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Exposure to waterborne Cu inhibits cutaneous Na^+ uptake in post-hatch larval rainbow trout (*Oncorhynchus mykiss*)

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ABSTRACT

In freshwater rainbow trout (*Oncorhynchus mykiss*), two common responses to acute waterborne copper (Cu) exposure are reductions in ammonia excretion and Na^+ uptake at the gills, with the latter representing the likely lethal mechanism of action for Cu in adult fish. Larval fish, however, lack a functional gill following hatch and rely predominantly on cutaneous exchange, yet represent the most Cu-sensitive life stage. It is not known if Cu toxicity in larval fish occurs via the skin or gills. The present study utilized divided chambers to assess cutaneous and branchial Cu toxicity over larval development, using disruptions in ammonia excretion (J_{amm}) and Na^+ uptake (J_{in}^{Na}) as toxicological endpoints. Early in development (early; 3 days post-hatch; dph), approximately 95% of J_{amm} and 78% of J_{in}^{Na} occurred cutaneously, while in the late developmental stage (late; 25 dph), the gills were the dominant site of exchange (83 and 87% of J_{amm} and J_{in}^{Na}, respectively). Exposure to 50 μg/l Cu led to a 49% inhibition of J_{amm} in the late developmental stage only, while in the early and middle developmental (mid; 17 dph) stages, Cu had no effect on J_{amm}. J_{in}^{Na}, however, was significantly inhibited by Cu exposure at the early (53% reduction) and late (47% reduction) stages. Inhibition at the early stage of development was mediated by a reduction in cutaneous uptake, representing the first evidence of cutaneous metal toxicity in an intact aquatic organism. The inhibitions of both J_{amm} and J_{in}^{Na} in the late developmental stage occurred via a reduction in branchial exchange only. The differential responses of the skin and gills to Cu exposure suggest that the mechanisms of J_{amm} and J_{in}^{Na} and/or Cu toxicity differ between these tissues. Exposure to 20 μg/l Cu revealed that J_{amm} is the more Cu-sensitive process. The results presented here have important implications in predicting metal toxicity in larval fish. The Biotic Ligand Model (BLM) is currently used to predict metal toxicity in aquatic organisms. However, for rainbow trout this is based on gill binding constants from juvenile fish. This may not be appropriate for post-hatch larval fish where the skin is the site of toxic action of Cu. Determining Cu binding constants and lethal accumulation concentrations for both skin and gills in larval fish may aid in developing a larval fish-specific BLM. Overall, the changing site of toxic action and physiology of developing larval fish present an interesting and exciting avenue for future research.

1. Introduction

Copper (Cu) is both an essential element and a persistent contaminant in aquatic systems that has toxic effects at elevated levels. Like most metals, Cu elicits toxicity by first binding to a ligand on the surface of aquatic organisms, usually considered to be located on the gills in fish, and acting either directly on the ligand or entering the organism and acting on downstream target organs (reviewed by Grosell, 2012). Cu exposure leads to a wide array of toxic effects including alterations in behaviour, olfaction, ionoregulation, nitrogenous waste excretion, and swimming performance (see Grosell, 2012). However, the key lethal mechanism of action of Cu exposure in freshwater fish is believed to be the reduction of whole-body Na^+ levels, caused by an inhibition of branchial Na^+ uptake (Lauren and McDonald, 1985, 1987a,b; Grosell and Wood, 2002). This understanding has led to the development of a Biotic Ligand Model (BLM) for the prediction of waterborne Cu toxicity (MacRae et al., 1999; Di Toro et al., 2001; Santore et al., 2001; Paquin et al., 2002; Niyogi and Wood, 2004).

Since Cu is potentially bioavailable in the environment to all aquatic organisms, a number of studies have focused on

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determining the most Cu-sensitive species and life stages (see Grosell, 2012). In general, it appears that aquatic invertebrates represent the most sensitive group of species (e.g., Santore et al., 2001), likely due to the small size of these animals. Indeed, animals with smaller sizes tend to have higher Na^+ turnover rates and, therefore, overall greater sensitivity to Cu (Grosell et al., 2002). In agreement, early life stages of fish, being smaller in size, appear to be overall more sensitive to Cu than fish in later life stages (McKim et al., 1978; Rombough and Garside, 1982).

In larval fish, it is unclear if Cu elicits its toxic effects exclusively at the gills or if the skin is also sensitive to exposure to Cu. Immediately following hatch, the branchial structures are undeveloped, having no lamellae and only small filament buds, leading to an overall small gill surface area (Gonzalez et al., 1996; Rombough, 1999). As such, normal branchial functions, such as ionoregulation, gas transfer, and nitrogenous waste excretion (see Evans et al., 2005) are minimal. Instead, larval fish utilize cutaneous surfaces for the bulk of physiological exchanges with the environment. Indeed, in rainbow trout (*Oncorhynchus mykiss*) the skin is known to be the primary site for oxygen uptake, Na^+ uptake, and ammonia excretion immediately following hatch (Wells and Pinder, 1996; Fu et al., 2010; Zimmer et al., 2014). As fish develop, the gills become functional and eventually become responsible for the majority of exchanges (Fu et al., 2010; Zimmer et al., 2014). Given that the primary site for Cu binding is generally considered to be the gill in fish (e.g., MacRae et al., 1999), Cu toxicity might be predicted to be a function of gill surface area. However, in larval topsmelt (*Atherinops affinis*), McNulty et al. (1994) could not attribute Cu sensitivity specifically to changes in gill surface area as both gill and skin surface areas increased over development, similar to previous observations in rainbow trout (Rombough, 1999). Conversely, the increase in sensitivity which occurs over larval development in fish (e.g., Chapman, 1978; McNulty et al., 1994) may be a function of mitochondrial rich cell (MRC) numbers which increase rapidly in the gills following hatch and are also present on skin surfaces of post-hatch fish (Rombough, 1999).

