Effects of prolonged copper exposure in the marine gulf toadfish (*Opsanus beta*) I. Hydromineral balance and plasma nitrogenous waste products

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

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

Abstract

Acute (96 h) and prolonged (30 days) copper exposure induced osmoregulatory disturbance and impaired nitrogenous waste excretion in the marine teleost, the gulf toadfish (*Opsanus beta*), which was found to be extremely tolerant to acute copper exposure with a 96 h LC50 exceeding 340 μM but exhibited disturbed mineral balance in response to both acute and prolonged exposure to ∼12 μM copper. The main cause of copper toxicity was found to be Na^+ and Cl^- regulatory failure leading to elevated plasma $[Na^+]$ and $[Cl^-]$ and osmolality which in turn led to fluid loss from muscle tissue. Analysis of intestinal fluid composition revealed a complicated pattern of effects of copper exposure. Intestinal transport physiology was directly influenced by copper exposure with Cl^- absorption being the most sensitive parameter. Evidence for increased Na^+ and fluid absorption when the fish exhibited elevated plasma osmolality indicates that the intestine may also exhibit a compensatory response to impairment of branchial transport processes, suggesting at least two target organs (gill and intestine) for copper toxicity in marine fish. Plasma Mg^{2+} was elevated from approximately 1.5 mM to as much as 4.0 mM, likely as a result of increased branchial permeability. While plasma [ammonia] clearly responded to copper exposure, plasma [urea] exhibited a much more sensitive and pronounced response to both acute and prolonged copper exposure, resulting in as much as a three-fold increase in circulating urea levels. This response is most likely the result of the unique ability of this teleost to convert ammonia to urea.
© 2004 Elsevier B.V. All rights reserved.

Keywords: Cu toxicity; Osmoregulation; Marine fish; Nitrogenous waste; Intestinal transport physiology

1. Introduction

Copper is an essential element required by all living organisms but can be toxic when present in excess. Toxicity arising from copper exposure is most frequently reported in aquatic environments and it

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

appears that water-borne exposure rather than exposure via the diet is most potent in exerting toxic effects during acute exposure. Whereas dietary bioavailability of copper to fish is low and toxicity arising from dietary exposure occurs only at very high exposure levels (Handy, 1996; Julshamm et al., 1988; Lanno et al., 1985), exposure to low concentrations of copper in the water can cause adverse toxic effects in aquatic organisms (Grosell et al., 2002).

Substantial efforts towards understanding the mechanism(s) of acute copper toxicity in freshwater fish have led to some understanding of copper uptake and accumulation in the gill, the primary target organs for acute toxicity, and the subsequent physiological effects [see (Grosell et al., 2002; Wood, 2001) for reviews]. Much less, however, is known about the mechanism of uptake, chronic copper toxicity, and the potential effects of copper in marine fish.

In freshwater fishes, copper exposure clearly results in rapid copper accumulation by the gill (Grosell et al., 1998a; Grosell and Wood, 2002; Grosell et al., 1997;Lauren and McDonald, 1985, 1987a,b) followed by disturbance of especially Na^+ and Cl^- homeostasis (Grosell et al., 2002; Wood, 2001). While limited information is available about the mechanisms of copper toxicity in saltwater fish, it seems that osmoregulatory failure is again the mechanism of acute copper toxicity (Stagg and Shuttleworth, 1982a,b; Wilson and Taylor, 1993a).

Osmoregulation in marine fish involves multiple organs which could all be potential targets during acute and chronic copper exposure. Marine fish combat water loss to the surrounding marine environment by continuous drinking of seawater (Smith, 1930). The imbibed seawater is desalinated in the esophagus before entering the intestine. In the intestine, fluid absorption follows the uptake of Cl^- and Na^+ and both these ions are subsequently extruded across the gill (Evans, 1993; Loretz, 1995; Shehadeh and Gordon, 1969). As a consequence of the high concentrations of SO_4^{2-} and Mg^{2+} in the imbibed seawater and the NaCl and fluid absorption, SO_4^{2-} and Mg^{2+} ions are highly concentrated in the intestinal fluids (Grosell et al., 2001a; Shehadeh and Gordon, 1969). As a result some SO_4^{2-} and Mg^{2+} are unavoidably taken up across the intestinal epithelium in excess of daily requirements and are subject to renal excretion (Evans, 1993; Hickman, 1968; Karnaky, 1998).

In addition to the gill which is exposed and accumulates copper when water-borne copper concentrations are elevated (Grosell et al., 2003; Stagg and Shuttleworth, 1982a), the gastro-intestinal tract is also in direct contact with copper as a result of drinking. The gastrointestinal tract is thus a potential target for toxicity as observed after water-borne silver exposure (Grosell et al., 1999; Grosell and Wood, 2001). Despite this potential, no studies have examined the effects of water-borne copper on gastro-intestinal transport physiology in marine teleost fish. Internalization of copper during prolonged exposure may also target other organs like the kidney. Freshwater fish, typically accumulates copper in the liver rather than in the kidney (Buckley et al., 1982; Grosell et al., 1996, 2001b, 1997, 1998b; Lauren and McDonald, 1987a; McCarter and Roch, 1984) but marine fish exhibit renal copper accumulation (Stagg and Shuttleworth, 1982a), making the kidney another potential target for copper toxicity.

Copper has a clear effect on circulating levels of ammonia in freshwater fish (Beaumont et al., 1995; Lauren and McDonald, 1985; Wang et al., 1998; Wilson and Taylor, 1993b) even though the mechanism is far from understood. The reason for this seems to be a combined effect of elevated, stress induced, ammonia production and an unchanged excretion despite an elevated plasma-to-water gradient (Beaumont et al., 2000; Grosell et al., 2002; Taylor et al., 1996).

In the present study we set out to first identify the key mechanism(s) of acute copper toxicity in a marine teleost and then to examine if similar effects occurred during prolonged copper exposure. Osmoregulatory disturbance and altered nitrogenous waste excretion were hypothesized to be the main effects of acute and prolonged copper exposure. Major plasma electrolytes and nitrogenous waste products were measured at the end of a 96 h acute copper challenge test and throughout 30-day copper exposures. In addition, muscle water content was measured during the prolonged exposures. Potential effects on intestinal transport physiology were investigated by analyzing intestinal and rectal fluid composition throughout the exposure. Effects of the prolonged copper exposure on copper homeostasis, branchial and intestinal Na^+/K^+-ATPase activity and drinking rate are reported in an accompanying study (Grosell et al., 2004a).

The present study was performed on the gulf toadfish which is a well characterized model with respect to physiology, easily obtainable and thriving under laboratory conditions.

2. Material and methods

2.1. Experimental animals

Gulf toadfish (*Opsanus beta*) were obtained as by-catch from local shrimp boats operated from Jimbo's on Virginia Key, FL. Upon arrival to the laboratory, fishes were exposed to freshwater for 3 min and then to a formalin/malachite green solution (in seawater, $0.1\,ml\,l^{-1}$, AquaVet) for an additional 3 h. This treatment was employed to minimize problems with ecto-parasites and bacterial infection. Subsequently, fish were sorted by size (to avoid cannibalism) and kept in glass aquaria at $\sim 23\,^\circ C$ with a flow-through of aerated Bear cut seawater (approximately 30‰) at ambient temperature. No sand or gravel was present in the holding tanks, but the toadfish were offered shelter in the form of short lengths of PVC tubing of various diameters and were fed squid once weekly.

