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Review

Sodium and chloride transport in soft water and hard water acclimated zebrafish (*Danio rerio*)

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Abstract

While the zebrafish is commonly used for studies of developmental biology and toxicology, very little is known about their osmoregulatory physiology. The present investigation of Na^+ and Cl^- transport revealed that the zebrafish is able to tolerate extremely low ambient ion concentrations and that this is achieved at least in part by a greatly enhanced apparent uptake capacity and affinity for both ions. Zebrafish maintain plasma and whole body electrolyte concentrations similar to most other freshwater teleosts even in deionized water containing only 35 μM NaCl, i.e soft water. We recorded an extremely low transport affinity constant (K_m) of 8 ± 1 μM for the active uptake of Cl^- in soft water acclimated fish, while other transport kinetic parameters were in agreement with reports for other freshwater organisms. While both Na^+ and Cl^- uptake in soft water clearly depends on apical proton pump activity, changes in abundance and possibly localization of this protein did not appear to contribute to soft water acclimation. Active Cl^- uptake was strongly dependent on branchial carbonic anhydrase (CA) activity regardless of water type, while the response of Na^+ transport to a CA inhibitor was more variable. Differential response of Na^+ uptake to amiloride depending on acclimation medium suggests that different Na^+ transport mechanisms are employed by zebrafish acclimated to soft and hard water.

Keywords: Osmoregulation; Proton pump; Carbonic anhydrase; Transport kinetics; Rapid regulation of ion transport

1. Introduction

In freshwater teleosts active branchial uptake of especially Na^+ and Cl^- is necessary to counterbalance the continuous diffusive ion loss to the hypo-osmotic environment.

Tolerance to low external ion concentrations varies greatly among fish species. The concentrations of NaCl in freshwater are often below 1000 μM and can be subject to large variations due to the nature of catchment areas and changes in precipitation [1]. The acclimation of freshwater teleosts to reduction in ambient NaCl concentrations has been subject to several investigations, but few studies have considered potential changes in mechanisms of apical Na^+ and Cl^- uptake under these conditions. In the present study, we investigated the potential involvement of the proton pump in uptake of both Na^+ and Cl^- in zebrafish (*Danio rerio*) acclimated to different levels of ambient NaCl. The zebrafish is an increasingly popular model for genetic studies in part because of the ease with which they are kept and bred in captivity. Furthermore, the entire zebrafish genome is in the process of being sequenced, and thus offers a powerful tool for studies of the molecular basis of development and physiological adaptation [2]. In the wild, the zebrafish has a wide geographical distribution in streams and rivers of the Indian subcontinent and is naturally exposed to large variations in environmental conditions including water chemistry [3]. In tributaries of the River Ganges, the natural habitat of the zebrafish, Na^+ and Cl^- concentrations as low as 74 and 32 μM, respectively, have been recorded [4]. Despite the extensive use of the zebrafish as an embryonic development model [5], and its more recent important role as a test organism in standardized toxicity evaluations [6], hardly anything is known about its osmoregulatory physiology. Consequently, one objective of the present study was to evaluate the tolerance of zebrafish to a low NaCl concentration.

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With respect to Na^+ uptake, the classical model of Na^+/H^+ exchange, first proposed by Krogh [7] and since attributed to a $Na^+/H^+(NH_4^+)$ exchanger, has been challenged in recent years. While the involvement of this exchange mechanism in Na^+ uptake cannot be dismissed, an alternative model has gained support at least in certain freshwater species [8–10]. According to this model, a (V-type) H^+ ATPase extrudes protons across the apical membrane which hyperpolarizes this membrane, and thus favors Na^+ entry into the gill epithelial cell through an apical channel, presumably the epithelial Na^+ channel (ENaC). Immunohistochemical observations have revealed that the V-type proton pump is located in the apical membrane of at least rainbow trout (*Oncorhynchus mykiss*) [11–13] and an ENaC-like protein has been identified to co-localize with the proton pump in the rainbow trout and tilapia (*Oreochromis mossambicus*) [14].

There seems to be a general consensus that Cl^- is transported across the gills by an apical Cl^-/HCO_3^- exchanger [14–17]. It is, however, less clear how Cl^- is driven across the apical membrane against a considerable chemical gradient, which in some cases represents an up to 1000-fold concentration difference. To date, a single study [8] has investigated the potential involvement of an apical proton pump in branchial Cl^- uptake in freshwater fish. This study revealed reduced Cl^- uptake in the presence of bafilomycin A1, a proton pump inhibitor, and is in agreement with reports from isolated amphibian skin where active Cl^- uptake is driven by apical proton pump activity [18–20]. The extrusion of protons by the apical proton pump presumably results on build-up of cytosolic HCO_3^-, which in turn is available for Cl^-/HCO_3^- exchange. Recently, an additional link between branchial proton pump activity and Cl^- uptake has been suggested. The extrusion of protons acidifies the boundary layer at the gill surface and this may effectively titrate HCO_3^- in this microenvironment [21]. This removal of external HCO_3^- would aid HCO_3^- extrusion via the Cl^-/HCO_3^- exchanger and thus facilitate Cl^- uptake.

Since zebrafish exhibit high tolerance to extremely low ambient Na^+ and Cl^- concentrations, a second goal of this study was to characterize Na^+ and Cl^- uptake kinetics in fish held in soft water (low Na^+ and Cl^-) and hard water (relatively high Na^+ and Cl^-). Acclimation to soft water resulted in substantial increases in affinity and capacity of both Na^+ and Cl^- uptake, which lead us to consider altered ion uptake mechanisms possibly involving alterations in proton pump-mediated transport, carbonic anhydrase (CA)-mediated reactions and apical Na^+ channel entry. Na^+ and Cl^- uptake was measured in the presence and absence of bafilomycin A1, ethoxzolamide and amiloride derivatives in both soft water and hard water acclimated fish. In addition, the proton pump protein abundance in crude gill homogenates was evaluated and subcellular localization of the proton pump was examined.

2. Materials and methods

2.1. Experimental fish

Zebrafish (*D. rerio*) were obtained from a local aquarium store and were kept in two 115-l aerated glass aquariums (500 fish per tank), with a 12 h:12 h light/dark photoperiod, at 26–27 °C. The fish were divided into two groups: one of which was acclimated to Copenhagen City tap water (referred to as hard water in the following); the other group was acclimated to soft water generated by reverse osmosis for a minimum of 40 days prior to experimentation unless stated otherwise (for water chemistries in the acclimation tanks, see Table 1). Acclimation to soft water was initiated by placing fish in a 63-l aquarium (maximum of 200 fish per tank) with a 40-l content of 50:50 hard and soft water. Fish were slowly acclimated to low ion levels by exchanging 10 l of the volume of the aquarium water with soft water every day during a 7-day period after which 20 l of water was renewed daily. In hard water tanks, 20 l of water was renewed three times a week. For both water types, aquaria were fitted with a biological filter and fish were fed commercial fish food (Tetramin) three times a week.

