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Integrated physiological response of tilapia, *Oreochromis mossambicus*, to sublethal copper exposure

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Abstract

Juvenile and mature tilapia (*Oreochromis mossambicus*) were exposed to a range of sublethal copper (Cu) concentrations for 6 days to examine the mechanisms underlying the acclimation to the toxic effects of the metal. The study focuses on the gills, the primary target for waterborne pollutants. To obtain a comprehensive picture of the branchial acclimation processes operating, multiple biochemical and morphological parameters were studied. At all concentrations tested, Cu exposure resulted in the accumulation of the metal in mature fish. At 100 and 200 μg Cu l^{-1} only, chloride cell proliferation was observed, which was accompanied by an increase in average cell diameter in these groups. Whole body flux measurements in juvenile fish demonstrated a decrease in Na influx in fish exposed to 200 μg l^{-1} Cu, in the absence of an effect on Ca influx. Gill Na^+/K^+-ATPase activity was also decreased in the crude branchial homogenates of the mature fish exposed to the highest Cu concentration only, but not in the purified branchial vesicle preparations of these fish, which may indicate reactivation of in vivo Cu-inhibited ATPase activity during the isolation process. Plasma pH, Na, Cl, K, glucose and ceruloplasmin concentrations were also affected in the 200 μg Cu l^{-1} group exclusively. In accordance with the gill accumulation data, plasma Cu levels were clearly elevated in all groups exposed to the metal. The results underscore the integrated response of the gills to Cu, which, however, does not come into play until challenged by relatively high ambient concentrations. These results indicate that, in comparison to the Cu-sensitive rainbow trout, tilapia is more Cu-tolerant. The most sensitive parameters affected by Cu are gill and plasma metal levels, followed by chloride cell number and diameter.

Keywords: Copper; Chloride cells; Na^+/K^+-ATPase activity; *Oreochromis mossambicus;* Physiological response; Ceruloplasmin

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1. Introduction

Copper (Cu) plays an essential role in cellular metabolism (Prasad, 1984; Cousins, 1985), but becomes toxic at elevated levels. As in mammals, in most fish the liver is an important storage organ for excess Cu (Buck, 1978; Shearer, 1984). However, the major difference in Cu toxicity between mammals and fish concerns the uptake from the environment, which in fish almost exclusively occurs through the gills (Stagg and Shuttleworth, 1982a; Norey et al., 1990; Battaglini et al., 1993). This organ in fish is the primary site of toxic insult and considered the key organ when it comes to the initiation of compensatory responses (Laurén and McDonald, 1985, 1987). Although many studies have been performed into the toxic effects of Cu on gill function (McKim et al., 1970; Stagg and Shuttleworth, 1982a,b; Laurén and McDonald, 1985, 1986, 1987; Reid and McDonald, 1988; Sayer et al., 1991), information regarding acclimation of gill function to the metal (the ability to compensate for disturbances of the ion homeostasis) is more scarce, and largely limited to one species, *Oncorhynchus mykiss* (reviewed by McDonald and Wood, 1993). Most of the studies cited herein investigated one or two parameters of gill function, largely focusing on the chloride cells (Stagg and Shuttleworth, 1982a,b; Laurén and McDonald, 1985, 1986; Reid and McDonald, 1988). Because acclimation to Cu probably requires an integrated response involving multiple regulatory mechanisms, we were prompted to investigate the effects of Cu in a somewhat broader context, relating data on the accumulation of the metal in the gills, clearance of the metal in the blood (plasma Cu and ceruloplasmin levels), with biochemical and morphological data on gill function. For this study we used *Oreochromis mossambicus* (tilapia) for several reasons. Firstly, the picture for *Oncorhynchus mykiss* (McDonald and Wood, 1993) might not be representative for other fish species; a possibility underscored by the species differences in adaptive strategy to other environmental challenges, such as low pH (Balm and Pottinger, 1993). Secondly, previous work on tilapia has characterized branchial ion transport mechanisms (Flik et al., 1985), and has yielded a variety of in vitro and in vivo experimental approaches, which in this species can be applied to small sized specimens, thereby allowing the rapid collection of data and the reduction of experimental stress factors (Pelgrom et al., 1994). To further limit aspecific experimental influences (Pelgrom et al., 1994), fish were exposed to Cu gradually, rather than acutely, and were studied after 6 days. It was anticipated that at this time point Cu metabolism will have reached a new set point, judged by data from Carbonell and Tarazona (1994). Ultimately, the combination of experimental approaches should allow a comprehensive discussion into the physiological response of the gills of tilapia to copper.

2. Materials and methods

Fish

Tilapia, *Oreochromis mossambicus*, were obtained from our laboratory stock. Fish were grown and held under artificial freshwater conditions with undetectable Cu concentrations (detection level 0.1 μg l^{-1}). Artificial freshwater consisted of deminer-

alized water supplemented with 1.3 mM $NaHCO_3$, 0.5 mM $CaCl_2$, 0.06 mM KCl and 0.2 mM $MgCl_2$, at pH 7.8. Composition and preparation of the water was based on the EEC instructions for artificial water for use in toxicity studies in fish (EEC Directives 84/449/EEC Annex 5 method c1: Acute toxicity for fish). Water was continuously aerated, filtered and refreshed by means of flow-through. The light/dark regime was 12/12 h and the water temperature 26°C. Fish were fed commercial tropical fishfood Tetramin™, 2% (dw/ww) of their body weight per day. The Cu content of the food was 9.86 ± 0.16 μg Cu g^{-1} (means ± s.e.; $n = 10$).

Whole body Na and Ca fluxes

Pilot experiments were performed to determine optimal experimental conditions: Cu exposure regime, conditions of the fish (density, size, acclimation period), water quality during the flux periods (pH, temperature, nitrate and ammonium concentrations), anaesthetic concentration, tracer injection volume, interaction between $CaCl_2$ and Na_2CO_3, peroxide digestion and rinsing of the fish after radiotracer exposure (data not shown).

Three days before the start of the experiment, 12 groups of 9 tilapia (weighing 1–2 g, about 2 months old) were placed randomly in 3.2-l flux chambers filled with artificial freshwater. Fish were fed daily (2% dw/ww Tetramin™). The food was eaten within 1 min. During the acclimation period, the water in the flux chambers was continuously aerated and refreshed by means of a flow-through system (flow rate 0.24 l h^{-1}; 16-channel peristaltic pump, Watson Marlow). The exposure period started with the connection of each flux chamber to reservoirs filled with artificial freshwater each with a well-defined Cu concentration (added as nitrate, Spectrosol, BDH, UK). During the first 4 h of the exposure, the flow rate was 0.90 l h^{-1}, followed by a flow rate of 0.24 l h^{-1} during the rest of the exposure period. Cu concentrations in the reservoirs were monitored daily. The Cu concentrations in the flux chambers were monitored every hour during the first 6 h of exposure, and at least once a day during the rest of the exposure period. The Cu concentrations in the flux chambers were: 0 (control), 50 (50Cu) and 200 (200Cu) μg Cu l^{-1}. The actual Cu concentrations measured did not deviate more than 5% from the nominal Cu concentrations. After 6 days of Cu exposure, Na^+ and Ca^{2+} influx and efflux were determined by means of radiotracers.

