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Ca^{2+} *VERSUS* Zn^{2+} TRANSPORT IN THE GILLS OF FRESHWATER RAINBOW TROUT AND THE COST OF ADAPTATION TO WATERBORNE Zn^{2+}

CHRISTER HOGSTRAND, SCOTT D. REID AND CHRIS M. WOOD
McMaster University, Department of Biology, Hamilton, Ontario L8S 4K1, Canada

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Summary

Previous work suggested that Ca^{2+} and Zn^{2+} share a common uptake pathway in rainbow trout gills. We here report on relationships between the kinetic variables for unidirectional Ca^{2+} influx and unidirectional Zn^{2+} influx during a 1 month exposure of freshwater rainbow trout to Zn^{2+} ($150\,\mu g\,l^{-1}$=$2.3\,\mu mol\,l^{-1}$ as total zinc, Zn). Initial exposure to Zn^{2+} caused a large competitive inhibition of Ca^{2+} influx, as indicated by a threefold increase in apparent K_m for Ca^{2+} (measured in the presence of Zn^{2+}). There was also a smaller non-competitive inhibition (50 % decrease in J_{max}) of the Ca^{2+} transport system, which was abolished after 1–2 weeks of exposure. The K_m, measured in the absence of Zn^{2+}, decreased dramatically (i.e. elevated affinity) on days 1–4 but increased thereafter; both true and apparent K_m finally stabilized significantly above control levels. However, the K_m values for Ca^{2+} (<$200\,\mu mol\,l^{-1}$) were low relative to the Ca^{2+} level in the water ($1000\,\mu mol\,l^{-1}$), and therefore the changes did not influence the actual Ca^{2+} influx of the fish, which tracked J_{max}. In contrast, water [Zn^{2+}] ($2.3\,\mu mol\,l^{-1}$ as total Zn) was close to the reported apparent K_m ($3.7\,\mu mol\,l^{-1}$) for Zn^{2+} influx in the presence of $1000\,\mu mol\,l^{-1}$ Ca^{2+}. Unidirectional Zn^{2+} influx increased during the first week of exposure to waterborne Zn^{2+}, followed by a persistent reduction to about 50 % of control levels, effects that may be largely explained by the observed changes in true K_m for Ca^{2+}. We speculate that the initial response of the fish to elevated [Zn^{2+}] is to compensate for a reduced availability of Ca^{2+} by markedly increasing the affinity of a dual Ca^{2+}/Zn^{2+} transporter. Once the Ca^{2+} influx is 'corrected' by restoration of functional transport sites (J_{max}), the system is tuned to limit the influx of Zn^{2+} by a persistent reduction in the affinities for both ions.

The changes in influx characteristics for Ca^{2+} and Zn^{2+} were correlated with internal physiological alterations indicative of adaptation to Zn^{2+} and increased metabolic cost. Depressed plasma [Ca] was corrected within 1 week, and there were no effects on whole-body [Ca] or [Zn]. A slight accumulation of Zn in the gills was associated with increased branchial metallothionein levels. Rates of protein synthesis and degradation in the gills were initially increased and whole-body growth was transiently impaired, effects which were reversed after 18 days of exposure. Sublethal challenge with Zn^{2+} (at $450\,\mu g\,l^{-1}$=$6.9\,\mu mol\,l^{-1}$ as total Zn) always depressed plasma [Ca] in control fish, but by 1 month of exposure to Zn^{2+} at $150\,\mu g\,l^{-1}$ (as total Zn), experimental fish were resistant to challenge. However, the fish did not acquire increased survival tolerance (LT_{50}) to a lethal concentration of Zn^{2+} ($4\,mg\,l^{-1}$=$61\,\mu mol\,l^{-1}$ as total Zn).

Key words: fish, rainbow trout, *Oncorhynchus mykiss*, gills, Zn^{2+}, Ca^{2+}, transport kinetics, acclimation, tolerance, adaptation, metallothionein, protein synthesis, RNA, growth rate, energetics.

Introduction

Zinc is a micronutrient for fish, but elevated concentrations of waterborne Zn^{2+} can impair the branchial uptake of Ca^{2+} (Spry and Wood, 1985, 1988; Sayer *et al.* 1991; Hogstrand *et al.* 1994). In brown trout (*Salmo trutta*), a lethal concentration of waterborne Zn^{2+} caused a net loss of Ca^{2+} by affecting both influx and efflux variables (Sayer *et al.* 1991). Recently, we found that a sublethal level of waterborne Zn^{2+} competitively inhibited the branchial Ca^{2+} influx of freshwater rainbow trout (*Oncorhynchus mykiss*), suggesting that Zn^{2+} and Ca^{2+} may compete for the same uptake sites (Hogstrand *et al.* 1994). These data confirm and extend previous findings that Ca^{2+} is a competitive inhibitor of Zn^{2+} influx through the gills (Spry and Wood, 1989). A simple competitive interaction between Ca^{2+} and Zn^{2+} at the gill surface could also explain the well-known protective effect of increased water hardness on fish exposed to Zn^{2+} (Pagenkopf, 1983).

Disturbances of branchial ionoregulation are common effects of metal exposure, although the underlying mechanisms may differ between metals (Wood, 1992). During chronic exposure to sublethal concentrations of metals, fish often adapt and normal rates of ion transport are restored (McDonald and Wood, 1993). However, adaptation to an elevated Zn^{2+} concentration does not seem to be associated with a complete restoration of normal Ca^{2+} influx kinetics: during prolonged

exposure (60 days) of hardwater-acclimated rainbow trout to waterborne Zn^{2+}, the apparent K_m for Ca^{2+} influx was chronically elevated and Zn^{2+} influx was reduced (Hogstrand *et al.* 1994). Bradley *et al.* (1985) also observed that the rate of Zn accumulation in gills was lower in Zn^{2+}-exposed rainbow trout. Hogstrand *et al.* (1994) reasoned that the K_m for Ca^{2+} was kept elevated to reduce the influx of Zn^{2+} and that this, in itself, could serve as a mechanism of adaptation. However, the influx of Zn^{2+} was only measured at the end of the 60-day exposure period and a relationship between Zn^{2+} influx and the affinity of the Ca^{2+} transporter has yet to be demonstrated.

The first major objective of the present study was, therefore, to examine the relationship between the kinetic variables of unidirectional Ca^{2+} influx and the influx of Zn^{2+} in freshwater rainbow trout. In our previous study (Hogstrand *et al.* 1994), the Ca^{2+} influx kinetics of Zn^{2+}-exposed fish was analyzed, but only in the presence of the inhibitor (Zn^{2+}). Therefore, it could not be determined whether the chronically elevated K_m for Ca^{2+} was due to the continuing competitive inhibition by Zn^{2+} or whether the properties of the carrier(s) *per se* had also changed. To address this question, the Ca^{2+} influx kinetics of rainbow trout chronically exposed for 1 month to Zn^{2+} ($150\,\mu g\,l^{-1}$=$2.3\,\mu mol\,l^{-1}$ as total Zn) was studied both in the presence and absence of the inhibitor (Zn^{2+}), yielding values of the apparent J_{max} and K_m as well as the true J_{max} and K_m for Ca^{2+} influx. The rate of Zn^{2+} influx into the whole animal and the Zn accumulation rate in gill tissue were also analyzed throughout the experimental period. These measurements allowed a continuous comparison between the kinetic variables for Ca^{2+} influx and the influx of Zn^{2+}. As measures of the degree of adaptation to an elevated Zn^{2+} concentration, plasma and whole-body levels of Ca and levels of Zn in the gills and whole body were monitored during the exposure. Periodic challenges with a higher, but still sublethal, concentration of Zn^{2+} were performed to assess whether the Ca^{2+} regulatory system was becoming resistant to the hypocalcaemic effect of Zn^{2+} challenge. Acute toxicity tests (LT_{50}) to Zn^{2+} were conducted regularly to give evidence for any change in tolerance to a lethal concentration of Zn^{2+}. Finally, the branchial intracellular sequestering capacity for Zn was assessed by analysing the concentrations of the metal-binding protein metallothionein (MT), using a specific and sensitive radioimmunoassay (Hogstrand and Haux, 1990).

