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Dietary sodium inhibits aqueous copper uptake in rainbow trout (*Oncorhynchus mykiss*)

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Summary

Ours is the first study to demonstrate an influence of dietary sodium on waterborne copper uptake in fish. We examined possible interactions between dietary sodium and the response of freshwater rainbow trout (*Oncorhynchus mykiss*) to waterborne copper in light of recent evidence of interactions between sodium and copper metabolism in the gills. Trout were maintained for 6 days on one of four diets of increasing sodium concentration (0.25 mmol g^{-1}, 0.51 mmol g^{-1}, 0.76 mmol g^{-1} and 1.27 mmol g^{-1}, which corresponds to 0.6%, 1.2%, 1.8% and 3% sodium by mass, respectively). At the end of 7 days, fish were exposed for 6 h to waterborne copper spiked with ^{64}Cu to determine if the dietary sodium affected responses to a subsequent short-term waterborne copper exposure. The radiotracer allowed us to distinguish between Cu occurring in fish tissues before the experiment and 'newly accumulated' Cu arising from the experimental exposure. Dietary sodium concentrations of 1.8% or 3% reduced newly accumulated copper concentrations in gill (from 93.9 ng g^{-1} in control to 38.9 ng g^{-1} and 20.0 ng g^{-1} in fish fed 1.8% or 3% Na^{+}-supplemented diets, respectively), liver (from 64.3 ng g^{-1} to 23.1 ng g^{-1} and 7.5 ng g^{-1}, respectively), kidney (from 29.3 ng g^{-1} to 11.7 ng g^{-1} and 7.8 ng g^{-1}, respectively), plasma (from 64.7 ng g^{-1} to 21.5 ng g^{-1} and 10.7 ng g^{-1}, respectively) and gut (from 6.8 ng g^{-1} to 3.4 ng g^{-1} and 2.2 ng g^{-1}, respectively) by 50.0–88.2%. The 3% Na^{+}-supplemented diets also increased plasma and gut sodium concentrations by 38.1% (from 137.1 μmol g^{-1} to 189.3 μmol g^{-1}) and 104.3% (from 56.5 μmol g^{-1} to 115.4 μmol g^{-1}), respectively, relative to fish maintained on untreated diets. Whole body uptake rates of both sodium and copper were significantly reduced, and highly correlated (r=0.97) with one another, in fish fed high-sodium diets relative to controls. Moreover, sodium efflux was 12% and 38% higher in fish fed 1.8% and 3% sodium-enriched diets, respectively. Fish fed high-sodium diets also drank more water, but the contribution of drinking to waterborne copper uptake was negligible. From these results, we speculate that, at least in part, aqueous sodium and copper share a common branchial uptake route, probably through an apical sodium channel. According to this hypothesis, as the channel is downregulated with increasing internal sodium concentrations, both sodium and copper uptake from the water are inhibited.

Key words: dietary sodium, aqueous copper uptake, fish, rainbow trout, *Oncorhynchus mykiss*.

Introduction

Although copper (Cu) is an essential nutrient for normal metabolic functioning (Mertz, 1981; Watanabe et al., 1997), it can be an important waterborne toxicant to aquatic organisms, particularly fish, when ambient concentrations exceed physiological thresholds (Wilson and Taylor, 1993; Taylor et al., 2000). Many studies have demonstrated a modifying influence of water quality on Cu toxicity to fish. For example, pH affects Cu speciation, which in turn affects bioavailability (Cusimano et al., 1985); calcium (Ca^{2+}) associated with water hardness tends to reduce toxicity by competitively inhibiting Cu binding to fish gills (Pagenkopf, 1983; Laurén and McDonald, 1986; Playle et al., 1992; Erickson et al., 1996); and increasing concentrations of dissolved organic matter sequester waterborne Cu from biological uptake (Playle et al., 1993; Hollis et al., 1997). Although much is known about the modifying factors associated with water quality, almost nothing is known about the effects of diet quality on the response of fish to waterborne Cu.

The primary mechanism of Cu toxicity to fish results from the combined effects of a reduction in sodium (Na^{+}) influx and an increase in Na^{+} efflux, giving rise to a net reduction of

plasma and whole body Na^+ (Laurén and McDonald, 1986, 1987b; Reid and McDonald, 1991). Reduced Na^+ influx is thought to be associated with non-competitive binding of Cu ions to the basolateral Na^+-pump, Na^+/K^+-ATPase, resulting in lower Na^+ uptake rates into the blood (Laurén and McDonald, 1987a; Pelgrom et al., 1995; Li et al., 1996, 1998). Massive 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^+ (Laurén and McDonald, 1986; Evans, 1987; McDonald and Wood, 1993). The result of this net loss of Na^+ is an increase in blood viscosity and blood pressure, a compensatory tachycardia and, under acutely toxic conditions, cardiac failure (Wilson and Taylor, 1993).

The etiology of waterborne Cu toxicity to freshwater fish, as described above, is similar to that demonstrated in fish exposed to acidic water (Milligan and Wood, 1982; McDonald, 1983; McDonald and Prior, 1988; McDonald et al., 1989a,b). Under low pH conditions, fish demonstrate a similar ionoregulatory disturbance that leads to a net reduction in whole body Na^+. Sadler and Lynam (1987) first suggested that a pH-induced ionoregulatory disturbance may be ameliorated by making use of dietary ions. Studies by Dockray et al. (1996), Wilson et al. (1996) and D'Cruz et al. (1998) implicated an ameliorative role of diet because satiation-fed fish exposed to acidic water demonstrated little or no stereotypical ionoregulatory disturbances when exposed to acidic water, contrary to findings in other studies where fish were maintained on limited (or no) rations. Moreover, acid-exposed fish had greater appetites relative to fish maintained under circumneutral conditions (Dockray et al., 1996). These results prompted a subsequent study that identified dietary Na^+ content, rather than dietary energy content, as the key component that reduced ionoregulatory disturbances in acid-exposed fish (D'Cruz and Wood, 1998).

It seems from these studies that dietary Na^+ plays some role in reducing stereotypical ionoregulatory disturbances in fish exposed to acidic water. The purpose of the present study was to determine if dietary Na^+ plays a similar type of protective role in rainbow trout (*Oncorhynchus mykiss*) exposed to waterborne Cu and, if so, to understand the mechanism(s) involved. This was achieved by feeding fish increasing concentrations of dietary Na^+ for one week, then challenging them with a short-term (6 h), sublethal exposure to waterborne Cu ($20\,\mu g\,l^{-1}$) to study the effect of dietary Na^+ on Cu uptake, distribution and effect on ionoregulatory processes such as Na^+ flux, Na^+/K^+-ATPase activity and drinking rates.

