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Comparative evaluation of Na^+ uptake in *Cyprinodon variegatus variegatus* (Lacepede) and *Cyprinodon variegatus hubbsi* (Carr) (Cyprinodontiformes, Teleostei): Evaluation of NHE function in high and low Na^+ freshwater

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ABSTRACT

The euryhaline pupfish, *Cyprinodon variegatus variegatus* (*Cvv*), can successfully osmoregulate in ≥ 2mM Na^+ and a freshwater population (*Cyprinodon variegatus hubbsi; Cvh*) osmoregulates at ≥0.1mM Na^+. We previously demonstrated that *Cvv* relies on an apical NKCC and NHE in the gill for Na^+ uptake in high (7mM) and intermediate (2mM) Na^+ concentrations, while *Cvh* relies only on NHE for Na^+ uptake. This study investigated whether differential NHE isoform use explains differences in Na^+ uptake kinetics between these two populations. We further studied whether *Cvh* uses a NHE-Rh metabolon or carbonic anhydrase (CA) to overcome thermodynamic challenges of NHE function in dilute freshwater. Transfer to more dilute freshwater resulted in upregulation of *nhe-2* (*Cvv* only) and *nhe-3* (*Cvv* and *Cvh*). Relative expression of *nhe-3* compared to *nhe-2* was 2-fold higher in *Cvv*, but 200-fold higher in Cvh suggesting that nhe-3 expression is an important freshwater adaptation for *Cvh*. Simultaneous measurement of Na^+ and T_{amm} flux under various conditions provided no support for a NHE-Rh metabolon in either population. Carbonic anhydrase activity in *Cvv* was comparable in 7 and 2mM Na^+ acclimated fish. In *Cvh*, CA activity increased by 75% in 0.1mM Na^+ acclimated fish compared to 7mM Na^+ fish. Ethoxzolamide had variable effects, stimulating and reducing Na^+ uptake in *Cvv* acclimated to 7 and 2mM Na^+, while reducing Na^+ uptake in 7 and 0.1mM Na^+ acclimated *Cvh*. This suggests that CA plays important, but different roles in regulating Na^+ uptake in *Cvv* and *Cvh*.

1. Introduction

In freshwater, teleost fish are hyperosmotic relative to their environment and therefore suffer from diffusive salt loss and water gain. To combat this, fish actively take up osmolytes (primarily Na^+ and Cl^-) at the gill against their concentration gradients and efficiently reabsorb salts at the kidney producing copious volumes of dilute urine (Marshall and Grosell, 2006). Common to all fish, Na^+ uptake at the gill is largely driven by a basolateral Na^+/K^+-ATPase. In contrast, a number of different transport proteins can be involved in Na^+ uptake at the apical membrane of the gill. There is variation in use of these different proteins both between species and within species under different environmental conditions. In general, there are three types of transport proteins: Cl^--dependent Na^+ uptake via a Na^+:Cl^- co-transporter (NCC) or Na^+:K^+:2Cl^- co-transporter (NKCC), a putative Na^+ channel linked to a H^+-ATPase that generates the electrochemical gradient for the channel, and several isoforms of Na^+/H^+ exchangers (NHEs) (Hwang et al., 2011).

Several studies have now shown that NCC may be involved in Na^+ uptake in zebrafish embryos in relatively low (0.8 mM) Na^+ water via multiple lines of evidence: inhibitor studies, gene expression, and immunohistochemistry (Wang et al., 2009; Kwong and Perry, 2013). Available evidence in pupfish (*Cyprinodon variegatus*) indicates that NKCC operates in relatively saline freshwaters (≥7 mM Na^+) (Brix and Grosell, 2012), but this tentative finding is based only on inhibitor experiments and awaits confirmation of both gene and protein expression. The Na^+ channel/H^+-ATPase system appears to function over a relatively wide range of ambient Na^+ concentrations (0.05–>1 mM Na^+), depending on the species (Hwang et al., 2011). A recent study indicates that the

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identity of the putative Na^+ channel in rainbow trout is an acid-sensing ion channel (ASIC) (Dymowska et al., 2014). There are two main NHE isoforms (NHE-2 and NHE-3) identified as contributing to apical Na^+ uptake in freshwater fish. Both isoforms are found in a range of species, often operating in conjunction with one or more of the other apical transport proteins. Based on mRNA expression studies, NHE-3 tends to be the dominant isoform expressed under low Na^+ conditions in most species. For example, NHE-3 has been shown to contribute to Na^+ uptake for several species (*Danio rerio*, *Oryzias latipes*) (Yan et al., 2007; Wu et al., 2010; Kumai and Perry, 2011) at low ambient Na^+ concentrations and low pH (*D. rerio*, *Tribolodon hakonensis*) (Hirata et al., 2003; Kumai and Perry, 2011).

Based on our current understanding of intracellular pH and Na^+ concentrations, operation of NHE-3 in low Na^+ or low pH environments should not be possible from a thermodynamic perspective (Parks et al., 2008). In fact, under these conditions, NHEs should operate in the reverse direction exporting Na^+ and taking up H^+. Fish have evolved at least two methods for dealing with these thermodynamic constraints. In the Japanese dace (*T. hakonensis*) which live in water with ~1 mM Na^+ and pH 3.5, carbonic anhydrase (CAc; cytosolic CA (Esbaugh and Tufts, 2006)) mediated hydration of CO_2 produces H^+ and HCO_3^-. The HCO_3^- is exported across the basolateral membrane via a Na^+:HCO_3^- co-transporter (NBC) leaving a large intracellular H^+ pool to drive operation of NHE-3 under low pH conditions (Hirata et al., 2003). An analogous system appears to operate in the euryhaline fish *Fundulus heteroclitus* between NHE-2 and CAc, allowing for Na^+ uptake at concentrations as low as 0.17 mM in this species (Patrick et al., 1997; Scott et al., 2004, 2005).

More recently a series of elegant experiments demonstrated that *D. rerio* and *O. latipes* (low pH and low Na^+) accomplish Na^+ uptake via NHE-3 in conjunction with the Rh glycoprotein Rhcg-1, which is located in the apical membrane of ionocytes (Wu et al., 2010; Kumai and Perry, 2011; Lin et al., 2012; Shih et al., 2012). The exact mechanism by which this metabolon works is currently not clear. Ammonia is transported from the blood into the ionocyte via the basolateral Rhbg and through the apical Rhcg-1 in ionocytes and Rhcg-2 in pavement cells (Nakada et al., 2007a, 2007b; Nawata et al., 2007). Ammonia is thought to bind to Rhcg as NH_4^+, but pass through the channel as NH_3 (Nawata et al., 2010). This aids in the forward operation (i.e., Na^+ inward, H^+ outward) of NHEs in several possible ways. First, proton trapping by NH_3 is likely to occur in the ionocyte boundary layer providing localized alkalinization of the external environment, making forward operation of NHE-3 more favorable. Second, intracellularly, the export of NH_3 by Rhcg-1 may cause a localized $NH_3-NH_4^+$ disequilibria resulting in the continuous formation of H^+ which consequently causes a localized acidification on the intracellular side of the apical membrane, again creating a favorable gradient for forward operation of NHE-3. Finally, it was recently demonstrated that NHE-3b in *D. rerio* can act as a Na^+/NH_4^+ exchanger (Ito et al., 2014), so that when fish experience high internal ammonia loads, NHE function in dilute water could be directly driven by ammonia excretion.