The goal of the present study was to determine the site-dependent responses (gills versus skin) to waterborne Cu exposure in larval rainbow trout over development, using the inhibitions of ammonia excretion (J_{amm}) and Na^+ uptake (J_{in}^{Na}) as toxicological endpoints. These endpoints were chosen because disruptions in both Na^+ and ammonia balance are two of the most commonly observed effects of Cu exposure in freshwater fish (see Grosell, 2012 for review), the two processes are thought to be mechanistically linked, at least in adult trout (Tsui et al., 2009; Wright and Wood, 2009), and both J_{amm} (Zimmer et al., 2014) and J_{in}^{Na} (Fu et al., 2010; Zimmer et al., 2014) are known to progress from the skin to the gills during larval development over a similar time frame.

Larval trout were exposed to two sublethal concentrations of waterborne Cu at three stages of development following hatch but prior to complete yolk sac absorption to determine the effects of Cu on whole-body J_{amm} and J_{in}^{Na}. Additional experiments were conducted to determine gill and skin-specific responses to Cu exposure using divided chambers identical to those used in previous studies (Fu et al., 2010; Zimmer et al., 2014), which allowed for the estimation of cutaneous and branchial fluxes. We hypothesized that if Cu exposure inhibited either J_{amm} or J_{in}^{Na} immediately following hatch, that this would occur via blockade of cutaneous exchanges as the skin accounts for the majority of both processes at this stage (Fu et al., 2010; Zimmer et al., 2014). Alternatively, Cu may elicit its effects solely via the gills, inhibiting the small proportion of exchange occurring branchially at this stage. If the skin proves to be responsive to waterborne Cu exposure, this may have important implications for the development of a BLM for the prediction of Cu toxicity (Di Toro et al., 2001) which is specific for larval fish.

2. Materials and methods

2.1. Fish

Rainbow trout (*Oncorhynchus mykiss*) were obtained by donation from the Vancouver Island Trout Hatchery in Duncan, BC, Canada. Trout were received at three developmental stages, eyed embryos (230 accumulated thermal units (ATUs)), post-hatch larvae (420 ATUs), and larvae near-yolk sac absorption (349 ATUs). All fish were held in soft, dechlorinated Vancouver Metro tap water (in mM: Na^+, 0.06; Cl^-, 0.05; Ca^{2+}, 0.03; Mg^{2+}, 0.007; K^+, 0.004; dissolved organic carbon (DOC), 2–3 mg C/l; alkalinity, 3.0 mg/l $CaCO_3$; hardness, 3.3 mg/l $CaCO_3$; pH 7.0) at 10 °C at the University of British Columbia in Vancouver, BC. Approximately 200 trout embryos or larvae of each developmental stage were maintained in 3 separate 3-l containers with aerated, dechlorinated Vancouver tapwater exchanged daily. Fish were never fed exogenously and were held in these conditions for at least 5 days prior to experimentation. Experiments were performed on fish at the following developmental stages, referred to subsequently as: early: 3 days post-hatch (dph) (330 ATUs); mid: 17 dph (470 ATUs); and late: 25 dph (550 ATUs).

2.2. Experimental Design

2.2.1. Series 1

An initial series of experiments was conducted to determine the effects of sublethal Cu exposure (50 μg/l Cu as $CuSO_4$, nominal concentration) on whole-body ammonia excretion (J_{amm}) and Na^+ uptake (J_{in}^{Na}) at the different stages of larval development. Fish in the early, mid, and late developmental stages were placed individually in small (25 ml) plastic beakers containing 5 ml (early and mid) or 10 ml (late) water containing nominal 0 (control) or 50 μg/l Cu. Fish were pre-exposed to these conditions for a 3-h period to allow for the full inhibitory effects of Cu prior to flux measurements; water was aerated for the duration of this period. Following the pre-exposure period, ^{22}Na (Amersham Pharmacia Biotech Inc.) was added to the water to a concentration of 0.05 μCi/ml and allowed to mix via aeration for 5 min, after which an initial 1-ml sample was taken for the determination of ^{22}Na radioactivity, [Na], and total ammonia levels (T_{amm}) (see below). Following a 1.5-h flux period, a final 1-ml sample was then taken and fish were euthanized with a lethal dose of neutralized MS-222, rinsed 3 times with 5 mM NaCl to removed surface-bound isotope, weighed, and counted for ^{22}Na radioactivity. A 0.25-ml aliquot of each water sample was used for counting ^{22}Na radioactivity and measuring [Na], while the remaining sample was frozen at −20 °C until later T_{amm} analyses. All subsequent experiments followed the same 3-h pre-exposure, 1.5-h flux exposure protocol.

2.2.2. Series 2

A separate set of control experiments was performed to assess the effects of clove oil anaesthesia which was used in Series 3 (see below) to calm larvae in divided chambers. These experiments were performed in the exactly same manner as those described for the divided chamber protocol in Series 3 except that fish were left in the pre-exposure containers for the 1.5-h flux measurement period, not loaded into divided chambers. In these experiments only nominal 0 and 50 μg/l Cu concentrations were tested; all other experimental protocols followed those described below for the divided chamber experiments in Series 3.

2.2.3. Series 3

A third set of experiments was conducted to assess the effects of exposure to 20 and 50 μg/l Cu on branchial and cutaneous J_{amm} and J_{in}^{Na}. The experimental series utilized a divided chamber system

similar to those employed previously (Fu et al., 2010; Zimmer et al., 2014). These chambers require fish to be lightly anaesthetized in order to prevent struggling and experimental stress. As such, fish from the early, mid, and late developmental stages were initially anaesthetized using a 50–100 mg/l clove oil solution to reach stage 3 anaesthesia (McFarland, 1959). Following this, fish were placed in small plastic beakers (25 ml) containing 5 ml (early and mid) or 10 ml (late) of aerated dechlorinated tapwater containing 10 mg/l clove oil to maintain anaesthesia, and nominal 0 (control), 20, or 50 μg/l Cu. Fish were exposed to these media for 3 h and were then loaded into divided chambers, comprising two half-chambers separated by a latex dam. The fish was pushed through a small hole in the dam such that its head, opercula, and pectoral fins were separated spatially from the rest of its body. The latex dam and fish were then loaded between the two 5-ml half-chambers which were secured together using elastic bands. During this process, fish were kept submerged at all times in "loading containers" (plastic wash basins) containing 5-l of water dosed with 10 mg/l clove oil and the same nominal Cu concentrations used in the corresponding pre-exposure. Both half-chambers were filled completely with the corresponding exposure medium and fitted with aeration lines. The fish were allowed to adjust to this set up for 15 min prior to experimentation.

