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# EFFECTS OF ZINC ON THE KINETICS OF BRANCHIAL CALCIUM UPTAKE IN FRESHWATER RAINBOW TROUT DURING ADAPTATION TO WATERBORNE ZINC

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## Summary

The effects of sublethal waterborne $Zn^{2+}$ (150 $\mu$g $l^{-1}$=2.3 $\mu$mol $l^{-1}$) on the kinetics of unidirectional $Ca^{2+}$ influx were studied in juvenile freshwater rainbow trout during chronic exposure (60 days) at a water [$Ca^{2+}$] of 1.0 mmol $l^{-1}$. An unexposed group held under identical conditions served as control. The presence of $Zn^{2+}$ in the water increased the apparent $K_m$ for $Ca^{2+}$ influx by up to 300 % with only a small inhibitory effect (35 % at most) on the maximum rate of uptake ($J_{max}$). These results, in combination with earlier data showing that $Ca^{2+}$ competitively inhibits $Zn^{2+}$ uptake, suggest that $Zn^{2+}$ and $Ca^{2+}$ compete for the same uptake sites. Acute withdrawal of $Zn^{2+}$ after 3 h of exposure resulted in a 23-fold reduction in $K_m$ for $Ca^{2+}$, but a persistent small depression of $J_{max}$. During prolonged exposure to $Zn^{2+}$, the apparent $K_m$ for $Ca^{2+}$ remained greatly elevated and $J_{max}$ remained slightly depressed. The actual $Ca^{2+}$ influx in hard water ([$Ca^{2+}$]=1.0 mmol $l^{-1}$) decreased marginally and paralleled the small changes in $J_{max}$. The increases in apparent $K_m$ had a negligible influence on the actual $Ca^{2+}$ influx because $K_m$ values (38–230 $\mu$mol $l^{-1}$), even when elevated by $Zn^{2+}$, remained below the water [$Ca^{2+}$] (1000 $\mu$mol $l^{-1}$). Rainbow trout exposed to $Zn^{2+}$ exhibited a slower rate of protein synthesis in the gills (measured on day 23) and an increased tolerance to $Zn^{2+}$ challenge (measured on both days 27 and 50). Unidirectional $Zn^{2+}$ influx, measured at the end of the exposure period, was significantly reduced in the $Zn^{2+}$-exposed fish. There were no changes in hepatic or branchial $Zn^{2+}$, $Cu^{2+}$ or metallothionein concentrations. We hypothesize that, during exposure to sublethal [$Zn^{2+}$] in hard water, the fish may change the $K_m$ for a mutual $Ca^{2+}/Zn^{2+}$ carrier so as to reduce markedly $Zn^{2+}$ influx without greatly altering $Ca^{2+}$ influx. This reduced $Zn^{2+}$ influx, rather than metallothionein induction, may be the basis of adaptation to elevated concentrations of waterborne $Zn^{2+}$.

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## Introduction

According to the present model of branchial $Ca^{2+}$ uptake in freshwater fish, $Ca^{2+}$ transport occurs *via* the mitochondria-rich 'chloride' cells (Perry and Wood, 1985; Flik *et al.* 1985; Perry and Flik, 1988). Calcium passes the apical membrane of the chloride cells through voltage-independent $Ca^{2+}$ channels, driven by its electrochemical gradient. Inside the chloride cell, $Ca^{2+}$ binds to proteins, such as calmodulin, thus keeping the intracellular $Ca^{2+}$ activity low. $Ca^{2+}$ is then transported over the basolateral membrane by a high-affinity $Ca^{2+}$-ATPase.

The primary effect of an acute increase in waterborne [$Zn^{2+}$] on freshwater fish is believed to be an impaired branchial $Ca^{2+}$ influx that, in turn, leads to hypocalcaemia (Spry and Wood, 1985). Chemically, $Zn^{2+}$ is closely related to $Cd^{2+}$, which also causes hypocalcaemia, primarily by inhibiting the $Ca^{2+}$-transporting ATPase in the basolateral membrane of the chloride cells (Verbost *et al.* 1987, 1988, 1989*b*; Fu *et al.* 1989). At present, it is not known how $Zn^{2+}$ interferes with branchial $Ca^{2+}$ uptake.

Fish exposed to sublethal levels of metals are often able to adapt, i.e. to make physiological adjustments which compensate for the altered environmental condition and confer increased tolerance to the metal (Chapman, 1985; McDonald and Wood, 1993). At least in freshwater fish, the mechanisms responsible for this adaptation are likely to act at the gill. The gill is the primary site of ionoregulation, acid–base balance, nitrogenous waste excretion and gas exchange; these systems, in particular ionoregulation, are especially sensitive to metal toxicity (Wood, 1992). Effects at the level of the gills may take place before the metals are distributed to other parts of the body. McDonald and Wood (1993) have proposed that metal adaptation results from a two-step process, involving initial damage to the gill epithelium followed by a period of tissue repair, during which the actual mechanisms responsible for the adaptation are expressed. Such mechanisms could include a reduced metal uptake, induction of the heavy metal binding protein metallothionein (MT), compensatory synthesis of ion-transporting ATPases and a proliferation or increased turnover of ionocytes (Hogstrand and Haux, 1991; Roesijadi, 1992; McDonald and Wood, 1993).

There is indirect evidence suggesting that induction of branchial MT could be involved in the adaptation of freshwater rainbow trout, *Oncorhynchus mykiss*, to $Zn^{2+}$ and other metals (Kito *et al.* 1982; Benson and Birge, 1985; Bradley *et al.* 1985; Olsson *et al.* 1989; Norey *et al.* 1990). The recent development of a highly specific radioimmunoassay (RIA) system for MT in fish (Hogstrand and Haux, 1990) should facilitate future research in this area. There is direct evidence for a reduced rate of accumulation of $Zn^{2+}$ in gills of adapted fish (Bradley *et al.* 1985). Similarly, adaptation to $Cd^{2+}$ could be associated with MT induction and a reduced uptake of $Cd^{2+}$ by the gills (Kito *et al.* 1982; Benson and Birge, 1985; Verbost *et al.* 1989*b*; Wicklund Glynn and Olsson, 1991).

Increased rates of protein synthesis in the gills might well accompany such processes of damage repair and induction of specific proteins. To our knowledge, protein synthesis rates have not been measured during prolonged exposure to sublethal metal stress, though a highly sensitive technique for such measurements is now available (Houlihan *et al.* 1986). Whatever the mechanisms responsible for adaptation to metals, they are likely to

carry a significant metabolic cost (Calow, 1991). Thus, during the adaptation process, less energy may be available for growth, locomotion, reproduction and other important variables determining the fitness of fish. For example, it has recently been shown that an increased tolerance to aluminium is accompanied by a reduced growth rate and critical swimming speed (Wilson and Wood, 1992; Wilson *et al.* 1993*a*,*b*).

Unidirectional flux measurements (using radiotracers) and kinetic analysis have proved to be valuable tools to characterize branchial ion-transport systems in fish (Wood, 1992). These techniques can cast light on the nature of both transport and inhibitory mechanisms. For example, linear kinetics (a proportional relationship between uptake and substrate concentration) suggest diffusive pathways, whereas Michaelis–Menten kinetics suggest carrier- or channel-mediated transport. When the latter processes apply, increases in $K_m$ (inverse of affinity) in the presence of the putative inhibitor are diagnostic of competitive inhibition, while decreases in $J_{max}$ (maximum transport rate) are indicative of non-competitive inhibition. During prolonged exposures, changes in $K_m$ may indicate adaptive alterations in the affinity of the carrier system, while changes in $J_{max}$ may signal a restoration of the absolute numbers of carrier sites. Such experiments have been used to investigate the effects of aluminium (McDonald and Milligan, 1988; McDonald *et al.* 1991), $Cu^{2+}$ (Laurén and McDonald, 1985, 1987; Reid and McDonald, 1988), $Zn^{2+}$ (Spry and Wood, 1985, 1988, 1989*a*; Bentley, 1992) and $Cd^{2+}$ (Verbost *et al.* 1987, 1989*b*; Reid and McDonald, 1988) on branchial uptake and excretion processes. Kinetic analysis of the effects of $Ca^{2+}$ on $Zn^{2+}$ uptake has clearly shown that increasing concentrations of $Ca^{2+}$ in the water elevate the $K_m$ for $Zn^{2+}$ influx, and the inhibitor constant ($K_i$) for this inhibition is close to the $K_m$ for $Ca^{2+}$ uptake (Spry and Wood, 1989*a*). These findings suggest that $Ca^{2+}$ and $Zn^{2+}$ may compete for the same uptake sites at the gills.

