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Copper metabolism in actively growing rainbow trout (*Oncorhynchus mykiss*): interactions between dietary and waterborne copper uptake

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Summary

Juvenile rainbow trout *Oncorhynchus mykiss* were exposed to diets with low (12.6 nmol g^{-1}), normal (50.4 nmol g^{-1}) or elevated (4437.5 nmol g^{-1}) Cu concentrations in combination with either low (5.8 nmol l^{-1}) or normal (48.5 nmol l^{-1}) waterborne Cu levels over a 50-day period, during which body mass increased up to fivefold. A nutritional requirement for Cu was demonstrated based on growth response and whole body and tissue Cu status. Simultaneous low Cu levels in both the water and the diet depressed growth by 31 % over 7 weeks. There were reductions in both specific growth rate (SGR, 1.95 *versus* 2.55 % day^{-1}) and food conversion efficiency (FCE, 53–59 % *versus* 75–80 %) over weeks 0–4, but these effects disappeared in weeks 4–7. Elevated concentrations of dietary Cu did not affect SGR or FCE. Low levels of dietary and waterborne Cu decreased, and high levels of dietary Cu increased, the Cu concentrations in whole body, liver, carcass, gut and gills. Copper levels in the liver strongly reflected the exposure conditions with a corresponding fivefold decrease and a 22-fold increase in Cu concentration. Restricting available Cu caused an exponential decline in whole body Cu concentration from 0.0175 to 0.0069 μmol g^{-1} and increased the uptake of waterborne Cu (measured with ^{64}Cu) by the gills. Conversely, high levels of dietary Cu caused a linear increase in whole body Cu concentration to approximately 0.170 μmol g^{-1} and depressed the uptake of waterborne Cu. Waterborne Cu uptake contributed the majority (60 %) of the body's Cu accumulation under Cu-deficient conditions while dietary Cu contributed the majority (99 %) at high dietary levels of Cu. True bioavailability of dietary Cu decreased with increasing levels of dietary Cu concentration, although the absolute amount retained increased. These findings demonstrate an important interaction between dietary and waterborne Cu uptake in fish and provide compelling evidence of a key role for the gill in Cu homeostasis.

Key words: Cu homeostasis, Cu deficiency, waterborne Cu uptake, dietary Cu uptake, gill, rainbow trout, *Oncorhynchus mykiss*.

Introduction

Copper is essential for the survival of all organisms, including fish (Ogino and Yang, 1980; Satoh et al., 1983). It is a cofactor for several proteins that carry out fundamental functions in growth and development (Linder, 1991; Fairweather-Tait, 1997; Uauy et al., 1998). However, Cu is also a very potent toxicant when allowed to accumulate in excess of cellular needs (Harris, 1991; Pena et al., 1999). Consequently body Cu levels should be subject to tight homeostatic control in order to guard against deficiency and toxicity. The maintenance of Cu balance involves the strict regulation of uptake, distribution, detoxification and excretion. Two genetic diseases of Cu metabolism in man, Menke's and Wilson's diseases (Linder and Hazegh-Azam, 1996), present as failure of these processes. Susceptibility to Cu (as well as other trace elements) deficiency or toxicity depends on species, age and diet, a reflection of variation in efficiency of absorption and excretion (Baker, 1986; Bremner, 1998; Uauy et al., 1998). Young animals are apparently more prone to deficiency or toxicity because of increased demands for growth and because they have a high efficiency of absorption coupled with immaturity of the excretion system.

Despite extensive studies (Harris, 1991; Linder and Hazegh-Azam, 1996; Pena et al., 1999), the exact mechanisms of Cu homeostasis in mammals are not well understood. Much less is known about Cu metabolism and regulation in fish, although in contaminated environments fish may take up Cu through both the gut and the gills (Dallinger et al., 1987). Despite substantial literature pertaining to Cu uptake *via* either gills or gut (McDonald and Wood 1993; Handy, 1996), the interactions between the two routes of uptake are yet to be

clearly determined. One study (Miller et al., 1993) did examine this potential interaction in rainbow trout but started with the assumption that uptake from the water was zero at control (low) waterborne Cu levels of 79–205 nmol l^{-1}, an assumption which is not substantiated by the present study. The assessment of Cu requirement in fish is much more complex than in mammals because of this potential for extra-intestinal Cu uptake *via* the gills, and the fact that Cu is ubiquitously present in the aquatic environment as a result of both natural and anthropogenic processes. While acknowledging a possible complication due to branchial Cu uptake, previous studies that have determined Cu requirements (Ogino and Yang, 1980; Satoh et al., 1983; Lorentzen et al., 1998) failed to assess the potential contribution of waterborne Cu.

Although previous studies have independently assessed toxic effects (for reviews, see McDonald and Wood, 1993; Handy, 1996) or nutritional requirements (Ogino and Yang, 1980; Murai et al., 1981; Satoh et al., 1983; Lorentzen et al., 1998), no study has simultaneously investigated Cu metabolism in states of experimental deficiency and sublethal loading in fish. In particular, the interaction of dietary and waterborne Cu uptake has yet to be unequivocally demonstrated, a finding that would allow the determination of the relative contributions of waterborne and dietary Cu in nutrition and toxicity.

This study was therefore conducted to investigate Cu metabolism during both Cu restriction and elevated levels of dietary Cu exposure in juvenile rainbow trout. Firstly, we set out to establish conditions under which Cu deficiency could be induced in fish and to determine whether they could obtain their Cu requirement from water. Secondly, we assessed the effects of Cu restriction and excess levels of dietary Cu exposure on growth and whole body and tissue Cu reserves. Thirdly, we used direct measurements of ^{64}Cu fluxes to quantify waterborne Cu uptake in fish in which whole body Cu had been depleted or elevated, and were thereby able to separate quantitatively Cu uptake from diet and from water in order to determine their relative contributions. Finally, we assessed possible interactions between dietary and waterborne Cu uptake.

Materials and methods

Experimental animals and diet

Fingerling rainbow trout *Oncorhynchus mykiss* L. were obtained from Humber Spring Trout Hatchery, Mono Mills, Ontario, Canada. Prior to beginning the experiment, the fish were acclimated to laboratory conditions by holding them in one large tank supplied with aerated flow-through Hamilton tapwater [moderately hard water from Lake Ontario; Na^+, 0.6 mmol l^{-1}; Cl^-, 0.7 mmol l^{-1}; Ca^{2+}, 1.0 mmol l^{-1}; HCO_3^-, 1.9 mmol l^{-1}, pH 7.9-8.2; dissolved organic carbon (DOC), 3 mg l^{-1}; background Cu, 4.72 nmol l^{-1} (3 μg l^{-1}) at 14 °C]. The fish were maintained on a commercial fish starter diet at a daily ration of 4 % wet body mass and attained the targeted starting wet body mass of 0.5 g within 1 month. By the end of this period the fish had become used to the laboratory diet and were consuming all of it within 1 h. The pre-experimental diet was a regular commercial trout starter diet (Martin Feed Mills) that contained 330±10 nmol g^{-1} Cu (20.95±0.64 μg g^{-1}).

Cu-supplemented and Cu-deficient diets were prepared at the West Vancouver Laboratory, Department of Fisheries and Oceans, West Vancouver, British Columbia. The diet composition (Table 1) was based on known requirements for rainbow trout (NRC, 1993) and the only variable was the Cu content. This diet fulfilled the criteria necessary for diets intended for nutrient requirement studies (Baker, 1986).

Table 1. *Compositions of test diets containing different supplemental amounts of copper*

Ingredients (g kg^{-1} dry mass)	Diet*		
	Low Cu	Normal Cu	High Cu
Copper premix; I-cellulose carrier[a]	0	37.3	37.3
$CuSO_4 \cdot 5H_2O$ (nmol g^{-1})	0	47.2	4720.7
I-cellulose	37.3	0	0

The diets received either no supplemental Cu (low Cu, i.e. approximately 3.15 nmol g^{-1} from casein), the required dietary level of Cu of 47.21 nmol g^{-1} (normal Cu) or a high dietary Cu level of 4720.69 nmol g^{-1} (high Cu). Normal dietary Cu levels were based upon the known Cu needs of rainbow trout (NRC, 1993).