Following this settling period, ^{22}Na was added to either the anterior (for determining anterior J_{in}^{Na}) or posterior (for determining posterior J_{in}^{Na}) half-chamber to a final concentration of 0.05 μCi/ml and, after 5 min of mixing by aeration, initial 1-ml samples were taken from both half-chambers. Note that it was necessary to add the isotope to only one half-chamber per flux (i.e., anterior or posterior, not both) as uptake was calculated as ^{22}Na appearance in the fish, and sampling of the non-labelled chamber was performed to detect leaks (see below). Following a 1.5-h flux period, final anterior and posterior samples were taken and fish were removed from the chambers and allowed to recover in dechlorinated tap water for a maximum of 5 min. This was done in order to verify that fish were able to fully recover from anaesthesia and divided chamber treatments within 5 min, which was a requisite for an acceptable flux. Fish were then euthanized with a lethal dose of neutralized MS-222, rinsed 3 times with 5 mMNaCl to remove surface-bound isotope, weighed, and counted for ^{22}Na radioactivity. Another requisite for acceptable flux was a <10% isotope leak from the loaded chamber to the unloaded chamber. In cases where fish did not recover from experimentation within 5 min, or there was more than 10% isotope leakage, data were rejected. The success rate of these divided chamber experiments was, on average, 85% across all developmental stages and treatments. Water samples from these experiments were handled in the same manner as those from the experiments conducted in Series 1.

In all experiments, final water samples were taken for the measurement of dissolved Cu concentrations. 1-ml samples were extracted from the final water volume (in Series 3, samples were taken from the non-isotope loaded side) and were filtered through 0.45 μm membrane filters (Acrodisc syringe filters, Pall Corporation, USA). These samples were then acidified to 1% HNO_3 and stored at 4 °C until further analysis. Water samples for the measurement of dissolved organic carbon concentrations (30 ml; filtered using 0.45 μm membrane) were taken from the water source used throughout the experiments periodically over the entire study period. These samples were stored at 4 °C for a maximum of 2 months prior to analysis.

2.3. Analytical techniques and calculations

Water samples were analyzed for water total ammonia concentration (T_{amm}) using the methods described by Verdouw et al. (1978). J_{amm} (μmol/g/h) was calculated using the following equation:

$$J_{amm} = \frac{(T_{amm}f - T_{amm}i) * V}{(M * t)}, \quad (1)$$

where $T_{amm}f$ and $T_{amm}i$ (μmol/l) are final and initial water total ammonia concentrations, V is volume (l), M is mass (g), and t is flux time (h).

Water and fish ^{22}Na gamma radioactivity (counts per minute; cpm) was measured using a gamma counter (PerkinElmer, Turku, Finland), while total [Na^+] of initial and final water samples was determined via flame atomic absorption spectrometry (Varian Australia Pty Ltd, Australia). J_{in}^{Na} (μmol/g/h) was calculated using the following equation:

$$J_{in}^{Na} = \frac{R_{fish}}{(SA_{average} * M * t)}, \quad (2)$$

where R_{fish} is the gamma radioactivity of the fish (cpm) and $SA_{average}$ is the average of the initial and final specific activities (cpm/μmol Na) of the exposure medium. Dissolved Cu concentrations were measured using graphite furnace atomic absorption spectrometry (SpectroAA220, Varian, Mulgrave, Australia). During determinations a National Research Council of Canada reference standard (TM-15.2; Ottawa, ON, Canada) was used to determine the percentage recovery of Cu, which was 92 ± 2% (n = 9) over all determinations. DOC concentration was measured using a Shimadzu TOC-$V_{CPH/CPN}$ total organic carbon analyzer (Shimadzu Corporation, Kyoto, Japan). Measurements were made against a prepared standard solution of potassium phthalate.

Table 1
Measured dissolved Cu concentrations in Series 1, Series 2, and 3-h pre-exposures from Series 3 (A) and in divided chamber exposures from Series 3 (B) (n = 15–40).

Nominal concentration (μg/l)	Measured dissolved concentration (μg/l)
(A)	
0	3.43 ± 0.47a
20	19.88 ± 0.91b
50	46.95 ± 1.00c
(B)	
0	2.94 ± 0.44a
20	14.33 ± 0.91b
50	34.70 ± 1.57c

Means not sharing the same letters indicate statistically significant differences among Cu exposure concentrations by a one-way ANOVA with a Holm–Sidak post hoc test.

2.4. Statistical analyses

All data are reported as mean values ± 1 standard error of mean (SEM). Comparisons between control (nominal 0 μg/l Cu exposure) means over development were made using a one-way ANOVA with a Student–Newman–Keuls post hoc test. Significant differences between means of animals exposed to 0 μg/l Cu versus means of Cu-exposed animals under the same conditions were determined using Student's unpaired t-tests. Comparisons among dissolved Cu concentrations were made using a one-way ANOVA with a Holm–Sidak post hoc test. Significance in all cases was accepted at the $P < 0.05$ level.

3. Results

3.1. Cu exposure concentrations

Measured dissolved Cu concentrations in Series 1, Series 2, and in the 3-h pre-exposures from Series 3 did not differ significantly from one another and were averaged together (Table 1A).