The second major objective of this study was to assess the metabolic cost associated with adaptation to sublethal levels of Zn^{2+}. It is generally believed that adaptation to any physiologically unfavourable environment is linked to increased metabolic expenditure, so that less energy is available for other processes such as feeding, growth and reproduction (Bayne *et al.* 1979; Widdows *et al.* 1990; Calow, 1991). Wilson *et al.* (1994*a*,*b*) have recently provided evidence for such energetic limitations in rainbow trout adapting to sublethal Al^{3+} concentrations. McDonald and Wood (1993) have further postulated that adaptation to waterborne metals involves a damage repair process in the gills, such that increased cost would be induced at this site. While damage repair would probably be associated with an increased rate of protein synthesis, evidence to date is equivocal. Indeed, Hogstrand *et al.* (1994) observed a slightly reduced protein synthesis rate in the gills of Zn^{2+}-exposed rainbow trout. However, in that study, the rate of protein synthesis was measured after increased tolerance to Zn^{2+} had developed (23 days), by which time any damage repair process may already have been completed. To investigate these questions, in the present study, the growth of fish on a fixed ration of food was monitored throughout the period of Zn^{2+} exposure, while protein and RNA concentrations in the gills, together with protein synthesis and degradation rates, were determined on days 9 and 18 of the exposure using the technique developed by Houlihan *et al.* (1986).

Materials and methods

Experimental animals

Approximately 1600 juvenile rainbow trout *Oncorhynchus mykiss* (Walbaum) (4.90±0.92 g; mean ± S.D., *N*=96) were obtained from a local fish hatchery (Spring Valley Trout Farm, Petersburg, Ontario). The fish were held in two 264 l fibreglass tanks (800 per tank), each supplied with 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) at a flow rate of 900 $ml\,min^{-1}$ and a temperature of 9 °C. Fish were fed dry trout pellets (Martin's Feed Mill Ltd, Ontario) at a ration of 1 % of their body mass per day.

Chronic Zn^{2+} exposure

After an acclimation period of 14 days, 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 control tank. The flow rate of the added Zn^{2+} stock ($45\,mg\,l^{-1}$=$690\,\mu mol\,l^{-1}$) was maintained at 3 $ml\,min^{-1}$, by a peristaltic pump, to reach a concentration of total Zn in the tank of $150\,\mu g\,l^{-1}$ ($2.3\,\mu mol\,l^{-1}$; measured range 132–184 $\mu g\,l^{-1}$). Using the MINEQL+ computer program for chemical equilibrium management (Schecher and McAvoy, 1991), it was calculated that the nominal $[Zn^{2+}]$ of the exposure water was $1.4\,\mu mol\,l^{-1}$. The day the Zn^{2+} exposure started is referred to as 'day 0' throughout the text. Water flow rate and dosing rate were checked daily and adjusted if necessary, and feeding was maintained at 1 % of the body mass per day. The experiment continued for 31 days, over May and June, during which the water temperature gradually increased from 9 to 13 °C due to ambient conditions. No mortality occurred during the Zn^{2+} exposure.

Water samples from the exposure tank were taken for analysis of total Zn every 1–2 days. Samples were acidified with HNO_3 (trace metal analysis grade, BDH Chemicals, was used in all procedures) to a final acid concentration of 1 % (w/w), and Zn was measured with an atomic absorption spectrophotometer (AAS; Varian AA-1275), using an air/acetylene flame.

Ca^{2+} uptake kinetics

The kinetics of unidirectional Ca^{2+} influx was determined on days −2 (i.e. 2 days before the treatment started in the experimental group), 17 and 31 in the control group, and on days 0 (i.e. after 4 h of exposure to Zn^{2+}), 1, 4, 7, 15 and 29 in the Zn^{2+}-exposed group. Kinetic analysis was performed as described by Hogstrand *et al.* (1994). In brief, eight fish from the control group were put into each of six polypropylene flux bags, containing 3 l of synthetic water ([NaCl]=0.7 mmol l^{-1}; [$KHCO_3$]=1.9 mmol l^{-1}; pH=8.0) with a designated concentration of Ca^{2+} reached by adding an appropriate volume of a $^{45}Ca(NO_3)_2$ stock solution (52 mmol l^{-1}; specific activity 35 kBq mol^{-1}). The six flux bags represented a geometric series of increasing [Ca^{2+}], with approximate values of 52, 104, 207, 413, 820 and 1615 μmol l^{-1}. In the flux medium, 99.4 % of the total Ca content was calculated to be present as Ca^{2+} (MINEQL+; Schecher and McAvoy, 1991). For the Zn^{2+}-exposed fish, two kinetic series (six flux bags each) were run in parallel. One series was performed identically to those for the control group (i.e. in the absence of Zn^{2+}), while the other was run in the presence of Zn^{2+} (150 μg l^{-1}=2.3 μmol l^{-1} as total Zn in each flux bag). The former yielded values of true K_m and J_{max} (no competitor present), and the latter yielded values of apparent K_m and J_{max} (i.e. determined in the presence of the competitor). On day 0 (first 4 h of exposure), only the latter series was performed. On all other days, both series were performed. The Zn^{2+}-exposed fish that were tested for Ca^{2+} influx in the absence of Zn^{2+} were rinsed in dechlorinated Hamilton tapwater for 5 min before they were introduced to the flux bags.

The unidirectional Ca^{2+} influx was calculated from the appearance of ^{45}Ca radioactivity in the whole body over a 4 h period. The fish were killed, rinsed in 10 mmol l^{-1} $Ca(NO_3)_2$ to displace surface-bound ^{45}Ca, and processed for counting as described by Hogstrand *et al.* (1994). Lineweaver–Burk plots were used to obtain values of J_{max} and K_m for the Michaelis–Menten equation. Note that the Lineweaver–Burk transformation yields asymmetrical S.E.M. values. The Michaelis–Menten equation was then used to interpolate the actual unidirectional influx of Ca^{2+}, J_{in}, for fish at the acclimation concentration of Ca^{2+}, 1.0 mmol l^{-1}. The body masses of the 48–96 fish used for analysis of Ca^{2+} influx were measured for estimation of growth in the two groups.

Influx of Zn^{2+} and Zn accumulation rate in gills

The unidirectional influx of Zn^{2+}, at the Zn^{2+} concentration of the exposure tank (150 μg l^{-1}=2.3 μmol l^{-1} as total Zn), was measured on days −2 (i.e. 2 days before the treatment started in the experimental group), 17 and 31 in the control group, and on days 0 (i.e. first 24 h of Zn^{2+} exposure), 1, 4, 7, 15 and 29, as described by Spry and Wood (1989) with the modifications used by Hogstrand *et al.* (1994). Black polypropylene bags were used as flux compartments. Each of the bags was filled with 20 l of dechlorinated Hamilton tapwater (see above) with 150 μg Zn l^{-1} (2.3 μmol l^{-1}) added as $ZnSO_4$. 5 min before the fish were introduced to the bags, 2.6 MBq of carrier-free ^{65}Zn was added to each bag. Ten fish from each tank were transferred to the flux bags and were then held there for 24 h. At the end of the flux period, the fish were lightly anaesthetized (MS 222, 20 mg l^{-1}) and individually transferred for 1 min to a beaker with tapwater containing MS 222 (1.0 g l^{-1}), and 'cold' Zn^{2+} (1.0 mg l^{-1} as total Zn) to displace surface-bound ^{65}Zn. The fish was blotted dry and a terminal blood sample (100 μl) was withdrawn with a heparinized Hamilton syringe from the caudal vessels. The blood was then centrifuged at 14 000 ***g*** for 3 min to obtain the plasma fraction. The gills were dissected out and the soft tissue was scraped from the cartilaginous tissue with two microscope slides. Plasma and gills were assayed for ^{65}Zn activity in a γ-counter (MINAXI γ Auto-Gamma 5000 Series, Canberra-Packard). Water samples were similarly counted for ^{65}Zn activity and total Zn in water was measured by atomic absorption spectroscopy (AAS) (Varian 1275). The influx rate of Zn^{2+} was calculated by the method established for rainbow trout by Spry and Wood (1989). The procedure was based on the relationship between the steady-state activity of ^{65}Zn in plasma after 24 h of exposure and the influx of Zn^{2+} (in nmol kg^{-1} h^{-1}). The calculation is not affected by the endogenous level of Zn in plasma (Spry and Wood, 1989). The accumulation rate of Zn in the gills was calculated from the ^{65}Zn activity in the gills, corrected for counts from blood trapped in the tissue (Munger *et al.* 1991), the specific activity of ^{65}Zn in the water and the mass of the soft gill tissue.

