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Waterborne iron acquisition by a freshwater teleost fish, zebrafish *Danio rerio*

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Summary

Waterborne iron accumulation by the gills of the zebrafish *Danio rerio* was assessed in ion-poor water. Branchial iron uptake, which comprises both the iron that has entered the gill cells and iron that is strongly bound to the epithelia, has high- and low-affinity components. At low nominal [Fe] (<40 nmol l^{-1}) the high-affinity component demonstrated saturation kinetics, with an apparent K_m of 5.9 nmol l^{-1} Fe and V_{max} of 2.1 pmol g^{-1} h^{-1}. Over a range of higher nominal [Fe] (40–200 nmol l^{-1}), branchial uptake was linear. In the presence of 2 μmol l^{-1} of the reducing agent dithiothreitol (DTT), branchial iron accumulation was significantly enhanced at [Fe]>15 nmol l^{-1}. The proton pump inhibitor bafilomycin A significantly reduced iron uptake in the presence of DTT. On the basis of these observations we conclude that branchial iron uptake at low [Fe] shows characteristics similar to those of other iron-transporting epithelia, coupling an apical membrane ferric reductase to a Fe^{2+}/H^+ symporter. Zebrafish branchial iron transport at 18.6 nmol l^{-1} was inhibited by 200 nmol l^{-1} Cd^{2+}. But, unlike other Fe^{2+}/H^+ symporters, iron uptake was not affected by other divalent metals (Co^{2+}, Ni^{2+}, Pb^{2+}, Cu^{2+}, Zn^{2+} and Mn^{2+}). Zebrafish loaded with ^{59}Fe from the water showed a loss of 7.9 pmol Fe g^{-1} body mass over the first day and a further loss of 5.7 pmol Fe g^{-1} body mass over the following 28 days. The depuration kinetics followed a two-component exponential model; for the short-lived component, $t_{1/2}$=0.31 days, and for the long-lived component, $t_{1/2}$=13.2 days. The daily iron loss by zebrafish can be compensated by iron uptake at exceedingly low water iron concentrations (uptake rate at 1.625 nmol l^{-1} Fe=0.425 pmol g^{-1} h^{-1}), demonstrating that uptake of iron from the water is potentially an important source of this nutritive metal in freshwater teleost fish.

Key words: iron bioavailability, teleost, divalent metal transporter (DMT), ferric reductase, ferroportin, IREG, fish nutrition, metal, zebrafish, *Danio rerio*.

Introduction

Iron is essential for life, being involved in cellular respiration in animals and photosynthesis in plants, as well as being an integral cofactor of ribonucleotide reductase. However, in excess iron is toxic, acting as a catalyst in the Fenton reaction generating free-radicals (Crichton et al., 2002). Consequently, organisms have to regulate iron uptake to prevent the detrimental effects of tissue iron loading.

Fish are unique amongst the vertebrates, because there is the potential for nutritive metal acquisition from the water in addition to metal absorption from the diet (Bury et al., 2003). However, the ability of the gills to absorb iron will be greatly influenced by geochemical factors that govern iron speciation. In oxic conditions and at circumneutral pH, Fe(II) is readily oxidised and forms colloidal hydrous iron oxides (Sturm and Morgan, 1996; Lienemann et al., 1999) that may be complexed with organic matter (Tipping, 1981). However, iron has seldom been found to limit phytoplankton growth in freshwater lakes (Hyenstrand et al., 1999). Many freshwater organisms have evolved mechanisms for mobilising and sequestering iron. A number of freshwater bacteria, algae and cyanobacteria produce organic compounds, siderophores, which are released to the environment (Wilhelm and Trick, 1994). These siderophores have exceptionally high binding affinity (~log$K$=19) for iron (Witter et al., 2000), thus maintaining iron in solution, and the iron siderophore complexes are taken up (Wilhelm and Trick, 1994; Cowart, 2002). Some algal species adopt a different mechanism, whereby a plasma membrane ferric chelate reductase utilises intracellular reducing power to liberate iron from its organic ligand and reduces Fe(III) to Fe(II), after which Fe(II) is subsequently transported into the cells (Robinson et al., 1999; Weger et al., 2002). In addition, photolysis of siderophore bound iron results in the formation of lower affinity Fe(III) ligands and the reduction of Fe(III), increasing the bioavailable Fe(II) fraction to organisms in the euphotic zone (Barbeau et al., 2001).