Materials and methods

Acclimation

Rainbow trout (*Oncorhynchus mykiss* Walbaum) were purchased from Humber Springs Fish Hatchery (Orangeville, Ontario, Canada). Fish were held in a 600-liter polypropylene tank supplied with aerated dechlorinated tapwater from Hamilton, Ontario ($[Na^+]$=0.6 mmol l^{-1}, $[Ca^{2+}]$=1.02 mmol l^{-1}, hardness=120 mg l^{-1} as $CaCO_3$, pH=7.6–8.0, background Cu concentration=3 $\mu g\,l^{-1}$, temperature=12–14°C) at a rate of approximately 1 l min^{-1}. Fish were fed Corey Hatchery Feed (Corey Feed Mills, Ltd, Fredericton, NB, Canada; ionic composition given below) at a rate of 2% total fish mass daily. Bulk fish masses, composed of a subsample of approximately 30 fish, were monitored weekly and ration was adjusted accordingly. Photoperiod was maintained at 12 h:12 h light:dark. After two weeks under these conditions, fish were gradually acclimated to soft water by mixing reverse osmosis water with dechlorinated Hamilton tapwater to achieve a final mixture of approximately 6:1 tapwater:reverse osmosis water. The final composition of the water at the end of the acclimation period was: $[Na^+]$=0.1 mmol l^{-1}, $[Ca^{2+}]$=0.1 mmol l^{-1}, hardness=16 mg l^{-1} as $CaCO_3$, pH=6.9–7.1, background Cu concentration=1.2 $\mu g\,l^{-1}$, and temperature remained constant between 12°C and 14°C. Fish were held under these conditions for at least two months prior to experimentation. All subsequent experiments were conducted in this reconstituted soft water.

Experimental design

Four experiments were conducted during this study: (1) to determine the effect of dietary Na^+ on subsequent waterborne Cu uptake; (2) to determine the effect of dietary Na^+ on whole body Na^+ concentrations and subsequent aqueous Na^+ uptake; (3) to determine the effect of dietary Na^+ on the simultaneous appearance of newly accumulated Cu and Na^+ in the gill and on Na^+/K^+-ATPase activity in gill tissue; and (4) to determine the effect of feeding and dietary Na^+ on drinking rate.

In the first two experiments, five fish (mass 9–12 g) were randomly assigned to each of four 20-liter experimental tanks, where they were held for 7 days. Each of these tanks was supplied with approximately 100 ml min^{-1} of aerated, reconstituted soft water (see above). During the first 6 days of the 7-day exposure period, fish in each tank were fed at a rate of 3% total fish mass per day. Fish were not fed during the final 24 h. Fish in each tank received a single diet, where each diet ranged from 0.6% (control) to 3% Na^+ by mass (see 'Diet preparation' below). Virtually all of the food provided was eaten within the first few minutes. Uneaten food and feces were siphoned from each tank 20 min after feeding. This cleaning regimen, in addition to the flow-through experimental design, ensured that excess Na^+ from Na^+-supplemented diets did not accumulate in the water.

In these first two experiments, at the end of 7 days, fish were transferred for six hours to 6-liter plastic flux chambers that contained either 20 μg Cu l^{-1} in vigorously aerated, reconstituted soft water spiked with 55.5 MBq l^{-1} ^{64}Cu (first experiment) or reconstituted soft water spiked with 0.93 kBq l^{-1} ^{22}Na (second experiment; see 'Copper and sodium fluxes' below). In neither case did the addition of radiotracer significantly change the Cu or Na^+ concentrations in flux chambers. At the end of the 6 h flux in each experiment, fish were sacrificed by an overdose of MS-222 and dissected to separate tissues (see 'Sampling and analysis' below).

In the third experiment, 72 fish (mass 60–105 g) were

randomly assigned to two 150-liter polypropylene tanks (i.e. 36 fish per tank). One tank received a normal diet (i.e. 3% total fish mass per day, untreated trout food), while the other tank received a 3% Na^+-supplemented diet at the same feeding rate. Fish were maintained under these conditions for seven days. On day eight (i.e. after a 24 h starvation period), 5–6 fish from each of the control and Na^+-diet exposed groups were moved into 20-liter plastic containers to create four waterborne Cu and feeding treatments, namely 'Fed', 'Fed+Cu', 'Na Fed' and 'Na Fed+Cu'. Copper treatment comprised 20 $\mu g\,l^{-1}$ labeled with ^{64}Cu (55.5 $MBq\,l^{-1}$, $CuNO_3$) for 6 h. In addition, water for all the groups was spiked with ^{22}Na (0.93 $kBq\,l^{-1}$). Simultaneous exposure to ^{64}Cu and ^{22}Na allowed for the measurement of newly accumulated Cu and Na^+ in the gill. Total Cu was also measured in gills. For each treatment group, in addition to the samples for radioisotope counting, a subsample of the gill (two middle gill arches) was dissected out and immediately frozen in liquid nitrogen for Na^+/K^+-ATPase activity analysis (see 'Na^+/K^+-ATPase activities' below).

In the fourth experiment, drinking rates were determined in three groups of fish (mass 60–105 g), namely 'Unfed control', 'Fed control' and 'Na-diet fed' (N=5–6) in the absence of waterborne Cu (see 'Drinking rates' below). For this experiment, fish were moved into flux tanks a day before the experiment, and feeding took place in the flux tanks in the presence of the drinking rate marker ([^{3}H]PEG-4000).

Diet preparation

All diets were prepared with granulated hatchery feed that had been ground to a powder {Corey Feed Mills, Ltd; manufacturer's specifications: [Na]=0.3 $mmol\,g^{-1}$ (6 $mg\,g^{-1}$; i.e. 0.6%); [P]=0.4 $mmol\,g^{-1}$ (11 $mg\,g^{-1}$); [Cu]=17.3 $\mu g\,g^{-1}$; crude protein=55%; crude fat=17%; crude fibre=2%}. Analytical grade NaCl was dissolved in 40% v/w distilled, deionized water and mixed into a pre-weighed sample of fish food to yield diets with 0.6% (control, no NaCl added but subjected to the same treatment as other diets), 1.2%, 1.8% and 3% Na^+ by mass. The resulting paste was extruded through a pasta maker, air-dried and broken into smaller pellets by hand. This method gave Na^+ concentrations that were very close to nominal values. Actual measured Na^+ concentrations in the four diets were: 0.25 $mmol\,g^{-1}$ (0.6%), 0.51 $mmol\,g^{-1}$ (1.2%), 0.76 $mmol\,g^{-1}$ (1.8%) and 1.27 $mmol\,g^{-1}$ (3%).

