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RESEARCH ARTICLE

Comparative characterization of Na^+ transport in *Cyprinodon variegatus variegatus* and *Cyprinodon variegatus hubbsi*: a model species complex for studying teleost invasion of freshwater

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SUMMARY

The euryhaline fish *Cyprinodon variegatus variegatus* is capable of tolerating ambient salinities ranging from 0.3 to 160 PSU, but is incapable of long-term survival in freshwater (<2 mmol l^{-1} Na^+). A population isolated in several freshwater (0.4–1 mmol l^{-1} Na^+) lakes in central Florida is now designated as a subspecies (*Cyprinodon variegatus hubbsi*). We conducted a comparative study of Na^+ transport kinetics in these two populations when acclimated to different ambient Na^+ concentrations. Results reveal that the two subspecies have qualitatively similar low affinity Na^+ uptake kinetics (K_m=7000–38,000 μmol l^{-1}) when acclimated to 2 or 7 mmol l^{-1} Na^+, but *C. v. hubbsi* switches to a high affinity system (K_m=100–140 μmol l^{-1}) in low-Na^+ freshwater (≤1 mmol l^{-1} Na^+). Inhibitor experiments indicate that Na^+ uptake in both subspecies is EIPA-sensitive, but sensitivity decreases with increasing external Na^+. EIPA induced a 95% inhibition of Na^+ influx in *C. v. hubbsi* acclimated to 0.1 mmol l^{-1} Na^+, suggesting that this subspecies is utilizing a Na^+/H^+ exchanger to take up Na^+ in low-Na^+ environments despite theoretical thermodynamic constraints. Na^+ uptake in *C. v. hubbsi* acclimated to 0.1 mmol l^{-1} Na^+ is phenamil-sensitive but not bafilomycin-sensitive, leading to uncertainty about whether this subspecies also utilizes Na^+ channels for Na^+ uptake. Experiments with both subspecies acclimated to 7 mmol l^{-1} Na^+ also indicate that a Cl^--dependent Na^+ uptake pathway is present. This pathway is not metolazone-sensitive (NCC inhibitor) in either species but is bumetanide-sensitive in *C. v. variegatus* but not *C. v. hubbsi*. This suggests that an apical NKCC is increasingly involved with Na^+ uptake for this subspecies as external Na^+ increases. Finally, characterization of mitochondria-rich cell (MRC) size and density in fish acclimated to different ambient Na^+ concentrations revealed significant increases in the number and size of emergent MRCs with decreasing ambient Na^+. A linear relationship between the fractional area of emergent MRCs and Na^+ uptake rate was observed for both subspecies. However, *C. v. variegatus* have lower Na^+ uptake rates at a given MRC fractional area compared with *C. v. hubbsi*, indicating that the enhanced Na^+ uptake by *C. v. hubbsi* at low ambient Na^+ concentrations is not strictly a result of increased MRC fractional area, and other variables, such as differential expression of proteins involved in Na^+ uptake, must provide *C. v. hubbsi* with the ability to osmoregulate in dilute freshwater.

Key words: Na^+ uptake, NHE, NKCC, mitochrondria-rich cells, osmoregulation.

INTRODUCTION

The invasion of ancient fish into freshwater environments represents one of the most physiologically significant adaptations in vertebrate evolution. It is generally accepted that early fishes first arose in marine environments and there is fossil evidence for multiple early groups (e.g. Anaspida, Placodermi and Osteichthyes) independently invading freshwater environments (Long, 2011). Basal teleost fish are thought to have arose in a brackish environment with multiple radiations of different lineages back into marine systems as well as into freshwater systems all retaining the highly conserved trait of maintaining an internal osmolality of ~300–400 mOsm (Evans et al., 2005). Internal osmolality in teleost fish is dominated by Na^+ and Cl^-, and there has been considerable study of ion homeostasis at the whole-animal, protein and gene expression levels of organization in several model fish (Evans et al., 2005; Hwang et al., 2011; Marshall and Grosell, 2006). However, there has been virtually no study of how these homeostatic mechanisms evolved during initial or subsequent re-invasion of freshwater environments by marine or estuarine fish.

To successfully osmoregulate in freshwater environments, fish need to compensate for diffusive loss of major osmolytes (primarily Na^+ and Cl^-) *via* active uptake against a large concentration gradient. Active Na^+ uptake at the fish gill is fueled primarily by a basolateral Na^+/K^+-ATPase. Three mechanisms have been hypothesized to accomplish apical entry of Na^+ at the gill in dilute freshwater. A Na^+ channel associated with a H^+-ATPase, which hyperpolarizes the membrane, is probably the most widely accepted model from a thermodynamic perspective, although molecular characterization of the Na^+ channel has been elusive. It has also been proposed that Na^+ entry at the apical membrane is accomplished by electroneutral Na^+/H^+ exchange (NHE) *via* one or more NHE isoforms. The functionality of an apical NHE in low-Na^+ environments (e.g. <1 mmol l^{-1} Na^+) is thermodynamically problematic (Parks et al., 2008), but there is increasing evidence of a metabolon involving NHE and Rhesus proteins transporting ammonia that could create a thermodynamically favorable environment for NHE function in low-Na^+ environments (Wu et al., 2010). Finally, it has been hypothesized that chloride-dependent uptake of Na^+ occurs at the apical membrane of some freshwater fish *via* either Na:K:2Cl (NKCC) and/or an apical Na^+-Cl^- co-transporter (NCC). Similar to NHEs, there are significant

thermodynamic questions regarding how such a system would function, but studies in goldfish (Preest et al., 2005) and tilapia (Hiroi et al., 2005) support the presence and function of NKCC whereas studies in zebrafish support the involvement of NCC (Esaki et al., 2007; Wang et al., 2009).

In addition to variations in the proteins involved in Na^+ acquisition in freshwater fish gills, a number of studies have documented a variety of mitochondria-rich cell (MRC) types and dynamic responses of these cell types to changes in external ion concentrations (Chang et al., 2001; Fernandes et al., 1998; Greco et al., 1996; Hirai et al., 1999; Lee et al., 1996). Several studies, particularly in tilapia, have also co-localized specific apical proteins to specific MRC types (Hiroi et al., 2005; Inokuchi et al., 2009), and in general a strong relationship between the fractional area of MRC on the gill and Na^+ uptake capacity has been demonstrated for a number of fish species (Perry et al., 1992).

