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Aquatic Toxicology 68 (2004) 263–275

AQUATIC TOXICOLOGY

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Effects of prolonged copper exposure in the marine gulf toadfish (*Opsanus beta*) II: copper accumulation, drinking rate and Na^+/K^+-ATPase activity in osmoregulatory tissues

M. Grosell [a,*], M.D. McDonald [a], P.J. Walsh [a], C.M. Wood [a,b]

[a] *Division of Marine Biology and Fisheries, RSMAS, University of Miami, 4600 Rickenbacker Causeway, Miami, FL 33149-1098, USA*
[b] *Department of Biology, McMaster University, Hamilton, Ont., Canada*

Received 14 October 2003; received in revised form 26 January 2004; accepted 3 March 2004

Abstract

Gulf toadfish were exposed to sublethal levels of copper (12.8 or 55.2 μM) for 30 days. Drinking in control fish averaged 1 ml kg^{-1} h^{-1} but exposure to 55.2 μM copper resulted in a complex biophasic pattern with initial (3 h and 1 day) inhibition of drinking rate, followed by an elevation of drinking rate from day 3 onwards. Drinking led to copper accumulation in the intestinal fluids at levels three to five times higher than the ambient copper concentrations, which in turn resulted in intestinal copper accumulation. The gill exhibited more rapid accumulation of copper than the intestine and contributed to early copper uptake leading to accumulation in internal organs. Muscle, spleen and plasma exhibited little if any disturbance of copper homeostasis while renal copper accumulation was evident at both ambient copper concentrations. The liver exhibited the highest copper concentrations and the greatest copper accumulation of all examined internal organs during exposure to 55.2 μM. Elevated biliary copper excretion was evident from measurements of gall bladder bile copper concentrations and appeared to protect partially against internal accumulation in fish exposed to 12.8 μM copper. No inhibition of Na^+/K^+-ATPase activity in either gills or intestine was seen despite copper accumulation in these organs. Calculations of inorganic copper speciation suggest that $Cu(CO_3)_2^{2-}$ complexes which dominate in seawater and intestinal fluids are of limited availability for uptake while the low levels of ionic Cu^{2+}, $CuOH^+$ and $CuCO_3$ may be the forms taken up by the gill and the intestinal epithelium.

Keywords: Cu uptake; Cu homeostasis; Cu speciation; Hepatobiliary excretion; Intestinal fluid Cu

1. Introduction

Copper is a co-factor for a number of enzymes including cytochrome C oxidase and is thus essential for respiration in all eukaryote cells (Linder et al., 1998). The redox nature of copper is also what makes this element a potent toxicant. Tight regulation of organ and cellular copper concentrations has therefore evolved to ensure sufficient copper for essential processes while at the same time preventing toxicity arising from copper excess (Pena et al., 1999). Aquatic organisms can take up copper directly from the water and elevated ambient copper concentrations can lead to excess copper accumulation in several tissues (Lauren

* Corresponding author. Tel.: +1-305-361-4623; fax: +1-305-361-4001.
E-mail address: mgrosell@rsmas.miami.edu (M. Grosell).

0166-445X/$ – see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.aquatox.2004.03.007

and McDonald, 1985, 1987a, 1987b; De Boeck et al., 1995; Grosell et al., 1997, 1998a, 2001b, 2003b; De Boeck et al., 2001; Grosell and Wood, 2002; Kamunde et al., 2002). Oxidative stress is generally accepted as one of the major effects of excessive cellular copper concentrations but other effects occur in osmoregulatory organs of aquatic organisms. Examples are the inhibition of Na^+ transport across the gills of freshwater fish which co-incide with inhibition of the Na^+/K^+-ATPase enzyme (Lauren and McDonald, 1985, 1987a, 1987b) and the inhibition of branchial carbonic anhydrase in crustaceans (Vitale et al., 1999). Osmoregulatory organs, of aquatic organisms, which are in direct contact with the environment are targets for these effects. In freshwater organisms osmoregulatory effects of acute copper exposure occur in the gills (Wood, 2001; Grosell et al., 2002) and can be related to the copper concentration in the gill tissue which in turn can be related to ambient copper concentration and water chemistry (Santore et al., 2001; Di Toro et al., 2001). The gills of marine fish also accumulate copper in response to elevated ambient concentrations (Stagg and Shuttleworth, 1982; Grosell et al., 2003b) but it is entirely unknown to what extent branchial copper accumulation reflects the degree of physiological disturbance during either acute or chronic exposure. In addition, the intestine of marine fish may accumulate copper from imbibed seawater. Drinking is a vital part of marine teleost osmoregulation (Smith, 1930; Loretz, 1995; Karnaky, 1998) and intestinal copper accumulation and perhaps associated osmoregulatory effects might therefore be expected during water-borne exposure. Despite earlier findings of osmoregulary effects of copper in marine teleost (Stagg and Shuttleworth, 1982; Wilson and Taylor, 1993) this issue has not been addressed for copper. Findings for silver, however, have demonstrated intestinal silver accumulation in marine fish exposed to water-borne silver and have also revealed that the silver accumulation in the intestine contributes significantly to whole animal osmoregulatory disturbance during both acute and Chronic exposure (Grosell et al., 1999; Hogstrand et al., 1999; Webb and Wood, 2000; Grosell and Wood, 2001; Webb et al., 2001).

The purpose of the present study was to characterize copper uptake, internal accumulation and potential excretion during exposure to two elevated but sublethal copper concentrations (12.8 and 55.2 μM) for up to 30 days in flow-through seawater. To achieve this goal, copper concentrations in gills, plasma, intestinal segments, liver, kidney, spleen, white muscle and gall bladder bile were monitored throughout 30 days of exposure. Furthermore, to ascertain intestinal copper exposure, drinking rate was measured and intestinal fluid samples were obtained for analysis of copper concentrations. Because both the gills and intestine accumulated copper during the 30-day exposures and because the fish exhibited significant osmoregulatory disturbance (Grosell et al., 2004), these tissues were also examined for Na^+/K^+-ATPase activity. By comparing the results of the present study with a parallel investigation of hydromineral balance during these prolonged copper exposures (Grosell et al., 2004) we were able to examine potential relationships between copper concentrations in osmoregulatory tissues, corresponding Na^+/K^+-ATPase activity and whole animal osmoregulatory disturbance.