Copper and sodium fluxes

Fish were exposed to radioactive Na^+ or Cu, as ^{22}Na ($t_{1/2}$=31.2 months; Amersham Pharmacia Biotech, Inc., Piscataway, NJ, USA) or ^{64}Cu (prepared at McMaster University Nuclear Reactor from $CuNO_3$, $t_{1/2}$=12.65 h), respectively. The use of radioisotopes allowed us to discriminate between newly accumulated Na^+ or Cu taken up by the fish during an experimental exposure from Na^+ or Cu already occurring in fish tissues before exposure to elevated (experimental) dietary Na^+ or waterborne Cu concentrations. Consequently, specific radioactivity corresponding to the Na^+ or Cu isotope in fish tissues after experimental exposures represents 'newly accumulated' Na^+ or Cu. Newly accumulated Cu or Na^+ was calculated by the following equation (Grosell et al., 1997):

$$M_{\mathrm{New}} = \frac{a}{\left(\frac{b}{c}\right)}, \qquad (1)$$

where M_{New} is the newly accumulated Cu or Na^+ concentration (measured in $ng\,g^{-1}$ or $\mu mol\,g^{-1}$, respectively), a is the number of γ-emissions per minute (i.e. c.p.m.) per gram of tissue or per liter as appropriate, b is the number of γ-emissions (c.p.m.) per liter of water, and c is the total Cu or Na^+ concentration of the water. Recent studies in our laboratory have demonstrated that ^{64}Cu uptake into fish tissues is linear for up to 12 h (Kamunde et al., 2001). Therefore, because Cu exposures in the present study were only 6 h in duration, uptake was linear with time. Vigorous aeration throughout the flux period ensured thorough mixing.

Unidirectional Na^+ and Cu uptake rates were determined by summing the uptake into all the individual tissues and dividing the result by the specific radioactivity in the environment, the fish's mass (in kg) and the length of the exposure period (6 h) to convert to a rate. Net Na^+ flux rates were calculated from the changes in total water Na^+ over the flux period by analyzing water samples taken 15 min after the start of the flux and at the end of the 6 h flux period for Na^+, as described in 'Sampling and analysis' below. Sodium efflux was calculated from the difference between net Na^+ flux and influx rates.

Sampling and analysis

In the first two experiments, gills, liver, kidney, gut [esophagus to rectum, rinsed in deionized water (18 mΩ Nanopure II, Sybron/Barnstead, Boston, MA, USA) to remove any partially digested food], plasma and carcass were dissected from fish. Gill sampling involved the removal of entire gill baskets, because the volume of cartilaginous material was small and it was impractical to separate it out in these juvenile fish. Whole blood was collected by caudal puncture using 1 ml heparinized syringes fitted with 23-gauge needles. Blood samples were immediately centrifuged at 10 000 $\boldsymbol{g}$ for 5 min to separate cellular material from plasma. Separated plasma was decanted from the cellular material and used in subsequent analyses. 10 ml water samples were collected from each flux chamber [one at the beginning (15 min) and the other at the end of the flux (6 h)] in all three experiments and acidified with 100 µl concentrated HNO_3 (trace metal grade, Fisher Scientific, Nepean, Ontario).

Whole body metal concentrations were calculated according to the following equation:

$$WB = \frac{\sum_{i=1}^{6} C_i m_i}{m_{\mathrm{WB}}}, \qquad (2)$$

where *WB* is the whole body Cu or Na^+ concentration in ng g^{-1} or µmol g^{-1}, respectively, i=1–6 represents individual tissues (gill, liver, kidney, plasma, gut and carcass) within a single fish, C_i is the concentration of either Cu (ng g^{-1}) or Na^+ (µmol g^{-1}) in tissue i, m is the mass (g) of tissue i, and m_{WB} is the total mass (g) of the fish.

For Na^+/K^+-ATPase activities determined in the third experiment, only gill tissue was analyzed. Rather than entire gill baskets as in the previous two experiments, two middle gill arches per fish were used. Gill filaments were immediately frozen in liquid nitrogen for subsequent analyses.

Radioactivity in tissue and water samples containing ^{64}Cu and ^{22}Na was measured on a Canberra-Packard Minaxi Auto-Gamma 5000 series gamma counter with on-board automatic decay correction for ^{64}Cu (Canberra-Packard Instruments, Meriden, CT, USA). In the third experiment, where fish were exposed to ^{64}Cu and ^{22}Na simultaneously, samples were counted immediately after dissection and were then stored for two weeks to allow the ^{64}Cu to decay to undetectable levels. Samples were then recounted for ^{22}Na and decay-corrected accordingly. The difference between the first and second count provided a measure of ^{64}Cu activity. Tissue and water [^{3}H]PEG-4000 activity was counted on a liquid scintillation counter (LKB Wallac 1217 Rackbeta; Pharmacia-LKB AB, Helsinki, Finland) using internal standardization.

After tissues (except plasma and water samples) were counted, they were digested in five volumes of 1 mol l^{-1} HNO_3 (trace metals grade; Fisher Scientific) at 70°C for 24 h and subsequently centrifuged for 5 min at 10 000 ***g***. A subsample of the supernatant (or whole plasma or water sample) was diluted appropriately in 0.5% HNO_3. Total Cu concentrations were measured by graphite furnace atomic absorption spectrophotometry (GFAAS; Varian 1275 AA with GTA-95 atomizer; Mississauga, Ontario) using a 10 µl injection volume and operating conditions as suggested by the manufacturer for Cu. Total Na^+ concentrations were measured using flame atomic absorption spectrophotometry (FAAS; Varian 1275). Certified analytical standards (National Research Council of Canada) analyzed simultaneously with experimental samples were within the specified range.