The euryhaline pupfish *Cyprinodon variegatus variegatus* (*Cvv*) occurs along the Gulf and Atlantic coasts of North America. *Cvv* tolerates salinities ranging from freshwater up to 167 g L^{-1} (Nordlie, 2006). However, previous studies indicate that *Cvv* does not survive (long-term), grow or reproduce in freshwater with <2 mM Na^+ (Dunson et al., 1998; Brix and Grosell, 2013b). A second population of *C. variegatus* currently given subspecies status (*Cyprinodon variegatus hubbsi; Cvh*) only occurs in eight freshwater lakes in central Florida (Carr, 1936; Guillory and Johnson, 1986). These lakes have ambient Na^+ concentrations of 0.4–1.0 mM Na^+, below the level typically tolerated by *Cvv*, suggesting that *Cvh* has adapted to this more dilute freshwater environment.

In a previous comparative study of Na^+ transport in the two populations, we demonstrated that *Cvv* and *Cvh* bred and raised under common garden conditions (freshwater with 7 mM Na^+) have similar low affinity Na^+ uptake kinetics (K_m = 7000–38,000 μM) when acclimated to 2 or 7 mM Na^+, while *Cvh* switches to a high affinity system (K_m = 100–140 μM) in low Na^+ freshwater (≤1 mM Na^+) which is characteristic of its native habitat (Brix and Grosell, 2012). We further demonstrated through a series of experiments with pharmacological inhibitors, that *Cvv* appears to utilize a combination of an NHE and apical NKCC for Na^+ uptake at 7 mM Na^+, but only an NHE at 2 mM Na^+. In contrast, *Cvh* appears to utilize only a low affinity NHE when acclimated to 2 or 7 mM Na^+, and a high affinity NHE when acclimated to 0.1 or 1 mM Na^+.

Given the above, we hypothesized that previously observed differences in Na^+ transport kinetics between *Cvv* and *Cvh* were the result of differential use of NHE isoforms. We further hypothesized that NHE function in *Cvh* under low Na^+ conditions was the result of an NHE-Rh metabolon, elevated CA activity, or a combination of these two mechanisms. To test these hypotheses, we conducted a series of comparative experiments at the whole animal and mRNA expression levels of organization. First, we evaluated simultaneous Na^+ uptake and total ammonia (T_{amm}) excretion in *Cvv* and *Cvh* acclimated to high and low Na^+ freshwater combined with a series of treatments in which Na^+ or T_{amm} gradients were experimentally manipulated. Next, the effect of the CA inhibitor ethoxzolamide on Na^+ uptake was examined and CA activity was characterized in fish acclimated to high and low Na^+ freshwater. Finally, we characterized changes in mRNA expression of key proteins (NKA, H^+-ATPase, NHE-2, NHE-3, Rhcg-1, Rhcg-2, CAc) potentially involved in Na^+ uptake in fish acutely transferred from 7 to 2 mM and 2 to 0.1 mM Na^+ freshwater.

2. Methods and materials

2.1. Animal holding

Adult *Cvv* were collected from a small pond on Key Biscayne, FL that is intermittently connected to Biscayne Bay. Salinity in this pond ranges seasonally from 12–39 g L^{-1}. Fish were held at the University of Miami in 110 L glass aquaria under flow-through conditions initially with filtered natural seawater (35 g L^{-1}) from Bear Cut, FL. Fish were acclimated to near freshwater conditions (0.3 g L^{-1}; 7 mM Na^+, pH 7.9) and held for >30 d. Dechlorinated City of Miami tapwater (~1.0 mM Na^+, 1.0 mM Cl^-, 0.5 mM Ca^{2+}, 0.2 mM Mg^{2+}, 0.5 mM SO_4^{2-}, 0.8 mM HCO_3^-, pH 7.9) was mixed with filtered natural seawater to achieve the desired salinity. After this holding period, fish were bred and offspring were hatched and raised to sexual maturity under the same near freshwater conditions (0.3 g L^{-1}; 7 mM Na^+, pH 7.9). This second generation was then bred under the same conditions to produce fish used for all experiments in this study. Throughout the acclimation and holding period, as well as during all experiments, *Cvv* were fed *Artemia* nauplii for 2 weeks post-hatch and then over a 1-week period gradually switched over to flake food (Tetramin™ Tropical Flakes).

Adult *Cvh* were originally collected from Lake Weir, Florida (0.9 mM Na^+, 1.1 mM Cl^-, 0.1 mM Ca^{2+}, 0.2 mM Mg^{2+}, 0.1 mM SO_4^{2-}, 0.2 mM HCO_3^-, pH 7.5). Fish were held at the University of Miami in 110 L glass aquaria under flow-through conditions with dechlorinated City of Miami tapwater. Adult fish were bred and offspring were hatched and raised in the same near freshwater conditions described for *Cvv*. This second generation was then bred under the same conditions to produce fish used for all experiments in this study. Throughout the holding period, as well as during all experiments, *Cvh* were fed *Artemia* nauplii for the first 2 weeks and then over a 1-week period gradually switched over to bloodworms (*Chironomus* sp.), as *Cvh* refused to eat the flake food diet fed to *Cvv*.

Cvv and *Cvh* were collected under Florida Fish and Wildlife Conservation Commission permit FNE-2010–09. Experiments were approved by the University of Miami animal care committee.

2.2. Experiments investigating NHE-Rh metabolon for Na^+ uptake

We used an approach conceptually similar to that devised by Kumai and Perry (2011) to evaluate the potential presence of an NHE-Rh metabolon in either *Cvh* or *Cvv*, manipulating independently or simultaneously Na^+ and T_{amm} gradients and then measuring the effects of these manipulations on concurrent Na^+ influx and T_{amm} excretion. The same treatments were used on *Cvv* and *Cvh* acclimated to 7 mM Na^+, and *Cvh* acclimated to 0.1 mM Na^+. Generally, for each treatment, 8 juvenile fish (50–250 mg) were placed individually in 20 mL of a defined media (480 μM $CaSO_4$, 150 μM $MgSO_4$, 100 μM $KHCO_3$, pH 7.0) to which NaCl was added to achieve either 0.1 or 7 mM Na^+. Fish were not fed for 48 h prior to the experiment. Test solutions were continuously aerated to maintain dissolved oxygen levels during the flux period. Fish were allowed to acclimate to this media for 10 min after which 1–2 μCi of ^{22}Na (depending on ambient Na^+ concentration) was added to the solution. The flux solution was sampled after 1 min for measurements of $[Na^+]$, ^{22}Na activity, and T_{amm}. The total flux exposure period ranged from 1–2 h, depending on the ambient Na^+ concentration being tested. In all cases, the internal specific activity was <1% of the external specific activity such that correction for backflux was unnecessary (Maetz, 1956). At the end of the exposure period, water samples for $[Na^+]$, ^{22}Na activity, and T_{amm} were again collected, fish were removed from the exposure media, double rinsed in a 100 mM Na^+ solution to displace any loosely bound ^{22}Na, blotted dry, weighed to the nearest 0.1 mg and then assayed individually for radioactivity.

We used the following treatments to elicit changes in the Na^+ and/or T_{amm} gradients to evaluate the potential presence of a NHE-Rh metabolon: Fed — Fish were fed to satiation twice per day for 2 d, including a meal 3 h prior to experimentation with the objective of increasing the efflux of T_{amm} from the fish; pH 4.5 — Fish were acclimated to pH 4.5 for 1 week and the exposure water pH was adjusted to pH 4.5 using HNO_3; HEA — Fish were exposed to high environmental ammonia (2 mM) as $(NH_4)_2SO_4$ during the Na^+/T_{amm} flux. We did not acclimate fish to this condition as our objective to simply provide an unfavorable gradient for T_{amm} excretion; MOPS — Fish were exposed to 5 mM of the buffer 3-(N-morpholino) propanesulfonic acid adjusted to pH 7.0 with KOH during the Na^+/T_{amm} flux, but were not acclimated to this condition; and EIPA — Fish were exposed to 5×10^{-5} M (5-(*N*-ethyl-*N*-isopropyl)-amiloride), which is a potent NHE inhibitor with low affinity for Na^+ channels (Kleyman and Cragoe, 1988), during the Na^+/T_{amm} flux, but were not acclimated to this condition. In all cases where the fish were not acclimated to treatment conditions, they were exposed to the treatment for 10 min prior to initiation of the flux. A separate carrier control was also performed for the EIPA experiment. It was found that both DMSO and ethanol were not suitable carriers as they interfered with the colorimetric assay used to measure T_{amm}. An alternate carrier (2-hydroxypropyl)-β-cyclodextrin was found to be suitable for both solubilizing EIPA and performing the ammonia assay.