2. Materials and methods

2.1. Experimental animals and copper exposure

The present investigation was conducted on the gulf toadfish (*Opsanus beta*) obtained and held in the laboratory as outlined in the accompanying article (Grosell et al., 2004). Fish were subject to prolonged copper exposure under flow-through conditions resulting in 12.8 ± 1.6 and 55.2 ± 5.0 μM copper in Bear cut seawater (30‰ and 23 °C) as previously outlined (Grosell et al., 2004).

2.2. Experimental protocol

2.2.1. Copper accumulation and Na^+/K^+-ATPase activity

To determine the effect of prolonged copper exposure on copper uptake, internal distribution and accumulation, fish ($N = 8$) were sampled from both copper concentrations at 0, 1, 3, 8, 16 and 30 days of exposure. Weekly feeding was scheduled to always precede sampling times by at least 48 h. For sampling, individual fish were transferred from the exposure tank to copper-free MS222 containing seawater ($0.2\ g\ l^{-1}$) and a spot sample of rectal fluids was obtained prior to blood sampling by caudal puncture as

already described (Grosell et al., 2004). Subsequently, fluid samples were obtained from the anterior, mid and posterior segment of the intestine and then gall bladder bile, liver, spleen, kidney, gill, muscle and intestinal tissue were obtained by dissection. Plasma, bile and tissues were placed in liquid nitrogen immediately after collection. Gill and intestinal tissues were transferred to an ultra-cold freezer (−80 °C) and stored for later analysis of Na^+/K^+-ATPase activity, while the remaining samples were stored at −20 °C until analysis of [copper].

2.2.2. Drinking rate experiments

When sampling fish exposed to 55.2 μM, we noted that initially the volume of intestinal fluid was low compared to non-exposed control fish. In addition, the volume tended to be higher in fish sampled towards the end of the 30 day exposure. Based on these observations, a separate experiment was performed to measure drinking rate in fish exposed to copper for 3 h, 1, 3, 8, 15 and 30 days. Drinking rate measurements were always conducted with a parallel non-exposed control group as follows. In each of two tanks (4 l) 8 fish were placed and allowed to recover from the handling for a minimum of 24 h. Both tanks were supplied with a flow-through seawater (>200 ml min^{-1}). For the copper exposed fish, copper was added as described above to yield a final nominal concentration of 50 μM. To initiate the drinking rate experiment, water flow was terminated and 10 μCi [^{3}H]polyethylene glycol-4000 (PEG-4000) l^{-1} (specific activity: 2050 μCi g^{-1}; NEN-Dupont), an extracellular marker, was added to each of the two tanks (staggered by 45 min to allow for sampling). Water samples were taken for radioactivity measurements at 0.25, 3.0 and 6.0 h after addition of the PEG-4000. After obtaining the last water samples, MS-222 was added from a neutralized stock solution to yield a final concentration of 0.2 g l^{-1} in the exposure tanks. Subsequently, individual fish were netted out of the exposure tanks, rinsed in non-radioactive seawater and weighed. Samples of rectal fluid and plasma were obtained to ensure that no PEG-4000 was absorbed into the blood or was leaving the gastro-intestinal tract through the rectum, respectively, at the end of the 6 h incubation period. No appreciable radioactivity was recorded in any of the plasma or rectal fluid samples. Subsequently, the gastro-intestinal tract (GIT) was exposed as above and was then clamped at the start of the esophagus and immediately anterior to the anus by hemostats to prevent loss of contents, carefully placed in a vial and weighed. The entire GIT was homogenized in 4 vol. of 10% perchloric acid. The homogenate was centrifuged (500 g for 5 min).

Initially, aliquots of 0.5, 1.0 and 1.5 ml of the clear, protein-free supernatant, 20 μl plasma and 20 μl of rectal fluid, from each fish, were prepared for determination of β radioactivity as described below. Having determined that the 1.5 ml supernatant volume was best suited for determining radioactivity in the gastro-intestinal tract, samples from subsequent experiments were prepared only using this volume.

Drinking rate was calculated by relating the counts from the GIT to the counts from the reference water samples, the weight of the individual fish and the PEG-4000 exposure time.

2.3. Analytical techniques

Sub-samples of all collected tissues, were prepared for analysis of copper concentration by adding approximately five times the volume of 1N HNO_3 (trace metal grade, Merck Chemicals). The samples were left overnight at 75 °C, vortexed and centrifuged. The supernatant together with water, plasma and bile samples were diluted appropriately prior to copper analysis on a graphite furnace atomic absorption spectroscope (Varian AA-1275 with GTA-9 atomiser) using a 10 μl injection volume, N_2 gas and standard operating conditions as documented by the manufacturer. Copper in seawater was determined using NH_4NO_2 as modifier and all copper analyses were referenced to a certified standard. Control copper concentrations were below detection limit of 12 nM.

[^{3}H]PEG-radioactivities in the water samples, plasma, rectal fluids and homogenized GIT samples from the drinking rate experiments were determined using liquid scintillation counting. All samples were made up to a total volume of 5 ml by addition of seawater, to which was added 10 ml ACS fluor (Amersham). Radioactivity was determined using a TmAnalytic BetaTract 6895 instrument and quench correction was performed manually by internal standardization. Drinking rates (ml kg^{-1} h^{-1}) were calculated from the radioactivity recorded in the gastro-intestinal tract taking into account the weight

of the fish, the [^{3}H]PEG incubation time, and the [^{3}H]PEG-specific activity of the water.

Na^+/K^+-ATPase enzyme activity was determined in gill filament samples and in samples of all intestinal segments by the micro-plate method (Mccormick, 1993).

