J. exp. Biol. 136, 243–258 (1988)
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PHYSIOLOGICAL CORRELATES OF INTERSPECIFIC VARIATION IN ACID TOLERANCE IN FISH

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Accepted 17 November 1987

Summary

This study investigated ion regulation in relation to water pH in three species of fish of differing tolerance to low pH (common shiners, *Notropis cornutus*, most sensitive; rainbow trout, *Salmo gairdneri*, intermediate; yellow perch, *Perca flavescens*, least sensitive). Increasing sensitivity to exposure to low pH was characterized by shorter survival times, greater losses of whole-body ions, more complete inhibition of Na^+ uptake, and greater stimulation of Na^+ efflux, the latter being the most important factor in determining survival. Interspecific variations in acid tolerance were also correlated with Na^+ transport characteristics at circumneutral pH; K_m was directly correlated and V_{max} inversely correlated with acid tolerance. In addition, there were large qualitative differences among the species in the Ca^{2+}-dependence of Na^+ efflux. Sodium efflux induced by low pH was markedly Ca^{2+}-dependent in both trout and shiners in a manner consistent with a simple competition between Ca^{2+} and H^+ for gill binding sites. The increased sensitivity of shiners relative to trout was related to lowered Ca^{2+}-binding activity. In contrast, Na^+ efflux in perch was virtually unaffected by water $[Ca^{2+}]$. Similarly, La^{3+} (a Ca^{2+} antagonist) stimulated higher Na^+ losses from shiners than from trout, but had little effect upon perch. Ionic losses produced by saturating La^{3+} concentrations were generally lower than those produced by H^+, suggesting that Ca^{2+} displacement is not the only mechanism for increased gill permeability at low pH. Nonetheless, the results obtained are consistent with the notion that acid tolerance may be related to Ca^{2+}-binding activity in some species (e.g. trout and shiners) although not in others (e.g. perch).

Introduction

It has long been recognized that dystrophic, acidic (pH 4·0–5·0) lakes possess small, characteristic assemblages of fish species (Jewell & Brown, 1924). Perch (*Perca flavescens*) and mudminnows (*Umbra* sp.) are generally abundant in these

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Key words: low pH, acid tolerance, sodium balance, calcium binding.

waters, whereas cyprinid minnows tend to be absent (Rahel & Magnuson, 1983; Frenette, Richard & Moreau, 1986). The banded sunfish (*Enneacanthus obesus*), another species endemic to naturally acidic waters, has been captured in ponds with pH levels as low as 3·7 (Graham & Hastings, 1984). In stark contrast, populations of salmonid and cyprinid fish disappear in lakes impacted by acidic precipitation (pH < 5·0) (Pauwels & Haines, 1986; Smith, Underwood & Ogden, 1986). Laboratory and field toxicity testing has demonstrated that the lack of a particular species in acidic surface waters is due to interspecific variation in the sensitivity of embryos, larvae and adults to acid stress. Perch, mudminnows, banded sunfish and certain Amazonian fish, all typical residents of acidic lakes and rivers, can survive prolonged exposure to pH levels less than 4·0 (Dunson, Swarts & Silvestri, 1977; Rask, 1984; Dederen, Leuven, Wendelaar Bonga & Oyen, 1986; Gonzalez & Dunson, 1987), whereas salmonids and cyprinids cannot generally survive below pH 4·5.

The toxicity of acidic water arises mainly from the disruption of ionic regulation at the gill. Active uptake of Na^+ and Cl^- is inhibited and the passive loss of these ions is stimulated (see review by McDonald, 1983*a*). During acute, lethal exposure, inhibition of Na^+ and Cl^- influx is quantitatively insignificant compared with their massive outward leakage. Plasma concentrations of Na^+ and Cl^- may be reduced by 50%, leading to fluid compartment disturbances, haemoconcentration, circulatory collapse and ultimately death (Milligan & Wood, 1982). Ion losses are thought to result from the leaching of Ca^{2+} bound to the surface of the branchial epithelium (McDonald, 1983*a*), because Ca^{2+} has a powerful influence on the permeability of biological membranes (Gordon & Sauerheber, 1982; Levine & Williams, 1982). During chronic, sublethal exposure, inhibition of Na^+ and Cl^- uptake assumes a more prominent role in reducing plasma ion concentrations for two reasons. First, the initial uptake blockade can occur at a higher pH than the acceleration of efflux (McDonald, 1983*a*; Freda & Dunson, 1984). Second, after prolonged exposure to acidic water, efflux may be reduced below control levels to compensate for net losses of ions, whereas influx only partially recovers (C. Audet, R. S. Munger & C. M. Wood, in preparation).

In view of the above, it seems likely that interspecific differences in acid tolerance are related to intrinsic differences in the properties of the ionoregulatory machinery. Thus the main objective of this study was to investigate specific aspects of ion regulation in fish species varying in acid tolerance. The species chosen (common shiners, *Notropis cornutus*; rainbow trout, *Salmo gairdneri*; yellow perch, *Perca flavescens*) represent a wide range in acid tolerance and in each species the following was investigated: (1) Na^+ exchange (i.e. influx and efflux) at circumneutral and low pH (3·0–4·0); (2) Na^+ influx kinetics at circumneutral pH; and (3) the interaction between branchial binding of Ca^{2+} and Na^+ efflux. The latter was accomplished by measuring Na^+ loss in relation to external Ca^{2+} concentration at low pH, and in relation to external La^{3+} concentration at circumneutral pH. Lanthanum is a specific Ca^{2+} antagonist which replaces Ca^{2+} at membrane binding sites, but does not assume any Ca^{2+}-related functions.