Na^+/K^+-ATPase activities

Na^+/K^+-ATPase activities were determined for two gill arches from fish after 7 days of exposure to dietary Na followed by 6 h of acute exposure to waterborne Cu (20 µg l^{-1}) using a slightly modified version of the microplate UV detection method described in McCormick (1993). Samples were frozen immediately in liquid nitrogen and subsequently stored at –70°C until analyzed for Na^+/K^+-ATPase activity. In this assay, the rate of hydrolysis of ATP to ADP in the presence and absence of oubain (Sigma, St Louis, MO, USA) was coupled to the oxidation of NADH to NAD^+. Changes in absorbance of the reaction mixture due to NADH oxidation were measured at 340 nm over 15 s intervals for 10 min. Na^+/K^+-ATPase activity was calculated as the difference in ATP hydrolysis in the absence and presence of oubain and normalized to total protein in each respective sample as determined by the Bradford (1976) method.

Drinking rates

Drinking rates were measured by the method of Wilson et al. (1996) in unfed controls, fed controls and in fish fed dietary Na^+ for 7 days. Fish were exposed to [^{3}H]PEG-4000 at a concentration of 185 MBq l^{-1} in the water for 6 h. This is less than the period of time (10 h) by which the tracer reaches the anus at this temperature (C. M. Wood, unpublished results). Water samples (10 ml) were taken 15 min after addition of [^{3}H]PEG-4000 and again after the 6 h exposure. Fish were then killed with an overdose of MS-222. The entire gastrointestinal tract was exposed by dissection, ligated at the esophagus and rectum, removed and homogenized in five volumes of 8% $HClO_3$. Homogenate was processed for scintillation counting according to Wilson et al. (1996), and a 1 ml sample was counted. Plasma was also counted to ascertain that the [^{3}H]PEG-4000 had been absorbed.

Drinking rate was calculated using the equation:

$$D = \frac{C}{MtW} \,, \qquad (3)$$

where D is the drinking rate in ml kg^{-1} h^{-1}, C is the number of counts (c.p.m.) in the entire gut, M represents counts (c.p.m.) per ml water, t is the time in hours, and W is the mass of the fish in kg.

Statistical treatment

All data are reported as means ± S.E.M. and were compared using analysis of variance (ANOVA). In cases where data did not meet normality or homogeneity of variance assumptions for ANOVA, significant differences were determined using a nonparametric Kruskall–Wallis rank sum test. Mean Na^+ and Cu concentrations in tissues of fish exposed to Na^+-supplemented diets in the first two experiments were compared with those of fish fed the control diet (i.e. normal, untreated trout food) using Dunnett's test. Mean Na^+/K^+-ATPase activities and mean drinking rates were compared among experimental treatments using Tukey–Kramer's honestly significant difference test. Mean differences were considered to be significant when P<0.05.

Results

Copper uptake

Copper uptake rates were significantly lower in rainbow trout fed 1.8% and 3% Na^+-enriched diets relative to controls (Fig. 1). Fish fed the 1.8% or 3% Na^+-supplemented diet took up waterborne Cu at a 52.9% or 75.0% lower rate, respectively, than fish fed the control diet.

Fish fed Na^+-supplemented diets of 1.8% Na^+ accumulated significantly less new Cu (as defined by equation 1) in a 6 h Cu flux period at 20 µg Cu l^{-1} than fish fed the control diet (0.6% Na^+) in gill, liver and gut tissues (Fig. 2). New Cu uptake into kidney and plasma was reduced, but not

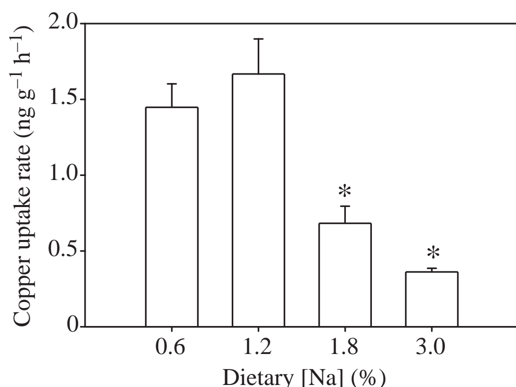

Fig. 1. New copper uptake rate (as defined by equation 1) into rainbow trout fed for 7 days on diets ranging in sodium concentration after a subsequent 6 h exposure to 20 μg l^{-1} of waterborne copper. Bars represent means ± S.E.M., N=4–5. Asterisks represent significant difference from control (0.6%) diet (P<0.05).

significantly different from fish fed the control diet. Similarly, fish fed the 3% Na^+ diet accumulated substantially and significantly less new Cu in gills, liver, kidney, plasma and gut relative to fish fed the control diet (in all cases P<0.05, N=4–5). Fish fed the 1.2% Na^+ diet accumulated a similar amount of new Cu relative to those fed the control diet (P>0.05), showing that the threshold for effect lay between 1.2% and 1.8% dietary Na^+.

Based on GFAAS analysis of total tissue Cu concentrations, only gill and liver tissues showed significantly lower total Cu concentrations in fish fed diets supplemented with 1.8% or 3% Na^+ relative to those fed the control diet (Fig. 3). Over the 7 day exposure, fish fed the 3% Na^+ diet exhibited a 25.1% reduction of Cu in the gills and a 44.5% reduction of Cu in the liver, relative to fish fed the control diet. In fish fed the 1.8% Na^+ diet, only the 36.6% reduction in total liver Cu burden was significant. Neither kidney, plasma, gut nor carcass showed elevated total Cu relative to controls in any of the Na^+-supplemented diet treatments (P>0.05).

Sodium uptake

After 7 days of the experimental feeding regime, total Na^+ concentrations were significantly higher in gut tissue and plasma of fish fed the 3% Na^+ diet relative to fish fed the control diet (Fig. 4). There were no significant differences in other tissues or at lower dietary Na^+ concentrations. As with waterborne Cu uptake rates, fish fed either 1.8% or 3% Na^+-supplemented diets demonstrated a significantly lower waterborne Na^+ uptake rate relative to fish maintained on the control diet (Fig. 5; P=0.02, N=4–5). Fish fed the 1.8% or 3% Na^+-supplemented diets took up waterborne Na^+ 40.8% or 44.0% slower than fish fed the control diet. Waterborne Na^+ and Cu uptake rates were strongly and positively correlated with one another (r=0.97, P=0.02, N=4–5). Na^+ efflux rates were also elevated in proportion to dietary Na^+ load, although these differences could not be evaluated statistically because they were measured on whole treatment groups, not individuals. Branchial Na^+ efflux rates were –0.41 μmol g^{-1} h^{-1}, –0.45 μmol g^{-1} h^{-1}, –0.47 μmol g^{-1} h^{-1} and –0.57 μmol g^{-1} h^{-1} in fish fed control (0.6%), 1.2%, 1.8% and 3% Na^+ diets, resulting in negative net Na^+ flux rates in the 1.8% and 3% Na^+ treatment groups.