2.4. Data presentation and statistical evaluation

Data are presented as means ± S.E.M. (N). [Copper] and Na^+/K^+-ATPase values from copper exposed fish were compared to their corresponding control values by a two-tailed Student's t-test with Bonferroni multisample comparison correction. Control animals were sampled both at the beginning and at the end of the 30 days of exposure and were not different. These two control samples have been combined and are presented as one control value at day 0 throughout. In addition, single factor ANOVA's were employed to evaluate trends in Na^+/K^+-ATPase activity values over time. Drinking rates from copper exposed fish were compared to the simultaneous control value using a simple two-tailed Student's t-test. In all cases, $P < 0.05$ was applied.

3. Results

3.1. Drinking rate

Drinking rate in control toadfish was greatly variable over time with values ranging from 0.5 to 1.5 ml $kg^{-1}\,h^{-1}$ illustrating the importance of simultaneous experimental and control measurements (Fig. 1). Exposure to 55.2 μM copper for 3 and 24 h resulted in a substantially reduced drinking rate whereas longer exposure periods resulted in drinking rates elevated as much as threefold above corresponding control rates (Fig. 1).

3.2. Copper accumulation

Gill filaments exhibited significantly elevated [copper] from day 1 of exposure to both ambient copper concentrations throughout the 30 days of exposure with a progressive, almost linear gradual increase over time. Interestingly, the approximately fivefold difference in exposure concentration did not lead to a fivefold difference in branchial copper concentrations, which differed by only 30% at the end of the 30-day exposure (Fig. 2). Only a very limited effect of exposure to elevated copper in the water was seen on plasma [copper] with levels being around 20 nmol ml^{-1} (Fig. 2). Both exposure concentrations resulted in a 20–25% increase in plasma copper after 1 day of exposure but values returned to control levels by days 3 and 8 for both groups (Fig. 2).

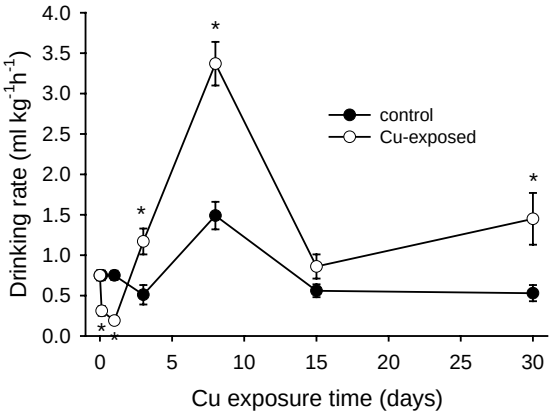

Fig. 1. Drinking rate in control toadfish (dark symbols) and in toadfish exposed to 55.2 μM copper (open symbols) for up to 30 days ($N = 8$). The symbol (*) denotes statistically significant difference from corresponding control ($P < 0.05$).

The other epithelial surface directly in contact with the copper containing environment, the intestine, showed a delayed onset of copper accumulation when compared to the gills (Fig. 3). The anterior segment of the intestine showed elevated copper concentration from days 3 and 8 onwards at exposure to 55.2 and 12.8 μM copper, respectively. The onset of copper accumulation was delayed even further in the more distal segments of the intestine where elevated copper concentrations were not seen until days 8 and 16 in the mid region and days 8 and 30 in the posterior region of the intestine during exposure to 55.2 and 12.8 μM copper, respectively (Fig. 3). It is interesting to note that the anterior intestine accumulated approximately 50% more copper in comparison to the branchial tissue from the same fish, and also that intestinal copper concentrations were lower in the distal segments than in the anterior segment of the intestine. Furthermore, the relative copper concentrations in the intestinal epithelium, at least during the first 16 days of exposure,

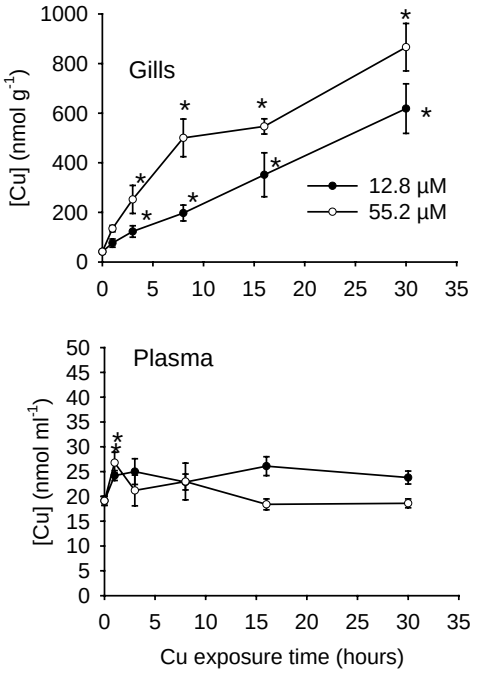

Fig. 2. Gill (top panel) and plasma (bottom panel) [copper] in toadfish exposed to 12.8 and 55.2 μM copper for up to 30 days ($N = 8$). The symbol (*) denotes statistically significant difference from the control value at day 0 (mean of controls obtained at days 0 and 30) ($P < 0.05$).

