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Food selection, growth and physiology in relation to dietary sodium chloride content in rainbow trout (*Oncorhynchus mykiss*) under chronic waterborne Cu exposure

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Abstract

Waterborne Cu is toxic to Na^+ and Cl^- regulation in freshwater fish, and Cu is taken up, at least in part, via the Na^+-transport pathway in the gills. Therefore, we hypothesized that freshwater fish may mitigate the toxic effects of waterborne Cu by selecting a NaCl-enriched diet over a normal diet. We tested this hypothesis in juvenile rainbow trout (*Oncorhynchus mykiss*) by offering them the choice between NaCl-enriched (1.9 mmol g^{-1} Na^+) and normal (0.2 mmol g^{-1} Na^+) diets under a chronic waterborne Cu exposure of 55 μg L^{-1} for a period of 28 days. Contrary to expectation, trout exhibited a preference for NaCl-enriched diet under control conditions, while exposure to chronic waterborne Cu severely disrupted their normal feeding pattern with an accompanying loss of preference for the NaCl-enriched diet. Waterborne Cu exposure also severely affected appetite and growth. Both appetite and growth gradually recovered with time, but remained significantly impaired relative to Cu-unexposed fish until the end of the exposure. Waterborne Cu exposure also significantly increased Cu accumulations in target organs (gill, liver, and gut), plasma and whole body. However, Cu accumulation decreased substantially towards the end of the exposure in target organs and whole body as well as in plasma in Cu-exposed fish with dietary choice relative to Cu-exposed fish with normal diet. These adjustments were concurrent with the gradual recovery of appetite, which also led to increased ingestion of the NaCl-enriched diet. Interestingly, this elevated dietary uptake of NaCl produced significant stimulation of Na^+ efflux in Cu-exposed fish. Subsequently, it also led to significant elevation of Na^+ levels in target organs and whole body, and restored the decrease of plasma Na^+ and Cl^- levels in Cu-exposed fish. The NaCl supplemented diet appeared to be beneficial in compensating Na^+ and Cl^- losses from the body induced by waterborne Cu. Overall, these results demonstrate that a NaCl-enriched diet, although consumed in relatively reduced quantities due to the impairment of food selection and appetite, can help to protect freshwater fish against chronic waterborne Cu toxicity.

Keywords: Rainbow trout; Dietary NaCl; Waterborne Cu; Feeding behavior; Na and Cu homeostasis

1. Introduction

Cu is an essential nutrient for normal metabolic functioning of all organisms, including fish (Ogino and Yang, 1980; Satoh et al., 1983). However, it can be highly toxic to aquatic organisms, particularly fish at concentrations above the physiological threshold levels. The primary mechanisms of Cu toxicity to freshwater fish result from the combined effects of a decrease in Na^+ influx and an increase in Na^+ efflux, which yield a net reduction of plasma and whole body Na^+ (Laurén and McDonald, 1986, 1987b; Reid and McDonald, 1991). In freshwater fish, Cu is believed to share a common route of uptake with Na^+, at least partially, in the gills (Grosell and Wood, 2002), and decreased Na^+ influx is reported to be associated with non-competitive binding of Cu to the basolateral Na^+-pump (Na^+-K^+-ATPase) in the chloride cells of the gills (Laurén and McDonald, 1987a; Pelgrom et al., 1995; Li et al., 1996, 1998). At higher waterborne Cu levels, greatly stimulated Na^+ efflux is thought to be associated with Cu-induced damage to gill epithelia that results in a reduction of the integrity of paracellular "tight-junctions", rendering the epithelium more permeable to internal Na^+ and Cl^- (Laurén and McDonald, 1986; Evans, 1987; McDonald and Wood, 1993).

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The result of this net loss of Na^+ and Cl^- is an increase in blood viscosity and blood pressure, and cardiac failure under acutely toxic conditions (Wilson and Taylor, 1993).

In freshwater fish, regulation of Na^+ uptake mechanisms, both from water and food, is believed to be one of the main strategies for acclimation to sub-lethal waterborne Cu exposure. Recovery of branchial Na^+ uptake occurs through the increase in Na^+-K^+-ATPase activity due to an increase in the number of enzyme units (Stagg and Shuttleworth, 1982; Laurén and McDonald, 1987a; McGeer et al., 2000a). Recent studies have shown that a NaCl-enriched diet reduces the uptake of Na^+ and Cu at the gills, and also reduces Cu accumulation in target tissues (gill and liver) in rainbow trout (*Oncorhynchus mykiss*) (Kamunde et al., 2003; Pyle et al., 2003). These studies strongly indicate that increased dietary uptake of Na^+ may also compensate the net Na^+ loss under sub-lethal waterborne Cu stress.

Since a diet with higher NaCl level is protective against waterborne Cu uptake (and possibly therefore toxicity), hypothetically fresh water fish could benefit by preferentially selecting a diet with higher NaCl content over their normal diet, when chronically exposed to waterborne Cu. Given the wide differences (three- to five-fold) in Na^+ contents of various natural diets (Smith et al., 1989), diet selection by feral freshwater fish can result in large differences in dietary Na^+ intake. Several empirical studies, both laboratory and field, have demonstrated that freshwater fish have the ability to select their prey/food (e.g., larger prey), when presented with a choice (e.g., Lazzaro, 1987; Hart and Gill, 1993; Hughes, 1997; Laberge and Hara, 2001; Gill, 2003). These studies suggest that freshwater fish tend to select diet based on several visual and/or olfactory factors such as size, shape, coloration, and smell. However, whether dietary quality (e.g., salt content) has any influence on diet selection in fish has not been investigated before, to our knowledge. The issue of dietary quality factors (e.g., higher NaCl content) evolves from the observation that, in natural conditions, predatory fish exhibit diet shifts from zooplankton through benthic invertebrates to predominantly fish in lakes with low to moderate metal contamination, whereas this phenomenon does not occur in highly contaminated lakes and the fish feed predominantly on zooplankton (Sherwood et al., 2000). This seemingly atypical feeding behavior of fish in highly contaminated lakes was explained on the basis that the diversity of food organisms was reduced by the toxicants in these lakes, resulting in a reduction in diet options available to fish. Whether fish in contaminated waters can select food based on higher mineral (e.g., Na^+, Cl^-, and/or Ca^{2+}) concentration remains an interesting hypothesis to be tested.

With this background in mind, the primary objective of our study was therefore to evaluate whether freshwater fish would prefer NaCl-enriched food to normal food, when exposed to chronic waterborne Cu. The difference in Na^+ level between the two experimental diets used in this study was approximately nine-fold, somewhat higher than the variability of Na^+ levels in natural fish diets (three- to five-fold) described before (Smith et al., 1989). Differential labeling of foods with ballotini beads, coupled with X-ray radiography (McCarthy et al., 1992) was used to assess dietary choice and quantify consumption of each diet type. We also wished to assess the subsequent effects of interactions between the chronic waterborne Cu exposure and the NaCl-enriched diet, consumed through selection, on different physiological endpoints, e.g., rate of food consumption, survival, growth, Cu accumulation in whole body and key target tissues, branchial transepithelial Na^+ transport, and Na^+ homeostasis.