2.2. Experimental protocol

2.2.1. Bioassays

Pilot studies revealed substantial mucus secretion by the gulf toadfish when exposed to copper. The secreted mucus evidently bound copper and formed blue-green stained mucus-copper precipitates in the exposure tanks. Consequently, static or semi-static exposure conditions were deemed unsuitable for bio-assays. Several 96 h LC50 studies were performed under flow-through conditions to finally determine copper concentrations high enough to cause physiological disturbance and significant mortalities. Six individual plastic tubs (8 l) containing 10 toadfish each (22–53 g) were provided a flow-through of seawater of at least $200\,ml\,min^{-1}$. One exposure tank received only seawater and served as control. The five remaining exposure tanks received seawater via a head chamber ensuring constant water pressure (and thus flow) draining into individual vigorously aerated mixing chambers before finally entering the exposure tanks. Copper was added as $CuSO_4 \cdot 5H_2O$

from concentrated stock solutions to each individual mixing chamber via a peristaltic pump. The stock solutions were made up to yield final nominal copper concentrations of 10, 50, 100, 200, and 500 µM copper l^{-1}. Mortalities were recorded and water samples were taken twice daily. The water samples were acidified with 1% HNO_3 (Merck trace metal grade) and stored at $-20\,^\circ C$ for subsequent analysis of copper concentration (see below).

Blood samples were taken from surviving fish at the end of the 96 h exposure period by caudal puncture with heparinized 1 ml syringes fitted with gauge 21 needles. Plasma was obtained immediately by centrifugation (10 000 g for 5 min) and stored on ice for a maximum of 1 h for analysis of total ammonia and urea concentration after which sub-samples were stored for later analysis of plasma osmolality and major plasma electrolytes as described below.

2.2.2. Prolonged copper exposures

To evaluate the effect of prolonged exposure to sub-lethal levels of copper, a glass tank of 160 l containing 40 toadfish (16–58 g) was supplied with copper containing seawater at a rate of $1.6\,l\,min^{-1}$. The seawater was delivered via a head chamber ensuring constant flow and a vigorously aerated mixing chamber to which copper was added from a concentrated stock solution of copper sulfate. The concentration of the copper stock solution was initially set to yield a final nominal concentration of 10 µM in the exposure tank. Since the fish exposed to this concentration, even for 30 days, exhibited only minor physiological disturbances, the experiment was repeated at 50 µM copper (nominal). Aside from the copper concentration and the total number of fish (60 fish ranging from 21 to 60 g), the two experimental series were similar. Fish were fed once per week. Flow rates were checked and adjusted if necessary on a daily basis. Water samples were taken every other day and acidified with 1% HNO_3 (Merck trace metal grade) and stored at $-20\,^\circ C$ for subsequent analysis of copper concentration (see below).

Groups of eight fishes were sampled at day 0, 1, 3, 8, 15, and 30 of exposure and an additional control group (non-exposed) was sampled at day 30. Weekly feeding was adjusted to always precede sampling times by at least 48 h. For sampling, individual fish were netted out of the exposure tank and anaesthetized

in copper-free seawater containing $0.2\,g\,l^{-1}$ MS-222. The area surrounding the anal opening was blotted dry with a paper towel and a sample of rectal fluid was obtained by a 1 ml syringe fitted with a gauge 21 needle and a short length of PE 60 tubing. Subsequently, a blood sample was obtained via caudal puncture using a 1 ml heparinized syringe fitted with a gauge 21 needle. Plasma was obtained by immediate centrifugation as above and stored on ice. The body cavity was exposed by a longitudinal incision and the gastrointestinal tract was tied off at the pyloric sphincter and at the rectum with a single 00 silk suture. Thereafter, two single 00 silk sutures were place approximately 1/3 and 2/3 thirds of the distance between the pyloric sphincter and the rectum separating the anterior, the mid and the posterior segments of the intestine. Next, the entire intestine was removed from the body cavity and the intestinal fluids were sampled by cutting each segment of the intestine distal to the proximal suture and letting the fluid drain into a pre-labeled tube. Each of the three intestinal segments were subsequently wrapped in aluminum foil and frozen in liquid nitrogen. Subsequently, the content of the gall bladder was sampled with a 1 ml syringe fitted with a gauge 26 needle. Samples of liver, kidney, spleen, white muscle, and gill filaments were then excised, blotted, and similarly frozen in liquid N_2. The gallbladder bile was transferred to a pre-labeled tube and frozen in liquid nitrogen.

Plasma was immediately analyzed for total ammonia and urea concentration and then stored at $-20\,^\circ C$ for later analysis of main plasma electrolytes. Samples of rectal and intestinal fluids were stored at $-20\,^\circ C$ for later analysis of ionic composition. All tissue samples were analyzed for copper concentration, muscle tissue was analyzed for water content, and gill filaments, and the three intestinal segments were also analyzed for Na/K-ATPase enzyme activity. Tissue copper concentrations and the findings from the enzyme analyses are presented in a separate report.

2.2.3. Analytical techniques

Urea concentrations in plasma and water were measured using the diacetyl monoxime method (Rahmatullah and Boyde, 1980). Plasma ammonia was measured using an enzymtic assay (SIGMA kit no. 171) modified for micro plates. The Cl^- concentrations of plasma, rectal and intestinal fluid samples

were determined using the colorimetric assay of (Zall et al., 1956), while cations were analyzed using a Varian 1275 atomic absorption spectrophotometer (AAS) with methods as documented by the manufacturer. Plasma osmolality was measured using a Wescor 5100C vapor pressure osmometer. White muscle water content was measured by weighing a sub-sample before and after 24 h drying at $60\,^\circ C$. Copper concentrations in seawater was determined by graphite furnace atomic absorption (Varian 1275 with GTA-9 atomizer) using NH_4NO_2 as modifier and certified copper standards as reference.

2.2.4. Statistical evaluation

All data are presented as means $\pm$ S.E.M. (N). Data obtained from the bio-assay were compared using two-tailed Student's t-test with Bonferroni multi-sample comparison correction. Data from the two 30-day copper exposure studies were first analyzed using a single factor (exposure time) ANOVA, after which individual means were compared using Bonferroni multi-sample correction. Control values obtained at the beginning and the end of the 30-day exposures were not significantly different and were therefore combined as one control measurement in the statistical analyses and data presentation. In all cases, differences were considered statistically significant at $P < 0.05$.

3. Results

3.1. Acute copper exposure

Copper additions resulted in concentrations of 11.3 ± 1.5, 88.1 ± 23.4, 105.0 ± 30.5, 340 ± 73.8, and $567.9 \pm 151\,\mu M$ ($n = 4$ in all cases) in the nominal 10, 50, 100, 200, and $500\,\mu M$ treatments, respectively, while control water–copper concentrations were below the detection limit ($\sim 10\,nM$ in seawater).