For measurement of Na^+ and Ca^{2+} influx, 1.0 MBq l^{-1} $^{24}Na_2CO_3$ (IRI, Delft, Netherlands) and 0.75 MBq l^{-1} $^{45}CaCl_2$ (Amersham, UK) were added to the flux chambers. $^{24}Na_2CO_3$ was neutralized to pH 7.5 with equimolar concentrations of hydrochloric acid. After 5, 20 and 45 min, water samples for tracer measurement were taken. After 45 min of tracer exposure, fish were quickly (within 1 min) anaesthetized with phenoxy-ethanol in a final dilution of 1:400. Of each flux chamber, all 9 fish were briefly (2 seconds) rinsed in artificial freshwater containing 5mM Ca and 10 mM Na, followed by a rinse in artificial freshwater. Subsequently, 4 fish were immediately killed in dry ice/acetone for determination of whole body Na^+ and Ca^{2+} influx.

To investigate Na^+ and Ca^{2+} efflux, the remaining 5 fish of each flux chamber were injected (i.p.) with 0.17 MBq $^{24}Na_2CO_3$ (neutralized with HCl to pH 7.5) and 0.12 MBq $^{45}CaCl_2$. Fish were allowed to recover from anaesthesia in freshwater with the

same Cu concentrations as they were exposed to, and put back in their flux chambers containing radiotracer-free artificial freshwater with Cu. Fish recovered from anaesthesia within 1 min after injection as indicated by a feeding response. The whole procedure took less than 5 min per flux chamber. Overnight, the flux chambers were continuously refreshed (0.24 l h^{-1}). During efflux measurement, the water-flow was stopped, and tracer appearance in the water was monitored for 4 h. After this period, fish were anaesthetized (phenoxy-ethanol 1:400) and rinsed in artificial freshwater. Blood from the caudal vessels was taken by means of heparinized mini-capillaries (Hirschmann). After centrifugation (3 min 18 000 *g*), plasma radiotracer concentration was determined in triplicate for each fish.

^{24}Na in whole fish, plasma and water was immediately determined in a γ-counter (LKB). After one week (11 times the half-life of ^{24}Na), no ^{24}Na could be detected in the samples. Then, scintillation fluid was added to the water and blood samples for ^{45}Ca determination. The fish were digested with peroxide (35%; 4 times 100 μl) at 40°C for 3 days, and 1 day at 60°C, and the digests were dissolved in scintillation fluid. ^{45}Ca was determined in water, blood and fish samples by means of a liquid scintillation counter (Pharmacia Wallac 1410).

Influx of Ca^{2+} and Na^{+} was calculated on the basis of the total body radioactivity after 1 h of exposure to ^{24}Na and ^{45}Ca, and the respective mean tracer specific activities in the water. For this calculation we assumed that during the influx period no significant backflux from the fish to the water occurred. Efflux of Ca^{2+} and Na^{+} was calculated from the tracer activities in the water and the specific Ca and Na activities in the plasma. In the exposed fish, the plasma concentrations of Ca and Na were determined to calculate the specific Ca and Na activities in the plasma. The net flux is given as the difference between average influx and efflux in each flux chamber.

Experimental design of experiments with mature fish

Six weeks before the start of the experiment four groups of 14 mature (mean weight 20 g) female tilapia were kept in 80-l aquaria with continuously filtered and refreshed artificial freshwater. The experiment started by connecting (by means of a 16 channel peristaltic pump; Watson Marlow) each aquarium to its own reservoir filled with artificial freshwater with or without (controls) a well-defined Cu concentration (added as nitrate; spectrosol, BDH, UK). During the first 6 h the flow rate was 4.5 l h^{-1}, followed by a flow rate of 1.5 l h^{-1}. In this way, the Cu concentrations in the aquaria were gradually raised, reaching a plateau after 18 h. Cu concentrations in both stock solutions and aquaria were monitored every hour during the first 6 h, and at least once a day during the rest of the exposure period. The nominal Cu concentrations in the aquaria were 0, 50, 100 and 200 μg l^{-1}, with the actual concentrations deviating maximally 5% from nominal concentrations. The exposure period lasted 6 days, and feeding was ended one day before sacrifice. At the end of the exposure period, blood samples were taken from the caudal blood vessels by means of heparinized capillaries, and fish were killed by spinal dissection. Blood cells and plasma were separated by centrifugation (3 min 18 000 *g*). The left opercula were prepared for chloride cell counting with DASPEI vital staining (Wendelaar Bonga et al., 1990), and the gill

arches were prepared for either Cu measurement or plasma membrane isolation. Dissection instruments were systematically cleaned with 0.1% HNO_3 and alcohol to prevent contamination.

Cu measurement

After determination of wet and dry weights, gills, blood cells and plasma were destructed with nitric acid (65% HNO_3 ultrapur, Merck), and stored in 0.2% HNO_3 at 4°C until analysis. Water samples were acidified with HNO_3 to a final concentration of 0.2%. Cu concentrations were determined with a flameless Atomic Absorption Spectrometer (AAS, Philips PU 9200) connected to an electrothermal atomizer (Philips PU 9390X).

Isolation of plasma membranes

Plasma membranes of the branchial epithelia were isolated at 4°C as described by Flik et al. (1985), with some adjustments. This procedure leads to a good enrichment of the Na^+/K^+-ATPase and Ca^{2+}-ATPase: the vesicles are leaky and the degree of mitochondrial contamination is low.