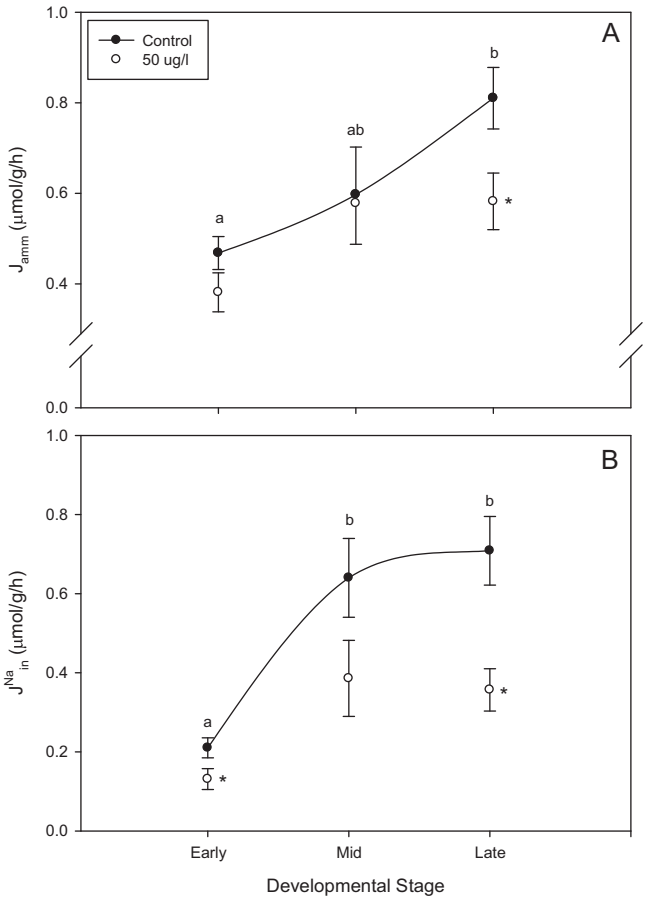

Fig. 1. Total ammonia excretion (A; J_{amm}; µmol/g/h; n = 6–12) and sodium uptake rates (B; J^{Na}_{in}; µmol/g/h; n = 6–12) in larval trout from Series 1 (unrestrained and non-anaesthetized) exposed to nominal 0 (control) or 50 µg/l Cu in early, mid, and late developmental stages. Means not sharing the same letters indicate statistically significant differences with development. Asterisks represent statistically significant differences between 50 and 0 µg/l Cu exposure at a given developmental stage.

Dissolved Cu concentrations in these experiments were all significantly different from one another and were close to nominal values (Table 1A). In the divided chamber exposures from Series 3, Cu concentrations were generally lower (~70% of nominal values) than in the exposures described above. However, a significant difference between each nominal exposure concentration was still observed (Table 1B).

3.2. Developmental patterns of J^{Na}_{in} and J_{amm} and effects of divided chambers and clove oil

In Series 1, J_{amm} increased progressively from early to late developmental stages (Fig. 1A), whereas J^{Na}_{in} approximately tripled in the mid and late developmental stages (not significantly different from one another) relative to the early stage (Fig. 1B). Overall, fish in divided chambers (Series 3) exhibited significantly lower total rates of both J_{amm} and J^{Na}_{in} at all developmental stages compared to those of Series 1 where the larvae were not anaesthetized or restrained (Table 2). When averaged over all stages, the divided chamber system reduced J_{amm} by 53% and J^{Na}_{in} by 40%. In most cases, J_{amm} and J^{Na}_{in} were not significantly different between Series 2 (clove oil alone) and Series 3 (Table 2), indicating that the depressions of J_{amm} and J^{Na}_{in} in Series 3 were mainly due to anaesthesia rather than to restraint.

Table 2
Ammonia excretion rates (A; J_{amm}; µmol/g/h; n = 6–14) and sodium uptake rates (B; J^{Na}_{in}; µmol/g/h; n = 4–12) in developing rainbow trout larvae exposed to nominal 0 or 50 µg/l Cu in Series 1 (unrestrained and non-anaesthetized), Series 2 (clove oil alone), and Series 3 (total divided chamber plus clove oil).

Developmental stage	Exposure (µg/l Cu)	Series 1	Series 2	Series 3
(A)				
Early	0	0.47 ± 0.04a	0.29 ± 0.05b	0.15 ± 0.03c
	50	0.38 ± 0.04x	0.27 ± 0.03xy	0.15 ± 0.03y
Mid	0	0.60 ± 0.10a	0.56 ± 0.09a	0.31 ± 0.04b
	50	0.58 ± 0.09x	0.54 ± 0.10x	0.21 ± 0.04y
Late	0	0.81 ± 0.07a	0.63 ± 0.05ab	0.47 ± 0.05b
	50	0.58 ± 0.06*x	0.46 ± 0.03*x	0.24 ± 0.04*y
(B)				
Early	0	0.21 ± 0.03a	0.12 ± 0.02b	0.17 ± 0.01ab
	50	0.13 ± 0.03*x	0.07 ± 0.01*x	0.08 ± 0.01*x
Mid	0	0.64 ± 0.10a	0.31 ± 0.05b	0.29 ± 0.07b
	50	0.39 ± 0.10x	0.33 ± 0.08x	0.16 ± 0.04x
Late	0	0.71 ± 0.09a	0.53 ± 0.09ab	0.38 ± 0.06b
	50	0.36 ± 0.05*x	0.27 ± 0.04*x	0.20 ± 0.04*x

Means not sharing the same letters represent flux rates that are significantly different during exposure to nominal 0 (a,b,c) or 50 (x,y,z) µg/l Cu in Series 1, 2, and 3 within a given developmental stage of larval trout. Differences were determined using a one-way ANOVA with a Student-Newman–Keuls post hoc test. Asterisks represent means at 50 µg/l Cu that differ significantly from their respective 0 µg/l Cu compared using Student's t-test.

In Series 3, fish in the early developmental stage demonstrated posterior J_{amm} and J^{Na}_{in} which were significantly greater than respective anterior values, accounting for 95 and 78% of total exchange, respectively (Fig. 2). In contrast, in the late developmental stage, anterior exchange dominated, accounting for 83% and 87% of total J_{amm} and J^{Na}_{in}, respectively (Fig. 2). In the mid developmental stage, no significant differences existed between anterior and posterior J_{amm} or J^{Na}_{in} (Fig. 2).