2. Materials and methods

2.1. Experimental fish

Juvenile (18–20 g) rainbow trout, *O. mykiss*, were obtained from Humber Springs Trout Hatchery, Mono Mills, Ontario, and acclimated to laboratory conditions in a single 500-L plastic tank for 3 weeks. Laboratory conditions included a flow-through of dechlorinated Hamilton Municipal tap water from Lake Ontario (Na^+ 0.6; Ca^{2+} 1.0; Cl^- 0.7; Mg^{2+} 0.2; HCO_3^- 1.9, all in mmol L^{-1}; dissolved organic carbon 3.0 mg L^{-1}; hardness 120 mg L^{-1} as $CaCO_3$; pH 7.8–8.0; temperature 12 ± 1 °C). The fish were maintained on 1.5% daily ration (dry food/wet body weight) of commercial granulated 1.0 grade dry trout pellet (Corey Feed Mills, Fredericton, New Brunswick, Canada) during 3 weeks of laboratory acclimation. The commercial trout diet contained crude protein 54% (minimum), crude fat 19% (minimum), crude fiber 2% (maximum), Ca^{2+} 1.5% (actual), phosphorus 1.1% (actual), Na^+ 0.6% (actual), Vitamin A 10,000 i.u. kg^{-1} (minimum), Vitamin D 36,000 i.u. kg^{-1} (minimum), and Vitamin E 400 i.u. kg^{-1} (minimum). Measured concentrations of Cu in water and food were 2.81 μg L^{-1} and 9.64 μg g^{-1}, respectively. The mean fish weight at the beginning of the experiment was 19.9 g (wet weight).

2.2. Experimental set-up

The experiment described here was part of a larger study comprising six different treatments: dietary choice (between NaCl-enriched and normal diet) in the presence and absence of waterborne Cu; NaCl-enriched diet only in the presence and absence of waterborne Cu; and normal diet only in the presence and absence of waterborne Cu (control). Here we focus on four treatments (the two diet choice treatments, and the two normal diet treatments) that were designed to investigate the diet preference under chronic sub-lethal waterborne Cu exposure and the subsequent physiological consequences with respect to rate of food consumption, growth, Cu accumulation, branchial Na^+ transport, and Na^+ homeostasis. The results of other two treatments are the subject of a separate report (Kamunde et al., 2005).

Following 3 weeks of laboratory acclimation, fish were equally ($n = 55$–60) and randomly distributed into four 150-L experimental tanks. An exposure concentration of 55 μg L^{-1} waterborne Cu was achieved via a constant drip of 2 mL min^{-1} of a stock solution containing 68.75 mg L^{-1} Cu (as $CuSO_4 \cdot 5H_2O$; Fisher Scientific, Toronto, Ont.) from a Marriott bottle into a head tank receiving 2.5 L min^{-1} dechlorinated Hamilton tap water. The head tank supplied the two experimental tanks (dietary choice and normal dietary treatments, both under water-

borne Cu exposure) at a flow rate of 800 mL min^{-1}. Actual Cu concentration in the exposure water was 52.34 ± 4.71 μg L^{-1} ($n = 28$). The remaining two treatments of this study (control and diet choice treatments in the absence of Cu) were maintained on regular dechlorinated water at a flow rate of 800 mL min^{-1}. The background Cu concentration in dechlorinated water was 2.81 ± 0.76 μg L^{-1} ($n = 28$). All the treatments were continued for 4 weeks.

2.3. Diet preparation, fish husbandry, and sampling

The NaCl-enriched diet used in the dietary choice treatments was made in-house by supplementing commercial trout chow with NaCl (Fisher Scientific, Canada), as previously reported (Kamunde et al., 2003; Pyle et al., 2003), to achieve a nominal level of 1.84 mmol g^{-1} of Na^+ in the diet. Normal diet, used in all four experimental treatments, was taken through the same processing procedure except that no NaCl was added. The pellet size (length) of the two types of diets was kept similar. However, the shapes of the two pellets used in dietary choice treatments were made different (rectangular and cylindrical) assuming that fish would quickly learn to select the diet of their choice based on shape. The shapes were interchanged halfway through the exposure period (2 weeks) to differentiate the effect of shapes, if any, on the diet selection pattern. In contrast, the shape of pellet used in two exclusively normal dietary treatments was kept identical (cylindrical) during the entire experimental period.

Prior to our actual study, we carried out a preliminary experiment where rainbow trout were given a choice of selecting diets between normal and KCl-enriched (10%, w/w) for a period of 14 days. Similar to the actual experiment, the normal and KCl-enriched diets were made rectangular and cylindrical in shapes. Spiking the diet with KCl was based on the notion that it would make the diet less palatable to the fish. The results showed trout almost immediately started to select normal diet relative to KCl-enriched diet in an overwhelming manner (Table 1), indicating that trout can quickly learn to choose their diet on the basis of shape. The same phenomenon was also observed even when the shapes of the respective diets were interchanged after 7 days.

Table 1
Food consumption data from the preliminary experiment where juvenile rainbow trout (*Oncorhynchus mykiss*) were offered a choice of normal diet vs. KCl-enriched diet (10%, w/w) over a period of 14 days

Time	Normal diet (mg g^{-1} wet wt.)	KCl-enriched diet (mg g^{-1} wet wt.)
Day 1	30.5 ± 5.6	5.1 ± 1.7
Day 7	42.6 ± 6.1	3.7 ± 1.1
Day 14	34.7 ± 4.2	3.2 ± 1.6

The two diets were made in different shapes (rectangular and cylindrical), and the shapes were interchanged on Day 8. Fish were offered a 3% daily ration (dry food/wet body wt.), containing 1.5% of each diet type, twice every day. Fish were sampled on days 1, 7, and 14 to evaluate the consumption of each diet type in individual fish. On each sampling day, the two different diets used were spiked with two different Ballotini glass beads, and the trout were sacrificed 1 h post-feeding and X-rayed. The consumption of each diet in each fish was calculated by counting the glass beads from the X-ray films, see text for details. Data presented as mean ± S.E.M. ($n = 5$).

Actual Na^+ concentrations in the NaCl-enriched and normal diets were 1.89 ± 0.17 and 0.24 ± 0.02 mmol g^{-1}, respectively ($n = 6$). The diets were stored at −20 °C until used. In dietary choice treatments, a mixture of NaCl-enriched and normal diet (in 1:1 ratio, w/w) was offered twice a day in the morning and evening. On each occasion, the fish were offered diet rations well in excess of what was needed for satiation feeding. The fish under exclusively normal diet treatments, both in the presence and absence of waterborne Cu, were also fed to satiation with laboratory-made pellets of normal diet twice daily. The fish were allowed to feed for an hour, after which the unconsumed food was immediately removed from all treatment tanks by siphoning. Significant amount of unconsumed food (both pellet types in two dietary choice treatments) was noticed after each feeding episode in all tanks, which indicated satiation feeding in all treatments as well as no shortage of either diet type in two dietary choice treatments. Water samples collected after 1 h of feeding (once a week) were analyzed for total Na^+ concentration in both dietary choice treatments, and no significant leaching of Na^+ into the water was recorded on any occasion. All the fish were bulk-weighed weekly to determine growth.

Fish ($n = 7$) were sampled from each of the four treatments at days 4, 7, 14, 21, and 28. Prior to the start of the experiment (i.e., day 0), 10 fish from a combined control pool were similarly sampled. At all sampling times, the fish were allowed to feed on the ballotini bead spiked diets, allowed to settle for 1 h to prevent regurgitation, then transferred to individual flux boxes, allowed to settle for another 0.5 h, then utilized for 3 h measurements of unidirectional and net Na^+ fluxes as described below (except on day 21 when fish were fed with ballotini bead spiked diets to analyze food consumption and selection only, and no subsequent measurements were carried out). Thereafter (i.e., 4.5 h after feeding) they were immediately euthanized by an overdose of anaesthetic, blood-sampled, and stored frozen at −20 °C for later X-ray analysis to determine diet selection pattern, as well as for analysis of tissue levels of Na^+, Cl^-, and Cu as described below.

