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Dietary Na does not reduce dietary Cu uptake by juvenile rainbow trout

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Rainbow trout *Oncorhynchus mykiss* fry in moderately hard water were exposed to control or high levels of dietary Cu (*c.* 6 and 580 ug Cu g food^{-1}) at one of three levels of Na (1·5, 3·0 or 4·5%) in the diet, *i.e.* six experimental groups. Fish were fed a 4% body mass ration daily for 28 days and 10 individuals from each group were sampled every 7 days. Concentrations of Cu and Na were measured in the gills, liver, gut and remaining carcass of sampled fish. Growth was not affected and no consistent differences were found in mass, total lengths (L_T) or indices of body condition among any of the groups on any sampling day. Copper concentration was significantly higher in tissues of Cu-exposed groups, although within treatment types (control Cu *v.* high Cu diet), it did not differ consistently among groups that received different levels of dietary Na. Tissue Na concentration did not differ among any of the groups and did not show any marked changes over time. In Cu-exposed groups, the proportion of total body Cu burden contained in the liver approximately doubled over time, from *c.* 30% on day 7 to *c.* 60% on day 28. In unexposed fish, the liver maintained *c.* 25% of the total Cu burden throughout the experiment. In contrast, the proportion of the total body Cu burden contained in the gut decreased somewhat over time in Cu-exposed fish, from *c.* 40% on day 7 to *c.* 30% on day 28, and remained fairly stable at *c.* 25–30% in control groups, *i.e.* approximately equal to liver values. In all groups, the carcass contained by far the largest portion of the total Na content (>80%). Measurements made 36 h post-feeding indicated that all six groups had much higher Na efflux relative to influx, suggesting that the fish were eliminating excess Na taken up from the diet, and differences in Na influx rates were small. Na efflux rate was significantly higher in the high Cu and high Na group than in the high Cu and low Na group. The results indicate that at the concentrations used in this experiment, dietary Na has little effect on dietary Cu uptake by juvenile rainbow trout, and dietary Cu has little effect on Na homeostasis.

Key words: dietary Cu; dietary Na; homeostasis; metals; *Oncorhynchus mykiss*.

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INTRODUCTION

Industrial operations such as mining and smelting contribute to the mobilization of metals, including copper (Cu), into watersheds. Copper is an essential micronutrient required by most organisms for proper growth and development (Peña *et al.*, 1999), but it can be toxic at higher levels (Handy, 1996). Similarly, sodium (Na) is an essential macronutrient, but it can also have detrimental effects at levels that exceed biological requirements. The nutritional requirements for rainbow trout *Oncorhynchus mykiss* (Walbaum) are 3 mg Cu kg dry diet^{-1} and *c.* 6 g Na kg food^{-1} (Clearwater *et al.*, 2002; Fish Nutrition Research Laboratory, University of Guelph and Ontario Ministry of Natural Resources: www.uoguelph.ca/fishnutrition/feedint.html). While most animals acquire these nutrients only through the diet, fishes can take up both Cu and Na by two routes of exposure: through the diet as well as across the gills. Although the waterborne route is usually considered dominant for Na and the dietary route is considered dominant for Cu, both natural and experimental situations exist in which the two routes may become equally important (Na: Smith *et al.*, 1989; Cu: Kamunde *et al.*, 2002). For example, Kamunde *et al.* (2002) found that under conditions of low waterborne Cu and normal dietary Cu, the relative contribution to the total body Cu burden was approximately equal *via* the gills *v.* via the diet (*i.e.* gut), but with increasing dietary Cu, the gill became less important as a route of exposure.

Waterborne Na and Cu are thought to enter the fish gill, at least in part, through the same apical Na channel (Grosell & Wood, 2002). Laurén & McDonald (1985, 1987) reported that elevated waterborne Cu inhibits the uptake of Na *via* the gills. Similarly, Grosell & Wood (2002) provided evidence that slightly elevated levels of Cu can reduce the branchial affinity to take up Na, and that waterborne Na inhibits branchial transfer of Cu across the gills of rainbow trout. Therefore, changes in waterborne Na concentration may affect the amount of Cu taken up by fish *via* the gills.

Pyle *et al.* (2003) reported that increased dietary Na intake reduced the branchial uptake of Na, which was accompanied by a reduction in branchial Cu uptake. They found that rainbow trout fed high Na diets also accumulated less Cu in the liver, kidney, gut and plasma than control fish did. Similar observations were reported by Kamunde *et al.* (2003). These studies lend support to the hypothesis that Na and Cu share a common branchial uptake route.

Despite these recent findings on the role that waterborne and dietary Na play in reducing branchial Cu uptake, the effects of dietary Na on dietary Cu uptake by fishes remain unknown and the relationship, if any, between Na and Cu transport mechanisms in the gut remains unclear. In mammals, Na is thought to play a role in Cu uptake through the gut, *i.e.* through Na-mediated Cu transport across intestinal epithelia (Wapnir & Stiel, 1987; Wapnir, 1991), although the nature of the interaction is uncertain. In African walking catfish *Clarias gariepinus* (Burchell), removal of Na tends to slow Cu absorption in the intestine (Handy *et al.*, 2002), which suggests a shared mechanism for the uptake of these two nutrients, although Handy *et al.* (2000) noted that the removal of Na may alter the transepithelial potential, which in turn could alter the movement of Cu. As far as is known, however, the possible interactions

between dietary Na and dietary Cu in fishes have yet to be investigated *in vivo*. The objective of this study was therefore to characterize these interactions. Specifically, whether increased levels of Na in the diet of juvenile rainbow trout would lead to decreased Cu uptake from the diet, at either control or experimentally elevated levels of Cu in the food, was investigated.

METHODS

ACCLIMATION

Approximately 350 rainbow trout fry (mass <1 g, <50 mm total length, L_T) were obtained from Humber Springs Trout Hatchery (Orangeville, Ontario, Canada). Fish were initially acclimated to laboratory conditions in one large tank (*c.* 500 l) supplied with aerated, flow-through, dechlorinated municipal tap water from Hamilton, Ontario (moderate hardness: $Na^+ = 0{\cdot}5$–$0{\cdot}6\,mmol\,l^{-1}$, $Cl^- = 0{\cdot}7\,mmol\,l^{-1}$, $Ca^{2+} = 1{\cdot}0\,mmol\,l^{-1}$, hardness *c.* 140 ppm as $CaCO_3$, background $Cu = <1\,ug\,l^{-1}$, *c.* pH 7·9; temperature = 12° C, range ±2° C). During the 2 week acclimation period, fish were fed commercial fish feed (0·5-Gr Silver Cup for Salmon Fry, Nelson & Sons, Inc., Murray, UT, U.S.A.; 15 mg Na g^{-1}, 6 ug Cu g^{-1}) twice a day at a ration of 2% wet body mass per feeding. The manufacturer's specifications for this feed are as follows: (minimum %) crude protein: 52; crude fat: 14; (maximum %) crude fibre: 3; ash: 12; (minimum IU kg^{-1}) vitamin A: 10 000; vitamin D: 2400; vitamin E: 380; (minimum mg kg^{-1}) vitamin C: 200.