*All three diets contained the following ingredients (g kg^{-1} dry mass): casein (vitamin-free), 87.97; amino acid mix[b], 381.79; dextrin, 158.20; stabilized[c] sardine oil, 168.65; vitamin supplement[d], 39.87; choline chloride (60 %), 4.98; ascorbic acid, 1.50; mineral supplement[e], 89.71; finnstim™, 14.95; Santoquin, 0.11; carboxymethyl cellulose, 14.95.

[a]Diet palatability enhancer supplied by Finnsugar Bioproducts, Helsinki, Finland.

[b]All three diets contained the following levels of supplemental amino acids (g kg^{-1} dry diet): arginine-HCl, 26.32; histidine, 8.64; isoleucine, 16.24; leucine, 33.59; lysine-HCl, 31.98; methionine, 10.62; cysteine, 4.11; phenylalanine, 20.02; tyrosine, 13.76; threonine, 17.78; tryptophan, 3.47; valine, 22.47; glutamic acid, 37.30; glycine, 116.4; alanine, 2.45; proline, 16.64.

[c]Stabilized with butylated hydroxyanisole (0.225 g kg^{-1} oil).

[d]The vitamin supplement provided the following levels of nutrients (kg^{-1} dry diet): vitamin A acetate, 5000 i.u.; cholecalciferol (D_3), 2400 i.u.; DL-I-tocopheryl acetate (E), 300 i.u.; menadione, 18 mg; D-calcium pantothenate, 165 mg; pyridoxine HCl, 40 mg; riboflavin, 60 mg; niacin, 300 mg; folic acid, 15 mg; thiamine mononitrate, 50 mg; biotin, 1.5 mg; cyanocobalamin (B_{12}), 0.2 mg; inositol, 400 mg; *p*-amino-benzoic acid, 400 mg; butylated hydroxytoluene, 22 mg.

[e]The mineral supplement provided the following levels of minerals (kg^{-1} dry diet): Ca (as $CaCO_3$ and $CaHPO_4$), 9989 mg; P (as KH_2PO_4 and $CaHPO_4$), 7361 mg; Mg (as $MgSO_4 \cdot 7H_2O$), 1500 mg; Fe (as $FeSO_4 \cdot 7H_2O$), 200 mg; Zn (as $ZnSO_4 \cdot 7H_2O$), 96 mg; Mn (as $MnSO_4 \cdot H_2O$), 75 mg; Na (as NaCl), 2344 mg; K (as K_2SO_4, K_2CO_3 and KH_2PO_4), 8000 mg; I (as KIO_3), 10 mg; F (as NaF), 5 mg; Co (as $CoCl_2 \cdot 6H_2O$), 3 mg; Se (as Na_2SeO_3), 0.2 mg; Al (as $AlCl_3 \cdot 6H_2O$), 5 mg.

Table 2. *Levels of Cu exposure and measured Cu concentrations in the water (nmol l^{-1} and μg l^{-1}) and diet (nmol g^{-1} and μg g^{-1}) for each level*

Water			Diet			
[Cu]	Low	Normal	[Cu]	Low	Normal	High
nmol l^{-1}	5.82±0.47	48.47±0.94	nmol g^{-1}	12.60±0.55	50.40±0.74	4437.45±224.68
μg l^{-1}	0.37±0.03	3.08±0.06	μg g^{-1}	0.80±0.03	3.20±0.05	282.00±14.28

Values are means ± S.E.M.; *N*=35 for each waterborne Cu level and 10 for each dietary Cu level.

Experimental protocol

The experimental design consisted of three dietary Cu levels (low, normal and high) and two waterborne Cu levels (low and normal). The exposure system comprised a battery of fifteen 3 l tanks allowing for triplicates of five treatments of combinations of waterborne and dietary Cu concentrations: low waterborne Cu + low dietary Cu, low waterborne Cu + normal dietary Cu, normal waterborne Cu + low dietary Cu, normal waterborne Cu + normal dietary Cu, and normal waterborne Cu + high dietary Cu; measured concentrations are shown in Table 2. The experimental water used for both the low and normal waterborne Cu levels was generated by reconstituting deionized water produced by reverse osmosis with $NaHCO_3$ and $CaCl_2$ to bring the levels of these ions to those of Hamilton tapwater that was used during the 1 month acclimation period. The deionized water contained 0.4 mg l^{-1} DOC. For low water Cu, the Cu concentration was 5.82±0.47 nmol l^{-1} (0.37 μg l^{-1}), the level remaining after reverse osmosis treatment. For normal waterborne Cu levels, Cu was added as $CuSO_4{\cdot}5H_2O$ to raise the level to 48.47±0.94 nmol l^{-1} (3.1 μg l^{-1}), the ambient Cu level in Hamilton tapwater. Replacement Cu and salts were delivered from separate Mariotte bottles into two header tanks that supplied the experimental tanks. The tanks were supplied with flow-through aerated water thermostatically maintained at 14±1 °C throughout the 50-day experimental period. Flow rates to all the experimental tanks were set at 60 ml min^{-1}, which provided a 50 % turnover time of 34.7 min in the 3 l tanks. At the beginning of the experiment, fish were randomly separated into groups of 40 in each of the 15 tanks. All the groups were fed the designated diet (low Cu, normal Cu or high Cu) at a ration of 4 % wet body mass, delivered in two equal portions twice a day. All food was consumed within 1 h. Faecal material was siphoned off after 1 h of feeding.

Sampling

Sampling was done at the start of the exposure (week 0) and subsequently at weeks 2, 4 and 7 to assess tissue and whole body Cu status. A sampling time interval of 2–3 weeks was used to provide adequate time for physiological adjustments (e.g. acclimation) to occur within each exposure group before the subsequent sampling. Prior to sampling, all the fish were bulk-weighed on a per tank basis and starved for 2 days. During the starvation period, faecal material was siphoned from the tanks twice every 12 h to minimize any faecal ingestion. For weeks 0, 2 and 4, five fish per replicate (15 fish per treatment) were randomly netted from the experimental tanks and killed with an overdose of MS222. Gills, liver, gut (washed free of its contents) and the rest of the carcass were weighed and collected into separate pre-weighed scintillation vials or Eppendorf tubes. A further two fish per tank were collected at each sampling time and used for moisture content analysis, by drying to a constant mass at 70 °C. For week 7, a 12 h measurement of Cu uptake using ^{64}Cu preceded sampling as described below.

Waterborne Cu uptake kinetics

The effect of the exposure conditions on waterborne Cu uptake kinetics by gills was assessed at week 7 (day 50) over a range of waterborne Cu concentrations. Each treatment was divided into five groups (*N*=9); each group was then exposed to waterborne ^{64}Cu at a nominal total Cu concentration of either 31, 47, 79, 94 or 126 nmol l^{-1}. The radioisotope ^{64}Cu (as $CuNO_3$) was prepared at the McMaster University Nuclear Reactor. On the day of the experiment, 0.7 μCi l^{-1} of ^{64}Cu (specific activity 0.35 μCi μg^{-1}) was introduced into each experimental tank; the tanks had been pre-dosed with $CuSO_4{\cdot}5H_2O$ to bring the concentration to the nominal level. The radioisotope dosage administered added a total concentration of 3 nmol l^{-1} (0.2 μg l^{-1}) Cu to the water, and therefore did not substantially elevate the water Cu concentration. The fish were then exposed to the ^{64}Cu for 12 h under static water conditions. A 10 ml water sample was taken from each tank 15 min after introduction of ^{64}Cu and again after 12 h. Over this period the water ^{64}Cu activity and total Cu concentration changed by no more than 6.5 %.