2.4. X-ray radiography

In order to determine the feeding patterns and the rate of food consumption of individual fish in the dietary choice treatments, the sampled fish were X-rayed. On each sampling day, the fish in the dietary choice treatments were fed food that had been repelleted in the same manner as the regular diet but contained lead glass ballotini beads (McCarthy et al., 1992; Gregory and Wood, 1999). The two different diet types (NaCl-enriched and normal) were labeled at fixed density (1% by dry mass of food) with two different sized beads (diameter: 0.4–0.45 and 0.65–0.75 mm; Jencons Inc., Bridgeville, PA, USA), which could be clearly distinguished by X-radiography. The beads served to identify as well as quantify each of the two diet types consumed by any individual fish. The ballotini beads did not affect the palatability of the food, which was consumed in comparable quantities as in regular feeding, nor did they exert any chronic effect on the appetite of fish. It took approximately 48 h after consumption for the beads to be completely cleared out of the gut, and

initial tests demonstrated that there was no faecal loss or regurgitation with the protocol used here up to the time when the fish were sacrificed (4.5 h post-feeding) and stored frozen for later X-ray analysis. Later on, the frozen fish were X-rayed by using a single cabinet, 110 kV X-ray system (Faxitron X-ray Corporation, Illinois, USA) with a 75 kilovolts peak (kVP) and an exposure of 5 min. The X-ray films were developed and the number of each type of bead located in the digestive tract was counted for each fish. In the same manner, 2 g (taken randomly) of each type of beaded diets were also X-rayed in triplicate. The X-rays of the diets showed homogeneous distribution of respective beads in both diet types, and the number of beads per unit mass in each diet type was counted. The beaded diets were X-rayed and subsequently counted whenever those were prepared afresh.

2.5. Unidirectional and net Na^+ flux measurements

Unidirectional and net Na^+ flux measurements were carried out for capturing the effects of preferential selection of Na^+-enriched diet on branchial Na^+ transport under waterborne Cu exposure. All flux measurements were performed on individual fish for 3 h. Fish were transferred from treatment tanks exactly 1 h after feeding on each sampling day (including day 0) into individual polyethylene chambers containing 500 mL of dechlorinated Hamilton Municipal tap water with a level of Cu similar to that of the exposure (i.e., control or 55 $\mu g\,L^{-1}$). Each chamber was fitted with an airline to aerate and mix the contents. Fish were allowed to settle down in the polyethylene flux chamber for 30 min. Each flux chamber was kept covered with a black polyethylene sheet to minimize stress on individual fish during this period, and also during the flux experiments later on. It is important to note here that no regurgitation of food was noticed in any experimental fish either during the initial stabilization period or during the subsequent flux experiments. After the initial 30-min of stabilization period, 0.05 μCi of ^{22}Na was introduced to each flux chamber and two 10 mL water samples were taken from each chamber, one after 15 min (time = 0) and the other at the end of the 3 h flux period (time = 3 h). These water samples were initially counted for ^{22}Na gamma radioactivity using a Canberra-Packard MINAXI Gamma counter and subsequently analyzed for total Na and Cu as described below.

After the flux measurements, fish were killed rapidly with an overdose of neutralized MS-222 and a blood sample taken immediately by caudal puncture, centrifuged for 4 min at 10,000 × *g* to separate plasma, which was decanted into separate polyethylene centrifuge tubes. The fish were then rinsed in running deionized water, blotted dry, weighed, and stored at −20 °C. Following X-ray radiography, the fish were thawed, and gills, liver, gut, kidney, and the rest of carcass were subsequently dissected into pre-weighed scintillation vials. Before placement into scintillation vials, gills were rinsed in running deionized water for 30 s to remove surface bound ^{22}Na while the gut tissues were initially emptied of their contents, then similarly washed in deionized water, and blotted dry. All the tissues, including plasma and red blood cells were subsequently counted for ^{22}Na gamma radioactivity as described for water samples and stored at −20 °C, until analyzed for total Na^+, Cl^-, and Cu as described below.

2.6. Analyses

The scintillation vials and centrifuge tubes were re-weighed and the tissue weights obtained by subtracting the weight of the empty vials. All the tissues were then digested overnight at 70 °C with six times volume of 1N HNO_3 and sub-samples were pipetted into 2 mL bullet tubes and centrifuged for 4 min at 10,000 × *g*. The supernatants were then diluted appropriately with 1% HNO_3 and analyzed for total Na^+ and Cu concentrations using certified standards (Fisher Scientific Ltd., Canada). Total Cu concentrations were measured by graphite furnace Atomic Absorption Spectrophotometry (GFAAS; Varian Australia, Model GTA 110). Total Na concentrations were measured by flame Atomic Absorption Spectrophotometry (FAAS; Varian Australia, Model 220FS). Water and plasma samples were similarly analyzed for Na^+ and Cu after appropriate dilution. In addition, plasma was analyzed for Cl^- by the protocol of Zall et al. (1956) adapted for micro-plate reader. Analytical quality assurance and control were maintained using method blanks and sample duplicates, and validated with certified reference material (National Research Council of Canada, Ottawa, Ont.).

2.7. Calculations

The diet selection pattern in dietary choice treatments was evaluated by determining the consumption of any particular diet in individual fish using the equation given below:

$$C = \frac{[XA^{-1}]}{\mathrm{g}} \quad (1)$$

where C is the rate of consumption of any particular diet ($mg\,g^{-1}$ wet weight), X the total number of a particular type of bead counted in the digestive tract of a fish, A the average number of a respective bead per mg of a respective diet type, and g is the wet weight of the fish in g.

The total diet consumption ($mg\,g^{-1}$ wet weight) in any individual fish was calculated as the sum of the consumptions of the two separate diets. Na^+ influx, efflux, and net flux in fish were calculated as described in Pyle et al. (2003).

2.8. Statistics

Total diet consumption data were statistically analyzed using two-way Analysis of Variance (ANOVA) with time and level of waterborne Cu exposure as independent variables. Diet selection data in Cu-unexposed and Cu-exposed fish were also analyzed by two-way ANOVA with dietary Na^+ and time as independent variables. All other data were analyzed by a three-way ANOVA with time, dietary Na^+ and waterborne Cu levels as independent variables. Tukey's honest significant difference (HSD) analysis incorporating Bonferroni's correction for multiple comparisons was used to delineate differences in mean values. The assumptions of ANOVA, i.e., homogeneity of variances and normality

of distribution, were tested using Bartlett and χ^2 tests. All data met these assumptions. Mean values were considered different at $P < 0.05$.

3. Results

3.1. Food consumption and food selection pattern

Between the two dietary choice treatments, food consumption rate was severely impaired in Cu-exposed fish relative to Cu-unexposed fish (Fig. 1). The impairment was at maximum during the first 7 days of the exposure followed by a gradual recovery with time. However, the food consumption rate in Cu-exposed fish still remained significantly reduced compared to Cu-unexposed fish until the end of the exposure.

In general, Cu-exposed fish did not show increased appetite for NaCl-enriched diet, but rather lost their apparent preference for it in concert with their overall loss of appetite, contrary to our proposed hypothesis. Furthermore, as appetite gradually recovered, there was no evidence that a preference for NaCl-enriched food also recovered. Cu-unexposed fish exhibited a preference for the Na^+-enriched diet as they consumed a significantly higher amount of Na^+-enriched diet relative to normal diet on 4 out of 6 sampling days (days 0, 4, 14, and 21; Fig. 2a). In contrast, no such pattern of dietary preference was observed on any sampling day beyond day 0 in Cu-exposed fish (Fig. 2b), indicating that exposure to sub-lethal waterborne Cu severely disrupted their normal diet selection pattern.

