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Calcium regulation in the freshwater-adapted mummichog

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In light of recent findings of an unusual pattern of ionoregulation (high Na^+ uptake and negligible Cl^- uptake) in the freshwater-adapted mummichog *Fundulus heteroclitus*, the pattern of Ca^{2+} regulation was examined. Under control conditions (water $Ca^{2+}=200$ μEq l^{-1}), unidirectional Ca^{2+} influx was 11 ± 4 nEq g^{-1} h^{-1}. Acute variation of external Ca^{2+} levels revealed a saturable Ca^{2+} uptake system with a relatively high affinity ($K_m=125\pm36$ μEq l^{-1}) and a transport capacity ($J_{max}=31\pm4$ nEq g^{-1} h^{-1}) comparable to those of other teleosts. Lanthanum (equimolar to [Ca^{2+}]) significantly blocked Ca^{2+} uptake by 67% whereas magnesium had no effect. Chronic low Ca^{2+} exposure (50 μEq l^{-1}) stimulated Ca^{2+} uptake almost three-fold above control values, whereas chronic high Ca^{2+} exposure (20 000 μEq l^{-1}) had no effect. Lanthanum and chronic low Ca^{2+} treatments disturbed the normally positive Ca^{2+} and Na^+ balances of the animals whereas acid-base balance and ammonia excretion were undisturbed. The results indicate that Ca^{2+} regulation by the mummichog conforms to the model for freshwater Ca^{2+} transport whereby chloride cells on the gills take up Ca^{2+} actively from the water. However, the absence of extra-intestinal Cl^- uptake and the recent demonstration of significant Ca^{2+} uptake by opercular epithelia raise questions about the relative roles of branchial and opercular epithelial chloride cells in freshwater *F. heteroclitus*.

Key words: fish; mummichog; calcium; *Fundulus heteroclitus*; ion fluxes.

INTRODUCTION

In a recent study, Patrick *et al.* (1993) demonstrated that the freshwater-adapted mummichog *Fundulus heteroclitus* (L.) exhibits very high rates of Na^+ turnover while Cl^- influx from the water is essentially zero. These findings deviate substantially from the current model of ion transport established for freshwater teleosts (McDonald *et al.*, 1989; Goss *et al.*, 1992; Wood & Marshall, 1994; Kirschner, 1996) and may be associated with the high degree of euryhalinity exhibited by *F. heteroclitus* (Griffith, 1972). The lack of Cl^- uptake was surprising considering the abundance of 'chloride cells' (mitochondria-rich cells) on both the gills and the opercular epithelium (Wood & Marshall, 1994). Current gill models attribute both Cl^- uptake (Goss *et al.*, 1992, 1995) and Ca^{2+} uptake (Flik & Verbost, 1993; Flik *et al.*, 1993, 1995) to the chloride cells, so the absence of Cl^- uptake raises questions about the nature and site of Ca^{2+} transport in the freshwater mummichog.

Only a few papers have examined Ca^{2+} uptake in the *F. heteroclitus* and of these, two *in vivo* studies (Pang *et al.*, 1980; Mayer-Gostan *et al.*, 1983), and a recent *in vitro* study, which utilized the opercular membrane (Marshall *et al.*,

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1995), are not consistent. The two former studies agree that Ca^{2+} enters the fish from the water via an extra-intestinal route and that the uptake rate increases after acclimation to lower environmental Ca^{2+} levels. However, in the more detailed study (Mayer-Gostan *et al.*, 1983), uptake was related linearly to external concentration under normal conditions, suggestive of a passive mechanism. The stimulated uptake after acclimation to low [Ca^{2+}] was not related linearly to external concentration, but did not follow classical Michaelis–Menten kinetics. In contrast, in the *in vitro* study (Marshall *et al.*, 1995) Ca^{2+} influx across the opercular epithelium of the *F. heteroclitus* exhibited saturable uptake kinetics.