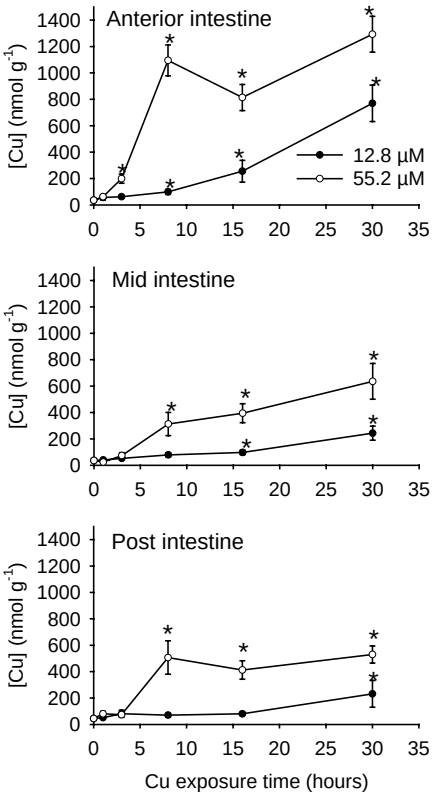

Fig. 3. Copper concentration in tissue obtained from the anterior (top panel), mid (middle panel) and posterior (bottom panel) region of the intestine in toadfish exposed to 12.8 and 55.2 μM copper for up to 30 days ($N = 8$). The symbol (*) denotes statistically significant difference from the control value at day 0 (mean of controls obtained at days 0 and 30) ($P < 0.05$).

seem to better reflect the difference in ambient copper concentrations than those seen in the branchial tissue.

While the kidney exhibited close to a doubling of tissue copper concentration from day 1 of exposure to 55.2 μM and at day 30 of exposure to 12.8 μM copper, the spleen and white muscle did not show copper accumulation during the 30 days of exposure to either copper concentration (Fig. 4). Copper levels in white muscle tissue were very low compared to all other examined tissues, even plasma, and even in control fish.

As could be expected, both hepatic and biliary copper levels were much higher (approximately one order of magnitude) than levels seen in any other tissues even in non-exposed fish. Hepatic copper was significantly elevated from day 3 onwards in fish exposed to 55.2 μM copper reaching levels seven to eight fold higher than control values by day 30. At the lower copper exposure (12.8 μM), the elevation in hepatic copper concentrations was much less, reaching about twofold the control values by day 30 (Fig. 5).

Onset of increased biliary copper concentration was not seen until day 3 of exposure but was of similar magnitude in the two groups of fish, largely independent of the exposure concentration. For both experimental groups biliary copper concentrations increased continuously over time to values seven to eight fold those of non-exposed fish (Fig. 5) and to values more than 100-fold higher than corresponding plasma values.

Spot samples of intestinal fluids obtained from fish exposed to both copper concentrations revealed copper concentrations up to fivefold higher than those of the surrounding and ingested seawater (Fig. 6). Furthermore, at least for fish exposed to 55.2 μM, copper levels in the gastro intestinal fluids tended to be

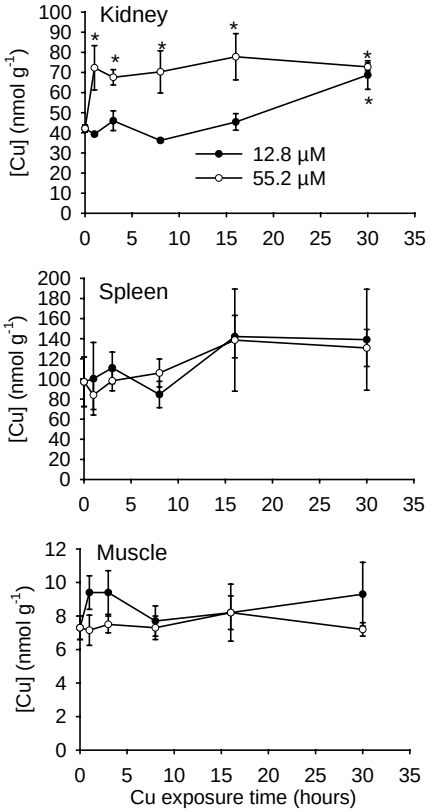

Fig. 4. Copper concentration in renal tissue (top panel), spleen (middle panel) and white muscle (bottom panel) from toadfish exposed to 12.8 and 55.2 μM copper for up to 30 days ($N = 8$). The symbol (*) denotes statistically significant difference from the control value at day 0 (mean of controls obtained at days 0 and 30) ($P < 0.05$).

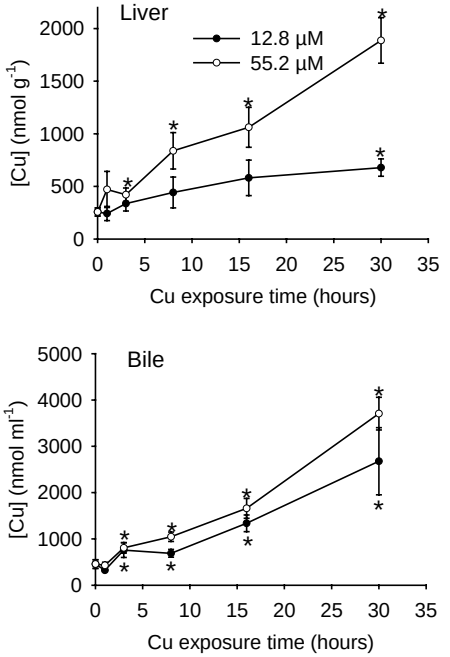

Fig. 5. Copper concentration in hepatic tissue (top panel) and in gall bladder bile from toadfish exposed to 12.8 and 55.2 μM copper for up to 30 days ($N = 8$). The symbol (*) denotes statistically significant difference from the control value at day 0 (mean of controls obtained at days 0 and 30) ($P < 0.05$).

higher in the more distal segments of the intestine. Notably, intestinal fluids of non-exposed fish contained relatively high copper concentrations on the order of 10 μM in the anterior region of the intestine and exhibited a trend towards reduced rather than increased concentrations in the distal gastro-intestinal regions (Fig. 6).

Despite substantial copper accumulation, no significant effect of copper exposure on branchial Na^+/K^+-ATPase activity was observed, although fish exposed to 55.2 μM may have exhibited a trend towards reduced levels from days 3–16, with re-established normal levels at the end of the 30-day exposure (Fig. 7). Na^+/K^+-ATPase activities were two- to threefold higher in the intestinal tissues but a similar lack of effect of copper exposure on Na^+/K^+-ATPase activity in the anterior intestine was evident (Fig. 8), however, both the mid and posterior region of the intestine exhibited an overall significant (ANOVA), and perhaps surprising, increase in Na^+/K^+-ATPase activity during the 30-day exposure to both copper concentrations (Fig. 8).