Na^+/K^+-ATPase activities

Na^+/K^+-ATPase activities in gill filaments (Fig. 6) varied significantly among experimental treatments. Generally, gills of fish exposed to waterborne Cu had lower Na^+/K^+-ATPase activities than those that were not exposed to Cu. Moreover, fish fed Na^+-supplemented diets showed higher gill filament Na^+/K^+-ATPase activity than those fed regular diets. Consequently, fish that were fed a Na^+-supplemented diet and were exposed to waterborne Cu showed gill Na^+/K^+-ATPase activity that was not significantly different from control fish (i.e. Fed in Fig. 6, representing normal diet and no waterborne Cu).

Newly accumulated Na^+ and Cu in the gills

Newly accumulated gill Na^+ and Cu varied among experimental treatments (Fig. 7). Gills of fish from the Na Fed+Cu treatment accumulated less than one-third the amount of new Na^+ than those from the Fed+Cu treatment (Fig. 7A; P<0.05, N=5–6). However, new gill Na^+ did not vary between fish from Fed and Fed+Cu treatments, nor between Fed and Na Fed + Cu treatments. Gills of Fed fish accumulated most new Cu,

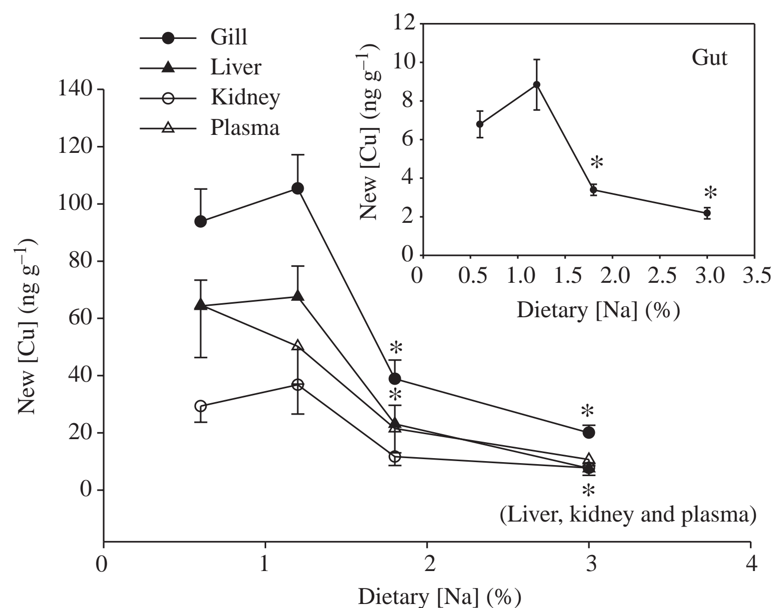

Fig. 2. Newly accumulated copper (as defined by equation 1) in gills, liver, kidney, blood plasma and gut (inset) of rainbow trout fed for 7 days on diets ranging in sodium concentration after a subsequent 6 h exposure to 20 μg l^{-1} of waterborne copper. Points represent means ± S.E.M., N=4–5. Asterisks represent significant difference from control (0.6%) diet (P<0.05).

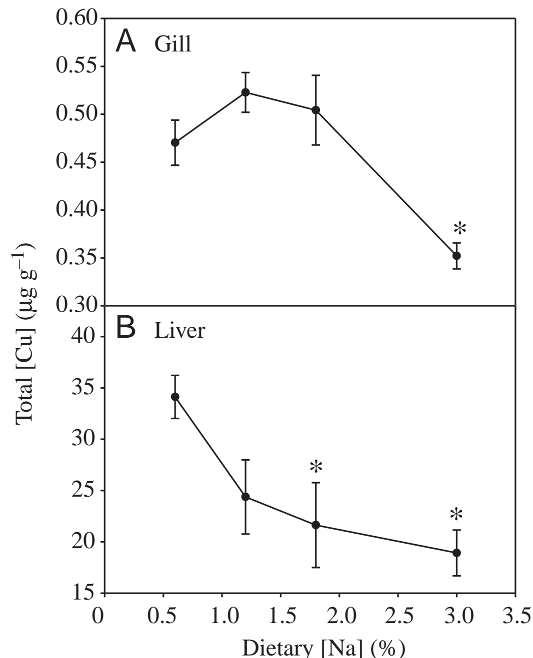

Fig. 3. Total copper concentrations in gills (A) and livers (B) of rainbow trout fed for 7 days on diets ranging in sodium concentration after a subsequent 6 h exposure to 20 µg l^{-1} of waterborne copper. Points represent means ± S.E.M., N=4–5. Asterisks represent significant difference from control (0.6%) diet (P<0.05).

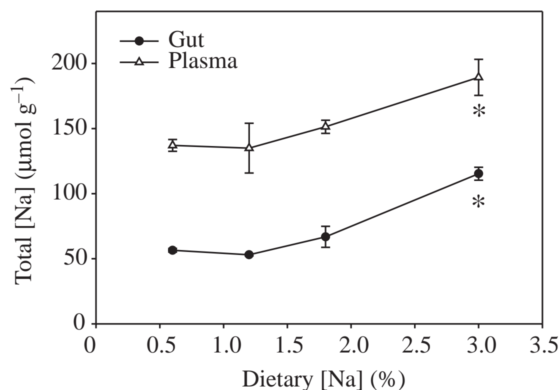

Fig. 4. Total sodium concentrations in gut tissue and plasma of rainbow trout fed for 7 days on diets ranging in sodium concentration after a subsequent 6 h exposure to 20 µg l^{-1} of waterborne copper. Points represent means ± S.E.M., N=4–5. Asterisks represent significant difference from control (0.6%) diet (P<0.05).