3.3. Effects of Cu exposure

Exposure to nominal 50 µg/l Cu in Series 1 led to a 24% inhibition of J_{amm} in the late developmental stage only, while J_{amm} in early and mid-developmental stages was not affected by Cu exposure (Fig. 1A). J^{Na}_{in}, however, was significantly inhibited by this level of Cu exposure at both the early (38% inhibition) and late (50% inhibition) developmental stages, while a non-significant inhibition was observed at the mid-developmental stage (P = 0.096; Fig. 1B). Though absolute values varied, these same stage-specific responses were also observed in both Series 2 and Series 3 (Table 2).

In Series 3, nominal 50 µg/l Cu led to a 53% inhibition of total J^{Na}_{in} in the early developmental stage, while in the late stage, Cu reduced both total J_{amm} and J^{Na}_{in} by 49% and 47%, respectively (Fig. 3C and Fig. 4C; Table 2). The mid developmental stage, again, was not affected by Cu exposure. An additional exposure in Series 3 using nominal 20 µg/l Cu revealed that only total J_{amm} in the late stage was significantly inhibited by this concentration of Cu (Fig. 3C). The specific site of the inhibitory action of Cu was always that which dominated exchange (i.e., posterior in early stage; anterior in late stage). When considering J_{amm}, the inhibitory effects of Cu seen at the late stage of development occurred via an inhibition of anterior J_{amm}, while posterior J_{amm} was never affected by Cu exposure (Fig. 3A and B). Inhibition of J^{Na}_{in} in the late stage also occurred via an inhibition of anterior uptake; however, inhibition in the early stage was driven by a reduction in posterior uptake (Fig. 4A and 4B).

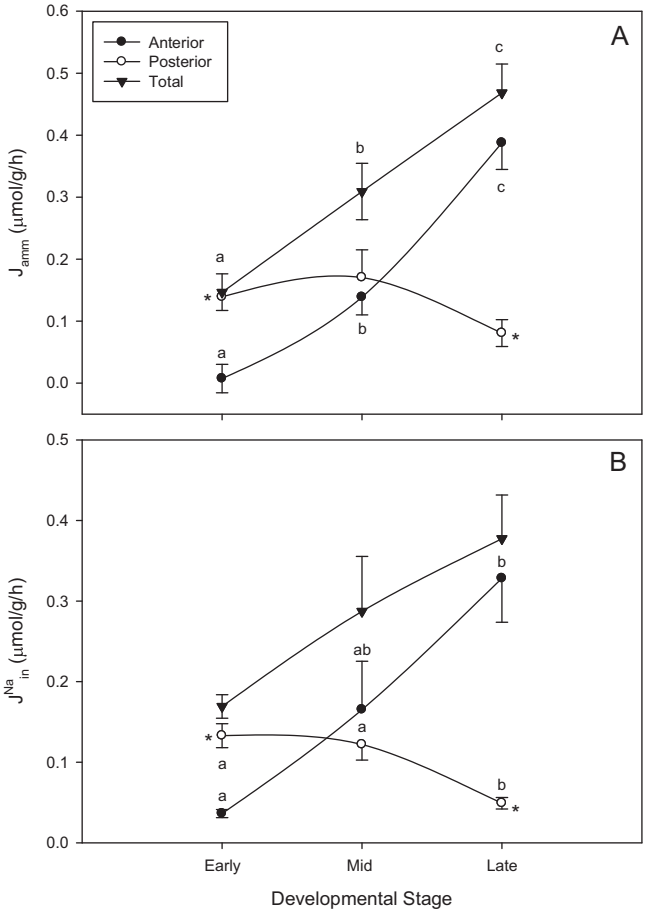

Fig. 2. Anterior (filled circles), posterior (open circles) and total (filled triangles) ammonia excretion (A; J_{amm}; µmol/g/h; n = 9–13) and sodium uptake rates (B; J^{Na}_{in}; µmol/g/h; n = 4–7) in control larval rainbow trout from Series 3 (divided chamber) at early, mid, and late developmental stages. Means not sharing the same letters indicate statistically significant differences among developmental stage within anterior, posterior, or total data sets. Asterisks represent posterior means which differ significantly from respective anterior means within a given developmental stage.