The euryhaline pupfish *Cyprinodon variegatus variegatus* Lacépède 1803 occurs along the Gulf and Atlantic coasts of North America and tolerates salinities ranging from near freshwater up to 167 PSU (Nordlie, 2006). Beyond data on its basic salinity tolerance, little is known about the osmoregulatory capacity of this species. With respect to its ability to tolerate freshwater conditions, *C. v. variegatus* does not survive, grow or reproduce at concentrations <2 mmol l^{-1} Na^+ (Dunson et al., 1998). A freshwater pupfish population currently given subspecies status (*C. v. hubbsi* Carr 1936) is found in five lakes in central Florida with ambient Na^+ concentrations of 0.4–1 mmol l^{-1}. *Cyprinodon variegatus hubbsi* is estimated to have been isolated from coastal populations of *C. v. variegatus* for 100,000–200,000 years (Darling, 1976; Guillory and Johnson, 1986).

Given the relatively recent divergence of *C. v. variegatus* and *C. v. hubbsi* and the apparent differences in their ability to osmoregulate in dilute freshwater, this species complex may provide a good model system for studying at least one way in which euryhaline fish have evolved to successfully invade freshwater systems. The objective of the present study was to provide an initial comparative characterization of Na^+ transport kinetics in freshwater-acclimated *C. v. variegatus* and *C. v. hubbsi*, begin to characterize the proteins that contribute to Na^+ uptake in these subspecies, and characterize whether any difference in MRC size and distribution have evolved between the two subspecies as a result of living in different osmoregulatory environments.

MATERIALS AND METHODS

Animal holding

Adult *C. v. variegatus* 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 to 39 PSU. Fish were held at the University of Miami in 110 l glass aquaria under flow-through conditions with filtered natural seawater (35 PSU) from Bear Cut, FL. Adult fish were bred and F1 offspring were hatched and raised in seawater until the late juvenile stage (~2 months old, 50–300 mg). Fish were fed *Artemia* nauplii for the first 2 weeks and then, over a 1 week period, gradually switched over to flake food (Tetramin™ Tropical Flakes, Blacksburg, VA, USA).

F1 fish were acclimated to near-freshwater conditions (0.3 PSU, 7 mmol l^{-1} Na^+, pH 7.9 or 0.1 PSU, 2 mmol l^{-1} Na^+, pH 7.9) for a minimum of 3 weeks prior to experimental use. Dechlorinated City of Miami tap water (~1.0 mmol l^{-1} Na^+, 1.0 mmol l^{-1} Cl^-, 0.5 mmol l^{-1} Ca^{2+}, 0.2 mmol l^{-1} Mg^{2+}, 0.5 mmol l^{-1} SO_4^{2-}, 0.8 mmol l^{-1} HCO_3^-, pH 7.9) was mixed with filtered natural seawater to achieve the desired salinity. Preliminary experiments indicated that 2 mmol l^{-1} Na^+ was the lowest salinity at which fish could be maintained and successfully reproduce, consistent with previous studies (Dunson et al., 1998). Fish were not fed for 2 days prior to experimental use.

Adult *C. v. hubbsi* were originally collected from Lake Weir, FL (0.9 mmol l^{-1} Na^+, 1.1 mmol l^{-1} Cl^-, 0.1 mmol l^{-1} Ca^{2+}, 0.2 mmol l^{-1} Mg^{2+}, 0.1 mmol l^{-1} SO_4^{2-}, 0.2 mmol l^{-1} 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 tap water. Adult fish were bred and F_1 offspring were hatched and raised in dechlorinated tap water until the late juvenile stage (~2 months old, 50–300 mg). Fish were fed *Artemia* nauplii for the first 2 weeks and then, over a 1 week period, gradually switched over to bloodworms (*Chironomus* sp.) as *C. v. hubbsi* refused to eat the flake food diet fed to *C. v. variegatus*. F_1 fish were acclimated to different Na^+ concentrations (0.1, 2 or 7 mmol l^{-1} Na^+) for a minimum of 3 weeks prior to experimental use. Dechlorinated tap water was diluted with nanopure water to create the 0.1 mmol l^{-1} Na^+ treatment and had a pH of 6.8.

Cyprinodon variegatus variegatus and *C. v. hubbsi* were collected under Florida Fish and Wildlife Conservation Commission permit FNE-2010-09. Experiments were approved by the University of Miami Animal Care Committee.

Characterization of Na^+ uptake kinetics and efflux rates

The Na^+ uptake kinetics of *C. v. hubbsi* were determined for fish acclimated to 0.1, 1, 2 and 7 mmol l^{-1} Na^+, whereas uptake kinetics for *C. v. variegatus* were determined for fish acclimated to 2 and 7 mmol l^{-1} Na^+. For each experiment, Na^+ uptake rates were measured at seven to 10 different ambient Na^+ concentrations ranging from 0.014 to 58.6 mmol l^{-1} Na^+ depending on the subspecies and Na^+ concentration to which they were acclimated. At each Na^+ concentration, eight juvenile fish (50–300 mg) were placed in 50 ml of a defined medium (480 μmol l^{-1} $CaSO_4$, 150 μmol l^{-1} $MgSO_4$, 100 μmol l^{-1} $KHCO_3$, pH 7.0) to which a targeted concentration of NaCl was added. Test solutions were continuously aerated to maintain dissolved oxygen levels during the flux period. Fish were allowed to acclimate to this medium for 10 min, after which the medium was replaced and 1–2 μCi of ^{22}Na (depending on ambient Na^+ concentration) was added to the solution. The flux solution (1 ml) was sampled after 1 min for measurements of [Na^+] and ^{22}Na activity. The total flux exposure period ranged from 0.5 to 3 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^+] and ^{22}Na activity were again collected, fish were removed from the exposure medium, double rinsed in a 100 mmol l^{-1} Na^+ solution to displace any loosely bound ^{22}Na, blotted dry, weighed to the nearest 0.1 mg and then assayed individually for radioactivity.

Characterization of Na^+ efflux rates was accomplished using adult (1.0–1.6 g) *C. v. hubbsi* and *C. v. variegatus* that had been acclimated for >3 weeks to 2 mmol l^{-1} Na^+ freshwater. Because of their small size, standard techniques of loading fish with ^{22}Na and measuring efflux rates by radioisotope were not considered logistically feasible. Instead, individual fish were initially held in 100 ml of this water with gentle aeration and allowed to acclimate for 1 h. After acclimation, the water was replaced with the previously described defined medium without NaCl. A water sample was immediately collected for measurement of water Na^+ concentrations. Subsequent water samples were collected at 2, 4, 8, 24 and 48 h for analysis of water Na^+ concentrations. After each sampling time point, test solutions were replaced with fresh media and the volume was

gradually increased over the 48 h experiment to accommodate longer flux periods. The flux periods and water volumes selected were based on preliminary experiments to ensure that water Na^+ concentrations did not exceed 20 μmol l^{-1} as a result of Na^+ efflux from fish. This minimized both the amount of waterborne Na^+ that the fish might take up and the changes in the diffusive Na^+ gradient over a given flux period.

