was whole-body sodium. The loss of whole-body sodium (expressed per gram of wet mass, rather than dry mass) has been reported as a sensitive indicator of ionoregulatory disruption resulting from exposure to mine-polluted waters [19,20]. Indeed, Croke et al. [21] compared sodium loss due to copper exposure at two different water calcium concentrations in RBT and fathead minnows and found that a 20 to 30% loss of whole-body sodium was the threshold for lethality. In that study, fathead minnows were the more sensitive species with regard to waterborne copper toxicity, and they showed greater rates of sodium loss, which was even more pronounced in the low-calcium water [21]. Similarly, we found a 30 to 40% loss in whole-body sodium at the toxicity threshold concentrations for RBT and YP, and the finding of an increased rate of sodium loss in the more sensitive species was duplicated in the present study.

Mechanism of copper tolerance

There are two possibilities, based on the result of whole-body sodium loss, by which greater copper tolerance may exist in different fish species: This tolerance may occur because the fish resists sodium loss (i.e., loss occurs, but at a higher Cu concentration) or because the fish tolerates sodium loss (i.e., the loss still occurs but with no mortality). Based on our findings, the first option apparently applies to YP. Yellow perch were able to resist sodium loss, since the same threshold for mortality (i.e., the loss of 20–40% whole-body sodium) occurred at a higher copper concentration than for RBT. The question of how YP are able to resist sodium loss due to copper exposure is an area that requires future research.

Clearly, YP have a higher threshold for the damage, which leads to sodium loss. Freda and McDonald [22] and McDonald et al. [23] characterized morphological differences between YP and RBT gills in the context of low pH (a toxicant that is often compared to copper because of the similar mechanisms of toxicity involved with the two toxicants). They attributed the ability of YP to resist net ion loss to the larger depth of tight junctions in the gills and to a reduced chloride cell proliferation. It is likely the YP may also use these physical differences to resist ion loss due to copper. Aside from morphological differences, perch also maintain lower whole-body sodium concentrations than do RBT, thereby reducing the Na^+ concentration gradient between the internal and external environment. The ability of the gills to prevent ion loss, thus providing tolerance, may also be the result of copper binding to the gills.

Gill–copper binding

Copper binding to the gills has been directly correlated with toxicity [13]. Therefore, it was not unreasonable to hypothesize that fish differing in their sensitivity to copper may have different gill–metal binding properties. At present, the Biotic Ligand Model predicts toxicity based on the rapid binding of copper on the gill [15]. The evaluation of gill–copper binding characteristics traditionally requires the conversion of total copper to free copper (i.e., Cu^{2+} ions). This was accomplished using the geochemical modeling program WHAM [16], which effectively models the interactions of metals with natural DOM (P.G.C. Campbell, Universite du Quebec, INRS-Eau, Ste-Foy, QC, Canada, personal communication). Previously in our laboratory we identified at least two types of binding sites in RBT in HW and SW [14]. Based on this result, we can characterize the binding of free copper to sites on the gill in the same manner (Fig. 5 [data from Fig. 3]). The fact that these two types of sites were also present in YP at the same copper concentrations was a novel finding. The saturable gill–copper binding sites can be described as high affinity and low capacity; beyond these, at 0.4 to 0.5 μM total copper, a second set of sites may exist (see data points beyond the saturation curves in Fig. 5). In SW, there was no statistical difference between RBT and YP newly accumulated gill copper; therefore, only one curve was fitted to the data. The maximum number of high-affinity binding sites (B_{max}) for both species was calculated at 1.88 nmol/g wet gill tissue. In contrast, YP saturated at slightly higher gill–copper concentrations than did RBT in HW. The B_{max} values for high-affinity binding sites in RBT and YP were 3.63 and 9.01 nmol/g of wet gill tissue, respectively. The affinity of these sites for free Cu^{2+} ions was distinctly different between the two water chemistries. In SW, the log*K* was 8.4 for both species, whereas in HW, the affinity had increased to log*K* values of 9.9 and 9.5 for trout and perch, respectively.