Analysis

Cu concentrations in water, tissue, and food samples were determined by atomic absorption spectroscopy (AAS; Varian AA-1275 with GTA furnace atomizer) using a 10 μl injection volume and the operating conditions for Cu specified by the manufacturer. Certified Cu standards (National Research Council of Canada) run at the same time were within the specified range. Water samples were acidified (0.5 % nitric acid), while solid samples were weighed and digested overnight at 70 °C with 6 volumes of 1 mol l^{-1} nitric acid (Fisher Scientific, trace metal grade), and then centrifuged for 4 min at 13000 ***g***. A subsample of the supernatant was diluted appropriately with 0.5 % nitric acid. For day 50, the tissues and

water samples were first measured for ^{64}Cu activity on a Canberra-Packard Minaxi Gamma counter with an on-board program for decay correction, and then analyzed as described above for determination of total Cu concentrations.

Calculations

Whole body total Cu concentration was calculated by dividing the sum of Cu contents (concentration multiplied by mass) of all the tissues plus the carcass by the sum of the masses of all the tissues plus carcass.

Whole body uptake of waterborne ^{64}Cu was calculated by adding ^{64}Cu activities (cts min^{-1}) in all tissues plus carcass. Fish masses were determined by summing up the masses of liver, gills, gut tissue (washed) and carcass for each fish. Whole body Cu uptake was then calculated from the formula

$$a(bc^{-1})^{-1}, \quad (1)$$

where a is the ^{64}Cu of fish (cts min^{-1} g^{-1}), b is the ^{64}Cu of water (cts min^{-1} l^{-1}) and c is the total Cu concentration in the water (nmol l^{-1}). The uptake was then divided by the time of exposure (12 h) to convert it into a rate. The resulting values were rather small, hence they are reported as pmol g^{-1} h^{-1}.

Specific growth rate (SGR) was calculated on a per tank basis for three growth periods of 2 or 3 weeks using the formula:

$$SGR = 100\{[\ln(m_2) - \ln(m_1)]/t\}, \quad (2)$$

Where m_1=mass at beginning of growth period (g), m_2=mass at end of growth period (g), t=duration of growth period in weeks).

Food conversion efficiency (FCE) was calculated on a per tank basis for growth periods 0–2, 2–4 and 4–7 weeks:

$$FCE\ (\%) = 100(\text{mass gain per tank/food eaten per tank}). \quad (3)$$

To calculate true bioavailability of dietary Cu, we first estimated Cu uptake from water over 7 weeks by adjusting waterborne Cu uptake rates measured at the end of week 7 for size using the mean fish masses determined for weeks 0–2, 2–4 and 4–7 using the Cu uptake rate *versus* body mass relationship determined by Kamunde et al. (2001). It was assumed that all the Cu accruing from waterborne uptake was accumulated.

True bioavailability of dietary Cu (%), defined as the percentage retention of Cu ingested *via* diet after subtracting the accumulation that occurred by waterborne uptake, was then calculated as:

$$100[(totCu_f - totCu_0) - Cu_{water}]/Cu_{diet}, \quad (4)$$

where $totCu_f$ and $totCu_0$ are whole body total Cu at the end and beginning of the experiment, respectively, Cu_{water} is the total Cu taken up from the water and Cu_{diet} is the total Cu ingested with the diet over the experimental period. Visual observation during feeding showed that all the food was ingested. Thus, to calculate Cu_{diet}, the total amount of food delivered (ration) and the Cu concentration of the food were used.

Relative contributions of dietary and waterborne Cu to the total body metal burden were calculated as:

$$\text{Relative contribution of water (\%)} = 100[Cu_{water}/(Cu_f - Cu_0)] \quad (5)$$

$$\text{Relative contribution of diet (\%)} = 100 - 100[Cu_{water}/(Cu_f - Cu_0)]. \quad (6)$$

The assumptions for this calculation were as for the bioavailability calculation (Equation 4).

Somatic indices for liver, gill and gut were calculated as:

$$100(x/\text{wet body mass}), \quad (7)$$

where x is wet mass of the organ or tissue of interest.

For gill the entire gill basket was used, whereas for the gut, gut contents and extraneous tissues such as fat were removed.

Statistical analysis

Data are presented as means ± S.E.M. (N). Effects of exposure conditions on growth, tissue Cu concentration and subsequent waterborne Cu uptake at each sampling point were assessed using a two-way analysis of variance (ANOVA) with time, diet and waterborne Cu concentrations as variables. Percentage data were subjected to arc-sin transformation prior to statistical testing. In all cases, significance was set at $P<0.05$. Student–Newman–Keuls pairwise multiple comparison procedure was used to make comparisons between measurements. One-way ANOVA and Bonferroni's test were used to compare changes in FCE and SGR at $P<0.05$ and curve fitting for whole body Cu concentration patterns over time was done with Statistica 5.1 using individual data points by the Quasi-Newton estimation method.

Results

Growth

Over the 7 week period, fish wet body mass increased by up to fivefold. Mortality was less than 2 % and was not related to conditions of exposure. Juvenile rainbow trout exposed to the combination of low levels of dietary and waterborne Cu were retarded in growth relative to all the other groups. Cumulative mass gain was lower at all times from 2 weeks onwards, and reduced by 31 % (18 g *versus* 26 g) over 7 weeks (Fig. 1A). Specific growth rate (data not shown) was significantly depressed at weeks 0–2 and 2–4 (approximately 1.95 % day^{-1} *versus* 2.55 % day^{-1} in both periods) though the effect had disappeared by weeks 4–7 (2.7 % day^{-1} *versus* 2.8 % day^{-1}). There were no significant differences in growth between any of the other treatment groups; fish receiving Cu *via* either one of the routes alone or in combination maintained normal growth. Growth retardation in the deficient group was associated with significantly decreased food conversion efficiency (53–59 % *versus* 75–80 %) during the first 4 weeks (Fig. 1B).

There were no significant differences over time or between groups in whole body moisture content, which remained between 74 % and 76 % throughout (data not shown). The hepatosomatic and gastrointestinosomatic indices increased from 1.3 % to 2.0 % and from approximately 9 % to 11 %,

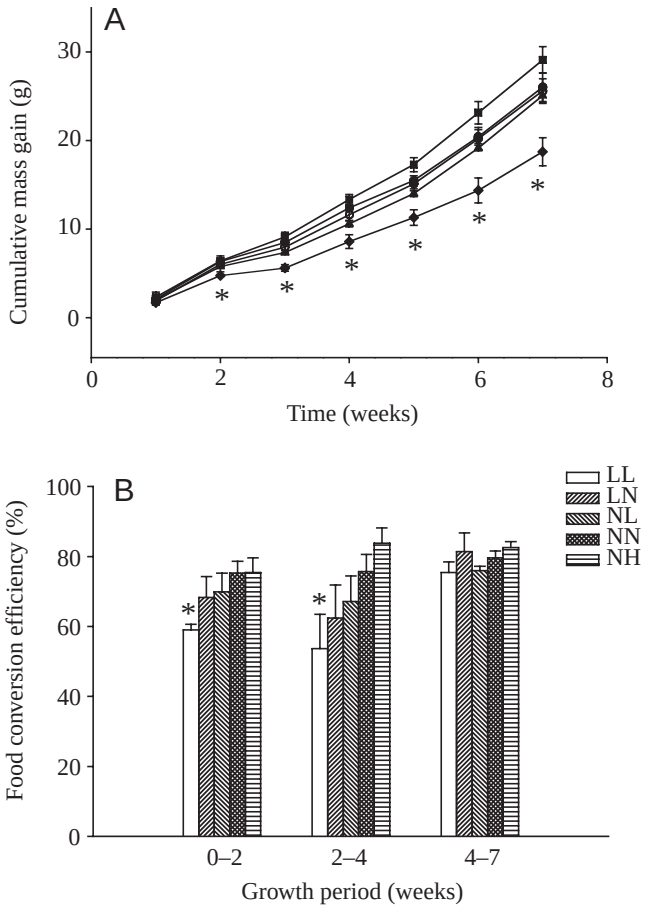

Fig. 1. (A) Effects on growth of exposure of juvenile rainbow trout to a combination of waterborne and dietary Cu levels ranging from deficient to excess. Values are cumulative mass gain per tank (means ± S.E.M., *N*=3). Diamonds, low waterborne Cu and low dietary Cu; triangles, low waterborne Cu and normal dietary Cu; open circles, normal waterborne Cu and low dietary Cu; filled circles, normal waterborne Cu and normal dietary Cu; squares, normal waterborne Cu and high dietary Cu level. (B) Effects of the exposure conditions on food conversion efficiency in actively growing rainbow trout. Values are means ± S.E.M. on a per tank basis, *N*=3 per data point. LL, low waterborne Cu and low dietary Cu; LN, low waterborne Cu and normal dietary Cu; NL, normal waterborne Cu and low dietary Cu; NN, normal waterborne Cu and normal dietary Cu; NH, normal waterborne Cu and high dietary Cu level. *Significant difference relative to group NN on normal water Cu and normal dietary Cu (ANOVA, $P<0.05$). No significant differences were observed with other comparisons of treatments.

respectively, while the branchiosomatic index decreased from approximately 4.5 % to 3.5 % over the 7 weeks (data not shown). There were no treatment-related effects on these indices. Consequently allometric equations for the growth of liver, gill, gut and carcass were derived from pooled data of these organs (Table 3). Body mass was well correlated with the mass of these organs and between 82 % and 99 % of the variance in growth of the organs could be explained by the change in body mass.