With this background in mind, the present study had three objectives. The first was to characterize the acute effect of waterborne $Zn^{2+}$ on the kinetics of unidirectional $Ca^{2+}$ influx (as measured with $^{45}Ca$) in juvenile freshwater rainbow trout. For this purpose, we developed a new *in vivo* method to measure $Ca^{2+}$ influx kinetics, based on the technique described by Laurén and McDonald (1987) for the analysis of $Na^+$ influx kinetics. The second was to see whether adaptive or non-adaptive changes in $Ca^{2+}$ kinetics occurred during chronic $Zn^{2+}$ exposure (60 days) and whether such changes could be correlated with the development of an increased tolerance to $Zn^{2+}$. Zinc was applied at a level ($150\,\mu g\,l^{-1}$=$2.3\,\mu mol\,l^{-1}$) below the reported $K_m$ for $Zn^{2+}$ uptake ($240\,\mu g\,l^{-1}$= $3.7\,\mu mol\,l^{-1}$) by rainbow trout in identical water quality (Spry and Wood, 1989*a*), and at only 0.2 % of the ambient $Ca^{2+}$ concentration ($1.0\,mmol\,l^{-1}$). This concentration of $Zn^{2+}$ is known to be within the nutritional range for rainbow trout in the water used, stimulating growth without effects on whole-body $Ca^{2+}$ and $Zn^{2+}$ concentrations (Spry and Wood, 1988). An increased survival time ($LT_{50}$) in a lethal concentration of $Zn^{2+}$ was used as an index of adaptation to $Zn^{2+}$. The third objective was to learn more about the possible costs and mechanisms of adaptation to $Zn^{2+}$ by measuring protein synthesis rates and RNA concentrations in gills, liver and the whole body (Houlihan *et al.* 1986), levels of MT and $Zn^{2+}$ in gills and liver (Hogstrand and Haux, 1990) and rates of unidirectional $Zn^{2+}$ influx (using $^{65}Zn$; Spry and Wood, 1989*a*) in $Zn^{2+}$-adapted fish.

## Materials and methods

### *Experimental animals*

Juvenile rainbow trout, *Oncorhynchus mykiss* (Walbaum) (4–40 g; mean 21 g) were obtained from a local hatchery (Rainbow Springs Hatchery, New Dundee, Ontario). The fish were held in two 264-l fibreglass tanks (350 per tank), each supplied at a rate of 900 ml $min^{-1}$ with a flow-through of dechlorinated, aerated Hamilton city tapwater ([$Na^+$] 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). The temperature gradually declined from 14 to 10 °C over the 2 month holding period prior to experiments. Fish were fed dry trout pellets (Martin's Feed Mill Ltd, Ontario) at a rate of 1 % of their body mass per day.

### *Chronic zinc exposure*

One tank was equipped with a dosing system that added $Zn^{2+}$, as $ZnSO_4 \cdot 7H_2O$ (BDH Chemicals), from a stock solution. The other served as a control tank. The flow rate of the added $Zn^{2+}$ stock (45.0 mg $Zn^{2+}$ $l^{-1}$=0.688 mmol $l^{-1}$) was maintained at 3 ml $min^{-1}$, by a peristaltic pump, to achieve a concentration of $Zn^{2+}$ in the tank of 150 $\mu$g $l^{-1}$±10 % (2.3 $\mu$mol $l^{-1}$). The exposure was initiated by adding a sufficient amount of $Zn^{2+}$ stock solution in the water to obtain the desired $Zn^{2+}$ concentration instantly. Water flow and dosing rates were checked daily and adjusted if necessary. The exposure to $Zn^{2+}$ continued for 60 days, during which the temperature was 8–10 °C. No mortality occurred during the experiment.

Water samples from the exposure tank were taken for analysis of zinc levels before and after each change of stock solution (every fourth day). Samples were acidified with $HNO_3$ (trace metal analysis grade, BDH Chemicals, was used in all procedures), and zinc was measured with an atomic absorption spectrophotometer (Varian AA-1275), using an air/acetylene flame.

### *Calcium uptake kinetics*

A method was developed to measure the kinetics of unidirectional $Ca^{2+}$ influx *in vivo* in which each fish provided one data point in a terminal measurement. $Ca^{2+}$ influx was calculated from the appearance of $^{45}Ca$ radioactivity in the whole body of the fish over a 4 h period (cf. Perry and Wood, 1985). The flux chambers used were 5 l polypropylene bags (black) filled with 3 l of synthetic $Ca^{2+}$-free water ([NaCl] 0.7 mmol $l^{-1}$; [$KHCO_3$] 1.9 mmol $l^{-1}$; pH 8.0) and equipped with an airline. The bags were placed in polyvinylchloride supports in a waterbath at the same temperature (8–10 °C) as the holding tanks. In total, at each time point, 12 flux-bags were used, representing six different water $Ca^{2+}$ concentrations for the $Zn^{2+}$-exposed fish and six for the controls. Five minutes before the fish were introduced to the flux-bags, a sample of a $^{45}Ca/Ca(NO_3)_2$ stock solution [$Ca^{2+}$ (52 mmol $l^{-1}$); specific activity 35 kBq $mol^{-1}$] was added to each of the bags. The volumes added to the bags were 3, 6, 12, 24, 48 and 96 ml, giving approximate $Ca^{2+}$ concentrations of 52, 104, 207, 413, 820 and 1615 $\mu$mol $l^{-1}$, respectively.

Eight fish were added to each flux-bag at the start of each flux period. Quadruplicate

water samples of 5 ml were withdrawn at 0, 2 and 4 h for later analysis of $^{45}Ca$ and $Ca^{2+}$. After 4 h in the flux-bags, the fish were given an overdose of anaesthetic (MS 222, 1 g per flux-bag), rinsed for 1 min in 10 mmol $l^{-1}$ $Ca(NO_3)_2$ to displace surface-bound $^{45}Ca$, blotted dry, weighed, and individually wrapped in aluminium foil. The fish and the water samples were frozen immediately after the experiment and kept at −20 °C until analysis.

The frozen fish were transferred to liquid nitrogen and ground to a fine powder with a mortar and pestle. The powder was weighed out in triplicate samples of 0.5 g in glass scintillation vials. Each tissue sample was digested with 2.0 ml of liquid tissue solubilizer (NCS, Amersham) for 48 h at 45 °C. The samples were then neutralized with 20 $\mu$l of glacial acetic acid, diluted with 10 ml of scintillation fluor (OCS, Amersham) and counted in a scintillation counter (LKB 1217 Rackbeta, Pharmacia-LKB AB).

Duplicate water samples were analyzed for calcium by atomic absorption spectroscopy (Varian AA 1275), using an air/acetylene flame. The remaining duplicate water samples (5 ml each) were diluted with 10 ml of scintillation fluor (ACS, Amersham) and counted for $^{45}Ca$.