DIET PREPARATION

All experimental diets were prepared using the above fish feed, ground to fine powder in a commercial blender. Analytical grade NaCl (BioShop Canada, Burlington, Ontario, Canada) was dissolved in deionized NANOpure-II water (Sybron/Barnstead, Boston, MA, U.S.A.) and mixed into pre-weighed fish food using a Popeil™ automatic pasta maker (Ronco Inventions, LLC, Chatsworth, CA, U.S.A.) to yield diets containing nominally 1·5 (control food: no NaCl added, but treated the same as the other diets), 3 and 4·5% Na by mass. This allowed for comparisons to previous studies (Kamunde *et al.*, 2003) that used similar dietary Na. $CuSO_4{\cdot}5H_2O$ (Fisher Scientific, Toronto, Ontario, Canada) was dissolved in deionized water and mixed into pre-weighed food to yield a nominal concentration of 500 ug Cu g $food^{-1}$, which again was a level comparable to previous studies of sublethal Cu toxicity (Handy *et al.*, 1999). Thus, experimental groups were fed one of the following six nominal diet formulations: no added Cu + 1·5% Na by mass (control), no added Cu + 3·0% Na, no added Cu + 4·5% Na, 500 ug Cu g $food^{-1}$ + 1·5% Na, 500 ug Cu g $food^{-1}$ + 3·0% Na or 500 ug Cu g $food^{-1}$ + 4·5% Na.

Food paste was mixed in the pasta maker for at least 15 min to ensure thorough incorporation of Cu and Na into the diet. The paste was then extruded, air-dried overnight and broken by hand to yield smaller pellets (*c.* 1 mm in diameter and < 5 mm long). All diets were frozen at −20° C until use. Five samples from each diet group were digested in six volumes of 1N HNO_3 (trace metal grade, Fisher Scientific, Nepean, Ontario, Canada) and Cu was analysed by graphite furnace atomic absorption spectroscopy [GFAAS; Varian SpectrAA-220 with graphite tube atomiser (GTA-110), Mulgrave, Australia] following manufacturer specifications for Cu-analysis. National Research Council of Canada-certified analytical standards were run simultaneously with the samples for quality control. To determine Na of the food, samples were analysed by flame atomic absorption spectroscopy (FAAS; Varian SpectrAA-220FS, Mulgrave, Australia), again following manufacturer specifications and using appropriate standards (Radiometer, Copenhagen, Denmark).

Actual mean ± S.E. measured concentrations of Na for control, medium and high Na groups, respectively, were 15·21 ± 0·27 g Na kg dry $food^{-1}$ (0·66 mmol g^{-1}, 1·52%; $n = 10$), 27·87 ± 0·39 g Na kg $food^{-1}$ (1·21 mmol g^{-1}, 2·79%; $n = 10$) and 47·15 ± 0·56 g

Na kg food^{-1} (2·06 mmol g^{-1}, 4·72%; $n=10$). Actual mean ± S.E. Cu was 579·12 ± 9·03 ($n=15$) and 6·14 ± 0·09 ug Cu g food^{-1} ($n=15$) in the Cu-treated and control groups, respectively. Daily doses of Cu (Clearwater *et al.*, 2002) at a daily food ration of 4% were therefore *c.* 0·25 ug Cu g fish^{-1} day^{-1} in control groups and *c.* 23·2 ug Cu g fish^{-1} day^{-1} in Cu-exposed groups. To determine moisture content of the dry pellets, *c.* 5 g of food were oven dried from each treatment group at 60° C for 1 week. Mean ± S.E. moisture content of the food was 8·49 ± 0·39% ($n=6$).

EXPERIMENTAL DESIGN

Following acclimation to laboratory conditions, fish were divided into six 11·3 l polyethylene tanks (Rubbermaid Home Products, Wooster, OH, U.S.A.), with drainage holes drilled at the 8 l mark, such that each tank contained 52 fish (initial density = 6·5 fish l^{-1}). Water temperature in the tanks was maintained at 12° C, range ±2° C throughout the 28 day experiment, and water flow was held constant at *c.* 700 ml min^{-1}. Composition of the water used in the experimental tanks was the same as in the acclimation tank, and each experimental tank contained an airstone to ensure proper aeration.

Fish in each tank were fed one of the six experimental diets twice a day at a ration of 2% wet body mass per feeding (*i.e.* 4% day^{-1}). Fish were weighed in bulk once each week and ration was adjusted accordingly. The animals became accustomed to the feeding regime within the first 2 days of the experiment and ingested all food within minutes of feeding. Faecal matter was siphoned two to three times daily to minimize build-up at the bottom of the tanks. Prior to a sampling day, fish were not fed for 36 h to clear the gut of ingesta; tanks were thoroughly siphoned several times during this period.

Fish were sampled on days 0, 7, 14, 21 and 28 of the experiment. Prior to separating fish into the six experimental tanks on day 0, 15 individuals were sacrificed with an overdose of tricaine methanesulphonate (MS-222, 1 g l^{-1}; Fisher Scientific). On subsequent sampling days, 10 fish were removed from each tank and sacrificed in the same manner. Each individual was blotted dry and weighed, and L_T recorded. Whole gill 'baskets' were then excised and liver and whole gut (*i.e.* oesophagus, stomach, pyloric caecae, anterior intestine, mid-intestine, posterior intestine and rectum) were removed. Guts were emptied of faeces and undigested food as thoroughly as possible, rinsed in deionized NANOpure-II water to remove remaining particulate matter, and blotted dry.

Individual tissues and remaining carcasses were then placed into pre-weighed 1·5- or 2·0 ml polypropylene tubes (tissues) or 20 ml glass scintillation vials (carcasses) and frozen until use. Tissue samples were digested in 12 volumes (liver) or six volumes (gill, gut and carcass) of 1N HNO_3 at 70° C for at least 24 h. Following digestion, samples were vortexed, centrifuged at 11 000*g* for 10 min, and a sub-sample of the supernatant was diluted in an appropriate volume of 1% HNO_3 (×10–40 dilution for unexposed fish tissues; ×30–200 for exposed groups). Total Cu and Na of the tissue samples were then determined by GFAAS and FAAS, respectively, as described for food analyses.