In the present study, Ca^{2+} uptake from the water was characterized *in vivo* in freshwater-adapted mummichog to determine if it, like Cl^- uptake, is unusual in this fish and departs from the currently accepted model and secondly, to help clarify the inconsistencies between the studies mentioned above. Calcium influx rates were determined by the appearance of radiolabelled Ca^{2+} in the fish (cf. Hogstrand *et al.*, 1994). Various experiments examined the external concentration dependence of calcium influx (' kinetics '), the influence of adaptation to high and low [Ca^{2+}], the effects of two known inhibitors of Ca^{2+} transport—La^{3+} (Verbost *et al.*, 1987, 1989; Perry & Flik, 1988) and Mg^{2+} (Hartzell & White, 1989), and possible linkages with Na^+, ammonia, and acid-base balance.

MATERIALS AND METHODS

EXPERIMENTAL ANIMALS

Mummichogs weighing 2·7–11·7 g were collected in summer from a brackish estuary near Antigonish, Nova Scotia and were held in 500-l fibreglass tanks containing aerated, charcoal-filtered 10% sea water at ambient temperature (18–25° C) and a 15L : 9D photoperiod. Ten days prior to the experiment, a maximum of 25 fish was placed in 80-l glass aquaria containing acclimation water which was aerated and filtered. Composition (all in µEq l^{-1}) was: Na^+, 1000; Cl^-, 1000; Ca^{2+}, 200; Mg^{2+}, 120; K^+, 20; titration alkalinity to pH = 4·0, 280 µEq l^{-1}; pH = 6·8–7·3. Water was replaced every 3 days. Fish were fed daily (a 1 : 2 mixture of Tetramin/Tetramarin) until 4 days before the experiment. All experiments were performed in the month of July.

EXPERIMENTAL METHODOLOGY

Calcium uptake kinetics

The rate of whole body Ca^{2+} influx (J^{Ca}_{in}) was measured in seven Ca^{2+} concentrations ranging from 50 to 3200 µEq l^{-1}, following a protocol developed by Hogstrand *et al.* (1994, 1995). Each of seven 3-l black polypropylene flux chambers, equipped with individual aeration, were filled with 1·5 l of Ca^{2+}-free water (distilled water supplemented with NaCl, KCl and $NaHCO_3$ to approximate the levels of these ions in the acclimation water). Appropriate aliquots of a $^{45}Ca/Ca(NO_3)_2$ stock solution (608 µEq l^{-1} specific activity = 74 kBq µEq $^{-1}$) were added to adjust the water Ca^{2+} levels to 50, 100, 200, 400, 800, 1600, and 3200 µEq l^{-1}, and allowed to mix for 15 min. The experiment was initiated by placing eight fish into each of the seven flux chambers. Water samples (30 ml) were taken from each chamber at 0, 2 and 4 h for subsequent analysis of ^{45}Ca and [Ca^{2+}]. After 4 h, fish were placed, one at a time, into a freshwater medium containing 20 000 µEq l^{-1} $Ca(NO_3)_2$, to displace any surface bound ^{45}Ca, and a terminal overdose of MS-222 (1·0 g l^{-1} MS-222). After 1 min, the fish were removed, blotted dry, weighed, wrapped in aluminum foil, and either processed immediately or stored at − 20° C.

The effects of external La^{3+}, Mg^{2+} and exposure to high and low $[Ca^{2+}]_{ext}$

Whole body Ca^{2+} uptake was measured during acute exposure to 100 μmol l^{-1} $MgSO_4$ or $LaCl_3$ (equimolar to the Ca^{2+} level in the medium) and following chronic (10 day) high and low Ca^{2+} exposure. Net Ca^{2+}, Na^+ ammonia, and acid-base fluxes were monitored also. The holding conditions for control, La^{3+}, and Mg^{2+} treatment groups were the same as described above. The low Ca^{2+} holding medium was prepared from distilled water and supplemented with NaCl, $Ca(NO_3)_2$, KCl and $NaHCO_3$ to approximate the composition described earlier except Ca^{2+} concentration was set to 50 μEq l^{-1}. High Ca^{2+} holding water was achieved by supplementing the defined freshwater medium with $Ca(NO_3)_2$ to raise the Ca^{2+} level to 20 000 μEq l^{-1}. Batches of control, Mg^{2+}, and La^{3+} experimental water (all with 200 μEq l^{-1} Ca^{2+}), and low Ca^{2+} experimental water (50 μEq l^{-1} Ca^{2+}) were made by adding the appropriate amount of the $^{45}Ca/Ca(NO_3)_2$ stock solution to the Ca^{2+}-free medium (above), supplemented with 100 μmol l^{-1} $MgSO_4$ or 100 μmol l^{-1} $LaCl_3$ as appropriate.