The inward flux ($J_{in}$) for $Ca^{2+}$ (in $\mu$mol $l^{-1}$ $kg^{-1}$ $h^{-1}$) was calculated according to the following formula:

$$J_{in} = \frac{ACT}{SA \times CE \times t} , \quad (1)$$

where ACT is the average counts in the tissue samples (cts $min^{-1}$ $kg^{-1}$), SA is the measured mean specific activity of $^{45}Ca$ in the water (cts $min^{-1}$ $\mu mol^{-1}$; essentially constant over the flux periods), CE is the relative counting efficiency of the 'tissue–NCS–OCS system', compared to the 'water–ACS' system, and $t$ is time (h).

Initially, Michaelis–Menten analyses of the relationships between $J_{in}$ and $[Ca^{2+}]$ were performed using both Eadie–Hofstee and Lineweaver–Burk linear regressions. The latter yielded generally higher correlation coefficients ($r$) and were therefore used throughout (e.g. Fig. 1) to yield values of $J_{max}$, $K_m$ and their S.E.M. values for the Michaelis–Menten equation (equation 2). Note that the Lineweaver–Burk transformation yields asymmetrical S.E.M. values:

$$J_{in} = \frac{J_{max}[Ca^{2+}]}{K_m + [Ca^{2+}]} . \quad (2)$$

In turn, Lineweaver–Burk conversion of the Michaelis–Menten equation was used to calculate the actual $J_{in}$ for $Ca^{2+}$ of fish at the acclimation concentration of $Ca^{2+}$, 1.0 mmol $l^{-1}$.

*Calcium uptake kinetics in the presence and absence of zinc*

One day before the start of exposure to $Zn^{2+}$, a control experiment with fish from control and experimental tanks was run to determine the kinetics of $Ca^{2+}$ influx in the absence of $Zn^{2+}$. Next, analyses of $Ca^{2+}$ influx kinetics were performed on $Zn^{2+}$-exposed fish (at the exposure concentration) and control fish (no $Zn^{2+}$ present) after 2, 5, 9, 15 and 50 days of exposure. For the $Zn^{2+}$-exposed fish, 3.0 ml of a $ZnSO_4$ stock solution

(150 mg $Zn^{2+}$ $l^{-1}$=2.3 mmol $l^{-1}$) was added to each of the flux-bags to give a final $Zn^{2+}$ concentration of 150 $\mu$g $l^{-1}$ (2.3 $\mu$mol $l^{-1}$).

In addition, after the first 3 h of exposure to 150 $\mu$g $l^{-1}$ $Zn^{2+}$, a similar $Ca^{2+}$ kinetic analysis to that described above was performed, but in the absence of $Zn^{2+}$. This experiment was designed to give information about any $Zn^{2+}$-induced short-term alterations in the number of $Ca^{2+}$ uptake sites and their affinity for $Ca^{2+}$ (i.e. changes in true $K_m$, rather than apparent $K_m$, which is the value measured in the presence of $Zn^{2+}$).

*Influx of zinc*

The unidirectional influx of $Zn^{2+}$, at the $Zn^{2+}$ concentration of the exposure tank (150 $\mu$g $l^{-1}$=2.3 $\mu$mol $l^{-1}$), was measured for $Zn^{2+}$-exposed fish and for control fish at day 56. The flux chambers used were two large black polypropylene bags, each filled with 20 l of dechlorinated Hamilton tapwater (see above) with 150 $\mu$g $Zn^{2+}$ $l^{-1}$ (2.3 $\mu$mol $l^{-1}$) added as $ZnSO_4$. The flux-bags were equipped with an airline and placed in a tub with a flow-through of water at the same temperature as the holding tanks. Five minutes before the fish were introduced to the bags, 2.59 MBq of carrier-free $^{65}Zn$ was added to each bag.

Ten fish from each tank were transferred to the flux-bags and were held there for 24 h. Quadruplicate water samples were withdrawn from the flux-bags at 0, 12, 18 and 24 h. At the end of the flux period, the fish were lightly anaesthetized (MS 222, 20 mg $l^{-1}$) and transferred, one at a time for 1 min, to a beaker with tapwater containing MS 222 (1.0 g $l^{-1}$). The fish were blotted dry and a terminal blood sample (100 $\mu$l) was withdrawn with a heparinized Hamilton syringe from the caudal vessels. The blood was centrifuged and the separated plasma assayed for $^{65}Zn$ activity in a $\gamma$-counter (MINAXI $\gamma$ Auto-Gamma 5000 Series, Canberra-Packard). Water samples were similarly counted for $^{65}Zn$ activity and total zinc was measured as above by atomic absorption spectroscopy.

The influx of $Zn^{2+}$ was calculated by the method described by Spry and Wood (1989*a*). The procedure was based on the relationship between the steady-state activity of $^{65}Zn$ in plasma after 24 h of exposure ($C_{ss}$ in units of nmol $l^{-1}$ of exogenous $Zn^{2+}$ per ml plasma, as calculated from the external specific activity of $Zn^{2+}$) and the influx of $Zn^{2+}$ (in nmol $kg^{-1}$ $h^{-1}$) established for rainbow trout by Spry and Wood (1989*a*):

$$J_{in} = 4.215 \times C_{ss} + 2.470 . \quad (3)$$

The calculation is not affected by the endogenous level of $Zn^{2+}$ in plasma. The $Zn^{2+}$ influx was only measured at one concentration of the substrate ($Zn^{2+}$).

*Acute toxicity tests*

$LT_{50}$ tests of $Zn^{2+}$-exposed fish and controls were performed at days 27 and 50. On both test occasions, ten fish from each group were transferred to a 14-l black acrylic box. The test chamber was equipped with airlines and divided in the middle by a plastic mesh to allow separate exposure of the two groups to identical water quality. To ensure an efficient water turnover, the inflow and the drain were at opposite ends. The water came from the same source and had an identical temperature to that in the holding tanks. A $ZnSO_4$ stock (350 mg $Zn^{2+}$ $l^{-1}$=5.35 mmol $l^{-1}$) was added at 5 ml $min^{-1}$ to the incoming water (500 ml $min^{-1}$) by a peristaltic pump and mixing funnel, yielding a measured $Zn^{2+}$

concentration in the test chamber from both tests of 3.4±0.3 mg $l^{-1}$ (52±5 $\mu$mol $l^{-1}$) (mean ± S.E.; *N*=12). Mortality was monitored throughout the first day of exposure, and four times daily thereafter for 6 days (144 h). $LT_{50}$ values ±95 % confidence limits were calculated from plots of probit mortality against log time by the methods of Litchfield (1949).

### *Levels of metallothionein, copper and zinc*

Sixty days after the start of the exposure, 10 fish from each group were sampled for analysis of branchial and hepatic levels of metallothionein (MT), copper and zinc. Copper was measured because MT binds both copper and zinc, and therefore an altered zinc content of a tissue could change the level of copper. The fish were killed by a blow to the head, and gills and liver were dissected. Soft tissue of the gill filaments was scraped from the cartilaginous tissue with two microscope slides. The samples were transferred to cryostatic vials, frozen in liquid nitrogen and then stored at −70 °C.

The frozen tissues were later weighed and homogenized individually in 50 mmol $l^{-1}$ Tris–HCl, pH 8.0, at 0 °C, using a glass–Teflon homogenizer. The volumes of buffer added in the homogenization step were 1.5 ml for liver and 1.0 ml for gill. A sample of 450 $\mu$l from each homogenate was stored at −20 °C for subsequent analysis of zinc and copper. The remainder was centrifuged at 10 000 ***g***, 4 °C, for 20 min; the supernatant was decanted, frozen in liquid nitrogen, and stored at −70 °C until used for measurement of MT. MT levels were analyzed with a double antibody radioimmunoassay (RIA), using rabbit antiserum raised against MT from perch, *Perca fluviatilis*, as the first antibody, $^{125}$I-labelled rainbow trout MT as tracer, and goat anti-rabbit IgG as the second antibody (Hogstrand and Haux, 1990). The MT (I and II) from rainbow trout, used as tracer, was purified according to Olsson and Haux (1985) with the modifications described by Hogstrand and Haux (1990) for perch MT. A 10 000 ***g*** supernatant prepared from the livers of $Cd^{2+}$-injected rainbow trout was used as the MT standard. The MT content of the standard was calibrated against a standard curve prepared from purified rainbow trout MT (Olsson and Haux, 1985; Hogstrand and Haux, 1990). The working range of the RIA was 10–100 ng rainbow trout MT per assay tube, which corresponds to 0.6–6 $\mu$g $g^{-1}$ liver wet mass.