Copper exposure for 96 h to concentrations less than the $340 \pm 73.8\,\mu M$ did not lead to mortality, but no survival was seen after exposure to $567.9 \pm 151\,\mu M$ indicating a fairly narrow window for toxicity. Except for plasma $[Ca^{2+}]$, all measured parameters were affected by acute copper exposure (Fig. 1). Plasma $[Mg^{2+}]$ and plasma [urea], followed by plasma osmolality and plasma [ammonia] appeared to be the most
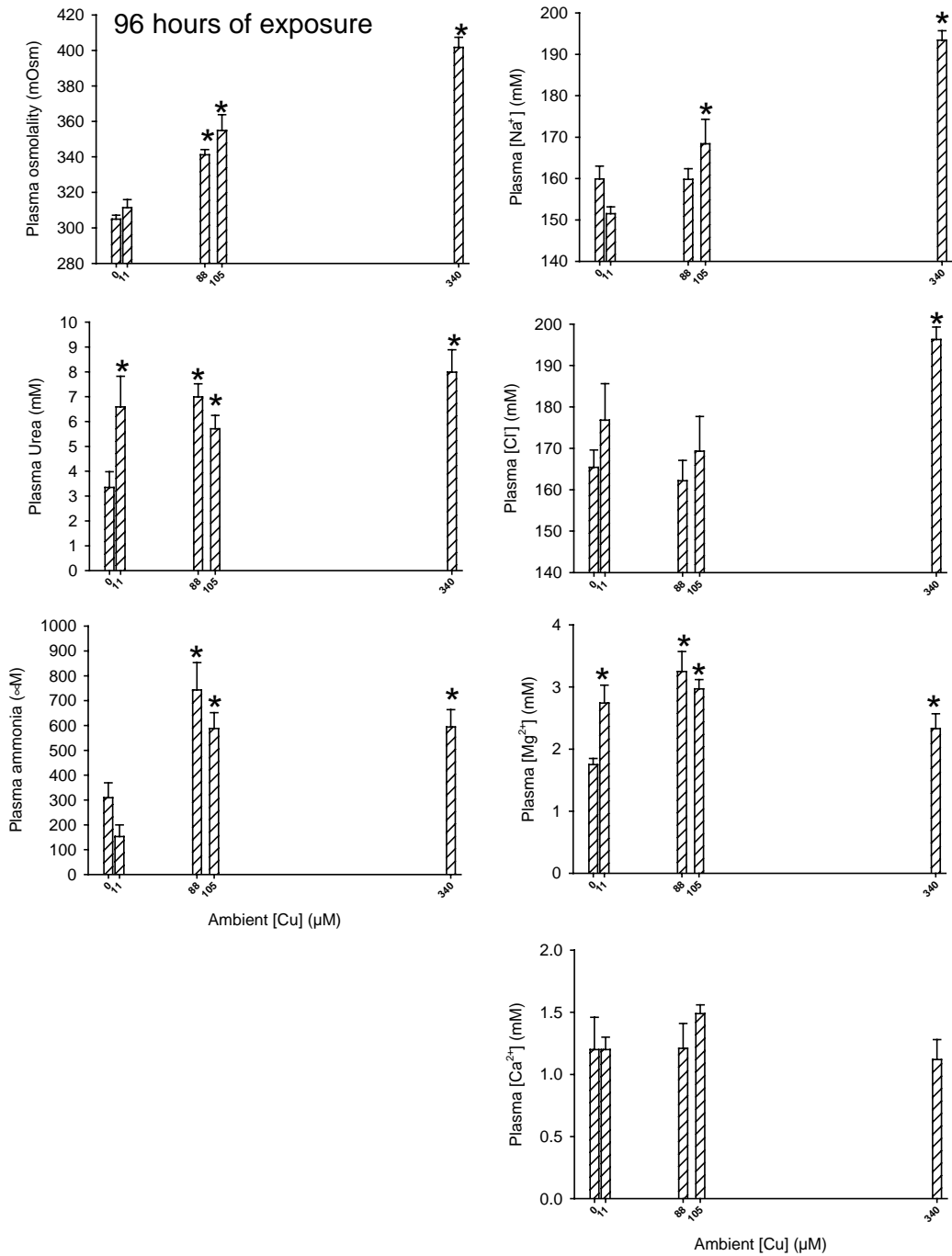
Fig. 1. Gulf toadfish plasma osmolality, [urea], [ammonia], [Na$^+$], [Cl$^-$], [Mg^{2+}], and [Ca^{2+}] after 96 h of exposure to a range of measured copper concentrations. (*) Denotes statistically significant difference from the corresponding control value ($P < 0.05$). $N = 10$ in all cases.

sensitive parameters since they were elevated at the lowest copper exposure concentration. However, whereas plasma concentrations of Mg^{2+}, urea and ammonia appeared to be most sensitive to copper exposure, only plasma osmolality and the concentrations of Na^+ and Cl^- exhibited a copper concentration-dependent response.

3.2. Prolonged copper exposure

3.2.1. Plasma electrolytes and N-products

The copper exposure concentrations in both 30-day exposures were constant over time (not shown) at 12.8 ± 1.6 and $55.2 \pm 5.0 \mu M$ ($N = 15$ and 18, respectively) and control water–copper concentrations were below the detection limit.

Plasma osmolality was 304.9 ± 2.3 mOsm in control fish samples and was constant over the 30-day experiment. Exposure to $12.8 \mu M$ copper did not influence plasma osmolality significantly (Fig. 2). However, exposure to $55.2 \mu M$ resulted in an initial rise in plasma osmolality by day 3 (although not statistically significant) and an apparent transient recovery by day 8 of the exposure. Measurements at day 16 and 30 revealed significant elevation of plasma osmolality to 350–360 mOsm (Fig. 2).

Plasma Na^+ and Cl^- concentrations in fish exposed to $55.2 \mu M$ copper exhibited good correlation and generally matched the pattern seen for plasma osmolality
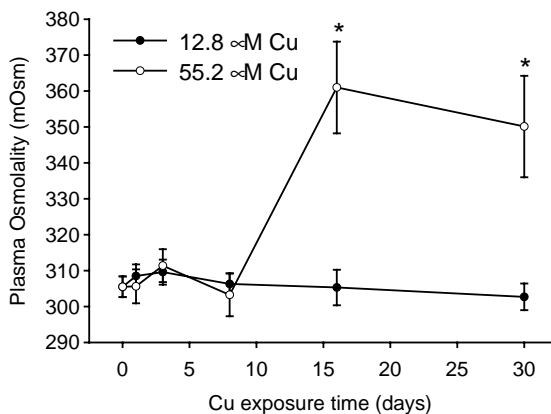

Fig. 2. Gulf toadfish plasma osmolality during a 30-day exposure to 12.8 and $55.2 \mu M$ copper. (*) Denotes statistically significant difference from the corresponding control value (mean of day 0 and terminal control) ($P < 0.05$). $N = 8$ in all cases.
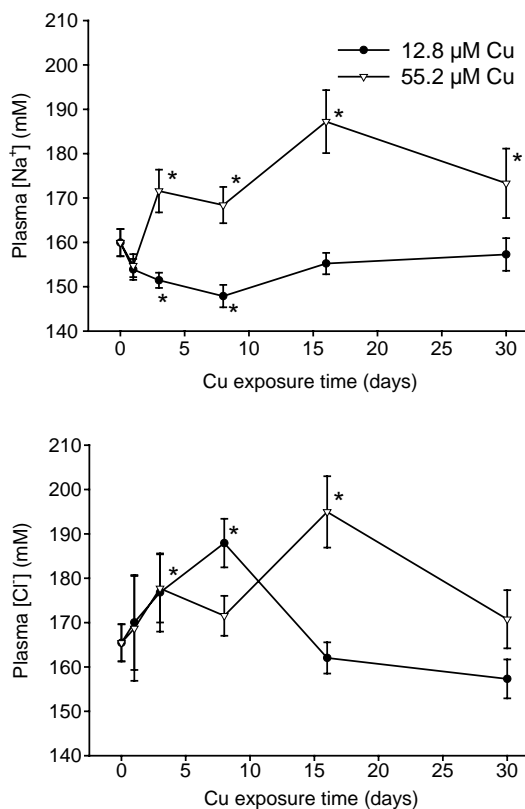

Fig. 3. Gulf toadfish plasma $[Na^+]$ (top panel) and $[Cl^-]$ (bottom panel) during a 30-day exposure to 12.8 and $55.2 \mu M$ copper. (*) Denotes statistically significant difference from the corresponding control value (mean of day 0 and terminal control) ($P < 0.05$). $N = 8$ in all cases.