In the vertebrate small intestine, non-haem bound iron is reduced *via* a membrane-bound ferric reductase (McKie et al., 2001) and ferrous iron enters the cell *via* a proton/Fe^{2+} symporter (Gunshin et al., 1997). This latter protein belongs to the family of proteins termed natural resistance associated macrophage

proteins (Nramp), or solute carrier 11 type 2a (Sla 11 2a). However, the protein is more commonly known as the divalent metal transporter (DMT), because it has been shown to transport other divalent cations (Gunshin et al., 1997; Tallkvist et al., 2001; Bannon et al., 2002). cDNA clones with sequence similarity to members of the Sla112a family of proteins have been identified in a number of fish species (see review by Bury et al., 2003). mRNA for one of the rainbow trout *Oncorhynchus mykiss* Nramp isoforms has been shown to be prevalent in the gills, intestine and kidney epithelia of freshwater-adapted fish, suggesting its involvement in ion acquisition (C. A. Cooper and N. R. Bury, personal observation). In zebrafish, iron leaves intestinal cells *via* a protein termed ferroportin (Donovan et al., 2000). Consequently, based on molecular evidence, the genes encoding proteins involved in epithelial iron uptake appear to be present in teleost freshwater fish.

Two studies have shown that the gill is potentially a site of iron uptake (Roeder and Roeder, 1966; Andersen, 1997). Roeder and Roeder (1966) demonstrated that growth was reduced in swordtail *Xiphodphorus helleri* reared in iron-poor water and fed an iron-restricted diet. Growth rate was restored if $FeSO_4$ (7.4 mg l^{-1}, 134 μmol l^{-1}) was added to the water. Andersen (1997) showed that start-fed brown trout larvae, a stage at which gills are formed and the maternal source of nutrients the yolk-sac has been absorbed, accumulate radiolabelled Fe when added as $FeCl_3$ and at a concentration of 35 mg l^{-1} Fe (627 μmol l^{-1} Fe) into the water. At this developmental stage there is also an increase in whole body expression of the iron binding protein transferrin (Andersen, 1997). To date, there is no information on the mechanism of the branchial iron uptake. Consequently, the present study characterises the iron uptake from the water in freshwater zebrafish *Danio rerio*, and assesses the contribution that this route of iron acquisition makes to whole body iron homeostasis.

Materials and methods

Fish husbandry

Juvenile zebrafish *Danio rerio* (Hamilton-Buchannan) of 51–566 mg body mass were obtained from a local pet store in Copenhagen, Denmark. A maximum of 500 fish were placed into glass tanks containing 40 litres of Copenhagen dechlorinated tapwater. This water was continuously filtered through a biological filter and maintained at 28°C. The ionic composition of this water (in mmol l^{-1}) is: Na^+, 1.48; K^+, 0.1; Ca^{2+}, 3.25; Cl^-, 1.63; PO_4^-, 0.79, pH 8.15. Each day 50% of the water was exchanged for deionized water (by reverse osmosis), gradually acclimating fish to ion-poor water of the following composition (in mmol l^{-1}): Na^+, 0.035; Ca^{2+}, 0.0044; Cl^-, 0.034; pH 6.76. During this period fish were feed fish food flakes once every 2 days to satiation.

Iron flux experiments

All iron uptake measurements were performed by assessing the accumulation of radiolabeled ^{59}Fe in the gills, body or whole body. Radiolabelled ferric chloride ($^{59}FeCl_3$) dissolved in 0.5 mol l^{-1} HCl was obtained from Amersham Pharmacia Biotech (327 MBq ml^{-1}, 552 μg Fe ml^{-1}). All radioactivity was counted on a Canberra Packard Minaxi auto gamma 5000 series gamma-counter using the manufacturer's guidelines.

All uptake experiments were run in daylight, in polyethylene bags containing 200 ml of deionised water at 28°C. Radioisotope was added 1 h before the addition of the fish, allowing for iron binding to the walls of the containers, which was approximately 50% of that originally added. Over the 4 h flux period applied in most experiments, counts in the water decreased on average by 13±1.6% (N=56), which equates to radioactivity taken up by the organism as well as that adsorbed to the surface of the polyethylene bags. To maintain constant pH an equal volume of 0.5 mol l^{-1} NaOH was added to the volume of 0.5 mol l^{-1} HCl added with the $^{59}FeCl_3$. The final pH of the water was 6.34–6.78. A 1 ml water sample was taken for radioactive counting before the addition of 8–10 fish to the vessels. At the end of the flux period a second 1 ml water sample was taken and the fish underwent a wash protocol of 2× 1 min rinses in tapwater (total iron content 0.46 μmol l^{-1}) and were then killed by an overdose of MS-222. The branchial basket was removed, and the masses of the gills and body after blotting dry with a paper towel recorded. This procedure removed radioactivity loosely bound to mucus and epithelia. Consequently, throughout this manuscript, branchial and body iron uptake refers to iron that has been transported into the cells or body, as well as that strongly bound to the epithelia following the wash protocol. Radioactivity present on the gills and in the body was measured separately. The time-course experiments were performed at a nominal iron concentration of 16.5±0.1 nmol l^{-1}.