Materials and methods

Experimental animals

Three species of fish were chosen for experimentation on the basis of their sensitivity to acidic water. The acid-tolerant yellow perch (Percidae: *P. flavescens* Mitchill) and the common shiner (Cyprinidae: *Notropis cornutus* Mitchill) represent opposite extremes in sensitivity. Rainbow trout (Salmonidae: *Salmo gairdneri* Richardson) are intermediate in sensitivity and would also act as an internal control since much has been reported about their ion regulation at low pH (McDonald, 1983*a*). Experiments were carried out during the winter and autumn of 1986. Fish were obtained from different sources for the two experimental periods and will be referred to as 'winter' and 'autumn' fish, respectively. Winter trout were obtained from Goosen's trout hatchery (Ottersville, Ontario), and winter perch and shiners from a local bait dealer. Autumn trout were obtained from Spring Valley trout farm (Petersberg, Ontario) and autumn perch were from Clear Waters Farms hatchery (Pickering, Ontario). Autumn shiners were captured from a local pond. All fish were reared in or captured in circumneutral (pH 7·0–8·0), hard water (Ca^{2+}: 1–1·5 mequiv l^{-1}). The trout and perch were juveniles, and shiners were adults: all fish ranged from 5 to 15 g. Once returned to the laboratory, fish were kept in artificial soft water (ASW, Table 1). All fish received at least a 2-week exposure to soft water before experimentation and, in each experiment, each species had spent similar periods in soft water (±3 days). ASW was made by dilution (1:40) of dechlorinated tap water (Table 1) with ion-free water obtained from an ion-exchange column. Sodium chloride was also added to the water (85 mequiv l^{-1}). Ion concentrations were never a strict (1:40) dilution of tap water because of minor contamination from extraneous sources (e.g. KCl from pH electrodes etc.). Temperature of holding and experimental water was maintained at 15°C and ambient lighting was used. Animals were fed commercial fish food every other day and fasted the day before experiments.

Analytical techniques

Body ions

Instrumental neutron activation analysis was used to determine the body concentrations (expressed per kg wet mass) of Na^+, K^+, Ca^{2+}, Mg^{2+} and Cl^-. Fish were removed from holding tanks or experimental chambers, blotted dry, and dried to a constant mass at 95°C. The difference in mass (0·001 g accuracy) before

Table 1. *Chemical composition of water used for holding of fish and experimentation*

		Ion concentration (mequiv l^{-1})				
Water	pH	Na^+	K^+	Ca^{2+}	Mg^{2+}	Cl^-
Tap	7·5	0·68	0·03	2·30	0·62	0·10
ASW	6·5	0·10–0·12	0·02	0·06–0·07	0·02–0·04	0·12–0·14

and after drying was used to determine water content. Samples were irradiated with thermal neutrons at the McMaster University Nuclear Reactor. After a 1-min delay, samples were counted for 10 min with a hyperpure germanium detector coupled to a Canberra multichannel analyser (series 40 or 90). The peaks for each ion and their energies (keV) were ^{24}Na (1368·4), ^{42}K (1524·6), ^{49}Ca (3084·4), ^{27}Mg (1014·5) and ^{38}Cl (1647·2). Concentrations were calculated by comparison with known standards (NBS citrus leaf standard no. 1572 and NRC marine fish tissue reference standard) and using routine equations (Desote, Gijbels & Hoste, 1972). A more detailed description of this technique can be found in C. M. Wood, D. G. McDonald, C. G. Ingersoll, D. R. Mount, O. E. Johansson, S. Landsburger & H. L. Bergman (in preparation).

Flux measurements

The exchange of Na^+ between fish and water was measured in darkened 300 ml polyethylene containers. Each container had an overflow port (on one side) and three holes in the lid for an aeration line (PE 50 tubing), water supply line (gravity fed, 100 ml min^{-1}) and a sampling port for the removal of water or addition of ions or isotopes. Up to 30 containers could be used simultaneously, receiving water from the same head tank. The pH of the head tank was continuously monitored with a Markson model 88 pH meter and Cole Palmer combination electrode. The pH was adjusted with dilute H_2SO_4. Fish were kept in the containers for 24 h prior to experimentation. At the start of an experiment, the input tube was removed and the overflow port plugged. Net flux of Na^+ was determined by measuring the change in Na^+ concentration of the water. The concentrations of Na^+ and other cations were determined by atomic absorption spectroscopy (Varian model 1275). Sodium influx was determined by one of two methods depending on the particular experiment. In method 1, ^{24}Na (12 kBq) was added to the container and 5 ml bath samples were collected at the beginning and end of the experiment. The disappearance of ^{24}Na was used to calculate Na^+ influx according to the equation:

$$J_{in} = (\ln Q^*_{out0} - \ln Q^*_{out1})Q_{out}/TM,$$

where Q^*_{out0} and Q^*_{out1} are the total counts min^{-1} in the flux chambers at the beginning and end of the flux period, respectively. Q_{out} is the average Na^+ content of the bath, T is the time (h), and M is the wet mass of the fish (kg). The specific activity within fish never exceeded more than 1 % of that of the bath so back flux corrections were not needed. In method 2, fish were removed from the containers at the end of the experiment, rinsed for 30 s in ASW, and directly assayed for ^{24}Na activity. Fish were laid flat in a polyethylene container and placed on top of a NaI detector connected to a scaler/timer and counted for 1 min. Samples of bath water (5 ml) were placed in small vials, approximating the length and thickness of fish, and counted in such a way that counting geometry would be similar. Efflux was calculated as the difference between net flux and influx. All measurement periods were 1 h.