(Figs. 2 and 3). An initial increase in both ions at day 3, followed by what may be a partial recovery at day 8, was seen for both ions (Fig. 3). Highest concentrations of plasma Na^+ and Cl^- were recorded in samples obtained at day 16 and values were also elevated at day 30 of the exposure (significant only for Na^+) (Fig. 3).

Plasma $[Ca^{2+}]$ was not influenced by copper exposure (with the exception of a fall at day 16 at $12.8 \mu M$) but plasma $[Mg^{2+}]$ was clearly influenced by exposure to both copper concentrations. During exposure to $12.8 \mu M$ copper plasma $[Mg^{2+}]$ was significantly elevated by day 1 and steadily increased for the duration of the 30-day exposure, approaching levels that were two-fold greater than the corresponding control values
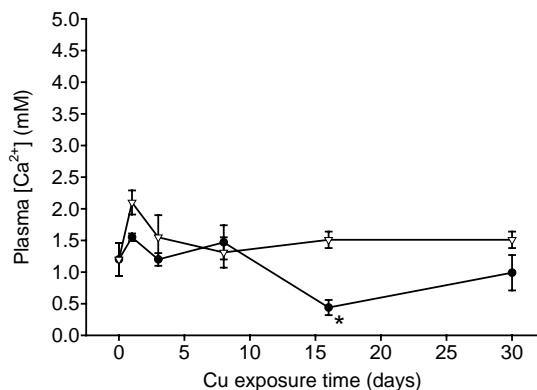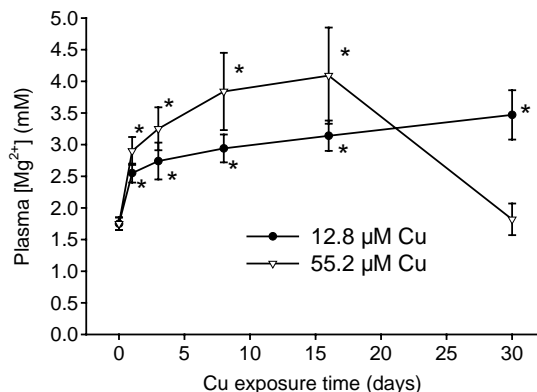
Fig. 4. Gulf toadfish plasma $[Mg^{2+}]$ (top panel) and $[Ca^{2+}]$ (bottom panel) during a 30-day exposure to 12.8 and 55.2 μM copper. (*) Denotes statistically significant difference from the corresponding control value (mean of day 0 and terminal control) ($P < 0.05$). $N = 8$ in all cases.
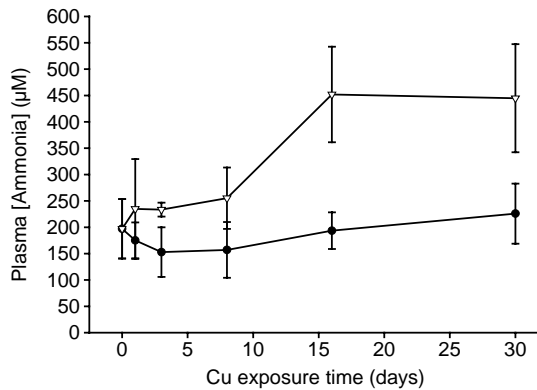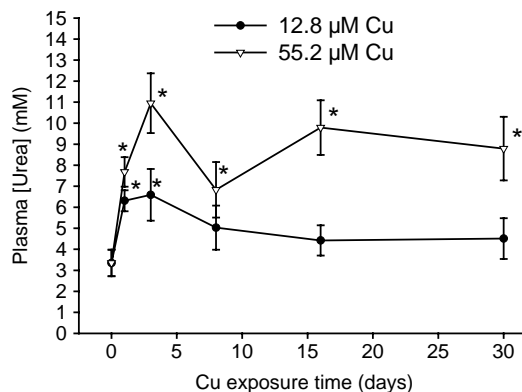

Fig. 5. Gulf toadfish plasma [urea] (top panel) and [total ammonia] (bottom panel) during a 30-day exposure to 12.8 and 55.2 μM copper. Note different units for [urea] and [ammonia]. (*) Denotes statistically significant difference from the corresponding control value (mean of day 0 and terminal control) ($P < 0.05$). $N = 8$ in all cases.

(Fig. 4). This pattern was even more pronounced in fish exposed to the high copper concentration (55.2 μM) with levels reaching 2.5-times the control values at day 16. However, unlike fish exposed to the lower copper concentration, these fish exhibited full recovery of plasma $[Mg^{2+}]$ by day 30 (Fig. 4).

Plasma [urea] concentrations were also influenced by exposure to both copper concentrations with an immediate increase to twice the control values at day 1 (Fig. 5.). While the fish exposed to 12.8 μM copper exhibited maximal values after 3 days of exposure followed by an apparent recovery, fish exposed to 55.2 μM copper showed a continued elevation in plasma urea concentrations at approximately three-fold the corresponding control value for the

duration of the 30 day exposure. Plasma [ammonia] was not influenced by exposure to 12.8 μM, but exposure to 55.2 μM resulted in an overall significant increase in circulating ammonia levels (by ANOVA) (Fig. 5).