3.2. Mortality and growth

Significantly lower mortality (13%) was observed in Cu-exposed fish maintained under dietary choice in comparison to Cu-exposed fish maintained under normal dietary treatment (26%), and all the mortalities occurred within the first 7 days of the exposure. No mortality was recorded among Cu-unexposed

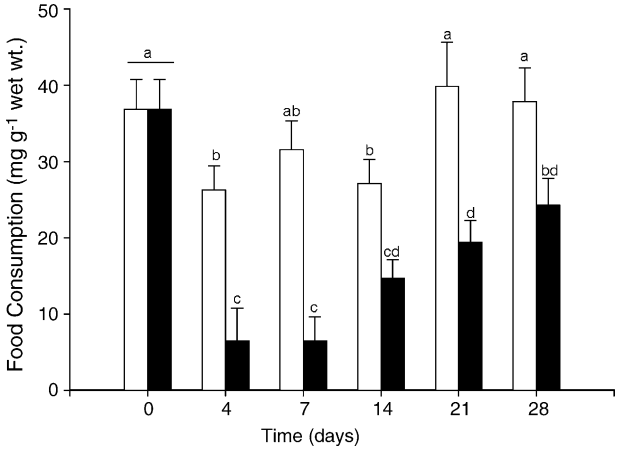

Fig. 1. Effect of sub-lethal waterborne Cu exposure (55 μg L^{-1}) on food consumption over a period of 28 days. The data presented as mean ± standard error of mean (S.E.M.) and $n = 7$ (except day 0 where $n = 10$). White and black bars represent Cu-unexposed and Cu-exposed fish, respectively. Bars with different letters are significantly different (Tukey's HSD, $p < 0.05$).

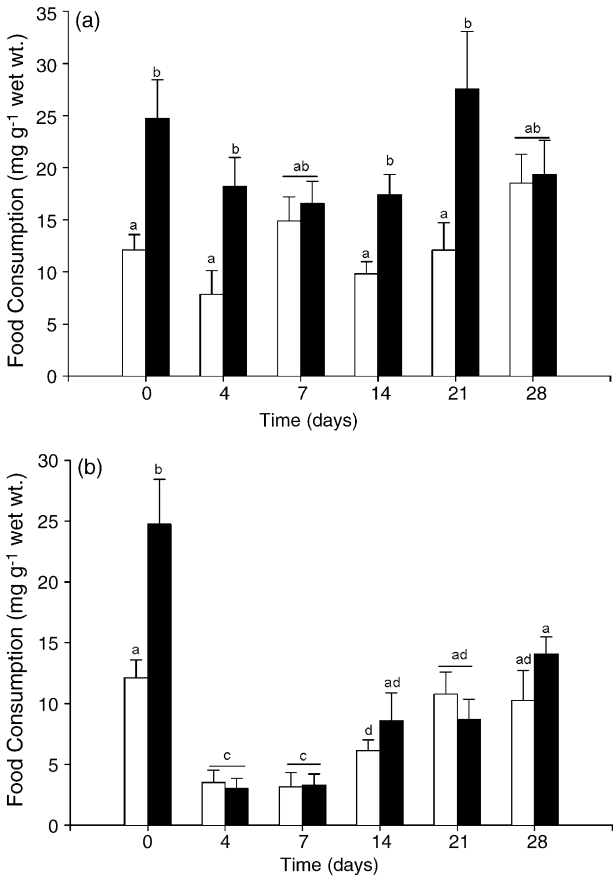

Fig. 2. Selection pattern between normal diet and Na^+-enriched diet over a period of 28 days in (a) Cu-unexposed and (b) Cu-exposed (55 μg L^{-1}) juvenile rainbow trout. The data presented as mean ± standard error of mean (S.E.M.) and $n = 7$ (except day 0 where $n = 10$). White and black bars represent normal and Na^+-enriched diet, respectively. Bars with different letters are significantly different (Tukey's HSD, $p < 0.05$).

fish, either under dietary choice or normal dietary (control) treatment.

Time had a significant effect on growth and there was a significant interaction between time and waterborne Cu as well as dietary NaCl and waterborne Cu. No significant differences were observed in growth at any sampling day between the two treatments of Cu-unexposed fish (Fig. 3). In contrast, growth was severely impacted in both groups of Cu-exposed fish at the early stages of the exposure. Although a gradual recovery was recorded with time in fish with dietary choice relative to fish maintained under normal diet, growth in the former group still remained significantly impaired till the end of the exposure relative to control fish (Fig. 3). At day 28, mean fish weight was approximately 20% and 49% lower in Cu-exposed fish with dietary choice and normal dietary treatments, respectively, relative to control fish.

3.3. Unidirectional and net Na^+ fluxes

In general, time and dietary NaCl had significant effects on Na^+ influx, efflux, and net flux, and there were significant three-way interactions between dietary Na^+, waterborne Cu and time. Na^+ influx, efflux and net fluxes for all four treatment groups

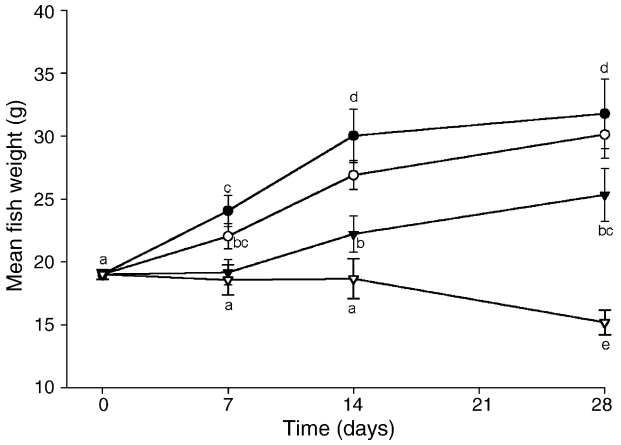

Fig. 3. Effects of interaction between sub-lethal waterborne Cu exposure and dietary selection on mean fish weight (growth). The data presented as mean ± standard error of mean (S.E.M.) and $n = 7$ (except day 0 where $n = 10$). Filled circles represent control treatment, open circles represent dietary choice in the absence of waterborne Cu, filled triangles represent dietary choice in the presence of waterborne Cu (55 μg L^{-1}), and open triangles represent normal diet and waterborne Cu (55 μg L^{-1}). Points with different letters are significantly different (Tukey's HSD, $p < 0.05$).