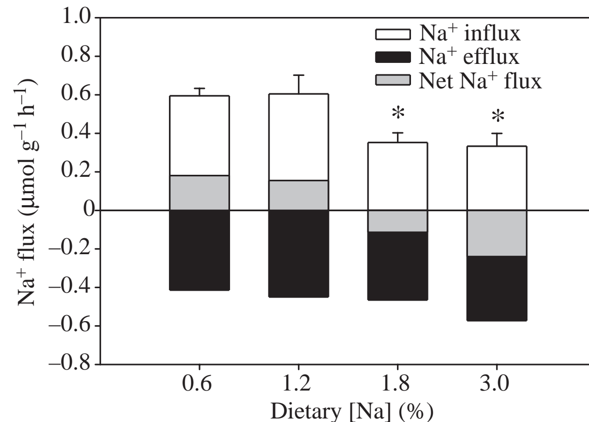

Fig. 5. Sodium influx, efflux and net flux rates into juvenile rainbow trout fed for 7 days on diets ranging in sodium concentration after a subsequent 6 h exposure to 20 µg l^{-1} of waterborne copper. Sodium influx rates were determined on individual fish, which facilitated the calculation of means and S.E.M. (bars; N=4–5) and statistical comparisons (asterisks indicate statistical significance at P<0.05). However, efflux rates were determined on groups of fish that precluded statistical comparisons among treatment groups for efflux or net flux data.

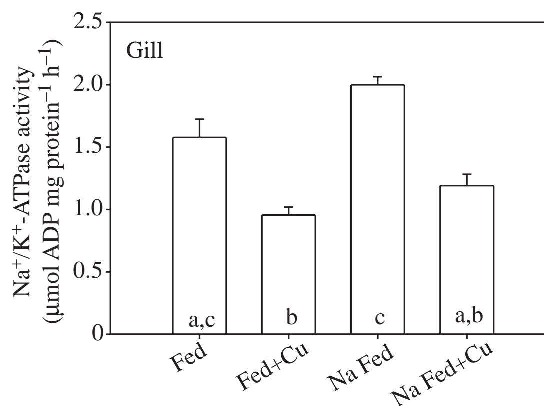

Fig. 6. Branchial Na^{+}/K^{+}-ATPase activity in rainbow trout exposed to different feeding regimes and waterborne copper concentrations. Experimental treatments included feeding fish 3% of their total body mass per day on untreated food ('Fed') or feeding fish 3% of their total body mass per day on food that was supplemented with 3% sodium by mass ('Na Fed'). Fish were then exposed to either no copper in the water or to 20 µg l^{-1} of dissolved copper (Cu) for 6 h. Bars represent means ± S.E.M., N=5–6, bars sharing the same letter are not significantly different from one another (P>0.05).

which was not significantly different from those of Fed+Cu treatment (P>0.05) but was 59.6% higher than in gills of fish from the Na Fed+Cu treatment (Fig. 7B; P<0.05, N=5–6).

Drinking rates

Mean drinking rates were low and never exceeded 2 ml kg^{-1} h^{-1} but still varied by treatment (Fig. 8). Drinking rates were not significantly different between fed and unfed fish (P>0.05). However, they did significantly differ between fish fed a normal diet (i.e. Fed) and those fed a 3% Na^{+}-supplemented diet (P<0.05, N=5). Fish fed the Na^{+}-supplemented diet demonstrated the highest drinking rates, which were 58.8% greater than in unfed fish.

Discussion

Ours is the first study to demonstrate an influence of dietary Na^{+} on waterborne Cu uptake in fish. Results reported here demonstrate that fish exposed to elevated dietary Na^{+} take up

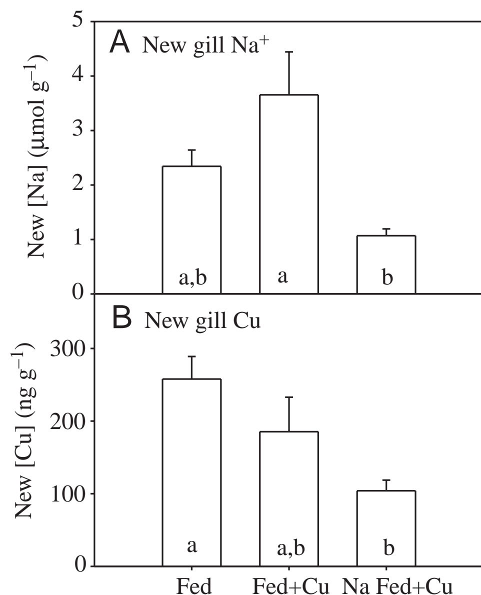

Fig. 7. New sodium (A) and copper (B) accumulation (as defined by equation 1) in gills of rainbow trout exposed to a normal diet and no waterborne Cu ('Fed'), a normal diet and 20 µg l^{-1} waterborne Cu ('Fed+Cu') or a diet supplemented with 3% Na^+ by mass and 20 µg l^{-1} waterborne Cu ('Na Fed+Cu'). Bars represent means ± S.E.M., N=5–6, bars sharing the same letter are not significantly different from one another (P>0.05).

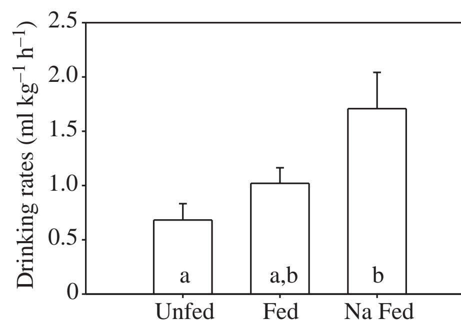

Fig. 8. Drinking rates in rainbow trout that were either starved ('Unfed'), fed 3% of their total mass on a normal diet ('Fed') or fed 3% of their total mass on a diet supplemented with 3% Na^+ by mass ('Na Fed') for 7 days. Bars represent means ± S.E.M., N=5. Bars sharing the same letter are not significantly different from one another (P>0.05).

significantly less waterborne Cu into their tissues, and at a slower rate, than fish maintained on a control diet.

Previous studies have shown that a dietary source of Na^+ can be just as important as a waterborne source for meeting physiological requirements in rainbow trout (Smith et al., 1989, 1995; D'Cruz and Wood, 1998). Almost 100% of the Na^+ taken up from the diet is absorbed though the gut and taken up into the plasma (Smith et al., 1995). Fish can lose Na^+ through their gills, liver (*via* biliary excretion) and kidneys, although Na^+ loss through the gills is much more important than other routes (Smith et al., 1989). Fish maintain Na^+ homeostasis by modulating influx and efflux, primarily at the gills, as appropriate. Once plasma concentrations are elevated beyond the needs of the fish, branchial Na^+ efflux is stimulated and influx is inhibited to ensure that electrolyte balance is maintained (Salman and Eddy 1987).