3.2.2. Intestinal fluids and muscle water content

N-numbers for measurements of gastro-intestinal fluid samples are generally lower than the $N = 8$ for plasma data described above due to limited sample volume in many cases. Gastro-intestinal fluid volume was especially low during the initial phase of the exposure to 55.2 μM copper but tended to be higher later during the exposure. *N*-numbers for individual means are given in Figs. 6 and 7.
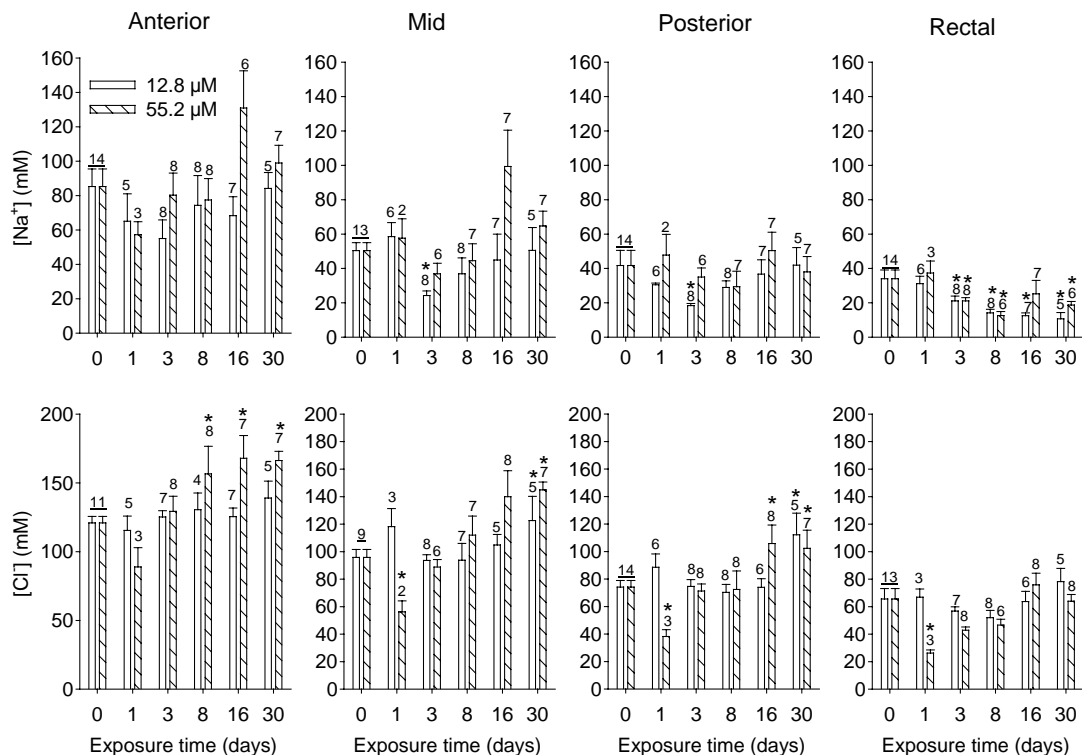
Fig. 6. $[Na^+]$ (top panels) and $[Cl^-]$ (bottom panels) in fluids obtained from the anterior, mid and posterior intestine as well as from the rectum of gulf toadfish exposed to 12.8 and 55.2 μM copper for up to 30 days. (*) denotes statistically significant difference (*t*-test) from the corresponding control value (mean of day 0 and terminal control plotted as double bar at day 0) ($P < 0.05$). *N*-numbers are given for each individual measurement. An ANOVA revealed a significant overall effect of copper exposure on rectal fluid $[Na^+]$ at both copper concentrations, and also a significant effect of 55.2 μM copper on $[Cl^-]$ concentrations in fluids obtain from all segments of the intestine and the rectal fluid of fish exposed ($P < 0.05$).

The concentration of Na^+ did not exhibit overall effects of copper exposure in the anterior, mid and posterior segments of the intestine but was reduced from day 3 onwards in rectal fluids from fish exposed to both copper concentrations (ANOVA). Comparing individual means however, revealed significantly reduced $[Na^+]$ also at day 3 of exposure to 55.2 μM copper in fluids obtained from the mid and posterior intestinal segments (Fig. 6). An overall effect of 55.2 μM copper was seen on $[Cl^-]$ in fluids obtained from all segments of the intestine and from the rectum (Fig. 6). The effect of this copper exposure appeared to be bi-phasic with an initial reduction in $[Cl^-]$ seen in all segments at day 1 (although not statistically significant for the anterior segment of the intestine). This initial reduction was followed by an elevation in lu-

minal $[Cl^-]$ by day 8, 16, and 30 to values above control levels in the anterior, mid and posterior segments while rectal fluids exhibited an apparent recovery to normal $[Cl^-]$. In addition, exposure to the lower copper concentration tended to elevate $[Cl^-]$ in the three intestinal segments by day 30, but this was statistically significant only in the posterior segment (Fig. 6).

Neither Mg^{2+} nor Ca^{2+} concentrations in fluids obtained from the three intestinal segments were affected by copper exposure, but both ions exhibited an overall elevation in rectal fluids as a result of exposure to 55.2 μM copper (Fig. 7). Mg^{2+} levels were significantly elevated from day 3 onwards while Ca^{2+} levels were elevated at day 1, 8, and 16. Fishes exposed to 12.8 μM copper exhibited significantly elevated Mg^{2+} at day 1 and Ca^{2+} at day 30.
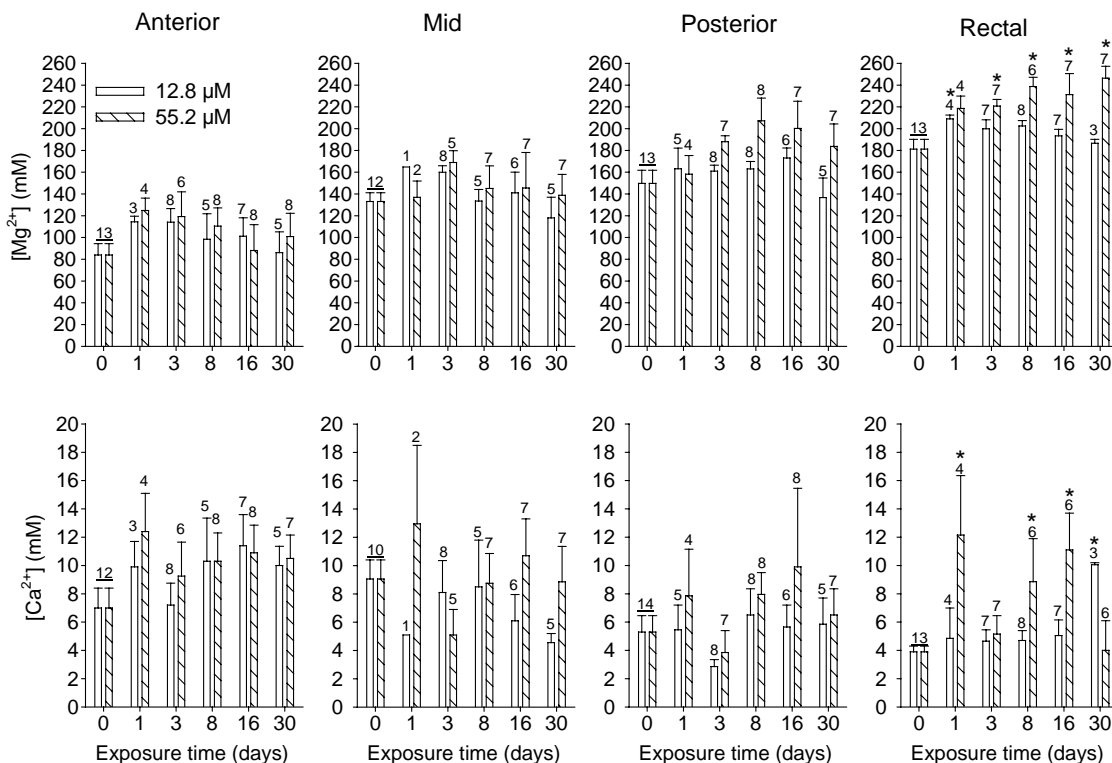
Fig. 7. $[Mg^{2+}]$ (top panels) and $[Ca^{2+}]$ (bottom panels) in fluids obtained from the anterior, mid, and posterior intestine as well as from the rectum of Gulf toadfish exposed to 12.8 and 55.2 μM copper for up to 30 days. (*) Denotes statistical significant difference (*t*-test) from the corresponding control value (mean of day 0 and terminal control plotted as a double bar at day 0) ($P < 0.05$). *N*-numbers are given for each individual measurement. An ANOVA revealed an overall significant effect of 55.2 μM copper exposure on rectal fluid $[Mg^{2+}]$ and $[Ca^{2+}]$ ($P < 0.05$).

Muscle water content exhibited a slight but significant elevation after 16 and 30 days of exposure to 12.8 μM copper while fish exposed to 55.2 μM exhibited a more pronounced reduction in water content

Table 1
Muscle water content in toadfish during 30 days of exposure to 12.8 and 55.2 μM copper

Days of exposure	12.8 μM copper	55.2 μM copper
0	78.1 ± 0.2	
1	78.4 ± 0.5	78.1 ± 0.2
3	78.6 ± 0.4	77.9 ± 0.4
8	78.7 ± 0.2	78.1 ± 0.4
16	80.0 ± 0.4*	73.2 ± 3.2*
30	80.5 ± 0.5*	76.4 ± 5.3

$N = 8$ in all cases.