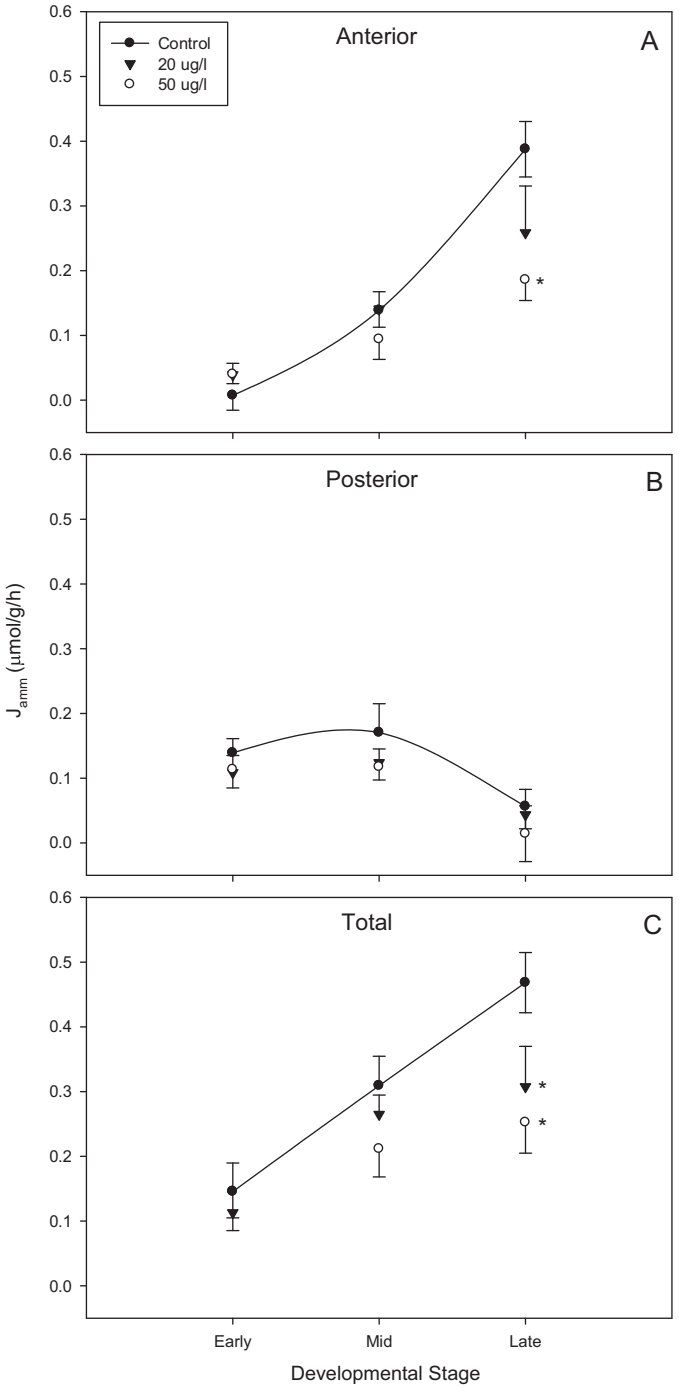

Fig. 3. Anterior (A), posterior (B), and total (C) ammonia excretion rates (J_{amm}; µmol/g/h) in larval rainbow trout from Series 3 (divided chambers) exposed to nominal 0 µg/l (control; filled circles), 20 µg/l (filled triangles), and 50 µg/l Cu (open circles) in early, mid, and late developmental stages. Asterisks represent means from Cu-exposed fish (20 or 50 µg/l Cu) which are significantly different from respective 0 µg/l Cu control values at a given developmental stage within anterior, posterior, or total data sets (n = 9–14).

4. Discussion

4.1. Overview

The present study demonstrates that larval rainbow trout, similarly to juvenile and adult animals, respond to waterborne Cu exposure with a reduction in both J_{amm} and J^{Na}_{in} (Fig. 1). In juvenile and adult trout, this toxicity is believed to be mediated via an inhibition of transport processes at the gill. The same was true of larval trout in the late developmental stage (25 dph) where Cu led to the inhibition of both J_{amm} and J^{Na}_{in} across the gills (Figs. 3 and 4). While J_{amm} was only affected in the late stage, J^{Na}_{in} was also inhibited by Cu at the early developmental stage (3 dph; Fig. 1). This inhibition occurred via a reduction in cutaneous J^{Na}_{in} (Fig. 4), demonstrating, to the best of our knowledge, the first evidence of cutaneous metal toxicity in an intact aquatic organism. Interestingly, our results suggest that the mechanisms of J_{amm} and J^{Na}_{in} and/or of Cu toxicity differ between the gill and skin of larval trout. These results have important implications in our current understanding and prediction of metal toxicity in developing larval fish.

4.2. Validation of divided chamber tests

The Cu concentrations used in the present study (Table 1) were chosen based on preliminary results (data not shown) and a 96-h LC50 of 125 µg/l for rainbow trout in dechlorinated Vancouver tapwater predicted by the Biotic Ligand Model (BLM version 2.2.3, HydroQual, 2007). This relatively high prediction value, given the extremely soft water of Vancouver (~3 mg/l $CaCO_3$), was likely due to the substantial DOC present in the water (2.5 mg C/l). All concentrations reported are dissolved Cu concentrations (Table 1) and, for Series 1, Series 2, and the 3-h pre-exposure from Series 3, values were very close to nominal concentrations (Table 1A).