Liver and gill homogenates were digested in acid-washed glass tubes for 1 h with 5 vols of 70 % $HNO_3$ at 120 °C. The samples were then cooled to room temperature and 0.75 vols of $H_2O_2$ was added. The digests were evaporated to dryness at 120 °C. 5 ml of 1 % $HNO_3$ was finally added to the digestion tubes and copper and zinc were analyzed by atomic absorption spectroscopy as described above. No solid material was present in the final digest.

### *Protein synthesis*

A protein synthesis experiment was run on day 23. Four days prior to this, 10 fish from the control group had been placed into a separate tank and starved. This group was used as a positive control, since starvation is known to reduce protein synthesis relative to that in fish fed daily (Houlihan *et al.* 1989). On the experimental day, each fish (10 starved controls, 18.12±1.98 g; 10 fed controls, 15.18±3.23 g; 10 fed $Zn^{2+}$-exposed fish,

18.96±1.59 g) was quickly dried, weighed in air to the nearest 0.01 g and then injected *via* the caudal vessels with 150 mmol $l^{-1}$ phenylalanine, pH 7.5 (1 ml 100 $g^{-1}$ fish), containing 3.7 MBq $ml^{-1}$ of L-2,6-[$^3$H]phenylalanine. Following injection, the fish were put into individual 250-ml black beakers with fitting lid and airline, containing the same water as before. After 1 h, they were killed by a blow to the head, and the gills and liver were dissected out. Soft gill tissue was immediately scraped from the cartilaginous filament and frozen in liquid nitrogen. Livers and the remaining carcass were also frozen in liquid nitrogen as quickly as possible. The samples were stored at −70 °C for later analysis.

Whole-body content of protein and RNA, and fractional rate of protein synthesis, $K_s$ (% $day^{-1}$), in gills, liver and whole body were analyzed as detailed in Houlihan *et al.* (1986). Briefly, tissue samples were homogenized in perchloric acid (PCA) and the denatured proteins were separated by centrifugation. PCA in the supernatant was precipitated with tripotassium citrate and centrifuged down, leaving the free phenylalanine in solution. Sample phenylalanine was converted to $\beta$-phenylethylamine, by L-tyrosine decarboxylase, extracted using *n*-heptane, and analyzed by a ninhydrin reaction. The content of [$^3$H]phenylalanine was measured by liquid scintillation counting. Sodium hydroxide was used to resuspend the PCA-extracted tissue pellet and duplicate samples were taken for analysis of protein content (Lowry *et al.* 1951). The remaining suspension was acidified with PCA and centrifuged, and the resultant supernatant was analyzed for total RNA, by the orcinol assay (Munro and Fleck, 1966). The pellet, containing protein and DNA, was washed twice with PCA and then hydrolysed in HCl. An air flow was used to removed the acid. The free amino acids were resuspended in sodium citrate buffer, and phenylalanine in the samples was determined as described above. The protein synthesis rate, $K_s$ (% $day^{-1}$), was calculated as:

$$K_s = \frac{SA_p}{SA_f} \times \frac{1440}{t} \times 100, \quad (4)$$

where $SA_p$ is the protein-bound specific radioactivity (disints $min^{-1}$ $nmol^{-1}$), $SA_f$ is the specific activity of the pool of free amino-acids (disints $min^{-1}$ $nmol^{-1}$), 1440 is the number of minutes in a day, $t$ is the time from [$^3$H]phenylalanine injection to tissue sampling (min), and 100 is the conversion constant to percentage.

### *Statistical methods*

Significant differences between the control and $Zn^{2+}$-exposed groups for $Ca^{2+}$ influx, and the $K_m$ and $J_{max}$ values for $Ca^{2+}$ influx, were evaluated by Student's *t*-test (two-tailed, unpaired). All statistical tests on these data were based on $N$=6 per treatment, rather than $N$=48, because the eight fish in each test concentration were not entirely independent, but had been exposed to an identical water quality in a single flux chamber. The Mann–Whitney *U*-test was used to detect differences between $Zn^{2+}$-exposed fish and controls in tissue levels of copper, zinc, MT and total RNA, $Zn^{2+}$ influx and protein synthesis rate. Groups were considered significantly different at $P$<0.05. In the acute toxicity tests, the groups were considered different if the 95 % confidence limits for the $LT_{50}$ values did not overlap (Litchfield, 1949).

## Results

Calcium influx in juvenile rainbow trout obeyed Michaelis–Menten kinetics (Fig. 1). One day before the start of $Zn^{2+}$-exposure, the $K_m$ in the absence of $Zn^{2+}$ was 104 $\mu$mol $l^{-1}$ and the $J_{max}$ was 62 $\mu$mol $kg^{-1}$ $h^{-1}$ (Figs 2 and 3). During the experimental period, both the $K_m$ and $J_{max}$ values for the control group exhibited significant fluctuations. These were more pronounced for $K_m$, which varied from 38 to 93 $\mu$mol $l^{-1}$ (Fig. 2), whereas the lowest and highest values for $J_{max}$ were 40 and 69 $\mu$mol $kg^{-1}$ $h^{-1}$, respectively (Fig. 3).

Despite these variations in the controls over time, exposure to waterborne $Zn^{2+}$ caused consistent changes in the kinetics of $Ca^{2+}$ uptake. $Zn^{2+}$ markedly increased the $K_m$ for $Ca^{2+}$ influx (by up to 300 %), while there was only a small depressant effect (35 % at most) on $J_{max}$ (Fig. 1). With the exception of day 15, the elevations in $K_m$ were highly significant on all days relative to the simultaneous controls (Fig. 2), whereas the reduction in $J_{max}$ was significant only on days 9 and 15 (Fig. 3). These results were indicative of a large competitive component (increased $K_m$) and a small non-competitive component (decreased $J_{max}$) to the inhibitory effect of $Zn^{2+}$ on $Ca^{2+}$ uptake.

Despite this highly effective competitive inhibition by very low levels of $Zn^{2+}$ (150 $\mu$g $l^{-1}$=2.3 $\mu$mol $l^{-1}$), the actual reductions of $Ca^{2+}$ uptake experienced by the fish