  * Denotes significant difference from control at $P < 0.05$.

after 16 days of exposure with partly recovered values at day 30 (Table 1).

4. Discussion

4.1. Acute toxicity

Toadfish survived 96 h of exposure to 340 μM copper but showed 100% mortality at 568 μM. These values thus bracket the 96 h LC50 and are 3–4 orders of magnitude higher than values obtained from freshwater fish which are typically in the low micromolar range (Grosell et al., 2002) and high even compared to seawater 96 h LC50 values from other marine teleosts (Steele, 1983a,b). Copper appears to be less toxic to fish in seawater than in freshwater. This is probably

attributable in part to differences in ionoregulatory strategies and thus gill physiology and is also likely attributable to copper speciation differences and the high ionic strength of the marine environment offering cations competing for copper uptake pathways and anions for complexation. The protective effects of Ca^{2+} and Na^+ on copper toxicity and uptake respectively has been documented for freshwater fish (Grosell and Wood, 2002; Pagenkopf, 1983; Santore et al., 2001), and the high concentration of these ions in seawater is likely part of the reason for the low sensitivity seen in marine teleost fish. The toadfish in particular is tolerant to harsh environmental conditions including elevated ambient ammonia, air exposure (Walsh et al., 1990, 1994) extreme salinities (McDonald and Grosell, personal observations) as well as temperature, so it is perhaps not surprising that it also exhibits high copper tolerance. Sloughing of vast quantities of mucus that evidently bound copper as observed during exposure to the higher concentrations in the acute challenge test may also contribute to the extreme acute copper tolerance.

While plasma urea and Mg^{2+} were the most sensitive indicators of acute copper exposure (i.e. elevated at the lowest concentrations), these parameters did not exhibit copper concentration-dependence. Plasma osmolality (mostly comprised of NaCl), $[Na^+]$, and $[Cl^-]$ however, exhibited clear dependence on ambient copper concentration, strongly arguing for NaCl regulation failure as the parameter leading to mortality during acute copper challenge tests.

4.2. Effects of prolonged copper exposure

Nominal 10 and 50 μM (actual 12.8 and 55.2) copper were chosen for the prolonged exposures because 10 μM represented the lowest concentration in the acute challenge test at which any effect was observed and 50 μM because this was the lowest copper concentration to cause obvious osmoregulatory disturbance.

4.2.1. Osmoregulatory disturbance

Both copper concentrations resulted in osmoregulatory disturbance in the gulf toadfish but the effects were more pronounced at 55.2 μM copper. At this concentration both plasma $[Na^+]$ and $[Cl^-]$ were increased, resulting in elevated plasma osmolality

(Figs. 2 and 3). When most extreme, the elevated plasma osmolality resulted in a fluid loss from white muscle tissue as evident from Table 1. The rise in plasma [NaCl] could be the result of either reduced branchial NaCl extrusion or/and reduced intestinal fluid absorption. Both these processes are influenced by silver in marine teleosts (Grosell et al., 1999; Grosell and Wood, 2001) and the similarity between the toxic mechanism of these two metals in freshwater fish (Grosell et al., 2002) suggests that this might also be the case for copper. Indeed, based on intestinal fluid composition it appears that gastro-intestinal transport may be influenced by copper exposure (Figs. 6 and 7). Intestinal fluid $[Na^+]$ was reduced at day 3 of the exposure in the mid and the posterior segment of the intestine as well as in the rectal fluids where lower $[Na^+]$ in fish exposed to 55.2 μM copper persisted throughout the 30 days of exposure. In addition, intestinal fluid $[Na^+]$ tended to be elevated in the anterior and mid segment of the intestine at day 16 of exposure. Reduced $[Cl^-]$ in the intestinal fluids was also observed but at day 1 of exposure as opposed to day 3 for Na^+. This reduction was followed by an elevation after 8, 16, and 30 days of exposure in all intestinal segments, but not in the rectal fluids (Fig. 6).

The interpretation of the intestinal fluid data is complicated because fluid composition is the function of both drinking rate and ion and fluid transport across the gastro-intestinal tract. Drinking rate was clearly influenced by exposure to 55.2 μM copper as reported in an accompanying paper (Grosell et al., 2004a), but it appears that intestinal transport physiology may also have been influenced by the copper exposure.

It is clear from the differential effect on Na^+ and Cl^- concentrations that the transport of these ions is not strictly coupled as would be expected from the generally accepted model of intestinal transport physiology which involves Na^+:Cl^- and Na^+:K^+:$2Cl^-$ co-transporters (Loretz, 1995). This observation is in agreement with the recent demonstration of an additional and significant Cl^-/HCO_3^- exchange component to intestinal Cl^- absorption (Grosell et al., 2001a; Grosell et al., 2004b; Wilson et al., 2002; Wilson and Grosell, 2003). The observation of more pronounced effects of copper on Cl^- than Na^+ concentrations in the intestinal fluids furthermore suggests that the

anion-exchange component to Cl^- absorption is more sensitive than the NaCl co-transport systems.

The initial drop in Cl^- and Na^+ concentrations in the intestinal fluids (Fig. 6) would be consistent with both the reduced drinking rate as seen after 1 day of exposure and elevated drinking rate after 3 days of exposure (Grosell et al., 2004a). In the situation of reduced drinking rate, more of the imbibed fluid can be expected to be absorbed from the intestinal lumen to still meet the whole animal water requirements. Since fluid absorption is driven by Na^+ and especially Cl^- absorption, reduced luminal Cl^- concentrations under these conditions are not suprising. The elevated drinking rate by day 3 of exposure, which preceded severe intestinal copper accumulation ((Grosell et al., 2004a) can be interpreted as a compensation for elevated plasma osmolality and $[Na^+]$ (Figs. 2 and 3), so increased intestinal fluid absorption might be expected. Increased fluid absorption would again be driven by increased ion absorption, in this case evidenced by decreased intestinal fluid $[Na^+]$. Therefore, both of the initial effects on $[Na^+]$ and $[Cl^-]$ in the intestinal fluids may be of compensatory nature for reduced drinking or elevated plasma osmolality (perhaps caused by impairment of branchial transport physiology). The lack of coupling between Na^+ and Cl^- responses indicate that the absorption of these ions not only occurs through different pathways, but also that these pathways are under differential regulation depending on the physiological state of the animal.

The later responses (day 8 and onward) to $55.2\,\mu M$ copper seen in intestinal fluid composition (Figs. 5–7) may reflect a direct effect on the intestinal epithelium, since copper concentrations were greatly elevated at this time (Grosell et al., 2004a). Elevation of luminal Cl^- but not Na^+ concentrations in all intestinal segments suggests that the anion exchange component to Cl^- uptake is more affected than the NaCl co-transport systems by intestinal copper accumulation. Considering the elevated plasma osmolality and the probable reduction in intestinal Cl^- absorption via the anion exchange system, it is not surprising to see reduced $[Na^+]$ in the rectal fluids during copper exposure. These reduced rectal Na^+ levels suggest increased Na^+ and fluid absorption by the intestine, which can be interpreted as a compensatory response to the elevated

plasma osmolality. The increased Na^+ absorption could occur through NaCl co-transport systems and would thus be accompanied by Cl^-, which may compensate for the reduced exchange resulting in apparently normal Cl^- concentrations in the rectal fluids.