2.3. Experiments investigating NHE–CA model for Na^+ uptake

To investigate the NHE–CA model for Na^+ uptake, the effects of the CA inhibitor ethoxzolamide (6-ethoxy-1,3-benzothiazole-2-sulfonamide) on Na^+ uptake was characterized. These experiments were performed on juvenile (50–250 mg) *Cvh* acclimated to either 0.1 or 7 mM Na^+ and *Cvv* acclimated to either 2 or 7 mM Na^+. The same defined media and general protocol described above was used for these experiments except for each treatment 8 juvenile fish were exposed together in 50 mL of water. Treatment fish were exposed to 10^{-4} M ethoxzolamide dissolved in DMSO (0.1% final concentration). DMSO was added to the control groups at the same concentration.

In a second experiment, adult *Cvv* and *Cvh* were acclimated to 7 (*Cvv* and *Cvh*), 2 (*Cvv*), or 0.1 (*Cvh*) mM Na^+ for four weeks. After acclimation, fish were euthanized with an overdose of MS-222 after which fish gills were perfused with a 150 mM NaCl heparinized saline to remove blood. Fish gill baskets were then dissected out, flash frozen in liquid N_2, and stored at −80 °C until use in CA activity assays. Carbonic anhydrase activity was measured using the electrometric delta pH method (Henry, 1991). Gill tissue was homogenized in 1 mL of reaction buffer (225 mM mannitol, 75 mM sucrose, 10 mM Tris base; Sigma, MO, USA) on ice and briefly centrifuged (1 min × 10,000 rpm) to pellet cellular debris. The reaction medium consisted of 2.5 mL of buffer and 5 μL of the supernatant from the gill homogenate kept at 4 °C. The reaction was started by adding 100 μL of CO_2 saturated Milli-Q water using a gas tight Hamilton syringe and the reaction rate was measured over a pH change of 0.15 units (+10 mV). To calculate the true catalyzed reaction rate, the uncatalyzed reaction rate was subtracted, and the buffer capacity of the reaction medium was used to convert the rate from mV into mol H^+ per unit time. The pH was measured using a PHC4000 combined pH electrode (Radiometer Analytical, Lyon, France) attached to a PHM220 lab pH meter (Radiometer Analytical, Lyon, France). All results were normalized to total protein concentrations as detected using the Bradford assay (Sigma) with bovine serum albumin standards and measured using a plate spectrophotometer (Molecular Devices, Sunnyvale, CA).

2.4. Whole body Na^+ and mRNA expression experiments

To provide additional support for the above experiments, we conducted a series of acute salinity transfers and monitored changes in mRNA expression for genes of interest in fish gills. Adult *Cvv* and *Cvh* were acclimated to either 7 or 2 mM Na^+ freshwater for at least 4 weeks in 100 L aquaria. Water changes (~95%) were performed three times per week during the acclimation period. After acclimation, fish were acutely transferred from 7 to 2 mM Na^+ or 2 to 0.1 mM Na^+ freshwater by performing a water change with the appropriate more dilute media. At 0, 0.5, 1, 2, 4, 8 and 16 d after transfer, 8 fish were euthanized with an overdose of MS-222. Gill baskets were quickly dissected out, flash frozen in liquid N_2 and then stored at −80 °C until further processing. The remaining carcass was frozen at −20 °C for the analysis of whole body Na^+ content.

To determine whole body Na^+ content, fish were digested in ~5 volumes (fish mass: volume) of 1 N HNO_3 at 70 °C for 24 h. Digests were centrifuged at 2000 g for 10 min and the supernatant removed for Na^+ analysis by flame atomic absorption spectrophotometry (Varian 220) using an air/acetylene flame.

Total RNA was extracted from pupfish gill by first homogenizing tissue in RNA Stat-60 (Tel-test Inc., TX, USA) using an IKA-Werke Ultra-turax T8 tissue homogenizer (Wilmington, NC, USA). Samples were phenol/chloroform extracted and isopropanol precipitated. Precipitated RNA was washed with chilled 75% ethanol and dissolved in nuclease free water. RNA concentration was determined spectrophotometrically at 260 nm and then treated with DNase (Turbo DNA-free kit, Ambion, Austin, TX, USA) to remove any traces of genomic DNA. cDNA synthesis was performed using RevertAid MULV reverse transcriptase (Fermentas, MD, USA) according to the manufacturer's specifications.

A total of 7 genes (*CAc*, *h^+-atpase*, *nka α1a*, *nhe-2*, *nhe-3*, *rhcg-1*, *rhcg-2*) were targeted for cloning in *Cvv* and *Cvh*, along with a normalizing gene (*ef1α*). Additionally, blood CA (*CAb*) was also cloned to ensure that *CAc* specific primers were designed for RT-PCR. Conserved regions of other teleost fish were used for each gene to design degenerate primers for amplification of fragments of each target gene using the FastPCR freeware program. Initial fragments were amplified from the cDNA template using AmpliTaq Gold polymerase (Ambion, TX, USA) and TA-cloned into the pCR 2.1 vector (Invitrogen) according to manufacturer specifications and then sequenced.

To obtain poly(A) RNA as template for RACE reactions, gills from fish acutely transferred from high (7 mM Na^+) to low (0.1 mM Na^+) and low to high Na^+ water were pooled, 24 and 48 h post-transfer. Pooled samples were processed using the MicroPoly(A) Purist kit (Ambion, TX, USA). RNA was reverse transcribed and amplified using the BD

SMART RACE cDNA Amplification kit (BD Biosciences, NJ, USA) to produce 5′ and 3′ RACE ready cDNA. Both 5′ and 3′ gene specific and nested gene specific primers were designed from the initial cloned fragments for each gene. Touchdown PCR cycling conditions were as follows: 94 °C for 30 s, 69 °C for 30 s, and 72 °C for 3 min followed by 5 and 20–25 cycles at 65 to 67 °C annealing temperature, depending on the target gene.

Real-time PCR was performed on a Mx3000P real-time PCR system (Stratagene, CA, USA) using the Brilliant II SYBR green master mix kit (Stratagene, CA, USA). The thermocycle and reaction composition were performed according to manufacturer guidelines, and disassociation curves were used to assess primer specificity of each reaction. The PCR efficiency of each primer pair (Supplemental Table 1) was calculated using a cDNA standard curve and ranged from 82 to 106% across all genes and populations. Amplicon identities were confirmed by sequencing.

2.5. Analytical methods, calculations and statistical analysis

Total Na^+ in water samples was measured by atomic absorption spectrophotometry (Varian SpectraAA220, Mulgrave, Australia). Water and fish samples were measured for ^{22}Na activity using a gamma counter with a window of 15–2000 keV (Packard Cobra II Auto-Gamma, Meriden, Connecticut). Rates of Na^+ uptake as measured by the appearance of radioactivity in the fish (in nmol g^{-1} h^{-1}) were calculated using previously described methods (Boisen et al., 2003). Total ammonia (T_{amm}) in water was measured by a micro-modified colorimetric method (Verdouw et al., 1978). Ammonia excretion rates were calculated as described in Patrick and Wood (1999).