To determine whether leaching from faecal matter or uningested food affected the ambient water Na or Cu concentrations in the fish tanks, water samples were taken on 10 randomly selected days during the experiment, approximately halfway between the morning and the evening feeding, typically between 1400 and 1500 hours and just before the tanks were siphoned. In addition, on the first of these water-sampling days, a 15 ml water sample was taken from each tank immediately prior to the morning feeding and then again several hours post-feeding. All water samples were acidified with 16N HNO_3 to make a 1% HNO_3 solution and were analysed for copper by GFAAS. Additional 1 ml water samples were added to 4 ml of 1% HNO_3 and were analysed for Na by FAAS. Mean ± S.E. 'before' and 'after' values on the first water-sampling day were 536 ± 11 ($n=6$) and 554 ± 31 ($n=6$) umol l^{-1} Na, respectively, and <1 ug Cu l^{-1} at both times. Tank water Na and Cu averaged over the 10 days (*c.* 620 umol l^{-1} and <1 ug l^{-1}, respectively) did not differ among groups. Therefore, leaching of Na and Cu from food or faeces was not a concern in this study.

UNIDIRECTIONAL Na FLUX RATES

Following sampling on day 28, unidirectional Na flux rates (influx, efflux and net flux) with the water were measured on the remaining fish, which had been starved for the previous 36 h. Ten fish from each group were placed in pairs into plastic 60 ml chambers, which were aerated and held at the experimental temperature. ^{22}Na (Amersham Pharmacia Biotech Inc., Piscataway, NJ, U.S.A.) was then added to each chamber [*c.* 0·1 μCi (3·7 kBq) per chamber]. After 15 min, a 2 ml water sample was taken from each chamber. The fish were then covered, and after 3 h, an additional 2 ml water sample was taken from each chamber. Fish were then sampled as described above, except that only the gill 'basket' was removed and rinsed for 30 s with NANOpure-II water to remove any surface-bound radioactivity. Gills and carcass were frozen overnight. ^{22}Na activity of tissue (gills and carcass) and water samples was measured on the following day in a gamma counter (Canberra-Packard Minaxi Auto Gamma 5000 series, Meriden, CT, U.S.A.), and water Na was determined by FAAS as described above.

CALCULATIONS

Copper and Na of different tissues (liver, gill, gut and remaining carcass) were measured for each sample and whole-body (WB) Cu or Na concentration was estimated as: Cu_{WB} or $Na_{WB} = \{\sum[\text{Cu (or Na) content of all tissues + carcass}]\}\,[\sum(\text{mass of all tissues + carcass})]^{-1}$. Standard somatic indices of body condition (for liver, gills and gut) as well as specific growth rate (G) and food conversion efficiency (E_{FC}) were calculated as described by Kamunde & Wood (2003). An overall body condition factor (K) was estimated by the following index: $K = 100 M L_T^{-3}$, where M is wet mass (g) of the whole fish and L_T is in cm.

Sodium influx (*i.e.* appearance of Na in the fish; J_{in}^{Na}) was calculated as: $J_{in}^{Na} = C_{WF}(T_F A_s)^{-1}$, where C_{WF} is whole fish counts min^{-1}, T_F is flux time and A_S is specific activity. Specific activity was calculated from $A_S = 0{\cdot}5 R_{initial} C_{initial}^{-1} + R_{final} C_{final}^{-1}$, where R = (cpm per sample volume) × flux chamber volume and C = Na concentration of water × flux flux chamber volume. Net Na flux (J_{net}^{Na}) was calculated as: $J_{net}^{Na} = (C_{initial} - C_{final})\,(MT_F)^{-1}$, and Na efflux ($J_{out}^{Na}$) was determined by subtracting J_{in} from J_{net}.

To determine how much Cu and Na the fish retained (R) from the diet over 28 days, the following equation modified from Berntssen *et al.* (1999*a*) was applied: $R = 100(C_f - C_i)\,T_{Cu,Na}^{-1}$, where C_f = final whole-fish Cu or Na content on day 28, C_i = initial whole-fish Cu or Na content on day 0 and $T_{Cu,Na}$ = total amount of Cu or Na fed to each fish over 28 days.

STATISTICAL ANALYSES

Comparisons were made on each sampling day by treatment, *i.e.* among Cu-exposed and among unexposed groups. A one-way ANOVA was generally used to compare the groups, except when data did not meet the requirements of equal variance; in these cases, comparisons were made with a Kruskal–Wallis test. Means were compared with a Tukey HSD test or a non-parametric comparison of mean ranks where appropriate. Percentage data were arcsin-transformed prior to analysis. In some cases, where indicated, pair-wise comparisons (two-sample *t*-tests or Mann–Whitney tests, as appropriate) were made between Cu-exposed and unexposed groups that received comparable amounts of Na. Data are reported as means ± S.E., and differences were considered significant at $P < 0{\cdot}05$. Unless otherwise noted, reported results are from the ANOVAs.

RESULTS

GROWTH

Fish increased in size from *c.* 0·5 g on day 0 to *c.* 1·5–2·0 g on day 28, and no significant differences in M or L_T were found among groups on any of the

sampling days. No significant differences were observed among the groups in either mean G or mean E_{FC} over 28 days (Table I). Similarly, few differences were evident in tissue masses among groups (unpubl. data), and indices of body condition showed no consistent patterns (Table I). Mortality during this experiment was very low, at *c.* 3% ($n = 10$ of 312).

COPPER

As expected, Cu-treated fish had consistently higher tissue [Cu] than those that had received no additional dietary Cu. Liver [Cu] was six-fold to 10-fold higher in Cu-exposed groups than in unexposed groups [Fig. 1(a)]. Gill [Fig. 1(b)], gut [Fig. 1(c)] and carcass [Cu] remained $\times 1{\cdot}5$–2, $\times 4$–6 and up to $\times 2$ higher on average, respectively, in exposed groups than in unexposed groups throughout the study. Differences in gill [Cu] between treatments (Cu-exposed *v.* unexposed) were not as marked as differences in liver [Cu]. The guts of Cu-treated fish accumulated substantially more Cu than those of control Cu fish did, although the values were much lower than levels in the liver [Fig. 1(c)]. The carcass accumulated the lowest absolute [Cu] ($<0{\cdot}6$ ug Cu g^{-1} tissue in all six groups), and differences between treatments (Cu-exposed *v.* unexposed) were not as pronounced as in the organs (unpubl. data). Within treatments, differences in carcass [Cu] were negligible. Not surprisingly, whole-body [Cu] was substantially higher in Cu-fed fish than in animals that received no additional dietary Cu [Fig. 1(d)]. Among control Cu and high Cu groups, however, whole-body [Cu] exhibited only minor and inconsistent differences [Fig. 1(d)].