Pharmacological inhibitor experiments

Juvenile *C. v. hubbsi* and *C. v. variegatus* were bred and acclimated to different Na^+ concentrations as described above. Experiments were then performed in which Na^+ uptake was measured in the presence and absence of different pharmacological inhibitors. The Na^+ uptake experiments indicate that *C. v. hubbsi* exhibits a high affinity Na^+ transport system whereas *C. v. variegatus* exhibits a comparatively low affinity Na^+ transport system (see Results). It was hypothesized that *C. v. hubbsi* utilized a H^+-ATPase/Na^+ channel system to take up Na^+ across the apical membrane whereas *C. v. variegatus* relied on an NHE. Experiments with pharmacological inhibitors were designed to test this hypothesis and identify the most likely transport proteins involved in Na^+ uptake with the fewest experiments possible (i.e. paired experiments with both subspecies were not performed *a priori* for all inhibitors).

Initial experiments were conducted using amiloride (N-amidino-3.5-diamino-6-chloropyrazinecarbromide) on *C. v. hubbsi* acclimated to 0.1 and 2 mmol l^{-1} Na^+ and *C. v. variegatus* acclimated to 2 mmol l^{-1} Na^+. Amiloride inhibits both Na^+ channels and NHEs with a higher affinity for Na^+ channels (Kleyman and Cragoe, 1988). We therefore tested three amiloride concentrations (10^{-5}, 10^{-4} and 10^{-3} mol l^{-1}) in an attempt to distinguish effects between these different pathways. For the control and amiloride treatments, 10 juvenile *C. v. hubbsi* (8–43 mg) or *C. v. variegatus* (39–164 mg) were exposed in 30 ml of the water to which they were acclimated. Fish were allowed to acclimate for 10 min to the test system, after which the water was replaced with fresh solution. Amiloride dissolved in DMSO was then added at final concentrations of 10^{-5}, 10^{-4} or 10^{-3} mol l^{-1} amiloride and 0.1% DMSO, whereas for the control group only DMSO was added. After allowing 5 min for the drug to take effect, 0.2 μCi of ^{22}Na was added to each treatment and the fish were exposed for 1 h. At the beginning and end of the exposure period, a 1 ml sample was collected for measurement of [Na^+] and ^{22}Na activity. At the end of the exposure period, fish were treated as described in the Na^+ uptake experiments.

Similar experimental designs were used in subsequent inhibitor experiments. *Cyprinodon variegatus hubbsi* (9–36 mg) acclimated to 0.1 mmol l^{-1} Na^+ were exposed to 10^{-6} mol l^{-1} bafilomycin A1, a H^+-ATPase inhibitor (Boisen et al., 2003; Bury and Wood, 1999). Because of the toxicity of this inhibitor, fish were only exposed for 11 min. In another experiment, *C. v. hubbsi* (16–48 mg) acclimated to 0.1 mmol l^{-1} Na^+ were exposed to 10^{-4} or 10^{-5} mol l^{-1} phenamil, a potent Na^+ channel inhibitor with relatively low affinity for NHEs (Kleyman and Cragoe, 1988). The final experiment exposed *C. v. hubbsi* (16–132 mg) acclimated to 0.1, 2 and 7 mmol l^{-1} Na^+ as well as *C. v. variegatus* (31–175 mg) acclimated to 2 and 7 mmol l^{-1} Na^+ to 5×10^{-5} mol l^{-1} EIPA [5-(*N*-ethyl-*N*-isopropyl)-amiloride], which is a potent NHE inhibitor with low affinity for Na^+ channels (Kleyman and Cragoe, 1988).

Chloride-dependent Na^+ uptake

To test whether Na^+ uptake was chloride-dependent, juvenile *C. v. hubbsi* (52–137 mg) were acclimated to 0.5, 2 or 7 mmol l^{-1} Na^+ and juvenile *C. v. variegatus* (37–126 mg) were acclimated to 2 or 7 mmol l^{-1} Na^+. Fish were then transferred to the previously described defined media spiked with either NaCl or Na_2SO_4 to Na^+ concentrations equivalent to the acclimation water, and Na^+ uptake was determined using ^{22}Na as previously described. Apparent Cl^--dependent Na^+ uptake was observed under some conditions (see Results), leading to additional experiments. Because flux periods were ~1.5 h for each experiment, an acid–base disturbance in the 'Cl^--free' water could indirectly impact Na^+ uptake. To evaluate this, two additional experiments were performed (one each for *C. v. hubbsi* and *C. v. variegatus*) with high concentrations of ^{22}Na in which the flux period was reduced to 11–15 min, which would presumably minimize any acid–base disturbance. These experiments were performed on fish acclimated to 7 mmol l^{-1} Na^+ for both subspecies. Additional experiments with pharmacological inhibitors were also performed to evaluate the proteins involved in the apparent Cl^--dependent Na^+ uptake. The experimental design was the same as described for other inhibitors with fish exposed to 10^{-5} mol l^{-1} metolazone (NCC inhibitor) or 10^{-4} mol l^{-1} bumetanide (NKCC inhibitor) in separate experiments.

Characterization of gill morphology at different ambient Na^+ concentrations

A comparative analysis of apical crypt and emergent MRC density and size was undertaken for *C. v. hubbsi* and *C. v. variegatus*. For these experiments, adult *C. v. hubbsi* (0.3–1.0 g) were acclimated to 0.1, 2 or 7 mmol l^{-1} Na^+ freshwater whereas adult *C. v. variegatus* (0.5–2 g) were acclimated to 2 or 7 mmol l^{-1} Na^+ for at least 21 days. After acclimation, fish were euthanized by an overdose of tricaine methanesulfonate and the second branchial arch was immediately dissected from the fish. The whole gill arch was then rinsed in the treatment water to remove excess mucous and blood, and was then prepared for scanning electron microscopy. Gill arches were first placed in Karnovsky's solution (1% glutaraldehyde, 1% formaldehyde, 0.1 mol l^{-1} phosphate buffer) overnight for fixation. The following day, gills were triple rinsed in 0.1 mol l^{-1} phosphate buffer and then treated with a 1% OsO_4 solution in 0.1 mol l^{-1} phosphate buffer for 1 h. After osmication, gills were dehydrated in an ethanol series (30, 50, 70, 80, 90 and 100%) with two 10 min rinses at each concentration. Subsequently, gills were rinsed twice for 5 min in HMDS (1,1,1,3,3,3-hexylmethyldisilizane) and then air-dried overnight.