All values are expressed as means ± SEM throughout. Data were compared by Student's *t*-test or by ANOVA when multiple treatments were evaluated. For ANOVA, either Dunnett's or a Kruskal–Wallis test was used depending on whether the data met equal variance assumptions (Brown–Forsythe test). In some cases, data were log transformed to meet equal variance assumptions. All statistical analyses were performed using Graphpad Prism (version 6). Relative mRNA expression was calculated using the efficiency correction method (Pfaffl, 2001) with *ef1α* as an internal control and scaled relative to the T_0 sampling point.

3. Results

3.1. NHE-Rh metabolon experiments

For *Cvv* acclimated to 7 mM Na^+, Na^+ uptake increased significantly ($p < 0.05$) relative to the control in the fed and MOPS treatments, while Na^+ uptake was significantly inhibited in the pH 4.5 and EIPA treatments (Fig. 1). Corresponding measurements of T_{amm} excretion show that feeding significantly increased T_{amm} excretion rates, while all other treatments were unchanged relative to the control (Fig. 1). For *Cvh* acclimated to 7 mM Na^+, no treatments increased Na^+ uptake, but Na^+ uptake was inhibited in the pH 4.5 and EIPA treatments to a lesser and greater extent, respectively, than observed for *Cvv* acclimated to 7 mM Na^+ (Fig. 2). T_{amm} excretions rates for *Cvh* acclimated to 7 mM Na^+ increased significantly in the fed, pH 4.5, and HEA treatments. Finally, for *Cvh* acclimated to 0.1 mM Na^+, Na^+ uptake was inhibited in the pH 4.5, HEA and EIPA treatments, and stimulated in the MOPS treatment. Corresponding T_{amm} excretion rates were stimulated in the fed treatment, and unchanged in all other treatments (Fig. 3).

3.2. NHE–CA experiments

Ethoxzolamide had varying results on *Cvv* acclimated to 7 or 2 mM Na^+. In 7 mM Na^+ acclimated fish, Na^+ uptake was strongly (2-fold) stimulated while in 2 mM Na^+ acclimated fish it was strongly (58%) inhibited (Fig. 4A). In contrast, the response of *Cvh* to ethoxzolamide

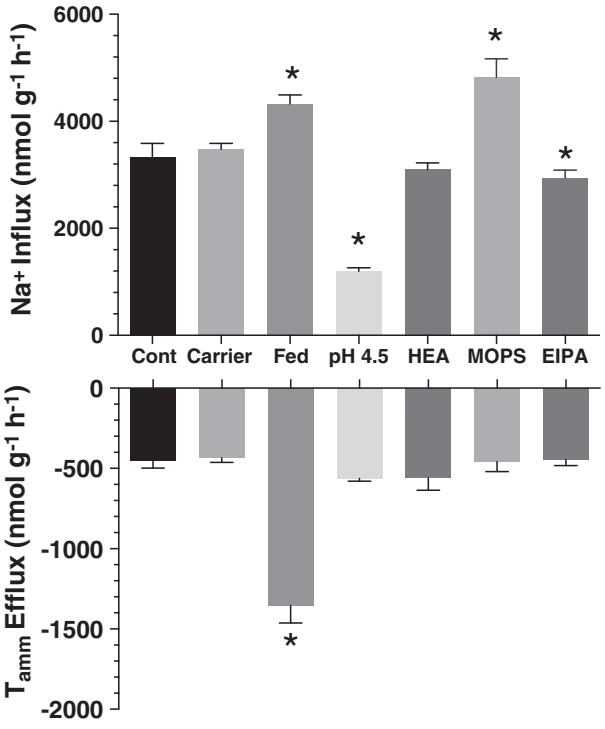

Fig. 1. Effects of different treatments on concurrent Na^+ uptake and T_{amm} excretion rates (nmol g^{-1} h^{-1}) in *Cvv* acclimated to 7 mM Na^+. See Methods and materials for description of each treatment. Mean ± SEM ($n = 8$). *$p < 0.05$. Difference compared to control or carrier control (EIPA treatment only).

was relatively consistent between acclimation salinities, with significant reductions in Na^+ uptake of 19% and 31% at 7 and 0.1 mM Na^+, respectively (Fig. 4B).

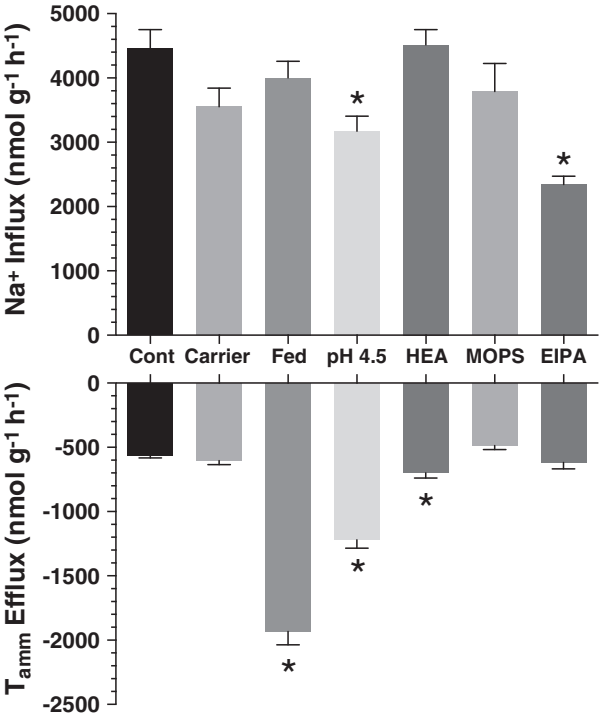

Fig. 2. Effects of different treatments on concurrent Na^+ uptake and T_{amm} excretion rates (nmol g^{-1} h^{-1}) in *Cvh* acclimated to 7 mM Na^+. See Methods and materials for description of each treatment. Mean ± SEM ($n = 8$). *$p < 0.05$. Difference compared to control or carrier control (EIPA treatment only).

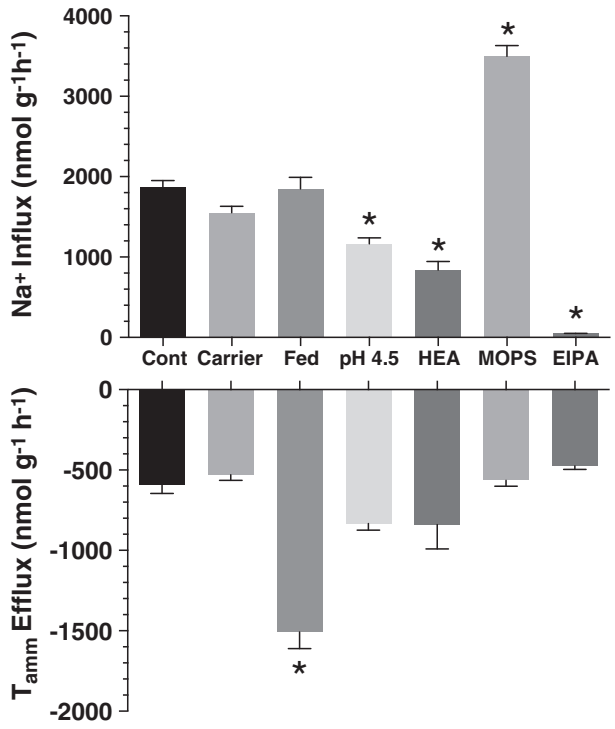

Fig. 3. Effects of different treatments on concurrent Na^+ uptake and T_{amm} excretion rates (nmol g^{-1} h^{-1}) in *Cvh* acclimated to 0.1 mM Na^+. See Methods and materials for description of each treatment. Mean ± SEM (n = 8). *p < 0.05. Difference compared to control or carrier control (EIPA treatment only).

Measurement of CA activity in gills of fish acclimated to different Na^+ concentrations revealed no difference in activity between *Cvv* acclimated to 2 and 7 mM Na^+ (Fig. 5A). In contrast, CA activity in *Cvh* acclimated to 0.1 mM Na^+ was significantly (75%) higher than observed in *Cvh* acclimated to 7 mM Na^+ (Fig. 5B).