2.2. Determination of plasma and whole body ion concentrations

For determination of whole body ion concentrations, fish from the soft water and hard water groups were anesthetized in 0.3 g MS222 l^{-1}, blotted dry and individually wrapped in tin foil and submerged in liquid nitrogen until frozen. Subsequently, manually pulverized fish were transferred to preweighed vials and weighed. One milliliter of 8% perchloric acid was added to each vial and samples were allowed to digest overnight. The resulting homogenates were vortexed briefly and centrifuged prior to analysis of ionic composition. To determinate the plasma ion concentration, 10 fish from each group were anesthetized as above and a blood sample was taken by inserting a heparinized capillary tube into the heart. Plasma was obtained by centrifugation and diluted to 1.5 ml in nanopure water.

2.3. Determination of Na^+ and Cl^- influx kinetics

Ten fish (mean weight 0.391 ± 0.0088 g, range 0.183–0.666 g) from each water type were placed in individual 10-

Table 1
Water composition (in μM) and pH of the two acclimation and test water types

	$[Na^+]$	$[Cl^-]$	$[Ca^{2+}]$	pH
Soft water	35 ± 1	43 ± 2	4.4 ± 1.4	6.00
Hard water	1480 ± 85	1625 ± 6	3246 ± 2	8.15

Samples were obtained from the acclimation tanks at random intervals, $n = 5$.

ml flux chambers set up in a temperature-controlled soft water bath maintained at 27 °C. Each flux chamber was aerated and supplied with water from the bath by a pump. The fish were allowed to recover from handling for a minimum of 45 min prior to experimentation. To ensure low NaCl concentrations, the water in the bath and flux chambers was then exchanged twice with fresh soft water. During measurements on fish acclimated to the hard water, the Ca^{2+} concentration of the water was adjusted to 3.2 mM by addition of Ca-gluconate. This was done to prevent excessive branchial loss of NaCl due to the low level of Ca^{2+} found in soft water [1]. Prior to the flux measurements, water and air flow were terminated and the chamber volume was adjusted to exactly 10 ml; after which aeration was reestablished.

To obtain the external target concentrations as displayed in Figs. 1 and 3, NaCl from a 100 mM stock was added to each chamber, as well as 0.1–0.3 μCi of either ^{22}Na or ^{36}Cl, unless otherwise stated. A 10-min period was allowed for equilibration before taking the first water sample of 1 ml while the second sample was obtained after 1–2.5 h, depending on water chemistry. After the second water sample was taken, NaCl was added again to elevate the concentration and the above procedure was repeated. Pilot studies revealed lower than expected Na^+ uptake rates by soft water fish when subjected to three or more subsequent flux measurements. For this reason, all presented values are obtained from a first or second flux period. To test whether this was the result of a down-regulation of Na^+ uptake, in response to ambient concentrations much above those in the holding medium (35 μM), the time course of Na^+ uptake in soft water zebrafish, exposed to 1200 μM NaCl, was followed for four subsequent 1-h flux periods as described above.

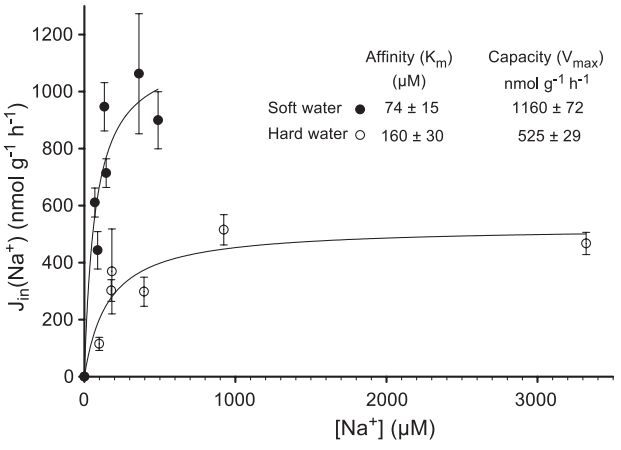

Fig. 1. Na^+ influx kinetics as a function of ambient Na^+ concentration in zebrafish acclimated to soft or hard water. The K_m and J_{max} for soft water and hard water were calculated from the Michaelis–Menten equation yielding the fitted curves (SigmaPlot 4.0 for Windows), $r^2 = 0.870$ and 0.866, respectively. Values are means ± S.E. ($n = 8–10$).

2.4. Measurements in nanopure water

Due to an unexpectedly high branchial affinity for Cl^- in soft water acclimated fish, it was necessary to measure Cl^- influx in nanopure water, which contains an undetectable level of NaCl. The same procedure as described above was used for these experiments using fish with a mean weight of 0.369 ± 0.0147 g (range 0.323–0.460 g). To maintain a low Cl^- concentration, however, only 0.05 μCi ^{36}Cl was added to each chamber and the experimental setup was rinsed three times with nanopure water prior to measurements. To mimic the conditions of acclimation, Ca^{2+} levels were adjusted to 4.2 μM by adding Ca-gluconate ($C_{12}H_{22}CaO_{14}$) to the medium water.

2.5. Inhibition of the proton pump with bafilomycin A1

To test the involvement of a proton ATPase in branchial Na^+ and Cl^- uptake, fish were treated with bafilomycin A1, a specific V-type proton ATPase inhibitor [22]. Due to the expense of this drug juvenile zebrafish were used for this experiment. Larvae were hatched in hard water, as breeding was largely unsuccessful in soft water, and after having reached the free-swimming stage, half of these were placed in an aquarium containing soft water. The juvenile fish were acclimated to the respective water types (Table 1) for 4 weeks, during which time they were fed every day with live artemia. At the time of experimentation the fish weighed 0.00937 ± 0.000386 g (range 0.0031–0.0219 g). A total of eight 10-ml beakers containing 8 ml of aerated water were set up in a temperature-controlled bath (27 °C), after which 10 fish were added to each beaker. Bafilomycin A1 dissolved in a final concentration of 0.05% DMSO was added to four of the beakers to yield a final concentration of 10^{-6} M, which has been reported to inhibit ion fluxes in other freshwater fish [8–10]. To the remaining four control beakers 0.05% DMSO was added. All beakers were either spiked with 1 μCi ^{22}Na or 3 μCi ^{36}Cl. After 1 min of equilibration the first water sample (750 μl) was taken. After 12 min, a second water sample was taken and the fish were rinsed briefly in 200 mM of nonradioactive NaCl to displace any radioactive surface bound ions, after which they were then anesthetized as described above, blotted dry and weighed. The fish containing ^{22}Na were placed directly in counting vials and assayed for gamma radioactivity while fish from beakers spiked with ^{36}Cl were placed in 1.5-ml test tubes containing 1 ml of 8% perchloric acid. Fish were subsequently homogenized with a small pestle and left overnight. The homogenates were vortexed briefly and centrifuged for 10 min at 10,000 × *g* and a sub-sample of the supernatant (800 μl) was transferred to a vial and assayed for beta radioactivity after an addition of 5 ml of scintillation cocktail. Of the water samples taken, 100 μl was assayed for beta radioactivity (700-μl 8% perchloric acid and 5-ml scintillation cocktail was added to each vial) while the remaining sample was analyzed for [Cl^-].