Table 3. *Allometric equations and correlation coefficients for the relationships between wet body mass and masses of gills, liver, gut and carcass*

Organ/tissue	Allometric equation	r^2	*P* value
Gills	$0.0379W^{0.8662}$	0.82	<0.0001
Liver	$0.0182W^{1.2027}$	0.90	<0.0001
Gut	$0.1041W^{1.0682}$	0.96	<0.0001
Carcass	$0.8394W^{0.9928}$	0.99	<0.0001

W, wet body mass.
N=144 per organ or tissue.

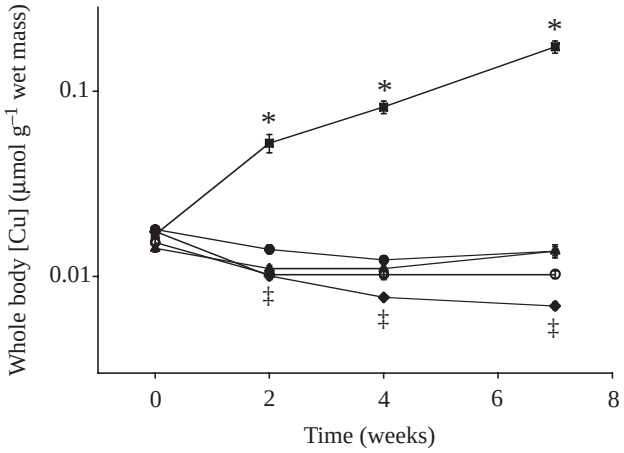

Fig. 2. Effects of dietary and waterborne Cu exposure conditions on whole body Cu concentration in actively growing rainbow trout. Values are means ± S.E.M., *N*=15 for weeks 0, 2 and 4, and *N*=9 for week 7 for each treatment. Diamonds, low waterborne Cu and low dietary Cu; triangles, low waterborne Cu and normal dietary Cu; open circles, normal waterborne Cu and low dietary Cu; filled circles, normal waterborne Cu and normal dietary Cu; squares, normal waterborne Cu and high dietary Cu level. *Significantly higher level, ‡significantly lower level relative to the group exposed to normal water Cu and normal dietary Cu (ANOVA, $P<0.05$).

Whole body Cu status

Whole body Cu concentration (initially approximately 0.0175 μmol g^{-1} wet mass) declined slightly to approximately 0.010 μmol g^{-1} in fish exposed to normal levels of Cu in water or diet, either in combination or separately (Fig. 2). However, fish deprived of Cu or exposed to high dietary Cu levels exhibited, respectively, much lower (0.0069 μmol g^{-1}) and higher (0.170 μmol g^{-1}) whole body Cu concentrations by week 7.

Fig. 3A analyses the pattern of whole body Cu concentration (y, μmol g^{-1} wet mass) over time (x, weeks) during exposure to the combination of low levels of Cu in water and in diet, while Fig. 3B analyses the corresponding pattern during exposure to normal Cu levels in water and elevated Cu concentration in the diet. In the former, the pattern was best explained by the negative exponential model, $y=0.4268+0.6879\exp(-0.5868x)$, $r^2=0.80$, with $t_{1/2}=1.18$ weeks. In contrast, during exposure to

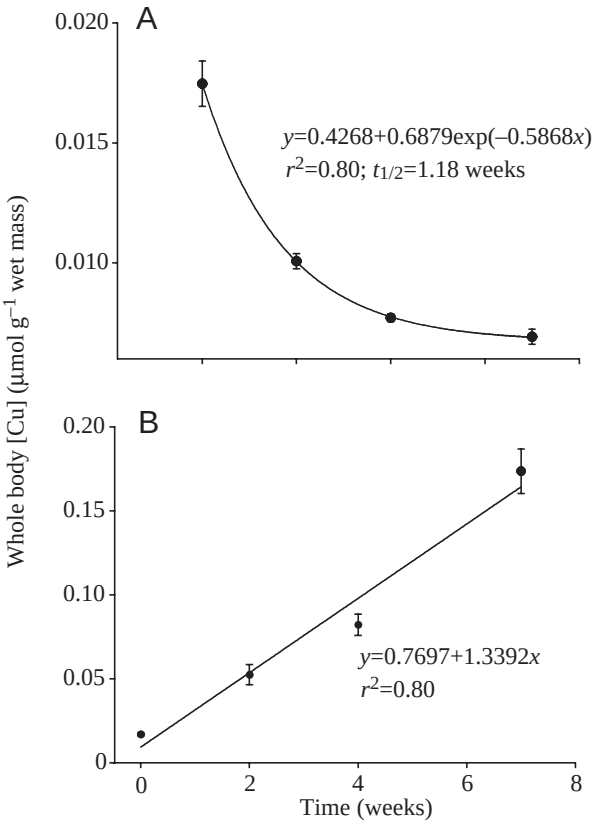

Fig. 3. Patterns of whole body Cu concentration (y, µmol g^{-1} wet mass) with time (x, weeks) during exposure to low waterborne and low dietary Cu levels (A), and to normal waterborne and high dietary Cu levels (B). Values are means ± S.E.M., N=15 for weeks 0, 2 and 4, and N=9 for week 7 for each treatment. In A the negative relationship is best described by the exponential equation y=0.4268+0.6879exp(–0.5868x), r^2=0.80, $t_{1/2}$=1.18 weeks; in B, the positive relationship is best described by the linear equation, y=0.5897+1.407x, r^2=0.80. Equations were derived from individual data points.

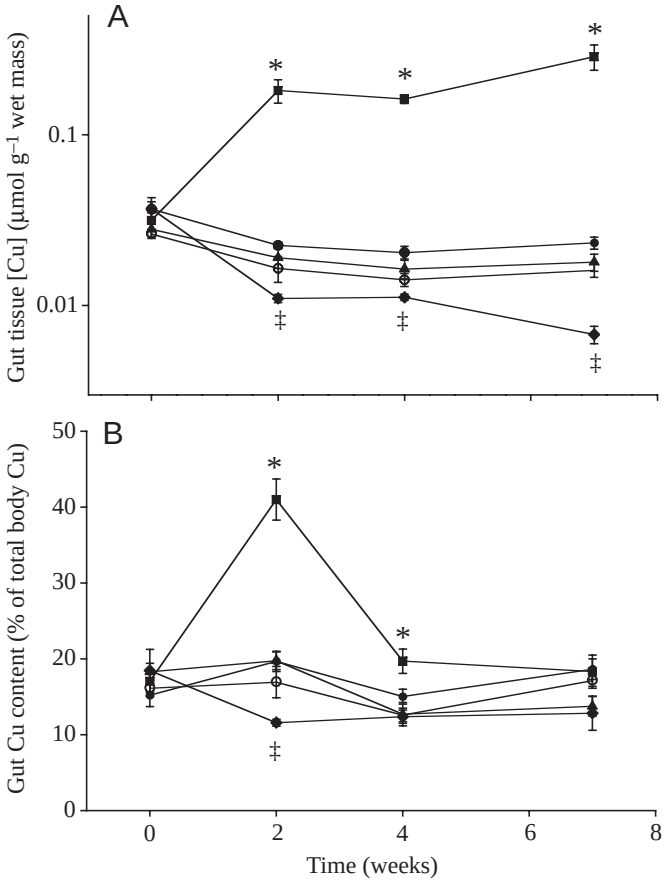

Fig. 4. Gut tissue Cu concentration (µmol g^{-1} wet mass) over the exposure period and the proportional contribution (%) to total body Cu burden. Percentage data were transformed to arc sin for statistical analysis. Values are means ± S.E.M.; N=15 for weeks 0, 2 and 4, and N=9 for week 7 for each treatment. Diamonds, low waterborne Cu and low dietary Cu; triangles, low waterborne Cu and normal dietary Cu; open circles, normal waterborne Cu and low dietary Cu; filled circles, normal waterborne Cu and normal dietary Cu; squares, normal waterborne Cu and high dietary Cu level. *Significantly higher level, ‡significantly lower level relative to the group on normal levels of water Cu and dietary Cu (ANOVA, P<0.05).