3.3. Whole body Na^+ and mRNA expression experiments

Acute transfer of *Cvv* and *Cvh* from 7 to 2 or 2 to 0.1 mM Na^+ had no significant effect on whole body Na^+ concentrations over time except for *Cvv* transferred from 2 to 0.1 mM Na^+ (Fig. 6). In this treatment, whole body Na^+ declined significantly from 57 to 37 mM Na^+ over the first 4 d of exposure and mortality began 5 d post-transfer with 100% mortality observed 7 d after transfer.

We cloned full or partial cDNA sequences for 7 genes of interest (*CAc*, *h^+-atpase* (beta subunit), *nka α1a*, *nhe-2*, *nhe-3*, *rhcg-1*, *rhcg-2*) along with the normalizing gene *ef1α*. Partial sequences were of sufficient length to identify the genes with a high level of confidence based on homology with target genes of related species. All sequences are deposited in Genbank (see Supplemental for accession numbers).

Due to the mortality observed after 4 d for *Cvv* transferred from 2 to 0.1 mM Na^+, we restricted our mRNA expression analysis to time points collected up to 4 d post-transfer. Because of this mortality, mRNA expression data for this treatment should be treated with caution, particularly for the Day 4 results as mortality began the following day in this treatment.

Expression of the normalizing gene, *ef1α*, was stable over time within a treatment. In general, transfer of fish to a more dilute Na^+ concentration elicited a significant change in expression levels for all of the target genes in at least one of the treatments (Fig. 7). In many cases, changes in mRNA expression were similar between the two species, although there were several differences. The mRNA expression for *CAc* and *rhcg-2* were significantly downregulated in all species-treatment combinations,

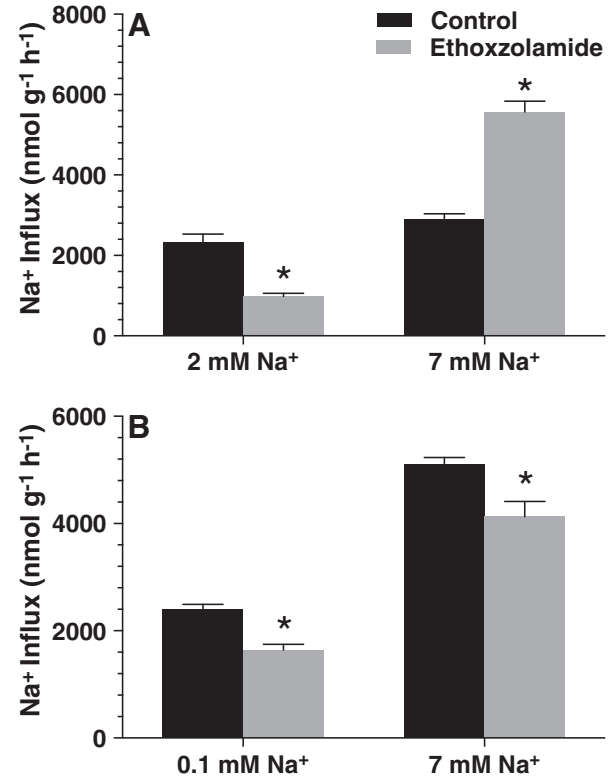

Fig. 4. Effects of 1×10^{-4} ethoxzolamide on Na^+ uptake rates (nmol g^{-1} h^{-1}) in *A. Cvv* acclimated to 2 or 7 mM Na^+; *B. Cvh* acclimated to 0.1 or 7 mM Na^+. Mean ± SEM (n = 8). *p < 0.05. Difference compared to corresponding control.

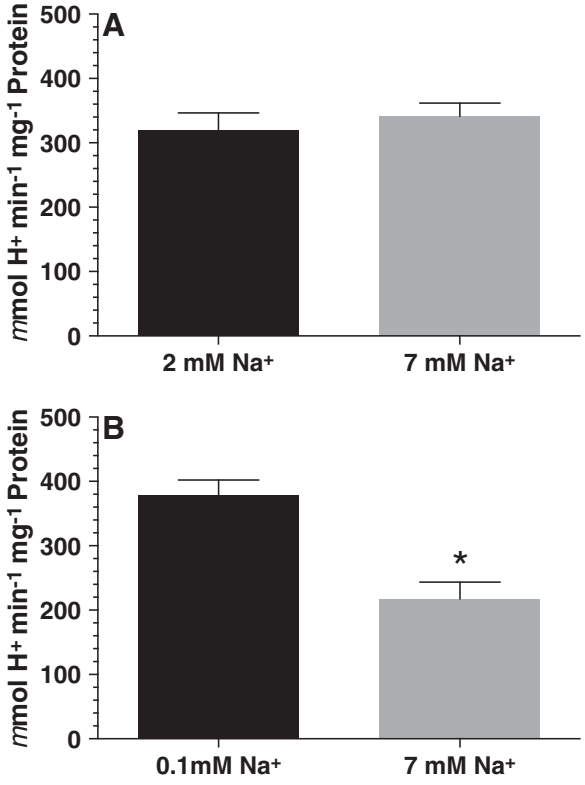

Fig. 5. Carbonic anhydrase activity (μmol H^+ min^{-1} mg^{-1} protein) in *A. Cvv* acclimated to 2 or 7 mM Na^+ and *B. Cvh* acclimated to 0.1 or 7 mM Na^+. Mean ± SEM (n = 8). *p < 0.05. Difference compared between low and high Na^+ water.

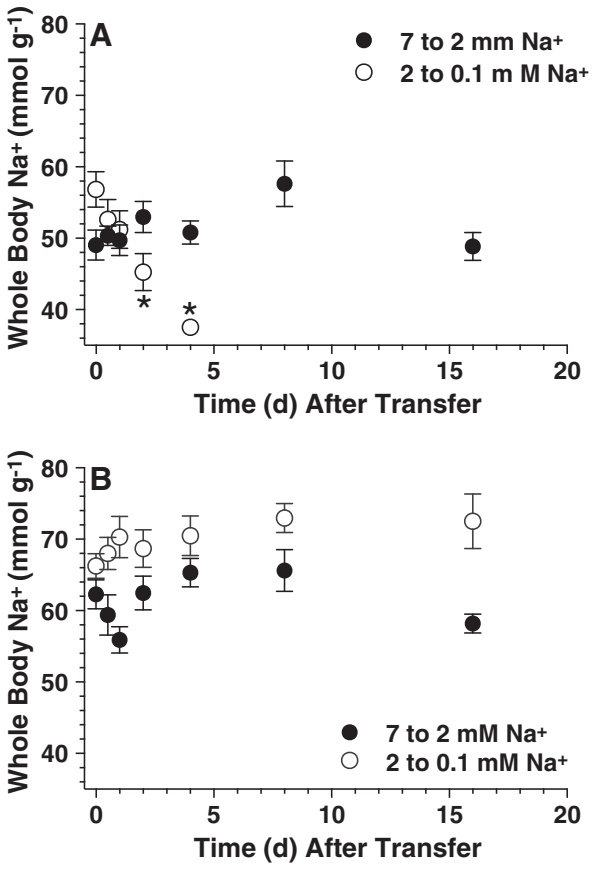

Fig. 6. Whole body Na^+ (mmol g^{-1}) concentrations over time in *A. Cvv* and *B. Cvh* after acute transfer from either 7 to 2 mM Na^+ or 2 to 0.1 mM Na^+. Mean ± SEM (n = 10). *$p < 0.05$. Difference compared to T_0.

while *nka α1a* was significantly upregulated. Expression of *h^+-atpase* was unchanged except in the *Cvv* 2 to 0.1 mM Na^+ treatment where it was modestly (~20%) downregulated ($p < 0.05$). Similarly, *rhcg-1* was intermittently downregulated in *Cvv* and in one instance in *Cvh*, but the pattern was not as robust or consistent as observed for *CAc* or *rhcg-2*. For *nhe-2*, there was a significant up-regulation in *Cvv* transferred from 7 to 2 mM Na^+ and a strong down-regulation of *nhe-2* when *Cvv* was transferred from 2 to 0.1 mM Na^+ ($p < 0.05$). In contrast, no changes in *nhe-2* mRNA expression were observed in *Cvh* for either treatment. Finally, *nhe-3* was transiently upregulated in *Cvv* transferred from 2 to 0.1 mM Na^+ (2 d) and *Cvh* transferred from 7 to 2 mM Na^+ (0.5 d), but more consistently upregulated in *Cvh* transferred from 2 to 0.1 mM Na^+ (2 d and 4 d).