To test whether longer incubation periods would result in inhibition of Na^+ and Cl^- influx, a second set of experiments were performed. These experiments were conducted as above except eight groups of adult zebrafish (weight 0.238 ± 0.0266 g; range 0.04–0.809 g) in 80 ml of water in aerated plastic bags employing a final concentration of 5×10^{-8} M bafilomycin A1 dissolved in 0.025% DMSO and 2-h flux periods.

2.6. Western blot analysis and immunohistochemistry

For both Western blot analysis and immunofluorescence, a rabbit polyclonal antibody raised against the catalytic 70-kDa A-subunit of the bovine V-type H-ATPase was employed. The antiserum [14] was a kind gift from Jonathan Wilson. Abundance of V-type H-ATPase (proton pump) in gill tissue from hard and soft water acclimated zebrafish was evaluated by Western blot analysis. Eight samples consisting of gills obtained from three individual fish from each group were homogenized in ice-cold lysis buffer (20 mM HEPES, 1% Triton X-100, 1 mM EDTA, 1 mM NaF, 1 mM Na_3VO_4, and a protease inhibitor tablet; Roche, Germany). After removal of cellular debris by centrifugation at $10{,}000 \times g$ for 10 min at 4 °C, the protein content in each sample was measured using Pierce Coomasie protein assay reagent according to the manufacturer's instructions (Pierce, Rockford, IL). Equal amounts of cell lysates were dissolved in 2× Laemmli buffer and subjected to SDS-PAGE. Subsequently, the proteins were transferred to nitrocellulose membranes (Scleicher & Schüll, Germany). Immunoreactive proteins were made visible using horseradish-peroxidase coupled secondary antibodies and enhanced chemiluminescence reagents according to the manufacturer's instructions (Amersham Biosciences, Uppsala, Sweden), followed by detection and quantification using an ImageStation 440 CF (Eastman Kodak, USA).

Gill baskets from zebrafish acclimated to both hard water and soft water were placed in PBS containing 4% formaldehyde and fixed for 48 h at 4 °C and dehydrated in ethanol and xylene prior to imbedding in paraffin. Five-micrometer sections were made, mounted on glass slides, dried overnight at 40 °C and kept at room temperature until use. Deparaffinized sections were blocked in 10% Normal Goat Serum (NGS) for 15 min followed by incubation overnight at 4 °C with primary antibody (diluted 1:500) in PBS containing 0.1% Triton X-100 and 0.25% BSA). After wash in PBS, sections were covered with secondary goat-anti-rabbit antibody conjugated to Alexa 568 and incubated for 1 h protected from light. After additional wash in PBS, sections were mounted and examined using a TCS NT/SP confocal laser scanning microscope equipped with ArKr laser and 20 × 0.7 NA and 63 × 1.2 NA PL APO objectives (Leica Micro Systems Heidleberg GmbH, Germany). Images were analyzed using Leica CLSM software or MetaMorph 5.0 sofware (Universal Imaging Corporation, West Chester, PA, USA).

2.7. Effects of ethoxzolamide on Na^+ and Cl^- uptake

Ethoxzolamide (6-ethoxy-2-benzothiazolesulfonamide 97%, Aldrich Chem. Co.), a membrane permeable inhibitor of CA, used at final concentration of 10^{-4} M, was used to test the importance of H^+ and HCO_3^- availability for apical exchange with Na^+ and Cl^-, respectively. A total of eight beakers, each containing 200 ml of aerated water (four of each water type), were placed in a temperature-controlled water bath (27 °C) and used for the Na^+ and Cl^- influx experiments. Four groups of zebrafish, two from each water type were treated with ethoxzolamide dissolved in 0.025% DMSO while the remaining four groups, which were exposed to 0.025% DMSO only, were controls (80 fish in total, mean weight $0.247 \pm 0.0099 \times g$, range 0.036–0.653 g). The fish were allowed a preincubation period of 30 min under these conditions, after which 0.2 μCi ^{22}Na or 2 μCi ^{36}Cl was added. At 10 min and again at 3 h of exposure to isotope, a water sample of 5 ml was obtained from each exposure beaker, after which fish were netted out of the beakers, rinsed, anesthetized and prepared for ^{22}Na or ^{36}Cl radioactivity, as outlined above.

2.8. Effects of amiloride and EIPA on Na^+ influx

In attempt to elucidate the mechanisms responsible for branchial Na^+ uptake in zebrafish, we tested the effect of Amiloride (*N*-amidino-3.5-diamino-6-chloropyrazinecarbromide $C_6H_8ClN_7$*HCl, Sigma) and EIPA (5-(*N*-ethyl-*N*-isopropyl)-amiloride, $C_{11}H_{18}ClN_7O$, Sigma) on branchial Na^+-uptake was investigated. Amiloride is known to inhibit both Na^+/H^+ exchangers and Na^+ channels in various epithelia [23]. Generally, Na^+ channels have a higher affinity for amiloride than the exchanger [23], and we therefore attempted to distinguish between the Na^+ channel and the exchanger by using two concentrations of amiloride: 10^{-5} and 10^{-4} M. Furthermore, EIPA, which more selectively targets Na^+/H^+ exchangers, was tested at a final concentration of 5×10^{-5} M. These Na^+-flux experiments were conducted as the ethoxzolamide experiments described above.

2.9. Analytical techniques, calculations and statistical analysis

Water samples from kinetic experiments on Na^+ uptake and samples of whole fish from pharmacological studies of Na^+ influx were assayed for gamma radioactivity on a Minaxiγ Autogamma® 5000 Series Gamma Counter. Water Samples from Cl^- uptake kinetic experiments and homogenates of whole fish from pharmacological studies were assayed on a TRI-CARB 2500 TR Liquid Scintillation Analyzer after an addition of 5-ml scintillation cocktail. All anion and cation concentrations in water, plasma and whole fish samples were measured on a Dionex Ion Chromatograph DX 120 after appropriate dilutions.

In kinetic studies, J_{in} of either Na^+ and Cl^- was calculated as

$$\text{Influx}, J_{in} = ((CPM_b - CPM_a)/(MSA))(V/W*t),$$

where CPM_a and CPM_b are measures of radioactivity (counts min^{-1}) in the flux chamber at the beginning and end of the flux period. MSA is the mean specific activity in the water during the flux period, V is the volume in the flux chamber (l), W is the mass of the fish (g) and t is time (h).