The data presented on Ca^{2+} and Zn^{2+} influx are likely to represent influx *via* the branchial pathway, because oesophageal ligation experiments have eliminated the intestinal route as a significant route of Ca^{2+} and Zn^{2+} uptake under identical conditions to the flux experiments performed here (Perry and Wood, 1985; Spry, 1987).

Levels of metallothionein, Cu, Zn and Ca

The levels of metallothionein (MT), Cu and Zn in gills, Ca in plasma and whole-body concentrations of Ca and Zn were analyzed at different points during the experiment. Cu in gills was measured because MT binds both Cu and Zn, and therefore an altered Zn concentration could change the Cu level. Exposed fish were sampled on days 1, 4, 7, 15 and 29, whereas controls were sampled on days −2 (i.e. 2 days before the exposure started in the experimental group), 17 and 28. On each sampling occasion, 10 fish were killed by a blow to the head and a blood sample from each fish was taken from the caudal vessels with a heparinized Hamilton syringe. The blood sample was centrifuged (14 000 ***g***, 3 min) and the plasma was separated, frozen in liquid nitrogen and stored at −70 °C for later analysis of Ca content by AAS. Gills were dissected out and the soft tissue of the gill filaments was scraped from the cartilaginous tissue with two microscope slides. The gill samples and the carcasses were frozen in liquid nitrogen and stored at −70 °C.

The frozen gill scrapings from fish sampled on days −2, 1, 15, 17, 28 and 29 were later weighed (typically, 50 mg of tissue

was obtained from each fish), thawed and homogenized individually in 1.0 ml of 50 mmol l^{-1} Tris–HCl, pH 8.0, at 0 °C, using a glass–Teflon homogenizer. A 450 μl sample from each homogenate was stored at −20 °C for subsequent analysis of Zn and Cu. The remainder was centrifuged at 10 000 ***g*** for 20 min at 4 °C; the supernatant was decanted, frozen in liquid nitrogen and stored at −70 °C until used for measurement of [MT]. Levels of MT 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 a 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 liver of cadmium-injected rainbow trout was used as a MT standard. The MT content of the standard was calibrated against a standard curve prepared from purified rainbow trout MT (Hogstrand and Haux, 1990). The working range of the RIA was 1.5–15 pmol of rainbow trout MT per assay tube, which corresponds to 92–920 pmol g^{-1} tissue wet mass.

For analysis of metals, 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 cooled to room temperature, 0.75 vols of H_2O_2 was added, and the samples were then evaporated to dryness at 120 °C. Finally, 5 ml of 1 % HNO_3 was added to the digestion tubes and Cu and Zn were analyzed by AAS as described above. No solid material was present in the final solution.

Whole carcasses (whole body minus gills) of fish sampled on days −2 (i.e. 2 days before the start of the exposure), 1, 15, 17, 28 and 29 were digested in 20 ml of 35 % HNO_3 at 85 °C for 2 h, using graduated plastic tubes with screw caps (Falcon). The tubes were cooled, 2.0 ml of 30 % H_2O_2 was added, and the digestion was then continued at 85 °C for another 12 h. The digest was then made up to the nearest 5 ml with deionized water (Nanopure). Samples for Zn analysis were further diluted 25 times with deionized water (Nanopure) and analyzed by AAS. Ca analysis was performed on digests diluted 3000 times with 0.2 % $LaCl_2$.

Sublethal Zn^{2+} challenge

The effect of a sublethal Zn^{2+} challenge on plasma Ca levels was studied on days −2 (i.e. 2 days before the start of exposure in the experimental group), 17 and 31 for control fish and on days 1, 15 and 29 for pre-exposed fish. In each challenge, 10 fish were transferred to a polypropylene bag containing 20 l of dechlorinated Hamilton tapwater with Zn^{2+} (450 μg l^{-1}= 6.9 μmol l^{-1} as total Zn), added as $ZnSO_4$. The bag was fitted with an air-line and submerged in a waterbath at the same temperature as the holding tanks. After 24 h, the fish were killed by a blow to the head and a blood sample was withdrawn from the caudal vessels. The blood was centrifuged (14 000 ***g***, 3 min) and the plasma separated and frozen in liquid nitrogen. Samples were stored at −70 °C until analysis of Ca content by AAS. The plasma [Ca] of the challenged fish was compared with that of untreated control fish. Sampling of untreated control fish is described above.

Acute toxicity tests

Median lethal time (LT_{50}) tests of both Zn^{2+}-exposed fish and controls were performed on days 1, 4, 7, 15 and 30. On these days, 10 fish from each group were transferred to a 14 l black acrylic box. The test chamber was equipped with air-lines and centrally divided by a plastic mesh to allow separate exposure of the two groups to identical water conditions. To ensure efficient water turnover, the inflow and the drain were at opposite ends of the chamber. The water was from the same source and was at the same temperature as the water in the holding tanks. A $ZnSO_4$ stock (160 mg Zn l^{-1}=2.45 mmol l^{-1}) was added at 3.0 ml min^{-1} to the incoming water (120 ml min^{-1}) by a peristaltic pump and mixing funnel, yielding a measured total Zn concentration in the test chambers averaging 4 mg l^{-1} (61 μmol l^{-1}). [Zn] in the water was monitored daily. The concentrations of total Zn (in mg l^{-1}) in the water at the different test occasions were 4.1±0.1 (*N*=4), 3.9±0.5 (*N*=5), 4.1±0.4 (*N*=5), 4.0±0.1 (*N*=3) and 4.1±0.3 (*N*=7) (mean ± S.D.), respectively. Mortality was monitored throughout the first day of exposure, and four times daily thereafter, for up to 10 days (226 h). The fish were not fed during the toxicity test. LT_{50} values ±95 % confidence limits were calculated from plots of probit mortality against log time by the methods of Litchfield (1949).

Protein turnover

Rates of protein synthesis and degradation, protein content and total RNA level in gills were analyzed on days 9 and 18. One day before the start of the Zn^{2+}-exposure (i.e. day −1), 20 fish from each group were lightly anaesthetized (MS 222), weighed to the nearest 0.01 g and labelled individually by freeze-branding (Mighell, 1969). Branded fish were put in a secluded compartment of each tank. The compartments were made from 8 l polypropylene buckets, each with a mesh bottom and lid. The buckets were submerged in the tanks and the incoming water was directed through the buckets into the tanks. Fish for protein turnover determination were fed daily and independently of the other fish in the tanks, at a rate of 1 % of their body mass (i.e. at the same rate as the rest of the fish).

On day 9, all of the branded fish were quickly and gently dried and weighed. Ten fish from each group were then injected *via* the caudal vessels with 150 mmol l^{-1} phenylalanine, pH 7.5 (1 ml per 100 g fish), containing 3.7 MBq ml^{-1} of L-2,6-[^{3}H]phenylalanine. Following injection, the fish were placed into individual 250 ml black beakers with a fitting lid and air-line, containing the same water as before. After 1 h, fish were killed by a blow to the head. The whole gill basket was immediately dissected out and frozen in liquid nitrogen. The samples were then stored at −70 °C for later analysis. On day 18, the remaining 10 fish from each group were weighed, injected with radioactive phenylalanine, and sampled as described above.

Gill content of protein and RNA and fractional rate of protein synthesis (K_s) in gills were analyzed as detailed in Houlihan *et al.* (1986) and Hogstrand *et al.* (1994). The protein degradation (K_d) rate was calculated by subtracting the fractional protein synthesis rate of the gills from the fractional growth rate of the gills multiplied by measured protein content.