In the dose-dependency experiment, nominal iron concentrations in the water below 50 nmol l^{-1} Fe consisted solely of radiolabelled $FeCl_3$, and desired concentrations higher than this were achieved by addition of 'cold' $FeCl_3$. The 'cold' $FeCl_3$ was made by dissolving $FeCl_3$ in Milli Q (18 Ω) water on the day of the experiment. To assess ferric *versus* ferrous iron uptake, zebrafish iron influx studies were performed in the presence of 2 μmol l^{-1} of the reducing agent dithiothreitol (DTT). In a preliminary experiment the proportion of 1 μmol l^{-1} $FeCl_3$ in the ferric or ferrous state following the addition of 3 μmol l^{-1} DTT was 49.5±5 and 50.5±5%, respectively (approximately 1:1), as determined by the spectrophometric phenanthroline method (Clesceri et al., 1998). In the absence of DTT the proportion of Fe^{3+} and Fe^{2+} was 81.3±6 and 18.8±6, respectively (approximately 4:1; C. A. Cooper and N. R. Bury, unpublished data). Sodium influx measurements in the presence of 2 μmol l^{-1} DTT were made in parallel to the iron flux experiments to assess the general effect of reducing conditions on ion uptake. All fluxes were performed in 200 ml of water ($[Na^+]$=30 μmol l^{-1}) to which was added 0.1 μCi of $^{22}Na^+$ (Dupont, Stockholm, Sweden; specific activity 11.2 MBq g^{-1} Na). 1 ml water samples were taken at the beginning and end of the experiment for radioactive counting. At the end of the experiment fish were

washed with tapwater, killed with an excess of MS-222, and whole body radioactivity recorded.

The influence of 50 nmol l^{-1} bafilomycin A (Sigma; Vejlegårdsveg, Denmark), a V-type ATPase inhibitor (Drose and Altendorf, 1997), on whole body iron influx at 18 nmol l^{-1} $^{59}FeCl_3$ was assessed in 10 fish over a 1 h exposure period and in a volume of 100 ml. Bafilomycin A would be expected to reduce acidification of the gill microenvironment by inhibiting H^+ extrusion across the gills.

Metal competition studies in the presence or absence of 2 μmol l^{-1} DTT were performed at 18.6±0.5 nmol l^{-1} Fe (N=16), in the presence of 200 nmol l^{-1} each of the following: $CoCl_2$, $NiCl_2$, $PbNO_3$, $CuCl_2$, $CdSO_4$, $ZnCl_2$, $MnCl_2$ or $FeCl_3$. All metal solutions were made by dissolving the appropriate salt in Milli Q (18 Ω) water on the day of the experiment.

Iron depuration was assessed by pre-loading zebrafish with $^{59}FeCl_3$ added to the water at a concentration of 21 nmol l^{-1} Fe for 24 h. After loading, 10 fish were taken, washed as described above and whole body radioactivity measured. The remaining fish were then placed in 10 litres of radioactive-free, deionised water at 28°C, and at 1, 7 and 28 days post-loading whole body radioactivity was determined. The fish were fed every other day during the depuration period and 50% of the water in the holding tank was replaced on alternate days. All calculations were decay corrected (^{59}Fe $t_{1/2}$=44.6 days) and there was no significance increase in fish mass over the 28-day period (t=0, 0.14±0.05 g; t=28, 0.15±0.06 g).

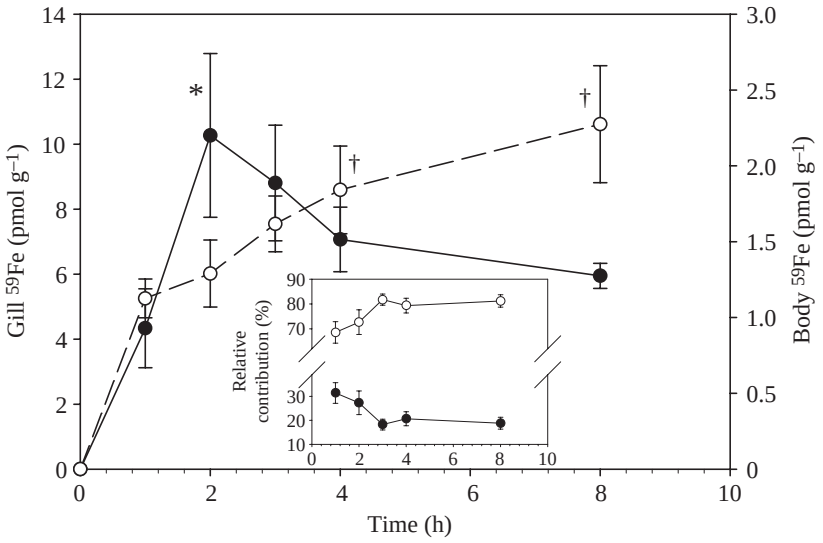

Fig. 1. Temporal pattern of gill (filled circles) and body (open circles) iron accumulation when iron is presented at 16.5 nmol l^{-1} in the form of $^{59}FeCl_3$. Values are means ± S.E.M., N=10–16. *Significant differences between the gill iron counts and †uptake into the body (P<0.05; ANOVA followed by a least-significance difference test). The inset illustrates the relative contributions that the gills and body make to the total ^{59}Fe accumulation during the exposure period.

