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Waterborne copper exposure inhibits ammonia excretion and branchial carbonic anhydrase activity in euryhaline guppies acclimated to both fresh water and sea water

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ABSTRACT

Inhibition of ammonia excretion (J_{amm}) is a common response to Cu exposure in freshwater (FW) and seawater (SW) organisms. To determine the mechanism of this response, a euryhaline species of guppy (*Poecilia vivipara*) was exposed to 20 μg Cu/l in FW (0 ppt) and SW (25 ppt) for 96 h. In both salinities, Cu transiently inhibited ammonia excretion (J_{amm}) followed by a full recovery by the end of the 96 h exposure. The activities of Na^+/K^+-ATPase, H^+-ATPase, and carbonic anhydrase (CA) were examined in the gills at 12 and 96 h of Cu exposure. In both salinity acclimations, CA activity was significantly inhibited following 12 h of Cu exposure in *P. vivipara*, marking the first *in vivo* evidence of Cu-induced inhibition of CA in fish. Moreover, the inhibition and recovery of this enzyme were correlated with the inhibition and recovery of J_{amm} in both salinity acclimations. The blockade of CA potentially acts as a common mechanism of J_{amm} inhibition in FW and SW. There were no significant effects on Na^+/K^+-ATPase or H^+-ATPase activity at either time point or salinity. However, H^+-ATPase activity was upregulated at 96 h relative to the 12 h time point, potentially involving this enzyme in re-establishing J_{amm}.

1. Introduction

Copper (Cu), while being an essential metal, also acts as a toxicant to both freshwater (FW) and seawater (SW) aquatic organisms and its toxicity has been demonstrated to be dependent upon salinity (Blanchard and Grosell, 2005, 2006; Grosell et al., 2007). Considering the effects of water chemistry alone, Cu is expected to be less toxic in SW due to increased inorganic metal complexation and cation competition (Paquin et al., 2002). However, several studies have now determined that physiology, rather than water chemistry alone, may be more important in predicting Cu toxicity in varying salinities. Since Cu acts as an ionoregulatory toxicant (e.g. Stagg and Shuttleworth, 1982a; Wilson and Taylor, 1993a, b; Blanchard and Grosell, 2006), it is expected that fish acclimated to salinities above or below the isosmotic point (~12 ppt), having to ionoregulate, will be more sensitive to Cu. In the euryhaline killifish (*Fundulus heteroclitus*), greater Cu-induced mortality is observed at salinity extremes and this was highly correlated to blood-to-water Na^+ gradients (Blanchard and Grosell, 2006; Grosell et al., 2007). Indeed, inhibition of Na^+ ionoregulation by Cu in FW has been shown in several studies (e.g. Laurén and McDonald, 1987b; Wilson and Taylor, 1993b; Blanchard and Grosell, 2006) and Grosell et al. (2002) have suggested that Cu-induced mortality in FW is greatly influenced by Na^+ turnover rates. In SW, however, this is not so clear. Though several studies demonstrate that Cu exposure in SW-acclimated fish results in a perturbation of Na^+ ionoregulation (e.g. Stagg and Shuttleworth, 1982a; Wilson and Taylor, 1993a; Larsen et al., 1997; Grosell et al., 2004a), Cu-induced mortality in SW-acclimated killifish occurred in the absence of a disruption in Na^+ ionoregulation (Blanchard and Grosell, 2006). Conversely, mortality in these fish was attributed to an inhibition of ammonia excretion (J_{amm}) which occurred only in SW-acclimated fish. In fact, inhibition of ammonia clearance (inhibition of J_{amm} or increase in plasma ammonia) is a common response to Cu exposure in both FW and SW (Laurén and McDonald, 1985; Wilson and Taylor, 1993a, b; Grosell et al., 2003, 2004a; Blanchard and Grosell, 2006), yet the mechanism for this inhibition is unknown. Also among the physiological effects of Cu exposure is the disruption of acid-base homeostasis (Wilson and Taylor, 1993b; Larsen et al., 1997; Wang et al., 1998). Similarly, the exact mechanism of this inhibition is unknown, though this effect is not as consistent as the inhibition of ionoregulation and ammonia clearance by Cu. Indeed, the disruption of ammonia clearance by Cu is perhaps the

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most consistently observed effect in both FW and SW-acclimated fish and may be a general physiological response to Cu exposure, potentially occurring via a common mechanism.

In the current model for ammonia excretion in FW (see Fig. 1A), the facilitated diffusion of ammonia in its unprotonated form (NH_3) across the gill epithelium via Rhesus (Rh) proteins is dependent upon apical gill boundary layer acidification by apical membrane-bound H^+-ATPase, Na^+/H^+-exchanger (NHE), and carbonic anhydrase (CA) (see Weihrauch et al., 2009; Wright and Wood, 2009 for review). Note that the former two transporters also facilitate Na^+ uptake, linking this process to ammonia excretion (see Evans et al., 2005; Weihrauch et al., 2009; Wright and Wood, 2009 for review). Cytosolic CA is also an integral part of the model, supplying intracellular H^+ for NHE and H^+-ATPase via the hydration of intracellular CO_2. Additional evidence suggests that basolateral Na^+/K^+-ATPase may also play a role in ammonia excretion in some species by substituting NH_4^+ for K^+, thereby transporting ammonia across the basolateral gill membrane (see Wilkie, 2002 for review). In SW (see Fig. 1B), branchial ammonia excretion is also dependent upon boundary layer acidification (see Weihrauch et al., 2009) and there is evidence that Na^+/K^+-ATPase also facilitates direct ammonia transport in the toadfish, *Opsanus beta* (Mallery, 1983). Similarly, Randall et al. (1999) demonstrated that branchial Na^+/K^+-ATPase from mudskippers (*Periophthalmodon schlosseri*) was activated *in vitro* by the substitution of physiological levels of NH_4^+ for K^+. Gill boundary acidification by SW fish, however, is believed to rely more upon NHE (utilizing an inwardly directed Na^+ gradient) than H^+-ATPase (Wilkie, 2002; Weihrauch et al., 2009; Wood and Nawata, 2011). In these fish, Na^+ is excreted at the gills to counteract diffusive ion gain from the water and this process is completely dependent upon the electrochemical gradients established by basolateral Na^+/K^+-ATPase (Evans et al., 2005) but is independent of ammonia excretion. Similarly in this model, cytosolic CA would play a role in supplying protons for boundary layer acidification.

Of the branchial transporters and enzymes described above, most studies have focused on the effects of Cu on Na^+/K^+-ATPase activity (Stagg and Shuttleworth, 1982b; Li et al., 1998; Ay et al., 1999; De Boeck et al., 2001; Grosell et al., 2003, 2004b; Blanchard and Grosell, 2006; Gagnon et al., 2006), with many studies attempting to link the inhibition of this enzyme to a disruption in Na^+ homeostasis. In FW, Cu exposure can cause an interruption of Na^+ balance with a concomitant inhibition of branchial Na^+/K^+-ATPase activity (Laurén and McDonald, 1987a, b; Blanchard and Grosell, 2006). In SW fish, however, Cu exposure often results in a disruption in Na^+ balance in the absence of an inhibition of branchial Na^+/K^+-ATPase activity (Stagg and Shuttleworth, 1982b; Grosell et al., 2003, 2004b; Blanchard and Grosell, 2006). Moreover, Blanchard and Grosell (2006) demonstrated that inhibition of J_{amm} in SW also occurred in the absence of an inhibition of Na^+/K^+-ATPase activity. Similarly, in FW-acclimated killifish, Cu exposure resulted in a reduction in Na^+/K^+-ATPase activity while J_{amm} was unaffected (Blanchard and Grosell, 2006). Overall, it appears that the inhibition of this enzyme cannot account for the reduction in ammonia clearance typically seen during Cu exposure in FW and SW-acclimated fish. Perhaps the direct role of Na^+/K^+-ATPase in ammonia excretion is a specialized adaptation seen only in certain species (e.g. Mallery, 1983; Randall et al., 1999) and may not be a common feature in the gills of other teleost species where this enzyme has been demonstrated to not be activated by NH_4^+ substitution (e.g., rainbow trout, *Oncorhynchus mykiss*, Wood and Nawata, 2011).