During pharmacological studies, Na^+ and Cl^- influx rates were calculated as follows:

$$\text{Influx}, J_{in} = ((CPMg^{-1})/MSA)/t$$

Were CPM (counts min^{-1}) is the final radioactivity in the fish, MSA and t have the same meaning as above.

Na^+ and Cl^- influx displayed apparent saturation kinetics. The Michaelis–Menten equation for nonlinear regression ($J_{in}[X] = J_{max}*[X]/K_m+[X]$), where X was Na^+ or Cl^-) was used to estimate the affinity (K_m) and maximum capacity (J_{max}) of the transport systems (SigmaPlot 4.0 for Windows).

Data are presented as means ± S.E. Statistical comparisons were made with a two-tailed t test for independent observations ($P < 0.05$).

3. Results

3.1. Plasma and body ions

Both plasma Na^+ and Cl^- concentrations were not statistically different in soft water and hard water acclimated fish; although concentrations in soft water fish appeared lower (Table 2). Similarly, whole body content of Na^+, Ca^{2+}, K^+ and Cl^- did not seem to differ between soft water and hard water acclimated fish (Table 3).

3.2. Na^+ influx kinetics

The influx of Na^+ in zebrafish acclimated to soft water (35 μM Na^+) or hard water (1480 μM Na^+) exhibited clear Michaelis–Menten saturation kinetics (Fig. 1). Both apparent Na^+ uptake affinity and capacity were significantly elevated about twofold in response to soft water acclimation. Interestingly, the rate of Na^+ uptake in fish at the Na^+ concentration to which they had been acclimated was approximately 500 (nmol g^{-1} h^{-1}) for both soft water and hard water acclimated fish. Soft water Na^+ uptake kinetics was calculated only from values obtained from flux measurements performed at Na^+ concentrations lower than 500 μM (>10 times the level of acclimation for soft water fish). At concentrations above 500 μM, soft water acclimated fish displayed large individual differences and at Na^+ concentration above 1000 μM, influx values were approximately 50% lower than what would have been expected according to the V_{max} (data not shown).

Table 2
Plasma Na^+ and Cl^- concentration (mM) in soft water and hard water acclimated zebrafish, mean ± S.E. ($n = 10$)

	$[Na^+]$	$[Cl^-]$
Soft water	123.3 ± 13.8	86.2 ± 6.4
Hard water	154.5 ± 8.8	105.7 ± 15.2

Table 3
Whole body ion concentration in soft water and hard water acclimated zebrafish (mmol kg^{-1}), mean ± S.E. ($n = 10$)

	$[Na^+]$	$[K^+]$	$[Ca^{2+}]$	$[Cl^-]$
Soft water	44.9 ± 1.5	81.9 ± 1.9	33.3 ± 1.6	23.8 ± 4.1
Hard water	49.6 ± 3.1	86.1 ± 2.4	35.2 ± 1.1	20.2 ± 2.0

3.3. Down-regulation of Na^+ transport

Soft water acclimated fish exposed to relatively high Na^+ concentrations (1200 μM) for more than two subsequent flux periods displayed a drop in the rate of Na^+ influx to less than half the value measured after one flux period (Fig. 2). Na^+ efflux was down-regulated in a similar manner resulting in no change in net Na^+ flux. In hard water acclimated fish, the Na^+ influx rate did not diminish as a function of time.

3.4. Cl^- influx kinetics

As for Na^+, Cl^- uptake in both soft water and hard water acclimated zebrafish exhibited saturation kinetics and the difference between Cl^- influx kinetic constants from the two groups of fish was statistically significant (Fig. 3). The apparent K_m for Cl^- influx in soft water acclimated zebra-

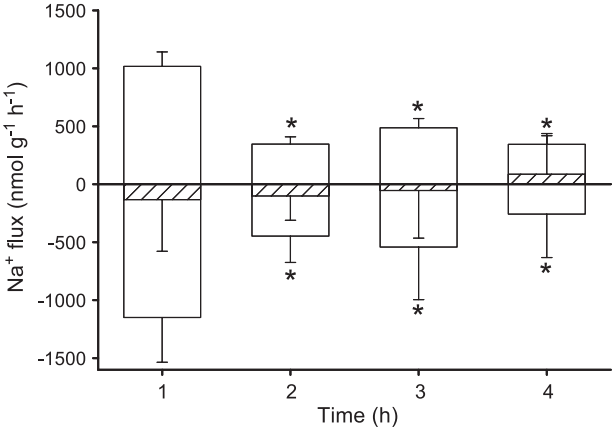

Fig. 2. Na^+ flux (nmol g^{-1} h^{-1}) measured after 1, 2, 3 and 4 h of exposure to an ambient Na^+ concentration of 1200 μM in zebrafish adapted to soft water. Open bars indicate unidirectional Na^+ flux (positive values indicate influx while negative values indicate efflux) and hatched bars indicate net flux. Asterisks signify a statistically significant difference between values after 1 h and subsequent hours, two-tailed Student's t test, unpaired ($P < 0.05$). Net flux values did not show any statistically significant difference from zero. All values are means ± S.E. ($n = 10$).

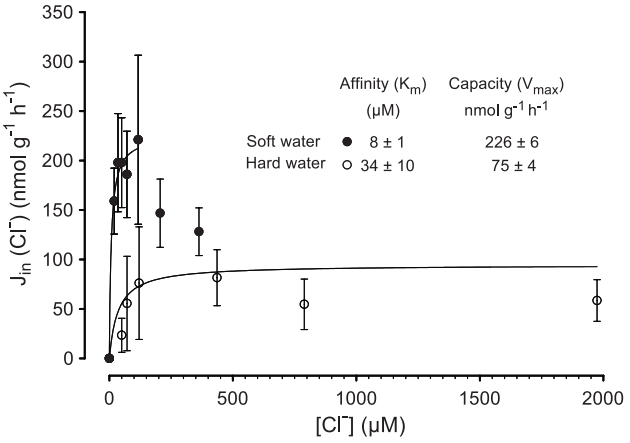

Fig. 3. Cl^- influx kinetics as a function of ambient Cl^- concentration in zebrafish adapted to soft or hard water. The K_m and J_{max} for soft water and hard water were calculated from the Michaelis–Menten equation yielding the fitted curves (SigmaPlot 4.0 for Windows), $r^2 = 0.982$ and 0.711, respectively. Values are means ± S.E. ($n = 3–10$).

fish was remarkably low at only 8 ± 1 μM and approximately fivefold lower than that of hard water acclimated fish. Similarly, the Cl^- uptake capacity was elevated approximately threefold as a result of soft water acclimation. For adult fish, the Cl^- transport capacity was much lower than that for Na^+, regardless of water type. That is, soft and hard water acclimated fish exhibited Cl^- uptake capacity amounting to 15% and 20%, respectively, of the corresponding Na^+ uptake capacity (Fig. 1 versus Fig. 3). As for Na^+ influx, soft water acclimated fish had Cl^- influx which was also lower than expected, based on the calculated V_{max}, at values greatly exceeding the acclimation Cl^- concentration (Fig. 3).