4. Discussion

4.1. Drinking rate

The initial inhibition of drinking rate in response to copper exposure is in agreement with several reports on reduced drinking as a result of acute silver exposure (Grosell et al., 1999; Hogstrand et al., 1999; Webb et al., 2001) and may be interpreted as a taste aversion. The control drinking rate in the gulf toadfish is at the lower end of the range of drinking rates reported from

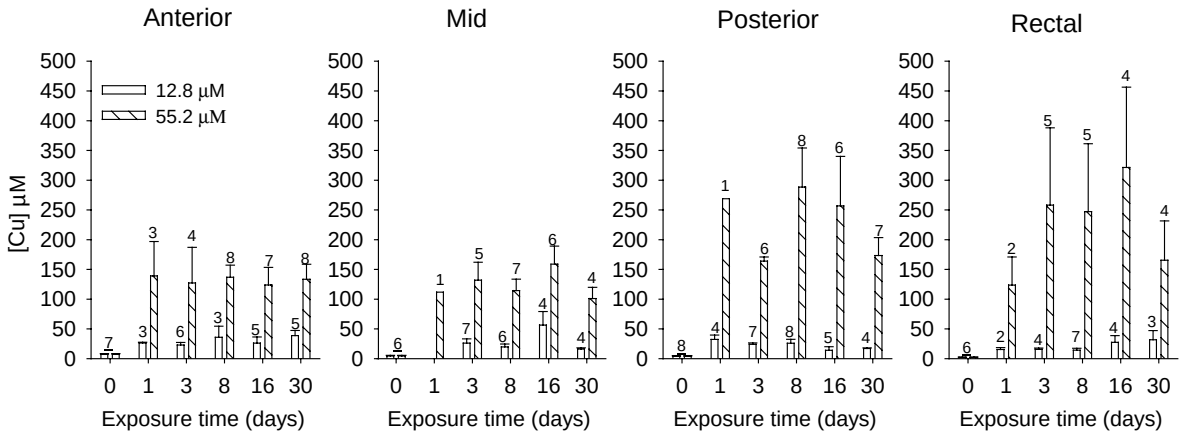

Fig. 6. Copper concentrations in fluid samples obtained from the anterior, mid and posterior segment of the intestine as well as from the rectum of toadfish exposed to 12.8 and 55.2 μM copper for up to 30 days ($N = 8$). All values were significantly different from the control value at days 0 (mean of controls obtained at days 0 and 30) ($P < 0.05$).

other teleosts (Shehadeh and Gordon, 1969; Carrick and Balment, 1983; Perrot et al., 1992; Wilson et al., 1996) and appears to vary greatly with time. From day 3 and onwards the drinking rate in copper-exposed fish was elevated above control values presumably in an attempt to increase intestinal fluid absorption to compensate for other copper induced effects. These effects could include reduced branchial ion extrusion as seen for Na^+ in silver-exposed marine fish (Grosell and Wood, 2001). In addition, increased osmotic fluid loss across the gill surface and reduced renal fluid reabsorption as a response to copper exposure cannot be dismissed as potential effects. In support of the latter was rapid renal copper accumulation (Fig. 4) during exposure to 55.2 μM copper which may have influenced kidney function. Elevated plasma Mg^{2+} as reported in the previous paper (Grosell et al., 2004) could be a consequence of both impaired renal Mg^{2+} excretion and increased branchial permeability. The potential effects of copper exposure on branchial permeability and on kidney function are clearly worthy of further attention. Indeed, it seems that renal copper accumulation is a feature common to only marine teleosts (Stagg and Shuttleworth, 1982; Grosell et al., 2003b). Regardless of which effect(s) lead to the increased drinking rate, it seems to have been a sufficient response only up until day 8 of exposure since plasma osmolality and NaCl concentration were elevated at days 16 and 30 (Grosell et al., 2004). Drinking rate was not measured in fish exposed to 12.8 μM copper, but it is clear that the gastro-intestinal tract must be involved in a compensatory response during exposure to this concentration since rectal fluid composition in these fish indicated increased fluid absorption (Grosell et al., 2004). In further support of the proposed compensatory role of the intestine is the overall increase in intestinal Na^+/K^+-ATPase activity during prolonged exposure (Fig. 8).

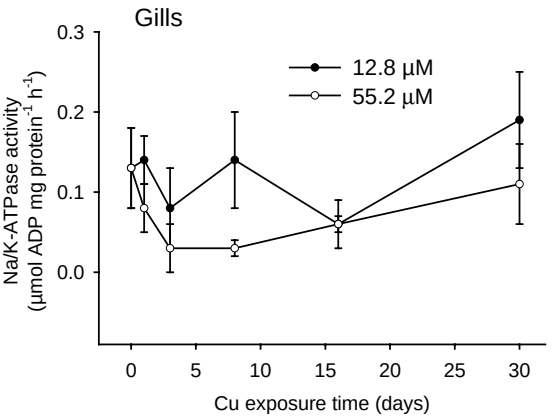

Fig. 7. Na^+/K^+-ATPase activity in gill filaments obtained from toadfish exposed to 12.8 and 55.2 μM copper for up to 30 days ($N = 8$). No significant differences among individual means were observed.