The proportion of whole-body Cu accounted for by the liver on day 28 of the experiment is shown in Fig. 2(a). Livers of Cu-treated fish accounted for an increasing proportion of body Cu (up to *c.* 60%) over time, whereas livers of control fish contained roughly the same percentage (*c.* 25%) of whole-body Cu over time. Gills contributed to 2–4 and 1–3% of the whole-body Cu burden in unexposed and Cu-exposed fish, respectively, with few differences among groups over time. The gut consistently accrued *c.* 20–30% of the whole-body Cu content in unexposed fish and decreased from 41 to 25% in Cu-exposed fish over time. Overall, the carcass contained a higher percentage of the whole-body Cu content in unexposed fish (42–50%) compared to exposed fish (15–25%).

Cu retention was about 11–15% of the total amount consumed over 28 days and did not appear to differ among the control Cu groups (Table I). In contrast, Cu retention was <1% among the high Cu groups, and was highest in the group that received the highest level of dietary Na (Table I). These results were not analysed statistically, as the Cu retention values represented means of means.

SODIUM

Sodium concentration in liver [Fig. 3(a)], gills [Fig. 3(b)] and gut [Fig. 3(c)] did not differ consistently among groups or over time. Carcass [Na] tended to decrease in most groups over time (from *c.* 48 to *c.* 41 umol Na g^{-1}), except in high Na groups, where levels remained elevated between *c.* 48 and 52 umol Na g^{-1} (unpubl. data). Whole-body [Na] remained fairly stable over time and

TABLE I. Mean specific growth rate (G) and food conversion efficiency (E_{FC}) over 4 weeks, as well as tissue condition indices (I_H = hepato-somatic index, I_B = branchio-somatic index, I_G = gastro-intestino-somatic index and K = condition factor) and mean whole-fish Cu and Na retention (%) of 10 juvenile rainbow trout from each treatment group sampled on day 28. Different lower case superscripts indicate groups that differed from one another by ANOVA with Tukey's HSD test ($P < 0{\cdot}05$). Note that means were used to generate retention values; they were therefore not analysed statistically

	Index							
Group	G (%)	E_{FC} (%)	I_H	I_B	I_G	K	Cu retention	Na retention
Control Cu								
1·5% Na	$3{\cdot}77 \pm 0{\cdot}49$	$138{\cdot}58 \pm 20{\cdot}32$	$0{\cdot}018 \pm 0{\cdot}001$	$0{\cdot}036 \pm 0{\cdot}01$	$0{\cdot}071 \pm 0{\cdot}04^{ab}$	$0{\cdot}902 \pm 0{\cdot}18$	14·85	5·97
3·0% Na	$3{\cdot}31 \pm 0{\cdot}57$	$120{\cdot}17 \pm 22{\cdot}55$	$0{\cdot}018 \pm 0{\cdot}01$	$0{\cdot}031 \pm 0{\cdot}02$	$0{\cdot}063 \pm 0{\cdot}04^{ab}$	$0{\cdot}937 \pm 0{\cdot}20$	11·25	3·46
4·5% Na	$3{\cdot}62 \pm 0{\cdot}38$	$131{\cdot}56 \pm 15{\cdot}82$	$0{\cdot}018 \pm 0{\cdot}01$	$0{\cdot}036 \pm 0{\cdot}01$	$0{\cdot}074 \pm 0{\cdot}03^{a}$	$0{\cdot}888 \pm 0{\cdot}22$	13·18	2·79
Cu-exposed								
1·5% Na	$3{\cdot}86 \pm 0{\cdot}15$	$140{\cdot}95 \pm 6{\cdot}00$	$0{\cdot}017 \pm 0{\cdot}01$	$0{\cdot}031 \pm 0{\cdot}02$	$0{\cdot}067 \pm 0{\cdot}04^{ab}$	$0{\cdot}922 \pm 0{\cdot}26$	0·55	5·80
3·0% Na	$3{\cdot}28 \pm 0{\cdot}27$	$117{\cdot}91 \pm 10{\cdot}80$	$0{\cdot}017 \pm 0{\cdot}01$	$0{\cdot}034 \pm 0{\cdot}01$	$0{\cdot}053 \pm 0{\cdot}04^{b}$	$0{\cdot}939 \pm 0{\cdot}17$	0·57	3·38
4·5% Na	$2{\cdot}96 \pm 0{\cdot}27$	$104{\cdot}69 \pm 10{\cdot}53$	$0{\cdot}018 \pm 0{\cdot}01$	$0{\cdot}030 \pm 0{\cdot}02$	$0{\cdot}066 \pm 0{\cdot}07^{ab}$	$0{\cdot}866 \pm 0{\cdot}19$	0·72	1·94