Calculations and statistics

Zebrafish iron or sodium uptake was determined from the following equation:

$$\text{Tissue Fe or } Na^+ \text{ uptake} = \text{c.p.m.} / (SA \times m \times t) ,$$

where c.p.m. is the counts min^{-1} in the tissue, SA is the specific activity of Fe (c.p.m. $pmol^{-1}$) or Na (c.p.m. $nmol^{-1}$) in the water, m (g) the mass of the fish, and t (h) the duration of the flux. For time-course experiments the time factor is removed.

A one-way analysis of variance (ANOVA) followed by a least-significance difference test was used to determine significant differences between gill or body iron uptake at different times during the time-course experiment. A Student's t-test was used to compare branchial, body or whole body iron uptake in control fish and those fish exposed to the various metals, or bafilomycin A. A Student's t-test was also used to compare whole body Na^+ uptake in controls and those exposed to DTT. To assess the differences between the uptake rate in the presence and absence of DTT we compared the slopes of the linear regression lines for uptake above 40 nmol l^{-1}. The 'best-fit' lines to the data points were based on the regression coefficients as calculated by Sigma Plot 2001 computer graphics package.

Results

Iron accumulation onto the gill reached a significant peak after 2 h of exposure to 16.5 nmol l^{-1} $FeCl_3$, after which levels on the gill decreased (Fig. 1). In contrast, the accumulation into the body continued to rise, being significantly greater at 4 and 8 h. The percentage contribution of the gill to total ^{59}Fe accumulation declined, whereas the percentage contribution of the body (without the gills) to total ^{59}Fe accumulation increased over the 8 h exposure period (Fig. 1 inset).

The kinetics of gill iron accumulation in the absence of DTT (e.g. mostly Fe^{3+}) showed two components. The first component, at concentrations below 40 nmol l^{-1} Fe, best fitted Michaelis–Menten kinetics with an apparent affinity K_m=5.9±1.7 nmol l^{-1} Fe and V_{max}=2.1±0.2 pmol g^{-1} h^{-1} (r^2=0.96). Above concentrations of 40 nmol l^{-1} the uptake rate increased in a linear manner (y=0.83–0.7+0.0537±0.006x, r^2=0.96) and did not saturate at 203 nmol l^{-1} Fe. In contrast, branchial gill accumulation of Fe in the presence of DTT (approximately 1:1 Fe^{3+}:Fe^{2+}) was linear over the whole range of Fe concentrations tested (y=0.15±0.3+0.13±0.001x, r^2=0.99, Fig. 2A). Below water [Fe]=15 nmol l^{-1} there was no significant difference between the gill accumulation of iron in the absence or presence of DTT. But, based on the linear regression lines for iron uptake in the presence or absence of DTT at [Fe]>15 nmol l^{-1}, uptake was significantly greater in the presence of DTT (P<0.05, Fig. 2A). That is, the higher [Fe^{2+}] resulted in increased ^{59}Fe uptake by the gills. Body Fe influx

