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Gill membrane remodeling with soft-water acclimation in zebrafish (*Danio rerio*)

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Craig PM, Wood CM, McClelland GB. Gill membrane remodeling with soft-water acclimation in zebrafish (*Danio rerio*). *Physiol Genomics* 30: 53–60, 2007. First published February 13, 2007; doi:10.1152/physiolgenomics.00195.2006.—Little is known regarding the ionoregulatory abilities of zebrafish exposed to soft water despite the popularity of this model organism for physiology and aquatic toxicology. We examined genomic and nongenomic changes to gills of zebrafish as they were progressively acclimated from moderately hard freshwater to typical soft water over 7 days and held in soft water for another 7 days. Gills were sampled daily and mRNA expression levels of gill Na^+-K^+-ATPase (NKA) α1a subunit, epithelium calcium channel (ECaC), carbonic anhydrase-1 and 2 (CA-1, CA-2), Na^+/H^+ exchanger (NHE-2), V-type proton (H^+)-ATPase, and copper transport protein (CTR-1) were quantified by real-time PCR. Changes in enzyme activities of gill NKA were determined and protein levels of NKA and ECaC were quantified by Western blotting. Levels of mRNA for ECaC increased fourfold after *day 6*, with an associated increase in ECaC protein levels after 1 wk in soft water. CA-1 and CA-2 exhibited a 1.5- and 6-fold increase in gene expression on *days 6* and *5*, respectively. Likewise, there was a fivefold increase in NHE-2 expression after *day 6*. Surprisingly, CTR-1 mRNA showed a large transient increase (over threefold) on *day 6,* while H^+-ATPase mRNA did not change. These data demonstrate a high degree of phenotypic plasticity in zebrafish gills exposed to an ion-poor environment. This not only enhances our understanding of ionoregulatory processes in fish but also highlights the need for proper experimental design for studies involving preacclimation to soft water (e.g., metal toxicity).

gene expression; gill; Na^+-K^+-ATPase; epithelium calcium channel; salinity transfer

THE ZEBRAFISH HAS A LONG HISTORY as a model organism in the study of developmental biology, but only recently has it gained acclaim for examining vertebrate physiology (e.g., Ref. 3). Despite its many advantages as a model organism, little is known regarding the physiology or ability of zebrafish to tolerate environmental stress. Although growing in popularity for both physiological (e.g., Ref. 1) and especially aquatic toxicology studies (24), few studies have examined the responses of zebrafish to different water chemistries. This has significant relevance to toxicology studies in particular where soft-water acclimation usually precedes toxic exposures.

Teleosts living in freshwater must constantly battle the loss of ions from the body to the surrounding environment. Many studies have examined changes in the mechanisms of gill transport that deal with salinity changes accompanying seawater to freshwater transfer, including recent studies examining gene expression (e.g., Refs. 31, 32). However, ion loss is further exacerbated in a soft-water situation where ion concentrations for Na^+ and Ca^{2+} can be 10 times less than measured in typical freshwater (Na^+ and Ca^{2+} ~1 mM, Mg^{2+} 0.4 mM), necessitating alterations in osmoregulatory physiology to maintain ion homeostasis in tolerant species. To maintain a high internal concentration of salts in an ion-deficient medium, teleosts must employ enzymes, such as basolateral Na^+-K^+-ATPase (NKA), apical Na^+/H^+ exchanger, and a (V-type) H^+-ATPase to enhance Na^+ uptake at the gills (16, 17, 18, 22, 32). Additionally, Ca^{2+} uptake occurs through epithelial calcium channels (ECaC) in the apical membrane, and through Ca^{2+}-ATPases or Na^+/Ca^{2+} exchangers in the basolateral membrane (8, 36). Anions like Cl^- appear to be taken up at the gill by an apical Cl^-/HCO_3^- exchanger assisted by cytoplasmic carbonic anhydrase (CA) catalyzing the conversion of CO_2 to HCO_3^- and a proton (27, 29, 37).

Zebrafish in the wild are found throughout the Indian subcontinent where they face a wide variety of environmental water chemistries and are known to be a soft water-tolerant species (15, 34). Their tolerance to ion-poor water, comprehensive genomic database, amenability to large-scale screening, and reverse genetics make the zebrafish an ideal model in which to investigate gill remodeling and ionregulatory physiology. These same reasons have also made them an increasingly popular model for aquatic toxicology. Surprisingly, very little is known regarding zebrafish osmoregulatory physiology. To date, only one other study has examined the effects of soft-water acclimation on zebrafish gill physiology, and none have looked at responses at the genomic level (2). Zebrafish are able to maintain their plasma and whole body ion composition in the face of exposure to extremely low ambient ion concentrations in soft water (2). This seems to be accomplished partly through a rapid change in Na^+ and Cl^- transport affinity and the regulation of transport processes. CA activity seems to play a critical role in Cl^- uptake, as demonstrated by Boisen et al. (2). When CA activity was inhibited, there was a complete blockade of Cl^- uptake. However, there is evidence that teleosts in freshwater employ apical epithelial Na^+ channels, and/or Na^+/H^+ exchangers, in association with cytoplasmic CA for Na^+ uptake (2, 28, 29, 32). In addition, studies on rainbow trout demonstrate that Ca^{2+} transport capacity is increased under conditions of low ambient Ca^{2+} concentrations (26). This suggests that soft-water acclimation is accompanied by gill remodeling at the level of membrane transporters and enzymes. However, to date there are limited data on the inducibility of gene expression for these components of zebrafish gill. It has been shown that salinity transfer (freshwater → seawater/seawater → freshwater) induces changes in gene expression for gill ion transporters in other fish species (31, 32, respectively). This suggests that transcriptional regulation of

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gill ion transporters may occur with soft-water acclimation in zebrafish. Although the change in "salinity" is relatively small compared with seawater/freshwater acclimation, soft-water acclimation represents an environmentally relevant stressor for freshwater fish.