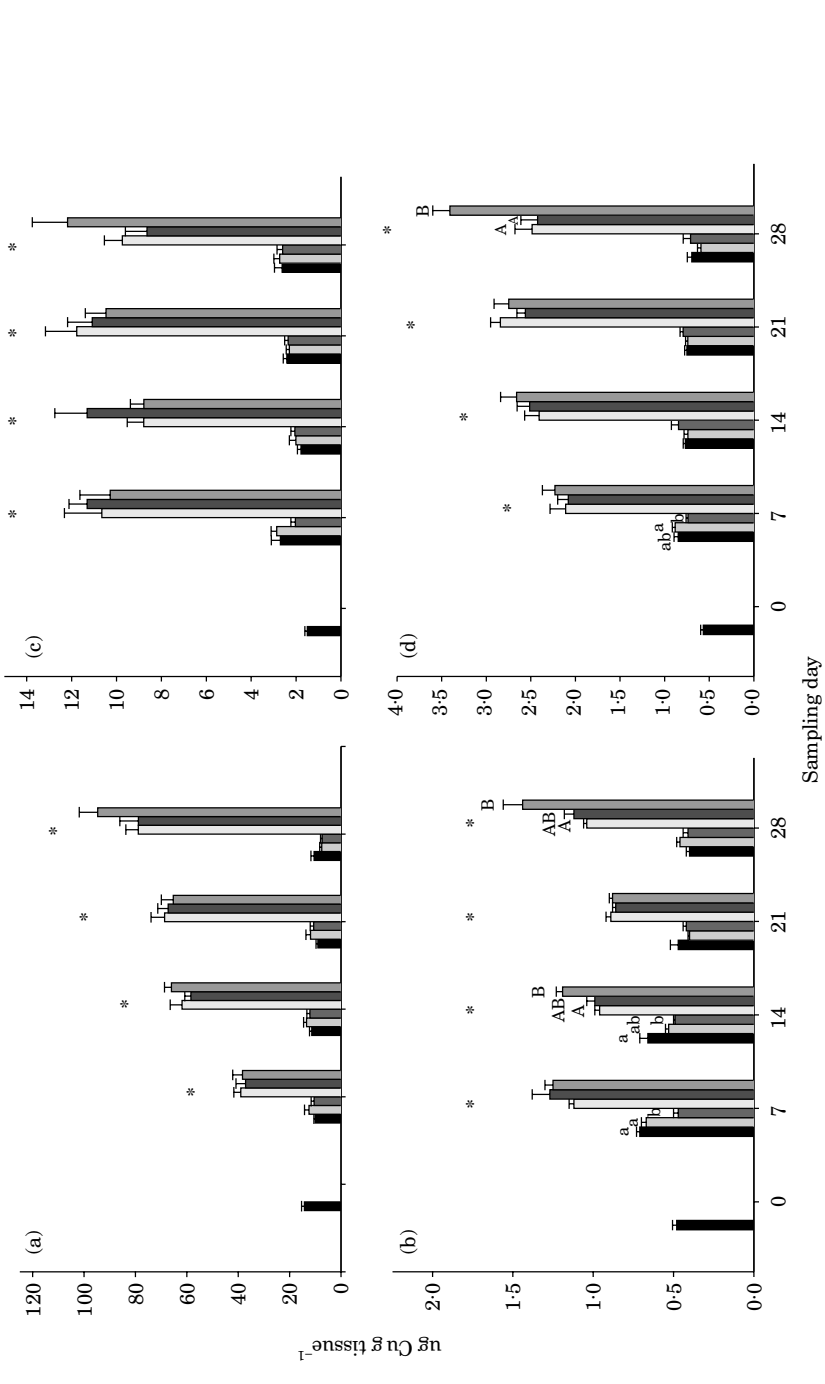

FIG. 1. Mean + S.E. (a) liver, (b) gill, (c) gut and (d) whole-body Cu concentrations of 10 juvenile rainbow trout per treatment group (■, Control Cu + 1·5% Na; □, Control Cu + 3% Na; ■, Control Cu + 4·5% Na; □, High Cu + 1·5% Na; ■, High Cu + 3% Na; □, High Cu + 4·5% Na) sampled on days 7, 14, 21 and 28. Day 0 represents 15 individuals that were sampled prior to experimentation. *, significant differences between Cu-exposed and unexposed fish. Different letters above bars (lowercase for unexposed groups and uppercase for exposed), groups that differed significantly within treatments (both comparisons: ANOVA or Kruskal–Wallis, $P < 0{\cdot}05$).

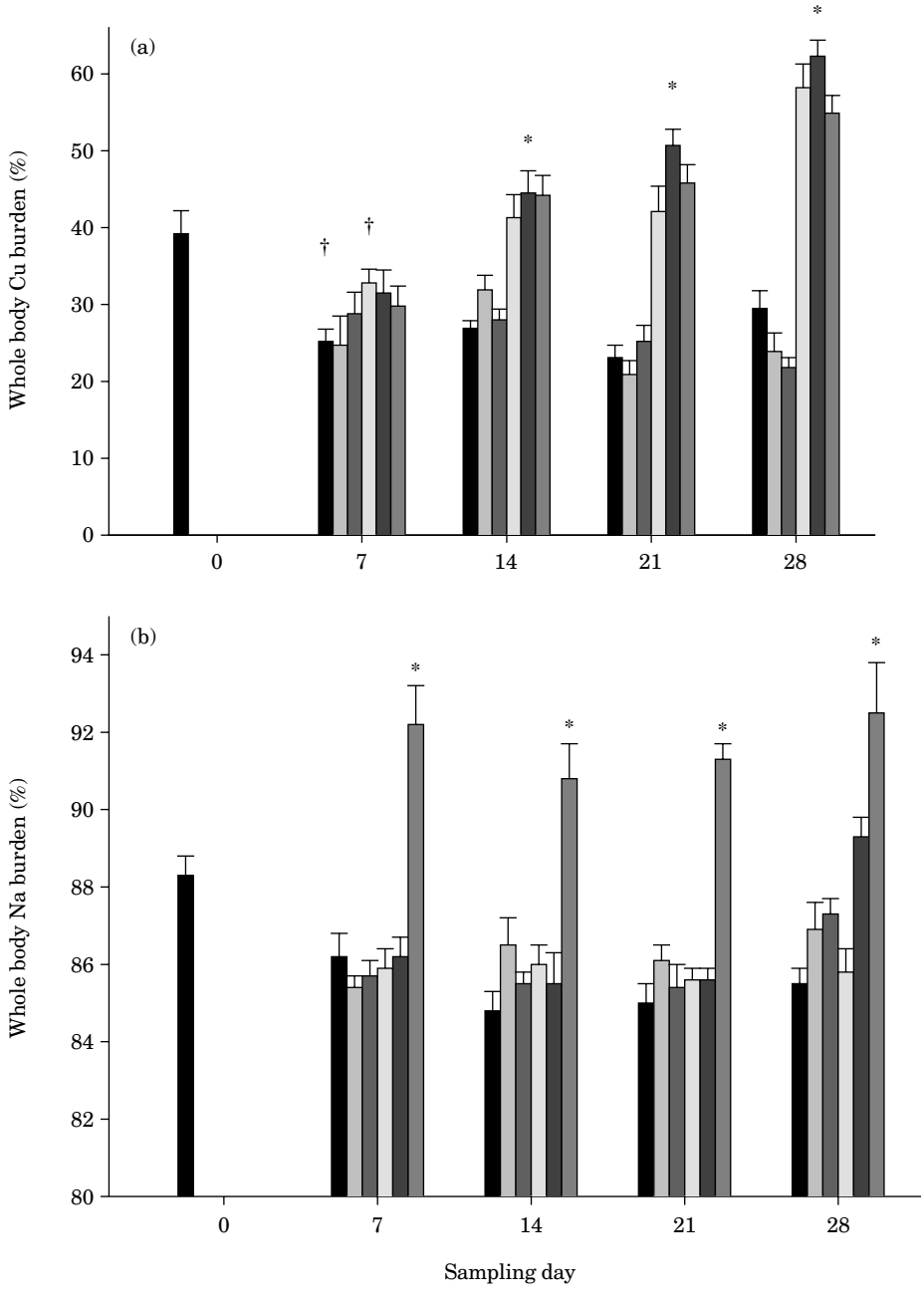

FIG. 2. Mean + S.E. per cent of whole-body (a) Cu burden contained in the liver and (b) Na burden contained in the carcasses of 10 juvenile rainbow trout per treatment group (■, Control Cu + 1·5% Na; □, Control Cu + 3% Na; ■, Control Cu + 4·5% Na; □, High Cu + 1·5% Na; ■, High Cu + 3% Na; ■, High Cu + 4·5% Na) sampled on days 7, 14, 21 and 28. Day 0 represents 15 individuals that were sampled prior to experimentation. (a) *, significant differences (ANOVA, $P < 0{\cdot}05$) between Cu-exposed groups and control groups; †, significant differences (*t*-test, $P < 0{\cdot}05$) between two groups. (b) *, significant differences (ANOVA, $P < 0{\cdot}001$) between the high Cu, high Na group and all other groups. Percentages were arcsin-transformed prior to analyses.