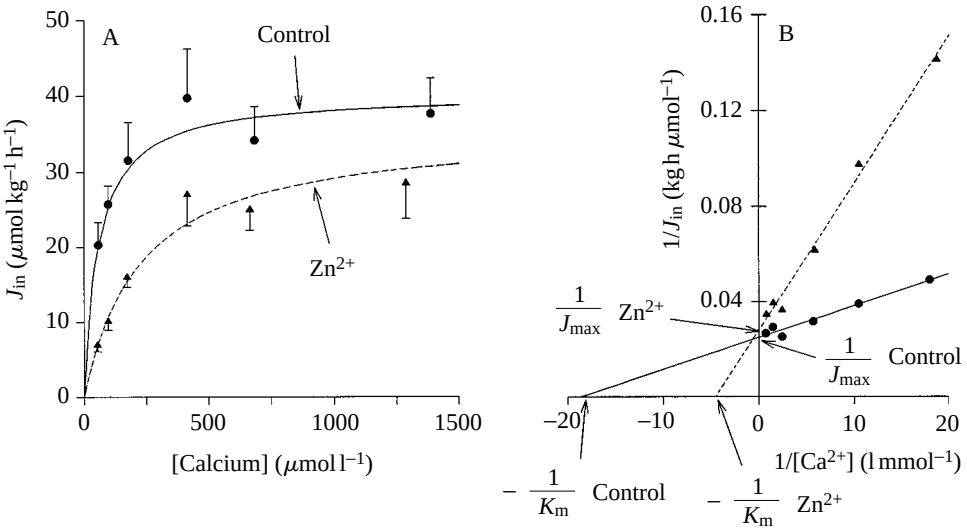


Fig. 1. (A) Michaelis–Menten kinetics of $Ca^{2+}$ uptake in freshwater rainbow trout and the inhibitory effect of sublethal waterborne $Zn^{2+}$ (150 $\mu$g $l^{-1}$=2.3 $\mu$mol $l^{-1}$). The solid line and filled circles show the unidirectional influx of $Ca^{2+}$ in control fish on day 50 at different concentrations of external $Ca^{2+}$. The dashed line and filled triangles show the $Ca^{2+}$ uptake in rainbow trout exposed for 50 days to a sublethal waterborne $[Zn^{2+}]$. Each point indicates the mean value for eight fish and vertical bars denote one-way S.E.M. The curves were generated by the Michaelis–Menten equation, using values of $K_m$ and $J_{max}$ obtained from the Lineweaver–Burk plots (B). Note the large increase in $K_m$ and very small decrease in $J_{max}$ in response to $Zn^{2+}$ exposure, indicative of a large competitive component and a small non-competitive component to the inhibition.

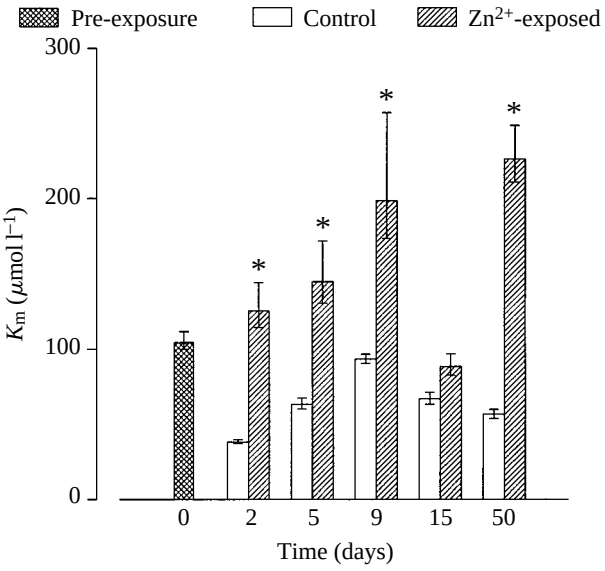


Fig. 2. Changes in the Michaelis–Menten constant $K_m$ for $Ca^{2+}$ uptake during 50 days of exposure of freshwater rainbow trout to sublethal waterborne [$Zn^{2+}$] (150 $\mu g\,l^{-1}$= 2.3 $\mu mol\,l^{-1}$). Means ±1 S.E.M. (six groups of eight fish each); asymmetrical S.E.M. derived from regression lines fitted to Lineweaver–Burk plots. The crosshatched bar shows the $K_m$ value for the fish 1 day before the start of the exposure. The hatched bars represent the $Zn^{2+}$-exposed fish and the open bars the simultaneous control group. $Zn^{2+}$-exposed means marked with an asterisk are significantly different ($P$<0.05) from the simultaneous control mean.

during the exposure were fairly modest (Fig. 4). This occurred because the $K_m$ for $Ca^{2+}$ uptake (38–230 $\mu mol\,l^{-1}$), even when greatly elevated by $Zn^{2+}$ competition, was so far below the available concentration of $Ca^{2+}$ (1.0 $mmol\,l^{-1}$) in the holding tanks. Indeed, at this acclimation level of $Ca^{2+}$ in the water, the actual $J_{in}$ (Fig. 4) closely paralleled the small alterations in $J_{max}$ (cf. Fig. 3).

Three hours after the onset of $Zn^{2+}$ exposure, $Ca^{2+}$ influx was analyzed for both controls and exposed fish in the absence of waterborne $Zn^{2+}$. A 3 h exposure to $Zn^{2+}$ caused a dramatic 23-fold decrease in $K_m$ for $Ca^{2+}$ (Table 1). There was also a much smaller, but still significant, decrease (20 %) in $J_{max}$ of exposed fish (Table 1) similar to that seen in the presence of $Zn^{2+}$ (cf. Fig. 3). Again, the $J_{max}$ remained the dominant factor determining the actual $J_{in}$ at the high water $Ca^{2+}$ level to which the fish were acclimated. Zinc-exposed fish exhibited a slightly decreased influx (15 %) of $Ca^{2+}$ after the removal of $Zn^{2+}$ from the water (Table 1). These results indicate that the actual competitive inhibition of $Ca^{2+}$ influx, at least during the early days of exposure, may have been even greater than indicated by the large increases in apparent $K_m$ shown in Fig. 2, which were determined in the presence of $Zn^{2+}$. The latter were occurring against a background of a compensatory decrease in the true $K_m$ determined in the absence of $Zn^{2+}$ in the experimental fish. In contrast, the small non-competitive inhibition appeared to be a

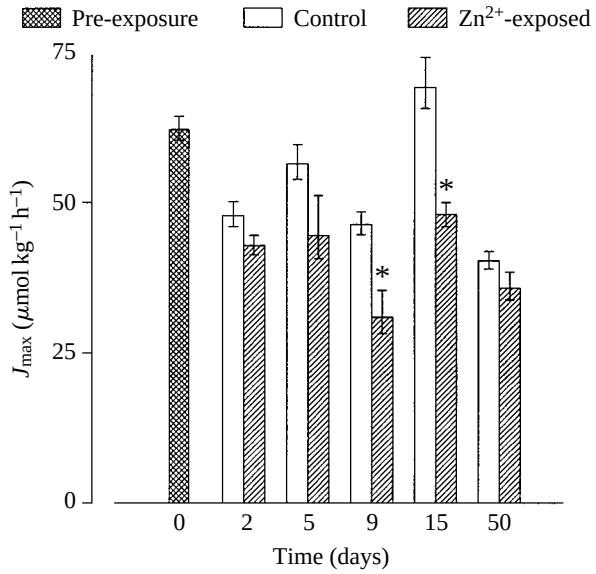


Fig. 3. Changes in the Michaelis–Menten constant $J_{max}$ for $Ca^{2+}$ uptake (maximal uptake rate) during 50 days of exposure of freshwater rainbow trout to sublethal waterborne [$Zn^{2+}$] (150 $\mu g\,l^{-1}$=2.3 $\mu mol\,l^{-1}$). Means ±1 S.E.M. (six groups of eight fish each); asymmetrical S.E.M. derived from regression lines fitted to Lineweaver–Burk plots. Other details as in legend of Fig. 2.

Table 1. *Kinetic constants ($K_m$, $J_{max}$) for the uptake of $Ca^{2+}$ and $Ca^{2+}$ influx rate at the acclimation water [$Ca^{2+}$] of 1.0 mmol $l^{-1}$ in the absence of waterborne $Zn^{2+}$*

| Treatment | $K_m$ ($\mu mol\,l^{-1}$) | $J_{max}$ ($\mu mol\,kg^{-1}\,h^{-1}$) | $J_{in}$ ($\mu mol\,kg^{-1}\,h^{-1}$) |
|---|---|---|---|
| Control | 92 (+8 –6) | 53 (+3 –2) | 50 (+2 –2) |
| Zinc-exposed | 4 (+0.5 –0.5)* | 42 (+1 –1)* | 42 (+4 –3)* |

The $Zn^{2+}$-exposed trout were exposed for 3 h to sublethal waterborne $Zn^{2+}$ (150 $\mu g\,l^{-1}$=2.3 $\mu mol\,l^{-1}$) and then immediately tested for $Ca^{2+}$ influx kinetics.