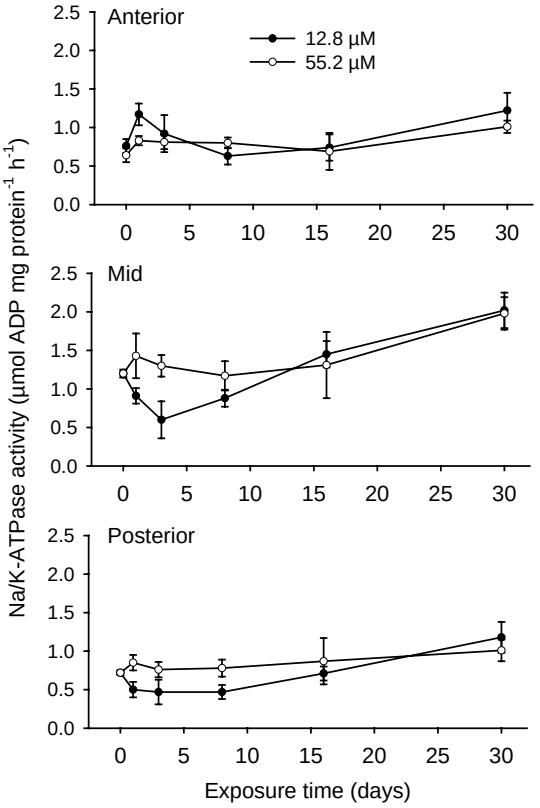

Fig. 8. Na^+/K^+-ATPase activity in tissue obtained from the anterior (top panel), mid (middle panel) and posterior (bottom panel) region of the intestine in toadfish exposed to 12.8 and 55.2 μM copper for up to 30 days ($N = 8$). No significant differences among individual means were observed but an overall effect of copper in the mid and posterior segment was revealed by ANOVA.

4.2. Copper homeostasis

4.2.1. Copper uptake and accumulation

Two main epithelia in marine fish are exposed to copper during water-borne exposure and may thus exhibit direct effects from contact with the metal-containing water and may also be involved in whole animal copper accumulation. Since both the gill and the intestine accumulate copper to concentrations several fold higher than control values (Figs. 2 and 3), these tissues could both contribute to whole body copper uptake. Internalization of copper in the toadfish was evident from kidney, liver and bile (Figs. 4 and 5).

Control gill copper concentrations are in agreement with previously reported values for teleost fish (Pelgrom et al., 1995; Grosell et al., 1996; Grosell et al., 1997; Taylor et al., 2000). Fish exposed to 12.8 μM copper exhibited a continuous linear increase in gill copper concentration over time. This was largely true also for fish exposed to 55.2 μM copper, although a tendency to reduced accumulation rate was seen from day 8 onwards. Notably, toadfish gill tissue accumulates copper to levels that are 10–15 times higher than background levels which is different from most teleosts (fresh water and marine) examined to date (Pelgrom et al., 1995; Grosell et al., 1996, 1997, 2003b; Taylor et al., 2000). One noteworthy exception is the freshwater acclimated European eel which can exhibit similarly elevated branchial copper concentrations when exposed to higher copper concentrations (Grosell et al., 1998a). It thus seems that greatly elevated gill copper concentrations are perhaps a species-specific response rather than a feature related to marine fish. It is interesting that the two species which exhibits the highest copper accumulation are also among the most copper tolerant (Grosell et al., 1997, 2004).

It is clear from the early accumulation in plasma and kidney (Figs. 2 and 4) at day 1 when copper is not elevated in the intestine (Fig. 3) but clearly elevated in the gill (Fig. 2) that the gill contributes to internalization of copper in marine teleosts. Copper accumulating in the gill is the product of net uptake from the water across the apical membrane and net extrusion across the basolateral membrane into the blood. Since copper continues to accumulate in the gill tissue during continuous exposure the basolateral membrane likely comprises the rate-limiting step for copper internalization across the gill.

The slightly elevated plasma copper concentrations observed at 1 day after onset of copper exposure (Fig. 2) must have been derived primarily from branchial uptake since gastro-intestinal exposure was limited during this period due to reduced drinking (Fig. 1). Plasma copper concentration had returned to normal values by day 3 of exposure and remained normal or only slightly elevated during the remainder of the 30 day exposure. This strong homeostatic control of plasma copper is in agreement with findings from several freshwater teleost but toadfish levels (20–25 nmol ml^{-1}) are slightly higher than in most other fish which typically exhibits plasma copper levels in the order of 8–10 nmol ml^{-1} (Buckley et al.,

1982; Pelgrom et al., 1995; Grosell et al., 1997; Kamunde et al., 2001; Grosell et al., 2001b, 2003b).

The intestinal epithelium did not exhibit significant copper accumulation until days 3–8 in fish exposed to 55.2 μM copper or until as late as day 30 for the posterior intestinal segment from fish exposed to 12.8 μM. This delayed intestinal copper accumulation was in contrast to intestinal fluid copper concentrations which were already elevated greatly above control values after 1 day of exposure. Unexpectedly, the copper concentrations in the anterior intestinal lumen were generally threefold higher than the corresponding copper concentration in the water. For fish exposed to 55.2 μM copper, this trend was even more pronounced in the distal segment of the intestine with values fivefold higher than water levels. Several conclusions can be made from this observation: Firstly, it is clear that fluid absorption across the intestinal epithelium renders copper more concentrated in the intestinal lumen. Secondly, considering the increase in copper concentrations as fluids are passing through the intestine (Fig. 6) and the lower tissue copper concentration in the more distal segments of the intestine (Fig. 3) it appears that copper becomes less bioavailable as it moves along the intestine. Indeed, gill copper levels are twice those found in the posterior intestine despite the fact that the posterior intestine is exposed to 150–250 μM which is three- to fivefold higher than the corresponding water copper concentration (55.2 μM). Although these differences in copper accumulation could be due to one or several of the many physiological difference between these two epithelia, it is conceivable that different chemistry in intestinal fluid compared to seawater could be part of the reason for the observed differences. Finally, billiary excretion of copper, perhaps in a form which is unavailable for intestinal uptake, could contribute to the highly elevated concentrations in the intestinal fluids.