Experimental series

The influence of water chemistry on body ions and water

Body ion concentrations were measured in winter fish maintained in tap water, ASW (2 weeks) and pH 4·0 ASW. The last treatment was lethal to trout and shiners but not to perch. Consequently, the latter were also exposed to pH 3·5 ASW so that body ion concentrations at death could be measured.

Na^+ exchange at pH 4·0

Sodium exchanges were monitored simultaneously in five individuals of each species (winter fish) exposed to pH 4·0 over a 48 h period (0, 1, 3, 6, 24, 48 h). Time zero measurements were controls at pH 6·5. After this first measurement period, containers were flushed with water for 1 h and the head tank was then acidified to pH 4·0. It took approximately 10 min for the measurement containers to reach the target pH values, and measurements commenced 50 min later. Water pH drifted upwards by less than 0·05 units during measurement periods. Between each measurement period, containers received a continuous flow of pH 4·0 ASW.

Na^+ uptake kinetics at pH 6·5

The kinetics of Na^+ uptake in the three species (autumn fish) were determined by measuring Na^+ influx (method 2) over a range of environmental Na^+ concentrations (0·025–0·400 mequiv l^{-1}). Five to 10 different fish of each species were exposed to one of 6–8 different concentrations. To adjust Na^+ concentration, varying amounts of a labelled NaCl stock solution (12 kBq ^{24}Na per 100 μl of 1 mol l^{-1} stock) was added to each container.

Na^+ loss in water with elevated Ca^{2+} or La^{3+}

Sodium exchange was measured in autumn fish over a range of pH (3·0–4·0) and environmental Ca^{2+} concentration. Different individuals of each species were exposed to each pH and concentration of Ca^{2+}. Influx was estimated using method 1. Calcium concentration was manipulated by the addition of varying amounts of a $CaCl_2$ stock solution. In a similar series of experiments, autumn fish were exposed to a range of $LaCl_3$ concentrations at pH 6·5. In addition, a separate group of fish was exposed to a range of La^{3+} concentrations using radiolabelled ^{140}La (0·16 kBq mequiv $^{-1}$). After 15 min, fish were rinsed for 1 min in ASW and their gills were excised and assayed for ^{140}La activity (Nuclear Chicago model 1085 gamma counter).

Statistical analysis

All data are expressed as means ± 1 S.E.M. K_m and V_{max} of Na^+ uptake were determined with an SAS non-linear regression package (SAS, 1982) using the equation:

$$Na^+ \text{ influx} = (V_{max} \times [Na^+]/(K_m + [Na^+]) .$$

This same equation was used for non-linear regression of the effects of Ca^{2+} and La^{3+} concentration. Comparisons of two means were made with two-sample *t*-tests ($P < 0.05$).

Results

Species comparisons at circumneutral pH

At circumneutral pH, the concentrations of body ions and water were generally similar among all three species, except for Ca^{2+} which was substantially lower in trout (Table 2). In all three species, acclimation to ASW from tap water did not generally affect the concentrations of body ions or water. Furthermore, at pH 6·5 and 0·1 mequiv l^{-1} Na^+ (acclimation Na^+ concentration), all three species were in sodium balance (i.e. influx = efflux) and neither influx nor efflux values were significantly different among the three species (Figs 1, 2).

Na^+ exchange kinetics

Sodium influx of the three species increased as a function of environmental Na^+ concentration showing apparent Michaelis–Menton saturation kinetics (Fig. 1). This meant that transport activity could be expressed in terms of affinity (K_m) and transport maximum (V_{max}; Table 3). Perch had a significantly higher affinity (lower

Table 2. *Body ion and water concentrations of three species of fish exposed to different water chemistries*

Species / Treatment	*N*	Na^+	K^+	Ca^{2+}	Mg^{2+}	Cl^-	(H_2O)
		(mequiv kg wet mass^{-1})					(kg kg dry mass^{-1})
Shiner							
Tap	11	48 ± 2	59 ± 2*	476 ± 19	28 ± 8	33 ± 2	3·9 ± 0·2
ASW	4	51 ± 2	66 ± 1	502 ± 25	30 ± 2	38 ± 2	3·6 ± 0·1
pH 4·0 ASW	8	24 ± 2*	52 ± 6	454 ± 42	28 ± 3	14 ± 1*	4·4 ± 0·1*
Trout							
Tap	10	45 ± 1	83 ± 3	182 ± 6*	22 ± 2	41 ± 1	3·3 ± 0·1
ASW	10	43 ± 1	82 ± 1	222 ± 4	20 ± 2	42 ± 1	3·6 ± 0·1
pH 4·0 ASW	9	22 ± 1*	78 ± 2	222 ± 5	24 ± 1	18 ± 1*	3·9 ± 0·1*
Perch							
Tap	10	55 ± 2	68 ± 2*	540 ± 15	30 ± 1	42 ± 1	3·9 ± 0·1
ASW	10	58 ± 3	50 ± 4	602 ± 34	28 ± 1	45 ± 3	4·3 ± 0·1
pH 4·0 ASW	9	49 ± 2*	63 ± 2	602 ± 13	28 ± 1	39 ± 2	4·2 ± 0·1
pH 3·5 ASW	7	24 ± 1*	48 ± 2	544 ± 9	26 ± 1	13 ± 1*	5·0 ± 0·2*

All values are means ± S.E.M. (*N* = sample size).

