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Renal Cu and Na excretion and hepatic Cu metabolism in both Cu acclimated and non acclimated rainbow trout (*Oncorhynchus mykiss*)

M.H. Grosell[a,*], C. Hogstrand[b], C.M. Wood[c]

[a] *Riso National Laboratory, Environmental Science and Technology Department, P.O. Box 49, DK-4000 Roskilde, Denmark*

[b] *University of Kentucky, 101 Morgan Building, T.H. Morgan School of Biological Sciences, Lexington, KY 40506-0225, USA*

[c] *McMaster University, Department of Biology, 1280 Main Street West, Hamilton, Ont. L8S 4K1, Canada*

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Abstract

^{64}Cu and total Cu accumulation were measured in gills, plasma, liver, kidney, bile and urine during 72 h of exposure to ^{64}Cu at 20 μg Cu l^{-1}, in non-acclimated and Cu-acclimated (28 days of pre-exposure) rainbow trout (*Oncorhynchus mykiss*) fitted with urinary bladder catheters. Renal Cu excretion gradually declined from 0.03 μg Cu kg^{-1} h^{-1} in non-exposed fish to 0.01 μg Cu kg^{-1} h^{-1} after 28 days of Cu exposure. A comparison of the ^{64}Cu-labelled Cu and the total Cu excretion rates and the corresponding renal clearance revealed apparent differences in Cu binding to plasma protein depending on whether the Cu is derived from recent branchial uptake or is already present in the plasma prior to ^{64}Cu exposure. The plasma Cu pool derived from recent branchial uptake and the Cu pool present in the plasma prior to ^{64}Cu exposure is accessible to renal excretion to different extents, whereas the pools seem equally accessible to hepatic accumulation and elimination. The renal Cu excretion is of minor importance compared with the hepatic Cu excretion, which was estimated to be 0.5–0.75 μg Cu kg^{-1} h^{-1} and 1.1–1.6 μg Cu kg^{-1} h^{-1} for non-acclimated and Cu-acclimated fish, respectively. Based on the biliary Cu concentration, hepatic Cu elimination appeared to be stimulated in the Cu-acclimated relative to the non-acclimated fish. Only 17% and 12% of the hepatic Cu could be accounted for by metallothionein in the control and Cu-acclimated fish, respectively. Renal Na^+ efflux decreased by 40%, which was largely due to increased tubular Na^+ reabsorption. Renal compensation for the impaired branchial Na^+ uptake, seen during Cu exposure, thus seems to be involved in Cu acclimation in rainbow trout. © 1998 Elsevier Science B.V.

Keywords: Cu-acclimation; Rainbow trout (*Oncorhynchus mykiss*); ^{64}Cu; Cu turnover; Cu excretion; Cu and plasma–protein binding; Metallothionein; Renal Na^+ reabsorption

*Corresponding author. Tel.: +45 46 77 41 90; fax: +45 46 77 41 93; e-mail: martin.grosell@risoe.dk

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1. Introduction

Cu is an essential element required by all living organisms; around 30 enzymes are known to use Cu as a co-factor (Harris, 1991). Cu is classified as a trace metal and is thus found in all cells in the picomolar range, but Cu is also potentially highly toxic to most organisms, including freshwater teleosts (Chakoumakos, 1979; Cusimano et al., 1986). As a consequence, organisms would benefit from tight cellular Cu regulation and from systems that can transport Cu between organs to ensure optimal local cellular Cu concentrations. Such mechanisms have been studied intensively over the last decades, especially in mammals (for reviews see Cousins, 1985; Harris, 1991) and more recent experiments have shown that plasma Cu levels in the teleost fish *Oncorhynchus mykiss* are indeed under very tight regulation even during exposure to elevated ambient Cu concentrations (Grosell et al., 1997). Fish are more likely to experience rapid changes in Cu uptake than terrestrial vertebrates due to direct branchial uptake of waterborne Cu. Cellular Cu handling in teleost fish has been studied to some extent, and hepatic Cu binding by metallothionein (MT) has been argued to play an important role in acclimation to Cu (McCarter and Roch, 1983; McCarter and Roch, 1984; Laurén and McDonald, 1987b). Cu excretion by rainbow trout has been reported after termination of Cu exposure (Laurén and McDonald, 1987a). Grosell et al. (1996) have suggested that adaptation to Cu by the European eel (*Anguilla anguilla*) involves stimulation of Cu excretion. Furthermore, a recent study on rainbow trout has shown a very high turnover of renal Cu and a lower, but still considerable, turnover of a large hepatic Cu pool, suggesting both renal and hepatic Cu elimination (Grosell et al., 1997). Clearly, stimulation of such excretion mechanisms would be important to regulate body Cu concentrations during chronic Cu exposure. To examine whether such stimulation occurs, we have used a combination of chronic Cu exposure (28 days at a total Cu concentration of about 20 $\mu g\ l^{-1}$) and a more brief exposure to radioactive ^{64}Cu in rainbow trout acclimated and non-acclimated to Cu. We had three specific objectives. The first objective was to compare renal Cu excretion in non-acclimated and Cu-acclimated rainbow trout during this low-level, sublethal exposure. To do this we monitored the urine flow rate as well as the ^{64}Cu and total Cu in the urine of rainbow trout fitted with internal urinary bladder catheters. Our second objective was to compare the relative importance of hepatic and renal Cu elimination in rainbow trout during adaptation to Cu. For this purpose, levels of ^{64}Cu and total Cu in the liver and in the contents of the gall bladder were monitored at the end of each experiment. Our third objective was to test whether acclimation to Cu involves any renal compensation for the expected impaired branchial Na^+ uptake. To do this we monitored Na^+ concentrations in urine collected from non-acclimated rainbow trout prior to and during Cu exposure and from Cu-acclimated rainbow trout during Cu exposure.

Cu exposure has been demonstrated to impair branchial Na^+ uptake, leading to reduced plasma Na^+ concentrations (Laurén and McDonald, 1985). Acclimation to Cu has been reported to involve a restoration of branchial Na^+ uptake following physiological, biochemical and morphological modifications (Laurén and Mc-

Donald, 1987a,b; Pelgrom et al., 1995). Since resting renal Na^+ efflux rates are as high as 10–30% of the branchial unidirectional Na^+ efflux (reviewed by Wood, 1995), reduction of renal Na^+ loss might also be an important mechanism of adaptation.

2. Materials and methods

2.1. Fish

Rainbow trout, *Oncorhynchus mykiss* (133–339 g, mean 269 g) were obtained from Spring Valley Trout Farm, Petersburg, Ontario. The fish were held, fed and acclimated to 15 ± 1°C in dechlorinated Hamilton city tap water (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; background Cu, 0.89 μg Cu l^{-1}). One group was then chronically exposed for 28 days to (mean ± SEM, $n = 20$) 22.7 ± 1.5 μg Cu l^{-1} (added as $CuCl_2 \cdot H_2O$), while the other group was held under identical conditions without Cu addition (for further details see Grosell et al., 1996).