The main goals of this study were to determine during a progressive 7-day soft-water acclimation: *1*) the temporal pattern of gene expression for key ion channels, transporters, and enzymes in zebrafish gills, *2*) whole body changes of ion levels, and *3*) the effect of acclimation on changes in protein expression and enzyme activities for selected membrane components. These data will help identify the molecular changes involved in gill remodeling and ion homeostasis in zebrafish. They will also provide valuable information for the design of experiments where soft-water acclimation precedes treatment. Aquatic toxicology studies on waterborne metal exposures use a preacclimation to soft water to eliminate the confounding effects of protective ions (e.g., Refs. 14, 23). Any changes in gill phenotype that result from this preacclimation must be accounted for when interpreting metal-induced gene expression changes.

MATERIALS AND METHODS

Animals. Zebrafish (*Danio rerio*) were purchased from a local pet supply store (PetsMart, Canada) and housed in two 40-liter aquaria in dechlorinated Hamilton tap water, which is considered moderately hard (Na^+ 927 ± 16 μM, Ca^{2+} 946 ± 11 μM, Mg^{2+} 422 ± 17 μM, Cu^{2+} 2.1 ± 0.7 μg/l, pH 8.3), maintained at 28°C (hard water). The fish were allowed 1 wk to acclimate to the new tanks before experimentation. Tanks were aerated and filtered with Aqua Clear 150 aquarium filters (Hagen, Montreal, Canada). Fish were fed daily with a commercial tropical fish food (Topfin, Phoenix, AZ) and maintained on a 12-h light, 12-h dark photoperiod regime. All procedures used were approved by the McMaster University Animal Research Ethics Board and conform to the principles of the Canadian Council for Animal Care.

Experimental design. Over a period of 7 days, hard water was progressively removed and replaced with ion-poor reverse-osmosis water (~15–20% daily, over a period of 15 minutes), until ion levels in the water were reduced to 115 ± 3 μM Na^+, 51 ± 1 μM Ca^{2+}, 26 ± 1 μM Mg^{2+}, 1.8 ± 0.5 μg/l Cu^{2+}; pH 6.8. At 10:00 AM daily, a sample of zebrafish was removed from both tanks, terminally anesthetized in buffered tricaine methanesulfonate (MS-222; Sigma, St. Louis, MO), and weighed. A control group was kept in hard water throughout and sampled simultaneously. The gills were removed and either immediately frozen in liquid N_2 for mRNA extraction and Western blotting or immersed in 100 μl of ice-cold SEI buffer (150 mM sucrose, 10 mM EDTA, 50 mM imidazole, pH 7.3) for analysis of NKA activity. Whole zebrafish were also weighed and frozen in liquid N_2 for analysis of body ion concentrations. Additionally, water samples were taken daily for analysis of pH and ion composition.

Water and whole body ion composition. Whole zebrafish were first digested in 5 volumes of 1 N HNO_3 for 48 h at 60°C. Both whole body and water ion composition were measured by flame atomic absorption (Spectra AA 220FS: Varian, Palo Alto, CA) after 1:10 dilutions were made with 1% HNO_3 (Na^+) or 0.5% $LaCl_3$/1% HNO_3 (Ca^{2+}, Mg^{2+}) and verified using certified Na^+, Ca^{2+}, Mg^{2+} standards (1 mg/l diluted in 1% HNO_3 or 0.5% $LaCl_3$/1% HNO_3; Fisher Scientific).

NKA activity. Gill NKA activity was determined using the microassay method of McCormick (21). Both NKA activity and Bradford protein assays (Bio-Rad, Hercules, CA) were run in 96-well format on a SpectraMAX Plus 384 microplate reader using SOFTmax software 4.6 (Molecular Devices, Menlo Park, CA).

Gill CA activity. CA activity in the gill was measured by the electrometric pH method (12). Whole gill arches were homogenized in reaction buffer (225 mM mannitol, 75 mM sucrose, 10 mM Tris, pH 7.4) and briefly centrifuged, and the supernatant was diluted 25-fold and assayed for CA activity using 6 ml of reaction buffer at 4°C and 200 μl of CO_2-saturated distilled water to initiate the reaction. The reaction velocity was measured over a 0.15-unit pH change, and the final result was a measure of the observed rate minus the uncatalyzed rate. The pH of the reaction medium was measured by a GK2401C pH electrode (Radiometer, London, ON, Canada) connected to a PHM84 pH meter (Radiometer) and recorded by data acquisition software (Biopac with AcqKnowledge v. 3.7.3). The protein content of the supernatant was measured by the BCA protein assay.

Quantification of mRNA by real-time RT-PCR. Total RNA from the gill tissue was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA) based on the acid guanidinium thiocyanate-phenol-chloroform extraction method. Total RNA concentrations were quantified immediately by UV spectrophotometry at 260 nm. First-strand cDNA was synthesized from 1 μg of total RNA treated with DNase I (Invitrogen) and reverse transcribed to cDNA using SuperScript II RNase H-reverse transcriptase (Invitrogen). mRNA expression was quantified in duplicate on a Stratagene MX3000P real-time PCR machine using SYBR green with ROX as reference dye (Bio-Rad, Mississauga, ON, Canada). Each reaction contained 12.5 μl SYBR green mix, 1 μl of each forward and reverse primer (0.5 μM), 5.5 μl RNase/DNase-free H_2O, and 5 μl cDNA template. Cycling conditions were as follows: 3 min initial denaturation at 95°C, 40 cycles of 95°C for 15 s, 60°C for 30 s, 72°C for 30 s. This was followed by a melting curve analysis to verify the specificity of the PCR products. To account for differences in amplification efficiency between different cDNAs, standard curves were constructed for each target gene using serial dilutions of stock gill cDNA. To account for differences in cDNA production and loading differences, all samples were normalized to the expression level of the housekeeping gene elongation factor-1 alpha (EF1α), which did not change over the course of the experiment. Both water and nonreverse-transcribed RNA were assayed on each plate to ensure there was no contamination present in reagents or primers used. Primers were designed using Primer Express (Table 1; Applied Biosystems, Foster City, CA). Target genes of interest for this study were carbonic anhydrase-1 (plasma; CA-1), carbonic anhydrase-2 (cytoplasmic; CA-2), copper transporter 1 (CTR-1), ECaC, EF1α, H^+-ATPase, Na^+/H^+ exchanger 2 (NHE2), and NKA (α1a).