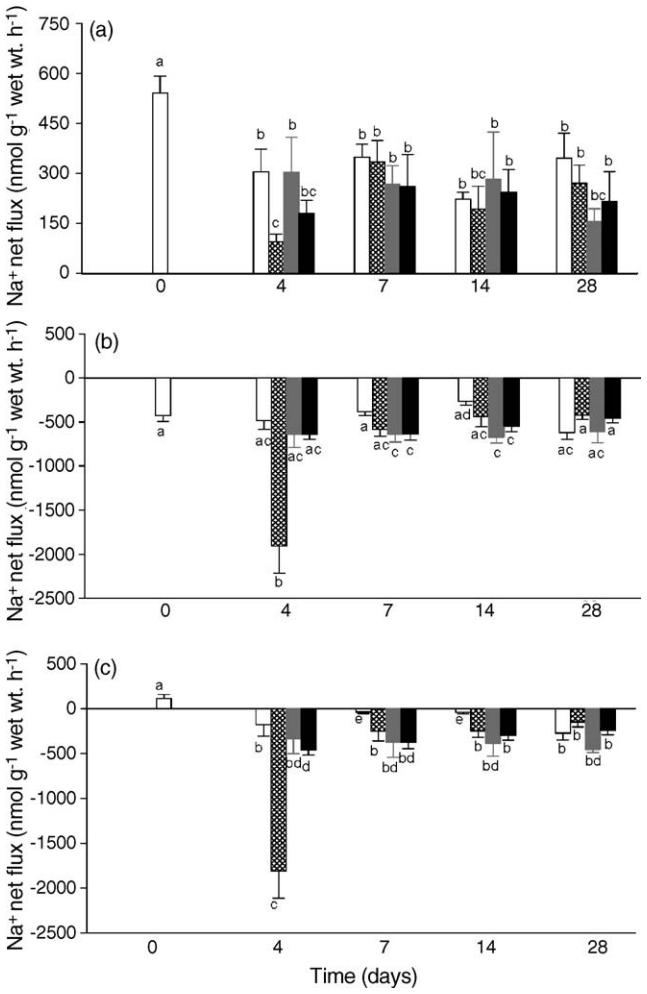

Fig. 4. Effects of interaction between sub-lethal waterborne Cu exposure and dietary choice on Na^+ influx (a), efflux (b), and net flux (c). The data presented as mean ± standard error of mean (S.E.M.) and $n = 7$ (except day 0 where $n = 10$). White bars represent control treatment, cross-hatched bars represent dietary choice in the absence of waterborne Cu, grey bars represent dietary choice in the presence of waterborne Cu (55 μg L^{-1}), and black bars represent normal diet and waterborne Cu (55 μg L^{-1}). Bars with different letters are significantly different (Tukey's HSD, $p < 0.05$).

are shown in Fig. 4a–c. Significant reduction of Na^+ influx rate was observed only in Cu-unexposed fish with dietary choice (day 4) as revealed by three-way ANOVA (Fig. 4a). In contrast, Na^+ efflux rates significantly increased in fish having increased dietary NaCl by choice, both in the presence (days 7 and 14) as well as in the absence (day 4) of waterborne Cu relative to Cu-unexposed fish maintained under normal diet (control; Fig. 4b). Similarly, significant increases in Na^+ efflux rates were also observed (days 7 and 14) in Cu-exposed fish with normal diet. However, these efflux stimulations were attenuated over time as no significant differences were observed on day 28 in both dietary choice treatments as well as in waterborne Cu with normal diet treatment, compared to control. Significant stimulations of net Na^+ flux rates were observed in dietary choice treatments both in the presence (days 7 and 14) and in the absence (days 4, 7, and 14) of waterborne Cu, as well as in waterborne Cu with normal diet treatment (days 4, 7, and 14), compared to control (Fig. 4c). Again, these increases in net Na^+ flux rates returned to control levels at the end of the exposure (day 28).

3.4. Tissue and whole body Cu accumulations

Time had significant effects on Cu accumulations in all tissues as well as whole body, and there were significant interactions between time and waterborne Cu as well as dietary NaCl and waterborne Cu. Waterborne Cu exposure significantly elevated the Cu concentrations in all tissues as well as in whole body (Fig. 5) both under dietary choice (except in kidney and carcass) as well as normal dietary treatments. Liver accumulated the greatest amount of Cu followed by gut, kidney, gill, and carcass in both groups of Cu-exposed fish. No significant differences were observed in tissue and whole body Cu levels between the two Cu-unexposed groups, maintained under dietary choice and normal dietary (control) treatments. Gill Cu concentration steadily increased by about four- to five-fold at day 14 in both waterborne Cu treatment groups relative to control. However, the branchial Cu concentration decreased to control level at the end of the exposure in fish having a choice of diet unlike in fish having a normal diet (Fig. 5a). A similar trend was also observed for Cu accumulation in the gut (Fig. 5d). Liver and whole body Cu levels (Fig. 5b and f) in both groups of Cu-exposed fish increased sharply relative to control. However, Cu accumulation in fish treated with dietary choice reached a steady-state within the first 7 days of the exposure that was maintained till the end of the exposure. Notably, Cu levels in both liver and whole body were significantly higher from day 14 until the end of the exposure in Cu-exposed fish having a normal diet in comparison to Cu-exposed fish with a choice of diet. Furthermore, in the kidney and carcass (Fig. 5c and f), a significant increase in the Cu level was observed only in Cu-exposed fish having a normal diet, and not in Cu-exposed fish offered a choice of NaCl-enriched diet.

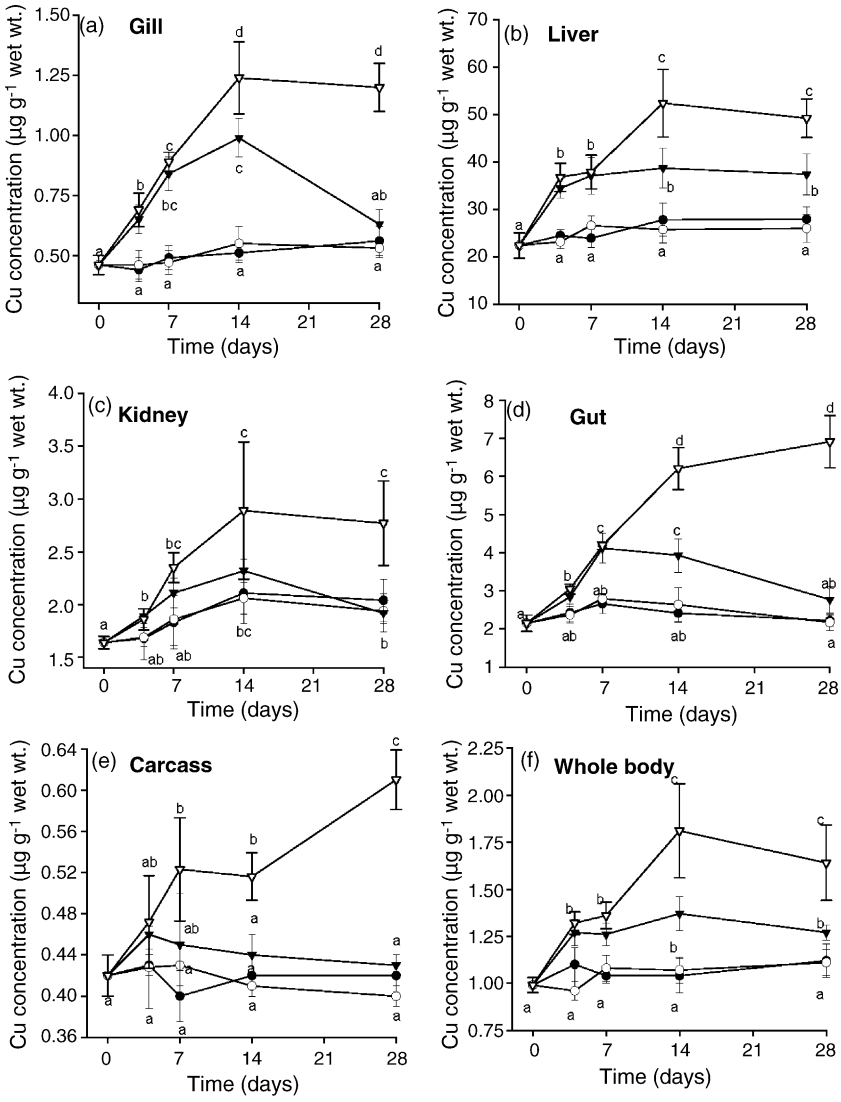

Fig. 5. Effects of interaction between sub-lethal waterborne Cu exposure and dietary selection on Cu accumulations in gill (a), liver (b), kidney (c), gut (d), carcass (e), and whole body (f). The data presented as mean ± standard error of mean (S.E.M.) and $n = 7$ (except day 0 where $n = 10$). Filled circles represent control treatment, open circles represent dietary choice in the absence of waterborne Cu, filled triangles represent dietary choice in the presence of waterborne Cu (55 μg L^{-1}), and open triangles represent normal diet and waterborne Cu (55 μg L^{-1}). Points with different letters are significantly different (Tukey's HSD, $p < 0.05$).