Fish can modulate branchial Na^+ influx by changing the activities of Na^+/K^+-ATPase in the basolateral membrane and the proton pump, H^+-ATPase, in the apical membrane (McCormick, 1995; Lin and Randall, 1995; Karnaky, 1997). Na^+/K^+-ATPase extrudes intracellular Na^+ from branchial epithelium into the blood, while H^+-ATPase in the apical membrane pumps protons out of the cell, which increases the electrochemical gradient between the external and internal environments, thereby creating conditions that favor waterborne Na^+ influx (Lin and Randall, 1995). Sodium efflux, on the other hand, is primarily diffusive and is modulated by changes in internal concentration, in transepithelial potential (and therefore in electrochemical gradient) and, most importantly, in gill permeability (McDonald and Prior, 1988; McDonald et al., 1989b). The latter may reflect changes in both transcellular and paracellular pathways.

Results from this study suggest that waterborne Cu uptake is strongly associated with waterborne Na^+ uptake and is therefore influenced by the same ionoregulatory mechanisms that control Na^+ homeostasis. Our results reveal that branchial Na^+ uptake was inhibited with increasing dietary Na^+ concentrations. This was apparent in the low branchial Na^+ uptake rates in fish fed diets containing 1.8% or 3% Na^+ (Fig. 5), which corresponds well with other studies investigating the ionoregulatory effects of dietary Na^+ (Salman and Eddy, 1987; Smith et al., 1995). Branchial Na^+ uptake was probably inhibited in these fish as a response to elevated plasma Na^+ concentrations (Fig. 4). At the same time, branchial Cu uptake was also inhibited in fish fed 1.8% or 3% Na^+ diets (Fig. 1). Moreover, branchial uptake rates for waterborne Na^+ and Cu were strongly and positively correlated with one another (r=0.97, P=0.02). Further evidence supporting a close relationship between aqueous Na^+ and Cu uptake is shown in Fig. 7, where Cu-exposed fish fed Na^+-supplemented diets accumulated significantly less branchial Na^+ and Cu than those fed normal diets. Therefore, the evidence collected in this study suggests that dietary Na^+ inhibits branchial Cu uptake. In fact, dietary Na^+ was such an effective branchial Cu uptake blocker that gills, livers, kidneys, plasma and guts of fish fed Na^+-enriched diets accumulated 50.0–88.2% less new Cu than fish maintained on a normal diet (Fig. 2).

Our results were based on food treated with NaCl in order to increase dietary Na^+ concentrations. We cannot rule out the possibility that reductions in branchial Cu uptake could be linked to dietary Cl^-. One possible way to determine if dietary Cl^- plays a role in reducing brachial Cu uptake is to repeat our experiments by supplementing food with a different salt, such as $NaSO_4$. However, given recent evidence reported by Grosell and Wood (2002) demonstrating a common branchial Na^+–Cu

uptake channel, we strongly suspect dietary Na^+, not Cl^-, inhibits branchial Cu uptake.

Radiolabeled ^{64}Cu was used in this study to distinguish between Cu taken up by fish during the 6 h exposure to elevated aqueous Cu (i.e. new Cu) and background Cu that occurred in tissues even before fish were exposed to elevated waterborne Cu (i.e. total Cu minus new Cu). An interesting effect of dietary Na^+ in these fish was the significant reduction of total Cu in gills of fish fed 3% Na^+-supplemented diets for the preceding 6 days, and in livers of fish fed either 1.8% or 3% Na^+-supplemented diets (Fig. 3). Newly accumulated Cu in gills of fish fed the 3% Na^+ diet accounted for only 5% of total gill Cu, whereas new Cu accounted for 0.11% and 0.04% of total Cu in livers of fish fed 1.8% and 3% Na^+ diets, respectively. Therefore, new Cu accounted for only a small fraction of the total Cu load, especially in livers. The significantly lower total gill and liver Cu concentrations in fish fed high-Na^+ diets suggest not only that normal Cu uptake was inhibited throughout the duration of the 6 day Na^+-diet feeding period, even before fish were exposed to elevated waterborne Cu in the 6 h flux, but also that net Cu efflux may have been stimulated in the Na^+-fed fish.

In addition to a reduction in branchial Na^+ influx, fish fed Na^+-supplemented diets also demonstrated high Na^+ efflux in order to maintain electrolyte balance. Sodium efflux rates were 12% and 38% higher in fish maintained on 1.8% and 3% Na^+-supplemented diets, respectively, resulting in negative net flux rates relative to fish maintained on the control diet. This result corroborates other studies that have demonstrated an increase in Na^+ efflux in fish maintained on Na^+-supplemented diets (Smith et al., 1995).

One of the ways that waterborne Cu causes ionoregulatory disturbances in fish is *via* non-competitive inhibition of branchial Na^+/K^+-ATPase activity (Laurén and McDonald, 1987a; Sola et al., 1995; Pelgrom et al., 1995; Li et al., 1998). Intracellular Cu competes with Mg^{2+} for binding sites on the ATP molecule and forms a physiologically inert ATP complex, Cu–ATP (Li et al., 1996). Normal functioning of Na^+/K^+-ATPase requires Mg–ATP. Once the basolateral Na^+/K^+-ATPase is inhibited by Cu, the branchial cell can no longer extrude intracellular Na^+ into the blood (i.e. influx is inhibited). Results reported here show Cu inhibition of branchial Na^+/K^+-ATPase in both fed fish and Na^+-fed fish when exposed to waterborne Cu. However, in the Na^+-fed fish, the inhibition was not significant relative to the fed-fish control (Fig. 6). This observation suggests that in fish fed high-Na^+ diets, Cu was less available in branchial epithelium to inhibit Na^+/K^+-ATPase activity.