Table 1. *Forward and reverse primers used for real-time PCR*

Gene	Accession No.	Primer	Amplicon
CA-1	NM131110	F-ccttgctgtggttggagttt	211
		R-acactgatcggctccttcaa	
CA-2	NM199215	F-tggacatacctgggctctct	179
		R-agtggctgaggaggacgata	
ECaC	AY383562	F-acttggtcaaccgcagaaag	197
		R-cagattccacttgagcgtga	
EF1α	NM131263	F-gtgctgtgctgattgttgct	201
		R-tgtatgcgctgacttccttg	
H^+-ATPase	NM182878	F-gtatgtagacagacagctgc	120
		R-atcagcgtgatctttgcgg	
NKA α1a	AF286372	F-gccccaaaaagagaggaaac	201
		R-tgcccttggactttgacttc	
NHE-2	XM686272	F-ccactcgacccttctttgaa	104
		R-gcagatggcaaatagggaga	
CTR-1	AY077715	F-cgtgttcttgttggctgtgt	209
		R-ccaccacttggatgatgtga	

CA, carbonic anhydrase; ECaC; epithelial calcium channel; EF, elongation factor; NKA, Na^+-K^+-ATPase; NHE, Na^+/H^+ exchanger; CTR, copper transporter; F, forward; R, reverse.

Genes were normalized to EF1α, and each value was expressed as a percentage of the hard-water control, which allowed for the calculation of SE.

Western blots. Whole gill arches were homogenized in buffer (100 mM imidazole, 5 mM EDTA, 200 mM sucrose, and 0.1% deoxycholate, pH 7.6) and centrifuged at 12,000 *g* for 10 min at 4°C. The supernatant was collected and diluted to 30 μg of protein in 4× loading buffer (48 mM Tris·HCl pH 6.8, 4% glycerol, 3.2% SDS, 600 mM 2-mercaptoethanol, 1.6% bromphenol blue). Samples were denatured in boiling water for 5 min and loaded onto a 7.5% SDS-polyacrylamide gel. Samples were electrophoresed for 1 h at 150 V. Samples were transferred to a polyvinylidene fluoride membrane (Bio-Rad), and blots were incubated overnight at 4°C in 5% skim milk + PBST (10 mM phosphate buffer, 0.09% NaCl, 0.05% Tween-20, pH 7.5). Blots were washed 3× 5 min in PBST and incubated at room temperature with the primary antibody diluted in 3% skim milk + PBST. Primary antibodies included rainbow trout ECaC (1:1,000), NKA (chicken; 1:500), and tubulin as a loading control (chick retina; 6G7, 1:500). Tubulin was used because of its availability and good reactivity with zebrafish tissues. Membranes were washed 3× 5 min in PBST and incubated for 1 h at room temperature with an horseradish peroxidase-conjugated anti-rabbit IgG (for ECaC, 1:25,000; Perkin-Elmer, Boston, MA) or anti-mouse IgG (for NKA and tubulin, 1:50,000). After 3× 5-min washes with PBST, proteins were visualized with a Western Lightning chemiluminescence kit, following the manufacturer's protocol (Perkin-Elmer). Blots and band density analysis were completed on a ChemiImager (AlphaInnotech, San Leandro, CA), which used pixel density to quantify band intensity. Bands were normalized to tubulin and expressed as a ratio of the hard-water control.

Statistical analysis. Statistical analysis was performed with Sigma Stat (SPSS, Chicago, IL). All data have been expressed as means ± SE. A one-way ANOVA and a Dunnett's test was used to test for significance relative to the control values, and a Student's *t*-test was used to compare between treatments and controls at any given time point ($P < 0.05$).

RESULTS

Whole body ion content. Despite the drop in ion concentration from hard water to soft water (Fig. 1), there were no significant differences found in whole body ions. Na^+, Ca^{2+}, and Mg^{2+} values fluctuated little from average values of 48.42 ± 7.61, 29.47 ± 4.43, and 20.67 ± 1.87 mmol/kg, respectively (Fig. 2).

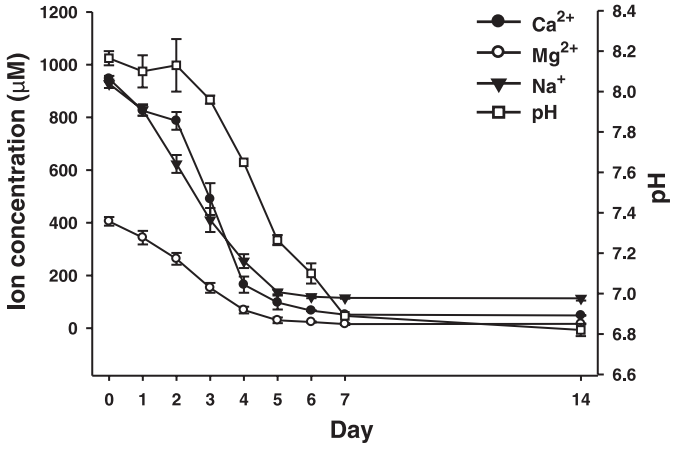

Fig. 1. Measured water pH and concentrations of Na^+, Ca^{2+}, and Mg^{2+} (means ± SE) during acclimation of zebrafish from hard water to soft water, over a period of 7 days, with a further acclimation for 7 days ($n = 2$).