It is interesting that reduced $[Na^+]$ in the rectal fluids, most likely reflecting increased fluid absorption, also occurs in fish exposed to $12.8\,\mu M$ copper (Fig. 6) which exhibit little, if any, osmoregulatory disturbance (Fig. 2). This may suggest that branchial ion extrusion or drinking rate was influenced by exposure to the lower copper concentration, but that the intestinal compensation is the reason for the limited effects on plasma osmolality and electrolyte concentrations.

The interpretation of elevated, compensatory fluid absorption by the distal segments of the intestine is supported by the increased $[Mg^{2+}]$ and $[Ca^{2+}]$ observed in the rectal fluids (Fig. 7). By far most of the divalent cations ingested with seawater are not absorbed across the intestine but are eliminated from the gastro-intestinal tract with the voided rectal fluids (Wilson and Grosell, 2003). It thus follows that increased fluid absorption would render these ions more concentrated in the luminal fluids and that this effect would be most pronounced in the rectal fluids since they would display the integrated result of transport across the entire length of the intestinal tract.

The elevated $[Ca^{2+}]$ measured in the rectal fluids (Fig. 7) also supports the suggestion of inhibited Cl^-/HCO_3^- exchange. Inhibition of Cl^-/HCO_3^- exchange would lead to reduced HCO_3^- secretion, reduced luminal $[HCO_3^-]$ and $[CO_3^{2-}]$ concentrations, and thus reduced $CaCO_3$ precipitate formation in the intestinal lumen [see (Wilson et al., 2002; Wilson and Grosell, 2003) for a detailed discussion of this phenomenon].

The elevated plasma $[Mg^{2+}]$ measured during exposure to both copper concentrations could be the result of increased branchial uptake, increased intestinal uptake and/or decreased renal excretion of Mg^{2+}. However, elevation of plasma $[Mg^{2+}]$ was seen during exposure to both copper concentrations but intestinal fluid $[Mg^{2+}]$ and renal copper concentrations were only severely elevated by exposure to the highest copper concentration (Grosell et al.,

2004a) it seems most likely that this elevation is due to increased branchial Mg^{2+} permeability.

4.2.2. Effects on nitrogenous waste products

An overall gradual elevation of plasma ammonia was observed in fish exposed to the highest copper concentration, but a dramatic effect on plasma urea concentrations was seen immediately after the onset of exposure to both copper concentrations (Fig. 5). To our knowledge this is the first report of effects of copper exposure on plasma urea concentrations in any fish, but it should be noted that the toadfish is unique among teleosts with respect to nitrogen metabolism. The toadfish is facultatively ureotelic, capable of switching from excreting nitrogenous waste as ammonia to excreting almost entirely urea when exposed to stressful conditions (Walsh et al., 1990, 1994; Walsh and Milligan, 1995). An increase in ammonia production as a result of metal induced stress together with an impaired ability to excrete ammonia across the gill is the typical response to copper exposure in freshwater fish and leads to elevated plasma ammonia levels (see Section 1). The ability of toadfish to convert ammonia to urea via their fully functional ornithine-urea cycle (Mommsen and Walsh, 1989) may therefore explain the more pronounced elevation in plasma [urea] than in [ammonia]. It is interesting to speculate that copper exposure may cause toadfish to switch from excreting mainly ammonia to excreting predominantly urea to avoid ammonia toxicity.

5. Conclusions

Copper exposure caused osmoregulatory disturbance in the gulf toadfish during both acute and prolonged exposure. This disturbance clearly involved alterations in intestinal transport physiology evident from intestinal and rectal fluid composition. These alterations seems to include both direct effects and effects of a compensatory nature, suggesting that branchial transport processes also may be influenced by copper exposure in marine fish. As in freshwater fish, increased plasma nitrogenous waste concentration was seen in response to copper exposure, only in the case of the facultatively ureotelic toadfish, this was manifested as elevated urea rather than ammonia concentrations.

References

Beaumont, M.W., Butler, P.J., Taylor, E.W., 1995. Exposure of brown trout, *Salmo trutta*, to sub-lethal copper concentrations in soft acidic water and its effect upon sustained swimming performance. Aquat. Tox. 33, 45–63.

Beaumont, M.W., Butler, P.J., Taylor, E.W., 2000. Exposure of brown trout, *Salmo trutta*, to a sub-lethal concentration of copper in soft acidic water: effects upon muscle metabolism and membrane potential. Aquat. Tox. 51, 259–272.

Buckley, J.T., Roch, M., McCarter, J.A., Rendell, C.A., Matheson, A.T., 1982. Chronic exposure of coho salmon to sublethal concentrations of copper-I: effect on growth, on accumulaton and distribution of copper, and on copper tolerance. Comp. Biochem. Physiol. C. 72, 15–19.

Evans, D.H. (Ed.), 1993. Osmotoc and ionic regulation. The Physiology of Fishes. CRC Press, Boca Raton, FL, pp. 315–341.

Grosell, M., Boetius, I., Hansen, H.J.M., Rosenkilde, P., 1996. Influence of preexposure to sublethal levels of copper on Cu-64 uptake and distribution among tissues of the European eel (*Anguilla anguilla*). Comp. Biochem. Physiol. C. 114, 229–235.

Grosell, M., De Boeck, G., Johannsson, O., Wood, C.M., 1999. The effects of silver on intestinal ion and acid–base regulation in the marine teleost fish, *Papophrys vetulus*. Comp. Biochem. Physiol. C. 124, 259–270.

Grosell, M., Hansen, H.J.M., Rosenkilde, P., 1998a. Cu uptake, metabolism and elimination in fed and starved European eels (*Anguilla anguilla*) during adaptation to water-borne Cu exposure. Comp. Biochem. Physiol. C. 120, 295–305.

Grosell, M., Laliberte, C.N., Wood, S., Jensen, F.B., Wood, C.M., 2001a. Intestinal HCO_3^- secretion in marine teleost fish: evidence for an apical rather than a basolateral Cl^-/HCO_3^- exchanger. Fish Physiol. Biochem. 24, 81–95.

Grosell, M., McDonald, M.D., Walsh, P.J., Wood, C. M., 2004a. Effects of prolonged copper exposure in the marine gulf toadfish (*Opsanus beta*). Part II. Drinking rate, copper accumulation and Na^+/K^+-ATPase activity in osmoregulatory tissues, Aquat. Tox. 68, 263–275

Grosell, M., McGeer, J.C., Wood, C.M., 2001b. Plasma copper clearance and biliary copper excretion are stimulated in copper-acclimated trout. Am. J. Phys. 280, R796–R806.

Grosell, M., Nielsen, C., Bianchini, A., 2002. Sodium turnover rate determines sensitivity to acute copper and silver exposure in freshwater animals. Comp. Biochem. Physiol. C. 133, 287–303.

Grosell, M., Wood, C.M., 2001. Branchial versus intestinal silver toxicity and uptake in the marine teleost *Parophrys vetulus*. J. Comp. Physiol. B. 171, 585–594.

Grosell, M., Wood, C.M., 2002. Copper uptake across Rainbow trout gills: mechanisms of apical entry. J. Exp. Biol. 205, 1179–1188.

Grosell, M., Wood, C.M., Walsh, P.J., 2003. Copper homeostasis and toxicity in the elasmobranch *Raja erinacea* and the teleost *Myoxocephalus octodecemspinosus* during exposure to elevated water-borne copper. Comp. Biochem. Physiol. C 135, 179–190.