3.5. Effects of proton pump inhibition on ion uptake

Soft water acclimated zebrafish treated with 1 μM bafilomycin for a short period of time (12 min) displayed a significant inhibition of Na^+ influx, while Na^+ uptake in hard water acclimated fish was unaffected (Fig. 4). In contrast, during bafilomycin treatment, Cl^- influx was not reduced in fish acclimated to either water type, but was increased in hard water acclimated fish (Fig. 4). More prolonged exposure (2 h) to a lower bafilomycin concentration (5×10^{-8} M) resulted in an inhibition of Cl^- but not Na^+ influx in the soft water acclimated fish. In hard water acclimated fish, Cl^- uptake was unaffected by this treatment whereas Na^+ influx appeared to be stimulated (Fig. 5).

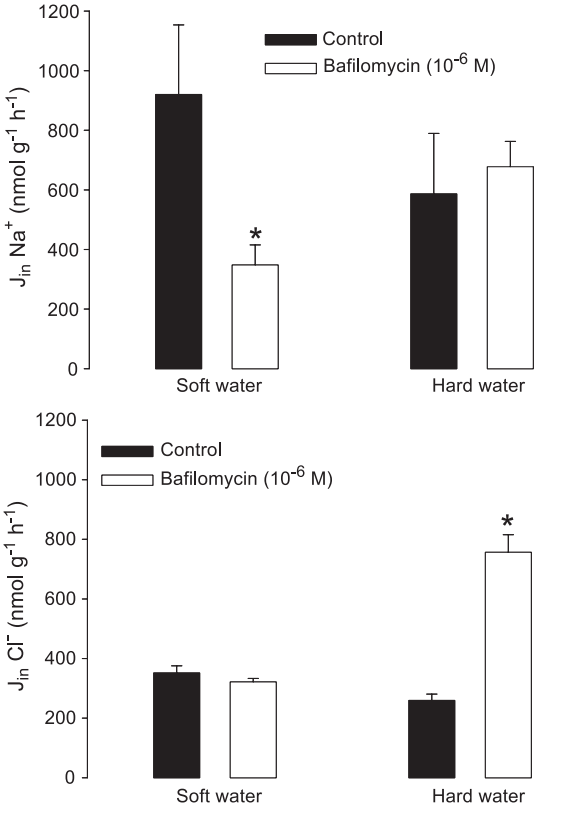

Fig. 4. Na^+ influx (nmol g^{-1} h^{-1}) (upper panel) and Cl^- influx (nmol g^{-1} h^{-1}) (lower panel) in juvenile zebrafish treated with 10^{-6} M bafilomycin for 12 min. Asterisks indicate significant difference from the corresponding control evaluated by Student's t test, unpaired ($P < 0.05$). All values are means ± S.E. ($n = 10$).

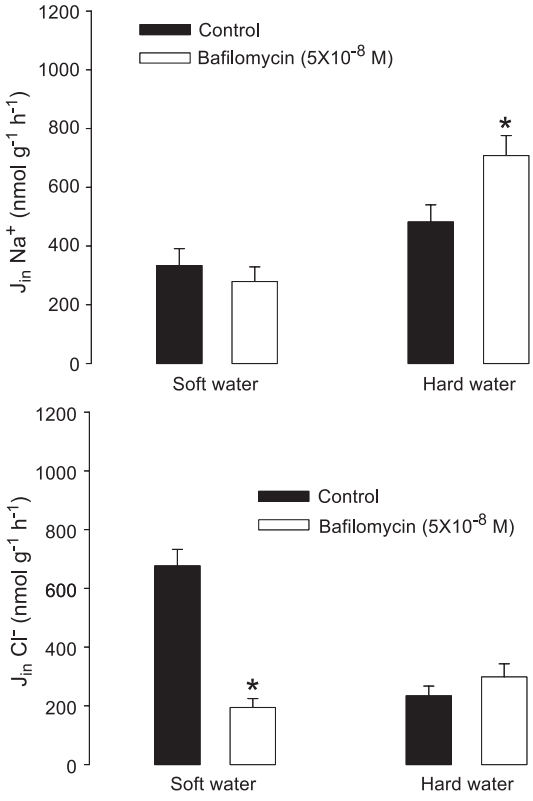

Fig. 5. Na^+ influx (nmol g^{-1} h^{-1}) (upper panel) and Cl^- influx (nmol g^{-1} h^{-1}) (lower panel) in zebrafish treated with 5×10^{-8} M bafilomycin for 2 h. Asterisks indicate significant difference from the corresponding control evaluated by Student's t test, unpaired ($P < 0.05$). All values are means ± S.E. ($n = 10$).