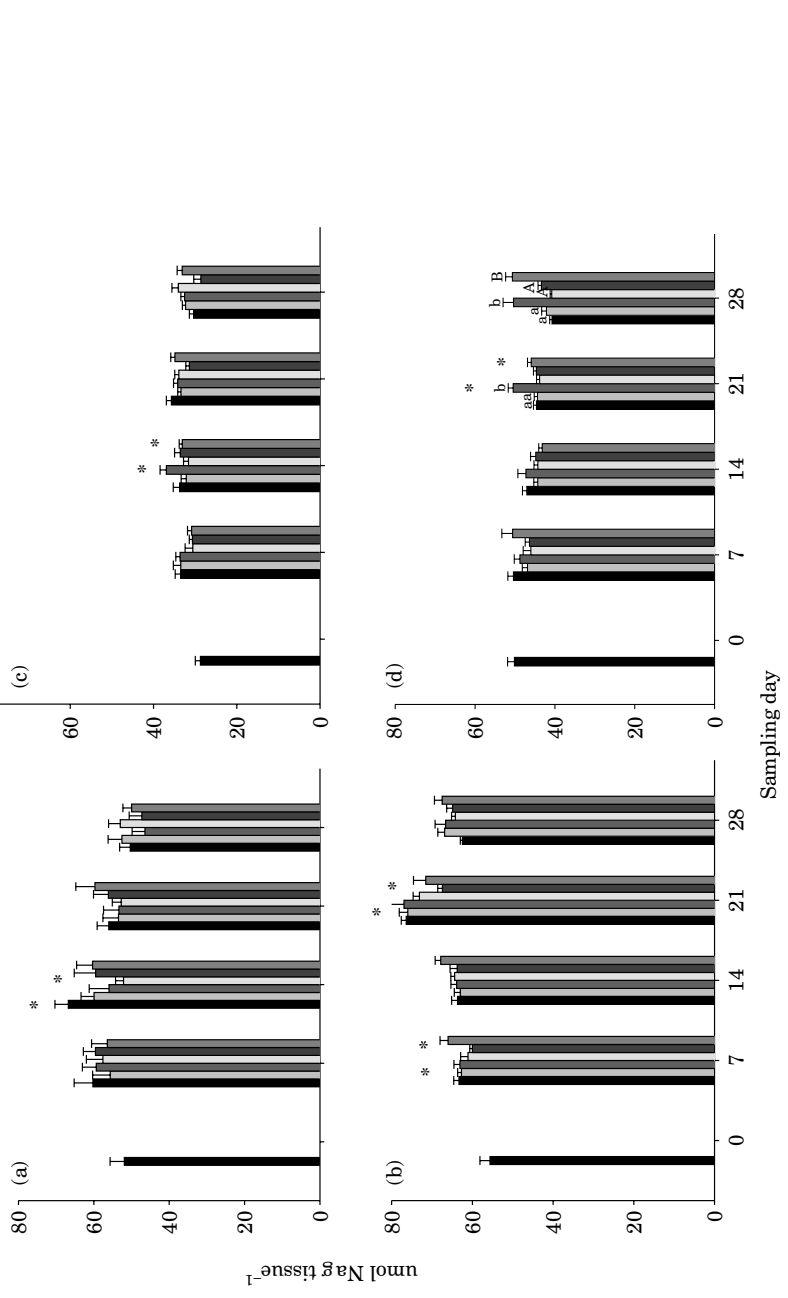

FIG. 3. Mean + S.E. (a) liver, (b) gill, (c) gut and (d) whole-body Na concentrations of 10 juvenile rainbow trout per treatment group (■, Control Cu + 1·5% Na; □, Control Cu + 3% Na; ■, Control Cu + 4·5% Na; □, High Cu + 1·5% Na; ■, High Cu + 3% Na; ■, High Cu + 4·5% Na) sampled on days 7, 14, 21 and 28. Day 0 represents 15 individuals that were sampled prior to experimentation. *, significant differences between Cu-exposed and unexposed fish. Different letters above bars (lowercase for unexposed groups and uppercase for exposed), groups that differed significantly within treatments (both comparisons: ANOVA or Kruskal–Wallis, $P < 0{\cdot}05$).

tended to be higher in fish fed the high-Na diet, although these differences were only significant on days 21 and 28 [Fig. 3(d)].

The bulk of the total Na burden was contained in the carcass, which accumulated between 85 and 88% of the total-body Na content over time in five of the six groups. Interestingly, the high Cu, high [Na] group had a significantly higher per cent carcass Na than all other groups on all sampling days, while the other five groups showed no consistent patterns [Fig. 2(b)].

Na retention over 28 days ranged from *c.* 2 to 6% and did not appear to be influenced by Cu content in the diet, but was related to [Na] of the diet. In both Cu-exposed and unexposed fish, Na retention was highest in the groups that received the lowest amount of dietary Na and lowest in the groups that received the highest amount of dietary Na (Table I). As with Cu, these data were not analysed statistically.

UNIDIRECTIONAL Na FLUX RATES

Within low Cu and high Cu treatment groups, J_{in}^{Na} did not differ, while J_{out}^{Na} and J_{net}^{Na} tended to be more negative with increased Na content in the diet, although this relationship was only significant for J_{out}^{Na} in the Cu-supplemented groups (Fig. 4). In pair-wise comparisons between Cu-treated and control groups fed comparable amounts of Na, no differences were found between any of the pairs, with one exception: J_{in} was higher in the high Cu, high Na group than in the control Cu, high Na group [Fig. 4(b)]. These effects were seen even though the fish had been starved for 36 h prior to the measurements.

DISCUSSION

DIETARY Na *V.* INTESTINAL Cu UPTAKE

In this study, in contrast to waterborne Na (Kamunde *et al.*, 2003; Pyle *et al.*, 2003), dietary Na did not reduce the Cu burden in fish exposed to relatively high [Cu] in the diet. This suggests that the mechanisms for Cu uptake at the gut differ from those at the gill, *i.e.* Cu transport *via* Na channels and other Na-transport pathways may not be involved in the gut (Handy *et al.*, 2002). Copper is therefore probably taken up *via* different routes in the gut, and copper-transporting proteins such as high affinity copper transporter 1 (Ctr1) and divalent metal transporter 1 (DMT1) are possible candidates (Handy *et al.*, 2002; Bury *et al.*, 2003). These conclusions, however, may be relevant only in ion-rich hard water in which this study was conducted, where the compensatory ability of the gill may mask any effects. It would be useful to repeat the experiment in Cu-deficient, low Na soft water, where ion flux rates at the gills and Cu availability at the gills may be too low for compensation to occur.