2.2. Experimental design

Ten non-exposed fish from the holding tanks were used as a control group (A) for tissue Cu and MT levels. Groups of ten non-acclimated (B) and ten acclimated (C) fish were anaesthetised with MS-222 from a stock solution (25 g l^{-1}; pH 7) at a final concentration of 0.1 g l^{-1}, fitted with internal urinary catheters (Wood and Randell, 1973) and placed in individual experimental chambers, of total volume 7.1 l. The experimental chambers were supplied with Hamilton city tap water at a rate of 200 ml min^{-1} from a recirculated system containing 500 l. The catheter tip was located within the urinary bladder, thus providing largely ureteral urine (cf. Beyenbach and Kirschner, 1975) and drained outside the fish chambers into covered vials by a 7 cm siphon (see diagram in McDonald, 1983). The fish were allowed to recover for at least 48 h prior to the start of the experiments. Total urine production was then collected over two successive 12 h control periods prior to ^{64}Cu exposure. In experiments with Cu-acclimated rainbow trout, the water in the recirculated system contained about 20 μg Cu l^{-1} during the recovery and control periods. Exposure to ^{64}Cu plus 22.3 ± 1.8 μg Cu l^{-1} (mean ± SEM, $n = 12$) (20 mCi in 500 l of water), was initiated as described by Grosell et al. (1996). During ^{64}Cu exposure, total urine production was collected over six successive 12 h periods. Each 12 h urine collection was analysed for volume, Na^+, ^{64}Cu (except for control periods) and total Cu. At the end of the 72 h ^{64}Cu exposure, the fish were anaesthetised, a blood sample of at least 0.5 ml was withdrawn from the caudal vessel with a heparinised Hamilton syringe and the fish was killed by a blow to the head. Bile, liver, kidney and gill filament samples were taken by dissection. Subsamples of livers were immediately transferred to cryostatic vials, frozen in liquid nitrogen and stored at −70°C until used. Subsamples of liver, kidney and gill filaments

were also freeze dried and the dry weights were determined at room temperature and humidity.

2.3. Newly accumulated Cu and renal efflux

The ^{64}Cu γ activity was determined in the reference water, the liver, kidney, gill filaments, plasma, bile and urine samples collected during ^{64}Cu exposure using a Canberra Packard MINAXI auto gamma 5000 series gamma-counter equipped with an on-board program for correction of radioactive decay. In this study, the term 'newly accumulated Cu' is used to designate that part of the Cu in one compartment which came from the 'previous' compartment, during the ^{64}Cu exposure period. The previous compartment for the gills is assumed to be the water; for the plasma, the gills; for the liver and kidney, the plasma; and for the bile, the liver as in Grosell et al. (1996). The previous compartment for the urine is assumed to be the plasma. Newly accumulated Cu was calculated by

$$a/(b/c) \quad (1)$$

where a is ^{64}Cu cpm g^{-1} tissue (dry weight) or ^{64}Cu cpm l^{-1}, b is ^{64}Cu counts in the previous compartment (cpm g^{-1} or μg^{-1}) and c is the total Cu concentration in the previous compartment (μg Cu l^{-1} or μg Cu g^{-1}).

2.4. Levels of MT and total Cu

The frozen liver samples were subsequently weighed and homogenised individually in 4 vol. 50 mmol l^{-1} Tris–HCl, pH 8.0, 0°C, using a glass–teflon homogeniser. Samples were centrifuged at $10\,000 \times g$, 4°C, for 20 min; the supernatant was decanted, frozen in liquid nitrogen, and stored at −70°C. MT levels were analysed by double antibody radioimmunoassay (RIA) as described by Hogstrand and Haux (1990).

After measurement of ^{64}Cu γ activity, urine samples were acidified with 1% HNO_3 (trace metal analysis grade, BDH Chemicals). For total Cu analysis, freeze-dried liver, kidney and gill filament samples were digested as described by Grosell et al. (1996). Total Cu was assayed in all water, plasma, urine and bile samples, as well as in the tissue digests by graphite furnace atomic absorption spectroscopy (Varian AA-1275 with GTA-9 atomiser) using 20 μl injection volume, N_2 gas, and standard operating conditions as documented by the manufacturer.

2.5. Plasma and urine Na^+

Na^+ was analysed in the plasma samples obtained from the control (A), non-acclimated (B) and acclimated (C) fish and in all urine samples collected during the control and ^{64}Cu exposure periods using the atomic absorption spectrophotometer Varian 1275.

2.6. Statistical methods

Significant differences in the kinetics of urine production, renal efflux of newly accumulated Cu, total renal Cu efflux and renal Na^+ efflux between groups B and C were evaluated in a multifactor ANOVA (Statgraphics for Windows version 1.3) with acclimation and ^{64}Cu exposure time as main variables. The starting points for the two data sets, i.e. the two sets of control values, were compared by means of Student's *t*-test (two-tailed, unpaired), and the same applied to a comparison between the starting points of group C and the end points (72 h of Cu exposure) of group B. Differences between data in groups B and C for total Cu concentration and newly accumulated Cu for all investigated tissues and bile samples, hepatic MT values and plasma Na^+ were also evaluated by Student's *t*-test (two-tailed, unpaired). In all cases, groups were considered significantly different at $P < 0.05$.

3. Results

The urine flow rate (UFR) (Fig. 1) in group C during the control period was 20–22% lower than that observed in B (2.4 ml kg^{-1} h^{-1}) (*t*-test, $P < 0.006$) and the total urine production remained generally lower throughout the ^{64}Cu exposure (ANOVA, $P < 0.01$). Groups B and C showed a general decrease, of 31% and 27%, respectively, in urine production over time, during the 72 h of ^{64}Cu exposure (ANOVA, $P < 0.003$). By the end of the 72 h exposure, the UFR in group B was identical to the starting UFR after 28 days in group C.

