The Journal of Experimental Biology 204, 3779–3787 (2001)
Printed in Great Britain © The Company of Biologists Limited 2001
JEB3677

Intestinal iron uptake in the European flounder (*Platichthys flesus*)

N. R. Bury[1,*], M. Grosell[2], C. M. Wood[2], C. Hogstrand[1], R. W. Wilson[3], J. C. Rankin[4], M. Busk[4], T. Lecklin[4] and F. B. Jensen[4]

[1]*Division of Health and Life Sciences, King's College London, 150 Stamford Street, London SE1 9NN, UK,* [2]*Department of Biology, McMaster University, 1280 Main Street West, Hamilton, Ontario, Canada, L8S 4K1,* [3]*School of Biological Sciences, University of Exeter, Hatherly Laboratories, Prince of Wales Road, Exeter, Devon EX4 4PS, UK* and [4]*Institute of Biology, SDU, Odense University, Campusvej 55, Odense, Denmark*

*e-mail: nic.bury@kcl.ac.uk

Accepted 31 July 2001

Summary

Iron is an essential element because it is a key constituent of the metalloproteins involved in cellular respiration and oxygen transport. There is no known regulated excretory mechanism for iron, and homeostasis is tightly controlled *via* its uptake from the diet. This study assessed *in vivo* intestinal iron uptake and *in vitro* iron absorption in a marine teleost, the European flounder *Platichthys flesus*. Ferric iron, in the form $^{59}FeCl_3$, was reduced to Fe^{2+} by ascorbate, and the bioavailability of Fe^{3+} and Fe^{2+} were compared. *In vivo* Fe^{2+} uptake was significantly greater than Fe^{3+} uptake and was reduced by the iron chelator desferrioxamine. Fe^{2+} was also more bioavailable than Fe^{3+} in *in vitro* studies that assessed the temporal pattern and concentration-dependency of iron absorption. The posterior region, when compared with the anterior and mid regions of the intestine, was the preferential site for Fe^{2+} uptake *in vivo*. *In vitro* iron absorption was upregulated in the posterior intestine in response to prior haemoglobin depletion of the fish, and the transport showed a Q_{10} value of 1.94. Iron absorption in the other segments of the intestine did not correlate with haematocrit, and Q_{10} values were lower. Manipulation of the luminal pH had no effect on *in vitro* iron absorption. The present study demonstrates that a marine teleost absorbs Fe^{2+} preferentially in the posterior intestine. This occurs in spite of extremely high luminal bicarbonate concentrations recorded *in vivo*, which may be expected to reduce the bioavailability of divalent cations as a result of the precipitation as carbonates (e.g. $FeCO_3$).

Key words: iron, European flounder, *Platichthys flesus*, bioavailability, intestine.

Introduction

Iron is an essential element involved in cellular respiration and oxygen transport. In higher vertebrates, there is no known regulated excretory mechanism for iron, and iron homeostasis is tightly controlled *via* its uptake. In the case of fish, iron is acquired predominantly from the diet, with the contribution from iron uptake from the water *via* the gills probably being negligible (Roeder and Roeder, 1966; Andersen, 1997). The estimated daily dietary requirement for iron of teleost fish ranges between 30 and 170 mg kg^{-1} (Watanabe et al., 1997), and aquacultural practice is to add iron to the feed. Deviations from this supplementation can compromise fish health, and iron-deficient diets result in a reduction in hepatic iron stores and haematocrit (Andersen et al., 1997), whereas iron-rich diets are toxic, causing reduced growth (Desjardins et al., 1987) as well as being linked to an increase in pathogen virulence (Fouz et al., 1994).

The mechanisms by which the teleost fish intestine absorbs iron are poorly understood, but bioavailability is influenced by the form in which iron is found in the diet (Andersen et al., 1997). In Atlantic salmon, $Fe(III)_2O_3$ is poorly absorbed (Maage and Sreier, 1998), whereas haem iron is more bioavailable than iron(II) sulphate, which in turn is more bioavailable than elemental iron (Andersen et al., 1997). In contrast, mammalian dietary iron uptake processes have been characterised, and there are two distinct forms of iron, haem and non-haem (Fe^{3+}). In the case of non-haem iron, the acidic environment of the stomach solubilises iron from its ingested matrix (Powell et al., 1999), probably in the ferric form (Fe^{3+}), and gastric and small intestine mucins bind this iron and maintain iron solubility at pH values approaching neutrality in the intestine (Whitehead et al., 1996). Ferric iron is reduced either *via* dietary compounds, such as ascorbate (Raja et al., 1992), or *via* a membrane-bound ferric reductase (McKie et al., 2001). The microclimate close to the tissue is maintained acidic and protected from the lumen *via* the mucus layer covering the mucosa. This environment helps solubilise ferrous iron and it is this form that enters the enterocyte *via* a pH-dependent Fe^{2+}/H^+ cotransporter, termed

a divalent cation transporter (DCT; Gunshin et al., 1997; Fleming et al., 1997). The DCT is part of the evolutionarily conserved family of Nramps (natural resistance-associated macrophage proteins) that are involved in membrane metal transport (Cellier et al., 1995; Orgad et al., 1998; Dorschener and Phillips, 1999; Portnoy et al., 2000; Thomine et al., 2000). Transfer of iron across the lateral membrane of the enterocyte into the circulation occurs *via* an iron-regulated transporter (IREG1). This transporter is closely linked to hephaestin, an integral membrane copper oxidase that oxidises Fe^{2+} to Fe^{3+} as it is released from IREG1, enabling Fe^{3+} binds to to transferrin (McKie et al., 2000). Haem-iron absorption is probably facilitated *via* a haem receptor, and a common intracellular pathway exists with inorganic iron (Conrad et al., 1999). The transferrin/iron complex circulates in the body, where it enters the cells by the well-characterised transferrin-receptor-mediated endocytosis (Andrews et al., 1999).

Evidence for a similar intestinal iron uptake pathway in fish comes from the recent cloning of cDNAs encoding for the Nramps in rainbow trout (Dorschener and Phillips, 1999) and the identification of ferroportin1, which is identical to the IREG1 protein (McKie et al., 2000), in zebrafish (Donovan et al., 2000). However, an interesting difference exists in the chemical composition of the lumen in freshwater and marine teleost intestines. Marine teleosts live in a hyperosmotic environment and thus, to prevent dehydration, they drink sea water. Conversely, freshwater fish reside in hypo-osmotic habits and thus need to minimise water uptake; consequently, they imbibe little water. Wilson (Wilson, 1999) observed that intestinal pH and bicarbonate/carbonate levels differ greatly between freshwater-adapted and seawater-adapted euryhaline teleost species. For example, freshwater rainbow trout or flounder have a mildly acidic intestine (pH 6.7 and 6.4, respectively) and low bicarbonate/carbonate levels (12 mmol l^{-1}, and undetectable, respectively), whereas in sea water they have an alkaline intestine (pH 9.1 and 8.4, respectively) with high bicarbonate/carbonate levels (120 mmol l^{-1} and 75 mmol l^{-1}, respectively). The increased base secretion into the intestine of seawater teleosts is believed to play a role in osmoregulation by facilitating the precipitation of divalent ions, such as Ca^{2+} and Mg^{2+} (Walsh et al., 1991; Wilson, 1999). On the basis of these findings, the intestine of seawater teleosts would appear to be an inhospitable environment for ferrous iron uptake *via* a DCT. The high levels of bicarbonate/carbonate cause precipitation of calcium and magnesium carbonate (Walsh et al., 1991). The solubility product (K_s) for $FeCO_3$ is lower than for $CaCO_3$ and $MgCO_3$, as reflected by pK_s ($-\log K_s$) values at room temperature of 10.5 for $FeCO_3$, 8.1 for $CaCO_3$ and 4 for $MgCO_3$ (Hägg, 1973). So, one might also expect Fe^{2+} to be precipitated as $FeCO_3$ and that the high pH would create a disadvantageous proton gradient. This study aimed to characterise inorganic iron bioavailability and uptake across the intestinal apical membrane of a marine teleost, the European flounder *Platichthys flesus*.