We also evaluated the relative mRNA expression of *nhe-2* and *nhe-3* at T_0 for both 7 and 2 mM Na^+ acclimated fish. This was accomplished by evaluating the data relative to expression levels observed for *nhe-2* in fish acclimated to 7 mM Na^+ (Fig. 8). Analyzing the data in this manner for *Cvv* indicates a comparable level of mRNA expression for *nhe-2* and *nhe-3* in 7 mM Na^+ acclimated fish with a 2-fold decrease and 3-fold increase in relative expression in fish acclimated to 2 mM Na^+. In contrast, for *Cvh* acclimated to 7 mM Na^+, *nhe-3* expression is 143-fold higher than *nhe-2*, and this increases to 207-fold higher in fish acclimated to 2 mM Na^+. There was no change in relative *nhe-2* mRNA expression in *Cvh* acclimated to 2 mM Na^+.

4. Discussion

We previously characterized Na^+ transport kinetics between *Cvv* and *Cvh* in freshwater, demonstrating that *Cvh* has a significantly higher affinity for Na^+ (~150-fold lower K_m) that allows it to ionoregulate in the relatively low Na^+ environments in which it occurs (Brix and Grosell, 2012). Based on results from experiments with pharmacological inhibitors that target specific proteins involved in Na^+ transport, we suggested that both *Cvv* and *Cvh* utilize one or more NHEs for apical Na^+ uptake in dilute freshwater and that *Cvv* also utilizes an apical NKCC for Na^+ uptake in relatively high Na^+ (≥7 mM) waters. These experiments also indicated that neither population utilized a Na^+ channel/ASIC linked to a H^+-ATPase as has been observed in many other species.

The mRNA expression experiments in the current study generally support our previous observations. As has been observed in other species (Bystriansky et al., 2007; Liao et al., 2009; Bystriansky and Schulte, 2011), transfer to more dilute freshwater elicited a significant upregulation of *nka α1a* in both populations (Fig. 7E). Given the similar levels of upregulation observed in the two populations, these results do not provide any insight into the mechanism(s) by which *Cvh* successfully ionoregulates in low Na^+ waters. However, the current analysis of *nka α1a* mRNA expression is relatively coarse as there are potentially a number of sub-isoforms (e.g., *nka α1a.1*, *nka α1a.2*) that may be differentially expressed between populations. In other species, these sub-isoforms are differentially expressed in response to changes in ambient Na^+ concentrations (Liao et al., 2009) and significantly affect Na^+ transport kinetics (Jorgensen, 2008). Given the variable number of sub-isoforms present in different species and the high degree of homology between sub-isoforms, a proper characterization of differential mRNA expression between sub-isoforms is constrained by the lack of a *Cyprinodon* genome available for primer design.

We previously demonstrated that Na^+ uptake in *Cvv* and *Cvh* was not bafilomycin-sensitive, suggesting that neither population used a Na^+ channel/H^+-ATPase system for apical Na^+ uptake (Brix and Grosell, 2012). This result for *Cvh* was confounded by the observation that Na^+ uptake was sensitive to phenamil, an inhibitor that is supposed to be specific to Na^+ channels (Kleyman and Cragoe, 1988). The absence of any upregulation of *h^+-atpase* mRNA expression in the current study (Fig. 7B) supports the hypothesis that neither population utilizes a Na^+ channel/H^+-ATPase system for apical Na^+ uptake. However, given that *h^+-atpase* regulation can occur at the protein level by trafficking from cytoplasmic vesicles to the cell membrane via microtubules (Weihrauch et al., 2002; Tresguerres et al., 2006), the lack of changes in mRNA expression does not definitively rule out the presence of a Na^+ channel/H^+-ATPase system in *Cyprinodon*. Additional immunohistochemistry studies and experiments on the specificity of phenamil to Na^+ channels in fish would be useful.

Results from this study support our previous hypothesis that in low Na^+ (≤2 mM) waters, both *Cvv* and *Cvh* rely on one or more NHE isoforms for Na^+ uptake at the apical membrane. We observed an upregulation in *nhe-2* mRNA expression, but only in *Cvv* transferred from 7 to 2 mM Na^+ (Fig. 7C). In contrast, *nhe-3* was upregulated in *Cvh* after transfer to both 2 and 0.1 mM Na^+, as well as in *Cvv* transferred to 0.1 mM Na^+ (Fig. 7D). Unlike *nhe-2* (and several other genes), no strong down-regulation of *nhe-3* was observed in *Cvv* 4 d post-transfer to 0.1 mM Na^+. We also observed that relative to *nhe-2* expression in 7 mM Na^+ acclimated fish, *nhe-3* expression was only modestly (2-fold) higher in *Cvv* acclimated to 2 mM Na^+ (Fig. 8A). In constrast, in *Cvh*, *nhe-3* expression was nearly 150-fold higher than *nhe-2* in fish acclimated to 7 mM Na^+ and >200-fold higher in fish acclimated to 2 mM Na^+. Overall, both the time course and relative expression data for acclimated fish suggest that differential regulation of NHE-3 isoforms likely contributes to the significant differences in Na^+ transport kinetics between *Cvv* and *Cvh* at ≤2 mM Na^+ and that this may be an important adaptation for *Cvh* to ionoregulate in dilute freshwater.

The related euryhaline fish, *F. heteroclitus*, has similar Na^+ transport kinetics (K_m = 1723 μM Na^+) to that observed in *Cvv*, when acclimated to 1 mM Na^+ (Patrick et al., 1997). Also, similar to *Cvv*, *F. heteroclitus* appears to rely heavily on NHE-2 for apical Na^+ uptake, although