The intestinal fluid has an osmolality generally comparable to that of the blood plasma (~300–320 mOsm) and thus much lower than the surrounding seawater (~1000 mOsm) but exhibits a rather unique chemistry. The continuous absorption of NaCl results in rather low Na^+ and Cl^- levels in intestinal fluids but leaves Mg^{2+} and SO_4^- concentrated much above the corresponding values in seawater. In addition, the intestinal epithelium secretes HCO_3^- at very high rates resulting not only in high HCO_3^- levels but also highly alkaline conditions with high CO_3^{2-} levels. Precipitation of calcium salt results, so dissolved Ca^{2+} concentrations are generally lower in the intestinal lumen than in seawater (10 mM) (Wilson, 1999; Grosell et al., 2001a; Wilson et al., 2002). Table 1 summarizes the toadfish intestinal fluid chemistry based on our previous report (Grosell et al., 2004) and unpublished observations (Grosell, McDonald and Walsh). The chemical composition listed in Table 1 was used together with a geochemical speciation computer program kindly provided by Dr. Millero (Millero and Pierrot, 1998) to calculate the inorganic chemical speciation of copper in seawater and in intestinal fluids (Table 2). Both seawater and intestinal fluids are clearly dominated by $Cu(CO_3)_2^{2-}$ and $CuCO_3$ an effect which is pronounced for $Cu(CO_3)_2$ in intestinal fluids due to the alkaline conditions. It is also clear that, although already low in seawater, the concentrations of ionic

Table 1
Concentration of major electrolytes (in mM) and pH in gastro-intestinal fluids of the gulf toadfish. Na^+, Mg^{2+}, Ca^{2+}, Cl^- values are from (Grosell et al., 2004) and remaining values are from Grosell, McDonald & Walsh (unpublished)

	Anterior	Mid	Posterior	Rectal
Na^+	85	50	41	35
Mg^{2+}	83	135	150	180
Ca^{2+}	7	9	5.5	4
K^+	5	5	5	5
Cl^-	120	97	75	65
SO_4^{2-}	83	125	130	112
HCO_3^-	50	60	60	70
pH	8.01	8.20	8.30	8.50

Table 2
Relative distribution of inorganic copper species in seawater and in gastro-intestinal fluids (% of total copper)

	Seawater	Gastro-intestinal fluids			
		Anterior	Mid	Posterior	Rectal
Cu^{2+}	0.27	0.08	0.03	0.02	0.01
$CuOH^+$	0.16	0.08	0.05	0.04	0.03
$Cu(OH)_2$	0.08	0.09	0.11	0.12	0.13
$CuHCO_3^+$	0.04	0.02	0	0	0
$CuCO_3$	25.44	15.87	8.16	6.65	5.08
$Cu(CO_3)_2^{2-}$	73.95	83.78	91.65	93.14	94.74

Speciation was calculated using a chemical speciation program based on natural waters (Millero and Pierrot, 1998) and was independent of copper concentration.

Table 3
Correlation coefficients (r) for relation between luminal concentration of various copper species and intestinal tissue copper concentration based on values obtained from days 16 and 30 of copper exposure

	12.8 μM	55.2 μM
Cu^{2+}	0.73	0.80
$CuOH^{+}$	0.55	0.41
$Cu(OH)_2$	0.02	−0.61
$CuCO_3$	0.65	0.61
$Cu(CO_3)_2{}^{2-}$	0.13	−0.56
Total copper	0.20	−0.29

Correlations are based on mean tissue and intestinal fluid concentrations from a total of 12 data sets.

Cu^{2+} and $CuOH^{+}$ are further reduced in the intestinal fluids.

A correlation analysis of luminal copper versus intestinal tissue copper concentration revealed a very low and even negative correlation suggesting that the majority of the copper is not available for uptake by the intestinal tissue (Table 3). A similar relationship is seen for the largely dominating $Cu(CO_3)_2{}^{2-}$ confirming that most of the copper is indeed of limited availability. In contrast however, Cu^{2+}, $CuCO_3$ and $CuOH^{+}$ all exhibit relative strong and positive correlations (most pronounced for Cu^{2+}) suggesting that these forms of copper are available for uptake by the intestinal epithelium. This is agreement with our understanding of copper toxicity in freshwater organisms where Cu^{2+} and to a lesser extent $CuOH^{+}$ are the most potent forms of copper when it comes to exerting acute toxicity at the gills (Chakoumakos et al., 1979; Lauren and McDonald, 1986). Less is known about uptake and toxicity of $CuCO_3$, however, although much less potent that Cu^{2+}, $CuCO_3$ have been found to exert acute toxicity to the freshwater cladoceran, *Daphnia magna* (De Schamphelaere et al., 2002). Recognizing the many differences between the gill and the intestinal epithelium, the gill copper vs. seawater copper speciation was not included in the correlation analysis. It should be noted however, that the above interpretations seem to extend also to the seawater–gill interface.

Although the intestine accumulates substantial amounts of copper, and although it seems that the intestinal transport processes are affected by copper exposure (Grosell et al., 2004), it is not clear to what extent intestinal copper uptake contributes to whole animal internalized copper and to overall osmoregulatory disturbance. This question could be resolved using an approach similar to that employed to assess the relative contribution of the gill versus the intestine to silver uptake and effects in marine teleosts (Grosell and Wood, 2001).

4.2.2. Fate of internalized copper

Spleen and white muscle did not accumulate copper during the 30 days of exposure. Muscle copper concentrations were low which is in agreement with previous reports from other teleosts (Stagg and Shuttleworth, 1982; Kamunde et al., 2001; Grosell et al., 2001b, 2003b). In contrast to freshwater teleosts, but in agreement with the marine fish examined to date, the toadfish accumulated significant amounts of copper in the kidney (Stagg and Shuttleworth, 1982; Buckley et al., 1982; Grosell et al., 1997, 1998a, 1998b, 2001b, 2003b; Kamunde et al., 2001).