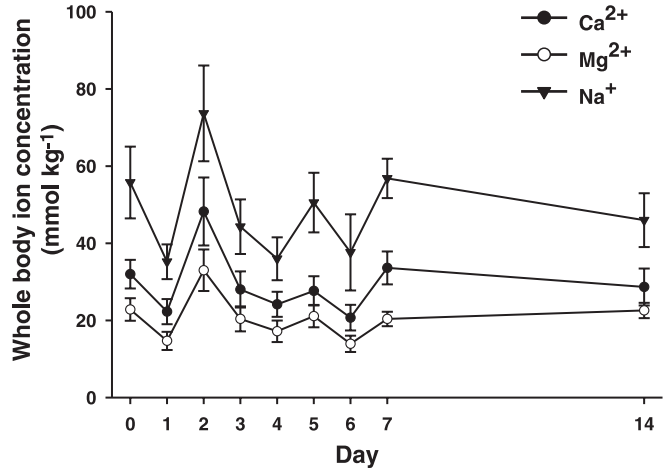

Fig. 2. Whole body concentrations of Na^+, Ca^{2+}, and Mg^{2+} (means ± SE) measured from zebrafish exposed to a progressive softwater transfer over 7 days, and acclimation in soft water for an additional 7 days ($n = 6$).

NKA and CA activity. Values of gill NKA activity in hard water-acclimated controls were 2.17 ± 0.38 μmol ADP·mg protein^{-1}·h^{-1}, and the activity increased ~2.5-fold to a maximum activity of 5.66 ± 0.55 μmol ADP·mg protein^{-1}·h^{-1} after 3 days of progressive water ion depletion (Fig. 3). The NKA activity decreased after 3 days to values not significantly different from hard water-acclimated controls (Fig. 3).

We measured gill CA activity on whole gill arches from fish exposed to hard water, and fish exposed to soft water acclimation on *day 7*, and after a further 1 wk in soft water. Initial gill CA activity was 865 ± 73 μmol·CO_2 mg^{-1}·min^{-1} in hard water, which increased slightly to 1,231 ± 211 μmol·CO_2 mg^{-1}·min^{-1} at *day 7* and 934 ± 151 μmol·CO_2 mg^{-1}·min^{-1} after 1 wk in typical soft water. These changes were not statistically significant.

Gene expression profile. Although NKA enzyme activity increased significantly during the first 3 days of soft-water

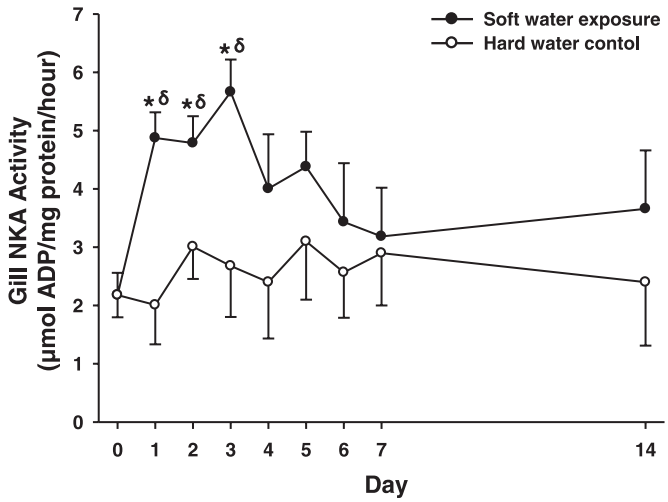

Fig. 3. Changes in gill Na^+-K^+-ATPase (NKA) activity in zebrafish during a progressive 7-day soft-water acclimation experiment and acclimation to soft water for an additional 7 days. *Significant difference from the *day 0* control value as determined by 1-way ANOVA and by Dunnett's test. δSignificant difference at a given time between hard-water and soft-water treatments as determined by Student's *t*-test ($n = 6–8$, $P < 0.05$).

acclimation, there was no significant change in the mRNA expression of the gill NKA isoform α1a gene over the entire 7-day period (Fig. 4). Similarly, there were no significant changes in H^+-ATPase mRNA expression over the duration of the experiment (Fig. 5). We did see significant changes in the mRNA expression of the ECaC gene (Fig. 6), which corresponded with decreasing waterborne Ca^{2+} concentrations (Fig. 1). After 6 days of progressive ion depletion, there was a dramatic fourfold increase in gill ECaC mRNA, which attenuated only slightly over *days 7–14* and remained significantly elevated up to the end of the experiment (Fig. 6). Likewise, we saw a significant increase in the mRNA expression of CA-1 and CA-2 within the gill after *days 6* and *5*, respectively (Fig. 7, *A* and *B*). It should be noted that the magnitude of the gene expression increased varied between CA-1 and CA-2 (1.5-fold vs. 6-fold, respectively). Furthermore, we saw a significant increase in NHE-2 gene expression after *day 6*, which remained elevated after 7 days in soft water (Fig. 8). Interestingly, there were changes in the mRNA expression of the Cu transport protein CTR-1, with rapid peak in expression after 6 days of ion depletion (Fig. 9). This was an ion-specific effect since waterborne copper analysis indicates that copper levels did not change throughout the course of the soft-water acclimation (1.93 ± 0.07 μg/l).

Western blot analysis. We examined the protein expression levels of gill NKA and gill ECaC to elucidate the effect of gene expression on protein levels in the gills. There were no significant changes in gill NKA protein levels over the course of the experiment (Fig. 10), despite the initial changes in enzyme activity (Fig. 3). Similar to ECaC mRNA changes, which peaked early in the acclimation period (Fig. 6), there was a progressive increase in ECaC protein expression, which increased threefold at *day 14* (Fig. 11).

DISCUSSION

This study demonstrates that zebrafish gills display a high degree of phenotypic plasticity to environmental challenge.