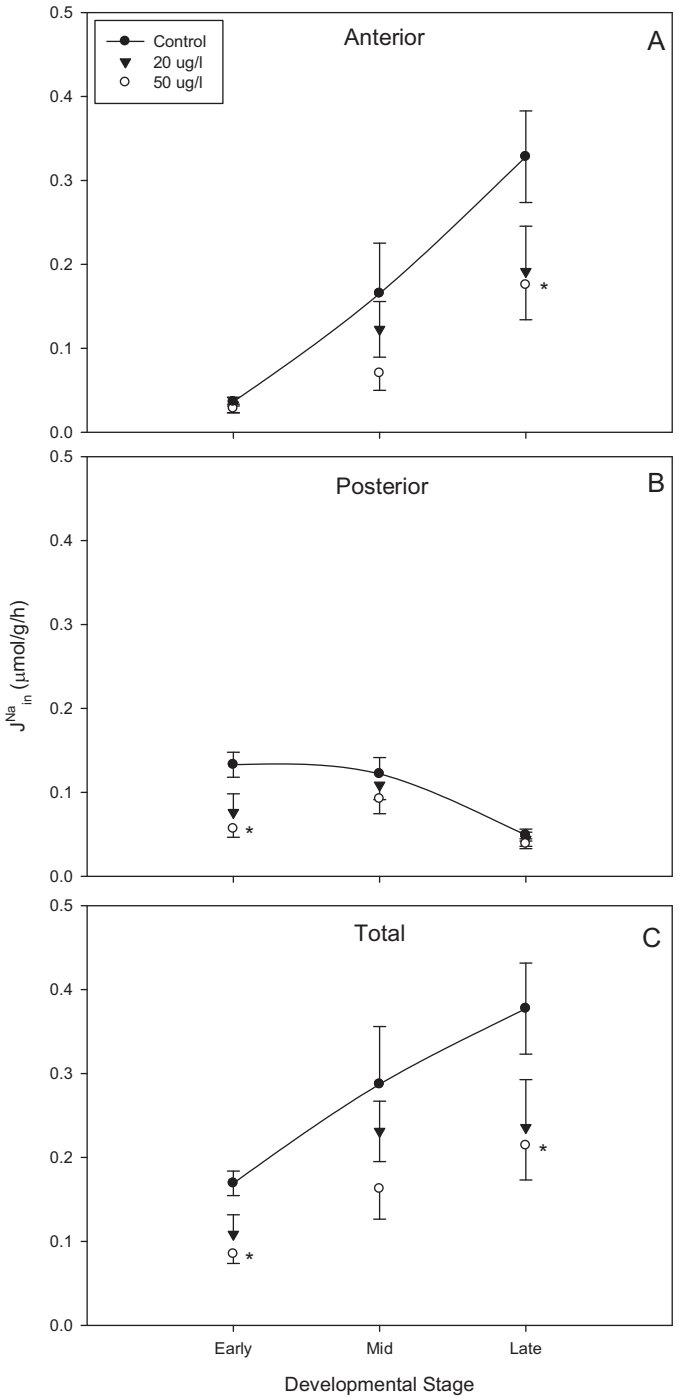

Fig. 4. Anterior (A), posterior (B), and total (C) sodium uptake rates (J^{Na}_{in}; µmol/g/h) in larval rainbow trout from Series 3 (divided chambers) exposed to nominal 0 µg/l (control; filled circles), 20 µg/l (filled triangles), and 50 µg/l Cu (open circles) in early, mid, and late developmental stages. Asterisks represent means from Cu-exposed fish (20 or 50 µg/l Cu) which are significantly different from respective 0 µg/l Cu control values at a given developmental stage within anterior, posterior, or total data sets (n = 4–8).

The measured concentrations in the divided chamber experiments from Series 3, however, were slightly lower than nominal concentrations (Table 1B), potentially due to the adsorption to the walls of the 5-l "loading containers" or to the latex dam. Regardless, the same pattern of inhibitory effects of Cu was seen across all exposure conditions (Table 2). Overall, clove oil (Series 2) tended to reduce J_{amm} and J^{Na}_{in}, similar to previous reports (Fu et al., 2010; Smith et al., 2012) and the resulting rates, for the most part, were not significantly different from the values measured in divided chambers (Series 3), suggesting that the anaesthetic was the cause of the reductions, but nevertheless was effective in reducing experimental stress. These results suggest that the use of divided chamber experiments in Cu toxicity testing is valid and, moreover, appears to be a useful tool in understanding the mechanisms of Cu toxicity in specific regions (i.e., skin versus gill) in larval fish.

As there is a significant amount of head skin in the anterior half-chamber (see Fu et al., 2010; Zimmer et al., 2014), potentially some of the fluxes measured in the anterior chamber are cutaneous, whereas in the posterior half-chamber some of the fluxes could be renal. Nevertheless, it seems very likely that the majority of the fluxes in the two sections are predominantly branchial and cutaneous, respectively. In the subsequent discussion, flux via the anterior half-chamber is considered to be branchial, while flux via the posterior chamber is considered to be cutaneous.

4.3. Cu inhibits branchial J_{amm} and J^{Na}_{in}

The disruption of both ammonia and Na^+ balance are two of the most common responses to acute Cu exposure in juvenile and adult freshwater fish (see Grosell, 2012 for review). This also appears to be the case in larval trout, where exposure to 50 µg/l Cu significantly impaired both J_{amm} and J^{Na}_{in}, regardless of experimental condition (e.g., Series 1,2, or 3; Table 2). As is believed to be the case in juvenile and adult fish, this response to Cu in the late developmental stage appeared to be mediated entirely by an inhibition of branchial exchange (Figs. 3A and 4A). The inhibition of ammonia excretion by Cu in fish is observed as either a decrease in J_{amm} (Lauren and McDonald, 1985; Blanchard and Grosell, 2006; Zimmer et al., 2012) or an increase in plasma ammonia levels (Lauren and McDonald, 1985; Wilson and Taylor, 1993a,b; Wang et al., 1998; Grosell et al., 2003, 2004). The mechanism of this inhibition, however, is not clear, but may be associated with inhibition of carbonic anhydrase activity (Vitale et al., 1999; Grosell et al., 2002; Zimmer et al., 2012), although there is no consensus in the literature (e.g., Blanchard and Grosell, 2006). The mechanism of inhibition of branchial J^{Na}_{in}, however, is usually attributed to competitive blockade of apical Na^+ uptake by Cu via an as yet uncharacterized epithelial Na^+ channel (Grosell and Wood, 2002) and/or by Cu inhibition of basolateral Na^+/K^+-ATPase activity (Lauren and McDonald, 1987b; Pelgrom et al., 1995; Sola et al., 1995). Since there appears to be a functional linkage between J^{Na}_{in} and J_{amm} at the gills of adult trout which is mediated via Rh proteins ("sodium-ammonium exchange complex or metabolon"; Tsui et al., 2009; Wright and Wood, 2009) and this is also seen in the gills of developing trout larvae (Zimmer et al., 2014), it is possible that inhibition of these processes by Cu may share a common mechanism which has yet to be elucidated. Two possibilities are (i) the inhibition of carbonic anhydrase activity (Zimmer et al., 2012; Grosell, 2012) or (ii) inhibition of ammonia-transporting Rhesus (Rh) proteins in the gill given their integral role in both ammonia and Na^+ transport (Wright and Wood, 2009; Kumai and Perry, 2011; Shih et al., 2012, 2013).

4.4. Cu Inhibits cutaneous J^{Na}_{in} but not cutaneous J_{amm}

In the early stage of development, rainbow trout larvae utilized their skin for the majority of both J_{amm} and J^{Na}_{in} (Fig. 2). This has been observed previously (Zimmer et al., 2014), and it is generally believed that the skin performs the majority of all physiological exchanges immediately following hatch (Wells and Pinder, 1996; Rombough, 2002; Fu et al., 2010; see Brauner and Rombough, 2012 for review). This reliance on cutaneous exchange is due to limited gill surface area and high skin mitochondrial rich cell (MRC) abundance in early stages of larval development (Gonzalez et al., 1996; Rombough, 1999). Thus, the response of the skin to contaminant

exposure is crucial to fully understand overall toxic responses at this stage. Moreover, it is known that larvae represent the most Cu-sensitive life stage in most fish (McKim et al., 1978; Rombough and Garside, 1982), likely due to the high Na^+ turnover rates of these animals associated with their small size (Grosell et al., 2002). Indeed, larval trout exposed to Cu experienced an inhibition of J_{in}^{Na} at all 3 developmental stages (though non-significantly in the mid-developmental stage; Fig. 1), which occurred via an inhibition of cutaneous uptake in the early stage, and branchial uptake in the late stage (Fig. 4). To the best of our knowledge, this represents the first in vivo evidence of the inhibition of cutaneous exchange processes by Cu in fish. Though the skin plays little to no role in Na^+ acquisition by the end of yolk sac absorption (Fig. 2B; Zimmer et al., 2014), these results may have important implications for earlier life stages which, in general, utilize cutaneous surfaces for Na^+ uptake (e.g., Fu et al., 2010; Wu et al., 2010; Shih et al., 2012).