Statistical methods

Owing to known temporal fluctuations in Ca^{2+} influx in juvenile rainbow trout (see Wagner *et al.* 1985; Hogstrand *et al.* 1994), statistical tests were only applied to samples taken within 3 day periods. Significant differences between the control and the Zn^{2+}-exposed groups in Ca^{2+} influx, and the K_m and J_{max} values for Ca^{2+} influx, were evaluated by Student's *t*-tests (two-tailed, unpaired). Statistical tests on these data were based on N=6 (six Ca^{2+} concentrations) per treatment, rather than N=48, because the eight fish in each test bag were not independent, but rather were exposed to identical water conditions in a single flux chamber. The Mann–Whitney *U*-test was used to detect differences between Zn^{2+}-exposed fish and controls for plasma [Ca] and tissue levels of Cu, Zn, MT and total RNA, Zn^{2+} influx, growth and protein turnover rate. Groups were considered significantly different at P<0.05. Within a treatment group, the significance of trends over time was assessed by analysis of variance (ANOVA). The Tukey HSD test was used to extend the ANOVA with *post-hoc* comparisons of means. In the acute toxicity tests, the groups were considered to be statistically different if the 95% confidence limits for the LT_{50} values did not overlap.

Results

Plasma Ca content was initially depressed by Zn^{2+} exposure (at 150 $\mu g\,l^{-1}$=2.3 $\mu mol\,l^{-1}$ as total Zn) but recovered within a week (Fig. 1). After 29 days of the experiment, the plasma [Ca] of exposed fish was significantly higher than that of the controls. There was also a slight time-dependent variation in plasma [Ca] within the control group; on day 17, the concentration of Ca in plasma was significantly lower than on day −2 (ANOVA, Tukey HSD, P<0.01). There were no differences in the whole-body Ca level that could be attributed to Zn^{2+} exposure (Table 1). However, there was a general increase in the whole-body Ca content of both groups over the experimental period (ANOVA, P<0.001).

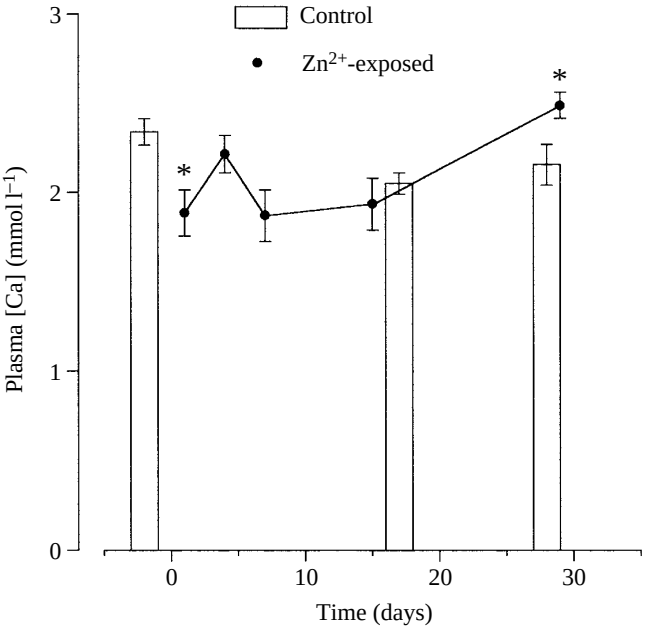

Fig. 1. Plasma Ca levels in juvenile rainbow trout during exposure to Zn^{2+} (150 $\mu g\,l^{-1}$=2.3 $\mu mol\,l^{-1}$ as total Zn). Values are means ±1 S.E.M. (N=10). Values for exposed fish (filled circles) obtained on days 1, 15 and 29 were tested statistically against values for control fish (open bars) sampled on days −2, 17 and 28, respectively. * indicates a significant difference from the control values at P<0.05.

There was a significant (ANOVA, P<0.05) increase in the Zn content of the gills of both groups of fish over the experimental period. Exposure to Zn^{2+} increased the Zn concentration in the soft tissue of the gills relative to control values, but this elevation in branchial Zn was only significant at the end of the experiment (day 29; Table 1). The whole-body

Table 1. *Levels of Zn and Ca in the whole body and Cu, Zn and metallothionein (MT) concentrations in gills of Zn^{2+}-exposed (at a concentration of 150 μg l^{-1} = 2.3 μmol l^{-1} as total Zn) rainbow trout and controls*

	Control					Zn^{2+}-exposed				
	Whole body		Gill			Whole body		Gill		
Day	Zn (nmol g^{-1})	Ca (μmol g^{-1})	Zn (nmol g^{-1})	Cu (nmol g^{-1})	MT (nmol g^{-1})	Zn (nmol g^{-1})	Ca (μmol g^{-1})	Zn (nmol g^{-1})	Cu (nmol g^{-1})	MT (nmol g^{-1})
–2 to 1	370±25	126±4	508±75	43±11	7.10±0.71	420±27	135±5	424±31	31±5	6.58±0.45
15 to 17	341±20	138±2	566±76	27±3	4.43±0.26	367±20	151±6	625±52	44±11	7.11±0.26*
28 to 29	348±15	152±7	783±86	34±4	4.86±0.31	361±17	152±7	1113±103*	29±3	8.33±0.62*

Exposed fish and controls were sampled on alternate days.
Values are means ± 1 S.E.M. (nmol g^{-1} wet mass or μmol g^{-1} wet mass), N=10.
* indicates a significantly different value compared to the control at P<0.05.
The day referred to in the table is the time elapsed from the onset of the chronic Zn^{2+} exposure until the start of the Zn^{2+} challenge.

Table 2. *The response of plasma [Ca] to a sublethal Zn^{2+} challenge (at 450 μg l^{-1} = 6.9 μmol l^{-1}, as total Zn, for 24 h) of Zn^{2+}-exposed (at 150 μg l^{-1} = 2.3 μmol l^{-1} as total Zn) rainbow trout and controls*

	Plasma [Ca] (mmol l^{-1})		
Day	Control (untreated)	Control (challenged)	Zn^{2+}-exposed (challenged)
–2 to 1	2.35±0.05	1.80±0.07†	1.95±0.15†
15 to 17	2.05±0.05	1.56±0.06†	1.95±0.23
29 to 31	2.15±0.11	1.88±0.05†	2.12±0.07*

† indicates groups significantly different (P<0.05) from the 'untreated control.'

* indicates a significant difference from the 'challenged' control.

The day referred to in the table is the time elapsed from the onset of the chronic Zn^{2+} exposure until the start of the Zn^{2+} challenge.

Exposed fish and controls were sampled on alternate days.

Values are means ± 1 S.E.M. (N=10).

content of Zn and branchial Cu levels remained unchanged throughout the experiment (Table 1). In the control group, the MT concentration of the gill was higher at the first sampling point on day −2 than in subsequent samplings (ANOVA, Tukey HSD, P<0.01). An increased level of branchial MT relative to controls was found after 15 and 30 days of Zn^{2+} exposure, indicating an increased capacity of the gills to immobilize Zn (Table 1). A 24 h sublethal challenge with Zn^{2+} (450 μg l^{-1}=6.9 μmol l^{-1} as total Zn) always significantly decreased the plasma [Ca] in fish that had not been previously exposed to Zn^{2+} [Control (challenged); Table 2]. At day 1, the plasma [Ca] was also reduced in fish that had been pre-exposed to Zn^{2+} (150 μg l^{-1}=2.3 μmol l^{-1} as total Zn), but not to the same extent as in controls. After 29 days of exposure, the pre-exposed fish were able to withstand the Zn^{2+} challenge without developing hypocalcaemia (Table 2).

In spite of the increased Ca^{2+} regulatory capability demonstrated by the sublethal Zn^{2+} challenge, the Zn^{2+}-exposed fish did not acquire an increased tolerance to a lethal concentration of Zn^{2+} (Table 3). After 30 days of exposure to Zn^{2+} (at 150 μg l^{-1}=2.3 μmol l^{-1} as total Zn), these fish had a lower LT_{50} when challenged with a lethal [Zn^{2+}] (4 mg l^{-1}=61 μmol l^{-1} as total Zn) than the controls. Before that, there was no significant difference in LT_{50} between the groups.

Table 3. *Median lethal time (LT_{50}) (in h) of Zn^{2+}-exposed (at 150 μg l^{-1} = 2.3 μmol l^{-1} as total Zn) rainbow trout and controls during a lethal challenge to Zn^{2+} (at 4 mg l^{-1} = 61 μmol l^{-1} as total Zn) at different times during the experimental period*

Day	Control	Zn^{2+}-exposed
1	50 (+8, –5)	43 (+3, –5)
4	35 (+7, –15)	38 (+9, –18)
7	61 (+9, –7)	68 (+24, –10)
15	33 (+1, –1)	29 (+10, –5)
30	74 (+16, –12)	33 (+14, –13)*

* indicates a value significantly different from the control at P<0.05.