Values marked with an asterisk are significantly different from ASW value (within a species ($P < 0.05$).

Values for trout and shiner at pH 4·0 and perch at pH 3·5 are for dead animals. Values for perch at pH 4·0 are for live animals after 7·7 h of exposure.

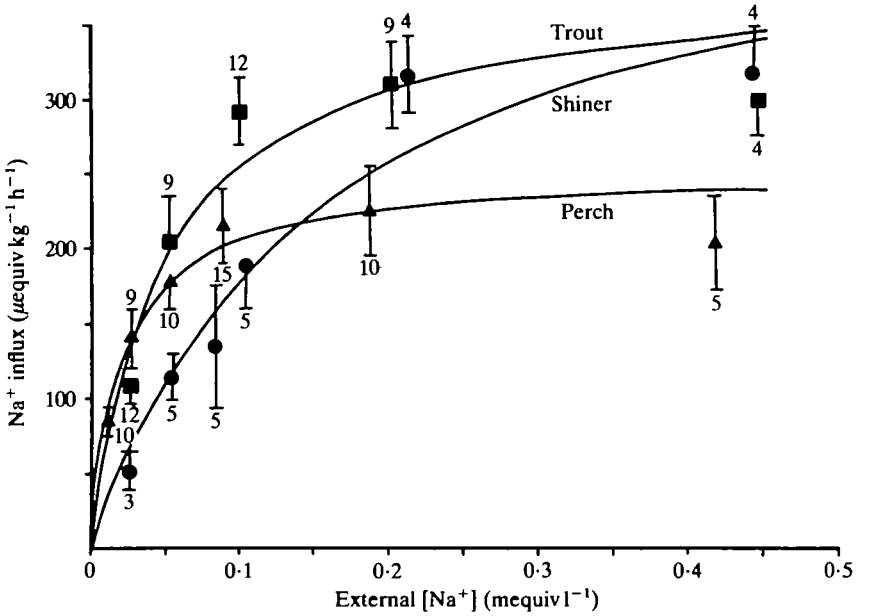

Fig. 1. Sodium influx over a range of environmental sodium concentration in three species of fish. All values are means ± S.E.M. (sample size indicated). Lines were drawn from the output of a non-linear regression program. Values for K_m and V_{max} for each line are listed in Table 3.

K_m) and lower transport maximum (V_{max}) for Na^+ than shiners. K_m and V_{max} for trout were intermediate, but not statistically different from either perch or shiners.

Interspecific variability in acid tolerance

The three species of fish displayed a wide range of acid tolerance. Shiners were the most sensitive and died after 4·6 ± 0·5 h ($N = 8$) of exposure to pH 4·0 ASW. Trout were intermediate in acid tolerance and lived for 6·5 ± 0·3 h ($N = 9$). Perch were extraordinarily tolerant and lived at pH 4·0 until the termination of the experiment (73 h, $N = 7$).

Table 3. *Sodium exchange kinetics of three species of fish*

Species	N	K_m (mequiv l^{-1})	V_{max} (μequiv kg^{-1} h^{-1})
Shiner	25	0·158 ± 0·054	460 ± 74
Trout	50	0·048 ± 0·013	379 ± 35
Perch	58	0·021 ± 0·008	249 ± 24

Values were calculated by non-linear regression of data illustrated in Fig. 1.

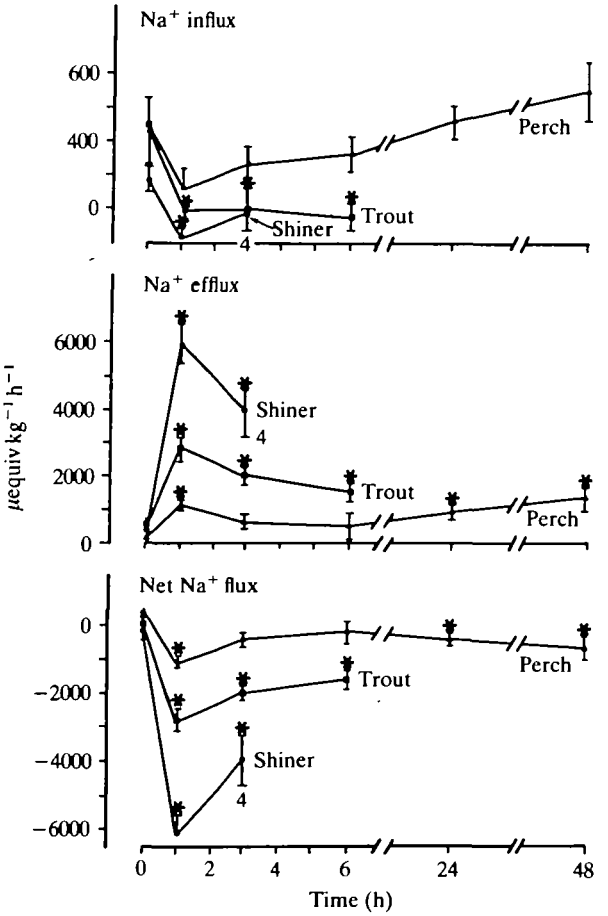

Fig. 2. Sodium influx, efflux and net flux of three species of fish exposed to pH 4·0. The pH at 0 h was 6·5. All values are means ± S.E.M. ($N = 5$ except where indicated). An asterisk indicates a significant difference ($P < 0{\cdot}05$) from the value at 0 h (within a species).