Under a dissecting microscope, gill arches were then cut into multiple pieces and mounted on to a single stub per gill arch. Pieces of gill arch were oriented so that the lateral sides of gill filaments were parallel to the stub face. Samples were sputter coated with Pd using a Cressington automated sputter coater (Watford, UK) and then digitally imaged in a Philips XL-30 ESEM FEG (Guildford, UK) set in SEM mode at 20 kV with a working distance of 15 mm. All images were taken at a fixed magnification of ×2000.

For each treatment, the second gill arch from five fish was sampled and five separate gill filaments were imaged for each arch. Filament images were taken in the mid-region of the afferent edge at least 80 μm distal from both where the filament joins the gill arch and the filament terminus, as MRC distribution appeared generally homogenous through this region. Each image was digitally analyzed using the free area analysis software ImageJ (http://rsb.info.nih.gov/ij). A 2000–3000 μm area of the afferent edge was delineated and the number of apical crypts and emergent MRCs was quantified. In images with emergent MRCs, the area of five MRCs per image was determined.

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, CT, USA). Rates of Na^{2+} uptake as measured by the appearance of radioactivity in the fish (nmol g^{-1} h^{-1}) were calculated using previously described methods (Boisen et al., 2003).

All values are expressed as means ± s.e.m. throughout. Most comparison data were analyzed by Student's *t*-test. In cases of unequal variance, a Mann–Whitney rank sum test was performed. When multiple treatments were evaluated (e.g. amiloride experiments), data were analyzed by ANOVA. All comparison analyses were performed using SigmaStat v3.5 (SPSS, 2006). Kinetic data were observed to fit a Michaelis–Menten function and estimates of the Michaelis constant (K_m) and maximum transport velocity (V_{max}) were determined in GraphPad Prism v5.0 (GraphPad Software Inc., 2007). Differences in K_m and V_{max} estimates for fish acclimated to different Na^+ concentrations were tested using an extra sum-of-squares *F*-test (Zar, 2009).

RESULTS

Na^+ influx and efflux experiments

Sodium uptake rates increased with increasing ambient Na^+ concentrations and followed a hyperbolic curve that approximated Michaelis–Menten saturation kinetics for both *C. v. hubbsi* and *C. v. variegatus* acclimated to different ambient Na^+ concentrations (Fig. 1). For *C. v. variegatus*, estimates of K_m and V_{max} were extremely high, indicating that this subspecies possesses a very low affinity and high capacity Na^+ uptake system (Table 1). Both K_m and V_{max} in *C. v. variegatus* acclimated to 2 mmol l^{-1} Na^+ were significantly lower (~50%) than values estimated at 7 mmol l^{-1} Na^+. For *C. v. hubbsi*, the K_m values for fish acclimated to 7 and 2 mmol l^{-1} Na^+ were similar, but the V_{max} of fish acclimated to 2 mmol l^{-1} Na^+ was significantly higher (~30%) than that observed for 7 mmol l^{-1} Na^+ acclimated fish. In contrast, the estimated K_m for *C. v. hubbsi* acclimated to 0.1 and 1 mmol l^{-1} Na^+ was significantly lower and relatively invariate (104–110 μmol l^{-1}; Table 1).

Measurement of Na^+ efflux rates over the course of a 48 h exposure to 'Na^+-free' water revealed that *C. v. hubbsi* and *C. v. variegatus* responded in a qualitatively similar manner. Initial efflux rates during the first 2 h were comparable (839–998 nmol g^{-1} h^{-1}) and then declined rapidly over the next 24 h, with efflux rates for the last flux period between 24 and 48 h also statistically similar (105–110 nmol g^{-1} h^{-1}) (Fig. 2). Efflux rates in *C. v. hubbsi* were significantly lower ($P \leq 0.05$) than those in *C. v. variegatus* 2 and 4 h after transfer to 'Na^+-free' water, indicating that *C. v. hubbsi* is able to reduce Na^+ efflux rates in dilute freshwater environments slightly faster than *C. v. variegatus*.

Pharmacological inhibitor experiments

Exposure of *C. v. hubbsi* and *C. v. variegatus* acclimated to 0.1 or 2 mmol l^{-1} Na^+ to increasing concentrations of amiloride resulted in sequentially increasing inhibition of Na^+ uptake (Fig. 3). *Cyprinodon variegatus hubbsi* acclimated to 0.1 mmol l^{-1} Na^+ were the most sensitive with $K_{0.5}=4.89\times10^{-6}$ mol l^{-1} amiloride, compared with fish acclimated to 2 mmol l^{-1} Na^+ where $K_{0.5}=3.57\times10^{-5}$ mol l^{-1} amiloride. *Cyprinodon variegatus variegatus* acclimated to 2 mmol l^{-1} Na^+ were the least sensitive with $K_{0.5}=2.96\times10^{-4}$ mol l^{-1} amiloride.

The next set of experiments involved using *C. v. hubbsi* acclimated to 0.1 mmol l^{-1} Na^+ and exposing them to bafilomycin A1 (a H^+-ATPase inhibitor) and phenamil (a Na^+ channel blocker). Exposure to bafilomycin resulted in no significant inhibition of Na^+ uptake (Fig. 4A) whereas exposure to phenamil induced significant reductions in Na^+ uptake: 71 and 25% at 10^{-4} and 10^{-5} mol l^{-1} phenamil, respectively (Fig. 4B).