TISSUE COPPER

As expected, fish fed high levels of Cu accrued more Cu in their tissues than control-Cu fish did. In both treatments, the liver accumulated the most Cu [Fig. 1(a)], although this percentage was substantially higher in Cu-exposed fish and increased over time in these fish. This was no surprise, as the liver is

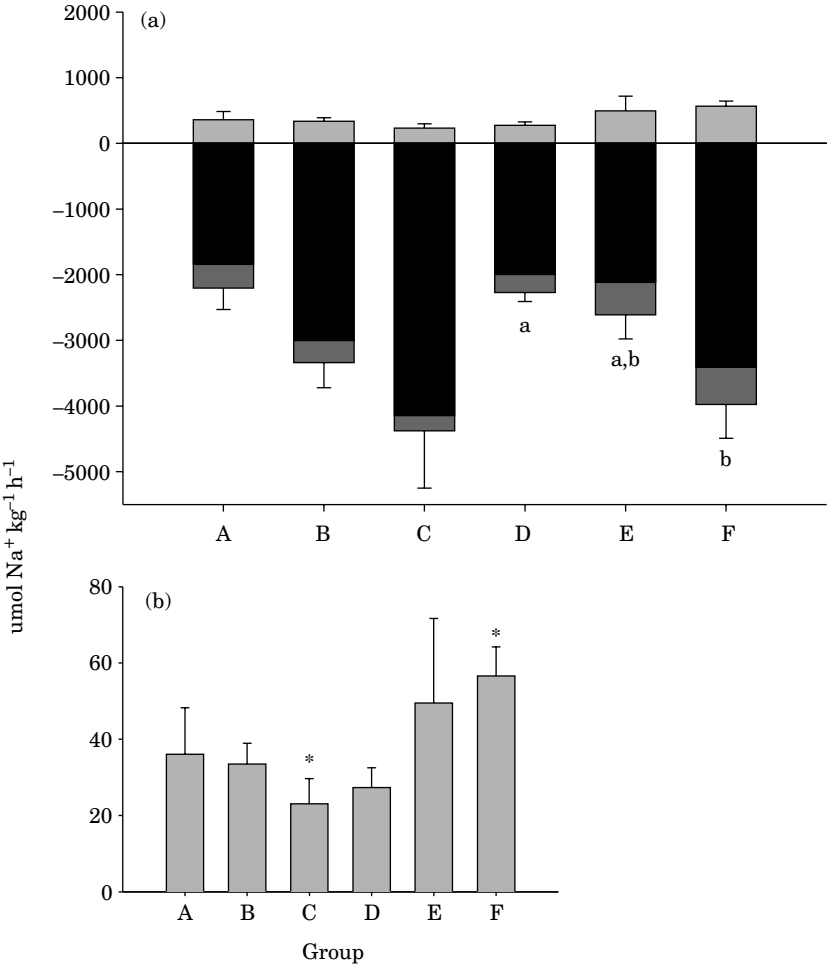

FIG. 4. (a) Mean + S.E. unidirectional Na influx (J_{in}; ▒, efflux (J_{out}; ▒)) and net flux rates (J_{net}; ■) of 10 juvenile rainbow trout per treatment group (A, Control Cu + 1·5% Na; B, Control Cu + 3% Na; C, Control Cu + 4·5% Na; D, High Cu + 1·5% Na; E, High Cu + 3% Na; F, High Cu + 4·5% Na) sampled on day 28. (b) J_{in} at a higher resolution. (a) Different lower case letters below bars indicate significantly different J_{out} values (ANOVA, $P < 0{\cdot}05$) within an exposure group. (b) *, significant differences between two groups (paired *t*-test, $P < 0{\cdot}05$).

considered the primary organ for maintenance of Cu homeostasis, and becomes a sink for excess Cu acquired through the diet (McGeer *et al.*, 2002; Kamunde & Wood, 2003, 2004). Differences in various tissue [Cu] showed no consistent patterns within treatment groups, suggesting that the supplementation of Na in the diet did not reduce Cu accretion in the tissues.

Kamunde *et al.* (2001) reported that the minimum dietary level of Cu that caused sustained significant accumulation in livers of juvenile rainbow trout at a 4% ration was $>300\,\mu g\,g^{-1}$. These authors also suggested that the uptake of dietary Cu may be less tightly regulated than waterborne Cu uptake, resulting in a continuous accumulation of Cu in the whole body or discrete tissues; thus

such an accumulation in the present experiment was expected in the high Cu-diet groups, which were fed $>500\,\mathrm{ug\,g^{-1}}$. While this was true in terms of absolute amounts of copper in the whole body, actual [Cu] did not consistently increase linearly over time in all tissues; rather, it remained relatively constant in gill and gut. This lack of Cu accumulation in certain tissues suggests that these organs were able to maintain Cu homeostasis (Berntssen *et al*., 1999*b*). The only tissue in which Cu did increase almost linearly over time was the liver, but only in those groups that had received additional Cu in the diet [Fig. 1(a)]. This again implicates the liver as a sink for excess Cu, and any ameliorative effects of dietary Na were expected to be evident in the liver. This was not the case.

In a review of dietborne Cu toxicity, Clearwater *et al.* (2002) noted that the patterns of Cu uptake and distribution in juvenile rainbow trout implicate the gut as the primary homeostatic organ for Cu. In another review of dietary metal uptake, Bury *et al.* (2003) reported that in the presence of excessive dietary levels, Cu is retained in the gut tissue and is prevented from entering other body compartments. Gut [Cu] should therefore be relatively high. Handy (1992) fed juvenile rainbow trout for 32 days with Cu-supplemented food ($200\,\mathrm{ug\,g^{-1}}$) and found that the fish accumulated 53% of the body Cu burden in the gut. In contrast, by day 28 in the present experiment, the gut and the liver contained roughly the same proportion of accumulated Cu, suggesting that the gut was not principally responsible for maintaining Cu homeostasis in these very young fish. The present data could reflect a sloughing of Cu-containing cells or increased rates of apoptosis, as described by Kamunde *et al.* (2001), which would also result in lower Cu accumulation in the gut.

From a study of Atlantic salmon *Salmo salar* L. parr, Berntssen *et al.* (1999*b*) concluded that dietary [Cu] would have to increase markedly over baseline levels to induce Cu accumulation in the liver. These authors (1999*a*, *b*) instead suggested that the intestine plays a strong regulatory role in relation to dietary metals. This again differs from the present findings in freshwater rainbow trout, where the liver accumulated the most Cu both in exposed and in non-exposed fish. These findings suggest that even during dietary exposures, the liver remains the principal internal sink for Cu, and by virtue of this storage ability, it allows the other organs to regulate their Cu levels. In general, the results are in accord with those of Handy *et al.* (1999) for fish fed a similar high Cu diet.