Sodium balance at low pH

After 1 h of exposure to pH 4·0, Na^+ influx in both shiners and trout was completely inhibited and was not significantly different from zero for the remainder of the experiment (Fig. 2). In contrast, perch showed an initial small decline in Na^+ influx, followed by an increase above control level at 24–48 h. However, none of these changes was significantly different from control measurements.

Sodium efflux followed a similar pattern in the three species (Fig. 2). Shiners experienced a 20-fold increase in Na^+ loss and trout a sixfold increase after 1 h of exposure to pH 4·0. Sodium efflux in both species declined slightly after 3 and 6 h of exposure but was still much higher than control levels. Again perch were the least affected. Efflux was significantly increased only during the 1, 24 and 48 h flux

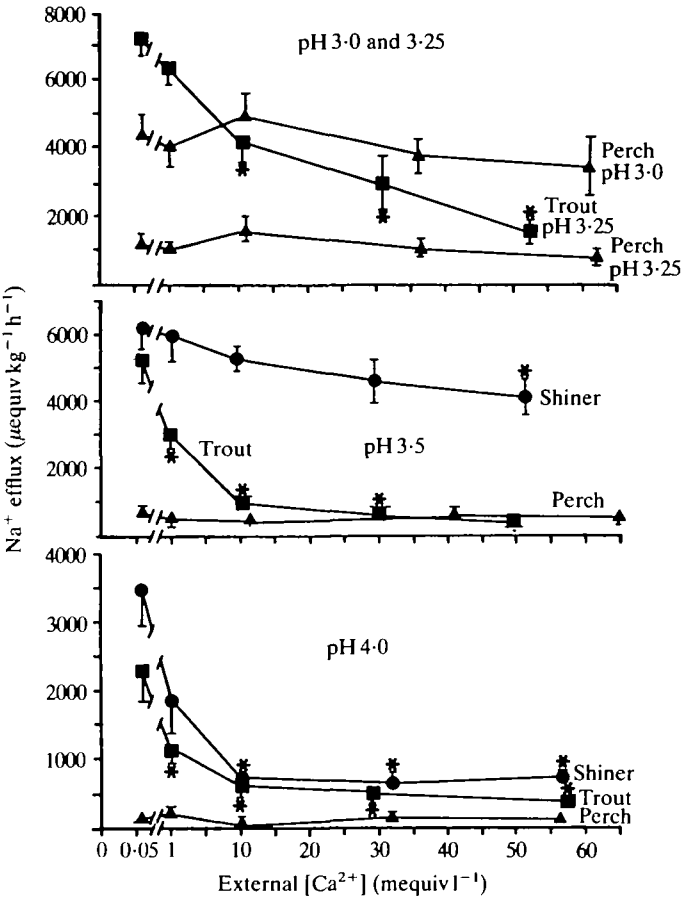

Fig. 3. The influence of external calcium concentration on sodium efflux at low pH (3·0–4·0). Values are means ± S.E.M. ($N = 5$). An asterisk indicates a significant difference ($P < 0{\cdot}05$) from the value at the control calcium concentration (0·07 mequiv l^{-1}) within a species.

periods and was only 1/3–1/6 of levels in trout and shiners. Sodium loss in perch was not significantly higher than control levels during the third and sixth hour of exposure. The effects on net flux mirrored the changes in efflux because the stimulation of efflux was quantitatively much greater than the inhibition of influx. Even at extremely low pH values (3·0–3·5) these relative differences in disruption of sodium balance remained qualitatively the same (Fig. 3). The pH had to be reduced to 3·0 before perch experienced Na^+ losses similar to those of trout and shiners at pH 4·0. It should be noted that autumn-collected fish (Fig. 3) were much less sensitive to low pH than winter-collected fish (Fig. 2) in terms of disruption of Na^+ balance or mortality at pH 4·0, but the relative differences among species remained the same.

Body ion and water concentrations

At death, significant amounts of Na^+ and Cl^- had been lost in trout (49 % and 57 %, respectively, of initial levels at 6·5 h) and shiners (53 % and 63 %, respectively, of initial levels at 4·6 h; Table 2). In perch, small but significant reductions in Na^+ concentration occurred after 7·7 h of exposure to pH 4·0. After 73 h no perch had died so the pH was lowered to 3·5, and death ensued after 25 ± 13 h ($N = 7$). At death, perch had lost 58 % and 71 % of initial body Na^+ and Cl^-, respectively. All three species also had significantly greater body water concentrations at death. In all species, the concentrations of K^+, Ca^{2+} and Mg^{2+} were unaffected by water of low pH.