Materials and methods

Experimental animals and solutions

European flounders *Platichthys flesus* L. (252–541 g) were caught in the Baltic Sea and kept in flowing ambient sea water (21 ‰) at Kerteminde Aquatic Research Centre, Funen, Denmark. Water temperature was 13 °C, and the fish were maintained in holding tanks for at least a week prior to experimentation. They were not fed during this period. Fish were killed with an overdose of anaesthetic (MS-222), and plasma and fluid from the stomach, anterior, mid and posterior intestine were taken for analysis of total CO_2 (Toledo-Corning 965 analyser), pH (Radiometer BMS3/PHM 73) and osmolarity (Wescor vapour pressure osmometer). Transepithelial resistance (TEP) was monitored using agar/salt bridges (3 mol l^{-1} KCl in 4 % agar) connected through Ag/AgCl electrodes (World Precision Instruments Inc.) to a Radiometer pHM 84 high-impedance electrometer. All TEP values were expressed relative to the mucosal side of the intestinal sac preparation as 0 mV while the sac was bathed on the mucosal and serosal surfaces with saline of the appropriate composition. Tip potential was routinely less than 1 mV, and the electrodes were checked for symmetry. The mucosal side was accessed *via* the cannulation catheter and the serosal side *via* the outside bathing solution. Triplicate measurements over a 5 min period were averaged. On the basis of a detailed analysis of blood plasma and intestinal fluid (M. Grosell, C. M. Wood, N. R. Bury, C. Hogstrand, J. C. Rankin, R. W. Wilson and F. B. Jensen, unpublished data), artificial salines were made to concentrations (in mmol l^{-1}) of, for intestinal saline, Na^+, 135.2; K^+, 2.5; Mg^{2+}, 1.5; Ca^{2+}, 3; Cl^-, 137; SO_4^{2-}, 1.5; PO_4^{3-}, 3; HCO_3^-, 1.2; glucose, 5.6, and for plasma saline, Na^+, 149; K^+, 5; Mg^{2+}, 0.9; Ca^{2+}, 1.6; Cl^-, 142.3; SO_4^{2-}, 0.9; PO_4^{3-}, 3; HCO_3^-, 11.9; glucose, 5.6.

Radiolabelled ferric chloride was purchased from Amersham International ($^{59}FeCl_3$ the specific activity of which was either 166 MBq ml^{-1}, 316 μg Fe ml^{-1} or 266 MBq ml^{-1}, 315 μg Fe ml^{-1} depending on the batch used). To assess whether the ferrous (Fe^{2+}) or ferric (Fe^{3+}) form of iron was more bioavailable, ferric iron was reduced by ascorbate using the methods of Randell et al. (Randell et al., 1994). Briefly, an appropriate quantity of $^{59}FeCl_3$ (depending on the concentration desired) was added to intestinal saline containing 20 mmol l^{-1} Hepes, pH 7.4, and then mixed with nitroacetic acid (NTA), forming Fe–NTA complexes, at a concentration four times that of iron. After mixing for 20 min, 1 mmol l^{-1} ascorbic acid was added. The solution was mixed for a further 15 min and then adjusted to pH 7.9. Under these conditions, 90 % of the iron is reduced to Fe^{2+} (Randell et al., 1994). For experiments assessing ferric (Fe^{3+}) iron uptake, the iron was prepared in a similar manner except that the ascorbate step was omitted. Throughout this article, the term ferrous iron will refer to that formed from ferric iron by the methods described above. The effect of the iron chelator desferrioxamine mesylate (DFO; Sigma) on *in vivo* iron uptake was assessed by the addition of 10 mmol l^{-1} DFO to the intestinal saline.

In vivo *uptake studies*

Flounders (mean mass 403.5±76.6, N=18) were anaesthetised (1 g l^{-1} MS-222), and a small incision was made in the body wall to expose the intestine. A 7–8 cm segment of the anterior, mid or posterior region was ligated with surgical thread, ensuring that no major blood vessels leading to the hepatic portal vein were trapped. Using a 25 gauge needle, 2 ml of intestinal saline, containing 2.4 μmol l^{-1} $^{59}Fe^{2+}$, was injected into the ligated region. The intestines were carefully replaced into the body cavity and the incision stitched. The fish were then placed into free-flowing sea water. After 8 h, the fish were killed with an overdose of anaesthetic, and 1 ml of blood was removed from the caudal vein *via* a heparinised 18 gauge needle. The ligated region of intestine containing the ^{59}Fe saline was excised, the mucosal fluid was removed and the surface area of the ligated region was determined by the methods of Grosell and Jensen (Grosell and Jensen, 1999). The intestinal tissues of the ligated region and the mucosal contents of this region were counted for radioactivity, as were subsamples of the blood, gill, liver, kidney, spleen, bile, intestine and muscle/skin, using a gamma counter (1480 Wizard 3 Automatic Wallac, Turku, Finland). The proportional mass of the tissues and blood as a percentage of the total mass of the fish was determined for six fish, and the counts present in the subsamples of the tissues were then multiplied by this value to give a total body count. Iron uptake rates (R) are calculated from the following:

$$R = T_{cpm}/(SA \times A \times M \times t), \quad (1)$$

where T_{cpm} is the total number of counts retrieved from the tissues, SA is the specific activity of the solution injected into the intestine (cts min^{-1} $nmol^{-1}$), A is the surface area of the ligated intestine (cm^2), M is the mass of the fish (kg) and t is the duration of the experiment (h).

In vitro *uptake studies*

The cost of the isotope prohibited further characterisation of *in vivo* iron uptake, and a widely accepted (Grosell and Jensen, 1999) *in vitro* approach was adopted. Flounder were killed with an overdose of anaesthetic, and the intestine was severed just below the pyloric caecum and above the rectal sphincter. The intestines were flushed with intestinal saline to remove food and faeces. Cleansed intestinal tracts were divided into three segments of approximately 2–4 cm in length, an anterior section, just below the pyloric caecae, a mid section, and a posterior section just above the rectal sphincter. One end of the segment was sealed tightly with surgical thread and, into the other end, a 5 cm piece of PE-50 tubing was inserted and tied to allow for administration and sampling of the intestinal saline. To assess the bioavailability of ferric and ferrous iron, salines were prepared as described above. In the case of the concentration study, the specific activity was adjusted by the addition of ferric chloride; thereafter, all procedures were the same as described above.