Other studies examining the ionoregulatory effects of dietary Na^+, albeit at much higher concentrations (i.e. 12% Na^+ w/w) than those used here, have demonstrated an increase in branchial Na^+/K^+-ATPase activity (Salman and Eddy, 1987), for which there was a non-significant tendency in the present study. Increasing Na^+/K^+-ATPase activity is commonly seen in freshwater-adapted euryhaline fish after being transferred to saltwater (McCormick, 1995). The increased Na^+/K^+-ATPase extrudes excess branchial Na^+ from the gills as a means of regulating Na^+ homeostasis. Possibly, increased Na^+/K^+-ATPase activity in gills of fish fed high-Na^+ diets may serve to regulate branchial Na^+ in the same way.

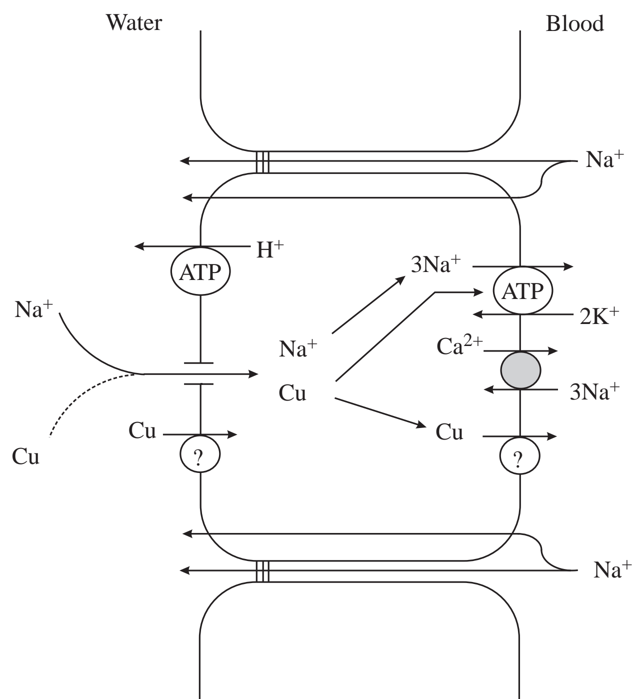

Fig. 9. Conceptual model of sodium and copper regulation in chloride cells of rainbow trout gills, including a common apical channel shared between sodium and copper (broken line). As sodium absorbed from the diet accumulates in these cells, the apical channel is downregulated. This results in reduced sodium and copper uptake, which is thought to be the mechanism by which dietary sodium protects against waterborne copper exposure. Details are given in the text.

Fish fed Na^+-supplemented diets showed significantly higher drinking rates than either fed or unfed fish (Fig. 8). Undoubtedly, these increased drinking rates represent another means by which Na^+-fed fish maintain osmoregulatory and ionoregulatory balance. Freshwater fish usually drink only small quantities of water because of the hypotonic nature of the dilute medium in which they reside, which causes a constant water influx across the gills and body surface (Karnaky, 1997). In order to compensate for this water influx, fish produce a copious amount of dilute urine and limit the amount of water they consume through drinking. However, as internal Na^+ concentrations increase with a high-Na^+ diet, freshwater fish will increase their drinking rates to dilute the extra Na^+ absorbed from the diet.

It could be argued that increased drinking rates in Na^+-fed fish might contribute to a higher waterborne Cu exposure through the gut, which could potentially offset any protection conferred by dietary Na^+ observed in the gills. On average, Na^+-fed fish drank 1.7 ml kg^{-1} h^{-1} of water that contained 20 µg Cu l^{-1} (Fig. 8). Therefore, these fish would consume

approximately 0.034 ng g^{-1} h^{-1}, which is at least 10-fold lower than Cu uptake rate across the gills (Fig. 1), so the contribution would be minor.

Taken together, results from this study suggest a common branchial uptake route shared between waterborne Na^+ and Cu (Fig. 9). Recently, Grosell and Wood (2002) have presented evidence for two high-affinity mechanisms for branchial Cu uptake in the gills of rainbow trout, one that directly competes for external Na^+ and another that is independent of external Na^+. In accord with this study, we speculate that this common route is probably in the form of an apical Na^+ channel, whose regulation depends on internal Na^+ concentrations, and a driving potential established by H^+-ATPase (Lin and Randall, 1995). Dietary Na^+ is taken up through the gut, causing an increase in plasma Na^+ concentrations. Plasma Na^+ can then be lost by passive diffusion to the water through the gills. Some of the excess Na^+ may be taken up by branchial cells *via* simple diffusion or by a Ca^{2+}/Na^+-exchanger on the basolateral membrane (Verbost et al., 1994). Sodium is also being taken up actively from the water through a putative Na^+ channel energized by the proton pump that it shares with Cu. The putative Na^+ channel would be downregulated in response to elevated intracellular Na^+ concentrations, resulting in a sharp reduction of aqueous Na^+ and Cu uptake. The net effect is an increase in branchial Na^+ efflux and a decrease in Na^+ and Cu influx.

Of course, the model we have proposed here does not preclude the other (Na^+-independent) branchial uptake routes for Cu characterized by Grosell and Wood (2002). Indeed, given that some new Cu accumulated in all tissues examined upon exposure to waterborne Cu for 6 h, regardless of Na^+ content in the food, Cu was probably being taken up from the water by some other route in addition to the shared Na^+ channel as suggested here. In this regard, Kamunde et al. (2001, 2002) have recently demonstrated that branchial Cu uptake is also responsive to internal Cu status of the fish. There is an interesting parallel here to cadmium metabolism, which is thought to be taken up across the gill *via* apical Ca^{2+} channels in the ionocyte (Verbost et al., 1989). Recently, Zohouri et al. (2001) reported that elevated dietary Ca^{2+} reduced but did not eliminate branchial cadmium uptake in rainbow trout.

In conclusion, dietary Na^+ effectively blocks waterborne Cu uptake in rainbow trout. Aqueous Cu uptake inhibition was closely associated with an inhibition of aqueous Na^+ uptake. Consequently, we propose that waterborne Na^+ and Cu share a common apical channel that is regulated, at least in part, based on internal Na^+ requirements. Although this study demonstrates the influence of dietary Na^+ on waterborne Cu uptake during short-term Cu exposures, the same principles seem to apply under chronic exposure conditions (Kamunde et al., in press). Possible implications to wild fish may include protective effects of high-Na^+ diets against waterborne Cu toxicity in nature, active dietary choice of high-Na^+ food items by Cu-stressed fish, and perhaps even Cu deficiency in fish raised on high-Na^+ diets in aquaculture. All these potential consequences deserve further investigation.

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