The liver and bile contained the highest copper concentrations of any tissue examined, which is in agreement with several previous reports from teleost fish (Stagg and Shuttleworth, 1982; Buckley et al., 1982; McCarter and Roch, 1984; Lauren and McDonald, 1987a; Grosell et al., 1998a, 1998b, 2001b) and in overall agreement with the homeostatic role of the liver in vertebrates in general (Cousins, 1985). Control liver copper levels were in agreement with values from other teleost and were increased several-fold during exposure to 55.2 μM copper but only slightly increased at 30 days of exposure to 12.8 μM copper. Gallbladder bile on the other hand was elevated five- to sevenfold at 30 days of exposure to either 12.8 or 55.2 μM copper, showing only a minor difference between the two exposure groups. From gallbladder bile copper concentrations it is evident that hepatobiliary copper excretion is involved in copper homeostasis. Whereas biliary copper excretion in fish exposed to 12.8 μM almost protects against hepatic and renal copper accumulation, this is not the case in fish exposed to 55.2 μM. Despite the fivefold difference in ambient copper concentrations and the three- to fourfold difference in hepatic copper between the two copper exposed groups at the end of the 30 days exposure, gall bladder bile copper remains similar. This finding demonstrates that whole animal copper uptake exceed the hepatobiliary copper excretion capacity in

fish exposed to 55.2 μM, but less so in fish exposed to 12.8 μM.

4.2.3. Na^+/K^+-ATPase activity and correlation between tissue copper and effects

The Biotic Ligand Model (BLM) has been refined to be highly successful in predicting *acute* copper and silver toxicity *in freshwater* based on the overall premise that short-term tissue metal accumulation equates to effect levels (Santore et al., 2001; Di Toro et al., 2001). It is therefore perhaps tempting to propose a similar relationship for tissue copper concentration and induced effects in *marine* fish during *chronic* exposure. A comparison of the osmoregulatory effects of the 30 day exposures reported in the previous study (Grosell et al., 2004) and the observed copper accumulation however, does not seem to support a simple relationship between copper concentration in osmoregulatory tissues and the observed whole animal effects. For example, comparing fish exposed to 55.2 μM copper for 16 days when osmoregulatory disturbance was most pronounced to fish exposed to 12.8 μM copper for 30 days illustrate this lack of relationship. Although accumulating the same amount of copper as the fish exposed for 16 day to 55.2 μM, fish exposed for 30 days to 12.8 μM exhibited no osmoregulatory disturbance. A similar case can be made for the anterior segment of the intestine although not for the mid and posterior segments where ambient copper concentrations were reflected by tissue copper accumulation at all times. It should be noted however that there was no, or a negative, general relationship between intestine luminal total copper concentration and intestinal tissue copper accumulation, making potential relationships between tissue copper and induced effect even more complicated.

The differences between the clear correlation of tissue copper accumulation and effect during acute exposure in freshwater and the lack thereof in the present prolonged exposure in seawater must be related to salinity and/or exposure duration. *First* considering the potential importance of different salinities, it is clear that the gill which is a target organ in both freshwater and marine fish, contributes significantly to osmoregulation in both environments, but it does so in entirely different ways. Not only is the direction of ion transport opposite in the two environments but it is also conducted through highly different pathways (Evans, 1993; Karnaky, 1998; Wood, 2001). Secondly, marine teleost osmoregulation involves more organs than in freshwater fish, in addition to a drinking response and these components seem be influenced in a complex manner including also compensatory responses (Grosell et al., 2004). *Second*, considering the exposure duration and the essentiality of copper it seems likely that homeostatic control mechanisms may have been activated during the prolonged exposure employed in the present study. Certainly at the whole animal level, this is evident from the highly elevated biliary copper concentrations. Furthermore, copper concentration increased in the anterior and posterior intestines of fish exposed to 55.2 μM copper during the first 8 days after which a stable level was maintained (Fig. 3). A similar pattern is evident in the gill (Fig. 2) where accumulation rate seemed to be reduced from day 8 and onwards. It is not possible from the present information to determine whether the homeostatic response involved reduced uptake, increased extrusion or both, but the response occurred after the duration of standard acute toxicity testing (96 h) and could thus be one reason for the dissimilarity between the metal-effect correlation seen when comparing acute and prolonged exposures.

Perhaps in contrast to expectation no or only little effect of copper exposure was seen on Na^+/K^+-ATPase enzyme activity levels in gills and intestinal segments. This is in clear contrast to observations from freshwater fish exposed to copper (Lauren and McDonald, 1987a) but is similar to reports from silver exposed marine fish (Grosell et al., 1999; Hogstrand et al., 1999). It is interesting that the distal intestinal segments exhibited a trend towards elevated enzyme activity as copper exposure progressed because it is in agreement with a compensatory response suggested based on intestinal fluid composition (Grosell et al., 2004). This might indicate that elevated Na^+/K^+-ATPase enzyme activity is involved in what appears to be a compensatory elevation of intestinal fluid absorption.

It is unknown why Na^+/K^+-ATPase enzyme activity seems to be insensitive to copper (and silver) exposure in seawater fish. The Na^+/K^+-ATPase enzyme is central to ion transport by the gills of both freshwater and marine fish and could therefore be expected to respond similarly to metal accumulation in the two environments. However, recent studies have shown that

in the gills of euryhaline fish different isoforms of this enzyme are expressed in response to salinity change (Richards et al., 2003) and it is conceivable that that different isoforms may exhibit differential sensitivity to copper.

While Na^+/K^+-ATPase enzyme activity in osmoregulatory tissues is not clearly influenced by copper exposure in marine fish, despite substantial copper accumulation, it is also clear that osmoregulation is impaired. This indicates that other components of ion transport processes may be the target for metal-induced osmoregulatory disturbance and clearly warrants attention. Furthermore, although we did not find a clear correlation between tissue copper concentrations and induced effects, it remains to be investigated whether effects of chronic copper exposure can be predicted from early copper uptake and accumulation.

Acknowledgements

Sincere thanks to Jimbo Luznar for supplying toadfish for the present study. Superb technical assistance by Christine Guadagnolo is greatly appreciated. MG was supported in part by a SETAC/Taylor-Francis advance training fellowship. CMW is supported by the Canada Research Chair Program and NSERC. MDM was supported by an OGS scholarship. Research was also supported by an NIEHS grant (ES 05705) from the Marine and Freshwater Biomedical Center at RSMAS, University of Miami.

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