Values are means ± 95 % confidence limits (in parentheses) (N=10).

Ca^{2+} influx through the gills obeyed Michaelis–Menten kinetics throughout the exposure period (see Fig. 1 from Hogstrand *et al.* 1994, for a typical relationship). The most pronounced effects of chronic Zn^{2+} exposure were on the kinetic variables (K_m, J_{max}) of Ca^{2+} influx. Initially, exposure to Zn^{2+} caused a 50 % reduction in the apparent J_{max} for Ca^{2+} influx (in the presence of Zn^{2+}) on day 0 during the first 4 h of Zn^{2+} exposure; i.e. a loss of available Ca^{2+} uptake sites (Fig. 2). This reduction in J_{max} persisted at 24 h (day 1) and was seen even when the treatment was withdrawn during the measurement of Ca^{2+} influx (in the absence of Zn^{2+}); i.e. both apparent J_{max} and true J_{max} were reduced (Fig. 2). Both J_{max} values were restored within a week and significantly elevated

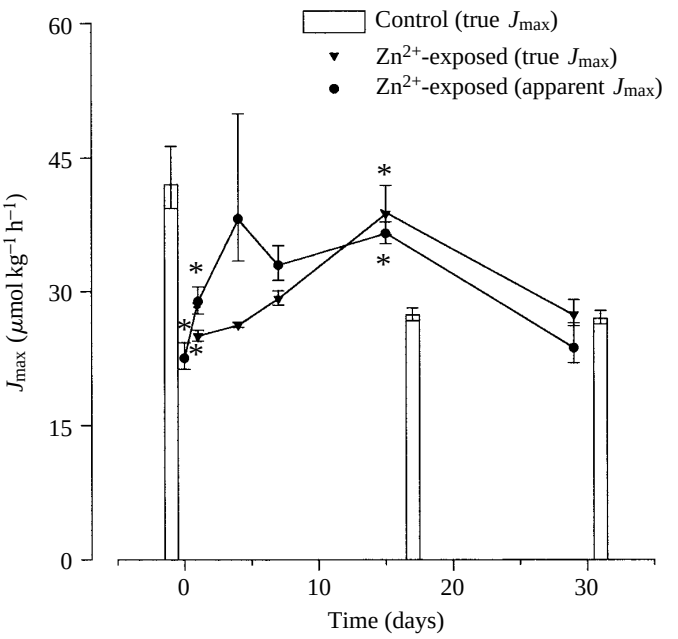

Fig. 2. Variation in the maximum rate of unidirectional Ca^{2+} influx (J_{max}) during chronic exposure of juvenile rainbow trout to Zn^{2+} (at 150 μg l^{-1}=2.3 μmol l^{-1} as total Zn), measured in the absence (true J_{max}) and in the presence (apparent J_{max}) of waterborne Zn^{2+}. Open bars show the J_{max} in control fish, filled triangles the true J_{max} (in the absence of Zn^{2+}) and filled circles the apparent J_{max} (in the presence of Zn^{2+}). Values are means ±1 S.E.M. (N=6 groups of eight fish each) derived from Lineweaver–Burk plots. Note that the Lineweaver–Burk transformation yields asymmetrical S.E.M. values. Values for exposed fish, obtained on days 1, 15 and 29, were tested statistically against values from control sampled on days −2, 17 and 31, respectively. Fish that were acutely exposed to Zn^{2+} for 4 h during the Ca^{2+} kinetics experiment on day 0 were tested statistically against the controls sampled on day −2. * indicates a significant difference from the control at P<0.05.

values of both apparent and true J_{max} were recorded on day 15. At the end of the experiment, the J_{max} values returned to the control level.

Acute exposure of 'Zn^{2+}-naive' fish to Zn^{2+} (150 μg l^{-1}=2.3 μmol l^{-1} as total Zn) on day 0 increased the apparent K_m for unidirectional Ca^{2+} influx by a factor of three; i.e. a large decline in Ca^{2+} affinity (Fig. 3). However, the apparent K_m returned to control values in fish that were exposed to Zn^{2+} for 24 h prior to the measurement of Ca^{2+} influx. 3 days later (day 4), the apparent K_m was found to be 4.5 times initial control values. After this peak, the apparent K_m declined and levelled out at a value about three times higher than that of the control for the remainder of the experiment. The true K_m for Ca^{2+} influx, as measured in the absence of the inhibitor (Zn^{2+}) in the water, decreased drastically after 24 h of exposure to Zn^{2+} from 50 to 0.75 μmol l^{-1}; i.e. a large increase in Ca^{2+} affinity. This effect was still present 3 days later, but after day 4 the true K_m increased and peaked on day 7. Later in the experiment, the true K_m stabilized at a level significantly above the control value (Fig. 3).

What is important for the fish, during chronic sublethal exposure to Zn^{2+}, is the actual influx of Ca^{2+} *at the [Ca^{2+}] of the ambient medium (1.0 mmol l^{-1})*. The actual Ca^{2+} influx (J_{in}) in the presence of Zn (150 μg l^{-1}=2.3 μmol l^{-1} as total Zn) was calculated from the Michaelis–Menten equation, using the measured values of apparent J_{max} and K_m (Fig. 4). The actual influx of Ca^{2+} (Fig. 4) closely followed the apparent J_{max} (Fig. 2), showing an initial decrease, followed by an 'overshoot' and a subsequent return to control values.

The unidirectional branchial influx of Zn^{2+} was measured for both Zn^{2+}-exposed fish and controls at the experimental concentration of Zn^{2+} (150 μg l^{-1}=2.3 μmol l^{-1} as total Zn; Fig. 5). It was found that the influx of Zn^{2+} was only about 0.3 % of that of Ca^{2+} (compare with Fig. 4). The Zn^{2+} influx of the control group fluctuated during the experimental period and was higher on day 17 than on days −2 and 31 (ANOVA, Tukey HSD, P<0.01). On day 1, the Zn^{2+} influx of pre-exposed fish did not differ from the control value for day −2. However, on days 15 and 29, the Zn^{2+} influx of pre-exposed fish was found to be half that of the control on days 17 and 31, respectively. The accumulation of Zn in the soft tissue of the gills, during the 24 h flux period, is shown in Fig. 6. With the exception for day 15, when the accumulation of Zn in gills of pre-exposed fish was the same as in controls, the Zn accumulation followed the Zn^{2+} influx. The Zn accumulation in gills of control fish exhibited an upward trend during the experiment and was significantly higher on days 17 and 31 than on day −2 (ANOVA, Tukey HSD, P<0.01).

The growth of fish over the 1 month Zn^{2+} exposure, determined from the masses of the 48–96 fish randomly sampled at the same times as the Ca^{2+} kinetics measurements, was similar for both groups (Fig. 7). However, the Zn^{2+}-exposed fish showed no increase in mass between days 7 and 15, with the consequence that Zn^{2+}-exposed fish sampled on day 15 were smaller than the controls sampled on day 17.

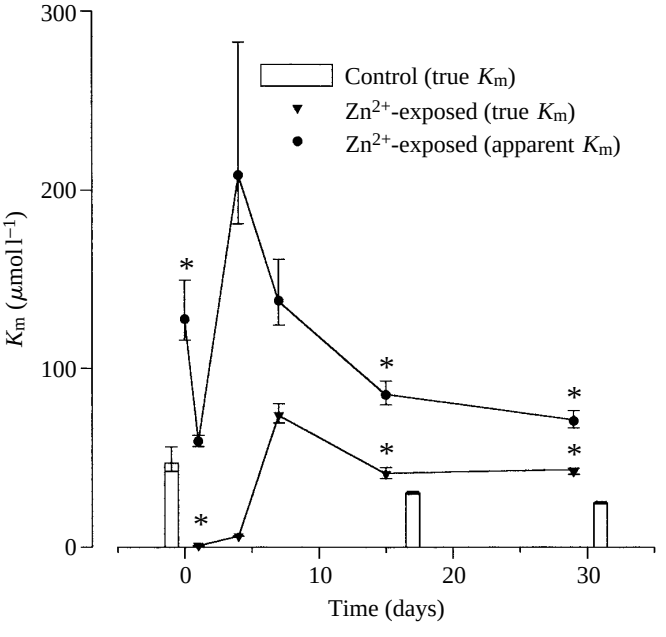

Fig. 3. Variations in the Michaelis–Menten constant (K_m) (inverse of Ca^{2+} affinity) of unidirectional Ca^{2+} influx during chronic exposure of juvenile rainbow trout to Zn^{2+} (at 150 μg l^{-1}=2.3 μmol l^{-1} as total Zn), measured in the absence (true K_m) and in the presence (apparent K_m) of waterborne Zn^{2+}. Open bars show the K_m in control fish, filled triangles the true K_m (in the absence of Zn^{2+}) and filled circles the apparent K_m (in the presence of Zn^{2+}). Other details as in legend of Fig. 2.