Briefly, the soft tissue of the gills was scraped off with a glass microscope slide, and carefully homogenized with a glass-to-glass Dounce homogenizer (10 strokes) in an isotonic buffer containing 250 mM sucrose, 12.5 mM NaCl, 5 mM HEPES/TRIS pH 7.5, 0.1 mM EDTA, 100 U ml^{-1} aprotinin (Sigma) and 50 U ml^{-1} heparin. Nuclei and cellular debris (pellet P_0) were separated from membrane fractions (supernatant H_0) by centrifugation for 10 min at 550 *g* (Hereus). After centrifugation of the supernatant H_0 (50 000 rpm, 30 min; Beckmann Ultracentrifuge, Ti 70 rotor), membranes were collected in a fluffy pellet (P_1). This pellet was resuspended with a glass-to-glass Dounce homogenizer (100 strokes) in an isotonic sucrose buffer containing 250 mM sucrose, 5 mM HEPES/TRIS pH 7.5 and 5 mM $MgCl_2$. The membrane suspension was centrifuged differentially: 10 min at 1000 *g* followed by 10 min at 9500 *g* (Sorval RC-5B). Finally, the supernatant was centrifuged for 15 min at 20 000 *g*, resulting in the final membrane fraction, pellet P_3. These pellets P_3 were resuspended by passage through a 23-G needle (10 times) in a buffer containing 20 mM HEPES/TRIS pH 7.4, 1.5 mM $MgCl_2$ and 150 mM KCl (for Ca^{2+} transport studies) or 150 mM NaCl (for Na^+/K^+- ATPase studies). Membrane preparations P_3 and crude membrane homogenates H_0 were quickly frozen in cold CO_2/acetone, and used the next day for determination of protein content, protein recovery in the P_3 fraction relative to the protein content in the H_0 fraction, enzyme activity and transport activity.

Na^+/K^+-ATPase activity

Na^+/K^+-ATPase activity in the H_0 and P_3 gill membrane fractions was determined by the method described by Flik et al. (1985). Routinely, 0.20 mg ml^{-1} saponin was added to optimize substrate accessibility. Membrane protein content was determined with a reagent kit (Biorad), using Bovine Serum Albumin (BSA, Sigma) as reference. Vesicles were incubated for 10 min at 37°C with medium containing 100 mM NaCl, 30 mM Imidazole, 0.1 mM EDTA, 5 mM $MgCl_2$ and either 15 mM KCl or 1 mM ouabain. Na_2ATP was added in a final concentration of 3 mM. The reaction was

stopped by adding ice-cold TCA solution. Inorganic phosphate (P_i) production, liberated from ATP, was measured by the colorimetric Fiske-Subbarow technique using a commercial (Sigma) phosphate standard (Flik et al., 1985). Total Na^+/K^+-ATPase activities (V_{tot}) are expressed as μmol P_i h^{-1} and specific activities (V_{spec}) are expressed as μmol P_i h^{-1} mg^{-1} protein.

Ca^{2+} transport

ATP-dependent Ca^{2+} transport was determined by means of a rapid filtration technique as described by Van Heeswijk et al. (1984). Ca^{2+} and Mg^{2+} concentrations were calculated according to Schoenmakers et al. (1992) using the computer program CHELATOR. Ca^{2+} transport was measured at a Ca^{2+} concentration of 10^{-6} M (V_{max}). Uptake of ^{45}Ca into membrane vesicles (P_3 fraction) was determined during 1-min incubations without or in the presence of 3 mM ATP (Tris-ATP). The reaction was stopped in ice-cold isotonic medium containing 0.1 mM $LaCl_3$, and the suspension was filtered (Schleicher & Schüll ME 25, pore size 0.45 μm). Filters were rinsed twice with ice-cold medium and transferred to counting vials and dissolved in Aqualuma®. ^{45}Ca was determined in a Pharmacia Wallac 1410 liquid scintillation counter.

Plasma

Plasma protein concentrations were determined by means of a reagent kit (Biorad) with BSA as reference. Plasma glucose was determined spectrophotometrically using a D-glucose kit (Boehringer Mannheim, UV method). Concentrations of plasma Na and K were measured with a flame-photometric Auto Analyzer (Model IV, Technicon), while the Cl concentration was determined spectrophotometrically by the forming of ferrothiocyanate. The cresolphthalein complexone method (Sigma Diagnostics) was used for the determination of total plasma Ca concentration. Ca^{2+} and pH were measured by means of an Ionic Calcium analyzer (Radiometer) as described by Fogh-Anderson (1981).

Plasma ceruloplasmin concentration was measured as *p*-phenylenediamine (PPD) oxidase activity, an assay based on the methods described by Houchin (1958) and Rice (1961). To validate the method of ceruloplasmin detection in plasma of tilapia, several parameters of the assay were tested: (a) substrates PPD and N-N-dimethyl-PPD; (b) incubation time (between 0 and 75 min); (c) the pH of the buffer (between pH 4 and 10); (d) incubation temperatures (4°, 20°, 26°, 37°C); (e) the plasma volume (between 0 and 100 μl).

From the results of these tests, ceruloplasmin concentrations in plasma of tilapia were measured by the following method. Plasma (15 μl) was mixed with 1 ml 1.2 M acetate/acetic acid buffer (pH 6.4) containing 0.1% PPD (Sigma) as substrate. To avoid non-specific substrate oxidation, incubation was carried out in the presence of 0.02 mM EDTA. Each plasma sample was incubated in duplicate. Concomitantly, each plasma sample was incubated in the presence of 1 ml 0.5% NaN_3 (azide blank). The mixtures were incubated for 30 min at 37°C. The reaction was stopped by the addition of 1 ml 0.5% NaN_3. Within 1 h, the absorption was measured at 550 nm (LKB spectrophotometer). Ceruloplasmin concentration was expressed as the difference in absorbance between the sample and its azide blank.

Statistics

Data are presented as means ± s.e. The Mann-Whitney U test was applied for statistical evaluation. Significant differences between control and Cu-exposed groups are indicated by asterisks with *: $P<0.05$; **: $P<0.02$; ***: $P<0.01$ and ****: $P<0.001$.