3.5. Tissue and whole body Na^+ concentration

There were significant interactions between dietary NaCl and time in all tissues (except kidney) as well as in whole body. Moreover, there were significant interactions between dietary NaCl and waterborne Cu in the kidney. The NaCl-enriched diet, offered as a choice, elevated Na^+ levels very significantly in all tissues (except kidney) as well as in whole body both in the presence and in the absence of waterborne Cu (Fig. 6). In both dietary choice treatments, gill and liver Na^+ levels increased rapidly and reached a plateau by day 4 (Fig. 6a and b). Na^+ levels in the carcass and whole body reached their peak by day 14 (Fig. 6e and f) in both dietary choice treatments relative to control. In contrast, Na^+ levels in the gut increased linearly in Cu-exposed fish with dietary choice unlike in Cu-unexposed fish where a plateau was observed by day 14. No significant differences were observed in kidney Na^+ levels during the entire exposure between the dietary choice treatments and control (Fig. 6c). Interestingly, a significant decrease in Na^+ levels in kidney was observed towards the end of the exposure in Cu-exposed fish maintained under normal diet.

3.6. Plasma Cu, Na^+, and Cl^- levels

Plasma Cu levels significantly increased in both groups of Cu-exposed fish relative to control, but decreased again by day 14 of the exposure in fish offered a choice of NaCl-enriched diet, unlike in fish under normal dietary treatment where plasma Cu remained elevated throughout the entire exposure (Fig. 7a). No significant differences were observed in plasma Cu level between the two Cu-unexposed groups. Time had significant effects on plasma Cu level, and there was also a significant inter-

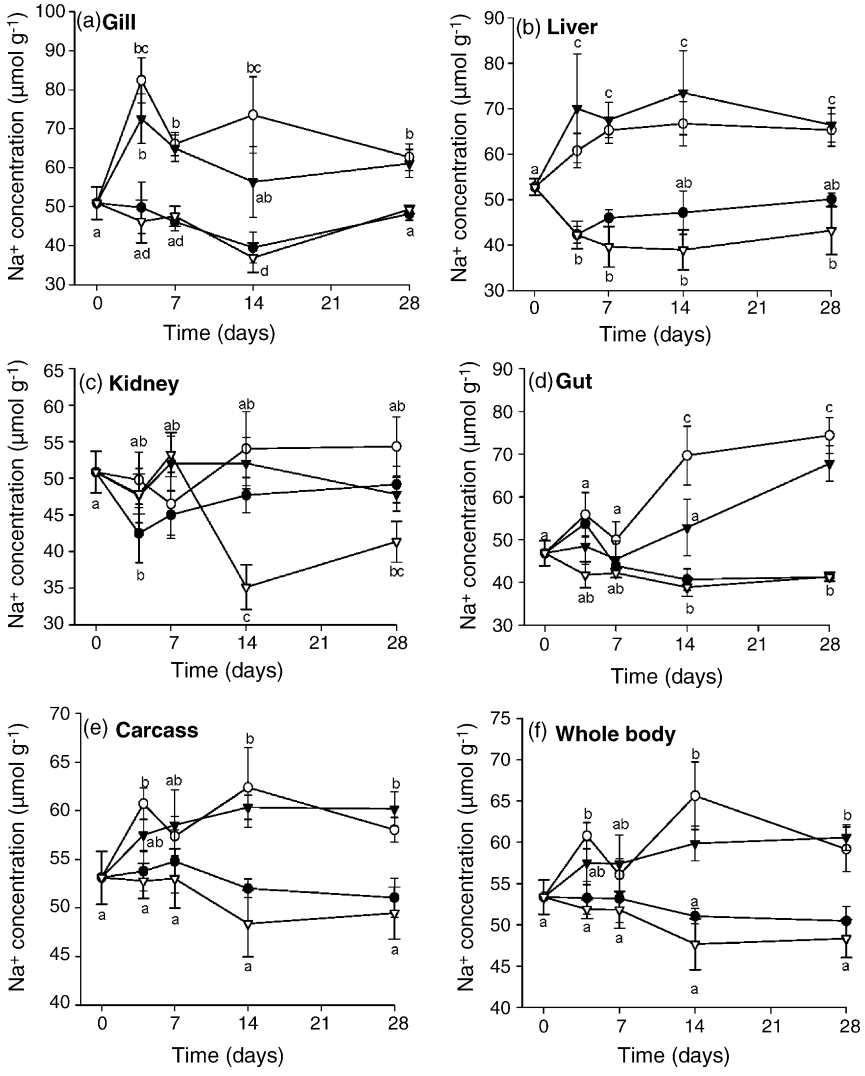

Fig. 6. Effects of interaction between sub-lethal waterborne Cu exposure and dietary selection on Na^+ levels in gill (a), liver (b), kidney (c), gut (d), carcass (e), and whole body (f). The data presented as mean ± standard error of mean (S.E.M.) and $n = 7$ (except day 0 where $n = 10$). Filled circles represent control treatment, open circles represent dietary choice in the absence of waterborne Cu, filled triangles represent dietary choice in the presence of waterborne Cu (55 $\mu g\,L^{-1}$), and open triangles represent normal diet and waterborne Cu (55 $\mu g\,L^{-1}$). Points with different letters are significantly different (Tukey's HSD, $p < 0.05$).

action between dietary Na^+ and waterborne Cu. Dietary uptake of NaCl significantly elevated plasma Na^+ levels (day 4) in the absence of waterborne Cu (Fig. 7b). In contrast, a significant decrease in plasma Na^+ levels was observed from day 7 until the end of the exposure in Cu-exposed fish under normal dietary treatment. Again, time had significant effects on plasma Na^+ level, and there were significant interactions between dietary Na^+ and waterborne Cu. Significant increases in plasma Cl^- level were observed only on day 4 in the dietary choice treatment without waterborne Cu relative to control (Fig. 7c). Waterborne Cu exposure significantly reduced plasma Cl^- levels (day 28) in fish under normal dietary treatment. Dietary NaCl had significant effects on plasma Cl^- level, and there were also significant interactions between dietary NaCl and time as well as dietary NaCl and waterborne Cu. No significant differences were observed in plasma Na^+ and Cl^- levels between Cu-exposed fish with dietary choice and control fish during the entire exposure period.

4. Discussion

4.1. Food consumption and feeding pattern

One of the first marked changes following exposure to 55 $\mu g\,L^{-1}$ waterborne Cu (approximately 1/2 of the 96-h LC50 in the ambient water chemistry; Taylor et al., 2000) was the immediate reduction of appetite in rainbow trout (*O. mykiss*). Although food consumption rate recovered gradually with time, it never reached the level of Cu-unexposed fish within a period of 28 days. Buckley et al. (1982) reported immediate reduction of food intake followed by gradual recovery in coho salmon (*Oncorhynchus kisutch*) exposed to both 70 and 140 $\mu g\,L^{-1}$ (i.e., 1/4 and 1/2 of 96-h LC50 concentrations, respectively) of waterborne Cu over a period of 100 days. Interestingly, they observed complete recovery of appetite only in fish exposed to 1/4 of 96-h LC50 concentrations of waterborne Cu, but not in the latter treatment. The same phenomenon of reduced appetite