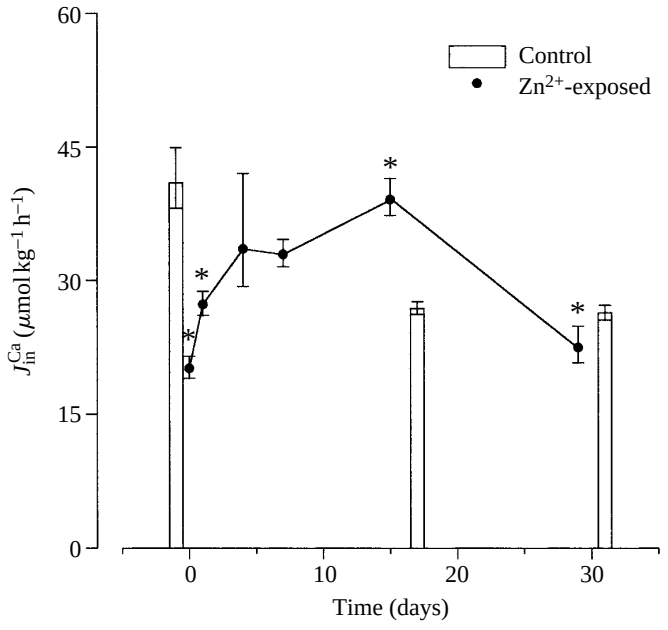

Fig. 4. Unidirectional influx of Ca^{2+} (J_{in}^{Ca}), in juvenile rainbow trout, at the acclimation Ca^{2+} concentration (1.0 mmol l^{-1}), during chronic exposure to Zn^{2+} (at 150 μg l^{-1}=2.3 μmol l^{-1} as total Zn). Open bars show the J_{in}^{Ca} of control fish and filled circles the J_{in}^{Ca} of Zn^{2+}-exposed fish. Means and asymmetrical S.E.M. values were derived from Lineweaver–Burk plots (N=6 groups of eight fish in each). Other details as in legend of Fig. 2.

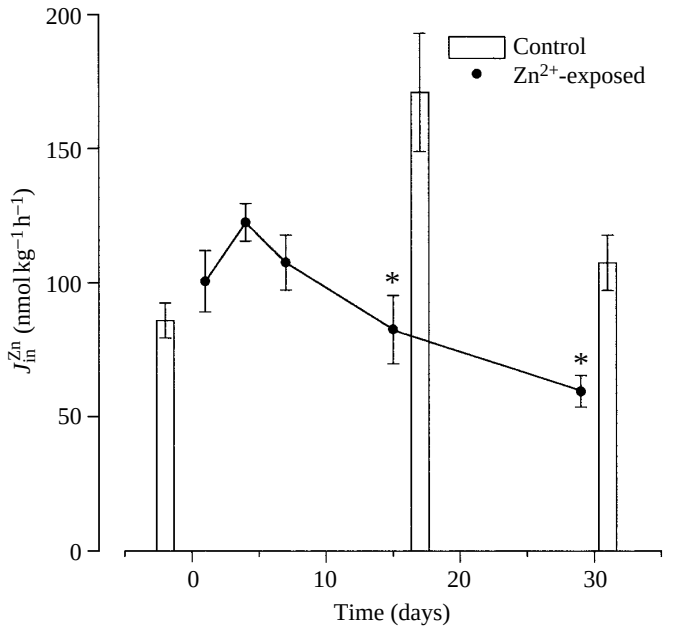

Fig. 5. Unidirectional influx of Zn^{2+} (J_{in}^{Zn}), at a total [Zn] of 150 μg l^{-1} (2.3 μmol l^{-1}), during chronic exposure of juvenile rainbow trout to Zn^{2+} at the same concentration. Open bars show the J_{in}^{Zn} in control fish and filled circles the J_{in}^{Zn} in chronically Zn^{2+}-exposed fish. Values are means ±1 S.E.M. (N=10). Values for exposed fish, obtained on days 1, 15 and 29, were tested statistically against values from controls sampled on day −2, 17 and 31, respectively. * indicates a statistically significant difference from the control at P<0.05.

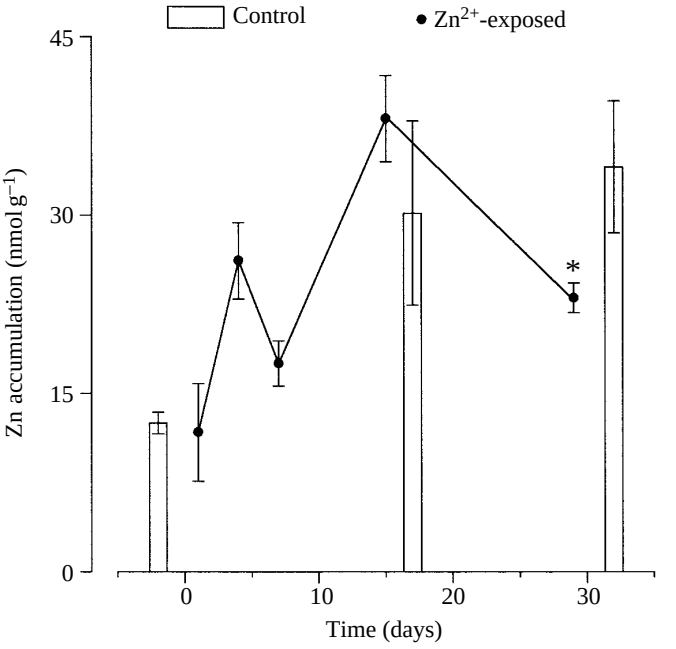

Fig. 6. Accumulation of Zn from waterborne Zn^{2+} in soft gill tissue during the 24 h Zn^{2+} influx experiments. Open bars show Zn accumulation in control fish and filled circles Zn accumulation in chronically Zn^{2+}-exposed fish. The data are expressed as nmol g^{-1} soft gill tissue (wet mass). Other details as in Fig. 5.

The fractional protein synthesis rate in the gills, K_s, was significantly elevated in Zn^{2+}-exposed fish on day 9, but after 18 days of exposure this difference was no longer present (Fig. 8A). The protein degradation rate of the gills, K_d, was approximately twice as high on day 9 as on day 18, and on day 9 the Zn^{2+}-exposed fish had significantly higher K_d than the control fish (Fig. 8B). There were no differences in the RNA content of the gills between groups or over time, suggesting that the maximal capacity for protein synthesis was not affected by the Zn^{2+} exposure (Fig. 8C). On both sampling occasions (days 9 and 18), Zn^{2+}-exposed fish had a lower branchial protein content than the control fish (Fig. 8D). The protein content within each group was not different between the sampling dates.