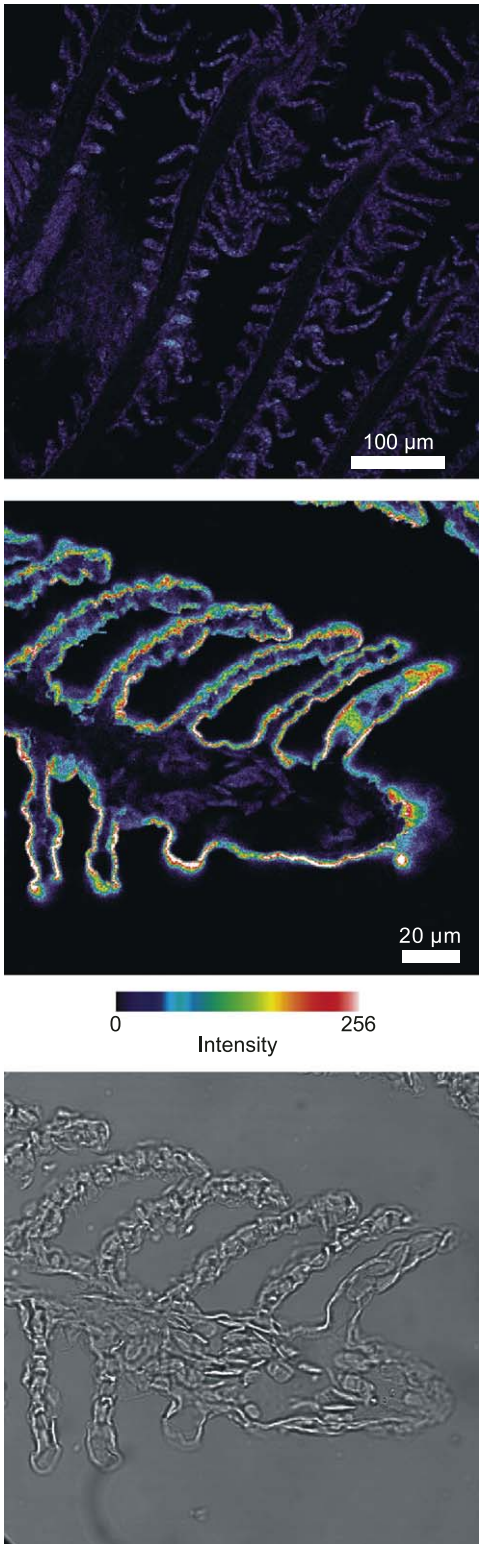

Fig. 6. Immunofluorescence in a representative gill section from a soft water acclimated zebrafish demonstrating abundant apical region localization (middle panel) with corresponding transmission image (bottom panel). Control sections (top panel) incubated without primary antibody revealed limited nonspecific fluorescence. No obvious difference in localization of immunoreactivity or intensity of the reaction was observed between hard water (not shown) and soft water acclimated fish.

3.6. Western blot analysis and immunohistochemistry

Immunofluorescence microscopy of gills demonstrated extensive apical region staining in both hard and soft water acclimated zebrafish (Fig. 6). However, there was no obvious difference in localization or intensity of immunoreactivity with the A-subunit of the H^+-ATPase between the two groups (data not shown). The latter was confirmed by Western blot analysis, which showed no difference in the relative amount of A-subunit immunoreactive protein in crude gill homogenates from soft and hard water acclimated fish (Fig. 7).

3.7. Effects of CA inhibition on ion uptake

The CA inhibitor ethoxzolamide (10^{-4} M) almost completely blocked Cl^- influx in both soft and hard water acclimated zebrafish, while Na^+ uptake was reduced in hard water acclimated fish only (Fig. 8). In soft water acclimated fish, Na^+ uptake appeared slightly stimulated by ethoxzolamide treatment.

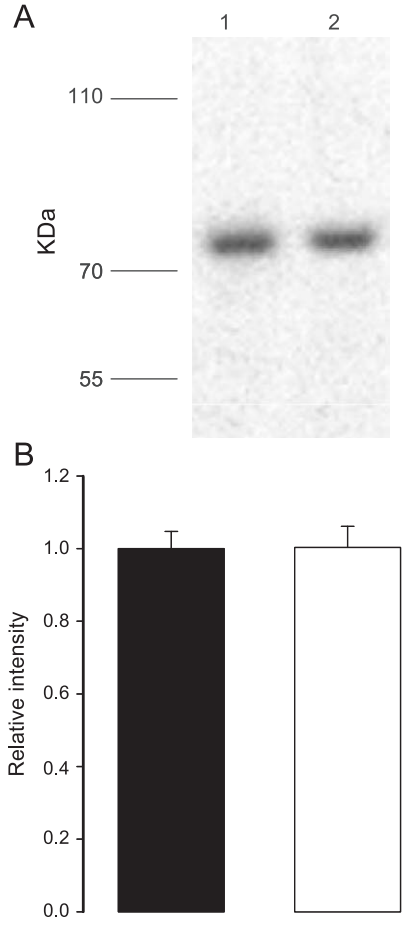

Fig. 7. Representative examples of H^+ pump antibody immunoreactive proteins (A) in hard water (lane 1) and soft water (lane 2) acclimated fish. Relative abundance (B) evaluated by Western blots of H^+ pump immunoreactive proteins in gill homogenates from hard water (dark bars) and soft water (open bars) acclimated fish ($n = 8$).

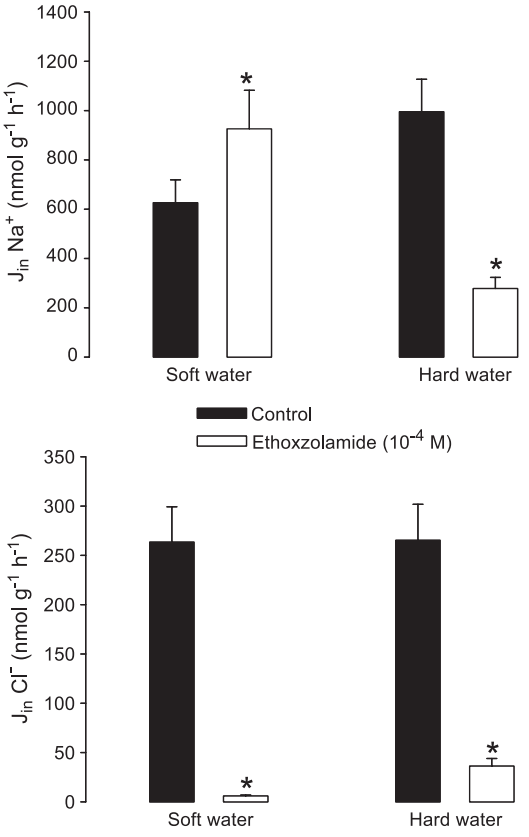

Fig. 8. Na^+ influx (nmol g^{-1} h^{-1}) (upper panel) and Cl^- influx (nmol g^{-1} h^{-1}) (lower panel) in zebrafish treated with 10^{-4} M ethoxzolamide. Asterisks indicate significant difference from the corresponding control evaluated by Student's *t* test, unpaired ($P<0.05$). All values are means ± S.E. ($n=10$).

3.8. Effects of amiloride and EIPA on Na^+ uptake

Surprisingly, soft water acclimated fish displayed no significant reduction in Na^+ uptake values when treated with 10^{-5} and 10^{-4} M amiloride (Fig. 9). In contrast, 10^{-4} M amiloride significantly reduced Na^+ uptake in hard water acclimated fish. The EIPA-treated fish displayed apparently increased influx rates (statistically significant only in soft water acclimated fish) compared to controls.

4. Discussion

As expected from the water chemistry of their natural habitat (c.f. Introduction), zebrafish were able to survive in the laboratory in very ion-poor water for several months. In the present study, zebrafish maintained close to normal plasma Na^+ and Cl^- concentrations and whole body ion concentrations in soft water (Tables 2 and 3). This appears to be largely due to a marked compensatory up-regulation of apparent branchial ion uptake affinity (K_m) and maximum uptake rate (J_{max}). Soft water acclimated fish used for these measurements had lived in holding tanks containing ~ 35 μM NaCl for several months, during which no mortalities or ill effects of the dilute medium were observed. Breeding in soft water, however, was largely unsuccessful due to a low number of eggs produced and poor survival of offspring.