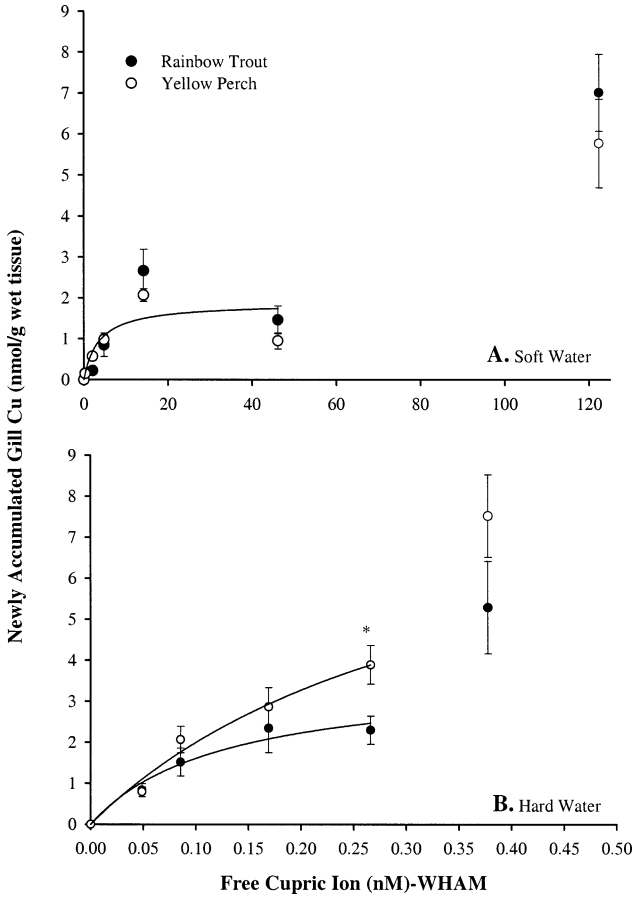

Fig. 5. The short-term (3-h) binding of free cupric ions to the gill in juvenile rainbow trout (RBT) and yellow perch (YP) in (**A**) soft water (SW) and (**B**) hard water (HW). Characterization of the saturable binding sites was by nonlinear regression; $r^2 = 0.67$ ($p < 0.05$) in SW for both species, and in HW, $r^2 = 0.97$ ($p < 0.01$) for RBT and $r^2 = 0.98$ ($p < 0.01$) for YP. The binding capacity (B_{max}) and affinity (log*K*) for cupric ions in SW was 1.88 nmol/g and 8.4 for both RBT and YP. In HW, B_{max}s were 3.63 and 9.01 nmol/g for RBT and YP, respectively. The affinity of the gill for cupric ions in HW was 9.9 and 9.5 for RBT and YP, respectively. Data represents means ± 1 standard error ($n = 10$). Free cupric ion concentrations were determined using the geochemical modeling program Windermere Humic Aqueous Model (WHAM). The asterisk indicates a significant difference between RBT and YP at that copper concentration ($p < 0.05$).

As in the case of ion loss, there are at least three explanations for differential copper tolerance, based on gill–copper binding characteristics; They are: (1) the gill may be less attractive to copper (i.e., a decrease in affinity [log*K*]), (2) the gill may saturate at a lower Cu^{2+} ion concentration (i.e., a decrease in capacity [B_{max}]), or (3) copper can accumulate in the gill but is not biologically reactive (e.g., through nonspecific binding and increased storage and elimination processes). Based on our findings, the first explanation may not apply. The affinity of the perch gill surface for cupric ions was lower than that for trout in HW; however, in SW, the affinity was equal between the two species. This does not agree qualitatively with the toxicity data, where the difference in toxicity was similar in HW and SW. The second reason explaining tolerance involves the saturation concentration of the gill. In SW, the binding capacity was equal in both species, and in HW, the perch gill Cu capacity was only somewhat higher than