Discussion

We have previously shown that Zn^{2+} is a competitive inhibitor of branchial Ca^{2+} uptake in freshwater-adapted rainbow trout (Hogstrand *et al.* 1994). This finding was substantiated in the present study, which demonstrates very large changes in K_m (Fig. 3) and smaller changes in J_{max}. This study provides details on the nature of the effects of Zn^{2+} on the Ca^{2+} influx kinetics and on their consequences for Ca^{2+} and Zn^{2+} uptake. During the first days of Zn^{2+} exposure, there was a loss of available Ca^{2+} uptake sites (decreased apparent J_{max}; Fig. 2) that was reflected in a reduced J_{in} for Ca^{2+} (Fig. 4) and a lowered plasma [Ca] (Fig. 1). The inhibition of Ca^{2+} transporting capacity was not ameliorated when Zn^{2+} was removed from the water (true J_{max}), which suggests a persistent binding of Zn^{2+} to allosteric inhibitory sites and/or permanent inactivation of Ca^{2+} carriers. Within 7 days of the exposure, the number of functional uptake sites for Ca^{2+} was restored and the plasma [Ca] seemed to be normalized within the first few days. There was a dramatic increase in affinity of the Ca^{2+} carriers (decreased true K_m) observed after 1 and 4 days of Zn^{2+} exposure (Fig. 3), a response that was also observed in our previous study (Hogstrand *et al.* 1994). We speculate that the increased affinity for Ca^{2+} is a compensatory response to the loss of functional uptake sites and to the competitive inhibition of Ca^{2+} influx by Zn^{2+}. However, from day 7 and through the rest of the experiment, the true K_m was increased compared with the control. This delayed decrease in affinity for Ca^{2+} might seem contradictory considering that the fish were already suffering a disturbance of Ca^{2+} homeostasis, but it may have served to limit the uptake of Zn^{2+}. It has previously been found not only that Zn^{2+} competitively inhibits the branchial Ca^{2+} influx (Hogstrand *et al.* 1994) but also that Ca^{2+} is a competitive inhibitor of Zn^{2+} influx at the gills (Spry and Wood, 1989). This information suggests that Ca^{2+} and Zn^{2+} partially or fully share a branchial uptake route (Spry and Wood, 1989; Hogstrand *et al.* 1994). Therefore, a decreased

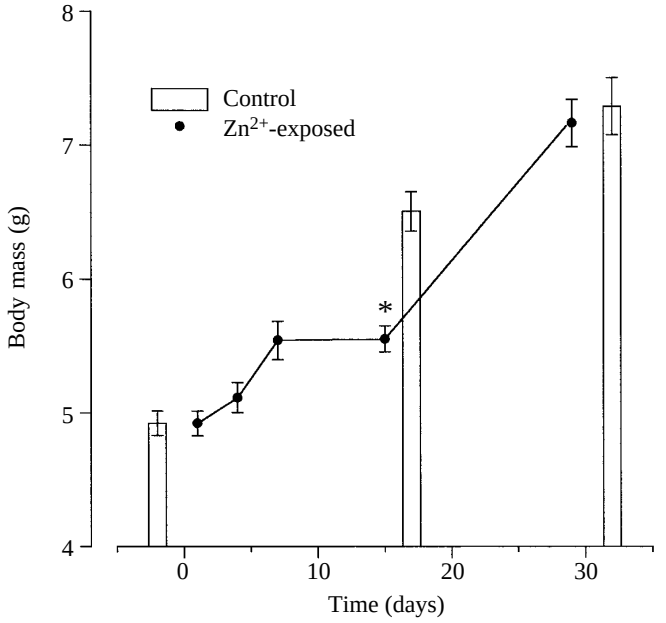

Fig. 7. Cumulative growth (measured as mass increase) of juvenile rainbow trout during chronic exposure to waterborne Zn^{2+} (150 $\mu g\,l^{-1}$=2.3 $\mu mol\,l^{-1}$ as total Zn). Open bars show the body mass of control fish and filled circles the mass of Zn^{2+}-exposed fish. Each point represents the mean ±1 S.E.M. of 48–96 fish. Values for Zn^{2+}-exposed fish, obtained on days 1, 15 and 29, were tested statistically against control values from controls sampled on days −2, 17 and 31, respectively. * indicates a statistically significant difference from control values at P<0.05.

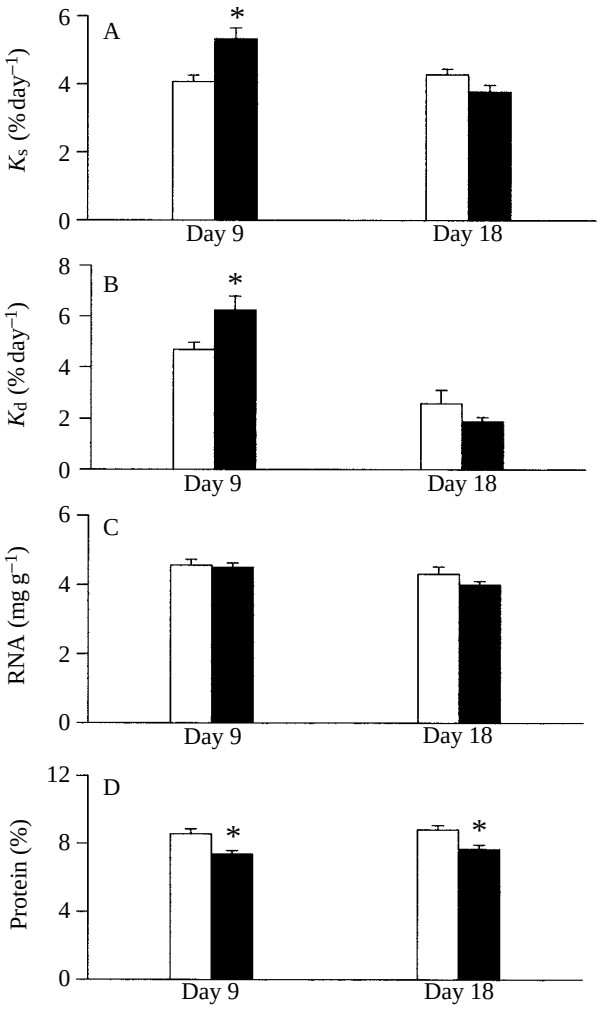

Fig. 8. Protein turnover in juvenile rainbow trout chronically exposed to Zn^{2+} (at 150 $\mu g\,l^{-1}$=2.3 $\mu mol\,l^{-1}$ as total Zn) on days 9 and 18 of the exposure. Open bars show the mean values (N=10) for controls and filled bars represent the Zn^{2+}-exposed fish. The error bars denote 1 S.E.M. (A) Fractional protein synthesis rate K_s in gills. (B) Fractional protein degradation rate K_d in gills. (C) Total RNA content, expressed as mg g^{-1} tissue (wet mass), of the gill. (D) Total protein content, expressed as a percentage of the tissue wet mass of the gills. * indicates a statistically significant difference from the control at P<0.05.

affinity of the carriers for Ca^{2+} could serve to reduce the Zn^{2+} influx.

Most of the Ca^{2+} transporting capacity (J_{max}) was restored during the first week of exposure, which would help the fish to maintain plasma [Ca] homeostasis even with a decreased affinity of the transporting sites. Furthermore, at the acclimation Ca^{2+} concentration of the water (1.0 mmol l^{-1}), the influx of Ca^{2+} was largely determined by the J_{max} (Figs 2 and 4) and the elevated K_m had a negligible effect on J_{in}. Zn^{2+} influx, however, should have been greatly influenced by a change in the affinity of the carrier, because the total Zn concentration in the exposure tank (150 $\mu g\,l^{-1}$=2.3 $\mu mol\,l^{-1}$) was below the measured apparent K_m for Zn^{2+} influx (240 $\mu g\,l^{-1}$=3.7 $\mu mol\,l^{-1}$) in identical conditions (Spry and Wood, 1989). Thus, we suggest that the initial response of the fish to Zn^{2+} is to compensate for the net Ca^{2+} loss by markedly increasing the affinity of the transporting sites. Once the J_{max} for Ca^{2+} has been ameliorated, and therefore the J_{in} for Ca^{2+} has been restored, the system is tuned to limit the influx of Zn^{2+} rather than to improve the Ca^{2+} uptake further.