To these 'gut sacs', 1 ml of radiolabelled intestinal saline (range of $^{59}Fe^{2+}$ or $^{59}Fe^{3+}$ used was 0.2–20.2 μmol l^{-1} depending on the experiment) was added, and a 50 μl sample was taken for initial counting. The PE tubing was sealed, and the sacs were placed into 18 ml of plasma saline that was constantly bubbled with a 99.5 % O_2/0.5 % CO_2 gas mixture delivered from a Wösthoff (Bochum, Germany) gas-mixing pump. Temperature was 13 °C (except for the experiment in which the effect of temperature was assessed). The osmolarities of the plasma saline and intestinal saline were similar, 286 mosmol l^{-1} and 291 mosmol l^{-1}, respectively. After a 3 h incubation (except where the temporal pattern of iron absorption was assessed), the mucosal fluid was removed, and a 50 μl subsample was taken for counting. A 10 ml sample of the plasma saline bath was also counted. The gut sac was cut open, and the mucosa was dried with tissue paper. The whole sac was then washed for 1 min in intestinal saline containing 1 mmol l^{-1} EDTA, pH 7.9, blotted dry and washed again for an additional minute. The mucosa was dried again by blotting, and the epithelium was scraped from the muscle layer, using a glass slide, placed into pre-weighed vials and the mass of the scrapings was determined. Preliminary tests demonstrated that the washing procedure removed essentially all the mucus and loosely bound ^{59}Fe, so the radioactivity incorporated into the epithelial tissue was considered to represent iron absorption. For example, the final wash solution from intestinal sacs (N=3) incubated for 3 h with $^{59}Fe^{2+}$ (at a count concentration of 281 000 cts min^{-1} ml^{-1}) possessed 1094±363 cts min^{-1} (N=3, means ± S.E.M.), the epithelial scraping from these samples possessed 1956±252 cts min^{-1}, the muscle layer, 247±39 cts min^{-1} and no counts were detected in the serosal bathing solution. In a small number of cases, counts were found in the serosal bath as a result of inadequate ligation. These preparations were discarded.

Epithelial iron absorption (E) was calculated as follows:

$$E = T/(SA \times M_s \times t), \quad (2)$$

where T is the total cts min^{-1} of the epithelial scrapings, SA is the specific activity of the ferrous or ferric solution (cts min^{-1} $nmol^{-1}$), M_s is the mass of the scrapings (g) and t is the duration (h) of the incubation.

Effects of temperature and pH on Fe^{2+} uptake

To investigate the effects of temperature on ferrous iron uptake (1 μmol l^{-1} iron), epithelial iron absorption was assessed in the anterior, mid or posterior regions of the intestine at ambient temperature (13 °C) and at 1 °C. The latter temperature was achieved by placing the containers possessing the gut sacs and serosal medium in an ice bath.

To assess the effects of pH on posterior intestinal ferrous iron absorption (1 μmol l^{-1} iron), the radiolabelled intestine saline was prepared as described above and then adjusted to pH 5, 6, 7 or 8 using H_2SO_4. The osmolarities of these salines were adjusted to parity to each other by the addition of mannitol, as was that of the serosal bathing medium.

Effects of reduced haematocrit on Fe^{2+} uptake

Flounders (252–504 g, mean mass 419±55 g, N=5) were anaesthetised, and 6–10 ml of blood was removed *via* a

heparinised 18 gauge needle and syringe inserted into the caudal artery/vein complex. The fish were then placed into flowing sea water to recover for 5 days, after which the blood haematocrit was determined by caudal artery/vein sampling and the regions of the intestine were prepared as intestinal sacs (described above) to assess *in vitro* Fe^{2+} absorption (1 μmol l^{-1} iron). The haematocrit and Fe^{2+} absorption of control fish (*N*=5) were also determined.

Statistical evaluation

All values are expressed as means ± S.E.M. Analysis of variance (ANOVA) followed by a least significance difference (LSD) test was used to assess differences between *in vivo* iron uptake and *in vitro* iron uptake at the different times in the three intestinal region and the *in vitro* iron absorption at different pH values. Student's *t*-tests were used to compare *in vivo* ferric (Fe^{3+}) and ferrous (Fe^{2+}) iron uptake rates, the effect of the iron chelator DFO on these rates, the differences between *in vitro* iron absorption when iron was present in the Fe^{3+} or Fe^{2+} form after different incubation periods and at different concentrations, and differences in rates of Fe^{2+} absorption between control and haemoglobin-depleted fish. Linear regression analysis was performed to determine the relationship between haematocrit values and *in vitro* iron absorption. Analysis was performed using the SPSS6 computer package, and $P<0.05$ was taken as the level of significance.

Results

The fluids collected along the tract from the stomach to the rectum were associated with a reduction in osmolarity from 510 to 318 mosmol l^{-1} (Table 1). At the same time, total CO_2 concentrations increased dramatically (stomach, 1.7±0.9 mmol l^{-1}; posterior intestine, 75±10 mmol l^{-1}) and pH increased to 8.45 (Table 1). The *in vitro* transepithelial potential of the anterior, mid and posterior gut regions, with plasma saline on the serosal surface, and intestinal saline on the mucosal surface was serosa-negative (Table 1).

Table 1. *Osmolarity, pH, total CO_2 content of the gut fluids and blood plasma and the* in vitro *transepithelial potential of the stomach and the anterior, mid and posterior regions of the intestine of European flounder acclimated to 21 ‰*

Region of intestine	Osmolality (mosmol l^{-1})	pH	Total CO_2 (mmol l^{-1})	TEP (mV)
Stomach	510±55		1.7±0.9	
Anterior	359±45	8.24±0.1	52±19	–13.7±1.8
Mid	332±30	8.35±0.1	63±16	–17.7±1.3
Posterior	318±22	8.45±0.1	75±10	–19.7±2.1
Plasma	323±24	7.79±0.1	10±1.1	

In vitro transepithelial potential (TEP) values are with plasma saline on the serosal surface and intestinal saline on the mucosal surface.

Values are the mean ± S.E.M. (*N*=5, except for the stomach where *N*=3).

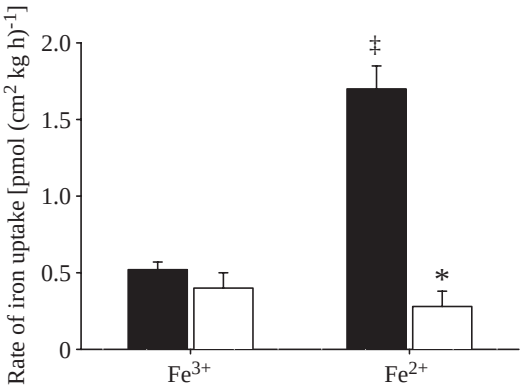

Fig. 1. *In vivo* ferric (Fe^{3+}) and ferrous (Fe^{2+}) iron uptake (filled columns) from the posterior region of the intestine of the European flounder and the influence of the iron chelator desferrioxamine (DFO; open columns) on this uptake rate. Values are means + S.E.M. (*N*=5–6). An asterisk indicates a significant difference between the control iron (Fe^{3+} or Fe^{2+}) uptake rate and the uptake rate in the presence of DFO, and a double dagger indicates a significant difference in uptake rate between the forms of iron, Fe^{3+} or Fe^{2+} (Student's *t*-test, $P<0.05$).