high dietary Cu, the data best fitted the linear model, y=0.7697+1.3392x, r^2=0.80, indicating continuous Cu accumulation above the normal body Cu concentration.

Tissue Cu status

In the intestinal tissue, Cu levels rose by week 7 from approximately 0.03 to 0.3 µmol g^{-1} wet mass, a tenfold increase, in the fish exposed to high dietary Cu concentration, and decreased fivefold to 0.007 µmol g^{-1} wet mass in the animals exposed to low Cu levels in both diet and water (Fig. 4A). In fish on normal Cu, either in the diet or in water, gut tissue Cu levels were similar to control levels and remained between 0.015 and 0.025 µmol g^{-1} wet mass. As a proportion of the total (Fig. 4B), Cu in the gut tissue depended on the level and period of exposure. For all the groups except the one on high level Cu diet, the proportion of Cu retained in the gut remained between 15 and 20 %. In contrast the group on high level Cu diet held more than 40 % of their total Cu in the gut tissue early in the exposure, but this declined to approximately 20 % later in the exposure.

The liver showed dramatic changes in Cu levels (Fig. 5A). Liver Cu concentration rose 22-fold in the fish exposed to high dietary Cu levels and fell by 80 % in fish on low dietary and waterborne Cu levels, relative to the values at the start of the experiment. Accumulation of Cu in the liver was continuous throughout the exposure whereas Cu depletion was initially rapid and slowed down over time. The proportion of whole body total Cu retained in the liver (Fig. 5B) gradually increased from approximately 20 % to approximately 75 % in the fish on high Cu dietary concentration. In all the other groups the proportion of Cu retained in the liver ranged between 10 and 30 %.

The Cu content of the gill was quite variable (Fig. 6) but was significantly elevated in the fish on high dietary Cu levels

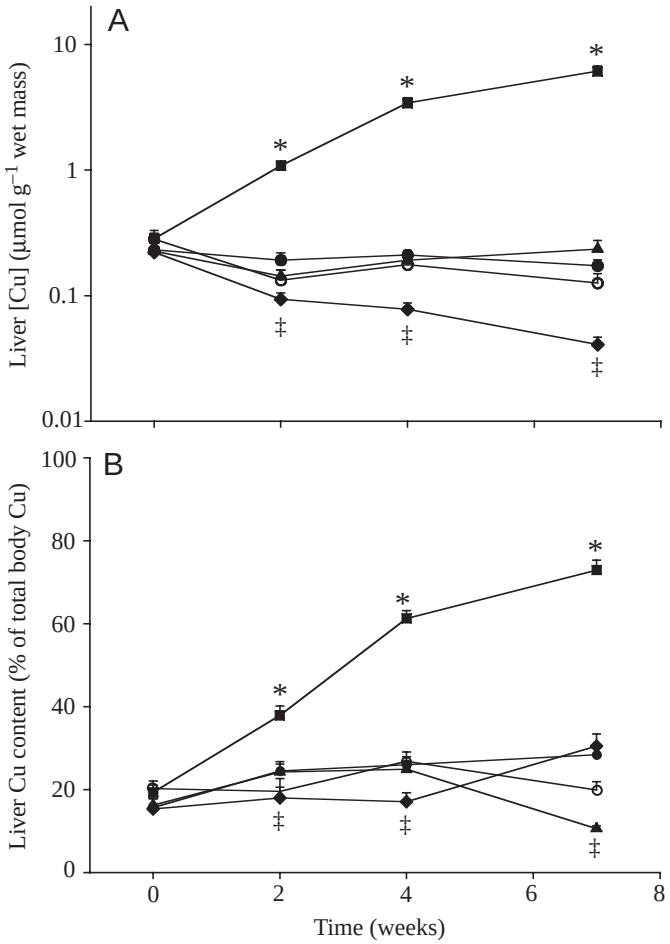

Fig. 5. Liver Cu concentration (μmol g^{-1} wet mass) during the exposure period (A) and the proportional contribution of liver Cu to total body Cu (B). Percentage data were transformed to arc sin for statistical analysis. Values are means ± S.E.M.; *N*=15 for weeks 0, 2 and 4, and *N*=9 for week 7 for each data point per treatment. Diamonds, low waterborne Cu and low dietary Cu; triangles, low waterborne Cu and normal dietary Cu; open circles, normal waterborne Cu and low dietary Cu; filled circles, normal waterborne Cu and normal dietary Cu; squares, normal waterborne Cu and high dietary Cu level. *Significantly higher level, ‡significantly lower level relative to group on normal levels of water Cu and dietary Cu (ANOVA, $P<0.05$).

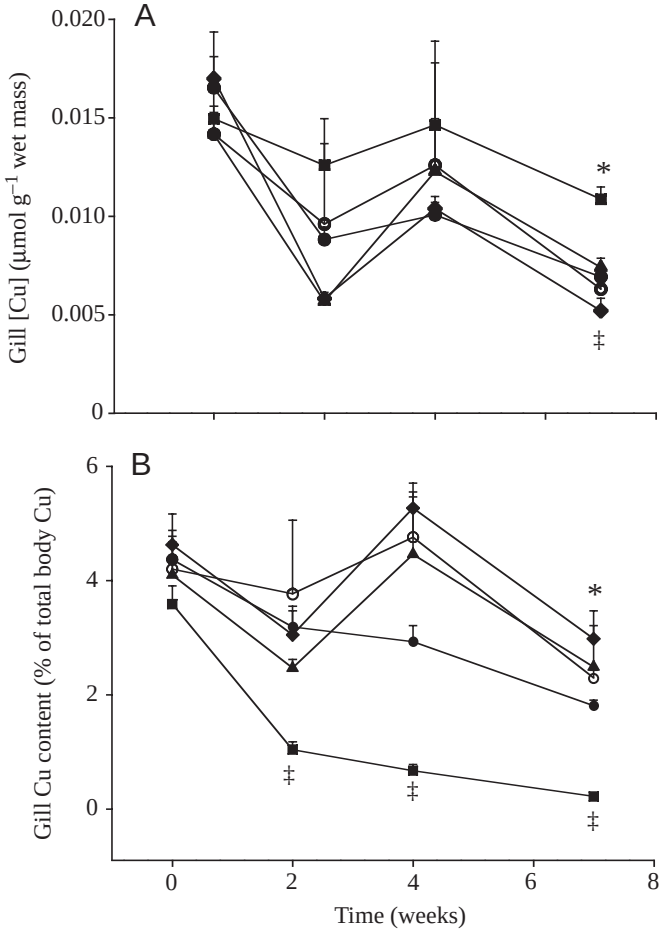

Fig. 6. Cu concentration in gills (μmol g^{-1} wet mass) during the exposure period (A) and the percentage contribution of the gill to total body Cu burden during the exposure period (B). Percentage data were transformed to arc sin for statistical analysis. All the values are means ± S.E.M., *N*=15 for weeks 0, 2 and 4, and *N*=9 for week 7 for each of the treatment. Diamonds, low waterborne Cu and low dietary Cu; triangles, low waterborne Cu and normal dietary Cu; open circles, normal waterborne Cu and low dietary Cu; filled circles, normal waterborne Cu and normal dietary Cu; squares, normal waterborne Cu and high dietary Cu levels. *Significantly higher level, ‡significantly lower level relative to the group on normal levels of water Cu and dietary Cu (ANOVA, $P<0.05$).

and significantly lower in the Cu-deficient group. The contribution of the gill to the total body Cu was 3–5 % in all the groups except the one on high dietary Cu concentration, where it fell to approximately 1 %.