Another set of inhibitor experiments involved the NHE-specific inhibitor EIPA using *C. v. hubbsi* acclimated to 0.1, 2 and

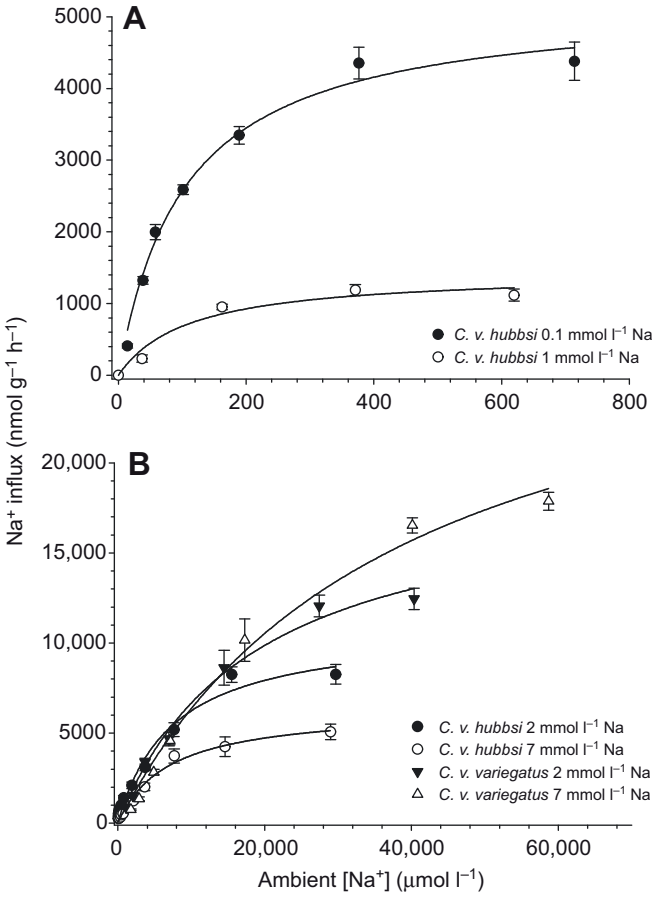

Fig. 1. Na^+ uptake rates (nmol g^{-1} h^{-1}) as a function of external Na^+ concentrations (μmol l^{-1}) for (A) *Cyprinodon variegatus hubbsi* acclimated to 0.1 and 1 mmol l^{-1} Na^+ and (B) *C. v. hubbsi* and *C. v. variegatus* acclimated to 2 and 7 mmol l^{-1} Na^+. Note the significantly different *x*- and *y*-axis scales for A and B. Data are means ± s.e.m.; *N*=8. See Table 2 for estimates of K_m and V_{max} for each treatment.

Table 1. Estimated Michaelis constant (K_m) and maximum transport velocity (V_{max}) for *Cyprinodon variegatus hubbsi* and *Cyprinodon variegatus variegatus* acclimated to different external Na^+ concentrations

Subspecies	Acclimation water [Na^+] (μmol l^{-1})	K_m (μmol l^{-1})	V_{max} (nmol g^{-1} h^{-1})
C. v. hubbsi	100	104±14^A	5232±234^A
	1000	110±52^A	1437±193^B
	2000	7464±1615^B	10878±904^C
	7000	6975±996^B	6370±348^D
C. v. variegatus	2000	18509±3342^C	18999±1560^E
	7000	38271±8321^D	30681±3393^F

Different letters equal significantly different values for each parameter (P<0.05).

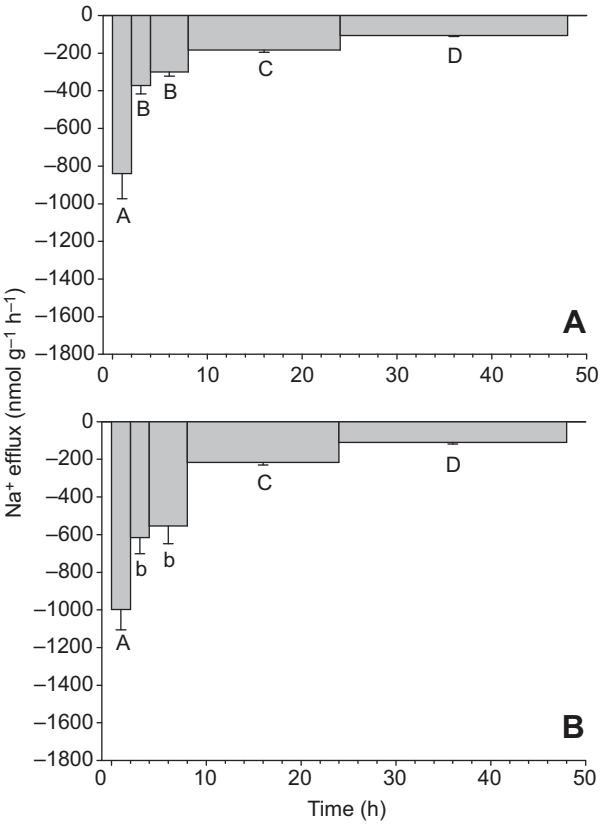

Fig. 2. Na^+ efflux rates (nmol g^{-1} h^{-1}) over time after transfer from 2 mmol l^{-1} Na^+ to 'NaCl-free' water for (A) *C. v. hubbsi* and (B) *C. v. variegatus*. Different widths of bars reflect different flux periods over the course of the 48 h experiment. Data are means ± s.e.m.; *N*=8. Different letters indicate significant differences (P<0.05) between flux periods within a subspecies. Different cases indicate significant differences between subspecies within a given flux period.