Flux measurements on the chronic low Ca^{2+} fish were performed in the low Ca^{2+} medium in order to determine the reference rate of J^{Ca}_{in} at 50 μEq l^{-1} Ca^{2+}. Fish acclimated to 200 μEq l^{-1} Ca^{2+} and acutely exposed to 50 μEq l^{-1} Ca^{2+} were used as a comparison to indicate flux rates in the absence of long-term exposure to low Ca^{2+}. Flux measurements on the chronic high Ca^{2+} fish were performed in the control medium (200 μEq l^{-1} Ca^{2+}) rather than at the holding Ca^{2+} level (20 000 μEq l^{-1}) as the amount of isotope required to maintain a water specific activity (0·07 kBq μEq $^{-1}$) similar to the other experiments would have been prohibitive.

Eight fish were used in each treatment group except control ($n=7$). For all treatments, the fish were placed in individual darkened flux chambers and held in a static, vigorously aerated 300-ml volume of the appropriate acclimation medium for no longer than 6 h. Following this overnight settling period, the flux chambers were flushed and 300 ml of fresh acclimation medium were added. Ninety minutes later, the chambers were flushed again. This time, 300 ml of the appropriate experimental media were added. Water samples (30 ml) were taken at 0-, 2- and 4-h intervals for subsequent analysis of ^{45}Ca, Ca^{2+}, Na^+, titratable alkalinity, and ammonia concentrations, after which the fish were killed and processed as described for the uptake kinetics study.

ANALYTICAL METHODS AND CALCULATIONS

Flux measurements

Individual frozen fish were diced and then ashed in closed ceramic crucibles at 750° C for a minimum of 4 h. The ash was dissolved subsequently with 2 N HCl; duplicate aliquots of fish ash digest and water samples were added to 10 ml scintillation cocktail (ACS, Amersham) and assayed with a Packard 2000 CA liquid scintillation counter. A preliminary test indicated that quenching was uniform, obviating the need for quench correction.

Water calcium and sodium concentrations were determined by atomic absorption (Varian 375 AAS), water total ammonia (T_{Amm}) by a micro-modification of the salicylate-hypochlorite assay (Verdouw *et al.*, 1978), water titratable alkalinity (TA) by titration of 10-ml volumes with 0·02 N HCL using ml burettes, and subsequent calculation of acid-base fluxes (J^{TA}_{net}, J^{Amm}_{net}, $J^{H^+}_{net}$) according to McDonald & Wood (1981). J^{TA}_{net} and $J^{H^+}_{net}$ measured in the $LaCl_3$ treatment are not reported as tests indicated that the water titration values were not reliable due to interference by $LaCl_3$.

J^{Ca}_{in} (nEq g^{-1} h^{-1}) was determined by the following equation:

$$J_{in}=\frac{c}{At}$$

where c is the average counts in the tissue samples (cpm/g) based on the volume of the fish ash-HCl solution counted, the amount of acid added to dissolve the ash and the weight of the fish prior to ashing, A is the mean specific activity of ^{45}Ca in the water (cpm nEq $^{-1}$) and t is time (h). The measurements were interpreted as extra-intestinal uptake rates since reported drinking rates for *F. heteroclitus* (Potts & Evans, 1967) could account for only about 10% of the measured J^{Ca}_{in}-values.