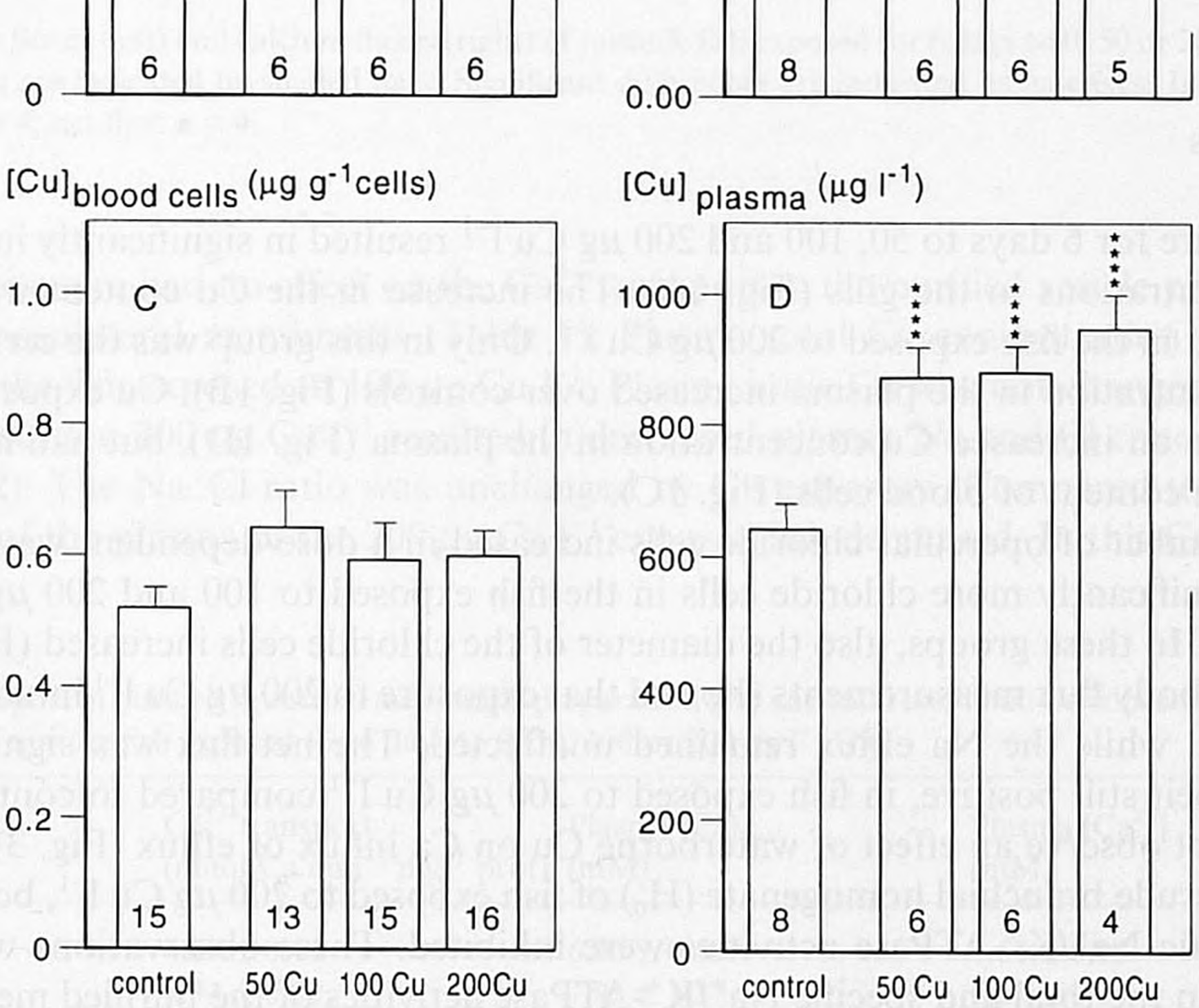

Fig. 1. Cu concentrations in the gills (A), plasma ceruloplasmin levels (B) and Cu concentrations in the blood cells (C) and in the plasma (D) of mature fish exposed for 6 days to 0, 50, 100 or 200 μg l^{-1} Cu. The number of fish per group is indicated in the bars. Significant differences are indicated by asterisks.

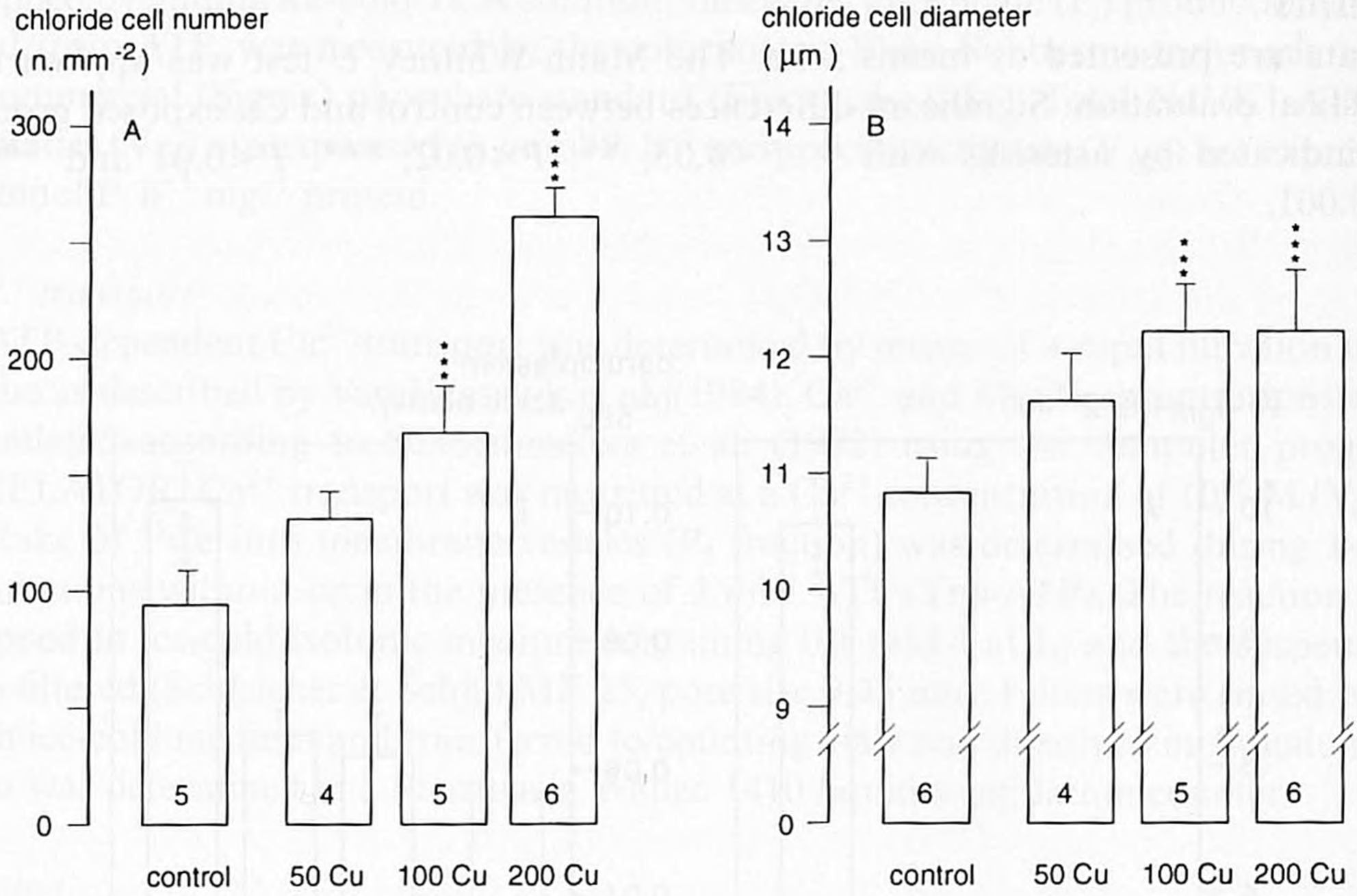

Fig. 2. Chloride cell number in the opercula (A) and diameter of the chloride cells (B) of mature fish exposed for 6 days to 0, 50, 100 or 200 μg l^{-1} Cu. The number of fish per group is indicated in the bars. Significant differences are indicated by asterisks.