Renal excretion of newly accumulated Cu was not different in non-acclimated

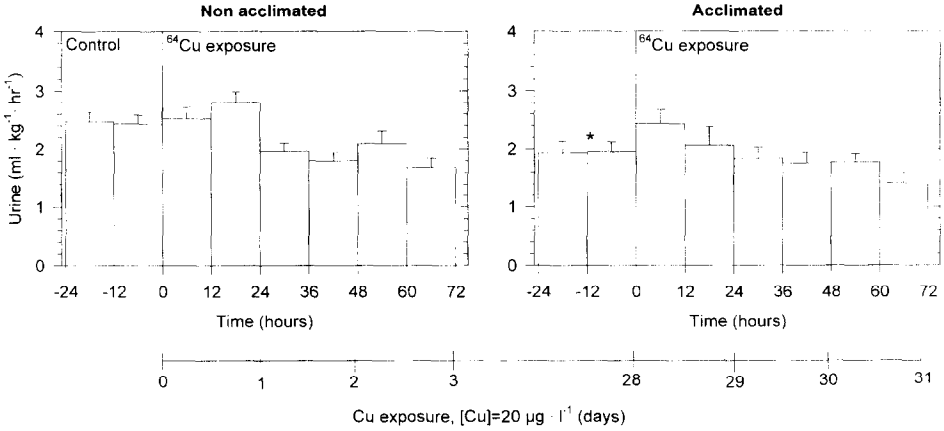

Fig. 1. Urine production in non-acclimated (group B, $n = 14$–16) and Cu-acclimated (group C, $n = 8$ 12) rainbow trout during 24 h of control and 72 h of ^{64}Cu exposure at about 20 µg Cu l^{-1}. Means ± SEM. Results for groups B and C were significantly different at $P < 0.0139$ and dependent on time at $P < 0.003$ (ANOVA). The two sets of control values were significantly different at $P < 0.006$, indicated by * (Student's *t*-test). The control values of acclimated fish are not significantly different from the 72 h values of non-acclimated fish.

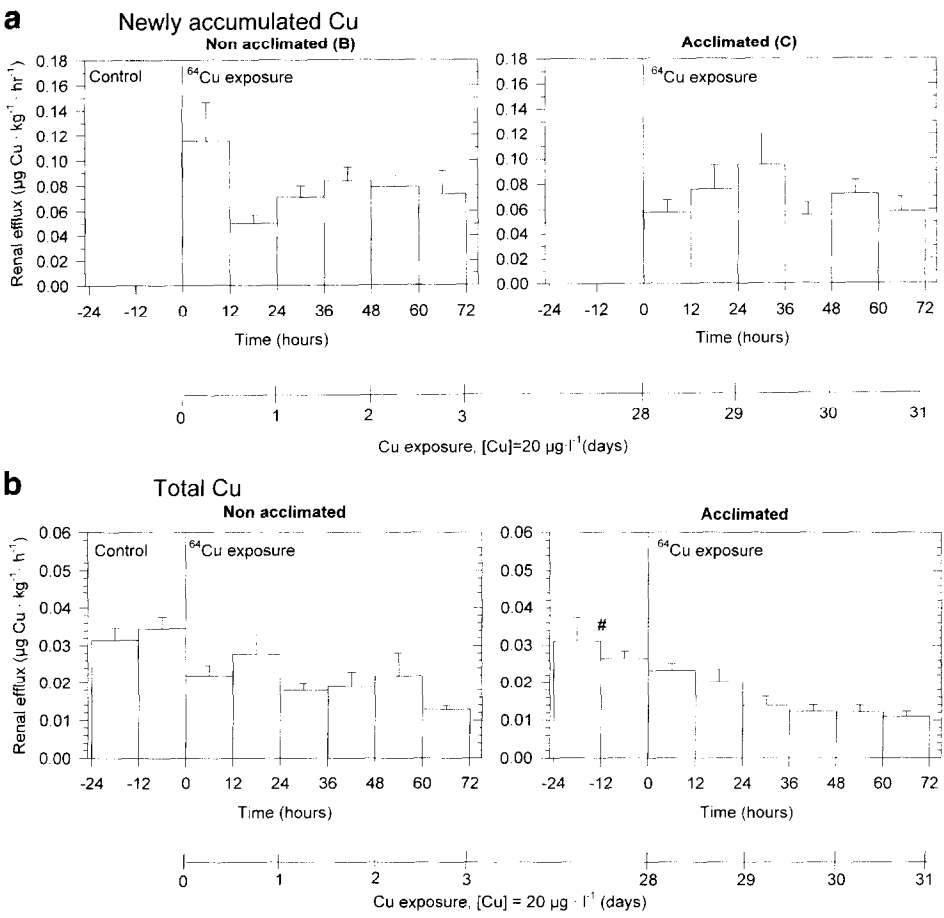

Fig. 2. (a) Renal newly accumulated Cu efflux in non-acclimated (group B) and Cu-acclimated (group C) rainbow trout during 72 h of ^{64}Cu exposure at about 20 μg Cu l^{-1}. Means ± SEM (for *n*, see legend to Fig. 1). Groups B and C were not significantly different (ANOVA). (b) Renal total Cu efflux in groups B and C during 24 h of control and 72 h of ^{64}Cu exposure at about 20 μg Cu l^{-1}. Means ± SEM (for *n*, see legend to Fig. 1). Groups B and C were significantly different at $P < 0.0131$ and were dependent on time at $P < 0.0005$ (ANOVA). The two sets of control values were not significantly different (Student's *t*-test). The control values of acclimated fish are significantly higher than the 72 h values of non-acclimated fish (*t*-test, $P < 0.0007$), indicated by #.

and acclimated fish (Fig. 2(a)), and no statistically significant trend over time was observed in either of the two groups. Group B exhibited a relatively high renal excretion of newly accumulated Cu of 0.12 μg Cu kg^{-1} h^{-1} during the first 12 h of exposure; thereafter, excretion was rather constant around 0.07 μg Cu kg^{-1} h^{-1} throughout the ^{64}Cu exposure period. Acclimated fish (C) exhibited a relatively constant excretion rate, around 0.07 μg Cu kg^{-1} h^{-1} throughout the ^{64}Cu exposure period.

Total renal Cu excretion was slightly lower in acclimated than non-acclimated

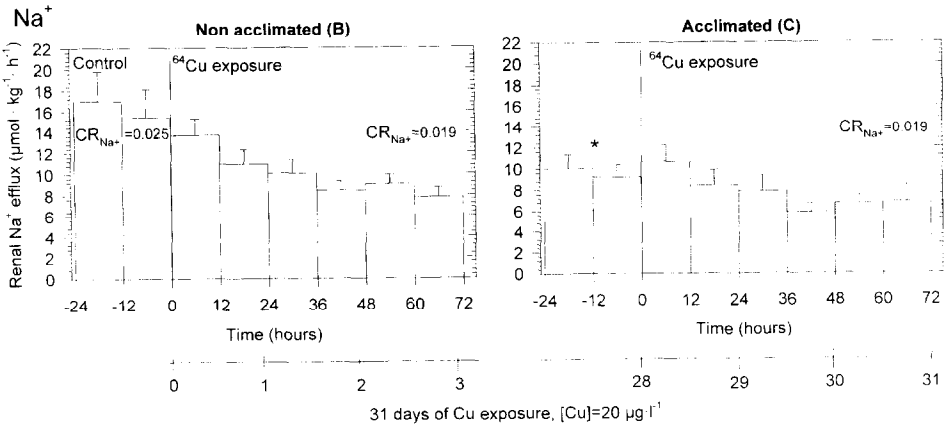

Fig. 3. Renal Na^+ efflux in non-acclimated (group B) and Cu-acclimated (group C) rainbow trout during 24 h of control and 72 h of ^{64}Cu exposure at about 20 μg Cu l^{-1}. Means ± SEM (for n, see legend to Fig. 1). Groups B and C were significantly different at $P < 0.009$ (ANOVA). The two sets of control values were significantly different at $P < 0.01$, indicated by * (Student's t-test). CR_{Na+}, the Na^+ clearance ratio is calculated for the non-exposed (control period of the non-acclimated fish), non-acclimated fish after 72 h of Cu exposure and in acclimated fish after 28 days + 72 h of Cu exposure assuming that GFR = 1.74 × UFR. The plasma data used are presented in Table 1 (see Section 4 for further details). The control values of group B are not significantly different from the 72 h values of group C (Student's t-test). Other details as in legend to Fig. 1.