Assessment of iron uptake *in vivo* showed that ferrous iron (Fe^{2+}), formed from the reduction of $FeCl_3$ by ascorbate prior to injection into the posterior region of the flounder intestine, was significantly more bioavailable than ferric iron (Fig. 1). Over an 8 h period, the influx rate of ferrous iron was significantly reduced by desferrioxamine mesylate (DFO), but DFO had no significant effect on ferric iron bioavailability (Fig. 1). A significantly greater ferrous iron uptake rate was observed in flounder in which radiolabelled iron had been injected into the posterior region of the intestine compared with either the mid or anterior regions (Fig. 2).

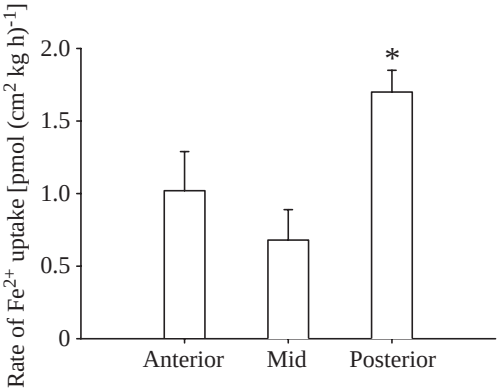

Fig. 2. *In vivo* ferrous (Fe^{2+}) iron uptake rate in the different regions of the intestine of the European flounder. Values are means + S.E.M. (*N*=5–6). An asterisk indicates a significance difference between the iron uptake rates from the three regions (ANOVA followed by an LSD test, $P<0.05$).

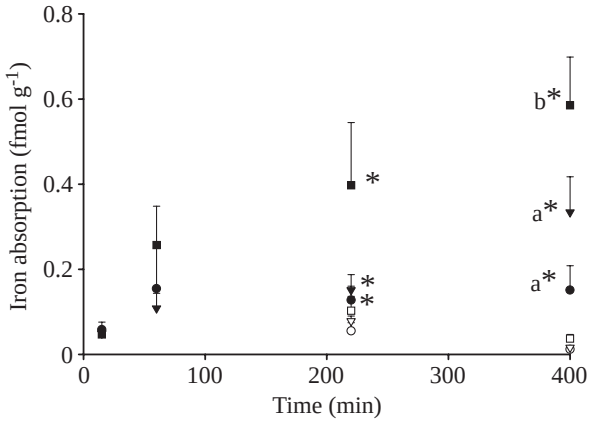

Fig. 3. Temporal pattern of *in vitro* ferrous iron (Fe^{2+}; filled symbols) and ferric iron (Fe^{3+}; open symbols) absorption at a concentration of 0.2 μmol l^{-1} iron in the three regions of the intestine of the European flounder; anterior (■, □), mid (○, ●) and posterior (▼, ▽). Values are means ± S.E.M. (*N*=3–4). An asterisk indicates a significant difference between the absorption of Fe^{3+} and Fe^{2+} at a particular time (Student's *t*-test, *P*<0.05), and values with differing letters indicate significant differences between Fe^{2+} absorption of the three regions at the 400 min incubation point (ANOVA followed by an LSD test, *P*<0.05). There are no significant difference between Fe^{2+} absorption of the three regions at the other time points.

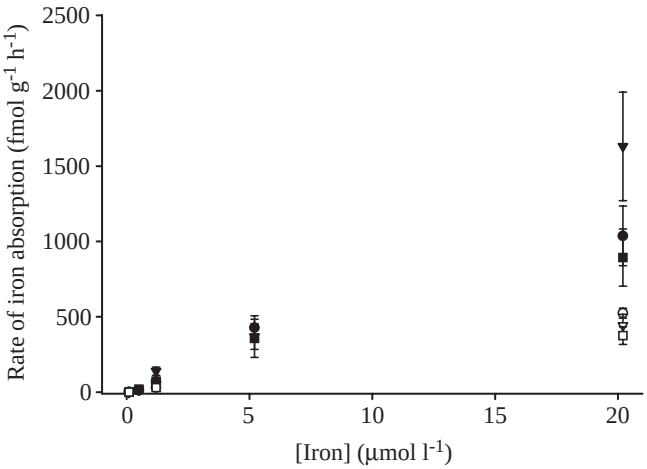

Fig. 4. Concentration-dependent *in vitro* ferrous (Fe^{2+}; filled symbols) and ferric (Fe^{3+}; open symbols) iron absorption in the three regions of the intestine of the European flounder, anterior (■, □), mid (○, ●) and posterior (▼, ▽). Values are means ± S.E.M. (*N*=3–6). At each concentration and in each region of the intestine, the rate of iron absorption in the presence of Fe^{2+} is significantly greater than that in the presence of Fe^{3+} (Student's *t*-test, *P*<0.05).

In vitro temporal iron absorption studies showed that the ferrous form of iron was more bioavailable than the ferric form in all regions of the intestine (Fig. 3). Ferrous iron absorption was 2.6, 4.2 and 5.8 times greater than ferric iron absorption in the anterior, mid and posterior regions, respectively. Therefore, at this low concentration (0.2 μmol l^{-1} iron), a significantly greater proportion of ferrous iron was absorbed in the posterior region compared with the anterior or mid regions after a 400 min incubation period (Fig. 3). The temporal pattern of ferrous iron absorption in the anterior and posterior regions reflected a rapid phase of absorption over the first 15 min followed by a plateau phase (anterior region) or a gradual slower rate of iron absorption (posterior region, Fig. 3). The mid region showed a more-or-less linear increase in iron absorption over time (Fig. 3).

The concentration-dependency of iron absorption showed a larger rate of absorption of Fe^{2+} than of Fe^{3+} in all regions of the intestine and at all iron concentrations tested (Fig. 4). Absorption of ferric and ferrous iron appeared to be linear with respect to the iron concentration of the lumen (Fig. 4), but there was no significant difference between the iron absorption rates of the three intestinal regions in this series (ANOVA follows by an LSD test, *P*=0.08).

Reducing the temperature to 1 °C reduced ferrous iron absorption by the epithelium in all regions of the intestine compared with iron absorption at 13 °C (Fig. 5). The calculated Q_{10} values were 1.5, 1.2 and 1.9 for the anterior, mid and posterior regions, respectively (Fig. 5).

Five days after removal of blood, the haematocrit was significantly reduced (5.4±1.2, *N*=5) compared with that of control fish (19±0.8, *N*=5; *P*<0.001). Ferrous iron absorption was significantly increased in the posterior intestine of anaemic flounder compared with control fish, and iron absorption correlated well with blood haematocrit (Fig. 6). However, in the anterior and mid segments of the intestine, the rates of Fe^{2+} absorption did not increase with reduced haematocrit, and the correlation with blood haematocrit was insignificant (Fig. 6).