3. Results

Exposure for 6 days to 50, 100 and 200 μg Cu l^{-1} resulted in significantly increased Cu concentrations in the gills (Fig. 1A). The increase in the Cu content was most prominent in the fish exposed to 200 μg Cu l^{-1}. Only in this group was the ceruloplasmin concentration in the plasma increased over controls (Fig. 1B). Cu exposure also resulted in an increased Cu concentration in the plasma (Fig. 1D), but had no effect on the Cu content of blood cells (Fig. 1C).

The number of opercular chloride cells increased in a dose-dependent way, resulting in significantly more chloride cells in the fish exposed to 100 and 200 μg Cu l^{-1} (Fig. 2A). In these groups, also the diameter of the chloride cells increased (Fig. 2B).

Whole body flux measurements showed that exposure to 200 μg Cu l^{-1} inhibited the Na influx, while the Na efflux remained unaffected. The net flux was significantly lower, albeit still positive, in fish exposed to 200 μg Cu l^{-1} compared to control fish. We did not observe an effect of waterborne Cu on Ca influx or efflux (Fig. 3).

In the crude branchial homogenate (H_0) of fish exposed to 200 μg Cu l^{-1}, both total and specific Na^+/K^+-ATPase activities were inhibited. These observations were not reflected in the total and specific Na^+/K^+-ATPase activities of the purified membrane fraction P_3. Cu exposure had no effect on the protein recovery during the process of membrane purification from H_0 to P_3, while the enzyme purification was doubled in fish from the highest Cu concentration when compared to controls (Fig. 4).

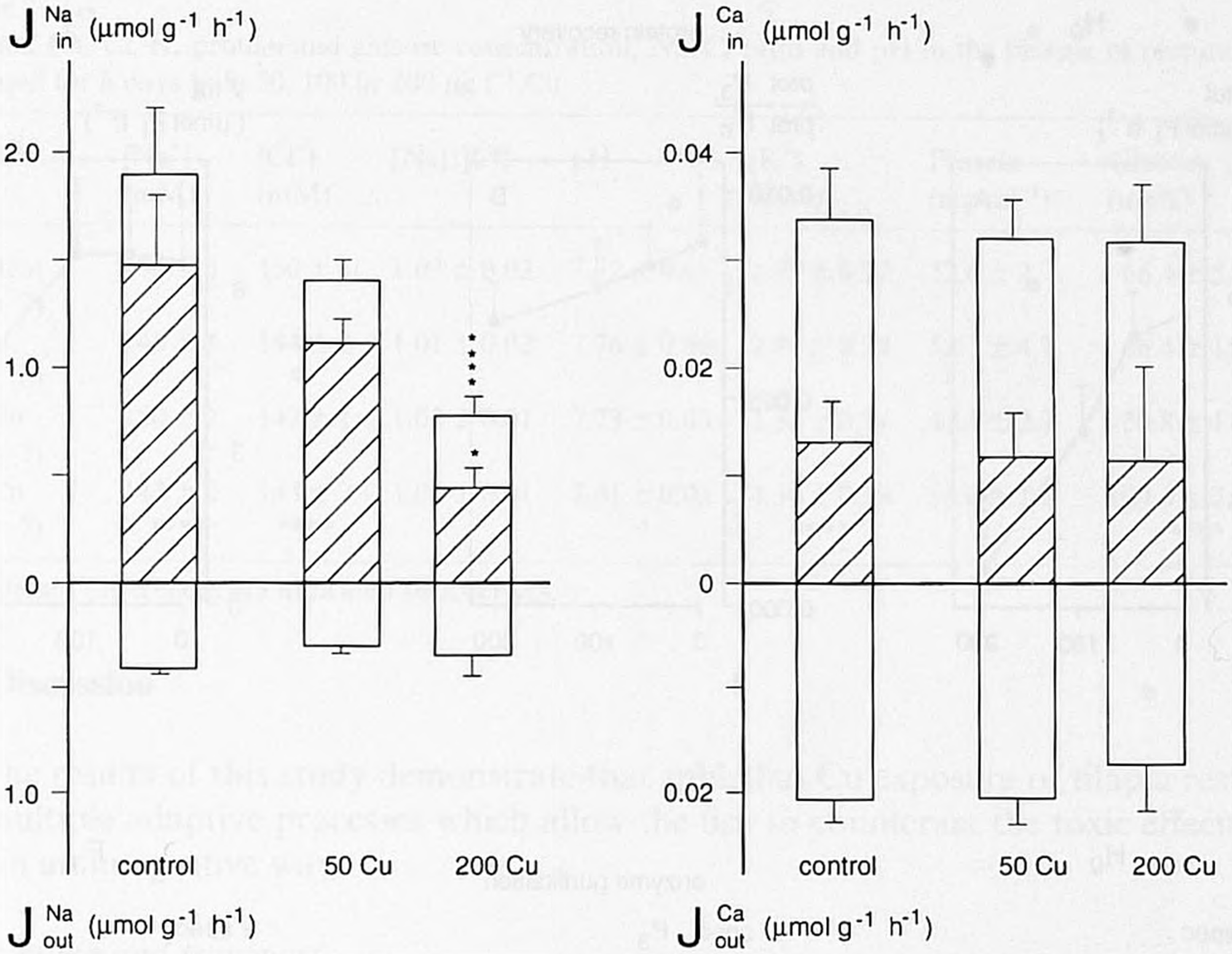

Fig. 3. Na fluxes (left) and calcium fluxes (right) of juvenile fish exposed for 6 days to 0, 50 or 200 μg l^{-1} Cu. Net fluxes are indicated by shaded bars. Significant differences are indicated by asterisks. Influx: $n = 12$; efflux: $n = 4$; net flux: $n = 4$.

Cu exposure had no effect on the Ca^{2+} transport in the purified vesicle preparation of gill basolateral membranes (Table 1). Plasma total Ca concentration decreased only in the fish exposed to 100 μg Cu l^{-1}. Plasma ionic Ca was not changed (Table 1).