Effects of calcium

The influence of Ca^{2+} on Na^+ efflux at low pH is illustrated in Fig. 3. At pH 4·0 and the lowest [Ca^{2+}] tested (0·07 mequiv l^{-1}), both shiners and trout experienced massive losses of Na^+, whereas perch were unaffected. Increasing [Ca^{2+}] to 1 mequiv l^{-1} reduced efflux by about one-half in trout and shiners, but had no effect on perch at pH 4·0 or at the lower pH values tested (3·0, 3·25, 3·5). Additional increments of [Ca^{2+}] to $\geq$10 mequiv l^{-1} further reduced Na^+ efflux in trout and shiners to levels only slightly above those observed in pH 6·5 ASW (Fig. 2). At pH 3·5, Ca^{2+} had an ameliorating effect on trout similar to that at pH 4·0, but was much less effective in shiners. At concentrations greater than 10 mequiv l^{-1} Ca^{2+}, efflux in trout levelled off near control rates, whereas efflux from shiners was still quite large. Most shiners died after 45–60 min of exposure to pH 3·5, but no trout or perch died during the 1 h flux period. At pH 3·25, Ca^{2+} was still able to reduce efflux in trout, but was much less effective at lower concentrations.

Using non-linear regression ([Ca^{2+}] *vs* reduction in efflux below efflux at control [Ca^{2+}]) we found that efflux reduction was half-maximal for trout at $0{\cdot}75 \pm 0{\cdot}22$ mequiv l^{-1} at pH 4·0 and $1{\cdot}35 \pm 0{\cdot}19$ mequiv l^{-1} at pH 3·5. In shiners half-maximal reduction was at $0{\cdot}95 \pm 0{\cdot}25$ mequiv l^{-1} at pH 4·0. We did not fit lines for trout at pH 3·25, or for shiners or trout at pH 3·5, because efflux reduction had not reached a plateau.

Effects of lanthanum

Exposure to La^{3+} stimulated Na^+ loss in all three species of fish and the magnitude of this loss was correlated to their respective sensitivities to low pH (Fig. 4A). At 3 and 6 mequiv l^{-1} La^{3+}, Na^+ effluxes in the three species were significantly different from one another. At 0·75 and 1·50 mequiv l^{-1} La^{3+}, Na^+ effluxes from trout and perch were not significantly different, but both species lost significantly less Na^+ than shiners. For trout and shiners, we computed the La^{3+} concentration at which stimulation of efflux was half-maximal by using non-linear

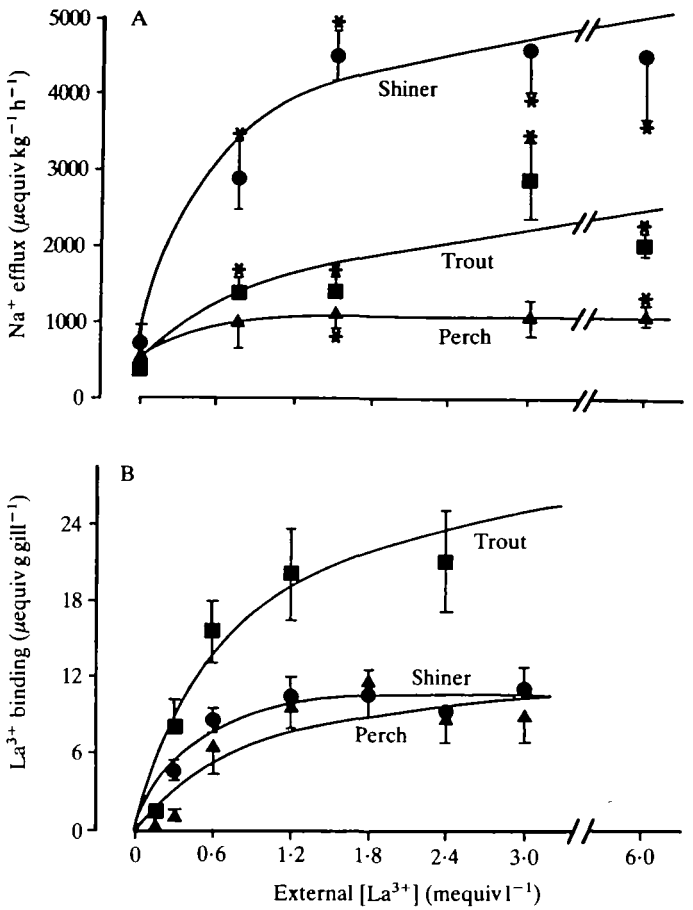

Fig. 4. The effects of external lanthanum concentration on (A) sodium efflux and (B) lanthanum binding in three species of fish. The curves illustrated in A were fitted by eye and the curves in B are the products of non-linear regression. All values are means ± S.E.M. An asterisk indicates a significant difference ($P < 0{\cdot}05$) from the value at 0 mequiv l^{-1} La^{3+} within a species (A only).

regression ([La^{3+}] *vs* stimulation of Na^+ efflux over control levels). The half-maximal values for trout and shiners were $1{\cdot}23 \pm 1{\cdot}07$ mequiv l^{-1}, $N = 20$ and $0{\cdot}53 \pm 0{\cdot}38$ mequiv l^{-1}, $N = 20$, respectively. A line could not be fitted for perch because of the small effect of La^{3+}.