Grosell et al. (2002) described a model whereby the physiological effects of Cu in FW-acclimated fish could all be related to an inhibition of CA. Indeed, the inhibition of this enzyme by Cu may explain an inhibition of ammonia excretion or sodium uptake (see Fig. 1) and may also account for the disruption of acid–base balance which may be observed during Cu exposure. Although Cu has been shown to inhibit CA *in vitro* (e.g. human CA: Magid, 1967; DiTusa et al., 2001; crab CA: Vitale et al., 1999; fish CA: Christensen and Tucker, 1976; Soyut et al., 2008; Ceyhun et al., 2011), inhibition of this enzyme has never been demonstrated *in vivo*. In fact, Blanchard and Grosell (2006) reported that in FW and SW-acclimated killifish exposed to Cu, CA activity was unaffected despite changes in Na^+ balance and J_{amm}. However, in a recent review, Grosell (2012) argued that this apparent lack of inhibition is a result of the delta pH method of assaying the activity of this enzyme (Henry, 1991) which requires dilution of sample to the point where Cu may become disassociated from CA, eliminating inhibition. In addition, to our knowledge, there have been no studies which have examined the effects of Cu exposure in fish on the activity of H^+-ATPase. Though this enzyme is not likely to play a role in Na^+ excretion or J_{amm} in SW-acclimated fish (see Wilkie, 2002), it is an ideal candidate for the inhibition of Na^+ ionoregulation and J_{amm} in FW. In zebrafish larvae exposed to bafilomycin, a H^+-ATPase inhibitor, there was a marked inhibition of both Na^+ influx (Esaki et al., 2007) and J_{amm} (Shih et al., 2008). Furthermore, internal acidosis in fish is associated with an increase in H^+-ATPase expression and Na^+-uptake, suggesting that this enzyme is integral to both acid–base regulation and ionoregulation (Perry and Fryer, 1997). To date, however, no studies have focused on H^+-ATPase as a potential target for Cu toxicity.

The goal of the present study was to assess the effects of a sublethal Cu exposure (20 μg Cu/l) on the mechanisms of J_{amm} in FW (0 ppt) and SW (25 ppt)-acclimated *Poecilia vivipara*, a euryhaline guppy found along the entire coast of Brazil. We hypothesized that this level of Cu would inhibit J_{amm} in both salinities over a 96 h exposure and that the inhibition of CA activity would act as a common mode of toxicity, in accordance with the arguments made by Grosell (2012). Moreover, we predicted that Cu exposure would result in a disruption of Na^+ homeostasis in both salinities but only in FW would this disruption occur concomitantly with inhibition of Na^+/K^+-ATPase activity. We also evaluated, for the first time to our knowledge, the effects of Cu on H^+-ATPase activity in both FW and SW-acclimated fish.

2. Materials and methods

2.1. Animals

Male and female *Poecilia vivipara* (0.1–1.1 g), a euryhaline cyprinodontiform, were collected from a freshwater stream which flows into Cassino Beach, Rio Grande, RS, Southern Brazil. Fish were allowed to adjust to laboratory conditions for 48 h before half of the fish were randomly selected for seawater (SW; 25 ppt) acclimation by increasing salinity by 5 ppt every 2 days while the other half remained in freshwater (FW; 0 ppt), resulting in a total acclimation period of approximately 2 weeks. Fish were held at 20 °C for the entire acclimation period and all subsequent experiments were conducted at the same temperature. See Table 1 for ionic composition of both media. Fish were considered fully acclimated when they would feed consistently for 5 consecutive days at the given salinity. Special attention was taken to ensure that female fish were not gravid before any experiment began.

2.2. Experimental design

The study consisted of two sets of experiments:

The goal of the first set of experiments was to follow J_{amm} in FW and SW-acclimated *P. vivipara* over 96 h of control or Cu exposure conditions and to determine terminal whole-body [Na^+] and gill Cu burden. Following a 72 h fasting period, fish were exposed to

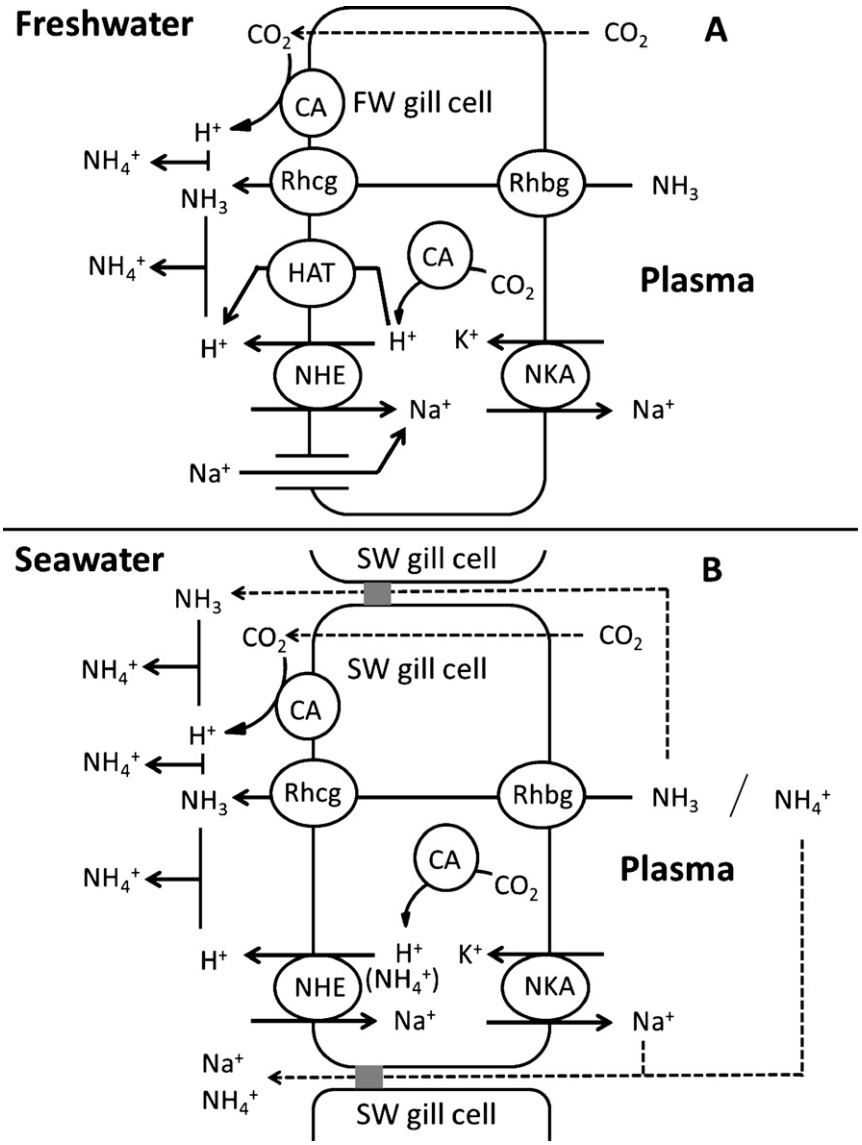

Fig. 1. Current models for ammonia excretion in freshwater (FW; A) and seawater (SW) fish (B). Demonstrated in both models are the potential targets for Cu (Na^+/K^+-ATPase, H^+-ATPase, and carbonic anhydrase) and their relation to ammonia excretion and Na^+ uptake. See text for details. CA, carbonic anhydrase; HAT, H^+-ATPase; NHE, Na^+/H^+-exchanger; NKA, Na^+/K^+-ATPase; Rh, Rhesus glycoprotein.

either control (0 μg/l nominal Cu) or exposed (20 μg/l nominal Cu) conditions in FW or SW over a 96 h period. Exposures and flux measurements for individual fish took place in beakers containing 100 ml of the test medium (1 fish per beaker) and were aerated continuously. As fish weight tended to vary significantly (up to 10-fold), water volume was adjusted to fish size during all experiments.