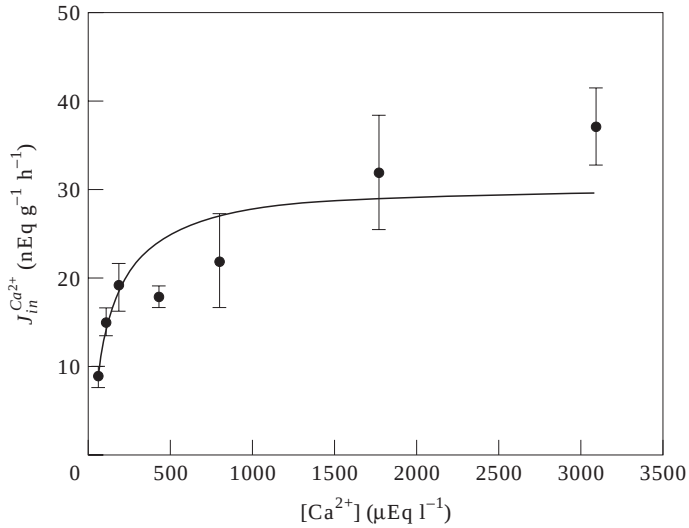

FIG. 1. The influence of external calcium on whole-body Ca^{2+} uptake ($J^{Ca}{}_{in}$) of the freshwater-adapted mummichog. Means ± S.E. $n=8$ for each $[Ca^{2+}]_{ext}$ tested. The line fitted to the data is a Michaelis–Menten equation with the values of K_m (125 μEq l^{-1}) and J_{max} (31 nEq g^{-1} h^{-1}) fitted by Eadie–Hofstee regression analysis.

The relationship between $[Ca^{2+}]_{ext}$ and Ca^{2+} uptake was examined using Michaelis–Menten analysis for first-order kinetics. Values of J_{max} (the maximum uptake rate) and K_m (the $[Ca^{2+}]_{ext}$ at which uptake is 50% of J_{max}) were calculated using Eadie–Hofstee regression analysis (Michal, 1985). To generate the curve fitted to the data, the J_{max} and K_m values were substituted into the Michaelis–Menten equation:

$$J_{in}=\frac{J_{max}[Ca^{2+}]_{ext}}{K_m[Ca^{2+}]_{ext}}$$

The net flux (J_{net}) for calcium, sodium, titratable alkalinity, and acidic equivalents was calculated by:

$$J_{net}=\frac{\text{volume}}{\text{weight}}\frac{1}{\text{time}}([\text{ion}]_1-[\text{ion}]_2)$$

where $[\text{ion}]_1$ and $[\text{ion}]_2$ are the water concentrations at the start and end of the flux period respectively. Net Ca^{2+} flux rate for the chronic high Ca^{2+} exposed fish was determined from the final 2-h flux period only so as to avoid possible contamination of the experimental medium with high Ca^{2+} water carried over by the fish.

STATISTICAL ANALYSES

All values are presented as mean ± 1 S.E. Multiple comparisons among treatment groups were performed by analysis of variance (ANOVA) followed by Fisher's test of least significant difference (LSD) in cases where the *F*-value indicated significance ($P \leq 0{\cdot}05$).

RESULTS

CALCIUM UPTAKE KINETICS

The rate of Ca^{2+} influx exhibited a hyperbolic relationship over the range of external $[Ca^{2+}]$ tested indicating that transport became saturated (Fig. 1). The

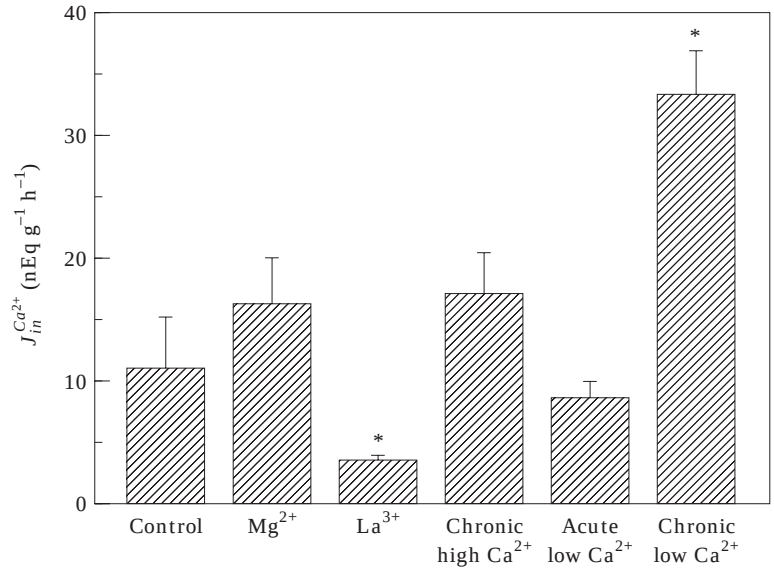

FIG. 2. The influence of external Mg^{2+} and La^{3+}, chronic high Ca^{2+} exposure, acute low Ca^{2+} and chronic low Ca^{2+} exposure on calcium uptake (J^{Ca}_{in}). Both Mg^{2+} and La^{3+} levels were set at 100 µmol l^{-1}. Low calcium concentration = 50 µEq l^{-1}; high calcium concentration = 20 000 µEq l^{-1}. See text for additional details. n = 8 for each of Mg^{2+}, La^{3+}, chronic high Ca^{2+}, chronic low Ca^{2+}, and acute low Ca^{2+} treatments; n = 7 for control. Means ± S.E. *Significant difference from control value ($P \leq 0{\cdot}05$).