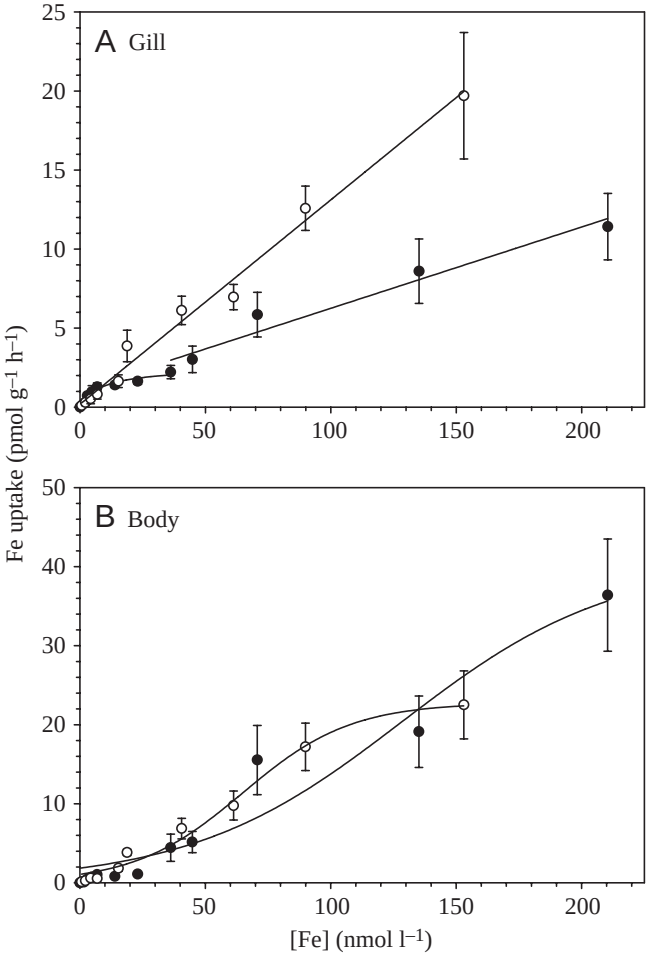

Fig. 2. Concentration-dependent iron accumulation in (A) the gills and (B) body of zebrafish in the absence (filled circles) and presence of 2 μmol l^{-1} DTT (open circles). Values are means ± S.E.M., N=10–32. Iron uptake into the gills in the absence of DTT at [Fe]<40 nmol l^{-1} showed Michaelis–Menten kinetics. Above [Fe]=40 nmol l^{-1} the branchial iron uptake was linear. The pattern of concentration-dependent branchial gill Fe accumulation in the presence of DTT best fitted a linear regression (see text). Concentration-dependent iron accumulation in the body of the zebrafish in the absence and presence of DTT followed a sigmoidal uptake pattern.

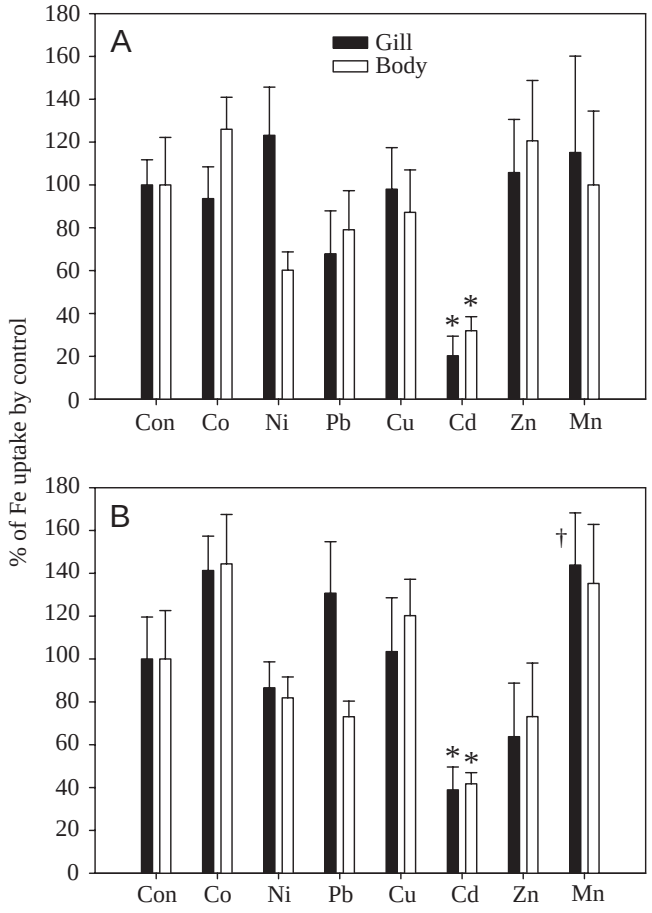

Fig. 3. The effects of various divalent cations (Co^{2+}, Ni^{2+}, Pb^{2+}, Cu^{2+}, Cd^{2+}, Zn^{2+}, Mn^{2+}) on iron uptake into the gills (filled bars) and body (open bars) of zebrafish in the absence (A) and presence (B) of 2 μmol l^{-1} DTT. The iron concentration was 18.6 nmol l^{-1} and nominal metal concentrations 200 nmol l^{-1}. Values are means ± S.E.M., N=10–18. *Significant reduction in iron uptake; †significant induction in iron uptake compared to control fish (Con) (P<0.05; Student t-test, using untransformed data).

both in the presence and absence of DTT showed a sigmoidal pattern of uptake (Fig. 2B). DTT did not significantly affect Na influx (control, 226±30 μmol Na kg^{-1} h^{-1}; in the presence of DTT, 155±14 μmol Na kg^{-1} h^{-1}, P=0.067, Student t-test). Iron uptake of fish treated with 50 nmol l^{-1} bafilomycin A, in the presence of DTT, showed a significant decrease in whole body iron accumulation (control, 0.028±0.003 pmol g^{-1} h^{-1}, N=10; *versus* bafilomycin A, 0.021±0.002 pmol g^{-1} h^{-1}, N=10; P=0.02, Student t-test) (not shown).