Exposure to 200 μg Cu l^{-1} resulted in decreased plasma Na and Cl concentrations (Table 2). The Na:Cl ratio was unchanged by Cu exposure. Compared to controls, the pH of the plasma of the 200 μg Cu l^{-1} exposed fish decreased. In this Cu-exposed

Table 1
Ca^{2+}-transport in purified branchial membrane preparation (P_3) and total and ionic Ca concentration in the plasma of mature fish exposed for 6 days to 0, 50, 100 or 200 μg l^{-1} Cu

	Ca^{2+} transport (nmol Ca min^{-1} mg^{-1} prot)	Plasma $[Ca]_{total}$ (mM)	Plasma $[Ca^{2+}]$ (mM)
Control	4.47 ± 0.40	6.96 ± 0.50	1.61 ± 0.05
50Cu	3.94 ± 0.52	7.93 ± 1.27	1.65 ± 0.04
100Cu	4.38 ± 0.59	4.78 ± 0.35***	1.71 ± 0.04
200Cu	3.91 ± 0.51	6.78 ± 0.78	1.70 ± 0.05

Significant differences are indicated by asterisks; $n = 6$.

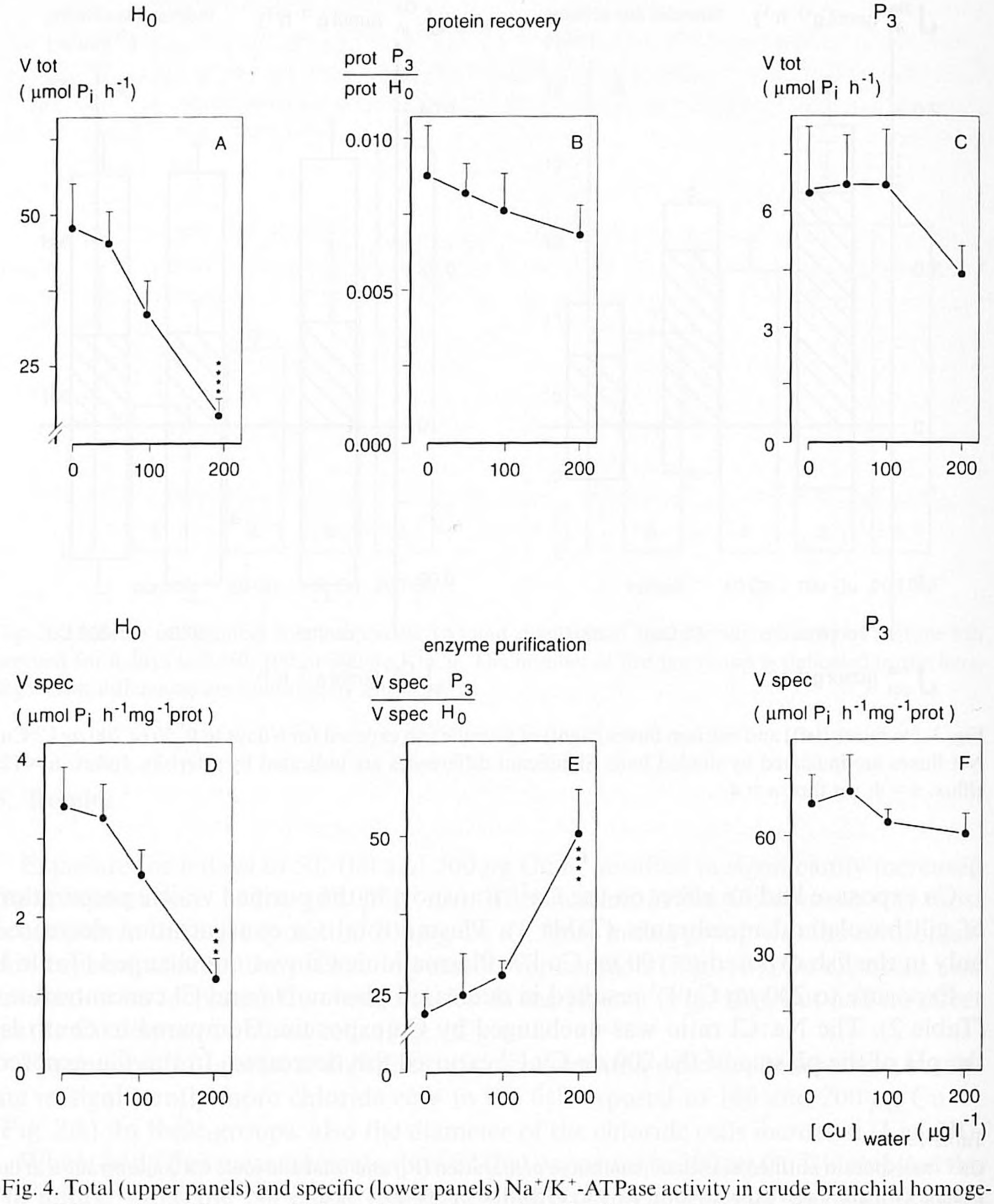

Fig. 4. Total (upper panels) and specific (lower panels) Na^+/K^+-ATPase activity in crude branchial homogenate (H_0) and in purified branchial membrane preparation (P_3). Protein recovery and enzyme purification are also indicated of mature fish exposed for 6 days to 0, 50, 100 or 200 μg l^{-1} Cu. Significant differences are indicated by asterisks. Control and 50Cu: $n = 6$; 100Cu and 200Cu: $n = 5$).

group, also the plasma K concentrations increased. Plasma protein concentrations did not change after Cu exposure. Only the glucose concentration in the plasma of the fish exposed to 200 μg Cu l^{-1} increased significantly as compared to controls.

Table 2
Plasma Na, Cl, K, protein and glucose concentration, Na:Cl ratio and pH in the plasma of mature fish exposed for 6 days to 0, 50, 100 or 200 μg l^{-1} Cu

Plasma	$[Na^+]$ (mM)	$[Cl^-]$ (mM)	[Na]:[Cl]	pH	$[K^+]$ (mM)	Protein (mg ml^{-1})	Glucose (mg%)
Control (n = 7)	154 ± 3	150 ± 1	1.03 ± 0.02	7.72 ± 0.03	2.73 ± 0.22	52.6 ± 2.7	66.4 ± 5.8
50Cu (n = 6)	145 ± 4	144 ± 2	1.01 ± 0.02	7.76 ± 0.06	2.47 ± 0.20	52.3 ± 4.7	66.4 ± 15.8
100Cu (n = 7)	150 ± 2	147 ± 1	1.02 ± 0.01	7.73 ± 0.03	3.37 ± 0.34	47.5 ± 2.7	50.8 ± 11.2
200Cu (n = 7)	142 ± 2 ****	143 ± 2 ****	1.00 ± 0.01	7.61 ± 0.03 *	4.34 ± 0.39 ****	54.9 ± 1.5	109.4 ± 7.2 ****

Significant differences are indicated by asterisks.

4. Discussion

The results of this study demonstrate that sublethal Cu exposure of tilapia results in multiple adaptive processes which allow the fish to counteract the toxic effects of Cu in an integrative way.