fish (ANOVA, $P < 0.01$) and there was a general reduction over time in the total renal excretion of Cu in both experimental groups (Fig. 2(b)) (ANOVA, $P < 0.0005$). Non-acclimated fish showed a marked reduction in Cu excretion during the first sampling period in response to Cu exposure and total Cu excretion remained lower than pre-exposure control values (0.033 μg Cu kg^{-1} h^{-1}) at around 0.02 μg Cu kg^{-1} h^{-1} throughout the ^{64}Cu exposure period (Fig. 2(b)). The control value of acclimated fish (0.029 μg Cu kg^{-1} h^{-1}) was not significantly different. The control values of acclimated fish were significantly higher than the 72 h values of non-acclimated fish (t-test, $P < 0.0007$). As in non-acclimated fish, there was a

Table 1
Plasma Na^+, total and newly accumulated Cu in control (group A, $n = 10$), non-acclimated (group B, $n = 14$) and acclimated (group C, $n = 12$) rainbow trout

Treatment group	Na^+ (mmol l^{-1})	Total Cu (μg Cu ml^{-1})	Newly accumulated Cu (μg Cu ml^{-1})
Control (A)	156.0 ± 6.4	0.749 ± 0.040	
Non-acclimated (B)	143.3 ± 2.5*	0.652 ± 0.033	0.076 ± 0.010
Acclimated (C)	148.1 ± 2.7	0.722 ± 0.045	0.048 ± 0.0080

* and indicate statistically significant differences, from control and between the two Cu-exposed groups, respectively (Student's t-test, $P < 0.05$).

reduction in total Cu excretion during the ^{64}Cu exposure period, in this case to 38% of the initial control value.

Renal Na^+ efflux (Fig. 3) was clearly different in groups B and C (ANOVA, $P < 0.009$). Furthermore, both these groups demonstrated a reduced renal Na^+ efflux with time throughout the experimental periods (ANOVA, $P < 0.0004$). This trend was expressed more strongly in the non-acclimated fish. Acclimated fish exhibited Na^+ efflux of only 9.7 μmol kg^{-1} h^{-1} during the control period, equivalent to 60% of the corresponding control values of the non-acclimated fish (Student's *t*-test, $P < 0.01$). Renal Na^+ efflux in non-acclimated fish fell to only 52% of the initial control value of 16.2 μmol kg^{-1} h^{-1} during the 72 h of ^{64}Cu exposure. Thus, by the end of the 72 h exposure period, urinary Na^+ excretion in non-acclimated fish was not significantly different from the starting value after 28 days in acclimated fish.

Plasma Na^+ was significantly reduced after 72 h of Cu exposure in non-acclimated fish (Table 1) but was (at least partly) restored after 28 days+72 h in acclimated fish. In agreement with earlier results (Grosell et al., 1997), total Cu concentration in the plasma of non-acclimated fish after 72 h and acclimated fish after 28 days+72 h of Cu exposure did not differ significantly from the controls (group A) (Table 1). Newly accumulated Cu in the plasma, however, was different in the two experimental groups, also in agreement with the previous study. Acclimated fish had accumulated only 63% of the concentration accumulated by non-acclimated fish after 72 h of ^{64}Cu exposure (Table 1).

Newly accumulated Cu in the livers of non-acclimated fish was in close agreement with earlier results (Grosell et al., 1997) and reached 153 ± 13.9 μg Cu g^{-1} (dry weight) (Fig. 4(a)). During the same period of exposure, the total Cu concentration in the livers of the same fish increased from 234.8 ± 42.2 in the controls to 462.4 ± 35.8 μg Cu g^{-1} (dry weight) in non-acclimated fish (Fig. 4(b)) ($P < 0.0004$), an absolute gain of 227.6 μg Cu g^{-1} (dry weight), which is in relatively close agreement with the newly accumulated Cu. Acclimated fish showed a slightly higher total Cu concentration of 584.2 ± 54.8 μg Cu g^{-1} (dry weight) and a slightly higher newly accumulated Cu level of 207.5 ± 37.5 μg Cu g^{-1} (dry weight) compared with non-acclimated fish. Acclimated fish showed an increased amount of hepatic MT (186.6 ± 43.6 μg Cu g^{-1}, wet weight) relative to control fish (97.5 ± 13.3) but this was not statistically significant.

Cu exposure for 72 h at 20 μg Cu l^{-1} increased the total Cu concentration in the gills of non-acclimated fish from 2.96 ± 0.42 μg Cu g^{-1} filaments, found in the controls to 5.07 ± 0.54 μg Cu g^{-1} filaments ($P < 0.006$) (Fig. 4(b)), which is in agreement with earlier results (Grosell et al., 1997). Of the 2.11 μg increase in total Cu concentration, only 0.84 ± 0.07 μg Cu g^{-1} filaments could be accounted for by the newly accumulated Cu during the same period (Fig. 4(a)). Further Cu exposure did not cause any significant increase in the total Cu concentration (Fig. 4(b)), but newly accumulated Cu in acclimated fish (1.40 ± 0.14 μg Cu g^{-1}) was found to be significantly higher ($P < 0.05$) than the corresponding values from non-acclimated fish (Fig. 4(a)).