Carcass (whole body less liver, gut and gills) Cu concentration (Fig. 7A) rose during exposure to high dietary Cu levels from approximately 0.012 to 0.017 μmol g^{-1}, and declined significantly in the fish exposed to conditions of Cu deficiency. In the groups receiving normal Cu *via* either or both routes, there were small but significant decreases in carcass Cu concentration at all the sampling times relative to day 0. The proportion of total Cu retained in the carcass (Fig. 7B) remained at approximately 60 % in the fish receiving normal levels of Cu in the diet and water but rose to 75 % in the Cu-deficient group. In contrast, the proportion of Cu in the carcass for the group on the high dietary Cu level declined to <10 % of the total by the end of the experiment.

Waterborne Cu uptake kinetics

Waterborne Cu uptake rates *via* the gills measured using ^{64}Cu over a range of waterborne Cu concentrations at week 7 of exposure are shown in Fig. 8. Fish exposed to low Cu either in the water and/or the diet had elevated rates of uptake of waterborne Cu at all the waterborne Cu concentrations tested. Fish exposed to a high dietary Cu concentration had decreased rates of waterborne Cu uptake. In all the groups the rate of

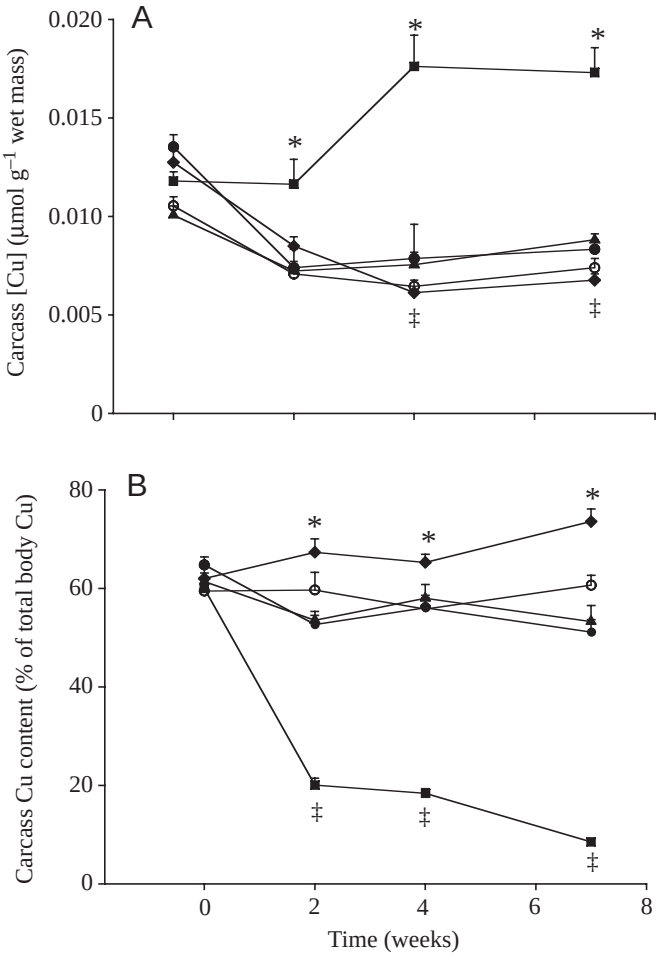

Fig. 7. Carcass Cu concentration (μmol g^{-1}wet mass) during the exposure period (A) and the proportional contribution of the carcass to the total Cu burden during the exposure period (B). Carcass comprised whole body less gill, liver and gut. Percentage data were transformed to arc sin for statistical analysis. All the values are means ± S.E.M., N=15 for weeks 0, 2 and 4, and N=9 for week 7 for each of the treatment. Diamonds, low waterborne Cu and low dietary Cu; triangles, low waterborne Cu and normal dietary Cu; open circles, normal waterborne Cu and low dietary Cu; filled circles, normal waterborne Cu and normal dietary Cu; squares, normal waterborne Cu and high dietary Cu level. *Significantly higher level, ‡significantly lower level relative to the group on normal levels of water Cu and dietary Cu (ANOVA, P<0.05).

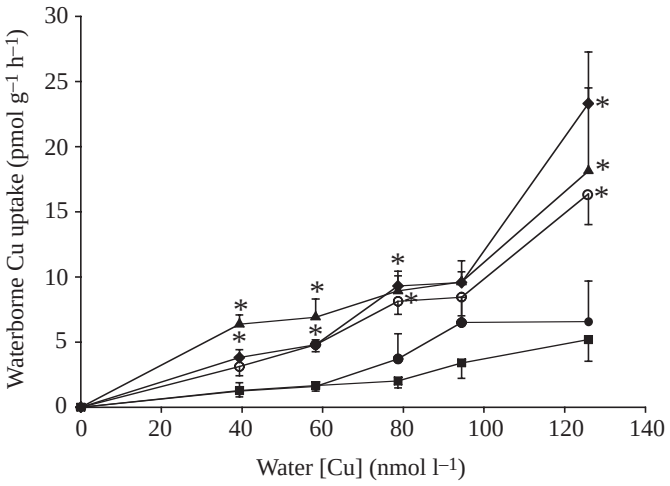

Fig. 8. Waterborne Cu uptake rates (means ± S.E.M., pmol g^{-1} h^{-1}, N=9 per data point), measured using ^{64}Cu at the end of the 7-week exposure for all the treatment groups. These measurements were carried out over a 12-hour period, at a range of waterborne copper concentrations. Diamonds, low waterborne Cu and low dietary Cu; triangles, low waterborne Cu and normal dietary Cu; open circles, normal waterborne Cu and low dietary Cu; filled circles, normal waterborne Cu and normal dietary Cu; squares, normal waterborne Cu and high dietary Cu level. *Significantly higher relative to the group on normal levels of water Cu and dietary Cu (ANOVA, P<0.05).

waterborne Cu uptake *via* gills increased with the water Cu concentration, a trend that was more marked in the Cu-deficient group.

Discussion

Growth and nutritional requirement for copper

Growth of juvenile rainbow trout on normal waterborne and dietary Cu regimes was within the expected range for the feeding and temperature regime (Brett and Groves, 1979). Copper is clearly an essential trace element in rainbow trout, based on the reduced growth associated with reduced food conversion efficiency in Cu-deficient animals. Copper deficiency in juvenile rainbow trout was induced by exposing fish to reduced Cu levels in both diet and water simultaneously. Ogino and Yang (1981) reported reduced growth in carp but not in rainbow trout exposed to low dietary Cu levels in water with normal Cu levels, whereas Satoh et al. (1983) observed growth depression in rainbow trout fed 22 nmol g^{-1} Cu in the diet. Gatlin and Wilson (1986) and Murai et al. (1981) did not find growth retardation in channel catfish fed diets containing 24 nmol g^{-1} or 14 nmol g^{-1} Cu. A fundamental difference between the present study and the previous ones is that in addition to receiving low dietary Cu, fish were exposed to water which was also deficient in Cu. Secondly, our fish were much smaller (starting mass 0.5 g). Our results clearly indicate that to induce Cu deficiency, the experimental fish need to be young, hence with a low basal Cu load. It is noteworthy that previous studies (Gatlin and Wilson, 1986; Murai et al., 1981) that did not find depressed growth used much larger fish (starting weights 10- to 30-fold higher than in the present study). Based on their growth response, fish given 12.6 nmol g^{-1} of Cu in their diet and normal levels of Cu in the surrounding water (48.5 nmol l^{-1}) had adequate amounts of Cu for normal growth, but the same amount of dietary Cu was inadequate if the water Cu concentration was deficient. Therefore, for determination of the minimum dietary requirement for Cu in fish, the waterborne Cu concentration must be taken into account.