4.1. Na^+ and Cl^- transport kinetics

Net Na^+ and Cl^- transport (later not shown) was essentially zero whenever measured and the measured Na^+ flux rates are within the range of values reported from freshwater animals of similar body mass [17] indicating that handling stress did not greatly influence ion flux rates in the present study.

The mechanism by which zebrafish acclimate to soft water seems to be an increase in Na^+ and Cl^- uptake at low ion concentration, possibly reflected by the overall number of carriers (J_{max}) and the overall apparent affinity of the carrier system (K_m) (Figs. 1 and 3). Thus, there are comparable Na^+ and Cl^- ion uptake rates in either soft water or hard water acclimated fish. In the case of Na^+, exposure to low-ion environments has been reported to cause an in-

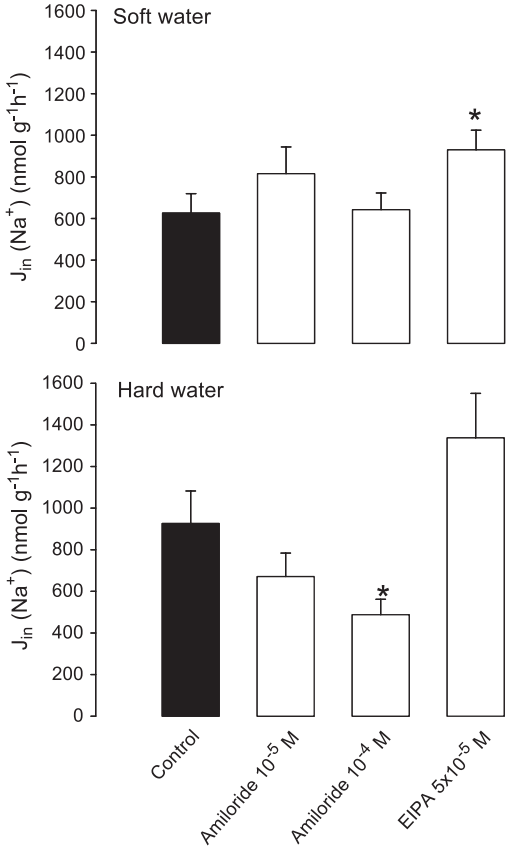

Fig. 9. Na^+ influx (nmol g^{-1} h^{-1}) in zebrafish treated with amiloride and EIPA. The upper panel and lower panel show results from soft water and hard water, respectively. Open bars indicate results from fish treated with amiloride 10^{-5} M, amiloride 10^{-4} M and EIPA 5×10^{-5} M while filled bars indicate control fish (0.025% DMSO). Asterisks indicate significant difference from the corresponding control evaluated by Student's *t* test, unpaired ($P<0.05$). All values are means ± S.E. ($n=10$).

crease in maximum uptake rate and/or affinity in several fish species [1,24]. The apparent Na^+ uptake kinetic constants of both groups of zebrafish in the present study compare well with previously reported values from other teleosts [10,24–28]. The observed increase in at least apparent Na^+ uptake affinity and capacity takes several days to develop (Bury and Grosell, unpublished observations); indicating that acclimation to soft water involves genomic regulation.

Saturable and thus carrier-mediated Cl^- uptake is not obligatory for osmoregulation in freshwater teleosts. Freshwater acclimated killifish (*Fundulus heteroclitus*) and members of the *Anguillidae* family do not appear to have the ability to take up Cl^- from freshwater [29–31]. In contrast to these fishes, zebrafish clearly exhibits carrier-mediated Cl^- uptake in freshwater (Fig. 3) and the greatest relative response to soft water acclimation was actually the increases in apparent Cl^- uptake affinity and capacity. Zebrafish generally exhibits a high affinity Cl^- uptake system when compared to other aquatic vertebrates and invertebrates. Indeed, zebrafish acclimated to soft water had a remarkably low K_m of only 8 μM, to our knowledge, the lowest ever recorded. Whether, the increase of apparent Cl^- uptake affinity and capacity is the result of genomic regulation and/or a faster non-genomic regulation waits to be investigated.

4.2. Rapid regulation of Na^+ and Cl^- transport

At high ambient Na^+ concentrations, there appeared to be a rapid down-regulation of Na^+ uptake, as well as Na^+ loss (Figs. 1 and 2). The mechanism of this rapid apparently non-genomic regulation, could be a Na^+-dependent inhibition of an epithelial Na^+ channel, as previously observed in experiments conducted on isolated frog skin [32,33]. Cl^- influx also seemed to be reduced when soft water acclimated fish were subjected to Cl^- concentrations above 200 μM (Fig. 3). As will be discussed in the following section, apical Cl^- uptake is suspected to be via Cl^-/HCO_3^- exchange, which expectedly would result in elevated Cl^- uptake rates at higher ambient Cl^- concentrations. Although cellular Cl^- uptake via certain conductive pathways appears to be regulated based on ambient Cl^- concentration [34–36], we are unaware of studies documenting down-regulation of Cl^-/HCO_3^- exchange in response to increased ambient Cl^- concentrations.

4.3. Na^+ and Cl^- uptake—the involvement of a proton pump

The presence of the H^+-pump in zebrafish was identified by use of a polyclonal antibody directed against the catalytic 70-kDa A-subunit. An immunoreactive band of approximately 75–80 kDa was observed in both soft water and hard water acclimated fish (Fig. 7A), which is in agreement with previous results obtained from rainbow trout [14]. Furthermore, immunofluorescence microscopy of gill tissue demonstrated abundant apical region immunoreactivity with no obvious differences in localization between fish acclimated to soft or hard water (Fig. 6). Since apical proton pump activity appears to be involved in both Na^+ and Cl^- uptake in soft water and since there is elevated transport capacity of both ions, one could expect increased proton pump abundance. Current preparation does not allow subcellular resolution, i.e. detecting differences between proton pumps inserted in the apical membrane and pumps located just under the apical membrane. It thus appears that the elevated ion uptake capacity in soft water fish cannot conclusively be explained by increased proton pump abundance, which is in agreement with observations of lack of increase in proton-pump mRNA expression and enzyme activity in response to soft water acclimation [37,38]. It is, however, possible that the increase in uptake capacity for both ions is due to an increase in the kinetic activity of the proton pump without a change in quantity or distribution [39]. The apical region localization observed in the present study on zebrafish is as mentioned in agreement with studies on rainbow trout [14] but is in contrast to a recent report of basolateral localization in the gills of freshwater acclimated killifish (*F. heteroclitus*) [40]. It should be noted, however, first, that killifish does not exhibit high affinity Cl^- uptake in freshwater [31] and second, that no studies of the functionality, with respect to ion uptake, of the basolateral proton pump were reported by Katoh et al. [40]. The apparent differences between zebrafish and killifish with respect to proton pump localization could well be related to their freshwater stenohalinity and euryhalinity, respectively.