Cadmium, at 200 nmol l^{-1}, was the only metal tested to significantly reduce both gill and body Fe uptake in presence and absence of DTT (Fig. 3A,B). Of the other metals tested, only Mn(II) had a significant effect on Fe uptake, stimulating gill Fe accumulation in the presence of DTT (Fig. 3B).

Zebrafish loaded with ^{59}Fe from the water then transferred to radiolabel-'free' water lost 7.9 pmol Fe g^{-1} over the first 24 h and then a further 5.7 pmol g^{-1} over the following 28 days (Fig. 4). The kinetics of iron depurations best fitted a two-component exponential model where $y=75.5(e^{-2.21t})+10.2(e^{-0.0525t})$ and r^2=1 (Sigma Plot 2001), and from which the radiotracer biological half-life for each component can be calculated (Bustamante et al., 2002). For the short-lived component, $t_{1/2}$=0.31 days, and the long-lived component, $t_{1/2}$=13.2 days.

Discussion

This study is the first to characterise the mechanism of branchial iron uptake (referred to herein as the iron that has entered the cell, as well as that which remains strongly bound to the epithelia following a rigorous wash protocol) by a

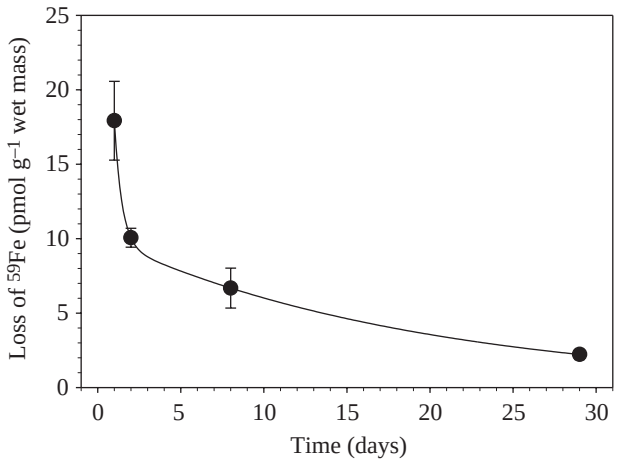

Fig. 4. Whole body depuration pattern of ^{59}Fe in zebrafish over a 28-day period. Fish were loaded for 24 h in water containing 21 nmol l^{-1} ^{59}Fe. These fish were then washed in isotope-free water and placed into clean water; they were fed every other day. Values are means ± S.E.M., N=10. The loss kinetics best fitted a two-component exponential model (Sigma Plot 2001), where y=75.5($e^{-2.21t}$)+10.2($e^{-0.0525t}$), r^2=1. The radiotracer biological half-life for the short-lived component, $t_{1/2}$=0.31 days, and the long-lived component, $t_{1/2}$=13.2 days.

freshwater teleost fish, the zebrafish. The temporal pattern of gill and body iron uptake is similar to that observed for other transition metals, such as copper and silver (Grosell et al., 1997; Bury and Wood, 1999), whereby a metal initially loads onto the gill, reaching a peak concentration after a few hours of exposure. There then follows a gradual decline in gill metal concentration, which eventually leads to a steady state level. In contrast, the entry of metal into the body (excluding gills) shows a steady increase throughout the exposure period (Fig. 1). This is reflected in the relative contribution that the gills and body make to the total ^{59}Fe accumulation (Fig. 1, inset). The kinetics of gill iron accumulation, when added to the water as $FeCl_3$, has two components: a saturable, high-affinity component at low iron concentrations (<40 nmol l^{-1}) with an apparent K_m=5.9 nmol l^{-1} and V_{max}=2.1 pmol g^{-1} h^{-1}, and a linear component at higher iron concentrations, up to at least 200 nmol l^{-1} (Fig. 2). The addition of 2 μmol l^{-1} of the reducing agent dithiothreitol (DTT) to the water increased iron uptake into the gills at concentrations of Fe >15 nmol l^{-1}. These results suggest that at low iron concentrations iron enters the gills in the ferrous (Fe^{2+}) state, and saturation kinetics indicate that the rate-limiting step is reduction of Fe^{3+} to Fe^{2+}.

The accumulation of Fe by zebrafish at exceedingly low concentrations (16.5–21 nmol l^{-1}), during the 4 h fluxes or the 24 h loading period, contrasts with the observation that swim-up brown trout fry do not accumulate iron over a 4-day exposure period to 6.4 μmol l^{-1} ^{59}Fe (Andersen, 1997). In that study, brown trout fry only took up iron from the water at a higher iron concentration of 640 μmol l^{-1} (Andersen, 1997). This disparity may reflect differences in the duration of the experiments, fish species and/or water chemistries. In freshwaters, formation of ferric oxyhydroxide occurs over a number of hours (Gunnars et al., 2002). Consequently, in the present flux experiments the majority of the iron probably remains in the dissociated Fe^{3+} state, whilst in longer exposure periods (i.e. Andersen, 1997) the iron may become unavailable. The quantity or nature of the organic material or the sulphide concentration, which both form complexes with iron (Rozan et al., 1999; Witter et al., 2000), were not measured in either study, but may also influence iron bioavailability.