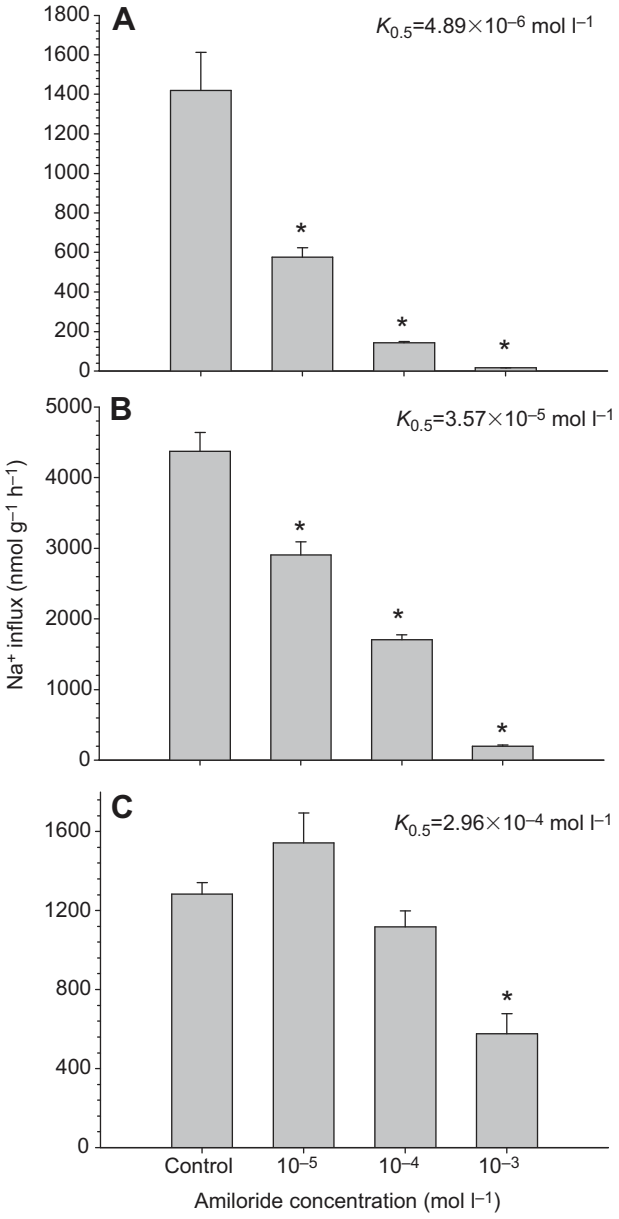

Fig. 3. Effect of increasing amiloride concentrations on Na^+ uptake rates (nmol g^{-1} h^{-1}) in *C. v. hubbsi* acclimated to (A) 0.1 mmol l^{-1} Na^+ and (B) 2 mmol l^{-1} Na^+, and *C. v. variegatus* acclimated to (C) 2 mmol l^{-1} Na^+. Controls include DMSO carrier. Data are means ± s.e.m.; *N*=8. Asterisks indicate significant differences compared with the control (*P<0.05).

7 mmol l^{-1} Na^+ and *C. v. variegatus* acclimated to 2 and 7 mmol l^{-1} Na^+. In fish acclimated to 7 mmol l^{-1} Na^+, a similar inhibition (29 and 21%, respectively) of Na^+ uptake relative to controls was observed (P≤0.05). In contrast, in 2 mmol l^{-1} Na^+ acclimated fish there appeared to be differential sensitivity to EIPA, with *C. v. variegatus* Na^+ uptake inhibited by 51% and *C. v. hubbsi* by 91%. In *C. v. hubbsi* acclimated to 0.1 mmol l^{-1} Na^+, inhibition of Na^+ uptake was 95% relative to control fish (Fig. 5).

Chloride-dependent Na^+ uptake experiment

Exposure of *C. v. hubbsi* acclimated to 0.5 or 2 mmol l^{-1} Na^+ and then acutely transferred to the same Na^+ concentration as either NaCl or Na_2SO_4 resulted in no significant difference in Na^+ uptake between the two treatments (Fig. 6). In contrast, *C. v. hubbsi* acclimated to 7 mmol l^{-1} Na^+ and *C. v. variegatus* acclimated to 2 or 7 mmol l^{-1} Na^+ both exhibited significantly reduced Na^+ uptake after transfer to 'Cl^--free' water (Fig. 6). Experiments to test whether flux duration influenced apparent Cl^--dependent Na^+ uptake *via* an acid–base disturbance showed similar reductions in Na^+ uptake in 'Cl^--free' water in experiments 10–11 min in duration compared with those 1.5 h in duration (Fig. 6). Additional experiments measuring Na^+ uptake in *C. v. variegatus* and *C. v. hubbsi* acclimated to 7 mmol l^{-1} Na^+ during exposure to metolazone (NCC inhibitor) or bumetanide (NKCC inhibitor) revealed no significant inhibition of Na^+ uptake in *C. v. hubbsi* exposed to either drug or *C. v. variegatus* exposed to metolazone. However, for *C. v. variegatus* exposed to bumetanide, there was a significant (36%) inhibition of Na^+ uptake (Fig. 7).

Characterization of gill morphology at different ambient Na^+ concentrations

Typical of most fish, MRCs were only observed along the afferent edge of the gill filament, with a smaller number of MRCs observed in the interlamellar spaces proximal to the afferent edge. No MRCs were observed on lamellae in any of the treatments. MRC distribution along the afferent edge appeared generally homogenous in terms of both density and relative contribution of apical crypt

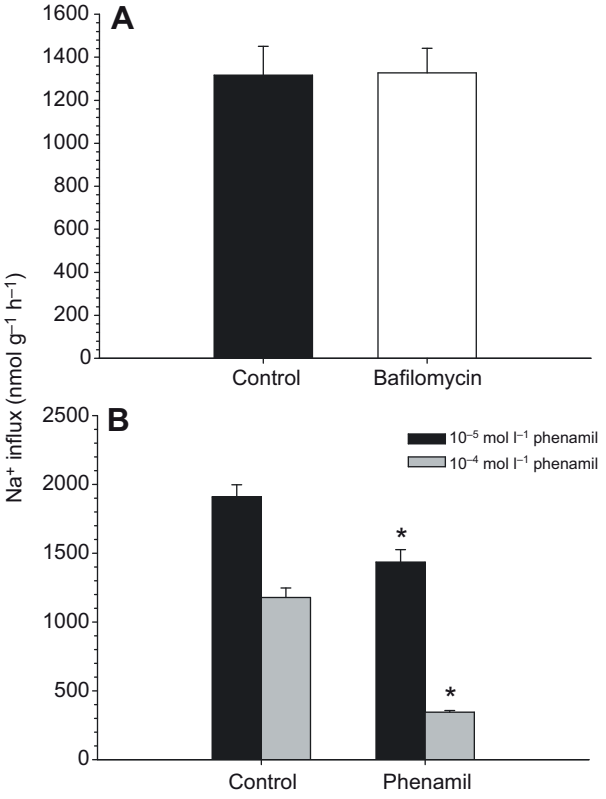

Fig. 4. Effects of (A) 10^{-6} mol l^{-1} bafilomycin and (B) 10^{-4} and 10^{-5} mol l^{-1} phenamil on Na^+ uptake rates (nmol g^{-1} h^{-1}) in *C. v. hubbsi* acclimated to 0.1 mmol l^{-1} Na^+. Phenamil experiments were performed at different times with separate control for comparison. Controls include DMSO carrier. Data are means ± s.e.m.; *N*=10. Asterisks indicate significant differences compared with the control (*P<0.05).