Table 1
Ionic composition of freshwater and seawater media employed to perform experiments with the guppy *Poecilia vivipara*.

	Freshwater (0 ppt)	Seawater (25 ppt)
Na^+ (mmol/l)	0.48	350
Cl^- (mmol/l)	0.55	392
Ca^{2+} (mmol/l)	0.33	8.6
K^+ (mmol/l)	0.02	6.8
Mg^{2+} (mmol/l)	0.08	25
SO_4^{2-} (mmol/l)	0.02	15
Alkalinity (mg/l as $CaCO_3$)	2.13	240
Dissolved organic carbon (mg C/l)	2.19	1.1
pH	7.0	7.8

During the first 12-h period, 10 ml water (FW or SW) was used for every 0.1 g fish weight. Over the first 12 h of exposure, 1-ml water samples were taken every 3 h to measure total ammonia (T_{amm}) in the water. After the initial 12-h time period, a water change was made and subsequent water changes were made every 24 h. During these 24-h periods, water volume was increased to 15 ml water for every 0.1 g fish weight. Following every water change, an initial 1-ml water sample was taken to measure T_{amm} followed by two others at 12 and 24 h post-water change. All water samples for T_{amm} measurement were immediately stored at −20 °C for later analysis. At the end of the 96 h experimental period, fish were sacrificed via a blow to the head and the gills were removed to measure gill [Cu] and [Na^+] while bodies were kept to measure whole-body [Na^+]. All samples were kept on ice then stored at −80 °C until analysis. Control samples for whole-body [Na^+] and branchial [Cu] were taken from fish which had been fasted for 72 h, taken directly from holding tanks, and sacrificed with a blow to the head. Water T_{amm} in all exposures ranged from approximately 10–90 μmol/l and initial T_{amm} (10–20 μmol/l) following each water change did not differ significantly throughout the duration of all exposures.

Table 2
Total and dissolved water Cu concentrations in freshwater and seawater control and exposed (20 μg Cu/l) experimental series.

Salinity	Experimental series	Total (μg/l)	Dissolved (μg/l)
Freshwater (0 ppt)	Control	BDL	BDL
	Exposed	21.17 ± 1.20*	22.17 ± 0.77*
Seawater (25 ppt)	Control	BDL	BDL
	Exposed	23.56 ± 1.52*	19.76 ± 1.11*

BDL, below detection limit.
* Exposed values which differ significantly from their respective control values. No significant differences occurred between total and dissolved values or between freshwater and seawater values.

The goal of the second set of experiments was to determine the branchial activities of Na^+/K^+-ATPase, H^+-ATPase, and CA following 12 and 96 h of Cu exposure. These experiments followed the same protocol described for the first set of experiments except that some fish were sacrificed following 12 h of exposure. Control enzyme activity was assessed in fish following 72 h of fasting and a 12 h control flux, following the same protocol described above for Cu exposed fish. Note that in all experiments, total and dissolved [Cu] were measured before and after all water changes and did not differ significantly. Therefore, these data are presented as total mean values in Table 2.

2.3. Analytical techniques and calculations

Ammonia flux rates (J_{amm}) were measured using the protocol described in previous studies (e.g. Nawata et al., 2007; Zimmer et al., 2010). Cu and Na concentrations in water, gill, and/or whole-body samples were measured by flame atomic absorption spectroscopy (AAS, Avanta, 932 Plus, GBC, Hampshire, IL, USA) against certified standards (Tritisol-Merck) adjusted to corresponding salinity and acid content. The detection limit was 2 μg/l. Gill tissues were digested in 250 μl 65% HNO_3 (Suprapur, Merck) while bodies were digested in 1 ml 65% HNO_3. Tissue concentrations are represented as μg Cu per wet weight of tissue (μg Cu/g wet weight) or as μmol Na per wet weight of tissue (μmol Na/g wet weight). Quality assurance controls were performed for Cu measurements and standard curves were built employing a standard Cu solution (Standard Reference Material® 3114) from the National Institute of Standards & Technology (Gaithersburg, MD, USA). For the analyses of gill samples, percentage of Cu recovery (94.2%) was calculated based on standard reference material (European Reference Material ERM®-CE278, Geel, Belgium) prepared as described for biological samples. For the analyses of water samples, procedures were followed as previously described (Pinho et al., 2007; Paganini et al., 2008; Lauer and Bianchini, 2010; Pinho and Bianchini, 2010; Martins et al., 2011a, b). In another study, measurements performed using this procedure were compared to those generated using the method described by Nadella et al. (2009) which removes salt ions, found at high concentrations in seawater, and no significant difference was observed (Prazeres et al., 2012).

Branchial Na^+/K^+-ATPase and H^+-ATPase activities in whole gill homogenates were measured in duplicate using a protocol similar to that described by Nawata et al. (2007). Activity is expressed as control rate (no inhibitor) minus inhibitor-treated rate (oubain for Na^+/K^+-ATPase and sodium azide and *N*-ethylmaleimide (NEM) for H^+-ATPase) measured at 340 nm using a kinetic microplate reader (ELx808 Biotek Instruments, USA) every 40 s for 20 min. Branchial CA activity in the same homogenates was determined by the delta pH method (Henry, 1991). Briefly, activity was measured (uncatalyzed, blank samples were measured in duplicate) in a pH 7.4 reaction buffer (225 mM mannitol, 75 mM sucrose, 10 mM Tris buffer, 10 mM NaH_2PO_4) kept at 2–4 °C by keeping the reaction buffer on ice throughout the assay. Reactions were performed in 20 ml scintillation vials (also kept on ice throughout the assay) and were mixed continuously using a magnetic stirrer. Assay reaction was initiated by the addition of 1 ml CO_2-saturated, double distilled water, and reaction rate was determined by measuring pH of the sample using a handheld pH meter and electrode (Lutron Electronic Enterprise Co., LTD, Taiwan) every 5 s for 30 s. Activity is represented as the measured rate divided by the rate of the uncatalyzed reaction (no sample homogenate added). Protein concentrations in all samples were measured using a colorimetric Biuret total protein assay kit (Doles, Goiânia, GO, Brazil).

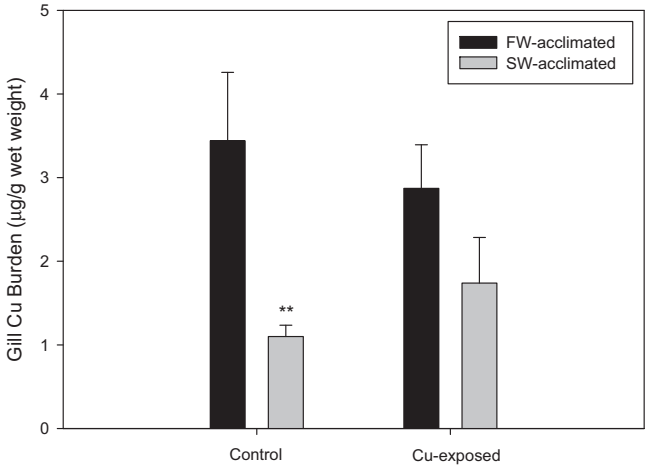

Fig. 2. Gill Cu burden in fish acclimated to FW (black bars; $N = 4–6$) SW (gray bars; $N = 6$) under control conditions or exposed to 20 μg Cu/l for 96 h. Fish in all groups were fasted 72 h prior to experimentation. Double asterisks represent a significant difference between FW and SW values as determined by an unpaired *t*-test ($P < 0.05$).