In the kidneys of control fish we found 7.42 ± 1.00 μg Cu g^{-1} (dry weight) and, as

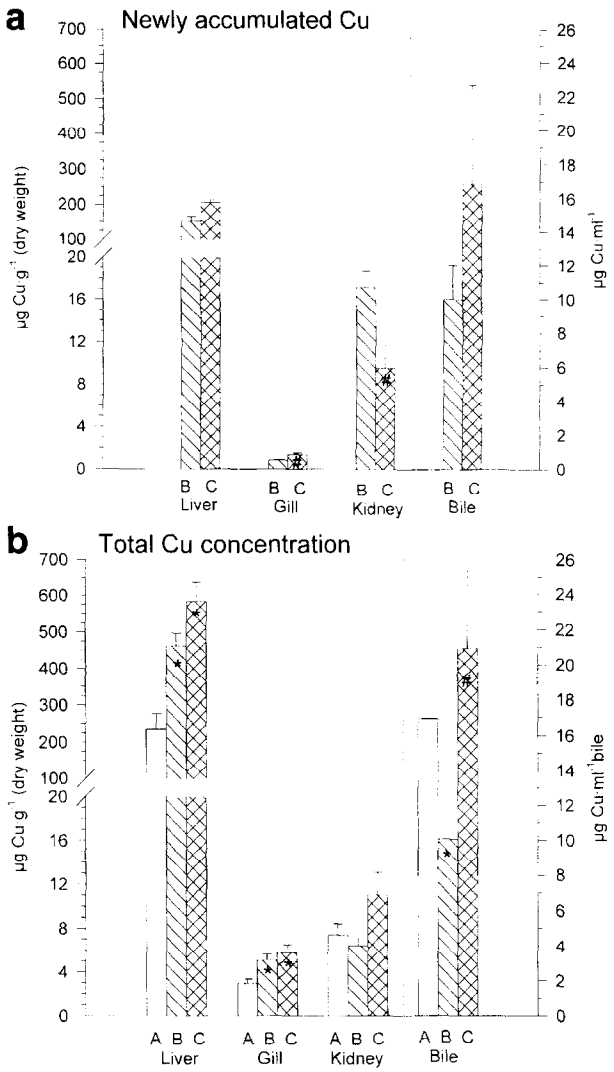

Fig. 4. (a) Newly accumulated Cu in liver, gill filaments, kidney and bile of non-acclimated (group B, hatched bars) and Cu-acclimated (group C, double hatched bars) rainbow trout after 72 h of ^{64}Cu exposure at about 20 μg Cu l^{-1}. Means ± 1 SEM (group B, $n = 14$; group C, $n = 12$). Values for group C were tested statistically against values for group B by an unpaired t-test. Significant differences between groups B and C, at $P < 0.05$, are indicated by #. (b) Total Cu concentrations in liver, gill filaments, kidney and bile of fish in group B (hatched bars) (after 3 days) and group C (double hatched bars) (after 31 days) after Cu exposure at about 20 μg Cu l^{-1}, as well as for the non-exposed controls (group A) (open bars). Means ± 1 SEM (group A, $n = 10$; group B, $n = 14$; group C, $n = 12$). Values for group C were tested statistically against those for group B by an unpaired t-test. A significant difference between groups B and C, at $P < 0.05$, is indicated by #. Values from groups B and C were tested against those from group A by unpaired t-tests; those found to be significantly different, at $P < 0.05$, are indicated by *.

previously reported (Grosell et al., 1997), Cu exposure caused no significant increase in the total Cu concentration (Fig. 4(b)). Despite the lack of total Cu accumulation in the kidneys of fish in both experimental groups we found newly accumulated Cu of 17.17 ± 1.54 μg Cu g^{-1} and 9.56 ± 2.14 μg Cu g^{-1} (dry weight) ($P < 0.05$) in groups B and C, respectively.

In the bile, the total Cu concentration was lowered from 16.93 ± 1.78 μg Cu ml^{-1} bile in the controls to 10.06 ± 0.43 μg Cu ml^{-1} bile in non-acclimated fish ($P < 0.0003$) after 72 h of exposure. Further Cu exposure increased the total Cu concentration in acclimated fish to 20.92+4.47 μg Cu ml^{-1} bile, which was significantly different ($P < 0.0001$) from that of non-acclimated fish, but not controls (Fig. 4(b)). Despite the decreased total Cu concentration observed in non-acclimated fish, there was a relatively high level of newly accumulated Cu of 10.00 ± 1.98 μg Cu ml^{-1} bile which could account for all the total Cu present. Acclimated fish showed 41% higher newly accumulated Cu than non-acclimated fish, but this increase was not statistically significant (Fig. 4(a)).

4. Discussion

The concept of clearance (Koch, 1965; Vander, 1975) is a useful tool for analysing the renal handling of plasma substances. The clearance ratio (CR) relates the clearance (C) of a substance (x) to the glomerular filtration rate (GFR), i.e. to the clearance of an inert marker passing the glomerular–tubular membrane, which is neither secreted nor reabsorbed by the tubules

$$CR_{[x]} = C_x/\mathrm{GFR} \tag{2}$$

where

$$C_x = ([x]\mathrm{u} \times \mathrm{UFR})/[x]\mathrm{p} \tag{3}$$

where u and p refer to urine and plasma, respectively. Thus, by substitution

$$Cr_x = ([x]\mathrm{u} \times \mathrm{UFR})/([x]\mathrm{p} \times \mathrm{GFR}) \tag{4}$$

Assuming that substance x is able to pass the glomerular–tubular membrane, the clearance ratio provides a direct index of how substance x is handled by the tubules. If $CR_x < 1$, then net reabsorption must occur; if $CR_x > 1$, then net secretion must occur, although the index does not quantitatively separate the relative contributions of simultaneous secretory and reabsorptive processes (Koch, 1965).

Freshwater fish in general show a linear relationship between GFR and UFR because a constant proportion of the water filtered at the glomeruli is reabsorbed by the tubules (Hickman, 1965; Hickman and Trump, 1969). This relationship has been validated in rainbow trout over a wide range of urine flows and metabolic rates (Hofmann and Butler, 1979). In the present study, we assumed $\mathrm{GFR} = 1.74 \times \mathrm{UFR}$, based on experiments conducted on trout obtained from the same supplier and kept under conditions similar to those used in the present inves-

tigation (Curtis and Wood, 1991). Note that the following conclusions are based on calculations using mean values and that the newly accumulated and total Cu clearance data were calculated from data obtained from two similar but separate sets of experiments (present study and Grosell et al., 1997). As a consequence, the individual CR and C values are presented without indications of variation and no statistical evaluation has been carried out.

Urinary Na^+ loss was reduced by 40% during chronic exposure to about 20 μg Cu l^{-1}—a decrease which was much greater than the observed 5–8% decrease in plasma Na^+ concentrations (Table 1). This suggests some adaptation in the renal tubular handling of Na^+. Renal clearance ratios for Na^+ calculated according to Eq. 3 are presented in Fig. 3. The control value of 0.025 means that there was a 97.5% net reabsorption of Na^+, which is in close agreement with earlier results obtained from freshwater rainbow trout (Wheatly et al., 1984). During the 72 h of Cu exposure of non-acclimated fish, CR_{Na+} was reduced by 24% to 0.019, indicating that increased reabsorption of Na^+ was the main factor responsible for the observed decrease in renal Na^+ loss. CR_{Na+} remained at 0.019 after an additional 28 days of exposure, indicating that the adaptation of tubular function was complete by 72 h of exposure. Since Cu exposure impairs branchial Na^+ uptake (Laurén and McDonald, 1985), the observed increased renal Na^+ reabsorption can be regarded as a way of restoring the reduced plasma Na^+ levels in the fish during chronic Cu exposure (Table 1). Thus, the reduction in renal Na^+ loss, and restoration of branchial Na^+ uptake (Laurén and McDonald, 1987a,b), are both important as part of the Cu acclimation process.