At [Fe]>40 nmol l^{-1} the linear, low-affinity component of zebrafish branchial Fe uptake (in the absence of DTT) may represent a non-specific uptake pathway. In contrast, the Michaelis–Menten kinetics of Fe uptake at lower Fe concentrations (<40 nmol l^{-1}) is indicative of a physiologically regulated transport protein. This pattern of gill metal uptake by freshwater fish has been observed for other metals (Comhaire et al., 1994; Hogstrand et al., 1996; Bury and Wood, 1999; Grosell and Wood, 2002). For example, copper uptake by rainbow trout shows saturation kinetics at low ambient copper concentrations and a linear uptake component at higher copper concentrations that are toxic (Grosell and Wood, 2002). The K_m for the high-affinity branchial iron transport is 25 times lower than that for iron uptake by the bakers yeast *Sacchoromyces cerevisiae* (Eide et al., 1992), and 325 times lower than *Xenopus* oocytes expressing Nramp2/DMT (Gunshin et al., 1997). The only other iron transport systems with affinity constants in the ≤nmol l^{-1} region are for the uptake of siderophore–Fe complexes by phytoplankton and the strategy II plants (Granger and Price, 1999; Guerinot, 2001). Evolution of an iron transporter with a high affinity for iron is probably driven by the freshwater Fe^{3+} concentration that the gill encounters.

The suggestion that it is the ferrous form (Fe^{2+}) of iron that enters the gills of fish is supported by the observation of Roeder and Roeder (1966), who showed that the reduced growth rate of swordtail fed an iron-poor diet and reared in ion-poor waters could be prevented if ferrous, and not ferric, salts were added to the water. Interestingly, intestinal uptake of iron by the European flounder *Platichthys flesus* is predominantly in the ferrous form (Bury et al., 2001). Ferrous iron is also the form in which iron is taken up by the strategy I plants (Thomine et al., 2000), some yeast (Cohen et al., 2000) and mammals (Gunshin et al., 1997). To obtain ferrous iron from the environment these organisms evolved a membrane ferric reductase associated with a ferrous iron transport protein. In strategy I plants, the ferric chelate reductase is capable of reducing ferric iron bound to organic matter in the soil (Robinson et al., 1999), whilst in mammals the ferric reductase, termed Dcytb, shares similarities with cytochrome *b* reductases (McKie et al., 2001). The enhanced uptake of iron in the presence of DTT at [Fe]>15 nmol l^{-1}, as well as the saturation kinetics exhibited by this system, demonstrate that the apical membrane is the rate-limiting step in iron uptake at low external iron concentrations, indicating the presence of a branchial ferric (chelate) reductase. However, the similarity between uptake in the absence and presence of DTT at

[Fe]<15 nmol l^{-1} suggests that the rate-limiting step is a combination of Fe^{3+} reduction and Fe^{2+} entry.

The V-type ATPase inhibitor bafilomycin A reduced whole body Fe uptake. Although an effect of apical membrane depolarization, caused by bafilomycin A, on Fe uptake cannot be completely ruled out, these results suggests that the Fe uptake is dependent on a proton gradient. A number of apical membrane ferrous iron transporters have been identified that act as Fe^{2+}/H^+ symporters (Gunshin et al., 1997; Andrews, 2000). Immunohistochemical evidence for a branchial apical membrane V-type ATPase has been documented (Perry 1997; Wilson et al., 2000). V-type ATPases are responsible for the acidification of the water that passes over the gill, and have been shown to govern zebrafish branchial Na^+ uptake in ion-poor water (A. M. Z. Boisen and M. Grosell, unpublished data). A partial cDNA sequence (accession number AF190508) with similarity to other members of the Nramp family of proteins has been identified in the zebrafish, and the proton pump has been localised in the zebrafish gill (A. M. Z. Boisen and M. Grosell, unpublished data). Consequently, the likelihood is that the branchial apical membrane ferrous iron uptake is *via* a divalent metal transporter (DMT).

The similar pattern of concentration-dependent iron transfer, in the presence or absence of DTT, from the gill into the body of zebrafish (Fig. 2) suggest that iron, whether in the ferric or ferrous form in the environment, is treated similarly intracellularly. At high external iron concentrations the transport step from the gill to the body becomes rate limiting and saturation of body iron uptake is observed. The transporter involved in this process is probably ferroportin, a basolateral ferrous iron transporter that has been identified in the intestine of zebrafish (Donovan et al., 2000). In other vertebrates, ferroportin is linked to a copper-containing ferrioxidase termed hephaestin (Vulpe et al., 1995), which oxidises Fe^{2+} so that iron circulates as Fe^{3+} bound to transferrin.