Furthermore, a significant proportion of Ca^{2+} uptake, which is inhibited by exposure to other divalent metals (e.g., Pb; Rogers et al., 2003), is believed to occur across the general body surface of adult rainbow trout (Perry and Wood, 1985), potentially implicating another role for cutaneous metal toxicity. Cutaneous metal toxicity may also be important in specialized epithelia such as the cleithrum skin (e.g., Marshall et al., 1992) and opercular epithelium (see Marshall, 2003) which contribute significantly to whole-body ionoregulation in some fish species. Indeed, Crespo and Karnaky (1983) demonstrated in vitro inhibition of Cl^- transport across the isolated opercular epithelium of killifish (*Fundulusheteroclitus*) by Cu, though this inhibition occurred only when Cu was applied to the serosal (blood-facing) side while Cu application to the apical (water-facing) side was without effect. Moreover, the skin of frogs, used extensively to model epithelial Na^+ transport in in vitro studies pioneered by Hans Ussing (Ussing and Zerahn, 1950), is also responsive to Cu exposure in vitro. Exposure to monovalent Cu^+ has been shown to inhibit Na^+ uptake across isolated frog skin in some studies (Skulskii and Lapin, 1992) and stimulate Na^+ uptake in others (Zadunaisky et al., 1963; Flonta et al., 1998); see Handy et al. (2002) for review. While it is clear that Cu can alter cutaneous Na^+ transport in post-hatch larval fish (designated here as "early"; Fig. 4B), it is unclear why this response is absent in later stages of development (mid and late stages). It may be a result of a reduction in yolk sac skin surface area, which seems to be the major site of cutaneous exchange (e.g., Shih et al., 2008) and MRC expression in larval fish (Rombough, 1999), however, further studies are required to address this.

Interestingly, Cu did not inhibit cutaneous J_{amm} at any developmental stage, despite reducing cutaneous J_{in}^{Na} in early stages (Fig. 4B) and branchial J_{amm} in the late stages of development (Fig. 3). Furthermore, Zimmer et al. (2014) did not find a relationship between J_{in}^{Na} and J_{amm} at the skin in larval trout, while a clear relationship between the two was observed at the gills. Taken together, these data indicate that the mechanism for J_{amm} and J_{in}^{Na} and/or the mode of toxic action of Cu is different between the skin and gills. More work is now needed to elucidate the cutaneous and branchial mechanisms of ammonia and Na^+ exchange in larval fish and the pathways by which Cu inhibits this exchange.

4.5. Perspectives and future directions

The depletion of whole-body Na^+ stores by inhibition of Na^+ acquisition is believed to be the lethal mechanism of action of Cu exposure in juvenile and adult freshwater fish (Grosell et al., 2002; Grosell, 2012). Understanding the mechanisms of Cu toxicity and lethality in larval fish is of particular importance due to the high sensitivity to Cu at this life stage (McKim et al., 1978). In general, Cu sensitivity increases following hatch (e.g., Stouthart et al., 1996), likely due to the shedding of the chorion, the outer egg capsule of embryonic fish, which is capable of binding metals (Guadagnolo et al., 2001; Brix et al., 2004), and continues to increase over larval development (e.g., Chapman, 1978; McNulty et al., 1994). Interestingly, the most sensitive endpoint statistically in the present study was the inhibition of total J_{amm} in the late developmental stage as it was responsive to 20 μg/l Cu, whereas this lower Cu concentration never significantly inhibited J_{in}^{Na} (Fig. 4). This may indicate that the lethal mechanism of action for Cu in larval fish is also different. Indeed, the large changes in nitrogen handling and metabolism which occur over this development stage (Wright et al., 1995; Essex-Fraser et al., 2005; Zimmer et al., 2014) may lead to greater susceptibility to detrimental disruptions in ammonia balance. Overall, the drastic physiological changes which occur over larval development, and the physiological differences between these fish and mature fish, warrant further investigation in understanding the nature of Cu toxicity in larval fish.

The BLM is used for a variety of scientific and regulatory purposes (Di Toro et al., 2001; Santore et al., 2001; Niyogi and Wood, 2004; Paquin et al., 2002; HydroQual, 2007; USEPA, 2007; Ng et al., 2010). The current Cu BLM model for rainbow trout (version 2.2.3, HydroQual, 2007) uses only gill lethal accumulation thresholds (LA50s) obtained from trout in later stages (MacRae et al., 1999) to predict toxicity. This may not be appropriate in immediately post-hatch larval fish lacking a functioning gill, where biotic ligands on the skin appear to be key targets of Cu toxicity. The skin is the major site of whole-body Na^+ uptake, and cutaneous J_{in}^{Na} was significantly inhibited by Cu (Fig. 4B). Future work should focus on characterizing and quantifying skin and gill Cu binding over larval development to determine how these relate to toxicity. A critical step in this regard will be to measure skin and gill Cu burdens associated with acute lethality (LA50 values; HydroQual, 2007). Overall, larval fish pose a new and exciting area of research in metal toxicity. Determining the possible toxicological implications of the changes in physiology and biotic ligand sites (i.e., skin to gills) which occur over larval development may lead to a more comprehensive understanding of metal toxicity in fish.

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