Cu uptake and transport

After 6 days of exposure to Cu, the accumulation of this metal in the gill tissue was most prominent in the 200 μg l^{-1} group. In a previous study, we observed no difference in Cu accumulation in the gills of fish exposed for either 6 or 11 days (Pelgrom et al., 1995). Therefore, in this study we decided to expose the fish for 6 days. In control fish, the Cu concentrations in blood plasma and blood cells are of the same magnitude, and comparable to concentrations reported for other fish species (600–1300 μg l^{-1}, Stagg and Shuttleworth, 1982a; Bettger et al., 1987). Our results show that during exposure to waterborne Cu only the blood plasma and not the blood cells display an elevated Cu concentration. This indicates that most of the Cu that enters the gills is transported via the blood plasma. Our results are in line with observations in mammals (Task Group on Metal Accumulation, 1973; Frieden, 1979; Cousins, 1985). Cu treatment of fish blood in vitro also resulted in association of Cu with plasma rather than with the blood cell fraction (Buckley et al., 1984). In the blood of mammals, Cu immediately binds to albumin and transcuprein, and is transported to the liver, where it is bound to ceruloplasmin, released into the blood, and distributed to other tissues (Weiss and Linder, 1985; Cousins, 1985). Cu bound to ceruloplasmin constitutes the larger part (90–95%) of plasma Cu, which makes ceruloplasmin the principal Cu transport protein in mammals (Frieden, 1979; Nederbragt et al., 1984). In a variety of vertebrate sera, the presence of this protein, as reflected by its *p*-phenylenediamine oxidase activity, has been reported (Frieden, 1979). In fish, ceruloplasmin has been demonstrated in plasma of carp (Yamamoto et al., 1977). Our results indicate that this protein is also present in the plasma of tilapia. However, we did not observe a direct relationship between the plasma ceruloplasmin concentration

and the water and/or plasma Cu concentration. A direct relationship between plasma Cu concentration and ceruloplasmin level was observed in mammals after parenteral Cu administration. In fish, however, waterborne Cu enters the general circulation primarily via the gills. Probably, only during exposure to high levels of Cu (200 μg Cu l^{-1}) is ceruloplasmin synthesis induced. Cu exposure had no effect on the protein concentration in the plasma. Comparable results are described in studies with brown bullhead and brook trout (McKim et al., 1970; Christensen et al., 1972).

Whole body ion-fluxes

Exposure to 200 μg l^{-1} Cu reduced the Na^+ influx, without affecting the Na^+ efflux. Consequently, the net Na^+ uptake decreased, although the Na balance was still positive. It should be noted that the measured whole body fluxes almost completely represent gill fluxes (Flik et al., 1985). The flux experiments were performed under conditions which cause minimal additional disturbance, characterized by using an acclimation period, gradual Cu exposure, normal feeding regime and housing the fish in groups. The advantage of our approach of ion-transport measurement is indicated by the high control net fluxes, as is demonstrated by a study of Dharmamba and Maetz (1972). In contrast, no positive net fluxes were observed in control rainbow trout and brown trout (Laurén and McDonald, 1986; Reader and Morris, 1988; Reid and McDonald, 1988). Generally, only influx and net flux are measured, with net fluxes determined from changes in the ion concentration in the water, whereas efflux is calculated from the difference between net flux and influx (Spry and Wood, 1985; Laurén and McDonald, 1986, 1987; Reader and Morris, 1988; Reid and McDonald, 1988). Branchial Na uptake is the result of Na^+/K^+-ATPase-dependent Na influx and Na efflux via passive diffusional losses (Mayer-Gostan et al., 1987; McDonald et al., 1989, 1991; Wood, 1992). The inhibitory effect of Cu on the Na influx may be due to the high affinity of Cu for -SH groups of transport enzymes such as Na^+/K^+-ATPase (Stagg and Shuttleworth, 1982b; Beckman and Zaugg, 1988). Cu-induced disturbances of the Na influx have also been observed in rainbow trout (Laurén and McDonald, 1986; McDonald et al., 1989). Exposure to high concentrations of Cu has been shown to cause histological alterations in the gills (Baker, 1969; Wilson and Taylor, 1993). Structural damage can explain increase in ionic permeability and reduction in transport function commonly seen during the early phase of metal exposure. The absence of an effect of Cu on Na^+ efflux observed in the present study might therefore indicate that the structural integrity of the gills, which determines the permeability to Na, is not affected by the Cu concentrations used. Interestingly, only in the fish exposed to the highest Cu concentration was the Na^+ influx affected, although increased Cu concentrations in the gills were observed after exposure to all Cu concentrations used. This suggests that fish can cope with a certain increased Cu concentration in the gills before dysfunction becomes apparent, which is still present after 6 days of exposure. The Cu-induced inhibition of Na^+ influx in these fish exposed to the highest Cu concentration was reflected in a reduction in plasma Na concentration in mature fish. The Na:Cl ratio was unchanged, as a result of the concurrent decrease in plasma Cl concentration. Observations in the present study are in line with other reports on Cu-exposed fish. In studies with flounder and rainbow trout, Na and Cl concentra-

tions were affected similarly by Cu exposure (Stagg and Shuttleworth, 1982a; Laurén and McDonald, 1985; McDonald et al., 1989; Wilson and Taylor, 1993).

In this study, Cu exposure had no effect on whole body Ca exchange with the water. This is in line with the unchanged plasma Ca concentration in mature fish observed in this study. Similar results have been reported for rainbow trout by Reid and McDonald (1988). A small and transient decrease in net Ca uptake was found in brown trout by Sayer et al. (1991). Influx rates of Ca^{2+} are usually substantially lower than those of Na^+ (Reid and McDonald, 1988). This was also observed in the present study with young fish. The branchial mechanisms regulating Na^+ and Ca^{2+} influxes are distinctly different from one another in hormonal control, ion-specific channels or carriers in the apical membrane and transport ATPases in the basolateral membrane of the ion-transporting cells (Flik et al., 1985; McDonald et al., 1989). This is also reflected by the present observation that active branchial Ca^{2+} uptake was, unlike Na^+/K^+-ATPase, not inactivated by Cu.