Overall, experiments with bafilomycin revealed that proton pump activity may be required for Na^+ and Cl^- uptake at least in soft water acclimated fish. Na^+ uptake in soft water but not hard water was inhibited by brief exposure to 10^{-6} M bafilomycin. Effects of bafilomycin on branchial Na^+ uptake in freshwater fish has been reported previously [8–10], but this is the first study to report differential effect of proton pump inhibition depending on acclimation conditions. It has been suggested that one function of the apical proton pump is to hyperpolarize the apical membrane to establish a sufficient electrochemical gradient for Na^+ uptake. Considering an expected apical membrane potential difference of at least −70mV and intracellular Na^+ concentrations of 20–50 mM [15,41–43], proton pump activity would only be necessary for Na^+ uptake in situations with low ambient Na^+ concentrations (see Ref. [20] in this issue for detailed discussion). In soft water containing very low Na^+ concentrations, the additional polarization of the apical membrane caused by the electrogenic proton pump may aid Na^+ uptake against the substantial chemical gradient. Our findings of reduced Na^+ uptake in soft water but absence of effect of bafilomycin in hard water, where the electrochemical Na^+ gradient generated by the Na^+/K^+-ATPase is sufficient to drive Na^+ uptake, is consistent with this proposed function of the proton pump. The reason for elevated Na^+ uptake in hard

water acclimated fish exposed for a longer period to a lower bafilomycin concentration is perhaps less clear (Fig. 5).

Cl^- uptake was inhibited by prolonged exposure to the lower bafilomycin concentration in soft water acclimated, but not hard water acclimated fish (Fig. 5). This observation is consistent with the hypothesized role of the proton pump in Cl^- uptake and is in agreement with previous observations in fish [8] and amphibians [18]. Inhibition of the proton pump would result in reduction of cellular HCO_3^- available for exchange with Cl^- and might also result in elevated HCO_3^- concentrations in the unstirred layer bathing the apical membrane (see Introduction). Taken together, these effects would reduce the HCO_3^- gradient across the apical membrane, which would result in impaired Cl^- uptake through Cl^-/HCO_3^- exchange especially in a low Cl^- environment. That the effect of bafilomycin is not evident in hard water acclimated fish may be explained by the higher ambient Cl^- concentration. At these higher Cl^- concentrations, less intracellular HCO_3^- is required for the anion exchange process to occur. It is conceivable that the effects of bafilomycin did not develop during the more brief exposure (12 min) to the higher concentration, because depletion of the apical HCO_3^- gradient is unlikely to occur instantaneously.

An unexpected stimulation of Cl^- uptake was observed during the brief exposure to the higher bafilomycin concentration. This effect cannot be explained by the current models of ion transport across fish gill epithelia, but it is interesting that Cl^- uptake appears to be stimulated in a situation where the apical membrane is expected to be depolarized due to the inhibition of the proton pump. Although unpreceded, at least in vertebrates, this could indicate electrogenic active uptake of Cl^- at least in hard water acclimated fish. This increase in Cl^- uptake is not seen in soft water during bafilomycin treatment, perhaps because the ambient Cl^- concentration was too low to allow an intracellular accumulation.

4.4. Na^+ and Cl^- uptake—the involvement of CA

Inhibition of CA-mediated CO_2 hydration with the lipophilic ethoxzolamide almost completely blocked Cl^- uptake in both soft water and hard water. This observation strongly supports the role of CA in providing substrate for Cl^- uptake via Cl^-/HCO_3^- exchange [44]. Our observations are in agreement with a previous study on rainbow trout [45] and also with several studies of active Cl^- uptake by isolated amphibian skin [46–48].

The corresponding results from measurements of Na^+ uptake are less clear; in hard water acclimated fish, inhibited Na^+ influx was seen during treatment with ethoxzolamide, while soft water acclimated fish exhibited a slightly stimulated Na^+ uptake. The lack of inhibitory effect of ethoxzolamide on Na^+ uptake may suggest that non-catalyzed CO_2 hydration results in sufficient H^+ supply for Na^+ uptake. In any case, the discrepancy between the response of Na^+ and Cl^- transport to CA inhibition illustrates a lack of coupling between H^+- and HCO_3^--mediated uptake of Na^+ and Cl^-, respectively. This may suggest that the effect of CA inhibition on Cl^- influx could be due to whole animal acidosis induced by the block of CA-mediated dehydration of H^+ and HCO_3^-. Such an acidosis could be conveyed to the intracellular compartment [49] and the blockage of Cl^- uptake could be a consequence of a pH regulatory mechanism to maintain HCO_3^- inside the cell. The slightly elevated Na^+ uptake in the soft water acclimated fish treated with the CA inhibitor might suggest that the above pH-regulatory mechanism is insufficient to prevent acidification of the gill cells under those conditions (low buffered environment), which would lead to elevated proton extrusion and thus Na^+ uptake.

4.5. Na^+ uptake and amiloride

Na^+ uptake by soft water acclimated zebrafish proved to be completely insensitive to concentrations of amiloride and EIPA which have successfully inhibited Na^+ influx in other species [50]. However, the highest concentration of amiloride employed (10^{-4} M) resulted in slightly reduced Na^+ influx in hard water acclimated fish. Apparent lack of, or low sensitivity to amiloride, has also been reported for the neon tetra (*Paracheirodon innesi*), another teleost capable of ionoregulation in highly dilute media [51]. Although insensitive to amiloride, Na^+ uptake in soft water acclimated fish appears to be dependent on presumed apical membrane hyper-polarization resulting from the proton pump (see above) and therefore seems to occur through a conductive pathway. The lack of amiloride sensitivity even at relatively high concentrations suggests that this conductive pathway may be distinct from the ENaC, otherwise presumed to be present in the teleost fish gill [15–17,21]. The response to 10^{-4} M amiloride seen in hard water acclimated zebrafish is consistent with Na^+ uptake via a Na^+/H^+ exchanger. This, together with the differential effect of proton pump inhibition on Na^+ uptake in soft water and hard water acclimated fish, indicates that two separate mechanisms for Na^+ uptake operate in the zebrafish depending on ambient conditions.

4.6. Summary/conclusions

In summary, our findings show that zebrafish maintain osmotic balance in an extreme, ion poor environment by enhanced apparent Na^+ and Cl^- uptake affinity and capacity and that transport of both ions can be subject to rapid regulation. Apical proton pump activity seems important for uptake of both ions in soft water, but increased protein abundance or localization does not seem to be the cause of increased apparent uptake capacity. CA activity appears critical to at least Cl^- uptake regardless of ambient Cl^- concentration. Differential effect of Na^+ uptake blockers in soft water and hard water acclimated fish strongly suggests

that fish under these different conditions employ different mechanisms for Na^+ uptake.

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