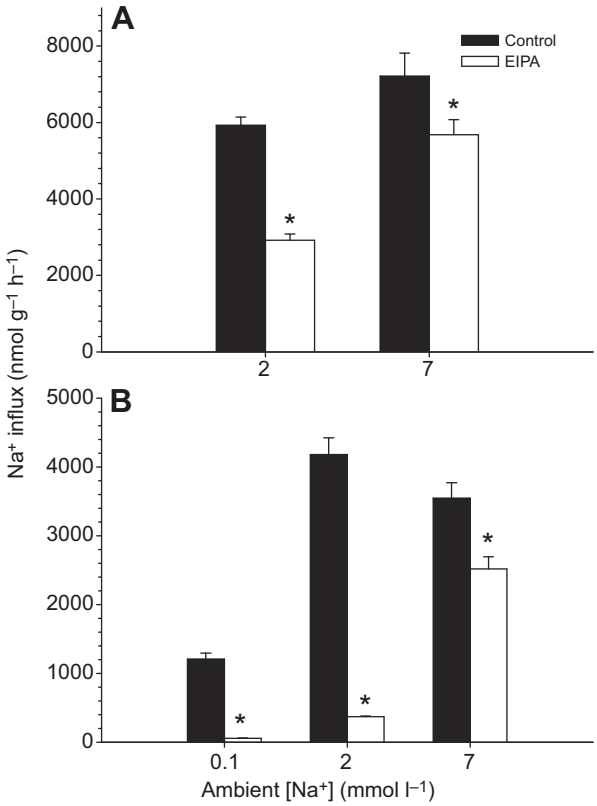

Fig. 5. Effect of 5×10^{-5} mol l^{-1} EIPA on Na^+ uptake rates (nmol g^{-1} h^{-1}) in (A) *C. v. variegatus* and (B) *C. v. hubbsi* acclimated to 0.1 (*C. v. hubbsi* only), 2 and 7 mmol l^{-1} Na^+. Controls include DMSO carrier. Data are means ± s.e.m.; *N*=10. Asterisks indicate significant differences compared with the control (*P<0.05).

and emergent MRCs within a given subspecies and treatment, except at the extreme basal and distal ends of gill filaments. At the distal end, relatively few MRCs were observed, similar to other fish. Interestingly, at the basal end of each filament for the first ~60 μm, only apical crypts were observed regardless of the ambient Na^+ concentration. This occurred even in *C. v. hubbsi* exposed to 0.1 mmol l^{-1} Na^+. Both of these regions were excluded from the analysis of changes in MRC density and type.

Representative scanning electron micrographs for each treatment are shown in Fig. 8. These micrographs show the general morphological appearance of fish gill filaments in each of the treatments. Changes in ambient Na^+ concentrations affected MRC type, density and size in both *C. v. hubbsi* and *C. v. variegatus*. The density of apical crypts increased with increasing ambient Na^+ concentration, ranging from ~500 mm^{-2} in *C. v. hubbsi* acclimated to 0.1 mmol l^{-1} Na^+ to ~3000–4000 mm^{-2} in fish acclimated to 7 mmol l^{-1} Na^+ (Fig. 9A). There were no significant differences in apical crypt density between subspecies in fish acclimated to 2 and 7 mmol l^{-1} Na^+. As would be expected, the density of emergent MRCs increased with decreasing ambient Na^+ in both subspecies, with fish at 7 mmol l^{-1} Na^+ having ~3000–6000 emergent MRCs per mm^2, reaching a maximum of ~11,000 emergent MRCs per mm^2 in *C. v. hubbsi* acclimated to 0.1 mmol l^{-1} Na^+ (Fig. 9B). *Cyprinodon variegatus variegatus* had a significantly ($P\leq0.05$) higher emergent MRC density than *C. v. hubbsi* when both subspecies were acclimated to 7 mmol l^{-1} Na^+, but emergent MRC densities were similar in fish acclimated to 2 mmol l^{-1} Na^+ (Fig. 9B).

The size of emergent MRCs increased with decreasing ambient Na^+ concentrations in both subspecies, ranging from 4.0±0.6 μm^2 $cell^{-1}$ in *C. v. hubbsi* acclimated to 7 mmol l^{-1} Na^+ to 16.6±2.1 μm^2 $cell^{-1}$ in *C. v. variegatus* acclimated to 2 mmol l^{-1} Na^+ (Fig. 9C). Within a subspecies, emergent MRC size increased significantly with each reduction in ambient Na^+ concentration. For fish acclimated to 2 and 7 mmol l^{-1} Na^+, emergent MRCs were significantly larger (approximately twofold) in *C. v. variegatus*, and emergent MRCs trended towards being larger (not significantly, P=0.16) in *C. v. variegatus* acclimated to 2 mmol l^{-1} Na^+ than *C. v. hubbsi* acclimated to 0.1 mmol l^{-1} Na^+.

A useful integrative metric is the MRC fractional area (MRCFA), which considers both cell density and size to estimate the fractional area of the gill filament occupied by emergent MRCs (Perry et al., 1992). Estimates of MRCFA followed the same general pattern observed for size of emergent MRCs (Fig. 10A). A plot of Na^+ uptake rates (using data from Fig. 1) as a function of MRCFA reveals strong linear relationships for both *C. v. hubbsi* and *C. v. variegatus*, with similar slopes but different intercepts (Fig. 10B).

DISCUSSION

Comparative Na^+ transport in *C. v. variegatus* and *C. v. hubbsi*

Characterization of Na^+ uptake and efflux in *C. v. variegatus* and *C. v. hubbsi* reveals that changes in the mechanism of Na^+ uptake is the principle means by which *C. v. hubbsi* has adapted to successfully regulate Na^+ in low Na^+ freshwater. Consistent with previous studies on tolerance to low-Na^+ environments, *C. v.*