Posterior gut sacs filled with saline adjusted to pH values of 5, 6, 7 and 8 had altered their pH to 7.1, 7.3, 7.6 and 7.8, respectively (Fig. 7), after a 3 h incubation. There were no significant differences in rates of Fe^{2+} absorption between these pH values (Fig. 7).

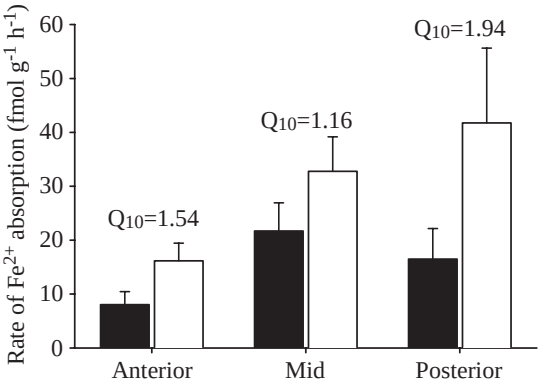

Fig. 5. *In vitro* ferrous iron (1 μmol l^{-1} Fe^{2+}) absorption in the posterior region of the intestine of the European flounder at 1 °C (filled columns) and 13 °C (open columns). Values are means + S.E.M. (*N*=5–6).

Discussion

This is the first study to measure iron uptake and absorption in a marine teleost intestine. The two main conclusions drawn are that, in the intestine of the European flounder, iron in the ferrous form (Fe^{2+}) is more bioavailable than iron in the ferric form (Fe^{3+}) and that *in vivo* iron uptake occurs to a greater extent in the posterior region of the intestine than in the anterior and mid segments.

These findings are remarkable because base secretion into the intestine of marine teleosts results in a progressive increase in pH and bicarbonate/carbonate concentrations towards the posterior region that would lead to an environment in which divalent metal ions would be predicted to precipitate (Table 1); see also Walsh et al. (Walsh et al., 1991), Wilson et al. (Wilson et al., 1996), Wilson (Wilson, 1999) and Grosell and Jensen (Grosell and Jensen, 2000). Indeed, precipitates of $CaCO_3$ and $MgCO_3$ are present in the intestine (Walsh et al., 1991) and, since the solubility product of $FeCO_3$ is even lower than those of $CaCO_3$ and $MgCO_3$, it is predicted that Fe^{2+} would also precipitate. The apical uptake of Fe^{2+} in mammals is *via* a divalent cation transporter (DCT) that avidly binds Fe^{2+} and transports iron under mildly acidic conditions (Fleming et al., 1997; Gunshin et al., 1997). Even though the total CO_2 content and pH of the marine teleost intestine, in principle, would reduce substrate availability and prevent apical metal/proton cotransport, there is molecular evidence for an analogous Fe^{2+}/H^+ cotransporter in fish. Clones of two cDNAs encoding for the two Nramp genes have been identified in freshwater rainbow trout (Dorschener and Phillips, 1999), and partial cDNA sequences have been detected in the zebrafish (GenBank accession number AF190508) and marine sea bass (GenBank accession number AY008746).

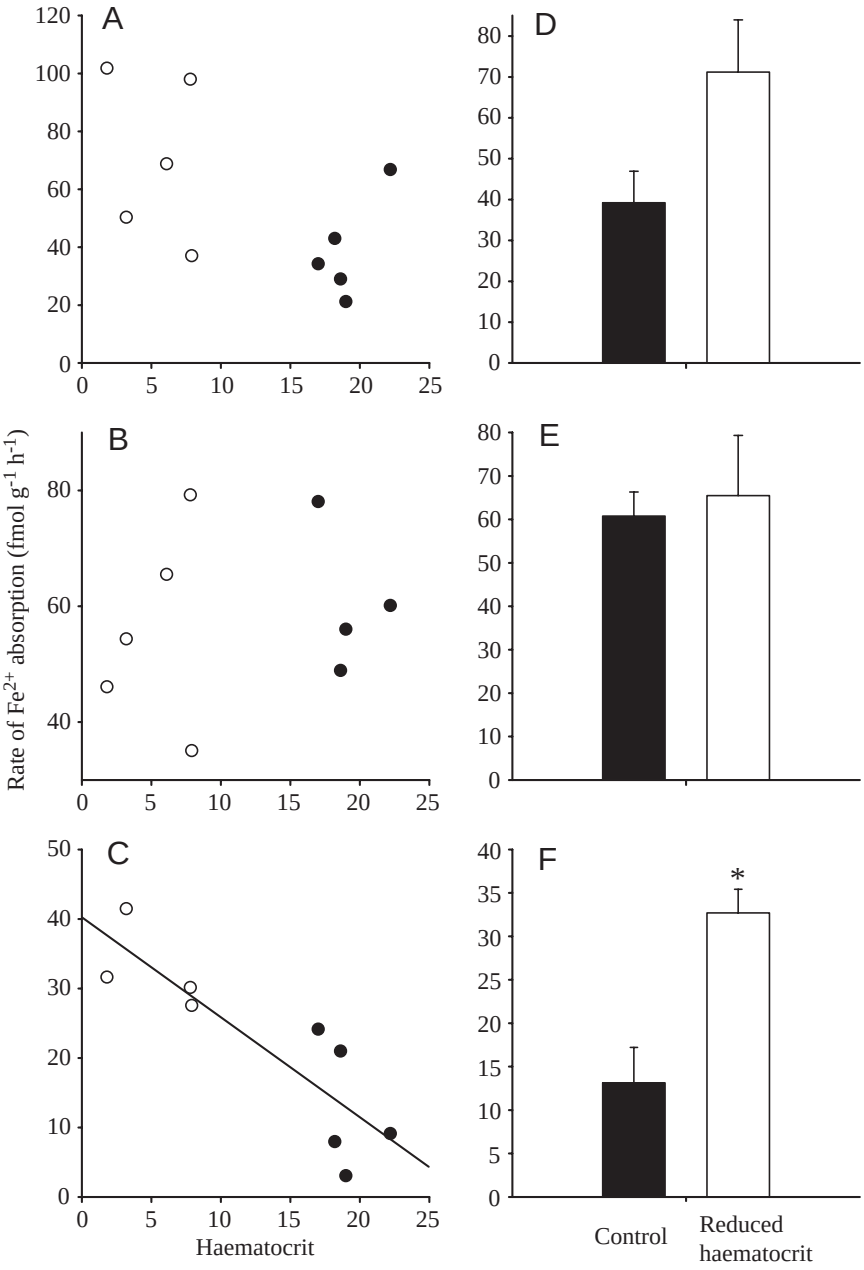

Fig. 6. *In vitro* rates of ferrous iron (Fe^{2+}) absorption at 1 μmol l^{-1} in the three regions of the intestine of the European flounder, (A,D) anterior, (B,E) mid and (C,F) posterior, of control (filled circles and open columns) and anaemic (open circles and filled columns) fish. Values are means + S.E.M. (N=4–5). An asterisk indicates a significant difference in the rates of iron absorption (Student's t-test, P<0.05). Linear regression analysis for iron absorption *versus* haematocrit shows r=–0.57, P=0.085 for the anterior region, r=0.10, P=0.63 for the mid region and r=–0.86, P=0.003 for the posterior region.