Chloride cells

In response to waterborne Cu we observed an increase in the number of chloride cells in the opercula which reflects the chloride cell density in the gills in tilapia (Wendelaar Bonga et al., 1990). Proliferation of chloride cells is a physiological response to agents affecting branchial ion uptake, such as Cd (Oronsaye and Brafield, 1984; Pratap and Wendelaar Bonga, 1993), and Cu (Baker, 1969). The increase in the number of chloride cells may be a compensatory response, playing a role in recovery from and acclimation to, heavy metals (Mallat, 1985; McDonald and Wood, 1993; Perry and Laurent, 1993). Because on average, the cells were also larger, we conclude that this increase could not be attributed to an increase of immature chloride cells, since immature cells are characterized by smaller cell diameters than mature chloride cells. The ion transport capacity is related to the fraction of the chloride cells in contact with the external environment only (mature chloride cells) rather than to the total number of epithelial chloride cells, which includes young or degenerating cell stages (Wendelaar Bonga et al., 1990). In a study on fish exposed to acid water (Wendelaar Bonga et al., 1990) the rapid increase in chloride cell numbers reflected a higher turnover rate of these cells. Most of the cells were degenerating or immature, as evidenced by smaller cell diameters, and therefore unlikely to contribute to ion transport. In our study, however, the increase in the number of chloride cells was unlikely to be the result of more small and immature cells, given the increased diameter after Cu exposure. These results therefore are more reminiscent of observations of chloride cell proliferation after exposure of trout to ion-deficient water (McDonald and Rogano, 1986; Perry and Laurent, 1989, 1993). In these studies, hyperplasia and hypertrophy of chloride cells resulted in an extension of the mean chloride cell area exposed to the water which coincided with an increased ion-transport activity of the gills (McDonald and Rogano, 1986; Perry and Laurent, 1989, 1993). However, our results on the effects of Cu demonstrate that an increase in the chloride cell number does not automatically imply an increase of the ion-transport capacity. Therefore, the increase in cell size and number observed in our experiment does not warrant restoration of the Na-transport activity.

Na- and Ca-transport mechanisms

The observed Cu-induced inhibition of Na^+ influx was not reflected in the specific Na^+/K^+-ATPase activity in membrane preparations of in vivo Cu-exposed fish. In vitro exposure to metals may cause a decrease in ATPase activity in membrane preparations (Stagg and Shuttleworth, 1982b). The mechanisms underlying an inhibition of ATPase-dependent ion transport in vivo will, however, be more complicated. Several mechanisms may interfere with the process of membrane purification and in vitro determination of active transport mechanisms. Firstly, the Na^+/K^+-ATPase activity in the gills may be regulated by changing the number of active enzyme units present, as suggested by Stagg and Shuttleworth (1982b). In the present study, the number of chloride cells was increased in Cu-exposed fish and this could account for the increase in Na^+/K^+-ATPase enrichment of the P_3 fraction in fish exposed to the highest Cu concentration. The increase in the number of chloride cells, however, is not reflected in the total Na^+/K^+-ATPase activity. In the crude membrane homogenate H_0, the total enzyme activity was even significantly decreased in these Cu-exposed fish. The second branchial mechanism in the process of compensation for Cu-induced ion losses might be a change in the activity per enzyme unit (Stagg and Shuttleworth, 1982b). During exposure, the Cu concentration in the gills increased significantly, which may also occur in the chloride cells, which may inactivate the Na^+/K^+-ATPase activity in these cells. Such an effect seems to be reflected only in the specific Na^+/K^+-ATPase activity in the crude homogenate (H_0), and not in the P_3 fraction. One might argue that this might indicate reactivation of in vivo Cu-inhibited ATPase activity during the isolation procedure, assuming that most of the accumulated Cu is lost in this process. However, the amount of Cu in the P_3 fraction relative to the amount of Cu in the H_0 fraction (recovery of Cu in the P_3 fraction) is the same in controls and fish exposed to 100 $\mu g\ l^{-1}$ Cu (39% and 35% respectively; Pelgrom et al., in prep.). Therefore, differences in total Cu concentration between gills of controls and Cu-exposed fish should equally influence the enzyme in H_0 and P_3 fractions. Thus the difference observed in Na^+/K^+- ATPase activity between H_0 and P_3 fractions cannot be attributed to reactivation due to loss of Cu from the membranes of the Cu-exposed fish during the in vitro isolation procedure.

Both mechanisms may be involved during in vitro determination of Na^+/K^+-ATPase activity after in vivo Cu exposure. In addition, in vitro ion-transport mechanisms are determined in the absence of hormonal factors, which likely differ between controls and fish exposed to Cu (Pelgrom et al., in prep.), and at optimal Na concentrations. It should also be noted that in most vertebrate tissues Na^+/K^+-ATPase is a heterogenous population of enzyme units. In a recent study of Middleton et al. (1993) it was demonstrated that not all forms of Na^+/K^+-ATPase in kidney cells were regulated by PKC phosphorylation, a mechanism which may be affected by Cu. Therefore, enzyme heterogeneity may contribute to the response diversity.

Few data are available on Cu-induced effects on branchial Na^+/K^+-ATPase activity after in vivo exposure. In flounder, Stagg and Shuttleworth (1982b) observed no effect after in vivo Cu exposure, whereas Laurén and McDonald (1987) observed in trout an inhibition of Na^+/K^+-ATPase specific activity, which was compensated by an

increase in the microsomal protein concentration. In the latter study, however, no Cu accumulation in the gills was measured.

To our knowledge, no data are available on the effects of in vivo Cu exposure on Ca^{2+} transport in gill membrane vesicles. In this study, Ca transport was not affected by Cu exposure and the increased Cu concentration in the gills. These observations are in line with the flux data, and confirm the specific action of Cu on Na.

Plasma ions and glucose

Our results demonstrate that the effects of Cu exposure on plasma are not limited to disturbed Na and Cl levels, confirming the complexity of ambient Cu on these fish. In the fish exposed to 200 μg l^{-} Cu, acid/base regulation was also disturbed, as indicated by a pH decrease. It has been suggested by Laurén and McDonald (1985) that Cu exposure leads to a general increase in the permeability of cell membranes, which partly explain the increase in the plasma K concentrations.

Hyperglycaemia is a common response to stressors in freshwater fish, and has been considered as an indicator of sublethal environmental pollutions (Hatting, 1976). In the present study, an increased concentration of glucose in the plasma was observed only in the fish exposed to the highest Cu level. This observation is in line with results from Cu-exposed brown bullhead (Christensen et al., 1972) and rainbow trout (Laurén and McDonald, 1985). A rise in the plasma glucose concentration indicates an activated carbohydrate metabolism, which in this species is under the control of cortisol (Christensen et al., 1972; Balm, 1986).

In conclusion, in tilapia, Cu levels in gills and plasma are primarily affected after sublethal waterborne Cu exposure for 6 days. At 100 and 200 μg Cu l^{-1}, chloride cell proliferation and an increase in the average cell diameter were observed. Secondarily to this response, Cu-induced disturbances of ionic regulation were noticed only in the fish exposed to 200 μgCu l^{-1}. These observations demonstrate that the mechanism of Cu toxicity in tilapia differs from that in rainbow trout (Laurén and McDonald, 1987) where in the absence of Cu accumulation branchial ionoregulatory disturbances were observed.

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