2.4. Statistical analysis

Data are presented as means ± 1 SEM (N = number of fish) and significance was accepted at $P < 0.05$. All statistical analyses were performed using SigmaStat Version 3.5 (Systat Software, Inc.). Specific statistical tests used for each different comparison are described within corresponding Figure captions.

3. Results

In all exposures, dissolved [Cu] did not differ significantly from total [Cu]. Both total and dissolved [Cu] were slightly greater than zero in control media in both salinities (Tale 2). In both FW and SW, exposure to 20 μg/l Cu (see Table 2 for measured values) for 96 h did not result in significant Cu accumulation in the gills of *P. vivipara* (Fig. 2). Interestingly, control fish acclimated to SW had a significantly lower gill Cu burden than control fish acclimated to FW (Fig. 2).

Under control conditions, there was no difference in J_{amm} between FW-acclimated *P. vivipara* and SW-acclimated *P. vivipara* over the first 9 h of measurements. However, a marked difference between these groups was consistently observed after 24 h (Fig. 3). Over the final 72 h of flux measurements, J_{amm} was approximately 3-fold greater in SW-acclimated fish (Fig. 3). J_{amm} in FW control fish was significantly lower over 48–84 h compared to the values observed over the first 24 h (Fig. 3).

When exposed to Cu, *P. vivipara* in FW demonstrated an immediate 40–90% inhibition of J_{amm} which persisted for the first 36 h of exposure (Fig. 4A). This was followed by a restoration of J_{amm} to control levels with a subsequent upregulation relative to control values of J_{amm} over the final 36 h of exposure (Fig. 4A) In SW, exposure to Cu also resulted in an inhibition of J_{amm} but the time

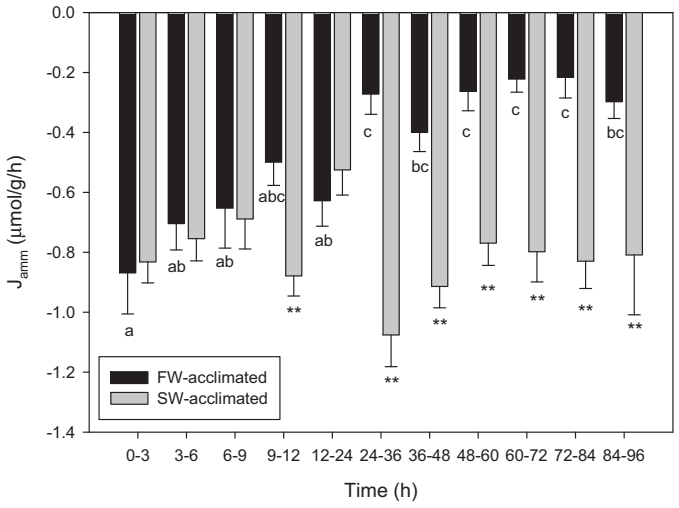

Fig. 3. Ammonia excretion rates (J_{amm}) in fish acclimated to FW (black bars; $N = 10–18$) and SW (gray bars; $N = 6$) under control conditions over 96 h. Fish in all groups were fasted 72 h prior to experimentation. Means for freshwater data sharing the same lower case letter do not differ significantly from one another as determined by an ANOVA on ranks with a Dunn's post hoc test. There were no significant differences among SW means, as determined by an ANOVA on ranks. Asterisks denote SW values which are significantly different than corresponding FW values at a given time period as determined by unpaired *t*-tests ($P < 0.05$).

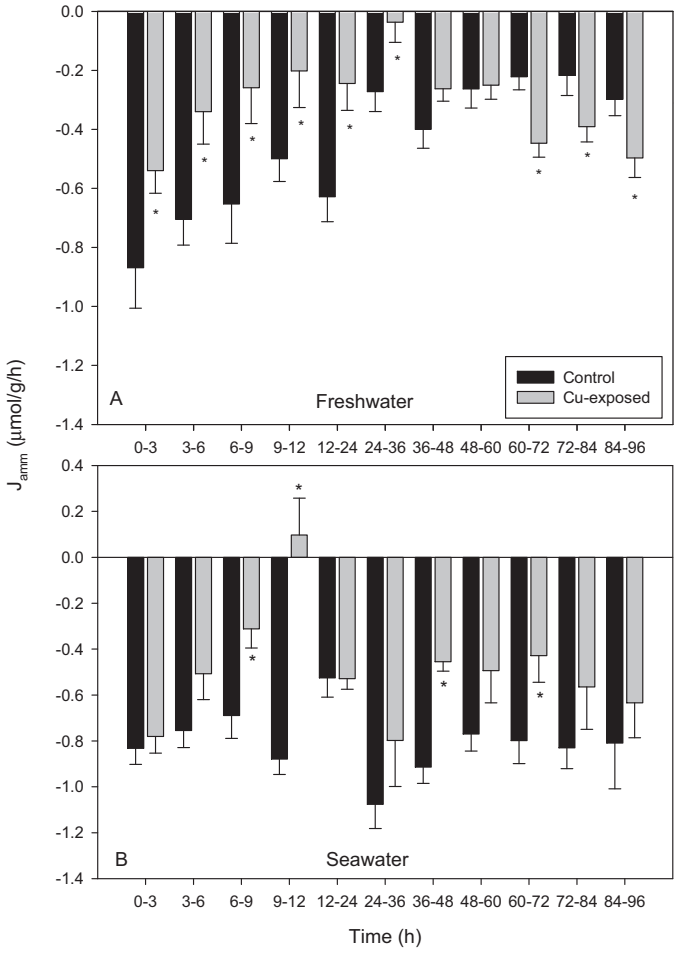

Fig. 4. Ammonia excretion rates (J_{amm}) of control (black bars) and Cu-exposed (gray bars; $N = 6–15$) fish acclimated to (A) FW and (B) SW over 96 h. Fish in all groups were fasted 72 h prior to experimentation. Asterisks represent Cu-exposed values which are significantly different from corresponding control values as determined by an unpaired *t*-test ($P < 0.05$).

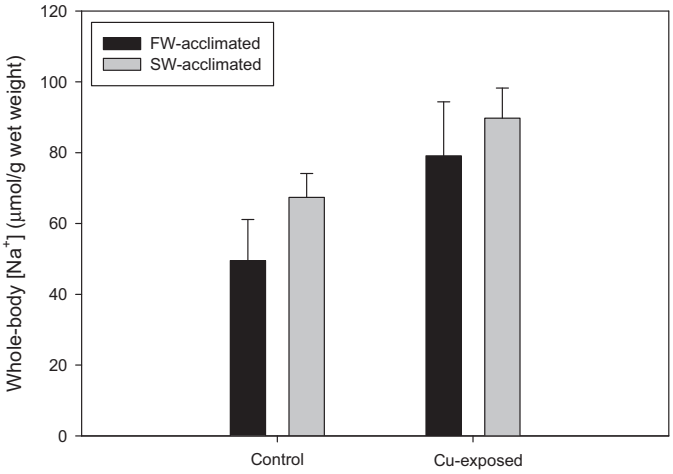

Fig. 5. Whole-body [Na^+] in fish acclimated to FW (black bars; $N = 4–6$) and SW (gray bars; $N = 6$) under control conditions or exposed to 20 µg Cu/l for 96 h. No significant differences occurred between FW and SW values or between control and exposed values as determined by unpaired *t*-tests ($P < 0.05$).

course of inhibition was different from that seen in FW (Fig. 4B). Noteworthy, however, were the reversal of J_{amm} at 9 h (at which time this value was not significantly different from zero) and the eventual recovery of J_{amm} by 72 h of exposure in these fish (Fig. 4B).

No significant differences in whole-body [Na^+] occurred between FW and SW-acclimation or between control and exposed fish (Fig. 5). There was, however, a slight, non-significant tendency for Cu-exposed fish to exhibit an increased whole-body [Na^+] in both salinities ($P = 0.153$ and $P = 0.089$ for FW and SW, respectively).