The maximum level of lanthanum binding over a range of La^{3+} concentrations was not correlated to low pH tolerance in the three species (Fig. 4B). Trout bound significantly more La^{3+} than either perch or shiners. Binding of La^{3+} by perch and shiners was similar except at 0·301 mequiv l^{-1} La^{3+}, where perch took up significantly less ($P < 0{\cdot}05$). The K_m and V_{max} values for La^{3+} binding are listed in Table 4. While the maximum binding of La^{3+} was not correlated to tolerance to low pH, the binding affinity for La^{3+} was inversely related to acid tolerance.

Table 4. K_m *and* V_{max} *for lanthanum binding in three species of fish*

Species	N	K_m (mequiv l^{-1})	V_{max} (μequiv g gill^{-1})
Shiner	30	0·333 ± 0·159	12·0 ± 1·2
Trout	21	0·795 ± 0·438	31·5 ± 8·1
Perch	32	0·930 ± 0·588	12·0 ± 3·3

Values were calculated by non-linear regression of data illustrated in Fig. 4B.

Discussion

Ion loss versus *mortality*

This study confirms the principle that the magnitude of ion loss is the major determinant of survival time or survival itself during exposure to water of low pH (Freda & Dunson, 1984; Gonzalez & Dunson, 1987). This principle applies not only to species that are sensitive to low pH but also to those that are extremely tolerant. The variation among species is not so much in the basic mechanism of H^+ toxicity but simply in the threshold at which large ion losses occur. Hence the net losses of body ions associated with mortality in perch at pH 3·5 were very similar to the losses seen in trout and shiners at pH 4·0 (Table 2) and in all cases were within the range of 50–60 %. Indeed, in every study investigating the lethal effects of low pH, an approximately 50 % loss of body Na^+ content at death is reported. This includes such diverse groups as air-breathing fish (Krout & Dunson, 1985), the acid-tolerant banded sunfish (Gonzalez & Dunson, 1987) and even amphibians (Freda & Dunson, 1984) and invertebrates (Hollett, Berrill & Rowe, 1986). The exact reduction at death can nonetheless vary and seems to be inversely related to the rate of loss of Na^+. In any case, it appears that the key to survival in acid waters is prevention of ion losses rather than simple toleration of large reductions in body electrolytes.

Mechanism of ion loss

Previous studies in our laboratory (McDonald, 1983*b*; McDonald, Walker & Wilkes, 1983; Booth, McDonald, Simons & Wood, 1988) have shown that the gills of fish are the primary target for H^+ and that the branchial response to acid exposure consists of two phases: an initial 'shock' phase, where there is usually fairly complete inhibition of influx and large abrupt increases in ionic efflux, and a 'recovery' phase, usually seen only in surviving animals, consisting of partial to complete recovery of efflux and a slower, less complete recovery of influx. During the recovery phase, efflux reduction is thought to result from reduction in branchial permeability (C. Audet, R. S. Munger & C. M. Wood, in preparation). Aspects of both phases are seen in the present study (at pH 4·0), although the recovery phase was quite truncated and lower in trout and shiner because of their rapid and complete mortality (Fig. 2). Here, the reduction in Na^+ efflux was probably due to a reduction in the diffusional gradient resulting from the large net

loss of Na^+ rather than any reduction in branchial permeability. Consistent with the general increase in branchial permeability during the shock phase, animals may also experience an osmotic gain of water (Table 2).

Initially, the net ion losses arose largely from the increase in ionic efflux. The contribution attributable to the inhibition of influx was, initially at least, insignificant relative to the stimulation in efflux. However, at less toxic pH values, where survival would be more prolonged, the persistent inhibition of influx would become quantitatively more important to ion balance as ionic effluxes returned to more normal values. Therefore, it is apparent that acid tolerance arises from two abilities: an ability to limit the increase in branchial permeability caused by low pH, and an ability of the ion transport mechanism to resist or to recover from low pH inhibition. Since the species we have examined show clear differences in acid tolerance and also in ion transport and permeability characteristics, we are now able to offer some insights into the physiological origin of such acid tolerance.

Branchial permeability

The leakiness of fish gills is, of course, largely a function of the physical dimensions of the gills; the diffusion distance, the thickness of the outer mucus layer and the intrinsic properties of the tight junctions which seal adjacent cells. However, it has long been thought that calcium has an important role to play in regulating branchial permeability by virtue of binding to and stabilizing fixed negative charges on the apical surface of cells and in paracellular channels (Gordon & Sauerheber, 1982). Furthermore, it has been suggested that the decrease in ionic and water permeability that is seen with adaptation to low-Ca^{2+} media is mediated, at least in part, by an increased Ca^{2+}-binding activity of the gills (McDonald, 1983*a*). Specific Ca^{2+}-binding proteins have been identified in gill mucus and have been shown to increase in response to reduced $[Ca^{2+}]$ in the water (Wendelaar Bonga, 1978; Flik, Vanrijs & Wendelaar Bonga, 1984). Finally, there is the suggestion that the increase in branchial permeability caused by acid exposure arises, at least in part, from the removal of calcium from binding sites on the gills (McWilliams, 1983; McDonald, 1983*a*). Evidence for this hypothesis comes from the observations that addition of Ca^{2+} to acidic water slows Na^+ loss (McDonald, Hôbe & Wood, 1980; Booth *et al.* 1988), and Ca^{2+} chelators (EDTA; McDonald & Rogano, 1986) and Ca^{2+} antagonists (La^{3+}; Eddy & Bath, 1979) stimulate Na^+ loss. This study confirms and extends this idea by showing, at least in trout and shiners, that Na^+ efflux at low pH was markedly Ca^{2+}-dependent. Furthermore, the fact that the Ca^{2+}-dependence saturated and had a half-maximal value directly related to the H^+ concentration (Fig. 3) suggests a simple competition between Ca^{2+} and H^+ for a finite number of binding sites controlling membrane permeability. Therefore, interspecific differences in branchial Ca^{2+}-binding activity may well be part of the basis for interspecific differences in acid tolerance. Certainly the data suggest that the less tolerant shiners had a lower Ca^{2+}-binding activity than trout; half-maximal reduction of efflux required a higher calcium concentration at pH 4·0 and particularly at pH 3·5. Indeed,