Whole body Cu status

Cu concentration data were expressed on a wet mass basis

since a previous study (Shearer, 1984) on rainbow trout of varying body size showed that whole body wet mass concentrations are more useful for comparison of trace elements than dry mass concentrations. In fact, since there were no treatment-related or time-related effects on moisture content, the same trends would have been seen even if the data had been expressed on a dry mass basis.

Although an ideal biomarker of Cu status in mammals has yet to be identified (Milne, 1998), several indicators have been used by different authors to assess Cu nutritional status. These include growth, activities of cuproenzymes, and plasma Cu concentration (Baker, 1986; Gatlin and Wilson, 1986; Turnlund et al., 1997, 1998). Based on previous studies (Grosell et al., 1997, 1998, 2001; Kamunde et al., 2001), plasma Cu concentration cannot be used as a sensitive indicator of Cu status in fish since it is very tightly regulated during waterborne and dietary Cu exposure. In this study whole body and liver Cu concentrations were sensitive indicators of Cu exposure. Baker (1986) pointed out that although growth data are in the long term the only defensible way to establish trace element requirement, the use of body stores also provides an important indicator in determining the nutrient requirement.

Whole body Cu concentration declined exponentially over time during deficiency, but increased linearly during exposure to high dietary Cu levels. Lauren and McDonald (1987) described a linear loss of whole body Cu after 28 days of exposure to high waterborne Cu levels. Although these authors used larger fish, there appear to be notable differences in the kinetics of elimination of abnormally high body Cu concentrations (depuration) (Lauren and McDonald, 1987) and the decline of normal body Cu concentrations in the face of deficiency (as in the present study). For actively growing juvenile rainbow trout, simple growth dilution was evident and could account for most of the decline in whole body Cu concentration. Fish mass increased by approximately 250 %, while whole body Cu concentration declined by 60 % over the same period, almost exactly the percentage that would be expected by growth dilution. Furthermore, growth of all the organs and tissues sampled was well correlated with body mass, independent of treatment. It is notable that body mass accounted for 90, 96 and 99 % of the change in liver, gut and carcass mass, respectively (Table 3). Since these organs were the main Cu reservoirs, a change in Cu concentration in the whole body due to growth dilution would reflect the change seen in these tissues. Overall, the decline in whole body Cu concentration fitted a one-compartment model (simple negative exponential), and the increase in whole body concentration during dietary loading was linear, an indication that the latter is not a well-regulated phenomenon.

Interestingly, despite the decline in whole body Cu concentration in the deficient fish, all the groups had significantly higher Cu levels per fish at the end of exposure compared to the levels at the beginning of the experiment (Fig. 9). Total Cu content in fish on a high dietary Cu level and normal water increased 65-fold, whilst for fish on inadequate Cu *via* both routes, only a twofold increase occurred (Fig. 9).

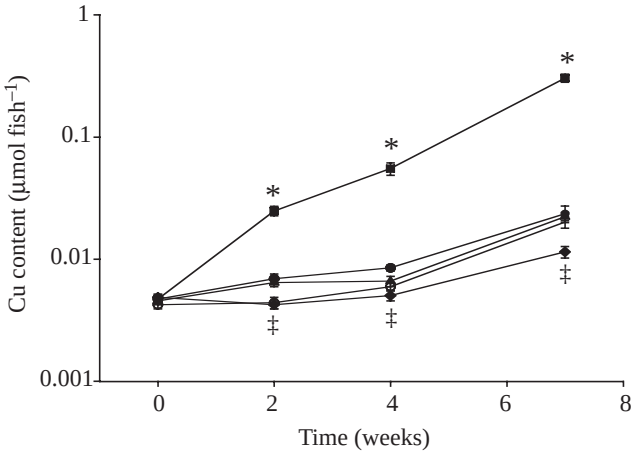

Fig. 9. Effects of dietary and waterborne Cu exposure conditions on fish Cu content. Values are means ± S.E.M., N=15 for weeks 0, 2 and 4, and N=9 for week 7 for each treatment. Diamonds, low waterborne Cu and low dietary Cu; triangles, low waterborne Cu and normal dietary Cu; open circles, normal waterborne Cu and low dietary Cu; filled circles, normal waterborne Cu and normal dietary Cu; squares, normal waterborne Cu and high dietary Cu level. *Significantly higher level, ‡significantly lower level relative to the group on normal levels of water Cu and dietary Cu (ANOVA, P<0.05).

For the groups receiving normal Cu levels in the diet or water in combination or separately, the Cu content increased fivefold. This increase occurred in the absence of changes in whole body moisture content. Thus in all the treatment combinations the fish extracted Cu from the water and their diet, although the amount obtained by fish exposed to low diet and low water Cu levels was not adequate to meet normal growth requirements or the normal tissue concentration. Nonetheless, this observation illustrates that both the gill and gut Cu uptake mechanisms are highly efficient.

Tissue Cu status

It has been demonstrated that the role of the liver is central in mammalian Cu metabolism (Cousins, 1985; Harris, 1991; Pena et al., 1999), and it appears to have a similar role in fish. Accumulation of high amounts of Cu during dietary exposure has been reported (Julshamn et al., 1988; Handy 1992, 1996; Kamunde et al., 2001). In the present study, liver Cu content clearly reflected the level of exposure. The role of the liver in Cu metabolism in fish can be viewed as concentrating Cu when fish are exposed to large quantities and mobilizing it when inadequate quantities are present in diet and water. In male Sprague–Dawley rats fed a Cu-deficient diet, liver Cu concentration was reported to decrease at a slow rate of approximately 4 % a week (Owen and Hazelrig, 1968). In this study, the decline in liver Cu concentration was relatively rapid, with more than half the Cu content lost in about a week [$t_{1/2}$=1.18 weeks (8.25 days), Fig. 3A], a reflection of both inadequate uptake and growth dilution. The decline in liver Cu concentration continued throughout the experimental period,

and only 20 % of the initial Cu concentration remained at the end of the experiment.

The concentration of Cu in the liver strongly influenced whole body Cu content although the liver represented only 1.3-2 % of the body mass. At the beginning of the exposure approximately 20 % of the body Cu burden was in the liver. This proportion remained between 10–30 % in all the groups except the group on a high dietary Cu level, which held 75 % of the body Cu in the liver by the end of the exposure. Chronic dietary Cu exposure is characterized by a continuous accumulation of Cu in the liver as seen in the present and previous studies (Handy, 1993; Kamunde et al., 2001). Although we noted massive accumulation of Cu in the liver in this study, there was no indication of toxicity since the fish grew at the same rate as the controls.

Cu content of gut tissue was greatly elevated in fish exposed to high dietary Cu but appeared to level out over time, an indication that this tissue effectively regulates its internal Cu levels, as suggested in previous studies (Berntssen et al., 1999; Kamunde et al., 2001). Furthermore, Cu build-up in gut tissue is diagnostic of dietary Cu exposure and does not occur during waterborne Cu exposure to any great extent (Kamunde et al., 2001). A common trend with Cu uptake kinetics and accumulation in gut is that early in the exposure, a high proportion of the metal burden is held within the gut tissue but subsequently this is mobilized into other tissues. Later in the exposure, the gut tissue attains steady state despite continued exposure to elevated dietary Cu levels, suggesting that prolonged exposure stimulates clearance of Cu from the gut to other tissue, increases loss through faeces and mucosal exfoliation, or decreases absorption. Our data suggest stimulated Cu mobilization into other tissues, especially the liver, under these conditions.

During elevated levels of dietary Cu exposure in normal water, the gills accumulated significant amounts of Cu, in agreement with previous studies (Miller et al., 1993; Kamunde et al., 2001), thus pointing to a potential role for the gills in Cu excretion. Although the changes in carcass Cu concentration during periods of Cu deficiency and exposure to elevated dietary Cu levels were small, the change in Cu content was enormous given the large mass that the carcass comprises. This compartment held the highest proportion of whole body Cu burden in all the groups except in the group receiving a high dietary Cu level, in which the liver was the dominant Cu reservoir.