Metal absorption and transfer across the teleost intestine are complex and involve an integration of biochemical and biophysical processes. *In vitro* transepithelial potential measurements, recorded under the conditions of the present experiments, show that all three regions of the flounder intestine are serosal-side negative (Table 1). This electrochemical gradient would encourage the absorption of metal ions into the intestinal mucosa. Furthermore, the lower electrical resistance of the anterior and mid regions of the marine teleost intestine compared with the posterior region demonstrates a 'leaky epithelium' (Loretz, 1995) that may allow the passage of iron *via* a paracellular route. This is the proposed route of entry for trivalent ions (Powell et al., 1999) and may, in part, explain Fe^{3+} uptake (Fig. 1). However, the uptake of divalent ions is generally believed to be *via* more specific transcellular routes (Whitehead et al., 1996) that enable physiological regulation of the uptake of essential, but potentially toxic, metals. This is supported by the limited number of studies on divalent metal

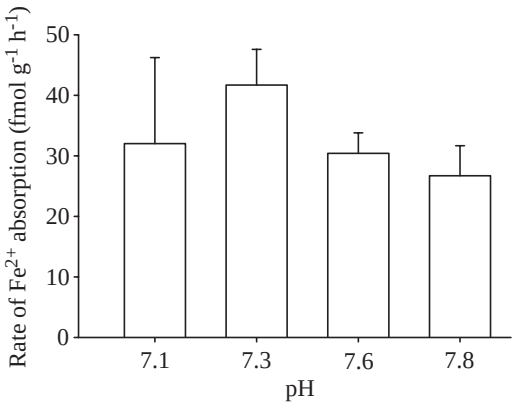

Fig. 7. *In vitro* rate of ferrous iron (1 μmol l^{-1} Fe^{2+}) absorption in the posterior region of the intestine of the European flounder at different pH values. The pH of the intestinal lumen changed over the experimental period; see text for details. Values are means + S.E.M. (*N*=5–6). There were no significant differences between rates of iron absorption.

ion uptake in the intestine of fish. Active transport and cation- or anion-dependent cotransport have been demonstrated for Mg^{2+}, Ca^{2+}, Cu^{2+} and Zn^{2+} in the fish intestine (Shears and Flectcher, 1983; Flik et al., 1990; Bijvelds et al., 1998; Handy et al., 2000) (C. N. Glover and C. Hogstrand, in preparation), and the presence of an Fe^{2+} uptake mechanism in flounder intestine confirms this hypothesis.

In vivo studies better mimic the situation in the intact animal because blood circulation is maintained and iron does not have to pass through the serosal membrane. Consequently, the iron-binding protein transferrin is present in the blood plasma to accept iron after basolateral transfer of Fe^{2+} by IREG1 and subsequent oxidation to Fe^{3+} (McKie et al., 2000). In these conditions, the posterior segment of the flounder intestine is the dominant site of Fe^{2+} uptake (Fig. 2). Support for regulated ferrous iron uptake in this region comes from the gut sac studies and, because *in vitro* there is no notable transfer of radiolabel into the serosal medium, the higher rate of transport in the posterior segment is due to an upregulation in an apical membrane-bound transport mechanism. The posterior intestine is the only region that has a Q_{10} approaching 2 (Q_{10}=1.94, Fig. 5), which suggests that, in addition to simple diffusion, there is also a carrier-mediated uptake process involved. It is also the only region in which iron absorption is significantly increased in response to a reduced iron status and that shows a strong negative correlation between blood haematocrit and iron absorption (Fig. 6).

Similarly, iron deficiency in mammals also induces an increase in iron uptake that is associated with a rise in duodenal DCT expression (Trinder et al., 1999). However, the situation is complicated by the fact that *in vitro* iron absorption in the posterior region was only significantly greater than in the other regions at low iron concentrations (Fig. 3, Fig. 4). Absorption will consist of a simple diffusion component and a facilitated uptake component, and this disparity between the *in vivo* and *in vitro* situations suggests that the other regions (anterior and mid) of the intestine possess a low-affinity/high-capacity transporter system or Fe^{2+} absorption is *via* simple diffusion and/or that, in these regions, there is a greater abundance of non-specific apical membrane divalent-cation-binding moieties. In contrast, the posterior region is the site of a high-affinity Fe^{2+} transporter that is upregulated in response to perturbed iron homeostasis.

In mammals, the anterior (duodenal) segment of the intestine is the site for dietary iron absorption (Gunshin et al., 1997). It is therefore surprising that the posterior intestine is the dominant, but not the only, site for iron uptake in marine fish (Fig. 2). The reason why flounder have evolved this strategy is unclear, and the situation is further complicated by the high concentrations of bicarbonate/carbonate that reduce divalent ion availability (see above). The marine fish must have developed specialised mechanisms to maintain metal bioavailability and ionic metal transport in these hostile conditions. Gastric and intestinal mucins and the mucous layer covering the intestinal wall probably play a pivotal role in metal availability and uptake (Whitehead et al., 1996; Powell et al., 1999). Mucins maintain iron solubility as the pH rises in the mammalian intestine (Powell et al., 1999). Specifically, the layer of mucus acts as a buffer between the lumen fluids and the tissues, generating a pH gradient with mildly acidic conditions close to the tissue, which aids metal–mucus dissociation and metal/proton cotransport (Whitehead et al., 1996). Interestingly, adjusting luminal pH had no effect on the rate of iron absorption (Fig. 7). In freshwater rainbow trout, intestinal mucus production is stimulated by perfusion with zinc (C. N. Glover and C. Hogstrand, in preparation). It awaits verification whether other metals stimulate mucus production, but this may be one way in which the marine flounder maintains Fe^{2+} soluble in the microclimate close to the intestinal transport site.

In the mammalian duodenum, dietary ferric iron is reduced to the bioavailable ferrous form by an apical-membrane-bound ferric reductase (McKie et al., 2001). The reduced bioavailability of ferric iron in the flounder intestine (Fig. 1) suggests that the level, or functional capacity, of a teleost ferric reductase is low. Under these conditions, the posterior positioning of the ferrous iron uptake processes may be beneficial because it will prolong the period that dietary reducing agents (e.g. ascorbate) have to convert dietary Fe^{3+} to Fe^{2+}. In addition, other physio-chemical factors may play a role. The autochthonous bacteria of fish intestines have been poorly assessed, but the population does contain anaerobic organisms (Ringo and Birkbeck, 1999). This indicates that favourable redox conditions may exist in some areas of the intestine that encourage Fe^{2+} formation.