An untreated group served as the simultaneous control.

Means ± 1 S.E.M. ($N$=6 groups of 8 fish each); asymmetrical S.E.M. derived from regression lines fitted to Lineweaver–Burk plots.

An asterisk denotes a statistical difference from the control ($P$<0.05).

relatively stable blockade or destruction of transport sites, which remained the same whether or not the $Zn^{2+}$ was still present.

The $Zn^{2+}$ influx at the exposure concentration (150 $\mu g\,l^{-1}$=2.3 $\mu mol\,l^{-1}$) was measured on day 56. The $Zn^{2+}$ influx into $Zn^{2+}$-exposed fish was 26.5±3.9 $nmol\,kg^{-1}\,h^{-1}$ (mean ± S.E., $N$=10), which was significantly lower than the influx, 43±5.9 $nmol\,kg^{-1}\,h^{-1}$ (mean ± S.E., $N$=10), measured for controls.

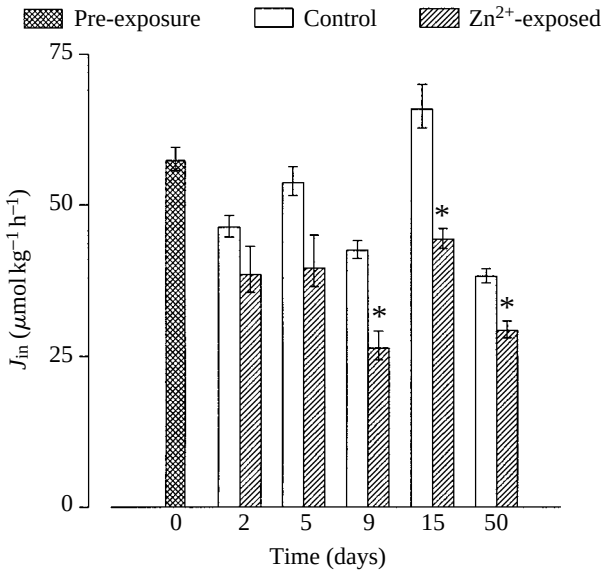


Fig. 4. Changes in the actual unidirectional influx rate of $Ca^{2+}$ ($J_{in}$) in hard water ($[Ca^{2+}]$=1.0 mmol $l^{-1}$), during 50 days of exposure of freshwater rainbow trout to sublethal waterborne $[Zn^{2+}]$ (150 $\mu$g $l^{-1}$=2.3 $\mu$mol $l^{-1}$). Means ±1 S.E.M. ($N$=6); asymmetrical S.E.M. derived from regression lines fitted to Lineweaver–Burk plots. Other details as in legend of Fig. 2.

$Zn^{2+}$-exposed fish developed increased tolerance to a potentially lethal concentration of $Zn^{2+}$. In the $LT_{50}$ test started on day 27, challenge with 3.4 mg $Zn^{2+}$ $l^{-1}$ resulted in an $LT_{50}$ of 45.5, +30.5, −18.3 h (mean, +, − 95 % confidence limits) for the control fish. The $LT_{50}$ for $Zn^{2+}$-exposed fish was greater than 144 h (the end of the test), by which time only 40 % of the fish had died. Consequently, no statistics could be carried out, but extrapolation of the probit mortality *versus* log time curve gave an $LT_{50}$ value for exposed fish of about 230 h. A final acute toxicity test, started on day 50, yielded $LT_{50}$ values for controls of 33.3, +8.0, −6.5 h and for pre-exposed fish of 49.5, +7.3, −5.9 h. These values were significantly different.

There were no differences between the groups in the levels of zinc, copper and MT in gills and liver at day 60 of the experiment (Fig. 5). The concentration of zinc in gills (about 75 $\mu$g $g^{-1}$ wet mass=1.1 $\mu$mol $g^{-1}$) was five times higher than in liver. In contrast, hepatic copper content was approximately 80 $\mu$g $g^{-1}$ (1.2 $\mu$mol $g^{-1}$), whereas the gill copper level was only about 1 $\mu$g $g^{-1}$ (15 nmol $g^{-1}$). The MT level was twice as high in the liver as in the gills.

Starvation of unexposed fish was employed as a positive control for the protein synthesis experiment. This treatment caused the predicted decrease in whole-body protein synthesis rate ($K_s$) and RNA content (Table 2). $K_s$ also declined significantly in the gills, but not in the liver, of starved fish. Twenty-three days of exposure to $Zn^{2+}$ also significantly reduced the $K_s$ in the gills by 11 %. With the exception of this

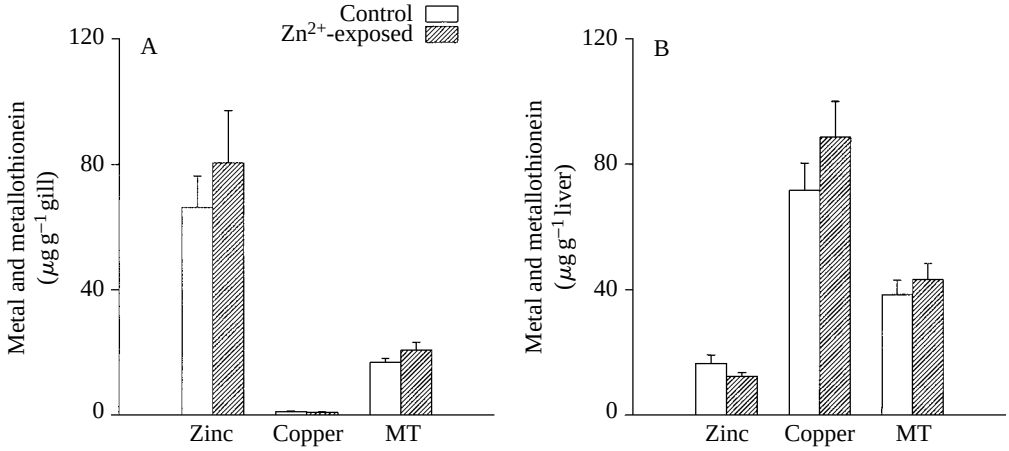


Fig. 5. Contents of zinc, copper and metallothionein (MT) in gills (A) and liver (B) of freshwater rainbow trout after 60 days of exposure to sublethal waterborne [$Zn^{2+}$] (150 $\mu g\,l^{-1}$=2.3 $\mu mol\,l^{-1}$). Means ±1 S.E.M. The hatched bars represent the $Zn^{2+}$-exposed fish and the open bars the simultaneous control group. There were no statistically significant differences ($P$>0.05) between the groups.

Table 2. *Protein synthesis rate ($K_s$), protein content and total RNA content in rainbow trout held under control conditions for 23 days, exposed to sublethal waterborne $Zn^{2+}$ (150 $\mu g\,l^{-1}$=2.3 $\mu mol\,l^{-1}$) for 23 days, or held under control conditions but starved for 4 days prior to test on day 23*

| | Whole body | | | | Gill | Liver |
|---|---|---|---|---|---|---|
| | RNA | | Protein | $K_s$ | $K_s$ | $K_s$ |
| Group | (mg $g^{-1}$ tissue) | (mg $g^{-1}$ protein) | (%) | (% $day^{-1}$) | (% $day^{-1}$) | (% $day^{-1}$) |
| Control | 2.93±0.08 | 28.3±1.4 | 10.8±0.4 | 0.89±0.12 | 2.57±0.11 | 9.81±0.95 |
| Zinc-exposed | 2.94±0.17 | 27.2±1.5 | 11.2±0.3 | 0.91±0.15 | 2.29±0.08* | 8.76±1.07 |
| Starved | 2.47±0.07* | 22.1±1.1* | 11.3±0.4 | 0.49±0.06* | 2.06±0.12* | 10.52±0.93 |

Means ± 1 S.E.M. ($N$=8–10).
An asterisk denotes a significant difference ($P$<0.05) from the control.

reduction, no other effects of $Zn^{2+}$ exposure were observed on protein synthesis or RNA levels.