Acclimation to Cu did not involve any increase in renal total Cu excretion, contrary to our hypothesis (Grosell et al., 1997; Section 1). Like the newly accumulated Cu in the kidney (Fig. 4(a)), the calculated newly accumulated Cu excretion in the urine (Fig. 2(a)) exceeded the total Cu excretion measured in the same samples (Fig. 4(b)Fig. 2(b), respectively). This means that the newly accumulated Cu pool was more accessible for renal accumulation and excretion than the Cu present in the plasma prior to ^{64}Cu exposure.

From higher vertebrates it is known that plasma Cu is distributed among three major constituents comprising two pools, between which it does not appear to be very exchangeable (Cousins, 1985).

1. Ceruloplasmin is a plasma protein which specifically binds Cu tightly and accounts for around 90% of the total plasma Cu in most species (Gubler et al., 1953; Henkins, 1974; Harris, 1991).
2. Albumin- and copper-binding amino acids constitute a less tightly bound pool and are believed to be the ligands that transport copper from the uptake sites (the gut in higher vertebrates) to the hepatocytes (Marceau and Aspin, 1973; Frieden, 1980; Weiner and Cousins, 1983; Cousins, 1985).

Evidence for two different plasma Cu pools in fish, which again do not appear to be exchangeable, has been given by Carbonell and Tarazona (1994), who reported two highly different elimination half-lives of 7.2 and 195.5 min for Cu after intravenous Cu injection of rainbow trout. Ceruloplasmin has been found in several fish species, including carp *Cyprinus carpio* (Siwicki and Studnicka, 1986), plaice *Pleuro-*

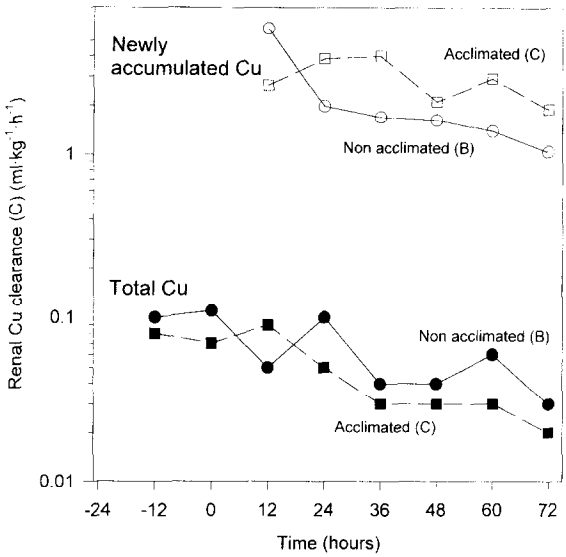

Fig. 5. Time-dependent alterations in the renal clearance of total Cu (■ and ●) and newly accumulated Cu (□ and ○) in non-acclimated (○ and ●) and Cu-acclimated (□ and ■) rainbow trout. The clearance calculations are described in the text. Plasma data were taken from Grosell et al. (1996). Note that the clearance scale is logarithmic.

nectes platessa (Syed and Coombs, 1986), mullet *Mugil cephalus* (Cogoni et al., 1990), tilapia *Oreochromis mossambicus* (Pelgrom et al., 1995) and European eel *Anguilla anguilla* (M.H. Grosell, 1995, unpublished data) so it is likely to be present in rainbow trout as well.

Therefore, much of the plasma Cu in rainbow trout is likely to be protein bound. As a consequence, we cannot calculate *CR* values for the newly accumulated and total Cu pools of the plasma, as we do not know the relative fractions which are filterable at the glomeruli. Instead, we have used the clearance (*C*) (Eq. 3) to evaluate the accessibility of the newly accumulated and total Cu pools to renal excretion; a logarithmic scale has been employed to encompass the wide range of *C* values observed (Fig. 5).

Renal clearances of newly accumulated Cu and total Cu were highly different (Fig. 5). The clearance of total Cu was very low (0.11–0.02) and did not differ substantially between non-acclimated and acclimated fish. The clearance of newly accumulated Cu was 10–300 times higher than the corresponding clearance of total Cu and appeared to be different between treatments, in contrast to the lack of effect of acclimation on the clearance of total Cu (Fig. 5). Non-acclimated fish exhibited a relatively high clearance of newly accumulated Cu during the first 12 h of exposure (6.0 ml kg^{-1} h^{-1}) and a reduced clearance with time during the entire 72 h of exposure. Acclimated fish had a relatively constant clearance of newly accumulated Cu (1.9–4.0 ml kg^{-1} h^{-1}) which was about twice as high as that of non-acclimated fish, apart from the first sampling period.

The large difference observed between the clearance of newly accumulated and total Cu pools probably reflects different protein binding of the two Cu pools. The low clearance of total Cu could be due to a tight binding to ceruloplasmin (as in higher vertebrates, cf. above) which is in close agreement with earlier findings (Grosell et al., 1997) showing that only 6–16% of plasma Cu was exchangeable. The higher clearance of the newly accumulated Cu pool probably reflects a weaker binding to albumin and amino acids (again referring to higher vertebrates), leaving the newly accumulated Cu pool more accessible to renal excretion. This would support the hypothesis that the newly accumulated Cu, recently taken up by the gills, is loosely bound to albumin and amino acids while the Cu already present in the plasma, prior to ^{64}Cu exposure, is tightly bound to ceruloplasmin.

Several factors can alter UFR; recovery from anaesthesia can, in some cases, lead to decreased UFR (for a review see Wood, 1995), which could be one reason for the generally reduced UFR during the experimental period in both experimental groups (Fig. 1). The experimental procedure could explain the generally reduced renal total Cu excretion during the experiment (Fig. 2(b)) in a similar way. Plasma levels of ceruloplasmin have been shown to be sensitive to a variety of factors in higher vertebrates, e.g. stress and exercise (for a review see Cousins, 1985). The handling of the fish involved in the present experiment could have increased the plasma ceruloplasmin levels and thereby reduced the accessibility of the total Cu to the kidney (by the tight Cu binding), leading to reduced renal total Cu excretion.