Desferrioxamine (DFO) treatment had no effect on Fe^{3+} bioavailability, but inhibited Fe^{2+} uptake (Fig. 1). This siderophore is secreted in its iron-free form by actinomycetes acting as an extra- or intracelluar chelator of iron (Richardson and Baker, 1992). In yeast, it can aid iron uptake by complexing environmental Fe^{3+} and either presenting Fe^{3+} to a membrane-bound reductase linked to a Fe^{2+} transport system

or the siderophore/iron complex is internalised by endocytosis (Yun et al., 2000). DFO has been used to stimulate iron uptake in mammalian cells (Richardson and Baker, 1990; Richardson and Baker, 1992). In the present study, DFO was added in excess (10 mmol l^{-1} DFO *versus* 1 μmol l^{-1} iron) and remained in solution for the 8 h experimental period. This time is insufficient to observe a positive effect of DFO on the rate of iron uptake (Richardson and Baker, 1994; Randell et al., 1994), and thus it is acting solely as an extracellular iron-chelating agent. The absence of effect of DFO on ferric iron accumulation indicates that this siderophore is not acting to enhance Fe^{3+} bioavailability in the flounder intestine, but other siderophores may operate in fish. However, the inhibition of intestinal Fe^{2+} uptake suggests that the flounder intestinal iron uptake pathway is at least in part *via* a ferrous iron transporter.

Aquacultural practice is to supplement feed with 60–100 mg iron kg^{-1}, and it is of interest in the light of the greater bioavailability of Fe^{2+} than of Fe^{3+} in the present study that this is in the form of ferrous salts (Andersen et al., 1996). However, Andersen et al. (Andersen et al., 1997) showed that the bioavailability of dietary haem-bound iron is greater than that of ferrous salts. Organically bound iron may therefore be an important source of dietary iron in fish. As alluded to above, dietary ferric iron must be converted by components within the diet and/or by reducing environments in the marine fish intestine. In the present study, ferrous iron was formed by chelation of ferric iron to nitroacetic acid and reduction by ascorbate, a method that has been used to assess Fe^{2+} uptake in mammalian systems (Randell et al., 1994). Ascorbate is routinely added to fish feeds in aquaculture, but its influence on iron status is variable. For example, iron methionine bioavailability is enhanced by ascorbate in catfish (Lim et al., 2000), whereas Fe^{2+} is readily bioavailable in salmonids and ascorbate either has little effect (Hilton, 1989; Andersen et al., 1997) or enhances iron status (Dabrowski et al., 1989). The importance of dietary reducing agents in fish intestine metal bioavailability requires further study.

The increased Fe^{2+} bioavailability and upregulation of iron-binding proteins in response to a physiological stimulus (haemoglobin depletion) suggests that a regulated divalent iron transport process is present in the intestine of the marine flounder. The evidence suggests that this transport process is probably *via* a protein that belongs to the Nramp gene family, a family of genes that has been shown to transport Fe^{2+} in plants and mammals (Conrad et al., 1999; Thomine et al., 2000), and analogous sequences have been cloned from rainbow trout (Dorschener and Phillips, 1999). However, the result of this study are of interest when placed in the context of the chemistry of the marine teleost intestine that would predict divalent ion carbonate precipitation and an unfavourable proton gradient for Fe^{2+}/H^{+} cotransport. Consequently, it will be important to determine the mechanisms by which the marine teleost intestine maintains iron and other essential metals bioavailable for uptake in spite of the chemical obstacles confronted.

This study was in part funded by a University Fellowship, University of Exeter, to N.R.B., a Royal Society travel grant to N.R.B. and R.W.W., an NSERC research grant to C.M.W., a Journal of Experimental Biology Travelling Fellowship to M.G. and the Danish Natural Science Research Council to F.B.J. C.M.W. is also supported by the Canada Research Chair Program. The authors wish to thank Annie Bach for excellent technical assistance.

References

Andersen, F., Lorentzen, M., Waagbo, R. and Maage, A. (1997). Bioavailability and interactions with other micronutrients of three dietary iron sources in Atlantic salmon, *Salmo salar* L. smolts. *Aquacult. Nutr*. **3**, 239–246.

Andersen, F., Maage, A. and Juhshman, K. (1996). An estimation of iron requirement of Atalntic salmon, Salmo salar L., parr. *Aquacult. Nutr*. **2**, 41–47.

Andersen, O. (1997). Accumulation of waterborne iron and expression of ferritin and transferrin in early developmental stages of brown trout (*Salmo trutta*). *Fish Physiol. Biochem*. **16**, 223–231.

Andrews, N. C., Fleming, M. D. and Gunshin, H. (1999). Iron transport across biologic membranes. *Nutr. Rev*. **57**, 114–123.

Bijvelds, M. J. C., van der Velden, J. A., Kolar, Z. and Flik, G. (1998). Magnesium transport in freshwater teleosts. *J. Exp. Biol*. **201**, 1981–1990.

Cellier, M., Prive, G., Belouchi, A., Kwan, T., Rodrigues, V., Chia, W. and Gros, P. (1995). Nramp defines a family of membrane proteins. *Proc. Natl. Acad. Sci. USA* **92**, 10089–10093.

Conrad, M. E., Umbreit, J. N. and Moore, E. G. (1999). Iron absorption and transport. *Am. J. Med. Sci.* **318**, 213–229.

Dabrowski, K., El-Fiky, N. and Kock, G. (1990). Requirement and utilization of ascorbic acid and ascorbic sulfate in juvenile rainbow trout. *Aquaculture* **91**, 317–328.

Donovan, A., Brownlie, A., Zhou, Y., Shepard, J., Pratt, S. J., Moynihan, J., Paw, B. H., Drejer, A., Barut., B., Zapata, A., Law, T. C., Brugnara, C., Lux, S. E., Pinkus, J. L., Kinsley, P. D., Palis, J., Fleming, M. D., Andrews, N. C. and Zon, L. I. (2000). Positional cloning of zebrafish *ferroportin1* identifies a conserved vertebrate iron exporter. *Nature* **403**, 776–781.

Dorschener, M. O. and Phillips, R. B. (1999). Comparative analysis of two Nramp loci from rainbow trout. *DNA Cell Biol*. **18**, 573–583.

Fleming, M. D., Trenor, C. C., Su, M. A., Foernzler, D., Beier, D. R., Dietrich, W. F. and Andrews, N. C. (1997). Microcytic anaemia mice have a mutation in Nramp2, a candidate iron transporter gene. *Nature Genet*. **16**, 383–386.

Flik, G., Schoenmakers, T. J. M., Groot, G. A., van Os, C. H. and Wendelaar Bonga, S. E. (1990). Calcium absorption by fish intestine: the involvement of ATP- and sodium-dependent calcium extrusion mechanisms. *J. Membr. Biol*. **113**, 13–22.

Fouz, B., Toranzo, A. E., Biosca, E. G., Mazoy, R. and Amaro, C. (1994). Role of iron in the pathogenecity of *Vibrio damsela* for fish and mammals. *FEMS Microbiol. Lett*. **121**, 181–188.

Grosell, M. and Jensen, F. B. (1999). NO_2^- uptake and HCO_3^- excretion in the intestine of the European flounder (*Platichthys flesus*). *J. Exp. Biol*. **202**, 2103–2110.