Whole body waterborne Cu uptake

Copper uptake rates were measured after 7 weeks of continuous exposure to constant conditions of dietary and waterborne Cu, by which time any acclimation process would presumably be complete. Fish deprived of Cu in the water or diet together or separately had high uptake rates at the low waterborne Cu concentrations (<100 nmol l^{-1} Cu), which increased dramatically above this concentration (Fig. 8). Two types of Cu binding sites, the high-affinity low-capacity binding sites, and the low-affinity high-capacity binding sites, have been recently described in trout gills (Taylor et al., 2000). These authors demonstrated saturation of the high-affinity low-capacity sites at <315 nmol l^{-1} Cu, and recruitment of low-affinity, high-capacity sites above this concentration. In the present study, which measured transport rather than binding, saturation of the high-affinity sites appeared to occur at much lower water Cu concentrations. The generally higher uptake rate at a waterborne concentration of 126 nmol l^{-1} may represent the point at which the low-affinity high-capacity sites start to be recruited. It appears that restriction of Cu in diet increases the capacity and affinity of both types of binding sites.

Uptake of waterborne Cu *via* gills has been studied mainly as it pertains to Cu toxicity (for a review, see McDonald and Wood, 1993), while a possible role for the gills in normal Cu metabolism has been largely disregarded. Gills play vital roles in gaseous exchange, acid–base balance, and ionoregulation; the present study suggests an additional, novel role of the gills in trace metal nutrition and homeostasis. We report for the first time that exposure of fish to conditions deficient in Cu causes an upregulation of branchial Cu uptake. Furthermore, there is reduced branchial uptake following pre-exposure to high dietary Cu (see also Kamunde et al., 2001). Thus fish respond to different levels of dietary Cu by varying the rate of Cu absorption from water. This strategy may serve to minimize or prevent the development of Cu deficiency when intake is low and, conversely, Cu toxicity when intake is high, and indicate that Cu is under tight homeostatic control.

These observations possibly suggest the presence of a Cu transporter in the fish gills that responds to body Cu status. Mammalian studies have shown several specific P-type ATPases that serve for Cu transport, e.g. the Menke's and Wilson's proteins, and are involved in Cu homeostasis (Bingham et al., 1998; Roft and Hediger, 1999). For fish, Campbell et al. (1999) demonstrated vanadate-sensitive Cu transport (indicative of the involvement of a P-type ATPase) in perfused whole gills of rainbow trout, and Bury et al. (1999) reported an ATP-dependent silver uptake by trout gill basolateral membrane vesicles. Silver can substitute for Cu in bacterial Cu-ATPase (Solioz and Odermatt, 1995) and silver transport in rainbow trout gills could thus well be *via* a Cu-ATPase.

Interactions between dietary and waterborne Cu

Only a few studies have assessed the interaction between dietary and waterborne metal uptake in fish. Miller et al. (1993) argued that Cu assimilated from either route partitioned into functionally independent compartments in rainbow trout. Furthermore, using whole body Zn burden, Spry et al. (1988) reported no interaction between dietary and waterborne Zn uptake in the same species. Both these studies based their conclusions, at least in part, however, on the assumption of zero uptake from their control water Cu (79–205 nmol l^{-1}) and Zn (107 nmol l^{-1}) levels. The current data (Fig. 8) show that this is clearly not the case for Cu at least. Furthermore, these measurements revealed a marked interaction between dietary

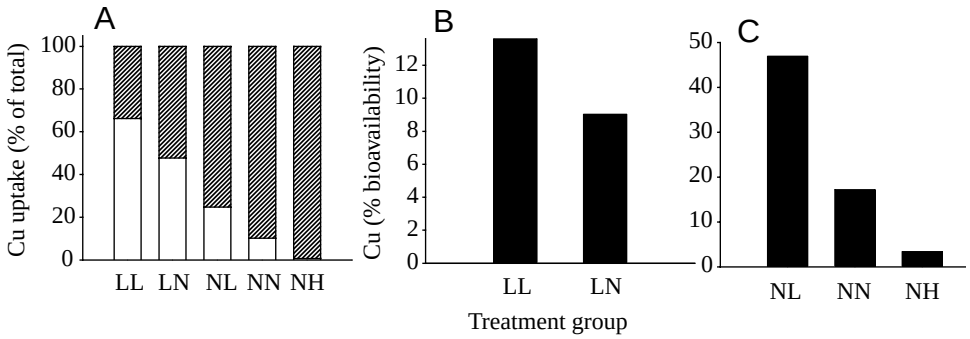

Fig. 10. (A) Relative contribution of dietary and waterborne Cu uptake to the total body Cu burden accumulated over 7 weeks of exposure to experimental regime. Open bars, contribution of the waterborne Cu uptake; hatched bars, contribution of the dietary Cu uptake; LL, low waterborne Cu and low dietary Cu; LN, low waterborne Cu and normal dietary Cu; NL, normal waterborne Cu and low dietary Cu; NN, normal waterborne Cu and normal dietary Cu; NH, normal waterborne Cu and high dietary Cu level. (B,C) True bioavailability of dietary Cu (% of Cu ingested in food and retained during the exposure period). (B) Bioavailability of dietary Cu in normal water. (C) Bioavailability of dietary Cu in low waterborne Cu. See text for details of the calculation

and waterborne Cu uptake geared toward maintaining Cu homeostasis during deficiency or excess Cu exposure.

We estimated the relative contribution of waterborne and dietary Cu uptake to the whole total body Cu load using measured waterborne Cu uptake rates, feeding rates and dietary Cu concentrations (see Materials and Methods). At low dietary Cu, water was clearly the main source of Cu, contributing 60 % of the total (Fig. 10A). With increasing dietary Cu, the contribution of dietary Cu increased whilst that of waterborne Cu decreased. In the group maintained on normal dietary and waterborne Cu, water contributed less than 10 % of the body Cu. At the highest dietary Cu concentration, diet was clearly the main source of Cu (99 %) and water contributed insignificant amounts to the total Cu burden. A previous study on relative contributions of waterborne and dietary Cu uptake to liver Cu concentration (Miller et al., 1993) showed increasing contribution of waterborne Cu uptake as waterborne Cu concentration increased.

In turn, this analysis allowed estimation of the true bioavailability of dietary Cu (see Materials and methods for definition), which decreased with increasing dietary Cu concentration (in general agreement with studies on humans (e.g. Turnlund et al., 1997, 1998), both in water with normal Cu concentration and water with low Cu concentration (Fig. 10B,C). Furthermore, waterborne Cu had an apparent stimulatory effect on the bioavailability of dietary Cu and *vice versa*. Possible explanations for this could be that the upregulation of gill uptake that occurs in the low Cu water entails a compensatory downregulation of intestinal uptake processes, or that gastrointestinal Cu absorptive mechanisms require some threshold in waterborne Cu for optimal performance and thus are less effective when water Cu levels are low. It is noteworthy that Spry et al. (1988) observed a similar stimulatory effect of waterborne Zn on the retention of dietary Zn in rainbow trout.

Murai et al. (1981) noted that the responsiveness of catfish to graded levels of dietary Cu was less pronounced than in most terrestrial animals and argued that Cu metabolism in catfish may have been affected by waterborne Cu. However, these authors did not provide any waterborne Cu uptake to support this insight. The present study not only provides this missing link (waterborne Cu uptake data) but ascribes to the gills a key role in normal Cu metabolism in fish. Branchial uptake contributed approximately 60 % of the body Cu load during deficiency, but diet was the preferred source of Cu under normal dietary and waterborne conditions, contributing more than 90 % of the body burden. These findings coupled with recent reports of branchial Cu excretion (Grosell et al., 2001; Kamunde et al., 2001) persuasively underline a key role of the gills in Cu homeostasis in fish and provide evidence of the gill as an organ of nutritional regulation.

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