The measured changes in Zn^{2+} influx fit with the hypothesis that Ca^{2+} and Zn^{2+} share a common uptake route through the gills. After 1 day of Zn^{2+} exposure, the Zn^{2+} influx was not different from that of control fish (Fig. 5). According to the Ca^{2+} influx kinetics, the affinity of the carriers increased, but there was also a decrease in the number of available uptake sites, leaving the Zn^{2+} influx unchanged. During the following few days, the number of carriers was restored and the Zn^{2+} influx was, therefore, increased. This restoration of the number of uptake sites was followed by a decrease in affinity of the Ca^{2+} transporting system, which would explain the subsequent reduction in Zn^{2+} influx. A decreased Zn^{2+} influx is likely to be an important physiological adaptation to an elevated water Zn^{2+} concentration. In a previous study (Hogstrand *et al.* 1994), we reported that the branchial influx of Zn^{2+} was decreased in rainbow trout exposed to Zn^{2+} during a 2 month period. The present study provides experimental evidence that

this reduction in Zn^{2+} influx is linked to a decreased affinity of a common Ca^{2+}/Zn^{2+} transporting system, but final proof requires Zn^{2+} kinetic studies.

With the exception of day 15, the pattern of Zn accumulation in the gills, during the 24 h flux period, paralleled the J_{in} values for Zn^{2+} (Figs 5 and 6). The disagreement between the day 15 and day 29 results, as to the effect of Zn^{2+} exposure on Zn accumulation in the gills, prevents any firm conclusion determining whether the apical entry step or the basolateral transfer of Zn to the blood is altered. However, there was a scattered, but highly significant, linear relationship between the Zn^{2+} influx and the accumulation of Zn in gills (r=0.420, P<0.0001, N=160). We therefore speculate that the rate-limiting step for Zn^{2+} influx is the transport through the apical membrane and that the influx of Zn^{2+} may be regulated by changing the permeability of the apical membrane to Zn^{2+}. The available evidence suggests that both Ca^{2+} and Cd^{2+} are transported through the gill epithelium by the chloride cells (Perry and Wood, 1985; Verbost *et al.* 1987, 1988, 1989; Perry and Flik, 1988; Flik *et al.* 1993; Perry *et al.* 1992; Marshall *et al.* 1992; McCormick *et al.* 1992). Both of these ions are thought to pass through the apical membrane of the chloride cells passively, through a voltage-independent Ca^{2+} channel, driven by their electrochemical gradients (Perry and Flik, 1988; Verbost *et al.* 1989). Furthermore, there is evidence that the influxes of Ca^{2+} and Cd^{2+} are subject to regulation by the hormone stanniocalcin at the level of the apical membrane (Lafeber *et al.* 1988; Verbost *et al.* 1989; Flik, 1990). In view of this information and the results from the present study, it seems quite possible that the branchial influx of Zn^{2+} can be regulated by a change in affinity of a common apical Ca^{2+}/Zn^{2+} channel that is also permeable to Cd^{2+}. More research is required to verify this hypothesis. Such experiments may include manipulation of the permeability of the apical membrane for Ca^{2+} and Zn^{2+} by lanthanum exposure and/or stanniocalcin treatment.

Both the present study and our previous investigation (Hogstrand *et al.* 1994) document temporal fluctuations in the kinetic variables for Ca^{2+} influx in untreated fish. Cyclic variations in branchial Ca^{2+} influx have been shown by Wagner *et al.* (1985, 1986), who also found a corresponding pattern of responsiveness to stanniocalcin. The effect of injected stanniocalcin was only found in the sequences of the cycle where Ca^{2+} influx was high (Wagner *et al.* 1986).

The Zn^{2+}-exposed rainbow trout, in the present study, did not accumulate any Zn or lose any Ca on a whole-body basis (Table 1). During the later part of the experiment there was, however, a slight increase in the branchial Zn content that correlated with an induction of MT. At this point, the increased accumulation of Zn by the gills did not seem to impair the health of the fish, as judged by normal plasma [Ca], protein turnover rates and growth. The increased MT concentration of the gills presumably helped the fish to immobilize the potentially toxic Zn (Hogstrand and Haux, 1991). It is interesting to note that the whole-body content of Ca and the concentration of Zn in gill tissue increased in the control group during the experiment. Furthermore, the increasing branchial Zn content was paralleled by an elevated branchial accumulation of Zn in gill tissue on days 17 and 31 compared with the value on day −2 (Fig. 6). We speculate that these effects are associated with normal growth in juvenile rainbow trout.

The rainbow trout in the present study did not show an increased tolerance in the acute Zn^{2+} toxicity tests (Table 3), in contrast to our earlier study (Hogstrand *et al.* 1994). However, in that study the fish were larger (21 g *versus* 4 g), the experiment was carried out in a different season (winter *versus* late spring) and MT induction did not occur. McDonald and Wood (1993) introduced the concept of the 'exposure window' within which an increased tolerance to metals can be conferred. The concept is based on the observation that increased tolerance to metals is preceded by initial physical damage to the gill structure followed by a repair process. If the exposure level is too low to cause substantial morphological damage (e.g. oedema, inflammation, cell sloughing), no increased tolerance will develop. It is quite possible that the level of Zn^{2+} exposure (150 $\mu g\,l^{-1}$=2.3 $\mu mol\,l^{-1}$ as total Zn) chosen by Hogstrand *et al.* (1994) and also used here was borderline between the nutritional and toxicological range for Zn^{2+} and caused only minor damage. Spry *et al.* (1988) found that the same Zn^{2+} concentration in identical water conditions stimulated the growth of juvenile rainbow trout without any effects on whole-body Ca and Zn levels. Although the fish from the present study did not exhibit an increased tolerance to lethal concentrations of waterborne Zn^{2+} (4 $mg\,l^{-1}$= 61 $\mu mol\,l^{-1}$ as total Zn), they did show a physiological recovery in terms of restored plasma [Ca] (Fig. 1) and there was no loss in whole-body [Ca] (Table 1). Furthermore, when challenged with a sublethal concentration of Zn^{2+} (450 $\mu g\,l^{-1}$= 6.9 $\mu mol\,l^{-1}$ as total Zn) three times higher than the pre-exposure level, they showed a significantly greater ability to maintain plasma [Ca] than the controls (Table 2). The conclusion that can be drawn from these results is that the mechanisms for toxicity at acutely lethal concentrations of waterborne Zn^{2+} are different from the effects on the Ca^{2+} homeostasis that can be found when fish are exposed to waterborne Zn^{2+} at sublethal levels. Our conclusion is consistent with previous experiments which have established acute hypoxia as the cause of death during exposure of rainbow trout to Zn^{2+} at 40 $mg\,l^{-1}$ (=610 $\mu mol\,l^{-1}$ as total Zn) and 1.5 $mg\,l^{-1}$ (=22 $\mu mol\,l^{-1}$ as total Zn) (Skidmore, 1970; Spry and Wood, 1984), whereas hypocalcaemia and acid–base disturbances are the major causes of toxicity at lower concentrations (800 $\mu g\,l^{-1}$=12 $\mu mol\,l^{-1}$ as total Zn) of waterborne Zn^{2+} (Spry and Wood, 1984, 1985). The results underline the importance on conducting studies of potentially toxic metals at environmentally relevant concentrations.

On day 9 of the experiment, the protein synthesis rate of the gills was significantly higher in Zn^{2+}-exposed fish than in controls (Fig. 8). We speculate that this enhanced protein synthesis rate reflected the repair mechanisms that were activated after the initial damage caused by Zn^{2+} exposure. The

Ca^{2+} kinetics data support this explanation by indicating that there was a regeneration of Ca^{2+} carriers during this part of the experimental period. Furthermore, the protein degradation rate was elevated in Zn^{2+}-exposed fish at the same time, reflecting the breakdown of damaged proteins. Associated with the faster protein turnover in the gills was, undoubtedly, an increased metabolic cost, which was likely to be the cause of the temporary attenuation of growth observed for Zn^{2+}-exposed fish in the middle of the experimental period (Fig. 7). On day 18, there were no longer any differences in the rates of protein synthesis and degradation between the treatment groups. In accordance with this observation, the Zn^{2+}-exposed fish were subsequently able to compensate for the lag in growth and, by the end of the experiment (day 29), fish from both groups were of similar masses. In a recent study, we found no increase in the fractional protein synthesis rates in gills, liver or whole body after 23 days of exposure to the Zn^{2+} concentration used here (Hogstrand *et al.* 1994). We speculated that no effects were seen because the analyses were performed late in the treatment, when the adaptation to Zn^{2+} had already been established. This corresponds well with the data obtained from the present study, where we show that adaptation to Zn^{2+} is initially associated with an elevated metabolic cost, but that the fish are able to compensate for their temporarily stalled growth rate within 1 month.

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