There were no significant differences in branchial Na^+/K^+-ATPase or H^+-ATPase activities between FW and SW-acclimated fish (Fig. 6A and B). Neither Na^+/K^+-ATPase nor H^+-ATPase activities were significantly affected by Cu exposure in either salinity acclimation relative to control values (Fig. 6A and B). Interestingly, H^+-ATPase activity was significantly greater following 96 h Cu exposure relative to the value observed after 12 h of Cu exposure (Fig. 6B). CA activity was significantly greater in SW compared to FW. Notably, Cu exposure significantly inhibited CA activity by 48% and 43% following 12 h in FW and SW, respectively (Fig. 6C). However, after 96 h of Cu exposure, CA activity was recovered to control values in both FW and SW-acclimated fish (Fig. 6C).

4. Discussion

4.1. Effects of Cu on FW-acclimated *P. vivipara*

Cu exposure in FW-acclimated *P. vivipara* resulted in an inhibition of J_{amm} (Fig. 4A), consistent with a number of studies showing that Cu disrupts the clearance of ammonia in FW (Laurén and McDonald, 1985; Wilson and Taylor, 1993b; Beaumont et al., 2003; Blanchard and Grosell, 2006). In the current model for ammonia excretion in FW (Wright and Wood, 2009), ammonia efflux is believed to be tied indirectly to Na^+ influx and some studies have demonstrated Cu-induced inhibition of ammonia clearance with a concomitant disruption in Na^+ ionoregulation (Laurén and McDonald, 1985; Wilson and Taylor, 1993b). Furthermore, Laurén and McDonald (1985) determined that this disruption in Na^+ regulation in FW rainbow trout occurs primarily via a decrease in active Na^+ influx rather than an increase in Na^+ efflux, indicating that Cu is acting upon active components of ionoregulation which may also be involved in ammonia excretion.

For the first time in fish, we have shown that Cu exposure can inhibit CA activity *in vivo* in FW (Fig. 6C), and that this inhibition

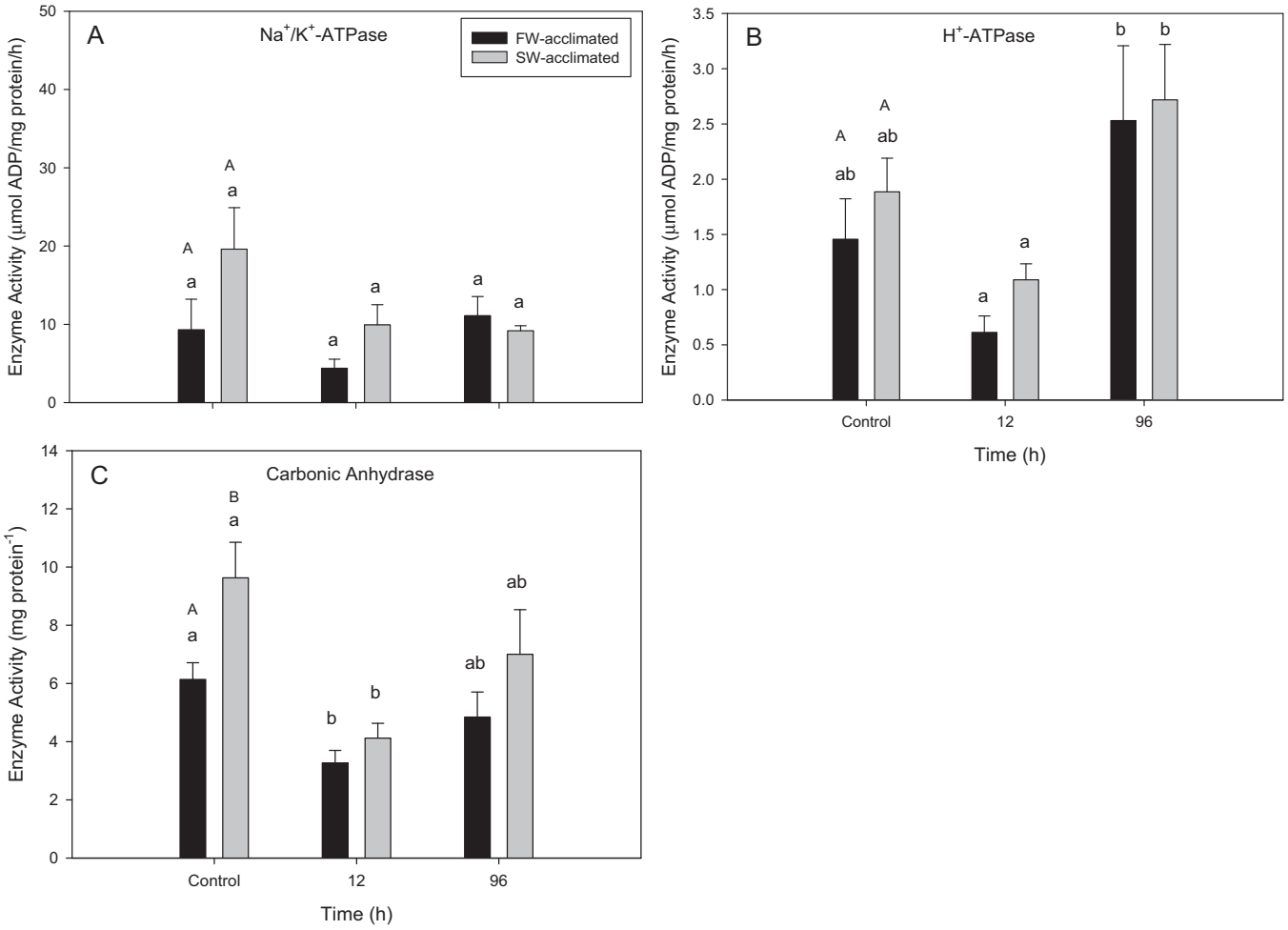

Fig. 6. Activities of (A) Na^+/K^+-ATPase, (B) H^+-ATPase, and (C) carbonic anhydrase in gills of fish acclimated to FW (black bars) and SW (gray bars) under control conditions or exposed to 20 μg Cu/l for 12 or 96 h. Fish in all groups were fasted 72 h prior to experimentation. Means sharing the same lower case letter are not significantly different from one another as determined by a one-way ANOVA with a Holm–Sidak post hoc test. Means sharing the same upper case letters are not significantly different from one another as determined by an unpaired *t*-test ($P < 0.05$, $N = 4–6$).

occurs concomitantly with a disruption of J_{amm}. This novel observation would also explain the commonly observed effects of Cu on Na^+ homeostasis as this enzyme provides protons for the electrogenic uptake of Na^+ powered by apical H^+-ATPase as well as for the apical Na^+/H^+ exchanger (see Evans et al., 2005; Evans, 2008 for review). In *P. vivipara*, Cu had no effects on whole-body [Na^+] following 96 h of exposure in FW (Fig. 5). This result is consistent with the branchial activity of all the enzymes measured and with J_{amm}, all of which recovered by 96 h of exposure. Moreover, J_{amm} was upregulated beyond control values in the final 36 h of exposure. Paralleling this trend was the significant increase in H^+-ATPase activity at 96 h relative to the depressed 12 h time point (Fig. 6B). This enzyme was the only one of those measured whose activity was significantly greater at 96 h compared to 12 h, potentially tying this enzyme to the active restoration of J_{amm} in these fish. The clear effects of Cu exposure on J_{amm} and lack of significant changes in Na^+/K^+-ATPase activity suggest that this enzyme does not play a role in the mechanism of Cu-induced J_{amm} inhibition in FW in *P. vivipara.* In fact, Wood and Nawata (2011) have recently demonstrated that in euryhaline rainbow trout, Na^+/K^+-ATPase activity cannot be activated by NH_4^+ substitution, though it can in some marine teleosts such as the toadfish (Mallery, 1983), mudskipper (Randall et al., 1999), and pufferfish (Nawata et al., 2010). Thus, this enzyme may not always play a strong role in ammonia excretion. In future studies, it will be interesting to determine the nature of NH_4^+ activation of Na^+/K^+-ATPase in *P. vivipara*. Overall, these fish appeared to be able to acclimate to Cu exposure by 96 h, showing no significant differences in enzyme activity or whole-body [Na^+]. Moreover, J_{amm} was upregulated following 60 h of Cu exposure, perhaps in response to a build-up of plasma ammonia caused by earlier inhibition by Cu. Unfortunately, due to the small size of the fish obtained from the field it was not possible to measure plasma ammonia.