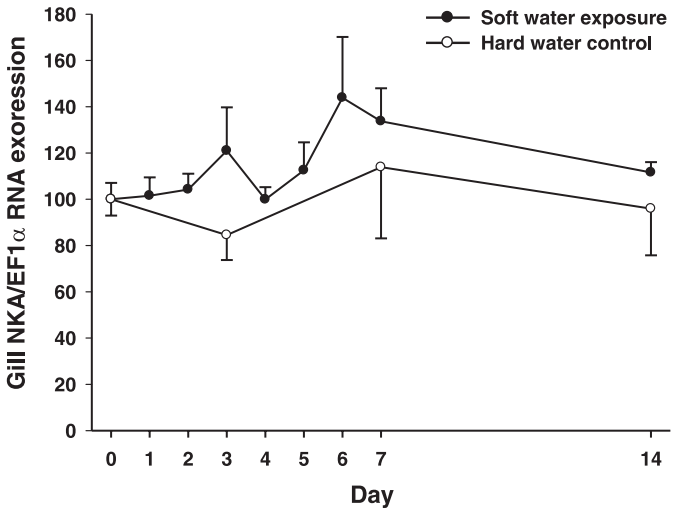

Fig. 4. mRNA expression of the NKA alpha isoform 1 (α1a) gene in the zebrafish gill during a progressive 7-day soft-water acclimation experiment and acclimation to soft water for an additional 7 days. Values are represented as means ± SE and based as a percentage of the control (100%). There were no significant differences between controls or treatments ($n = 5–6$, $P < 0.05$). EF1α, elongation factor-1 alpha.

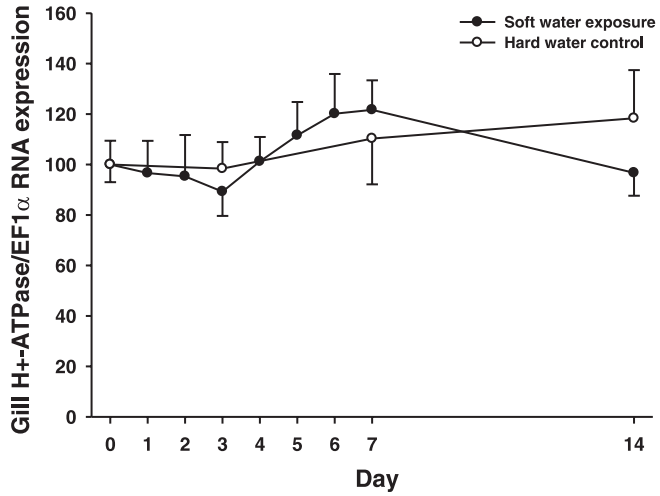

Fig. 5. mRNA expression of the H^+-ATPase gene in the zebrafish gill during a progressive 7-day soft-water acclimation experiment and acclimation to soft water for an additional 7 days. Values are represented as means ± SE and based as a percentage of the control (100%). There were no significant differences between controls or treatments ($n = 5–6$, $P < 0.05$).

Zebrafish respond to soft-water acclimation with the induction of gene expression and protein expression for key ion channels (such as ECaC). They also invoke the upregulation of NKA activity possibly through covalent modulation or translocation of the existing protein. These represent potential mechanisms which allow zebrafish to maintain ion homeostasis in an extremely low ion environment.

We assessed the osmoregulatory response of zebrafish to a step-wise acclimation to soft water over a period of 7 days by examining both changes in mRNA of known ion-responsive enzymes (NKA, H^+-ATPase, CA) and ion transport proteins, ECaC and NKA. This is an important factor to consider especially in the emerging field of toxicogenomics since there

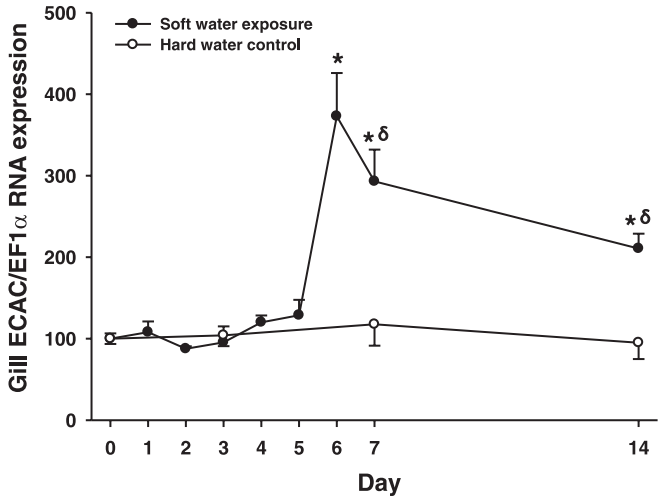

Fig. 6. mRNA expression of the epithelial calcium channel (ECaC) gene in the zebrafish gill during a progressive 7-day soft-water acclimation experiment and acclimation to soft water for an additional 7 days. Values are represented as means ± SE and based as a percentage of the control (100%). *Significant difference from the *day 0* control value as determined by 1-way ANOVA followed by a Dunnett's test. δSignificant difference at a given time between hard-water and soft-water treatments as determined by Student's *t*-test ($n = 5–6$, $P < 0.05$).

is the need to initially test metals in soft water where effects are not confounded by competing cations and complexing anions (5, 11, 23). Although this represents a limited number of total gill genes and proteins that may respond, they highlight the flexibility of this organ to environmental changes. Future studies can expand on the short list of genes examined here with the use of commercially produced microarrays. Other environmental stressors, such as heat or waterborne-metal stresses, are known to induce a number of genes and proteins in both the goby (*Gillichthys mirabilis*) and rainbow trout (*Oncorhynchus mykiss*), including genes involved in chloride and calcium transport, and NHE proteins (4, 13, respectively). We demonstrate here that care is needed in the design of toxicity experiments since soft-water acclimation alone can induce significant changes in gene and protein expression. Indeed changes in environmental ion concentrations resulted in changes in mRNA for an important metal transport protein (CTR-1; Fig. 8).