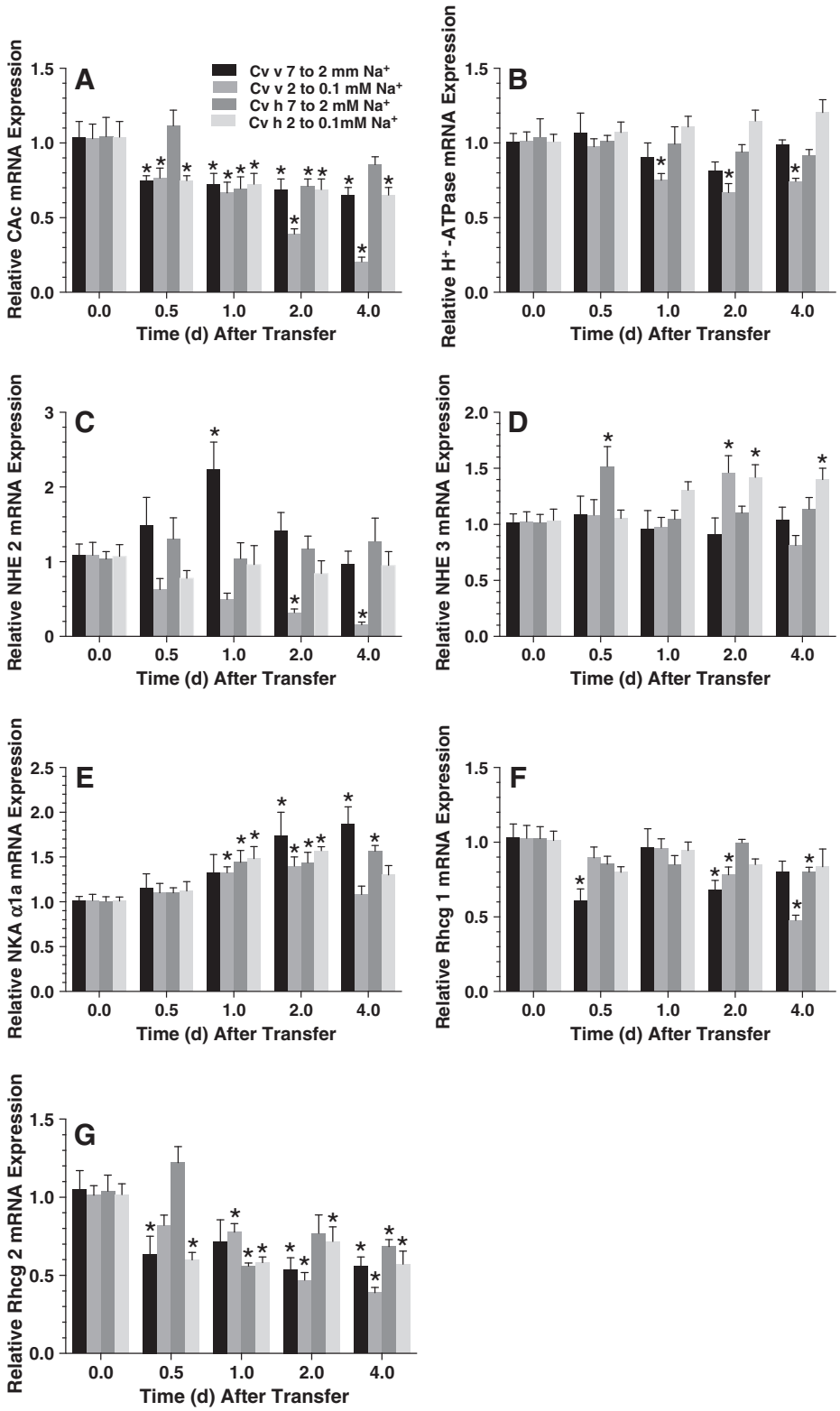

Fig. 7. The effects of acute transfer from 7 to 2 or 2 to 0.1 mM Na^+ freshwater on mRNA expression in the gills of *Cvv* and *Cvh* for *A.* CAc; *B.* H^+-ATPase; *C.* NKA α1a; *D.* NHE-2; *E.* NHE-3; *F.* Rhcg-1; and *G.* Rhcg-2. Mean ± SEM (n = 8). *p < 0.05. Difference compared to compared to T_0 within a given species-salinity treatment.

upregulation of *nhe-3* has been observed in the opercular epithelium of *Fundulus* after acute transfer from brackish to freshwater (Scott et al., 2005). Similarly, *nhe-3* is upregulated in both *D. rerio* and *O. latipes*, which are capable of ionoregulating in very low Na^+ water (≤0.1 mM Na^+), suggesting that it may be generally associated with apical Na^+ acquisition under these conditions (Wu et al., 2010; Shih et al., 2012). Our results for *Cvv* and *Cvh* are generally consistent with these observations for other species, with *Cvv* predominantly utilizing NHE-2, while *Cvh* appears to rely primarily on NHE-3. Given the thermodynamic constraints on NHE function in dilute freshwater (Parks et al., 2008), we next explored the mechanism(s) by which *Cvh* overcomes these constraints to allow for NHE-3 function in dilute freshwater.

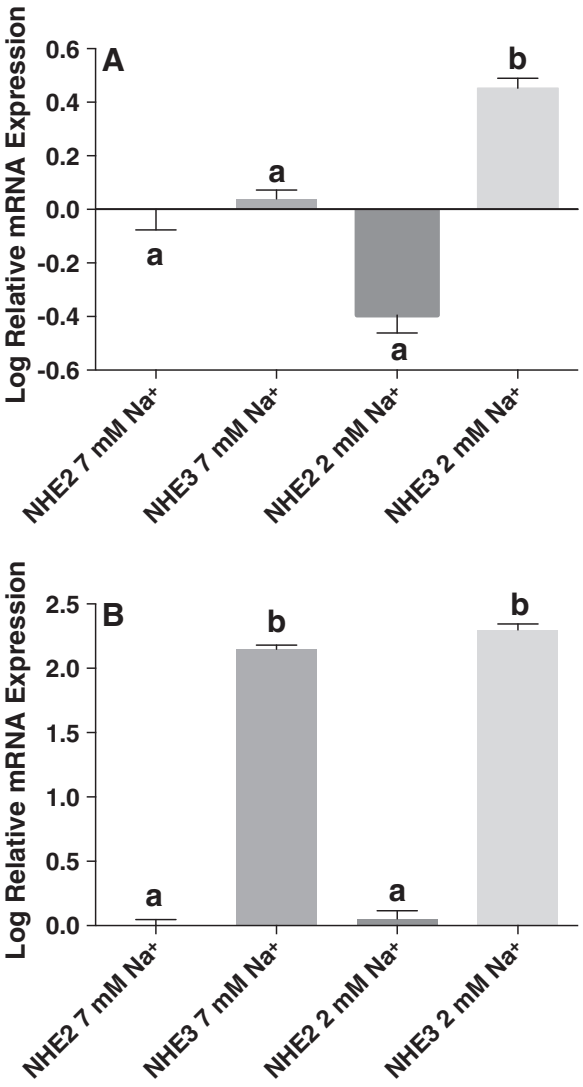

Fig. 8. The relative mRNA expression of NHE2 and NHE3 in the gills of *A. Cvv* and *B. Cvh*. All data from T_0 time points in the gene expression experiments and expressed relative to NHE2 in fish acclimated to 7 mM Na^+. Mean ± SEM (n = 8). Different letters denote statistically significant differences between mRNA expression levels ($p < 0.05$).

4.1. NHE-Rh metabolon experiments

We hypothesized that *Cvv* and *Cvh* acclimated to 7 mM Na^+ would show little or no linkage between Na^+ uptake and T_{amm} excretion, and therefore no evidence of an NHE-Rh metabolon, as these high Na^+ conditions are favorable for normal NHE function (Parks et al., 2008). We further hypothesized, that when *Cvh* are acclimated to 0.1 mM Na^+, evidence for an NHE-Rh metabolon would be present in the form of a strong linkage between Na^+ uptake and T_{amm} excretion. Contrary to these hypotheses, observed increases in Na^+ influx were linked to a corresponding increase in ammonia efflux in only one treatment (*Cvv* 7 mM Na^+ Fed), while treatments that caused a decrease in Na^+ influx never caused a corresponding decrease in ammonia efflux (Figs. 1–3).