Branchial newly accumulated Cu (Fig. 4(a)) values were comparable to values reported previously for non-catheterised rainbow trout, but considerably lower those from rainbow trout fitted with dorsal aorta catheters (Grosell et al., 1997). In the present investigation, chronic Cu exposure led to increased newly accumulated Cu, which has also been reported for coho salmon (McCarter and Roch, 1984). This is in contrast to earlier findings in rainbow trout, where acclimation to waterborne Cu exposure in one case did not influence, and in another case resulted in a decreased branchial newly accumulated Cu (Grosell et al., 1996). It is also in contrast to results from European eels, where branchial newly accumulated Cu was constant during 28 days of waterborne exposure to two different Cu concentrations (Grosell et al., 1996). Nothing conclusive can thus be said, but it appears that the increased tolerance to Cu during acclimation does not involve dramatic changes in the branchial Cu uptake in contrast to findings for zinc (Bradley et al., 1985; Hogstrand et al., 1994, 1995), cadmium (Wicklund-Glynn and Olsson, 1991) and aluminium (Reid et al., 1991). The total Cu concentration in the gills of non-acclimated and acclimated fish was in agreement with earlier findings (Grosell et al., 1997) and did not differ between the two experimental groups. This means that Cu in the gills was turned over at a high rate, since a constant net branchial Cu uptake did not lead to further Cu accumulation.

Also in agreement with earlier results (Grosell et al., 1997), the kidney did not exhibit an increase in total Cu concentration (Fig. 4(b)) despite the high level of newly accumulated Cu (Fig. 4(a)), which means that there was a rapid turnover of Cu in the kidney.

The increased hepatic total Cu concentration in non-acclimated fish could be

accounted for by the newly accumulated Cu, in agreement with earlier findings (Grosell et al., 1997). This suggests that the liver accumulates equally from two plasma Cu pools. As before, further Cu exposure did not increase the hepatic total Cu concentration (Fig. 4(b)) in acclimated fish, despite a high level of newly accumulated Cu (Fig. 4(a)), which showed that 36% of the hepatic Cu pool (newly accumulated [Cu]/total [Cu]) was exchangeable and available for elimination within 72 h. The hepatic MT concentration was increased by 52% in acclimated fish compared with the controls, in agreement with 46% in the earlier experiments (Grosell et al., 1997). The fraction of the hepatic Cu pool that can be accounted for by MT can be calculated as previously described by Grosell et al. (1996) to be no more than 12% in acclimated fish and 17% in controls, somewhat lower than values of 20–30% reported by Hogstrand (1991). These differences can be partly explained by the lower hepatic MT levels found in fish in the present study and by Grosell et al. (1997). Since the values reported by Hogstrand (1991) were obtained from fish exposed for up to 85 days, the lower MT values in the present experiment could perhaps reflect a shorter period of exposure.

Considering the large exchangeable hepatic Cu pool, it is not surprising to find high concentrations of both newly accumulated and total Cu in the bile. In the present study the newly accumulated Cu in the bile accounts almost perfectly for the total Cu concentration found in the same samples. The Cu in the bile is highly concentrated compared to the plasma; 13–28 and 130–430 times for the total and newly accumulated Cu, respectively, which means that hepatic Cu elimination and biliary Cu excretion can be very effective in restoring the initially elevated plasma Cu levels previously reported (Grosell et al., 1997). By using reported bile flow rates from rainbow trout of 50 to 75 $\mu l \cdot kg^{-1} \cdot h^{-1}$ (Schmidt and Weber, 1973; Gingerich et al., 1977) combined with the total Cu concentrations found in the bile in the present experiment, we can calculate the biliary hepatic elimination of Cu to be 0.5–0.75 and 1.1–1.6 $\mu g\ Cu \cdot kg^{-1} \cdot h^{-1}$ for the non-acclimated (B) and the acclimated fish (C), respectively. The hepatic Cu excretion thus seems to be 20–30 and 50–100 times higher than the renal Cu excretion presented in Fig. 2b in the experimental groups (B) and (C). Apparently the hepatic total Cu elimination decreases initially (group B) during Cu exposure (Fig. 4b) and then increases in the acclimated fish (C) in this case to values higher than those of the control (A). The initially decreased hepatic Cu elimination (and also the decreased renal Cu excretion) in the non-acclimated fish (B) could be due to an acutely increased hepatic Cu sequestration, as a primary defence system, elicited by Cu exposure, which initially immobilises hepatic Cu rendering it less available for biliary secretion. In the acclimated fish, on the other hand, the Cu taken up by the liver does not cause a significantly increased hepatic Cu concentration (Fig. 4a and 4b) and seems to be excreted to a higher degree.

The estimations of hepatic Cu excretion presented above are based only on the concentration of Cu in the bile and the bile flow but do not consider possible reabsorption or secretion by the intestine. Clearly, more detailed work is needed on the hepatic and gastrointestinal elimination of Cu.

The conclusions that can be drawn from the present study are that Cu metabolism in rainbow trout during Cu exposure involves renal and hepatic Cu excretion.

Renal Cu excretion was not stimulated during Cu acclimation and seems to be of minor importance compared with hepatic Cu excretion, which was apparently stimulated during acclimation. Plasma Cu seems to be distributed into at least two pools which are accessible to renal excretion to different extents but which are equally accessible to hepatic accumulation and elimination. Furthermore, Cu acclimation of rainbow trout involves decreased renal Na^+ excretion, largely due to increased tubular Na^+ reabsorption, which contributes to a restoration of normal concentrations of plasma Na^+.

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References

Beyenbach, K.W., Kirschner, L.B., 1975. Kidney and urinary bladder functions of the rainbow trout Mg and Na excretion. Am. J. Physiol. 229, 389–393.

Bradley, R.W., DuQuesnay, C., Sprague, J.B., 1985. Acclimation of rainbow trout, *Salmo gairdneri*, Richardson, to zinc: Kinetics and mechanism of enhanced tolerance induction. J. Fish Biol. 27, 367–379.

Carbonell, G., Tarazona, J.V., 1994. Toxicokinetics of copper in rainbow trout (*Oncorhynchus mykiss*). Aquat. Toxicol. 29, 213–221.

Chakoumakos, C., 1979. Toxicity of copper to cutthroat trout (*Salmo clarki*) under different conditions of alkalinity, pH and hardness. Environ. Sci. Technol. 13, 213–219.

Cogoni, A., Farci, R., Cau, A., Melis, A., Medda, R., Floris, G., 1990. Mullet plasma: serumamine oxidase and ceruloplasmin: purification and properties. Comp. Biochem. Physiol. 95C, 297–300.

Cousins, R.J., 1985. Absorption, transport, and hepatic metabolism of copper and zinc: Special reference to metallothionein and ceruloplasmin. Physiol. Rev. 65, 238–309.

Curtis, B.J., Wood, C.M., 1991. The function of the urinary bladder in vivo in the freshwater rainbow trout. J. Exp. Biol. 155, 567–583.

Cusimano, R.F., Brakke, D.F., Chapman, G.A., 1986. Effects of pH on the toxicities of cadmium, copper and zinc to steelhead trout (*Salmo gairdneri*). Can. J. Fish. Aquat. Sci. 43, 1497–1503.