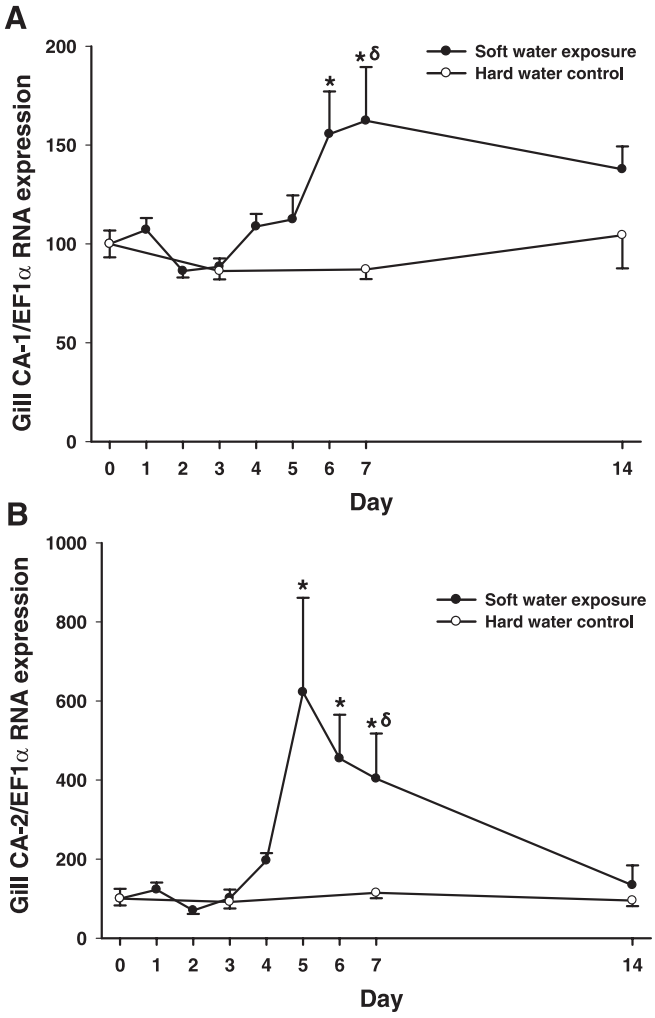

Fig. 7. mRNA expression of the carbonic anhydrase 1 (CA-1; *A*) and carbonic anhydrase 2 (CA-2; *B*) genes in the zebrafish gill during a progressive 7-day soft-water acclimation experiment, and acclimation to soft water for an additional 7 days. Values are represented as means ± SE and based as a percentage of the control (100%). *Significant difference from the *day 0* control value as determined by 1-way ANOVA followed by a Dunnett's test. δSignificant difference at a given time between hard-water and soft-water treatments as determined by Student's *t*-test ($n = 5–6$, $P < 0.05$).

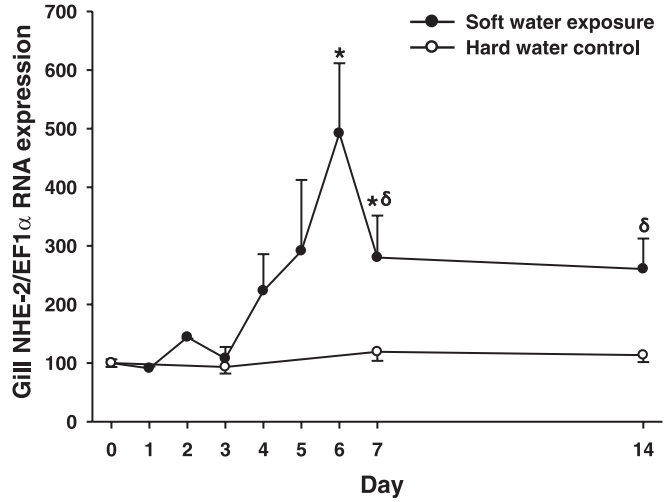

Fig. 8. mRNA expression of the Na^+-H^+ exchanger 2 (NHE-2) gene in the zebrafish gill during a progressive 7-day soft-water acclimation experiment and acclimation to soft water for an additional 7 days. Values are represented as means ± SE and based as a percentage of the control (100%). *Significant difference from the *day 0* control value as determined by 1-way ANOVA followed by a Dunnett's test ($n = 5–6$, $P < 0.05$). δSignificant difference at a given time between hard-water and soft-water treatments as determined by Student's *t*-test ($n = 5–6$, $P < 0.05$).

This is the first study to examine gill NKA enzyme activity in zebrafish and the changes in expression during soft-water acclimation. Enzyme activities initially rise upon soft-water acclimation and are elevated after 1 day of progressive soft-water acclimation (Fig. 3). We found there were no significant changes in the gene expression of the NKA isoform of the catalytic subunit α1a (Fig. 4), shown to be upregulated during sea water to freshwater acclimation in other species (19, 31). However, this is not surprising, since the magnitude in the change of salinity is only from 0.1 ppm to 0.01 ppm. We also saw no change in total protein expression (Fig. 9) using an