data were described satisfactorily by the Michaelis–Menten relationship. The maximum uptake rate (J_{max}) and affinity constant (K_m) were 31 ± 4 nEq g^{-1} h^{-1} and 125 ± 36 µEq l^{-1} respectively.

La^{3+}, Mg^{2+}, HIGH AND LOW Ca^{2+} EXPOSURE

Under control conditions, J^{Ca}_{in} was 11 ± 4 nEq g^{-1} h^{-1}. The addition of 100 µmol l^{-1} La^{3+} to the freshwater medium inhibited Ca^{2+} uptake by about 67%, while the same concentration of Mg^{2+} had no effect (Fig. 2). Chronic exposure to low Ca^{2+} (50 µEq l^{-1}) produced a threefold elevation of J^{Ca}_{in} relative to fish transferred acutely to this same medium (Fig. 2); both groups were tested in this low Ca^{2+} medium. Notably, this elevated rate was significantly higher than even that of the fish tested in the control medium (200 µEq l^{-1}). The rate in fish exposed acutely to low Ca^{2+} was only slightly lower (non-significant) than the control treatment and the chronic high Ca^{2+} group exhibited no change in J^{Ca}_{in}.

Although J^{Ca}_{net} did not differ significantly from zero in both the La^{3+} and Mg^{2+} groups, the fish were no longer in positive Ca^{2+} balance (Table I). The chronic high Ca^{2+} group, tested in normal Ca^{2+} media, showed a significant net Ca^{2+} loss as did the chronic low Ca^{2+} group tested in low Ca^{2+} media. While La^{3+} exposure did not reduce J^{Ca}_{net} significantly, it did affect Na^+ balance negatively (Table 1). Chronic exposure to low Ca^{2+} had a similar effect on J^{Na}_{net}, but none of the other treatments disrupted Ca^{2+} or Na^+ balance. None of these treatments altered ammonia excretion significantly or induced a disturbance of net acid-base flux (Table I).

TABLE I. Net Ca^{2+}, Na^{+}, titratable alkalinity (TA), ammonia (Amm), and acidic equivalent (H^{+}) fluxes (nEq g^{-1} h^{-1}) measured during various exposures

	J^{Ca2+}_{net}	J^{Na+}_{net}	J^{TA}	J^{Amm}		J^{H+}
Control	40·5 ± 6·6 (6)	159·8 ± 82·3 (7)	694·7 ± 67·3 (5)	638·8 ± 67·2		55·8 ± 112·8
$MgSO_4$	2·6 ± 29·0 (8)	34·8 ± 97·4 (8)	947·9 ± 98·0 (5)	867·7 ± 133·1		142·6 ± 142·8
$LaCl_3$	1·2 ± 18·6 (8)	− 185·9 ± 74·7*	—	446·7 ± 60·9	(8)	—
Chronic high Ca^{2+}	− 77·7 ± 40·9 (8)*	67·8 ± 54·9 (8)	480·7 ± 66·4 (8)	620·9 ± 46·8		− 137·5 ± 57·7
Chronic low Ca^{2+}	− 20·5 ± 6·1 (8)	− 186·6 ± 66·9 (8)*	788·3 ± 103·9 (8)	640·0 ± 101·7		186·5 ± 115·6

Means ± S.E. (n). n for J^{TA} is the same for J^{Amm} and J^{H+}.
*Significant difference from control ($P \leq 0{\cdot}05$).