Grosell, M., Wood, C.M., Wilson, R.W., Bury, N.R., Hogstrand, C., Rankin, J.C., Jensen, F.B., 2004b. Active bicarbonate secretion plays a role in chloride and water absorption of the European flounder intestine. Am. J. Phys. submitted.

Grosell, M.H., Hogstrand, C., Wood, C.M., 1997. Cu uptake and turnover in both Cu-acclimated and non-acclimated Rainbow trout (*Oncorhynchus mykiss*). Aquat. Tox. 38, 257–276.

Grosell, M.H., Hogstrand, C., Wood, C.M., 1998b. Renal Cu and Na excretion and hepatic Cu metabolism in both Cu acclimated and non acclimated Rainbow trout (*Oncorhynchus mykiss*). Aquat. Tox. 40, 275–291.

Handy, R.D., 1996. Dietary exposure to toxic metals in fish. In: Taylor, E.W. (Ed.), Toxicology of Aquatic Pollution: Physiological, Cellular and Molecular Approaches. Cambridge University press, Cambridge, UK, pp. 29–60.

Hickman, C.P., 1968. Ingestion, intestinal absorption, and elimination of seawater and salts in the southern flounder, *Paralichthys lethostigma*. Can. J. Zool. 46, 457–466.

Julshamm, K., Andersen, K.J., Ringdal, O., Brenna, J., 1988. Effects of dietary copper on hepatic concentration and subcellular distribution of copper and zinc in Rainbow trout (*Salmo gairdneri*). Aquaculture 73, 143–155.

Karnaky, K.J., 1998. Osmotic and ionic regulation. In: Evans, D.H. (Ed.), The Physiology of Fishes. CRC Press, Boca Raton, FL, pp. 157–176.

Lanno, R.P., Singer, S.J., Hilton, J.W., 1985. Maximum tolerable and toxicity level of dietary copper in Rainbow trout (*Salmo gairdneri* Richardson). Aquaculture 49, 257–268.

Lauren, D.J., McDonald, D.G., 1985. Effects of copper on branchial ionoregulation in the Rainbow trout, *Salmo-Gairdneri* Richardson—modulation by water hardness and pH. J. Comp. Physiol. B. 155, 635–644.

Lauren, D.J., McDonald, D.G., 1987a. Acclimation to copper by Rainbow trout *Salmo-Gairdneri*—biochemistry. Can. J. Fish. Aquat. Sci. 44, 105–111.

Lauren, D.J., McDonald, D.G., 1987b. Acclimation to copper by Rainbow trout, *Salmo gairdneri*—physiology. Can. J. Fish. Aquat. Sci. 44, 99–104.

Loretz, C.A., 1995. Electrophysiology of ion transport in the teleost intestinal cells. In: Wood, C.M., Shuttleworth, T.J. (Eds.), Cellular and Molecular Approaches to Fish Ionic Regulation, Fish Physiol. 14, 25–56.

McCarter, J.A., Roch, M., 1984. Chronic exposure of coho salmon to sublethal concentrations of copper-II: kinetics of metabolism of metallothionein. Comp. Biochem. Physiol. C. 77, 83–87.

Mommsen, T.P., Walsh, P.J., 1989. Evolution of urea synthesis in vertebrates—the piscine connection. Science 243, 72–75.

Pagenkopf, R.K., 1983. Gill surface interaction model for trace-metal toxicity to fishes: role of complexation, pH, and water hardness. Environ. Sci. Technol. 17, 342–347.

Rahmatullah, M., Boyde, T.R., 1980. Improvements in the determination of urea using diacetyl monoxime methods with and without deproteination. Clin. Chim. Acta 107, 3–9.

Santore, R.C., Di Toro, D.M., Paquin, P.R., Allen, H.E., Meyer, J.S., 2001. Biotic ligand model on the acute toxicity of metals. Part II. Application to acute copper toxicity in freshwater fish and daphnia. Environ. Tox. Chem. 20, 2397–2402.

Shehadeh, Z.H., Gordon, M.S., 1969. The role of the intestine in salinity adaptation of the Rainbow trout, *Salmo gairdneri*. Comp. Biochem. Physiol. 30, 397–418.

Smith, H., 1930. The absorption and excretion of water and salts by marine teleosts. Am. J. Physiol. 93, 480–505.

Stagg, R.M., Shuttleworth, T.J., 1982a. The accumulation of copper in *Platichthys flesus L.* and its effects on plasma electrolyte concentrations. J. Fish. biol. 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.). J. Comp. Physiol. 149, 83–90.

Steele, C.W., 1983a. Acute toxicity of copper to sea catfish. Mar. Pollut. Bull. 14, 168–170.

Steele, C.W., 1983b. Comparison of the behavioural and acute toxicity of copper to sheepshead, Atlantic croaker and pinfish. Mar. Pollut. Bull. 14, 425–428.

Taylor, E.W., Beaumont, M.W., Butler, P.J., Mair, J., Mujallid, M.S.I., 1996. Lethal and sub-lethal effects of copper upon fish: a role for ammonia toxicity? In Taylor E.W. (Ed.), Toxicology of Aquatic Pollution. Cambridge University Press, Cambridge, UK, pp. 85–113.

Walsh, P.J., Danulat, E., Mommsen, T.P., 1990. Variation in urea excretion in the gulf toadfish *Opsanus beta*. Marine Biol. 106, 323–328.

Walsh, P.J., Milligan, C.L., 1995. Effects of feeding and confinement on nitrogen-metabolism and excretion in the gulf toadfish *Opsanus beta*. J. Exp. Biol. 198, 1559–1566.

Walsh, P.J., Tucker, B.C., Hopkins, T.E., 1994. Effects of confinement/crowding on Ureogenesis in the gulf toadfish *Opsanus beta*. J. Exp. Biol. 191, 195–206.

Wang, T., Knudsen, P.K., Brauner, C.J., Busk, M., Vijayan, M.M., Jensen, F.B., 1998. Copper exposure impairs intra- and extra-cellular acid–base regulation during hypercapnia in the fresh water Rainbow trout (*Oncorhynchus mykiss*). J. Comp. Physiol. B. 168, 591–599.

Wilson, R.W., Grosell, M., 2003. Intestinal bicarbonate secretion in marine teleost fish—source of bicarbonate, pH sensitivity, and consequence for whole animal acid–base and divalent cation homeostasis. Biochim. Biophys. Acta. 1618 (2), 163–193.

Wilson, R.W., Taylor, E.W., 1993a. Differential responses to copper in Rainbow trout (*Oncorhynchus mykiss*) acclimated to seawater and brackish water. J. Comp. Phys B. 163, 239–246.

Wilson, R.W., Taylor, E.W., 1993b. The physiological-responses of fresh-water Rainbow trout, *Oncorhynchusm mykiss*, during acutely lethal copper exposure. J. Comp. Phys. B. 163, 38–47.

Wilson, R.W., Wilson, J.M., Grosell, M., 2002. Intestinal bicarbonate secretion by marine teleost fish-why and how? Biochim. Biophys. Acta. 1566, 182–193.

Wood, C.M., 2001. The toxic response of the gill. Target organ toxicity in marine and freshwater teleosts. In: Benson, H.W., Schlenk, D.W. (Ed.), Taylor & Francis, Washington, DC, pp. 1–87.

Zall, D.M., Fisher, D., Garner, M.D., 1956. Photometric determination of chlorides in water. Anal. Chem. 28, 1665–1678.