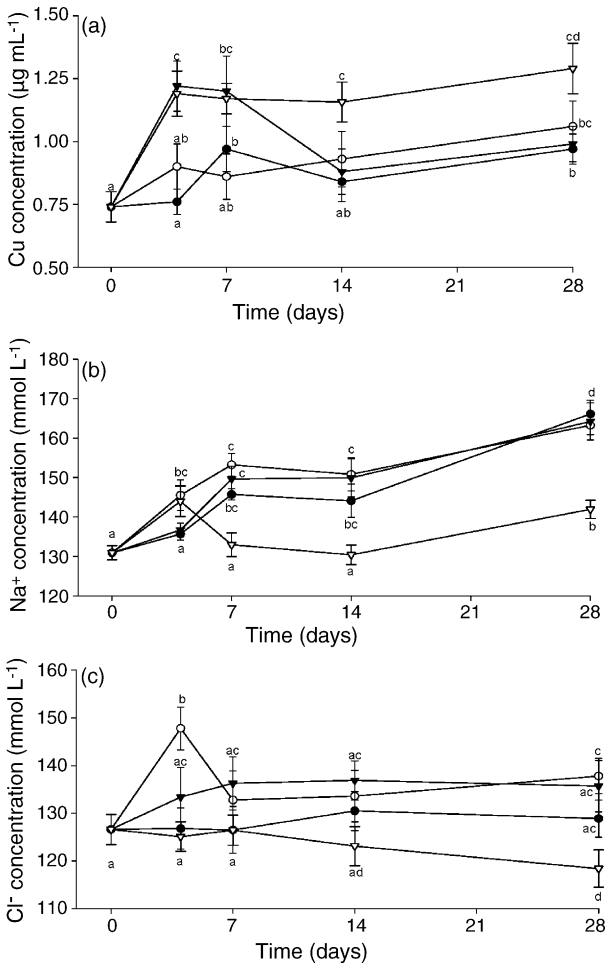

Fig. 7. Effects of interaction between sub-lethal waterborne Cu exposure and dietary selection on Cu (a), Na^+ (b), and Cl^- (c) levels in plasma. The data presented as mean ± standard error of mean (S.E.M.) and $n = 7$ (except day 0 where $n = 10$). Filled circles represent control treatment, open circles represent dietary choice in the absence of waterborne Cu, filled triangles represent dietary choice in the presence of waterborne Cu (55 $\mu g\,L^{-1}$), and open triangles represent normal diet and waterborne Cu (55 $\mu g\,L^{-1}$). Points with different letters are significantly different (Tukey's HSD, $p < 0.05$).

and gradual recovery over time in freshwater fish exposed to chronic waterborne Cu has also been reported by other studies (Lett et al., 1976; Collvin, 1985; De Boeck et al., 1997). Therefore, our result is in very good agreement with several other previous studies on the effect of waterborne Cu on feeding.

The diet selection process in fish is dependent on several factors related to food, e.g., size, shape, smell/olfaction, palatability, contrast, pigmentation and abundance (Lazzaro, 1987; Laberge and Hara, 2001). In our study, the only factors which were different between the two offered diet types, were shape, palatability and also probably smell (i.e., olfactory cues). However, interchanging of shapes of the pellets between the two diet types in both dietary choice treatments halfway through the exposure period did not produce any change in the food selection pattern in Cu-unexposed or Cu-exposed fish. Thus, it could be argued that palatability, and not the shape, was probably the factor responsible for the predominant preference for NaCl-enriched diet in Cu-unexposed fish. Palatability of natural diet has been reported to be an influencing factor in diet selection of salmonid fish (Ware, 1971). The preliminary experiment prior to our actual study described earlier, also demonstrated that rainbow trout tend to prefer normal diet almost immediately over KCl-enriched diet (less palatable) in an overwhelming manner, irrespective of shapes (Table 1). These findings also strongly indicate that fish can choose their diet based on palatability and suggest for the first time that dietary NaCl content can influence the palatability of diet in fish. Moreover, because freshwater fish are constantly losing Na^+ to the dilute external medium (Wood, 2001), selection of a NaCl-enriched diet would not only help replenish the lost Na^+ but also reduce the energetic cost associated with active branchial Na^+ uptake. Interestingly, D'Cruz and Wood (1998) have also reported that the higher NaCl content rather than the energy content in the diet provides better protection against the deleterious effects of low pH in rainbow trout because it enables them to replenish branchial ion losses induced by low external acidity.

Several laboratory (e.g., Wankowski and Thorpe, 1979; Hart and Connellan, 1984) and field (e.g., Mills and Forney, 1981; Rask and Hiisivuori, 1985) studies have demonstrated that fish consistently tend to select the larger food items available, and that fish consuming the largest food items have the fastest growth rates. Therefore, it appears that fish tend to select food in a way that benefits their bioenergetic status. This study is the first attempt to investigate whether freshwater fish have the ability to select their diet in a way that will enable them to mitigate the adverse physiological effects of a waterborne metal like Cu. We observed that, contrary to our hypothesis, exposure of rainbow trout to chronic waterborne Cu did not result in increased appetite for the NaCl-enriched diet. Instead, it severely impaired their apparent preference for the NaCl-enriched diet, despite the potential beneficial effects of NaCl-enriched diet against Cu toxicity in the form of reduced Cu uptake and accumulation, and better ability to maintain Na^+ homeostasis (Kamunde et al., 2003, 2005; Pyle et al., 2003).

This loss of choice might be due to the impairment of their olfactory mechanisms of food-sensing following exposure to chronic waterborne Cu. Hansen et al. (1999) have demonstrated that exposure to 25 $\mu g\,L^{-1}$ waterborne Cu for 4 h in soft water (hardness: 25 $mg\,L^{-1}$ as $CaCO_3$) significantly reduced the number of olfactory receptors in olfactory epithelium tissue by causing cellular necrosis, and resulted in impaired olfactory bulb electroencephalogram (EEG) responses to 10^{-3} L-serine (an olfactory cue) in rainbow trout. Saucier and Astic (1995) have also reported dose-dependent histo-pathological alterations and impaired olfactory discrimination ability in rainbow trout chronically exposed to 20 and 40 $\mu g\,L^{-1}$ waterborne Cu for 40 weeks (at a hardness of 61 $mg\,L^{-1}$ as $CaCO_3$). Olfactory receptors in salmonid fish are known to respond directly to NaCl in water (Shoji et al., 1996), however further research will be needed to determine whether freshwater fish can sense NaCl in food through olfaction and also whether waterborne Cu has any deleterious effects on that potential olfactory process.

4.2. Mortality and growth

We observed significant mortality, all within the first week of the exposure, and reduced growth in both groups of Cu-exposed fish relative to both treatments of non-exposed fish in our study. Fractional mortality and growth inhibition in freshwater fish following chronic waterborne Cu are fairly well documented (e.g., Marr et al., 1996; De Boeck et al., 1997; Kamunde et al., 2005). The Cu-exposed fish with dietary choice in our study did not show any significant weight gain during the first week of the exposure, a period that corresponded with their lowest food consumption rate or, in other words, reduced supply of energy. The significant weight gain in this treatment was only observed with subsequent recovery in appetite. Thus, the growth inhibition in our Cu-exposed fish with dietary choice could be predominantly explained by the lower energy availability in the body for normal metabolic processes (Buckley et al., 1982), and less likely caused by an increased metabolic load resulting from metal exposure as reported in common carp by De Boeck et al. (1997).

However, both survival and growth in Cu-exposed fish under dietary choice treatment, although significantly impaired relative to non-exposed fish, were significantly higher compared to fish exposed to the same concentration of waterborne Cu but fed exclusively with normal diet. In fact, growth rates in Cu-exposed fish maintained under dietary choice treatment were similar after 28 days relative to fish exposed to the same concentration of waterborne Cu but fed exclusively with NaCl-enriched diet of identical composition as used in this study (Kamunde et al., 2005). Therefore, it appears that NaCl-enriched diet, ingested either by exclusive choice or even through an impaired voluntary selection process, can help fish to recover growth, but only to a limited extent. Interestingly, there were no significant differences between the growth rates during the entire exposure period between the non-exposed fish maintained under normal dietary as well as dietary choice treatments, indicating no deleterious effects of voluntary selection of the NaCl-enriched diet on fish growth.