Frieden, E., 1980. Ceruloplasmin: a multi-functional metalloprotein of vertebrate plasma. In: Evered, D., Lawrenson, G., Biological Roles of Copper. Elsevier/North-Holland, New York, pp. 98–124.

Gingerich, W.H., Weber, L.J., Larson, R.E., 1977. Hepatic accumulation, metabolism and biliary excretion of sulfobromophthalein by rainbow trout (*Salmo gairdneri*). Comp. Biochem. Physiol. 58C, 113–120.

Grosell, M., Boëtius, I., Hansen, H.J.M., Rosenkilde, P., 1996. Influence of preexposure to sublethal levels of copper on ^{61}Cu uptake and distribution among tissues of the European eel (*Anguilla anguilla*). Comp. Biochem. Physiol. 114C, 229–235.

Grosell, M.H., Hogstrand, C., Wood, C.M., 1997. Cu uptake and turnover in both Cu acclimated and non acclimated rainbow trout (*Oncorhynchus mykiss*). Aquat. Toxicol. 38(4), 257–276.

Gubler, C.J., Lahey, G.R., Cartwright, G.E., Wintrobe, M.M., 1953. Studies of copper metabolism IX. The transport of copper in the blood. J. Clin. Invest. 32, 405–414.

Harris, E.D., 1991. Copper transport: an overview. Proc. Soc.Exp. Med. 192, 130–140.
Henkins, R.I., 1974. Metal–albumin–amino acid interactions: chemical and physiological relationships. In: Friedman, M. (Ed.), Protein–metal Interactions. Plenum Press, New York, pp. 299–328.
Hickman Jr., C.P., 1965. Studies on renal function in freshwater teleost fish. Trans. R. Soc. Can. 3, 213–236.
Hickman Jr., C.P., Trump, B.F., 1969. The kidney. In: Hoar, W.S., Randall, D.J. (Eds.), Fish Physiology, Vol. 1. Academic Press, New York.
Hofmann, E.L., Butler, D.G., 1979. The effect of increased metabolic rate on renal function in the rainbow trout, *Salmo gairdneri*. J. Exp. Biol. 82, 11–23.
Hogstrand, C., 1991. Regulation of metallothionein in teleost fish during metal exposure. Ph.D. Dissertation, University of Göteborg, Sweden, 176 pp.
Hogstrand, C., Haux, C., 1990. A radioimmunoassay for perch (*Perca fluviatilis*) metallothionein. Toxicol. Appl. Pharmacol. 103, 56–65.
Hogstrand, C., Wilson, R.W., Polgar, D., Wood, C.M., 1994. Effects of zinc on the kinetics of branchial calcium uptake in freshwater rainbow trout during adaptation to waterborne zinc. J. Exp. Biol. 186, 55–78.
Hogstrand, C., Reid, S.D., Wood, C.M., 1995. Ca^{2+} versus Zn^{2+} transport in the gills of the freshwater rainbow trout and the cost of adaptation to waterborne Zn. J. Exp. Biol. 198, 337–348.
Koch, A., 1965. Kidney function and body fluids. In: Ruch, T.C., Patton, H.D. (Eds.), Physiology and Biophysics. W.B. Saunders, Philadelphia.
Laurén, D.J., McDonald, D.G., 1985. Effects of copper on bronchial ionoregulation in the rainbow trout, *Salmo gairdneri* Richardson. J. Comp. Physiol. 155B, 635–644.
Laurén, D.J., McDonald, D.G., 1987a. Acclimation to copper by rainbow trout, *Salmo gairdneri*: Physiology. Can. J. Fish. Aquat. Sci. 44, 99–104.
Laurén, D.J., McDonald, D.G., 1987b. Acclimation to copper by rainbow trout, *Salmo gairdneri*: Biochemistry. Can. J. Fish. Aquat. Sci. 44, 105–111.
Marceau, N., Aspin, N., 1973. The intracellular distribution of the radiocopper derived from ceruloplasmin and from albumin. Biochem. Biophys. Acta 293, 228–350.
McCarter, J.A., Roch, M., 1983. Hepatic metallothionein and resistance to copper in juvenile coho salmon. Comp. Biochem. Physiol. 74C, 133–137.
McCarter, J.A., Roch, M., 1984. Chronic exposure of coho salmon to sublethal concentrations of copper—III. Kinetics of metabolism of metallothionein. Comp. Biochem. Physiol. 77C, 83–87.
McDonald, D.G., 1983. The interactions of calcium and low pH on the physiological responses of the rainbow trout, *Salmo gairdneri*, I. Branchial and renal net ion and H^+ fluxes. J. Exp. Biol. 102, 123–140.
Pelgrom, S.M.G.J., Lock, R.A.C., Balm, P.H.M., Wendelaar Bonga, S.E., 1995. Integrated physiological response of tilapia, *Oreochromis mossambicus*, to sublethal copper exposure. Aquat. Toxicol. 32, 303–320.
Reid, S.D., Rhem, R.G., McDonald, D.G., 1991. Acclimation to sublethal aluminum: modifications of metal–gill surface interactions of juvenile rainbow trout (*Oncorhynchus mykiss*). Can. J. Fish. Aquat. Sci. 48, 1996–2005.
Schmidt, D.C., Weber, L.J., 1973. Metabolism and biliary excretion of sulfobromophthalein by rainbow trout (*Salmo gairdneri*). J. Fish. Res. Board Can. 30, 1301–1308.
Siwicki, A., Studnicka, M., 1986. Ceruloplasmin activity in carp (*Cyprinus carpio* L.). Bamidgeh 38, 126–129.
Syed, M.A., Coombs, T.L., 1986. Copper metabolism in the plaice, *Pleuronectes platessa* (L.), some physiological properties of ceruloplasmin. Contemp. Themes Biochem. 6, 332–333.
Vander, A.J., 1975. Renal Physiology. McGraw-Hill, New York.
Weiner, A.L., Cousins, R.J., 1983. Hormonally produced changes in ceruloplasmin synthesis and secretion in primary cultured rat hepatocytes. Biochem. J. 212, 297–304.
Wheatly, M.G., Hobe, H., Wood, C.M., 1984. The mechanisms of acid–base and ion regulation in the freshwater rainbow trout during environmental hyperoxia and subsequent normoxia II. The role of the kidney. Resp. Physiol. 55, 155–173.

Wicklund-Glynn, A., Olsson, P.-E., 1991. Cadmium turnover in minnows, *Phoxinus phoxinus*, pre-exposed to waterborne cadmium. Environ. Toxicol. Chem. 10, 383–394.

Wood, C.M., 1995. Excretion. In: Groot, C., Margolis, C., Claike, W.C. (Eds.), Physiological Ecology of Pacific Salmon. U.B.C. Press, Vancouver, pp. 381–438.

Wood, C.M., Randell, D.J., 1973. The influence of swimming activity on water balance in the rainbow trout (*Salmo gairdneri*). J. Comp. Physiol. 82, 257–276.