## Discussion

The $K_m$ values measured in juvenile fish (average 21 g) under control conditions in the present study agreed well with previous measurements on adult trout (average 244 g) in the same water quality (Perry and Wood, 1985). However $J_{max}$ values and the actual $Ca^{2+}$ influx were two- to threefold higher than previous measurements on adult trout. This

difference probably reflects allometry plus the more intensive mineralization of bone that occurs in young fish. There was a considerable variation in $K_m$ and $J_{max}$ during the experimental period for both controls and exposed fish (Figs 2, 3). We have observed the same phenomenon in a separate study (C. Hogstrand, S. D. Reid and C. M. Wood, unpublished results). The $Ca^{2+}$ influx in juvenile rainbow trout appears to follow a biorhythm, a phenomenon first documented by Wagner *et al.* (1985, 1986), who observed that there could be a fivefold cyclic variation in the uptake of $Ca^{2+}$ with a periodicity of about 11 days.

The present study shows that waterborne $Zn^{2+}$ competitively inhibits the uptake of $Ca^{2+}$ (Fig. 1), whereas Spry and Wood (1989*a*) demonstrated that waterborne $Ca^{2+}$ competitively inhibited the uptake of $Zn^{2+}$ in rainbow trout under similar water quality conditions. This substantiates previous evidence that these two elements compete for the same uptake sites and routes (Spry and Wood, 1985, 1988, 1989*a*). For both elements, oesophageal ligation experiments have eliminated drinking as a significant route of influx in freshwater rainbow trout and the gills appear to be the principal site of entry for waterborne $Ca^{2+}$ and $Zn^{2+}$ (Perry and Wood, 1985; Spry, 1987). It remains to be established whether the competition between $Zn^{2+}$ and $Ca^{2+}$ takes place at the apical membrane, the basolateral membrane of the transport cells or at both sites. There is ample evidence depicting the chloride cell as the uptake site for $Ca^{2+}$ (Perry and Wood, 1985; Flik *et al.* 1985; Perry and Flik, 1988; McCormick *et al.* 1992; Perry *et al.* 1992; Marshall *et al.* 1992). The competitive interaction between $Zn^{2+}$ and $Ca^{2+}$ suggests that the branchial uptake of $Zn^{2+}$ also occurs *via* the chloride cell. Cadmium, which is a very potent inhibitor of $Ca^{2+}$ uptake, also seems to enter the gills through apical voltage-independent $Ca^{2+}$ channels of the chloride cells (Verbost *et al.* 1987, 1989*b*).

The primary toxic effect of $Cd^{2+}$ is probably an inhibition of the high-affinity $Ca^{2+}$-ATPase on the basolateral membrane rather than a competition at the apical channel (Verbost *et al.* 1987, 1988, 1989*a*,*b*). The inhibition of $Ca^{2+}$ uptake is not reversed within a 12 h period if the $Cd^{2+}$ is removed, which suggests that $Cd^{2+}$ causes permanent damage to the $Ca^{2+}$-ATPase (Reid and McDonald, 1988). In the present study, $Zn^{2+}$ exerted only a small inhibitory effect on $J_{max}$ (Fig. 3), implying that the non-competitive component was minor compared with the competitive inhibition. The inhibition of $J_{max}$ could possibly reflect allosteric site interaction. This small effect persisted when the waterborne $Zn^{2+}$ was acutely removed (Table 1), indicating either a strong binding of $Zn^{2+}$ to non-competitive inhibitory sites or a small reduction in the number of functional transporters. The important point is that $Zn^{2+}$, in contrast to $Cd^{2+}$, did not appear to cause any severe damage to the $Ca^{2+}$ uptake mechanism. The probable reason is that $Zn^{2+}$, unlike $Cd^{2+}$, is an essential micronutrient in fish, and that branchial $Zn^{2+}$ uptake is a normal phenomenon which becomes especially important when the diet is deficient in $Zn^{2+}$, i.e. the system is designed to take up $Zn^{2+}$ in moderate quantities (Spry *et al.* 1988). Only when the waterborne $[Zn^{2+}]$ becomes unusually elevated does the potential for damage become evident. Recently, Bentley (1992) demonstrated that the presence of $Cd^{2+}$ also reduces $Zn^{2+}$ influx. The nature of this inhibition is unknown, although the antagonizing effects of both $Ca^{2+}$ and $Cd^{2+}$ on the uptake of $Zn^{2+}$ fit well to the model that $Zn^{2+}$ influx occurs *via* the $Ca^{2+}$ uptake pathway. If $Zn^{2+}$ moves through the gill epithelium *via* the $Ca^{2+}$ pathway,

then the blockade of the common $Ca^{2+}/Zn^{2+}$ transport mechanism by $Cd^{2+}$ would inhibit $Zn^{2+}$ influx.

Fish exposed to $Zn^{2+}$ for 3 h showed a remarkable 23-fold increase in the affinity for $Ca^{2+}$ when they were returned to $Zn^{2+}$-free water (i.e. true $K_m$; Table 1). The response was not an artefact because we have confirmed its presence after 24 h of exposure in an entirely separate study at the same $Zn^{2+}$ concentration (C. Hogstrand, S. D. Reid and C. M. Wood, unpublished results). Interestingly, the response was similar to that observed when rainbow trout were transferred from the present acclimation water ($1000\,\mu mol\,l^{-1}$) to water containing a very low $Ca^{2+}$ concentration ($25\,\mu mol\,l^{-1}$) for 1 day (Perry and Wood, 1985). The explanation could be that the fish or the transport system 'senses' low $[Ca^{2+}]$ when $Zn^{2+}$ is added to the water, because less $Ca^{2+}$ is available for uptake or is bound to the gill surface. Consequently, the observed increase in affinity of the $Ca^{2+}$ uptake sites could represent an attempt to restore the normal $Ca^{2+}$ transporting activity of the gill. The mechanism of this response remains unknown; it could occur *via* a simple surface chemistry effect on the transport cells associated with the displacement of bound $Ca^{2+}$, *via* a cellular effect (e.g. at the channel, intracellular binding protein or basolateral enzyme) and/or *via* a hormonal effect. It is also unknown whether this decrease in true $K_m$ persisted throughout the exposure period. At the very least, an attenuation of the effect seems likely in view of the general increase in apparent $K_m$ with time during prolonged $Zn^{2+}$ exposure (Fig. 2).

It was surprising to find that $Ca^{2+}$ uptake kinetics exhibited no indication of recovery over time despite the development of increased $Zn^{2+}$ tolerance seen in the $LT_{50}$ tests. Indeed if anything, the apparent $K_m$ for $Ca^{2+}$ influx tended to increase during prolonged $Zn^{2+}$ exposure (Fig. 2). This contrasts with sublethal copper or aluminium concentrations, both of which cause an initial disturbance in $Na^+$ uptake followed by a restoration of branchial $Na^+$ transport as increased tolerance to the metals develops (Laurén and McDonald, 1987; McDonald *et al.* 1991). For example, Laurén and McDonald (1987) found an immediate increase in $K_m$ and decrease in $J_{max}$ for $Na^+$ influx in trout exposed to sublethal $[Cu^{2+}]$ for 24 h (i.e. mixed competitive and non-competitive inhibition). However, over 28 days of chronic exposure, $J_{max}$ recovered almost completely, while $K_m$ was only partially restored; changes in $J_{max}$ paralleled alterations in branchial $Na^+/K^+$-ATPase activity. The important difference may be that neither copper nor aluminium is taken up by the $Na^+$ transport pathway, whereas $Zn^{2+}$ seems to be taken up by the $Ca^{2+}$ transport pathway. A strategy to decrease the uptake of $Zn^{2+}$ might be to reduce the affinity of the $Ca^{2+}$ uptake sites. Indeed, the $Zn^{2+}$ influx of exposed fish, measured at day 56, was significantly decreased relative to the simultaneous control. In accord with these observations, Bradley *et al.* (1985) found that $Zn^{2+}$-exposed rainbow trout survived longer in $LT_{50}$ tests than did controls. When the pre-exposed fish finally died, their gills did not contain more $Zn^{2+}$ than the controls, although the former spent more time in the test medium before they died. Similarly, the uptake rate of $Cd^{2+}$ was decreased during chronic $Cd^{2+}$ exposure (Wicklund Glynn and Olsson, 1991), as well as by several pharmacological treatments known to reduce the apical permeability of the gill epithelium to $Ca^{2+}$ (Verbost *et al.* 1989*b*). Thus, one mechanism to alter the influx of both $Zn^{2+}$ and $Cd^{2+}$ could be to change the permeability of the apical membrane to $Ca^{2+}$.