4.3. Interactive effects between dietary NaCl uptake and waterborne Cu on Na^+ transport

In our study, dietary NaCl uptake by choice significantly inhibited the waterborne Na^+ influx in Cu-unexposed fish relative to control, although this effect was only observed at the earlier stages of the exposure. In freshwater fish, reduction of waterborne Na^+ influx has been proposed as an effective strategy to deal with the excess Na^+ when the Na^+ level in the body exceeds homeostatic requirements (Smith et al., 1989). Smith et al. (1995) reported a decrease in waterborne Na^+ influx in rainbow trout following exposure to NaCl-enriched diet (2.1 mmol g^{-1} Na^+) in the absence of waterborne Cu, which is in agreement with our findings. Interestingly, no significant decrease in waterborne Na^+ influx was observed in both treatments of Cu-exposed fish relative to control in our study. Laurén and McDonald (1987a) reported significant inhibition of Na^+ influx in trout during the first 14 days of the chronic exposure to 55 $\mu g\,L^{-1}$ of waterborne Cu at identical water chemistry to that used in the present study, however it should be noted that they used fish that were much younger (average weight: 2.7 g) relative to our fish (average weight: 19 g) and therefore probably more sensitive. The apparent contradiction between our findings and the study cited above could also be due to the stress induced on the fish by our handling, which might have increased Na^+ influx and efflux and thus likely masked any Cu-induced effects in our experiment. Nevertheless, Laurén and McDonald (1987a) did not observe any difference between Cu-exposed and non-exposed trout by day 21–28 of the exposure, which is in agreement with our findings.

In contrast, the Na^+ efflux rates in both Cu-exposed and Cu-unexposed fish under dietary choice as well as in Cu-exposed fish under normal dietary treatments relative to control were significantly greater, although the increases in efflux rates in all the treatments returned to control level at the end of exposure. Significant increases in Na^+ efflux rates following chronic exposure to NaCl-enriched diet in the absence of waterborne Cu have also been reported in rainbow trout by Smith et al. (1989, 1995). Acclimation to waterborne Cu can occur through the modulation of Na^+ influx and/or efflux rates, and also perhaps through the modulation of dietary Na^+ uptake, leading to conservation of Na^+. In the present study, Na^+ influx rates did not show any notable change whereas Na^+ efflux rates increased significantly, eventually leading to a significant increase in net negative flux in Cu-exposed fish with dietary choice as well as with normal dietary treatment. Interestingly, the changes in Na^+ efflux rates were more or less similar (except day 4) between the fish under dietary choice treatments irrespective of waterborne Cu exposure. These findings as well as highly significant elevation of whole body Na^+ levels in both dietary choice treatments indicate that efflux stimulation in those fish is geared primarily to the removal of excess Na^+ from the body accumulated by the voluntary ingestion of NaCl-enriched diet. However, the stimulation of Na^+ efflux and reduced plasma Na^+ levels in Cu-exposed fish fed a normal diet were probably due to the waterborne Cu-induced damage of the branchial epithelium (Laurén and McDonald, 1986; Evans, 1987; McDonald and Wood, 1993). Thus, our findings reveal that dietary uptake of NaCl, although consumed to a lesser extent due to impaired diet selection, could still be beneficial since it could prevent the net Na^+ loss induced by chronic waterborne Cu exposure.

4.4. Tissue and whole body Cu levels

Cu accumulation increased steadily in all tissues during the period (first 7 days) when feeding was at the minimum level in Cu-exposed fish with dietary choice, simultaneous with the Cu-exposed fish under normal dietary treatment. Thereafter, except in liver and whole body, Cu levels in Cu-exposed fish with dietary choice decreased with time in all other tissues, and returned to the levels of Cu-unexposed control fish, in contrast to the Cu-exposed fish under normal dietary treatment. The gradual decrease in tissue Cu levels (except liver and whole body) occurred with the gradual recovery of appetite (day 14 and onwards), which also led to the consumption of increased amounts of NaCl-enriched diet, although the prefer-

ential choice of NaCl-enriched diet over normal diet remained disrupted. Moreover, Cu levels in liver and whole body, although significantly higher than in the Cu-unexposed fish, remained significantly lower from day 14 onwards in Cu-exposed fish with dietary choice relative to Cu-exposed fish under normal dietary treatment. Interestingly, the Cu levels in gill, kidney and gut in Cu-exposed fish with dietary choice never attained saturation as observed in Cu-exposed fish maintained under normal diet and also reported by other workers (Grosell et al., 1997, 1998; McGeer et al., 2000b). In fact, Cu levels in all tissues of Cu-exposed fish with dietary choice in our study were quite similar at the end of the exposure relative to fish exposed to the same waterborne Cu level (55 $\mu g\,L^{-1}$) and fed exclusively with the same NaCl-enriched diet (Na: 1.89 $mmol\,g^{-1}$) as reported by Kamunde et al. (2005). Thus, our results indicate that elevated dietary NaCl, even in the limited quantities provided by the dietary choice regime, can still impede waterborne Cu accumulation in target organs and thus can protect freshwater fish against waterborne Cu toxicity.

4.5. Plasma ion levels and ionic homeostasis

Previous studies have demonstrated that waterborne Cu exposure reduces both plasma Na^+ and Cl^- levels in freshwater fish maintained under control diet (Christensen et al., 1972; Wood, 2001), and that Na^+ and Cl^- losses occur simultaneously (McDonald et al., 1989; Wilson and Taylor, 1993; Pelgrom et al., 1995). In the present study, significant decreases in plasma Na^+ and Cl^- levels were observed in the Cu-exposed fish under normal dietary treatment. In contrast, no significant reduction was observed either in plasma Na^+ or in plasma Cl^- levels in Cu-exposed fish with dietary choice. These findings suggest that increased dietary Cl^- uptake, in addition to increased Na^+ uptake, may account for part of the protection against chronic waterborne Cu exposure in fish. Interestingly, significant elevation of plasma Na^+ and Cl^- levels was observed only at day 4 in Cu-unexposed fish having NaCl-enriched diet by choice, thereby suggesting strong homeostatic control of Na^+ and Cl^- levels.

Several studies have reported a strong homeostatic control of plasma Cu levels in rainbow trout exposed to elevated waterborne and/or dietary Cu levels (Grosell et al., 1997, 1998; Clearwater et al., 2000; Kamunde et al., 2001, 2002, 2003). In the present study, the significant increase in plasma Cu levels in both groups of Cu-exposed fish relative to control fish indicated the disruption of Cu homeostasis. However, plasma Cu concentration decreased in Cu-exposed fish under dietary choice treatment by day 14, but not in Cu-exposed fish fed a normal diet. Interestingly, growth retardation also occurred simultaneously with the episode of plasma Cu elevation in the respective groups of Cu-exposed fish, thereby suggesting that the disruption of Cu homeostasis could lead to chronic toxicity.

5. Conclusion

In the present study, we have confirmed that chronic waterborne Cu exposure severely disrupts appetite, growth, Na^+, Cl^-, and Cu homeostasis, and significantly elevates the tissue and whole body Cu levels in rainbow trout. Most interestingly this study also demonstrated that chronic waterborne Cu exposure disrupts preferential ingestion of NaCl-enriched diet. However, a diet with higher NaCl content, even when consumed in relatively small proportion because of the aforementioned disruption reduces the Cu accumulation in gill, gut, and carcass, mitigates growth retardation, compensates the loss of Na^+ and Cl^- from the body, and thereby helps to maintain the Na^+ and Cl^- balance in fish and to protect against chronic waterborne Cu toxicity.

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