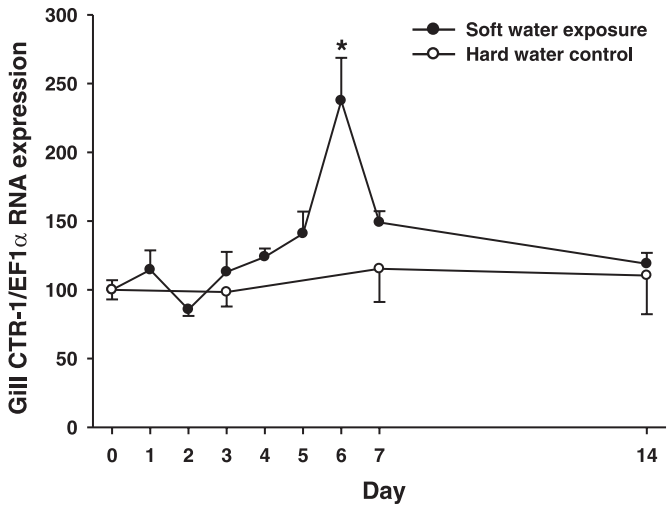

Fig. 9. mRNA expression of the copper transporter 1 (CTR-1) gene in the zebrafish gill during a progressive 7-day soft-water acclimation experiment and acclimation to soft water for an additional 7 days. Values are represented as means ± SE and based as a percentage of the control (100%). *Significant difference from the *day 0* control value as determined by 1-way ANOVA followed by a Dunnett's test ($n = 5–6$, $P < 0.05$).

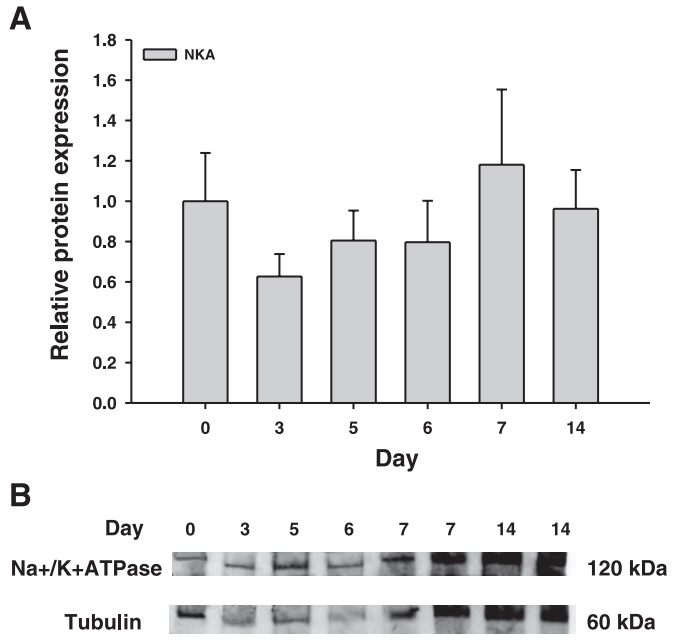

Fig. 10. Relative protein expression of gill NKA (*A*) and representative Western blot of gill NKA (*B*) in zebrafish during a progressive 7-day soft-water acclimation experiment and acclimation to soft water for an additional 7 days. Tubulin was also measured to account for differences in protein loading ($n = 4–5$). There were no significant differences in *A*. Values are represented as means ± SE and based as ratio of the control.

antibody that recognizes all α-subunits isoforms. These data suggest that NKA activity is not regulated by transcriptional means but, rather, is modified at the protein level in response to soft-water acclimation (Fig. 9). It also suggests that unlike the situation seen in trout with respect to changes in salinity (31), zebrafish do not switch α1a- and b-subunit isoforms of NKA to alter enzyme kinetics with changes in water hardness. Various hormones modulate NKA activity, and short-term regulation can have direct effects on the kinetics of enzyme activity or the translocation of NKA pumps between the plasma membrane and the intracellular stores (see Ref. 35 for review). This rapid response in NKA may explain how zebrafish are able to maintain ion balance even in the first days of acclimation before any changes in gene expression have occurred. In addition, the V-type H^+-ATPase may play a role in Na^+ uptake by modifying the electrochemical gradient of the apical membrane by extruding H^+ ions, which favors the absorption of Na^+ (16–18). We initially hypothesized there would be a change in the expression of the H^+-ATPase mRNA upon soft-water acclimation. However, there were no significant changes in expression (Fig. 5). Moreover, Boisen et al. (2) did not see any changes in H^+-ATPase protein abundance after soft-water exposure in zebrafish. This does not necessarily downplay the importance of H^+-ATPase in maintaining ion homeostasis in zebrafish exposed to soft water, since H^+-ATPase kinetics can increase without changes in protein abundance or location (9). Conversely, we did see a fivefold increase in NHE-2 gene expression after *day 6* (Fig. 8). NHE proteins have been characterized in the gills of many freshwater species of fish (7, 37), and their potential role in Na^+ uptake in the gill has been proposed (32). Certainly, NHE-2 may play an essential role in the uptake of Na^+ from the water into the gill cell. However, NHE-2 has yet to be localized in the gill of the zebrafish.

During soft-water acclimation, we saw a significant elevation in the gene expression of CA-1 and CA-2 in the gills of zebrafish after *days 6* and *5*, respectively (Fig. 7, *A* and *B*). However, CA-2 had a greater magnitude of gene expression than CA-1. When the CA isozymes were compared with the red blood cell (RBC) (accession no. AY307082) and cytoplasmic (accession no. AY514870) isozymes of trout CA, we found that CA-1 shared a 78% homology with the RBC isozyme, and CA-2 shared a 68% homology with the cytoplasmic isozyme. Due to the small size of the gill samples and the inability to flush out all of the contaminating blood, it was necessary to measure both CA isozyme expression levels to determine whether there were any contaminating effects of blood and to determine whether an ion-poor medium contributed to increased cytoplasmic CA expression. We did see a significant sixfold increase in CA-2 after *day 6*, indicating that cytoplasmic CA may aid in ion uptake. Despite this increase in gene expression, we did not see a significant elevation in CA enzyme activity on these days, although there was a slight but nonsignificant elevation on *day 7* of acclimation. Although this represents the combined activity of both CA isoforms, the cytoplasmic activity is expected to be threefold higher per unit gill protein (23). Cytoplasmic CA can provide bicarbonate for the Cl^-/HCO_3^- exchanger on the apical surface of the gill and