DISCUSSION

Although the pattern of Na^+ *v.* Cl^- uptake is most unusual in the freshwater-adapted mummichog (Patrick *et al.*, 1993), the present study indicates that the pattern of Ca^{2+} uptake is similar to that of other freshwater fish in that: (i) it is a saturable process conforming to standard Michaelis–Menten kinetics; (ii) uptake can be blocked by a known calcium channel blocker, La^{3+}; and (iii) it can be stimulated by long-term, low Ca^{2+} exposure. A recent *in vitro* study using opercular epithelial preparations (rich in chloride cells) of *F. heteroclitus* (Marshall *et al.*, 1995) reported results in agreement with (i) and (ii). Thus despite the absence of detectable Cl^- uptake from the water, Ca^{2+} uptake proceeds in a manner characteristic of chloride cell function in other freshwater teleosts (Flik *et al.*, 1993, 1995).

The present hyperbolic relationship between J^{Ca}_{in} and external Ca^{2+} (Fig. 1) is typical of the saturable, carrier-mediated process seen in most freshwater fish (Hogstrand *et al.*, 1994; Flik *et al.*, 1993, 1995). The K_m and J_{max} values estimated for *F. heteroclitus* indicate a transport system that has a relatively high affinity and a transport capacity that is comparable to that reported for rainbow trout *Oncorhynchus mykiss* (Walbaum) (Table II). In contrast, Mayer-Gostan *et al.* (1983) found a linear, non-saturable relationship in mummichogs held at a Ca^{2+} level of 3000 µEq l^{-1}, and a curious biphasic relationship in mummichogs acclimated to the same Ca^{2+} level as in the present study (i.e. 200 µEq l^{-1}). Furthermore, Mayer-Gostan *et al.* (1983) reported Ca^{2+} uptake rates of between 80 and 100 nEq g^{-1} h^{-1} in the two groups of fish at 3000 and 200 µEq l^{-1} Ca^{2+}, in contrast to present values of 11–19 nEq g^{-1} h^{-1} at the latter concentration. Pang *et al.* (1980) reported a rate of 65 nEq g^{-1} h^{-1} for *F. heteroclitus* held at 1300 µEq l^{-1} Ca^{2+}. The reason(s) for these differences are unknown, but variations in holding conditions, stocks and methodology could all contribute. Another factor could be cycles of regulating hormones such as stanniocalcin which are thought to be associated with fivefold variations of Ca^{2+} uptake in juvenile rainbow trout occurring over several weeks (Wagner *et al.*, 1993; Hogstrand *et al.*, 1994, 1995). In the present study, the potential for this effect was minimized by performing the various experiments (i.e. kinetics, blockers and low or high Ca^{2+} exposure) more or less simultaneously.

Marshall *et al.* (1995) examined Ca^{2+} transport *in vitro* in opercular epithelia of *F. heteroclitus* which were from the same stock and held under similar conditions as in the present study. The higher *in vitro* K_m relative to the present *in vivo* data (Table II) may represent the difference between the K_m of the apical membrane events (*in vivo* data) *v.* the transcellular events of apical and basolateral events in series (*in vitro* data). Alternatively, it may reflect a difference between transport sites on the opercular epithelium alone and those on the gills. A reasonable agreement was found between the present whole body estimate of J_{max} and our calculation of J_{max} using the *in vitro* value (Table II) suggesting that most or all of Ca^{2+} uptake *in vivo* could occur through the chloride cells of the opercular epithelia rather than those of the gills in freshwater *F. heteroclitus*.

The pronounced inhibition of Ca^{2+} uptake by external La^{3+} *in vivo* (Fig. 2) is in agreement with *in vitro* results on mummichog opercular membrane (Marshall

TABLE II. The acclimation calcium concentration, affinity constant, K_m and maximum transport rate, J_{max} for calcium uptake measured in freshwater-adapted mummichog *Fundulus heteroclitus*; a comparison with *in vitro* measurements on the opercular epithelium in this species and *in vivo* measurements on rainbow trout *Oncorhynchus mykiss*

	$[Ca^{2+}]$ (μEq l^{-1})	K_m (μEq l^{-1})	J_{max} (nEq g^{-1} h^{-1})
Mummichog (*in vivo*)*	200	125 ± 36	31 ± 4
Mummichog (*in vitro*)†	200	~696	~75‡
Rainbow trout (21 g)§	2000	184 ± 16	106 ± 5
Rainbow trout (244 g)¶	2000	280 ± 70	12 ± 2
Rainbow trout¶	50	230 ± 10	66 ± 13