McWilliams (1983) found that isolated gills of a tolerant strain of brown trout (*Salmo trutta*) lost surface-bound Ca^{2+} at a slower rate than a sensitive strain when exposed to acidic water. However, this is not a universal phenomenon as Na^+ efflux in perch was essentially Ca^{2+}-independent. Although ion losses increased in this species with declining pH (particularly between pH 3·25 and 3·0), at no pH did Ca^{2+} have a protective effect (Fig. 3). Clearly, in the perch, branchial permeability is maintained by a fundamentally different mechanism from that apparently shared by trout and shiners.

The role of Ca^{2+} in permeability control is further revealed by the branchial effects of La^{3+}. Lanthanum has an ionic radius similar to Ca^{2+}, a high affinity for Ca^{2+}-binding sites, binds irreversibly to these sites, and does not penetrate cell membranes (see review by Weiss, 1974). It thus offers two potential advantages as a probe of gill Ca^{2+} binding: it is a more specific Ca^{2+} antagonist than H^+ and a more surface-specific cation than calcium (i.e. no intracellular penetration). Indeed, we find that La^{3+} binding saturated on the gills in a manner consistent with binding to a finite number of surface sites (Fig. 4B) and stimulated, at least in trout and shiners, large increases in Na^+ efflux consistent with the removal of Ca^{2+} from the gills (Fig. 4A). The concentrations of La^{3+} needed for both half-maximal La^{3+} binding and half-maximal efflux stimulation were much lower for shiners than for trout, indicating again that Ca^{2+} was displaced more easily from shiners (Fig. 4; Table 4). Note, however, that the maximal Na^+ loss rates seen at saturating La^{3+} concentrations were much lower than those caused by low pH (pH < 4·0). If this reflects the maximum increase in permeability that can be caused by removal of surface calcium, then H^+ must increase permeability of the gills by an additional mechanism(s). The likely mechanism may be an alteration in branchial structure, similar to that seen in acid-exposed fish, i.e. epithelial sloughing, lamellar fusion and cellular destruction (Daye & Garside, 1976; Jagoe & Haines, 1983). The fact that La^{3+} had very little effect on Na^+ efflux in perch (Fig. 4A), despite binding to the gills (Fig. 4B), confirms the Ca^{2+}-independence of this species.

The maximum rates of Na^+ loss in the presence of La^{3+} also varied considerably among the three species in the same fashion as their respective acid tolerance. This indicates that, even when all surface-bound Ca^{2+} was displaced, the permeability characteristics of each species were still very different.

Ion transport

We have shown that acid tolerance is related not only to an ability to limit ionic leakiness but also to the basic characteristics of the Na^+ transport mechanism. The perch was the most tolerant and had the lowest V_{max}, i.e. the fewest ion transport sites, but these sites had the highest affinity (i.e. lowest K_m). The acid-sensitive shiners were the opposite extreme and trout were intermediate, in accord with their relative acid tolerance. A low K_m value may be related to an ability to resist low pH as perch were able to maintain Na^+ uptake at pH 4·0, while uptake in trout and shiners fell to zero. McWilliams (1982), in comparing acid-tolerant and acid-sensitive strains of the brown trout, found that the tolerant strain had a lower K_m

and suffered less inhibition of Na^+ uptake at pH 4·0. Similarly, tadpoles adapted to conditions that decreased K_m (distilled water, low pH) had the highest levels of influx when exposed to acidic water (Freda & Dunson, 1986). These observations suggest a competitive interaction between Na^+ and H^+ for the Na^+ transport site, an interaction which a high-affinity mechanism is best adapted to deal with. Although a low K_m value appears to be a common characteristic of acid tolerance, the maintenance of Na^+ influx is not necessarily a prerequisite for acid tolerance because influx remains completely inhibited at pH $\leq$ 4·0 in the banded sunfish, yet it can survive virtually indefinitely at pH 3·5 (Gonzalez & Dunson, 1987).

In summary, it appears that large qualitative and quantitative differences exist between species in terms of basic gill design, and that these differences mediate the magnitude of response to low environmental pH. We currently have studies under way exploring the morphological basis for these differences.

We thank Dr C. M. Wood and R. J. Gonzalez for useful discussions and V. Cavdek for excellent technical assistance. Financial support was provided by a strategic grant in environmental toxicology from the Natural Sciences and Engineering Research Council of Canada.

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