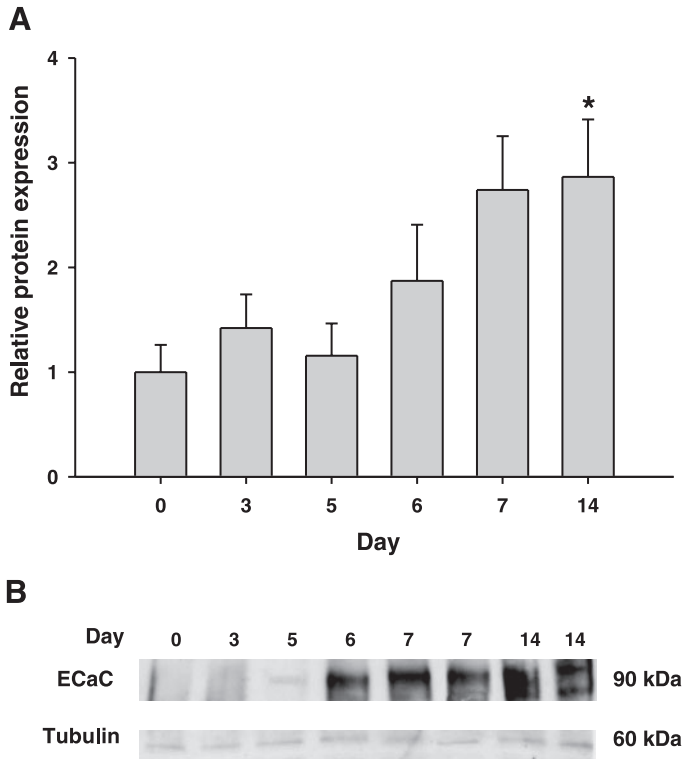

Fig. 11. Relative protein expression of gill epithelial calcium channels (ECaC; *A*) and representative Western blot of gill ECaC (*B*) in zebrafish during a progressive 7-day soft-water acclimation experiment and acclimation to soft water for an additional 7 days. Tubulin was also measured to account for differences in protein loading. In *A*, *significant difference from the initial hard-water value (*day 0*) as determined by 1-way ANOVA followed by a Dunnett's test ($n = 4–5$, $P < 0.05$). Values are represented as means ± SE and based as ratio of the control.

aid in Cl^- uptake (10). Generally there is evidence to support the role of cytoplasmic CA in ion uptake (2, 6). Furthermore, CA may provide H^+ ions for an apical Na^+/H^+-exchanger (32), which would allow for the passage of Na^+ ions into the gill of the zebrafish.

Aside from diet, a major source of Ca^{2+} for teleost fish comes from the surrounding medium. Ca^{2+} from the environment is taken up by ECaC and transported across the basolateral membrane via Ca^{2+}-ATPases or Na^+/Ca^{2+} exchangers (8, 36). Therefore, it is likely that changes in environmental Ca^{2+} levels will result in significant changes in ECaC to maintain adequate Ca^{2+} uptake. After *day 6* (80% soft water), there was 3.5-fold increase in the gene expression of ECaC, which remained elevated for the remainder of the experiment (Fig. 6). This coincided with a corresponding increase in the protein level, with a threefold increase after a 1-wk acclimation in soft water (Fig. 11; 51 $\pm$ 1.0 μM Ca^{2+}) demonstrating that ECaC is primarily transcriptionally regulated under these conditions. Similarly, Pan et al. (25) demonstrated an increase in the expression of gill and skin ECaC in zebrafish embryos after exposure to 20 μM Ca^{2+} and localized the ECaC to the mitochondrial rich cells. In addition, Shahsavarani and Perry (33) showed significant increases in ECaC gene expression (10-fold) and protein expression (2.5-fold) after a 5-day exposure of rainbow trout to low Ca^{2+} (20–30 μM). Combined, these results indicate that ECaC plays a key role in Ca^{2+} uptake from the surrounding medium, although how the cells sense Ca^{2+} concentrations is not fully understood. Potentially, the presence of a Ca^{2+} receptor might mediate changes in ECaC expression (30). There is a need to further examine changes in plasma ion levels and key ionoregulatory hormones that may affect changes in gill epithelial structure and ionoregulatory capacity during soft-water acclimation.

The primary goal of this study was to examine the effects of soft-water acclimation on the ionoregulatory capabilities of a tropical, soft water-tolerant fish. In addition zebrafish are becoming an important model in vertebrate physiology and aquatic toxicology, due to their publicly available genome and their ability to tolerate soft water environments. To this end, we examined a recently identified CTR-1 found in the gill epithelium of zebrafish (20). Since copper is an essential micronutrient obtained from the environment, we predicted that soft-water acclimation might affect CTR-1 expression. The mRNA expression for this transporter spiked (threefold increase) after 6 days of soft-water acclimation but declined to baseline 1 day later (Fig. 9) despite constant environmental copper content. This result suggests that the timing of soft-water acclimation might be crucial in studies which require a preacclimation period (e.g., metal toxicology). Despite the transient change in CTR-1 expression, zebrafish in soft water may have an increased sensitivity to copper, or to other metals, since many metals have shared pathways with Na^+ and Ca^{2+} transport across the apical surface of the gill (5, 11, 23).

The results of this study indicate a high degree of phenotypic plasticity in zebrafish gills during soft-water acclimation to maintain ion homeostasis. Zebrafish gills respond to soft-water acclimation through genomic (ECaC, CA-2, NHE) and nongenomic changes (NKA) in ionoregulatory machinery. These represent potential mechanisms that allow zebrafish to tightly regulate whole body ion levels, although further work is required to establish causations between these changes and ion transport needs to be established. The ability to apply molecular techniques such as reverse genetics to the zebrafish model will allow for direct connections to be made between gill remodeling and ion homeostasis.

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GRANTS

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