Copper retention did not appear to differ much among the control Cu groups, suggesting that dietary [Na] did not influence how much Cu was retained. In Cu-exposed groups, which were fed *c.* $\times 100$ more Cu than control fish, retention decreased some 25-fold to $<1{\cdot}0\%$ (Table I). Handy *et al.* (2000) suggested that declining absorption efficiency at higher Cu doses may function to protect the fish from absorbing excess Cu. Interestingly, among the Cu-exposed fish, the group that received the most dietary Na showed the highest Cu retention (Table I), and by day 28, the highest whole-body Cu [Fig. 1(d)], possibly reflecting positive interactions with Na. Overall, the Cu-retention values were only about half of those reported by Berntssen *et al.* (1999*a*) for Atlantic salmon fry. These authors found that control fish fed $5\,\mathrm{ug\,Cu\,g\,food^{-1}}$ retained *c.* 25%, whereas fish fed $500\,\mathrm{ug\,Cu\,g\,food^{-1}}$ retained *c.* $1{\cdot}1\%$. Neither study, however, measured Cu uptake from the water, which can account for anywhere from 1% to >60% of Cu accumulation by salmonids, depending on

its availability in the two media (Kamunde *et al.*, 2002; Kamunde & Wood, 2004).

TISSUE SODIUM

Sodium concentration did not differ much among groups or even among tissues within the same group, nor did it increase markedly over time, suggesting tight regulation of this macro-nutrient. The gut showed the lowest [Na], which was surprising given that this organ had the most immediate contact with dietary Na. This suggests fairly rapid mobilization from the gut into other tissues. Handy *et al.* (2002) reported a time course of <30 s for Na transfer from the gills to the blood; thus, it is plausible that [Na] transfer through the gut could be equally rapid. Alternatively, gut Na could have been lower than the other tissues as a result of having starved the fish for 36 h prior to sampling, as sampling after a period of starvation may not reveal the apparently transient changes in tissue Na levels that occur (Kamunde *et al.*, 2004). In contrast, Pyle *et al.* (2003) reported that the gut tissues of fish fed a 3% Na diet had significantly higher [Na] than tissues of control fish after only 7 days, including a 24 h starvation period prior to sampling. The highest dietary Na level was 4·5% in the present study, yet no marked increases in tissue [Na] were observed on any sampling day. The gill may be more important than the gut in Na homeostasis even in dietary exposures (Smith *et al.*, 1995; Kamunde *et al.*, 2003); this may explain the lack of accumulation in the gut observed during the present study.

In terms of overall whole-body Na burden, the carcass contributed the highest percentage by a very large margin. What is unclear is why the carcasses of fish in the high Na, high Cu group contained a significantly higher percentage of the whole-body Na burden than the other five groups [Fig. 2(b)]. The same pattern was observed under different water conditions (V.A. Kjoss, M. Grosell & C.M. Wood, unpubl. data), suggesting that the presence of Cu may facilitate Na movement into the muscle tissue under a high-Na feeding regime. It is also possible that an alteration of the muscle membrane potential caused by Cu exposure is involved (Beaumont *et al.*, 2000). Sodium retention did not appear to be affected by the amount of Cu in the diet, since high-Cu and control fish fed comparable amounts of Na had very similar retention values (Table I). Sodium regulation by the gill, however, was probably sufficient to mask any important influence of dietary Na on whole-body Na homeostasis.

UNIDIRECTIONAL Na FLUX RATES

A tendency was observed for greater negative net Na flux as a consequence of greater efflux in both Cu-exposed and unexposed fish (Fig. 4). This suggests that even at 36 h after their last meal, fish were eliminating excess Na taken up from the diet (Smith *et al.*, 1989, 1995). Wilson *et al.* (1985) suggested that excess dietary Na is either not assimilated or is readily excreted by rainbow trout. The present results support the latter hypothesis, as increasing efflux rates with higher levels of dietary Na were observed. This also corroborates the findings of Smith *et al.* (1995), who reported that Na efflux increased significantly in fish that were fed diets containing relatively high amounts of NaCl.

With one exception, the presence of Cu did not appear to affect Na influx or efflux. In pair-wise comparisons, the high Na, high Cu group showed significantly higher Na influx than its counterpart in the control Cu treatment. Although the explanation for this observation is unclear, it is consistent with higher Na retention in the fish in this group. Compared to efflux rates, however, these differences were negligible and suggest that when exposed to high levels of dietary Cu, fish were not consistently taking up Na in excess of the amounts taken up by control fish.

The lack of effect of dietary Cu on dietary Na transport suggests that dietary Cu did not impair Na homeostasis to a level that would warrant compensatory increase in uptake of dietary Na, either because the dose used was too low, or the mechanisms of dietary Cu toxicity do not entail interference with Na homeostasis as does waterborne Cu toxicity.

GROWTH AND CONDITION FACTORS

No consistent differences in G, E_{FC} or indices of body condition were evident among fish in different treatments. Even a relatively high concentration of Na (4·5% by mass) in the diet had no detrimental effects over 4 weeks. Furthermore, even at the relatively high Cu dose used in this experiment (*c.* 23 ug Cu g $fish^{-1}$ day^{-1}), dietary Cu did not negatively impact the growth and development of *O. mykiss* fry, at least over the short term. Overall, the results with regard to Cu are consistent with the analysis by Clearwater *et al.* (2002) that in rainbow trout, toxicity (*e.g.* growth inhibition) of dietary Cu occurs only at daily dosages of >35 ug Cu g $fish^{-1}$.

Lundebye *et al.* (1999) concluded that dietary exposure to Cu is less toxic than waterborne exposure, because the mucosal layer of the gut represents an effective barrier to metals. In addition, these authors suggested that the bioavailability of Cu is much lower in contaminated feed than in water although Clearwater *et al.* (2002) do not agree with these conclusions. Handy *et al.* (1999) found that dietary [Cu] of 500 ug g^{-1} caused only a minor reduction in mean growth rate after 3 months of exposure, and Kamunde *et al.* (2001) reported that dietary [Cu] of 1000 ug Cu g $food^{-1}$ produced no effects on growth, E_{FC}, or body condition of rainbow trout. Several researchers (McGeer *et al.*, 2002; Kamunde *et al.*, 2003) have therefore suggested that growth is a poor indicator by which to assess the effects of sublethal Cu exposure, although results from other studies have indicated that at higher doses, growth may be a useful indicator (Baker *et al.*, 1998; Lundebye *et al.*, 1999; Berntssen *et al.*, 1999*a*). With respect to dietary Na, Salman & Eddy (1988) reported that levels of 9·2 and 11·6% NaCl (*i.e.* 3·6 and 4·5% Na) supplementation in the diet adversely affected the growth of rainbow trout as a result of interference with protein and energy content of the diet. Although the highest level of dietary Na (4·5%) in the present study fell within this range, it clearly did not impact growth. Factors such as differences in growth stage, ration and food formulation may all contribute to differences among studies.

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