Results from this experiment differ significantly from similar experiments with *D. rerio* and *O. latipes* where it was concluded that an NHE-Rh metabolon is operating. For example, in *D. rerio* and *O. latipes* acclimated to low Na^+ water, acute exposure to high environmental ammonia (HEA) resulted in significant reductions in Na^+ uptake and T_{amm} excretion (Wu et al., 2010; Kumai and Perry, 2011; Shih et al., 2012). In contrast, when *Cvh* are acclimated to similar conditions and exposed to HEA, Na^+ uptake is reduced but T_{amm} excretion remains unchanged. Similarly, when *D. rerio* and *O. latipes* are exposed to EIPA, both Na^+ uptake and T_{amm} excretion are reduced (Wu et al., 2010; Shih et al., 2012), while in *Cvh* acclimated to 0.1 mM Na^+, EIPA exposure inhibits Na^+ uptake by 98%, but has no effect on T_{amm} excretion. Additionally, when *D. rerio* and *O. latipes* are acutely exposed to low pH, Na^+ uptake is stimulated and T_{amm} excretion is either unchanged or stimulated, while in both *Cvv* and *Cvh* Na^+ uptake is inhibited despite T_{amm} excretion generally being stimulated (Figs. 1–3) (Brix et al., 2013). Perhaps the most incongruent result is exposure to MOPS buffer. In *D. rerio*, MOPS inhibits both Na^+ uptake and T_{amm} excretion (Shih et al., 2012), while in *Cvh* acclimated to 0.1 mM Na^+, Na^+ uptake is significantly increased while T_{amm} excretion remains unchanged (Fig. 3). An important caveat to these comparative observations is that experiments on *D. rerio* and *O. latipes* were all performed on newly hatched larvae where Na^+ uptake and T_{amm} excretion are occurring primarily across the skin rather than the gills.

Consistent with whole animal measurements showing no linkage between Na^+ uptake and T_{amm} excretion, we observed either no change or strong downregulation of *rhcg-1* and *rhcg-2* when both populations were acutely transferred to more dilute water (Fig. 7F–G). Again, this is inconsistent with previous observations in *D. rerio* and *O. latipes* that showed an upregulation of *rhcg-1* upon transfer to low Na^+ water (Wu et al., 2010; Shih et al., 2012). *Rhcg-2* has been shown to be upregulated in response to feeding in rainbow trout (*Oncorhynchus mykiss*), with a corresponding upregulation of *nhe-2* and an increase in both Na^+ uptake and T_{amm} excretion (Zimmer et al., 2010). However, to the best of our knowledge, there have been no direct experiments evaluating the response of the putative NHE-2/Rhcg-2 metabolon in rainbow trout to low environmental Na^+. Interestingly, the one experiment showing a link between Na^+ uptake and T_{amm} excretion was in the feeding experiment with *Cvv* acclimated to 7 mM Na^+. Despite this result, and the upregulation of *nhe-2* by *Cvv* when transferred from 7 to 2 mM Na^+, *rhcg-2* was consistently downregulated in all of our experiments, perhaps reflecting the relative reduction in pavement cell surface area associated with proliferation and expansion of ionocytes in low Na^+ water (Brix and Grosell, 2012).

Overall, results from these experiments fail to support the presence of a NHE-Rh metabolon in either *Cvv* or *Cvh* under high or low Na^+ conditions. The observed correspondence in the *Cvv* 7 mM Na^+ fed treatment may simply be the result of increased metabolism from feeding and digestion, which would result in increased ventilation rates, increased diffusive Na^+ loss across the gills, and a compensatory response. Alternatively, this metabolon may be responsive to feeding and the corresponding increased ammonia load, but not responsive to low environmental Na^+.

4.2. NHE–CA experiments

We employed two approaches to investigating the importance of CA generating H^+ via CO_2 hydration in the function of NHE in *Cvv* and *Cvh*. First we treated *Cvv* acclimated to 7 and 2 mM Na^+ and *Cvh* acclimated to 7 and 0.1 mM Na^+ to ethoxzolamide, a membrane permeable CA inhibitor. We hypothesized that for both species, exposure to ethoxzolamide would result in a greater inhibition of Na^+ uptake at the lower acclimation salinity reflecting the greater importance of CA in generating H^+ under these thermodynamically less favorable conditions. We also directly measured CA activity in fish acclimated to these different salinities and hypothesized that it would be higher in fish at lower salinity.

For *Cvh* acclimated to 7 and 0.1 mM Na^+, we observed 19% and 31% reductions in Na^+ uptake when treated with ethoxzolamide (Fig. 4B). The results support a role for CA in NHE function, and perhaps even an increasing importance in low Na^+ waters. Consistent with this observation, CA activity was 75% higher in fish acclimated to 0.1 mM Na^+ compared to 7 mM Na^+ (Fig. 5B). Combined, these data support the hypothesis that CA plays an important role in NHE function in *Cvh* under low Na^+ conditions. However, contradicting these results was

the modest (~20%), but statistically significant, down-regulation of *CAc* in both *Cvh* experiments (Fig. 7A). It is also possible that an extracellular CA is contributing to Na^+ uptake as has been demonstrated for CA-15 in *D. rerio* (Lin et al., 2008), which would potentially confound interpretation of our observations. Regardless, given the incongruent mRNA expression results, it is not clear that that H^+ generated via CA-mediated CO_2 hydration is the primary mechanism allowing for NHE function in *Cvh* in dilute freshwater.

Surprisingly, inhibition of CA had a greater effect on Na^+ uptake in *Cvv* than in *Cvh*. In *Cvv* acclimated to 2 mM Na^+, Na^+ uptake was only 42% of the control in CA-inhibited fish suggesting that CA plays a stronger role in NHE function of *Cvv* acclimated to 2 mM Na^+ than for *Cvh* acclimated to 0.1 mM Na^+. This result and the corresponding upregulation of NHE-2 suggest that *Cvv* may be using the same NHE-2/CA system that has been proposed to operate in *Fundulus* at intermediate Na^+ concentrations (Scott et al., 2005).

Equally interesting, was the nearly 2-fold stimulation of Na^+ uptake in CA-inhibited *Cvv* acclimated to 7 mM Na^+, a considerably different result than observed at 2 mM Na^+ despite fish having the same CA activity when acclimated to these different salinities (Fig. 4A and 5). The stimulation in Na^+ uptake at 7 mM Na^+ is difficult to explain, but we hypothesize that this response is related to the putative use of an apical NKCC by *Cvv* (but not *Cvh*) at this salinity (Brix and Grosell, 2012). This hypothesis is supported by the observation that in the related pupfish, *Cyprionodon macularius*, which also does not use NKCC for Na^+ uptake, ethoxzolamide inhibits Na^+ uptake in fish acclimated to 7 mM Na^+ (Brix and Grosell, 2013a). CA has been linked to stimulation of apical NKCC in the marine fish intestine where HCO_3^- produced by CO_2 hydration activates soluble adenylyl cyclase, which in turn stimulates apical NKCC (Tresguerres et al., 2010), but if this pathway was operating in the *Cvv* gill, we would have expected a decrease rather than an increase in Na^+ uptake. However, it has also been demonstrated in mammalian systems that apical NKCC activity can be affected by numerous regulatory pathways including PKA-phosphorylation, PKA-mediated membrane insertion, and cAMP-mediated membrane insertion (Meade et al., 2003; Oritz, 2006; Caceres et al., 2009). The interactions of these various pathways with CA-facilitated H^+ or HCO_3^- have yet to be described. Clearly more study is needed to unravel the interactions between CA and the apical NKCC in *Cvv*.

5. Conclusions

In conclusion, we aimed to test whether differential NHE expression explained observed differences in Na^+ transport kinetics between *Cvv* and *Cvh*, and whether a NHE-Rh metabolon and/or NHE–CA system explained NHE function in *Cvh* in low Na^+ waters. Our results suggest that *Cvv* primarily relies on NHE-2 while *Cvh* relies on NHE-3 for apical Na^+ uptake and that these differences play an important role in the adaptation of *Cvh* to low Na^+ waters. Results provided strong evidence that an NHE-Rh metabolon is not operating in either population. For *Cvv*, CA appears to play an important role in Na^+ uptake, reducing uptake via a currently unknown signaling pathway at 7 mM Na^+ and contributing significantly, presumably through generation of H^+, to NHE operation at 2 mM Na^+. For *Cvh*, CA appears to have a relatively minor role in NHE function at 7 mM Na^+ and a more significant role at 0.1 mM Na^+. However, the possibility that an additional novel mechanism by which NHE functions at low Na^+ against its thermodynamic gradient in this population cannot be ruled out.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.cbpa.2015.04.002.

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