that of RBT, an argument against the second hypothesis. It is this last result that leads us to believe the third option may also apply to YP in HW. The copper was binding to the gill but must not have been biologically reactive, since it would not have had a negative impact on survival (i.e., the toxic range is far beyond the characterized sites in HW). MacRae et al. [13] also used a species comparison approach to evaluate gill–copper binding characteristics between RBT and brook trout (*Salvelinus fontinalis*) in SW. Brook trout were more tolerant to copper toxicity than were RBT, and the reduced sensitivity may have been related to a significantly lower affinity for copper (log*K* values were 7.14 and 7.56, also based on nonlinear regression). The SW log*K* for RBT in MacRae et al. [13] was lower than our log*K* (7.56 vs 8.4) and was likely the result of three factors, the first being a finer resolution in our study using radiolabeled ^{64}Cu (and consequently starting at a zero background); the second being the ability to work at lower total Cu concentrations (<0.16 μM), thereby reaching higher affinity sites, and the third being that MacRae et al. [13] reported 24-h gill binding, compared to the present 3-h gill binding time interval. The evaluation of binding capacity in MacRae et al. [13] revealed that brook trout had a significantly higher B_{max} than the RBT (63 vs 29 nmol/g, including background), which supports the notion that copper can bind to the gill but not necessarily be toxic. The possibility that a surplus of copper can bind to the gill but exhibit no toxic effect is one not currently taken into consideration by the Biotic Ligand Model, and this possibility affords an avenue for future research.

The accumulation of new copper on the gills at the LC50 concentration (defined as the 3-h LA50) provided toxicologically relevant information with which to compare the two species. In other words, the amount of copper bound to the gill in 3 h would be predictive of the percent mortality at 96 h. The calculated LA50 values for the two species in SW were 0.2 and 1.7 nmol/g for RBT and YP, respectively. These values were calculated from the equation of the saturation curve, since the LC50 values fell within the tested range. In striking contrast, the measured LA50 concentrations in HW were 3.1 and 27.8 nmol/g for RBT and YP at their LC50 concentrations, respectively. In comparison to their B_{max} values, the LA50 in SW occurred when approximately 11 and 90% of the sites were filled for RBT and YP, respectively. For YP in HW, the LA50 occurred beyond the characterized set of saturable sites. In other words, 100% of the high-affinity, low-capacity sites are filled, and in addition, an unknown percentage of the lower affinity, higher capacity sites were also filled.

MacRae et al. [13] measured gill–copper binding at 24 h during a 120-h (5-d) toxicity test. For brook trout and RBT in SW, the 24-h LA50 was 22 nmol/g, which included the background level of copper. If the background concentration of 12 nmol/g [5,13] is subtracted from this value, we can compare it to our newly accumulated 3-h LA50 for RBT and YP (i.e., 22–12 = 10 nmol/g). For both species in SW, our 3-h LA50 was approximately 0.2 to 2 nmol/g, which is approximately 2 and 20% of the MacRae et al. [13] 24-h LA50 of 10 nmol/g. In HW, the RBT 3-h LA50 was one third of 10 nmol/g, whereas the YP 3-h LA50 was three times higher, at 28 nmol/g. According to the Biotic Ligand Model, the gill LA50 for a particular species should occur at the same concentration independent of DOM, Ca^{2+} concentrations, or pH [15]. Overall, the gill binding characteristics were the same in SW between RBT and YP, and the difference in copper toxicity was small. In HW, the gill binding characteristics were only slightly different, and the toxicity difference was large. More importantly, the LA50s and LC50s were consistently different between the two species (i.e., YP LA50s and LC50s were nine and four times higher than similar values in RBT, respectively, in SW and HW).

CONCLUSIONS

The species comparison approach used in the present study is an effective way to distinguish possible mechanisms of copper tolerance. The resistance of sodium loss in YP may be attributable to differences in gill morphology (i.e., permeability) and reduced sodium gradients between the fish and its environment. The binding of copper to the gill did explain toxicity qualitatively but not quantitatively, which may have important implications for the acute Biotic Ligand Model. In our study, we show that burden does not necessarily translate into toxicity, but more likely the surplus Cu is dealt with by effective detoxification, storage, and elimination mechanisms. The influence of water chemistry, particularly SW acclimation, on the binding properties of the gill demonstrates the dynamic nature of the gill in maintaining ionoregulatory homeostasis. This type of gill behavior will become a key issue in the future development of the chronic Biotic Ligand Model, especially since the process of SW acclimation may only be relevant to fish kept in the laboratory rather than in their natural environment.

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