Grosell, M. and Jensen, F. B. (2000). Uptake and effects of nitrite in the marine teleost fish *Platichthys flesus*. *Aquat. Toxicol*. **50**, 97–107.

Gunshin, H., Mackenzie, B., Berger, U. V., Gunshin, Y., Romero, M. F., Boron, W. F., Nussberger, S., Gollan, J. L. and Hediger, M. A. (1997). Cloning and characterisation of a mammalian proton-coupled metal-ion transporter. *Nature* **388**, 482–488.

Hägg, G. (1973). *Allmän och Oorganisk Kemi*. Stockholm, Sweden: Almqvist & Wiksell.

Handy, R. D., Musonda, M. M., Phillips, C. and Falla, S. J. (2000). Mechanisms of gastrointestinal copper absorption in the African walking catfish: copper dose-effects and a novel anion-dependent pathway in the intestine. *J. Exp. Biol*. **203**, 2365–2377.

Hilton, J. W. (1989). The interaction of vitamins, minerals and diet composition in the diet of fish. *Aquaculture* **79**, 223–244.

Lim, C., Klesius, P. H., Li, M. H. and Robinson, E. H. (2000). Interaction

between dietary levels of iron and vitamin C on growth, hematology, immune response and resistance of channel catfish (*Ictalurus punctatus*) to *Edwardsiella ictaluri* challenge. *Aquaculture* **185**, 313–327.

Loretz, C. A. (1995). Electrophysiology of ion transport in teleost intestinal cells. *In Cellular and Molecular Approaches to Fish Ionic Regulation, Fish Physiology*, vol. 4 (ed. C. M. Wood and T. Shuttleworth), pp. 25–56. London: Academic Press.

Maage, A. and Sveier, H. (1998). Addition of dietary iron(III) oxide does not increase iron status of growing Atlantic salmon. *Aquacult. Int.* **6**, 249–252.

McKie, A. T., Barrow, D., Latunde-Dada, G. O., Rolfs, A., Sager, G., Mudaly, E., Mudaly, M., Richardson, C., Barlow, D., Bomford, A., Peters, T. J., Raja, K. B., Shirali, S., Hediger, M. A., Farzaneh, F. and Simpson, R. J. (2001). An iron-regulated ferric reductase associated with absorption of dietary iron. *Science* **291**, 1755–1759.

McKie, A. T., Marcini, P., Rolfs, A., Brennan, K., Wehr, K., Barrow, D., Miret, S., Bomfor, A., Peter, T. J., Farzaneh, F., Hediger, M. A., Hentze, M. W. and Simpson, R. J. (2000). A novel duodenal iron-regulated transporter IREG1 implicated in the basolateral transfer of iron to the circulation. *Mol. Cell.* **5**, 1–20.

Orgad, S., Nelson, H., Segal, D. and Nelson, N. (1998). Metal ions surpress the abnormal taste behaviour of the *Drosophila mutaner malvolio*. *J. Exp. Biol*. **201**, 115–120.

Portnoy, M. E., Liu, X. F. and Culotta, V. C. (2000). *Sacchatomyces cerevisiae* expresses three functionally distinct homologues of the Nramp family of metal transporters. *Mol.Cell. Biol.* **20**, 7893–7902.

Powell, J. J., Jugdaohsingh, R. and Thompson, R. P. H. (1999). The regulation of mineral absorption in the gastrointestinal tract. *Proc. Nutr. Soc.* **58**, 147–153.

Raja, K. B., Simpson, R. J. and Peters, T. J. (1992). Investigation of a role for reduction in ferric iron uptake by mouse duodenum. *Biochim. Biophys. Acta*. **1135**, 141–146.

Randell, E. W., Parkes, J. G., Olivier, N. F. and Templeton, D. M. (1994). Uptake of non-transferrin-bound iron by both reductive and nonreductive processes is modulated by intracellular iron. *J. Biol. Chem.* **269**, 16046–16053.

Richardson, D. R. and Baker, E. (1990). The uptake of iron and transferrin by the human malignant melanoma cell. *Biochim. Biophys. Acta*. **1053**, 1–12.

Richardson, D. R. and Baker, E. (1992). Two mechanisms of iron uptake from transferrin by melanoma cells. *J. Biol. Chem.*. **267**, 13972–13979.

Richardson, D. R. and Baker, E. (1994). Two saturable mechanisms of iron uptake from transferrin in human melanoma cells: the effect of transferrin concentration, chelators and metabolic probes on transferrin and iron uptake. *J Cell Physiol*. **161**, 160–168.

Ringo, E. and Birkbeck, T. H. (1999). Intestinal microflora of fish larvae and fry. *Aquacult. Res.* **30**, 73–93.

Roeder, M. and Roeder, R. H. (1966). Effect of iron on the growth rate of fishes. *J. Nutr.* **90**, 86–90.

Shears, M. A. and Flectcher, G. L. (1983). Regulation of Zn^{2+} uptake from the gastrointestinal tract of a marine teleost, the winter flounder (*Pseudopleuronectes americanus*). *Can. J. Fish. Aquat. Sci*. **40 (Suppl. 2)**, 197–205.

Thomine, S., Wang, R., War, J. M., Crawford, N. M. and Scroeder, J. I. (2000). Cadmium and iron transport by members of a plant metal transporter family in *Arabidopsis* with homology to Nramp genes. *Proc. Natl. Acad. Sci. USA* **98**, 4991–4996.

Trinder, D., Oates, P. S., Thomas, C., Sadleir, J. and Morgan, E. H. (1999). Localisation of divalent metal transporter 1 (DMT1) to the microvillus membrane of rat duodenal enterocytes in iron deficiency, but to hepatocytes in iron overload. *Gut* **46**, 270–276.

Walsh, P. J., Blackwelder, P., Gill, K. A., Danulat, E. and Mommsen, T. P. (1991). Carbonate deposits in marine fish intestine: a new source of biomineralization. *Limnol. Oceanogr.* **36**, 1227–1232.

Watanbe, T., Kiron, V. and Satoh, S. (1997). Trace minerals in fish nutrition. *Aquaculture* **151**, 185–207.

Whitehead, M. W., Thompson, R. P. H. and Powell, J. J. (1996). Regulation of metal absorption the gastrointestinal tract. *Gut* **39**, 625–628.

Wilson, R. W. (1999). A novel role for the gut of seawater teleosts in acid–base balance. In *Regulation of Tissue pH in Plants and Animals* (ed. S. Egginton, E. W.Taylor and J. A. Raven), pp. 257–274. Cambridge: Cambridge University Press.

Wilson, R. W., Gilmour, K. M., Henry, R. P. and Wood, C. M. (1996). Intestinal base excretion in the seawater-adapted rainbow trout: a role in acid–base balance? *J. Exp. Biol.* **199**, 2331–2343.

Yun, C.-W., Ferea, T., Rashford, J., Ardon, O., Brown, P. O., Botsein, D., Kaplan, J. and Philpott, C. C. (2000). Desferrioxamine-mediated iron uptake in *Saccharomyces cerevisiae*. *J. Biol. Chem.* **275**, 10709–10715.