Means ± S.E.
*Present study (n=7 groups of 8 fish).
†Marshall *et al.* (1995); (n=8–12 opercular epithelia).
‡Estimate based on J_{max}=188 nEq cm^{-2} h^{-1} and assuming a 2-cm^2 total area (two opercular epithelia) for an average fish weighing 5 g.
§Hogstrand *et al.* (1994) (n=6 groups of 8 fish).
¶Perry & Wood (1985) (n=7 for 2000 μEq l^{-1} acclimated, n=6 for 50 μEq l^{-1} acclimated for 7 days).

et al., 1995), and both *in vivo* (Perry & Flik, 1988) and perfused head studies on rainbow trout (Verbost *et al.*, 1987, 1989). Lanthanum is thought to block apical Ca^{2+} channels on the chloride cells (e.g. Perry & Flik, 1988). While Mg^{2+} can inactivate Ca^{2+} channels in other systems (e.g. Hartzell & White, 1989), it was ineffective on intact *F. heteroclitus* (Fig. 2), and only weakly effective at a 10-fold higher concentration on Ca^{2+} uptake across the opercular membrane *in vitro* (Marshall *et al.*, 1995). The only evidence that Mg^{2+} influences Ca^{2+} uptake in fish *in vivo* is a report that high water Mg^{2+} levels cause hypocalcaemia in tilapia *Sarotherodon mossambicus* (Peters) (Wendelaar Bonga *et al.*, 1983).

The threefold stimulation of J^{Ca}_{in} caused by long-term exposure to low Ca^{2+} water was in agreement (at least on a qualitative basis) with the earlier results of Mayer-Gostan *et al.* (1983) on freshwater mummichog, as well as studies on freshwater rainbow trout *in vivo* (Perry & Wood, 1985), and freshwater tilapia both *in vivo* (Flik *et al.*, 1986) and *in vitro* (McCormick *et al.*, 1992). Perry & Wood (1985) reported that upregulation of Ca^{2+} transport in trout held at low Ca^{2+} occurs by increasing the number of transport sites (i.e. increased J_{max}) rather than altering affinity (K_m) (see Table II for values), and there is now general accord that chloride cell proliferation is an important element of this response (Flik *et al.*, 1995). However following long-term exposure of mummichogs to high Ca^{2+} there was no reciprocal downregulation of J^{Ca}_{in} (Fig. 2), suggesting that the transport capacity of the uptake system was already at a low level under control conditions.

Branchial permeability to ions was apparently compromised by chronic high and low Ca^{2+} exposures and the La^{3+} treatment. The chronic low Ca^{2+} group were in negative Na^+ and Ca^{2+} balance (Table I) despite a significant elevation in the uptake of the latter cation (Fig. 2). The high Ca^{2+} exposed fish, which were tested in a lower Ca^{2+} water, maintained J^{Ca}_{in} (Fig. 2) yet experienced a net Ca^{2+} loss (Table I) suggesting that efflux had increased. Dharamba & Maetz

(1972) suggested that during low Ca^{2+} exposure, elevated branchial ion loss occurs through the paracellular pathways. Lanthanum exposure resulted in a negative J^{Na}_{net} (Table I) which agrees with earlier studies that reported increased paracellular permeability to ions with La^{3+} treatment (Eddy & Bath, 1979; Freda & McDonald, 1988). Net ammonia and acid excretion were unaffected by those agents which altered J^{Ca}_{in} and/or net Ca^{2+} and Na^{+} balance. At present, current models do not link Ca^{2+} uptake directly to acid-base balance, though there have been no previous direct tests on this topic to our knowledge.

Finally, based on the present measurements and the calculations in Table II, much or all of Ca^{2+} uptake could occur via chloride cells on the opercular epithelia (Marshall *et al.*, 1995), cells which appear to be at best only weakly active in Cl^{-} uptake (Wood & Marshall, 1994). This raises the possibility that opercular chloride cells are retained in fresh water primarily for the purposes of calcium regulation, and that gill chloride cells in freshwater mummichogs are essentially non-functional. Clearly, these ideas are speculative, and future experiments in which rates of Ca^{2+}, Cl^{-}, and Na^{+} uptake are partitioned between gills and opercular epithelia should help prove or disprove them.

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