The actual influx of $Ca^{2+}$ ($J_{in}$), at the water [$Ca^{2+}$] present in the holding tanks (1.0 mmol $l^{-1}$), was only moderately decreased by exposure to $Zn^{2+}$ (Fig. 4). This water $Ca^{2+}$ concentration gave $J_{in}$ values in the region close to saturation of the $Ca^{2+}$ uptake curve (cf. Fig. 1). Therefore, the $J_{in}$ was largely determined by the $J_{max}$ and changes in $K_m$ only marginally affected the actual influx. The apparent $K_m$ for $Zn^{2+}$ uptake, measured by Spry and Wood (1989*a*) in unexposed rainbow trout in the same Hamilton tapwater ([$Ca^{2+}$]=1.0 mmol $l^{-1}$), was 240 $\mu$g $l^{-1}$ (3.7 $\mu$mol $l^{-1}$). Since the water $Zn^{2+}$ concentration used in the present study (2.3 $\mu$mol $l^{-1}$) was below the $K_m$ for $Zn^{2+}$, a change in the affinity of the binding sites for $Zn^{2+}$ would probably alter the $J_{in}$ for $Zn^{2+}$ significantly without having much effect on the $J_{in}$ for $Ca^{2+}$. We suggest that, during exposure to sublethal [$Zn^{2+}$], the fish may manipulate the $K_m$ for a mutual $Ca^{2+}/Zn^{2+}$ carrier so as to reduce $Zn^{2+}$ influx markedly without greatly altering $Ca^{2+}$ influx. Measurements of the $K_m$ for $Zn^{2+}$ transport during prolonged $Zn^{2+}$ exposure and/or depletion will be required to test this hypothesis.

The $Zn^{2+}$-exposed fish in the present study displayed an increased tolerance to a lethal concentration of $Zn^{2+}$ from the first test at day 27. Since plasma $Ca^{2+}$ was not analyzed, it is not known whether the fish also showed a physiological recovery in terms of normalized plasma [$Ca^{2+}$]. During chronic exposure of tilapia to $Cd^{2+}$, the plasma [$Ca^{2+}$] was restored, a response which appeared to be correlated with increased prolactin cell activity, increased plasma cortisol concentration and a proliferation of chloride cells, though it was not clear whether unidirectional $Ca^{2+}$ influx, efflux or both were corrected (Fu *et al.* 1989, 1990). In the present study on rainbow trout, the $J_{max}$ of both controls and exposed fish declined during the experimental period. Thus, there was no evidence for an increased number of $Ca^{2+}$ uptake sites associated with the $Zn^{2+}$ adaptation.

Induction of metallothionein has often been depicted as a possible mechanism responsible for adaptation and development of increased tolerance to metals (Chapman, 1985; Hamilton and Mehrle, 1986; Roesijadi, 1992; McDonald and Wood, 1993). While there is convincing evidence that MT protects cells from metal toxicity, there are few experimental data directly supporting the idea that MT is the dominant factor for the development of increased tolerance to metals in fish (see Hogstrand and Haux, 1991). One of the few is the work of Bradley *et al.* (1985), who exposed rainbow trout to a much higher, but still sublethal, level of waterborne $Zn^{2+}$ (670 $\mu$g $l^{-1}$=10.2 $\mu$mol $l^{-1}$) and noted a direct correlation between the development of increased tolerance and the induction of a heat-stable sulphydryl-rich protein (‘putative MT’) in gills and liver. Spry and Wood (1989*b*) also found induction of a heat-stable metal-binding protein in the gills of trout exposed to 148 $\mu$g $Zn^{2+}$ $l^{-1}$ (2.3 $\mu$mol $l^{-1}$) for 16 weeks, but concluded that the protein was not MT. In the present study, MT was measured directly by a highly specific RIA (Hogstrand and Haux, 1990). The fish in our study exhibited an enhanced tolerance to $Zn^{2+}$ without any increase in branchial or hepatic MT (Fig. 5). Moreover, even if all of the MT in the gill (20.7 $\mu$g $g^{-1}$≈3.2 nmol $g^{-1}$) bound zinc only (i.e. 7 atoms of zinc per MT molecule; Olsson and Haux, 1985), the total zinc-binding capacity of the branchial MT would still be no more than 1.9 % of the zinc present in the gill (1.2 $\mu$mol $g^{-1}$). Even more interestingly, after 60 days of exposure, there was no accumulation of zinc in liver or gill tissue (Fig. 5). With no increased zinc content in these tissues, elevated levels of

MT would not be predicted. Bradley *et al.* (1985), using a much higher exposure level of waterborne $Zn^{2+}$, found only very modest levels of gill and liver $Zn^{2+}$ build-up, but apparently this was enough to induce 'MT' synthesis. The lack of zinc accumulation and MT induction, found in the present study, suggests that the $Zn^{2+}$ concentration used was within the nutritional range. If MT is involved in the process of $Zn^{2+}$ adaptation, it would be as a second line of defence when the level of $Zn^{2+}$ exposure is high enough to cause a net accumulation in tissues.

As the $Zn^{2+}$ adaptation develops and the mechanisms responsible for this are expressed, an enhanced metabolic activity could be predicted. An increase in metabolic activity would be reflected in elevated levels of RNA and an increased protein synthesis rate. However, the only significant change found in these variables in $Zn^{2+}$-exposed fish was a moderate decrease in the rate of protein synthesis in gill tissue. The lack of increased protein synthesis rates in response to the treatment again points towards the conclusion that the $Zn^{2+}$ exposure was below the toxic range.

As predicted, in starved fish there was a reduction in the RNA content of muscle and in the rate of protein synthesis in the gills and muscle. However, the protein synthesis rate of the liver remained unchanged. The latter observation, though somewhat surprising, agrees with the data of McMillan and Houlihan (1988), who found that protein synthesis rates were similar in the livers of fasted or fully fed rainbow trout.

In summary, the present study supports previous evidence that $Zn^{2+}$ and $Ca^{2+}$ compete directly for a common uptake route at the gills. The $K_m$ for $Ca^{2+}$ uptake is far below the normal water $[Ca^{2+}]$, so $J_{in}$ normally occurs at close to the maximum rate set by $J_{max}$. There is only a small non-competitive component (i.e. decrease in $J_{max}$), but a large competitive component (increase in $K_m$), to the inhibitory effect of $Zn^{2+}$ on $Ca^{2+}$ transport. During chronic sublethal $Zn^{2+}$ exposure, a significantly increased tolerance to $Zn^{2+}$ can develop without the induction of MT in gills or liver. During this period, the apparent affinity of the transport mechanism for $Ca^{2+}$ is greatly decreased, which may be a way to minimize $Zn^{2+}$ uptake *via* the same route without greatly affecting $Ca^{2+}$ uptake. This is supported by the findings that the $Zn^{2+}$ influx was decreased in $Zn^{2+}$-adapted fish and that there was no zinc build-up in gills or liver.

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