4.2. Effects of Cu on SW-acclimated P. vivipara

Cu exposure in SW-acclimated fish also resulted in an inhibition of J_{amm} (Fig. 4B), consistent with previous studies in SW-acclimated fish (Wilson and Taylor, 1993a; Grosell et al., 2003, 2004a; Blanchard and Grosell, 2006). Unlike the situation in FW, J_{amm} is not believed to be tied to Na^+ regulation (i.e., Na^+ excretion) mechanistically, though both Na^+ and NH_4^+ movement may occur via the same paracellular cation shunt in SW teleosts (Fig. 1). Despite this, some studies have demonstrated that Cu exposure can result in an accumulation of both ammonia and Na^+ in the plasma (Wilson and Taylor, 1993a; Grosell et al., 2004a). In previous studies, the inhibition of Na^+/K^+-ATPase has been hypothesized as the mechanism of this loss of Na^+ regulation, yet several studies show that Cu does not affect the branchial activity of this enzyme in SW-acclimated fish (Stagg and Shuttleworth, 1982b; Grosell et al., 2003, 2004b; Blanchard and Grosell, 2006). In the present

study, Na^+/K^+-ATPase activity was not significantly inhibited by Cu (Fig. 6A). CA activity was inhibited significantly following 12 h of Cu exposure in SW-acclimated *P. vivipara*, followed by a recovery by 96 h (Fig. 6C). The inhibition of this enzyme, similar to the case in FW, would result in a reduction of the protons available for gill boundary layer acidification and, in fact, correlated with an inhibition of J_{amm} (Fig. 4B). Note that despite the similar trends in H^+-ATPase activity in both FW and SW-acclimated *P. vivipara* in response to Cu exposure, this enzyme is not believed to play a role in ammonia excretion in SW-acclimated fish (see Wilkie, 2002; Weihrauch et al., 2009) and the activity of this enzyme in SW is likely representative of vesicular V-ATPase. As was the case in FW, *P. vivipara* acclimated to SW also appear to readily adapt to Cu following 96 h of exposure, demonstrating no significant differences in J_{amm}, whole-body [Na^+], or branchial enzyme activity between control or Cu-exposed conditions.

4.3. Comparison of FW and SW-acclimated P. vivipara

Under control conditions, J_{amm} in *P. vivipara* was higher in SW than in FW at 24–96 h (Fig. 3). In accordance, Wood and Nawata (2011) demonstrated that J_{amm} is greater in SW-acclimated rainbow trout (*Oncorhynchus mykiss*) than in FW-acclimated trout. This may not be the case in all species as Blanchard and Grosell (2006) did not find any difference in J_{amm} between FW and SW-acclimated killifish. In their study, however, J_{amm} was measured only for 4 h. In future studies, it will be interesting to determine how different salinity acclimations and salinity transfers affect J_{amm} in killifish and *P. vivipara*.

Aside from J_{amm}, the only other physiological difference between *P. vivipara* in FW and SW under control conditions was the activity of CA (Fig. 6C). This difference, with SW-acclimated fish displaying a greater gill CA activity, has been demonstrated previously in coho salmon smolts (Zbanyszek and Smith, 1984) and tilapia (Kültz et al., 1992), possibly representing a physiological adaptation to SW acclimation.

FW-acclimated *P.vivipara* displayed a greater gill Cu burden under control conditions than SW-acclimated fish, likely as a result of a lower cation competition in FW. Nonetheless, Cu did not accumulate in the gills following 96 h of exposure to 20 μg/l in either salinity (Fig. 2). This may suggest that toxic effects of Cu occurred in the absence of significant branchial Cu accumulation or that by 96 h, fish were able to re-establish control levels of branchial Cu, potentially alleviating any toxic effects. In future studies it will be interesting to examine a time-course of branchial Cu accumulation and relate this to physiological effects of the metal.

The speciation of Cu was undoubtedly different in our FW versus SW exposures. In both, DOC was likely the dominant factor affecting Cu bioavailability (Grosell, 2012). A standard speciation program (Visual MINTEQ ver. 3.0, KTH, Dept. of Land and Water Resources Engineering, Stockholm, Sweden), run with the present water chemistry (Table 1) suggests that in both media, Cu-DOC comprised the dominant fraction (>75%) and free ionic Cu^{2+} was only a very small fraction (<3%) of the total, while other species differed considerably between FW and SW. Regardless, the effects of Cu exposure on J_{amm} and CA activity were qualitatively similar in FW versus SW, suggesting that speciation in the water was not a critical parameter in the present study.

4.4. Implications of Cu-induced inhibition of branchial CA activity

To date, the only evidence of Cu eliciting effects on CA activity in aquatic organisms arises from *in vitro* studies. In a euryhaline crab species, Cu was demonstrated to inhibit branchial CA activity in an *in vitro* gill preparation (Vitale et al., 1999). In fish, Cu has been demonstrated to inhibit CA activity in lysed red blood cells (Christensen and Tucker, 1976), and to inhibit the activity of isolated CA purified from seabass liver (Ceyhun et al., 2011) and from rainbow trout brain (Soyut et al., 2008). Grosell et al. (2002) first proposed that the inhibition of CA by Cu would account for the suite of physiological effects which occur as a result of Cu exposure. In addition to impaired Na^+ regulation and J_{amm}, Cu exposure also causes impaired Cl^- balance (Stagg and Shuttleworth, 1982a; Laurén and McDonald, 1985; Wilson and Taylor, 1993a, b; Blanchard and Grosell, 2006) and acid–base balance dysfunction (Wilson and Taylor, 1993b; Larsen et al., 1997; Wang et al., 1998), all of these processes being tied to CA activity (e.g. Payan and Maetz, 1972; Perry et al., 1984, 1986; Wright et al., 1989). Recently, Grosell (2012) suggested that the lack of evidence for Cu-induced CA inhibition is a result of the high dilution of sample homogenates, necessitated by the delta pH method of measuring activity, which leads to a loss of inhibition. Despite this, we have demonstrated inhibition of CA by Cu in FW and SW-acclimated *P. vivipara* using this method. Moreover, if the delta pH method of assaying CA activity dilutes Cu inhibition, we may be underestimating the severity of this inhibition *in vivo*. In *P. vivipara*, inhibition of CA, at 12 h of exposure, was followed by a subsequent recovery of activity after 96 h exposure (Fig. 6C). This novel finding demonstrates that Cu has the capacity to inhibit this enzyme *in vivo* and that its inhibition and recovery can be related to the inhibition and recovery of J_{amm}. In killifish, exposure to 150 μg Cu/l did not result in CA inhibition in FW or SW-acclimated fish (Blanchard and Grosell, 2006). The lack of CA inhibition in their study could be a result of species differences, although these species are fairly closely related, and CA, a ubiquitous enzyme, is likely to be highly conserved among species. Potentially, the activity of CA during Cu exposure is recovered very quickly, given the importance of this enzyme to ionoregulation, acid-base balance and J_{amm}, and the time course of the study by Blanchard and Grosell (2006) missed its inhibition. Regardless, it is clear that Cu has the potential for CA inhibition and that this could act as a common mechanism for Cu-induced inhibitions of J_{amm} in FW and SW-acclimated fish as originally suggested by Grosell et al. (2002).