Of the divalent metals tested, only Cd^{2+} inhibited iron uptake into both the gills and body of zebrafish in the presence or absence of DTT (Fig. 3). The lack of effect of the metals, and indeed the stimulation by Mn(II), was a surprise because *Xenopus laevis* oocytes injected with cRNA for the mammalian DMT show, that in addition to Fe^{2+}, they are capable of transporting other divalent metals (Gunshin et al., 1997). DMT homologues in the plant *Arabidopsis thaliana* and the bakers yeast *Saccharomyces cerevisiae* also transport other divalent metals (Thomine et al., 2000; Cohen et al., 2000). In addition, cadmium and lead both inhibit iron uptake by various cell lines in culture (Tallkvist et al., 2001; Bannon et al., 2002), and lead interferes with iron uptake in the duodenum (Smith et al., 2002). The close coupling of the ferric reductase and DMT in other systems indicates that metals interact with iron at the Fe^{2+} transport site. However, metals may also interact with Fe^{3+} or Fe^{2+} binding to non-transport moieties on the apical surface. The calculated apparent gill iron-binding constant (log$K$) of 8.3 (based on the apparent K_m for iron uptake) is similar to that for Cd binding to fathead minnow gills (log$K$=8.6; Playle et al., 1993), and is at least an order of magnitude higher than the log$K$ values for other gill–metal interactions (see table 2 of Bury and Hogstrand, 2002). The competition between divalent Cd in the absence and presence of DTT shows that it is directly interacting with iron following reduction, suggesting that these metals share the same uptake route.

Depuration of radiolabelled iron following a loading from waterborne iron follows a two-component exponential model (see Fig. 4). The initial rapid decline may represent loss of loosely bound iron from the gills and skin. The degree to which this radiolabelled iron has been incorporated into 'new' proteins was not determined, but a loss rate of 0.53 pmol g^{-1} day^{-1} (i.e radiolabel lost over the whole depuration experiment) equates to 28.4 μg kg^{-1} day^{-1}, which is roughly equivalent to the daily iron lost by humans, 14–28 μg kg^{-1} day^{-1} (1–2 mg Fe is lost by a 70 kg male per day; Conrad et al., 1999). At the lowest iron concentration tested (1.625 pmol), the body iron uptake rate met this demand (0.47 pmol Fe g^{-1} h^{-1}). However, it must be stressed that the zebrafish in the present study were acclimated to deionised water, where iron levels were exceptionally low (below detection limits), which would be likely to result in an upregulation of branchial ion transporters. This upregulation would lead to an overestimation of the uptake rate from the gills when the water is supplemented with a metal or other ions. However, support for the importance of iron uptake from the water in contributing to iron homeostasis comes from the observations that growth rate in swordtail can be enhanced if $FeSO_4$ is added to the water (Roeder and Roeder, 1966).

In conclusion, we demonstrate that iron can be absorbed from the water in a teleost fish, the zebrafish. The branchial iron transport shows characteristics of other epithelial iron transport, coupling an apical membrane ferric reductase to a ferrous transporter. The ferrous iron transporter appears to be linked to a proton pump, suggesting that it probably belongs to the large Scl 11 2a family of proteins involved in iron transport. The affinity for this system is very high, with a K_m=5.9 nmol l^{-1}, which probably reflects the freshwater concentration of iron available to teleost fish. Similar to other Fe^{2+}/H^+ symporters, it appears that the zebrafish branchial iron transporter is inhibited by cadmium. No other divalent metals affected iron uptake at the same concentrations, suggesting that this iron transport process is relatively specific. The results obtained suggest that uptake of iron from the water may be adequate to compensate for daily iron loss, which fits with recent results demonstrating the contribution of branchial metal uptake in maintaining metal homeostasis during times of metal deprivation (Bury et al., 2003). For example, Kamunde et al. (2002) demonstrated that rainbow trout fed a low copper diet (0.8 μg Cu g^{-1}) and reared in copper-poor water (0.4 μg Cu g^{-1}) showed reduced growth, which could be reversed by addition of low levels of copper to the water. Based on radiotracer studies, 60% of the copper needs were met from uptake *via* the water in the fish fed copper-depleted diets (Kamunde et al., 2002). Rainbow trout thus have an integrative physiological mechanism whereby the capacity for branchial metal uptake is upregulated to meet copper demand. In the case

of iron, it will be important to determine the relative proportion of iron acquired from the diet or water in fish reared under different dietary or waterborne iron regimes.

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