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References

Ay, Ö., Kalay, M., Tamer, L., Canli, M., 1999. Copper and lead accumulation in tissues of a freshwater fish *Tilapia zillii* and its effects on branchial Na, K-ATPase activity. Bulletin of Environment Contamination and Toxicology 62, 160–168.

Beaumont, M.W., Butler, P.J., Taylor, E.W., 2003. Exposure of brown trout *Salmo trutta* to a sublethal concentration of copper in soft acidic water: effects upon

gas exchange and ammonia accumulation. Journal of Experimental Biology 206, 153–162.
Blanchard, J., Grosell, M., 2005. Effects of salinity on copper accumulation in the common killifish (*Fundulus heteroclitus*). Environmental Toxicology and Chemistry 24, 1403–1413.
Blanchard, J., Grosell, M., 2006. Copper toxicity across salinities from freshwater to seawater in the euryhaline fish *Fundulus heteroclitus*: is copper an ionoregulatory toxicant in high salinities? Aquatic Toxicology 80, 131–139.
Ceyhun, S.B., Şentürk, M., Yerlikaya, E., Erdoğan, O., Küfrevioğlu, Oi., Ekinci, D., 2011. Purification and characterization of carbonic anhydrase from the teleost fish *Dicentrarchus labrax* (European seabass) liver and toxicological effects of metals on enzyme activity. Environmental Toxicology and Pharmacology 32, 69–74.
Christensen, G.M., Tucker, J.H., 1976. Effects of selected water toxicants on the *in vitro* activity of fish carbonic anhydrase. Chemico-Biological Interactions 13, 181–192.
De Boeck, G., Vlaeminck, A., Balm, P.H.M., Lock, R.A.C., De Wachter, B., Blust, R., 2001. Morphological and metabolic changes in common carp, *Cyprinus carpio*, during short-term copper exposure: interactions between Cu^{2+} and plasma cortisol elevation. Environmental Toxicology and Chemistry 20, 374–381.
DiTusa, C.A., Christensen, T., McCall, K.A., Fierke, C.A., Toone, E.J., 2001. Thermodynamics of metal binding 1. Metal ion binding by wild-type carbonic anhydrase. Biochemistry 40, 5338–5344.
Esaki, M., Hoshijima, K., Kobayashi, S., Fukuda, H., Kawakami, K., Hirose, S., 2007. Visualization in zebrafish larvae of Na^+ uptake in mitochondria-rich cells whose differentiation is dependent on *foxi3a*. American Journal of Physiology: Regulatory, Integrative and Comparative Physiology 292, R470–R480.
Evans, D.H., 2008. Teleost fish osmoregulation: what have we learned since August Krogh, Homer Smith, and Ancel Keys. American Journal of Physiology: Regulatory, Integrative and Comparative Physiology 295, R704–R713.
Evans, D.H., Piermarini, P.M., Choe, K.P., 2005. The multifunctional fish gill: dominant site of gas exchange, osmoregulation, acid–base regulation, and excretion of nitrogenous waste. Physiological Reviews 85, 97–177.
Gagnon, A., Jumarie, C., Hontela, A., 2006. Effects of Cu on plasma cortisol and cortisol secretion by adrenocortical of rainbow trout (*Oncorhynchus mykiss*). Aquatic Toxicology 78, 59–65.
Grosell, M., 2012. Copper. In: Farrell, A.P., Wood, C.M., Brauner, C.J. (Eds.), Homeostasis and Toxicology of Essential Metals, Fish Physiology, vol. 31A. Academic Press, San Diego, pp. 54–110.
Grosell, M., Blanchard, J., Brix, K.V., Gerdes, R., 2007. Physiology is pivotal for interactions between salinity and acute copper toxicity to fish and invertebrates. Aquatic Toxicology 84, 162–172.
Grosell, M., McDonald, M.D., Wood, C.M., Walsh, P.J., 2004a. Effects of prolonged copper exposure in the marine gulf toadfish (*Ospanus beta*) I. Hydromineral balance and plasma nitrogenous waste products. Aquatic Toxicology 68, 249–262.
Grosell, M., McDonald, M.D., Walsh, P.J., Wood, C.M., 2004b. Effects of prolonged copper exposure in the marine gulf toadfish (*Ospanus beta*) II: copper accumulation, drinking rate and Na^+/K^+-ATPase activity in osmoregulatory tissues. Aquatic Toxicology 68, 263–275.
Grosell, M., Nielsen, C., Bianchini, A., 2002. Sodium turnover rate determines sensitivity to acute copper and silver exposure in freshwater animals. Comparative Biochemistry and Physiology. C: Comparative Pharmacology 133, 287–303.
Grosell, M., Wood, C.M., Walsh, P.J., 2003. Copper homeostasis and toxicity in the elasmobranch *Raja erinacea* and the teleost *Myoxecephalus octodecemspinosus* during exposure to elevated water-borne copper. Comparative Biochemistry and Physiology Part C 135, 179–190.
Henry, R.P., 1991. Techniques for measuring carbonic anhydrase activity *in vitro*: the electrometric delta pH and pH stat assay. In: Dodgson, S.J., Tashian, R.E., Gros, G., Carter, N.D. (Eds.), The Carbonic Anhydrases: Cellular Physiology and Molecular Genetics. Plenum, New York, pp. 119–126.
Kültz, D., Bastrop, R., Jürss, K., Siebers, D., 1992. Mitochondria-rich (MR) cells and the activities of the Na^+/K^+-ATPase and carbonic anhydrase in the gill and opercular epithelium of *Oreochromis mossambicus* adapted to various salinities. Comparative Biochemistry and Physiology. B: Comparative Biochemistry 102, 293–301.
Larsen, B.K., Pörtner, H.-O., Jensen, F.B., 1997. Extra- and intracellular acid-base balance and ionic regulation in cod (*Gadus morhua*) during combined and isolated exposures to hypercapnia and copper. Marine Biology 128, 337–346.
Lauer, M.M., Bianchini, A., 2010. Chronic copper toxicity in the estuarine copepod *Acartia tonsa* at different salinities. Environmental Toxicology and Chemistry 29, 2297–2303.
Laurén, D.J., McDonald, D.G., 1985. Effects of copper on branchial ionoregulation in the rainbow trout, *Salmo gairdneri* Richardson. Journal of Comparative Physiology B 155, 635–644.
Laurén, D.J., McDonald, D.G., 1987a. Acclimation to copper by rainbow trout, *Salmo gairdneri*: biochemistry. Canadian Journal of Fisheries and Aquatic Sciences 44, 105–111.
Laurén, D.J., McDonald, D.G., 1987b. Acclimation to copper by rainbow trout, *Salmo gairdneri*: physiology. Canadian Journal of Fisheries and Aquatic Sciences 44, 99–104.
Li, J., Quabius, E.S., Wendelaar Bonga, S.E., Flik, G., Lock, R.A.C., 1998. Effects of water-borne copper on branchial chloride cells and Na^+/K^+-ATPase activities in Mozambique tilapia (*Oreochromis mossambicus*). Aquatic Toxicology 43, 1–11.
Magid, E., 1967. The activity of carbonic anhydrases B and C from human erythrocytes and the inhibition of the enzymes by copper. Scandinavian Journal of Haematology 4, 257–270.
Mallery, C.H., 1983. A carrier enzyme basis for ammonium excretion in teleost gill. NH4+-stimulated Na-dependent ATPase activity in *Ospanus beta*. Comparative Biochemistry and Physiology Part A 74, 889–897.
Martins, C.M.G., Almeida, D.V., Marins, L.F.F., Bianchini, A., 2011a. mRNA expression and activity of ion-transporting proteins in gills of the blue crab *Callinectes sapidus*: effects of waterborne copper. Environmental Toxicology and Chemistry 30, 206–211.
Martins, C.M.G., Barcarolli, I.F., Menezes, E.J., Giacomin, M.M., Wood, C.M., Bianchini, A., 2011b. Acute toxicity, accumulation and tissue distribution of copper in the blue crab *Callinectes sapidus* acclimated to different salinities: *in vivo* and *in vitro* studies. Aquatic Toxicology 101, 88–99.
Nadella, S.D., Fitzpatrick, J.L., Franklin, N., Bucking, C., Smith, S., Wood, C.M., 2009. Toxicity of dissolved Cu, Zn, Ni and Cd to developing embryos of the blue mussel (*Mytilus trossolus*) and the protective effect of dissolved organic carbon. Comparative Biochemistry and Physiology. C: Comparative Pharmacology 149, 340–348.
Nawata, C.M., Hung, C.C.Y., Tsui, T.K.N., Wilson, J.M., Wright, P.A., Wood, C.M., 2007. Ammonia excretion in rainbow trout (*Oncorhynchus mykiss*): evidence for Rh glycoprotein and H^+-ATPase involvement. Physiological Genomics 31, 463–474.
Nawata, C.M., Hirose, S., Nakada, T., Wood, C.M., Kato, A., 2010. Rh glycoprotein expression is modulated in pufferfish (*Takifugurubripes*) during high environmental ammonia exposure. Journal of Experimental Biology 213, 3150–3160.
Paganini, C.L., Souza, M.M., Bianchini, A., 2008. Gill ion transport and copper accumulation in the blue crab *Callinectes sapidus*. Comparative Biochemistry and Physiology. C: Comparative Pharmacology 184, 461.
Paquin, P.R., Gorsuch, J.W., Apte, S., Batley, G.E., Bowles, K.C., Campbell, P.G.C., Delos, C.G., Di Toro, D.M., Dwyer, R.L., Galvez, F., Gensemer, R.W., Goss, G.G., Hogstrand, C., Janssen, C.R., McGeer, J.C., Naddy, R.B., Playle, R.C., Santore, R.C., Schneider, U., Stubblefield, W.A., Wood, C.M., Wu, K.B., 2002. The biotic ligand model: a historical overview. Comparative Biochemistry and Physiology. C: Comparative Pharmacology 133, 3–35.
Payan, P., Maetz, J., 1972. Branchial sodium transport mechanisms in *Scyliorhinus canicula*: evidence for Na^+/NH_4^+ and Na^+H^+ exchanges and for a role of carbonic anhydrase. Journal of Experimental Biology 58, 487–502.
Perry, S.F., Fryer, J.N., 1997. Proton pumps in the fish gill and kidney. Fish Physiology and Biochemistry 17, 363–369.
Perry, S.F., Malone, S., Ewing, D., 1986. Hypercapnic acidosis in the rainbow trout (*Salmo gairdneri*). I. Branchial ionic fluxes and blood acid–base status. Canadian Journal of Zoology 65, 888–895.
Perry, S.F., Payan, P., Girard, J.P., 1984. Effects of perfusate HCO_3^- and P_{CO2} on chloride uptake in perfused gills of rainbow trout (*Salmo gairdneri*). Canadian Journal of Fisheries and Aquatic Sciences 41, 1768–1773.
Pinho, G.L.L., Bianchini, A., 2010. Acute copper toxicity in the euryhaline copepod *Acartia tonsa*: implications for the development of an estuarine and marine Biotic Ligand Model. Environmental Toxicology and Chemistry 29, 1834–1840.
Pinho, G.L.L., Pedroso, M.S., Rodrigues, S.C., Souza, S.S., Bianchini, A., 2007. Physiological effects of copper in the euryhaline copepod *Acartia tonsa*: waterborne versus waterborne plus dietborne exposure. Aquatic Toxicology 84, 62–70.
Prazeres, M.F., Martins, S.E., Bianchini, A., 2012. Assessment of water quality in coastal waters of Fernando de Noronha, Brazil: biomarker analyses in *Amphistegina lessonii*. Journal of Foraminiferal Research 42, 56–65.
Randall, D.J., Wilson, J.M., Peng, K.W., Kok, T.W.K., Kuah, S.S.L., Chew, S.F., Lam, T.J., Ip, Y.K., 1999. The mudskipper, *Periophthalmodon schlosseri*, actively transports against a concentration gradient. American Journal of Physiology: Regulatory, Integrative and Comparative Physiology 277, R1562–R1567.
Shih, T-H., Horng, J-L., Hwang, P-P., Lin, L-Y., 2008. Ammonia excretion by the skin of zebrafish (*Danio rerio*) larvae. American Journal of Physiology: Cell Physiology 295, C1625–C1632.
Soyut, H., Beydemir, Ş., Hisar, O., 2008. Effects of some metals on carbonic anhydrase from brains of rainbow trout. Biological Trace Element Research 123, 179–190.
Stagg, R.M., Shuttleworth, T.J., 1982a. The accumulation of copper in *Platichthys flesus* L. and its effects on plasma electrolyte concentrations. Journal of Fish Biology 20, 491–500.
Stagg, R.M., Shuttleworth, T.J., 1982b. The effects of copper on ionic regulation by the gills of the seawater-adapted flounder (*Platichthys flesus* L.). Journal of Comparative Physiology B 149, 83–90.
Vitale, A.M., Monserrat, J.M., Castilho, P., Rodriguez, E.M., 1999. Inhibitory effects of cadmium on carbonic anhydrase activity and ionic regulation of the estuarine crab *Chasmagnathus granulate* (Decapoda, Grapsidae). Comparative Biochemistry and Physiology. C: Comparative Pharmacology 122, 121–129.
Wang, T., Knudsen, P.K., Brauner, C.J., Busk, M., Vijayan, M.M., Jensen, F.B., 1998. Copper Exposure impairs intra- and extracellular acid–base regulation during hypercapnia in the fresh water rainbow trout (*Oncorhynchus mykiss*). Journal of Comparative Physiology B 168, 591–599.
Weihrauch, D., Wilkie, M.P., Walsh, P.J., 2009. Ammonia and urea transporters in gills of fish and aquatic crustaceans. Journal of Experimental Biology 212, 1716–1730.
Wilkie, M.P., 2002. Ammonia excretion and urea handling by fish gills: present understanding and future challenges. Journal of Experimental Zoology 293, 284–301.
Wilson, R.W., Taylor, E.W., 1993a. Differential responses to copper in rainbow trout (*Oncorhynchus mykiss*) acclimated to sea water and brackish water. Journal of Comparative Physiology B 163, 239–246.

Wilson, R.W., Taylor, E.W., 1993b. The physiological responses of freshwater rainbow trout, *Oncorhynchus mykiss*, during acutely lethal copper exposure. Journal of Comparative Physiology B 163, 38–47.

Wood, C.M., Nawata, C.M., 2011. A nose-to-nose comparison of the physiological and molecular responses of rainbow trout to high environmental ammonia in seawater *versus* freshwater. Journal of Experimental Biology 214, 3557–3569.

Wright, P.A., Randall, D.J., Perry, S.F., 1989. Fish gill water boundary layer: a site of linkage between carbon dioxide and ammonia excretion. Journal of Comparative Physiology B 158, 627–635.

Wright, P.A., Wood, C.M., 2009. A new paradigm for ammonia excretion in aquatic animals: role of Rhesus (Rh) glycoproteins. Journal of Experimental Biology 212, 2303–2312.

Zbanyszek, R., Smith, L.S., 1984. Changes in carbonic anhydrase activity in coho salmon smolts resulting from physical training and transfer into seawater. Comparative Biochemistry and Physiology. A: Comparative Physiology 79, 229–233.

Zimmer, A.M., Nawata, C.M., Wood, C.M., 2010. Physiological and molecular analysis of the interactive effects of feeding and high environmental ammonia on branchial ammonia excretion and Na^+ uptake in freshwater rainbow trout. Journal of Comparative Physiology B 180, 1191–1204.