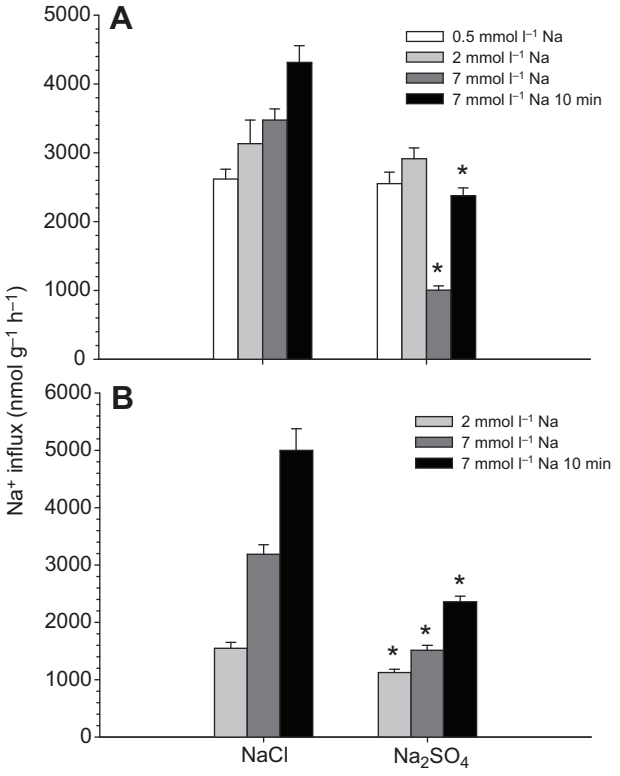

Fig. 6. Na^+ uptake (nmol g^{-1} h^{-1}) in (A) *C. v. hubbsi* acclimated to 0.5, 2 or 7 mmol l^{-1} Na^+ and (B) *C. v. variegatus* acclimated to 2 or 7 mmol l^{-1} Na^+. All flux experiments were ~1.5 h in duration except the 7 mmol l^{-1} Na^+ 10 min flux, which served as a method control to test for indirect inhibition of Na^+ uptake *via* an acid–base disturbance in Cl^--free water. Acclimated fish were exposed to the same Na^+ concentration as either NaCl or Na_2SO_4. Data are means ± s.e.m.; *N*=10. Asterisks indicate significant differences between the NaCl and Na_2SO_4 treatments (*P<0.05).

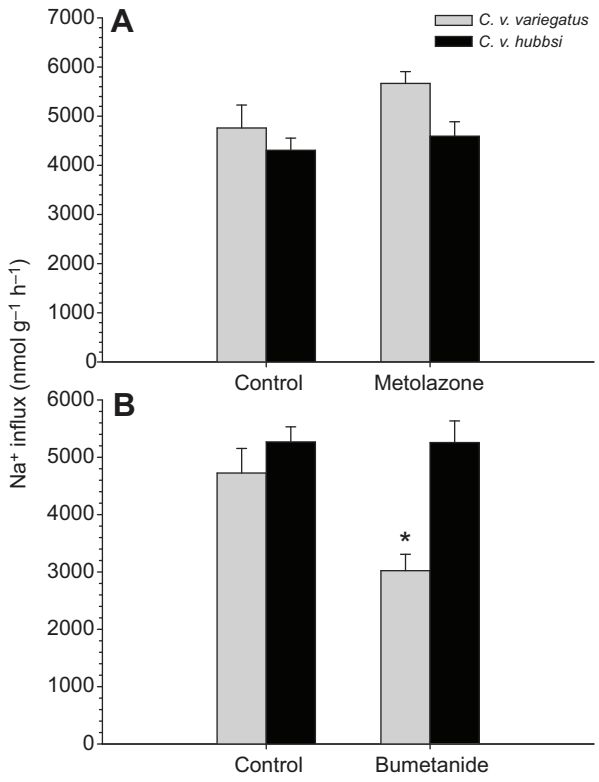

Fig. 7. Effect of (A) 1×10^{-5} mol l^{-1} metolazone and (B) 1×10^{-4} mol l^{-1} bumetanide on Na^+ uptake rates (nmol g^{-1} h^{-1}) in *C. v. variegatus* and *C. v. hubbsi* acclimated to 7 mmol l^{-1} Na^+. Controls include DMSO carrier. Data are means ± s.e.m.; *N*=8. Asterisks indicate significant differences compared with the control (*P<0.05).

variegatus exhibited a very low affinity, but high capacity Na^+ uptake system with estimated K_m values of 38,271 and 18,509 µmol l^{-1} in fish acclimated to 7 and 2 mmol l^{-1} Na^+, respectively (Table 1). This compares with K_m values of 1723 and 8000 µmol l^{-1} for two other euryhaline fish, *Fundulus heteroclitus* and *Poecilia reticulata*, respectively, acclimated to 1 mmol l^{-1} Na^+ freshwater (Evans, 1973; Patrick et al., 1997). In contrast, *C. v. hubbsi* exhibited a somewhat higher affinity Na^+ uptake system at both 2 and 7 mmol l^{-1} Na^+. Most interesting was the dramatic shift in K_m between fish acclimated to 2–7 mmol l^{-1} Na^+ and those acclimated to 0.1–1 mmol l^{-1} Na^+. The K_m for *C. v. hubbsi* did not change substantially in fish acclimated to Na^+ concentrations <1 mmol l^{-1} Na^+, although there was a compensatory increase in V_{max}. The change in K_m between 2 and 1 mmol l^{-1} Na^+ suggests that *C. v. hubbsi* utilize one or more different proteins to acquire Na^+ in dilute freshwater compared with more saline waters.

The significant increase in Na^+ affinity at 1 mmol l^{-1} Na^+ that occurs in *C. v. hubbsi* but is lacking in *C. v. variegatus* appears to be the primary mechanism by which *C. v. hubbsi* has evolved to survive in low-Na^+ environments, as efflux rates between the two subspecies were comparable (Fig. 2). Baseline Na^+ efflux rates were similar in the two subspecies when acclimated to 2 mmol l^{-1} Na^+ and both species rapidly reduced efflux rates to ~100 nmol g^{-1} h^{-1} in 'NaCl-free' water. Given the method used to measure efflux rates, these rates likely reflect the maximum reduction in efflux these fish are capable of rather than typical resting efflux rates. However, they are considerably lower than those of most fish, including those adapted to low-Na^+ waters. For example, at a comparable external Na^+ concentration to the current experiments, efflux rates on the order of 300–400 nmol g^{-1} h^{-1} were measured in two species of Amazonian fish (*Corydoras julii* and *Geophagus* sp.) adapted to low-Na^+ environments (K_m=56–112 µmol l^{-1} in these species) (Gonzalez et al., 2011). Unlike these fish, *C. v. variegatus* is unable to take up Na^+ in these environments (Table 1) and reduced Na^+ efflux is likely an adaptation to survive short-term excursions in low-Na^+ waters that might occur during tidal cycles high in an estuary. *Cyprinodon variegatus hubbsi* appears to have retained this adaptation despite having the ability to take up Na^+ in these environments.

Differences in MRC size and density do not explain differential Na^+ uptake

A second set of experiments evaluated whether potential differences in MRC size or density might explain observed differences in Na^+ uptake between *C. v. hubbsi* and *C. v. variegatus*. Exposure to lower ambient Na^+ concentrations resulted in an increase in emergent MRC density, approximately doubling the number of emergent MRCs over the range (0.1–7 mmol l^{-1}) of Na^+ concentrations evaluated (Fig. 9B). The extent of emergent MRC proliferation observed in *C. v. variegatus* and *C. v. hubbsi* is comparable to that observed in other euryhaline teleosts (Lee et al., 1996; Scott et al., 2004). The approximate 10-fold increase in apical crypts with increasing Na^+ (Fig. 9A) was also similar to that observed in *F. heteroclitus* when

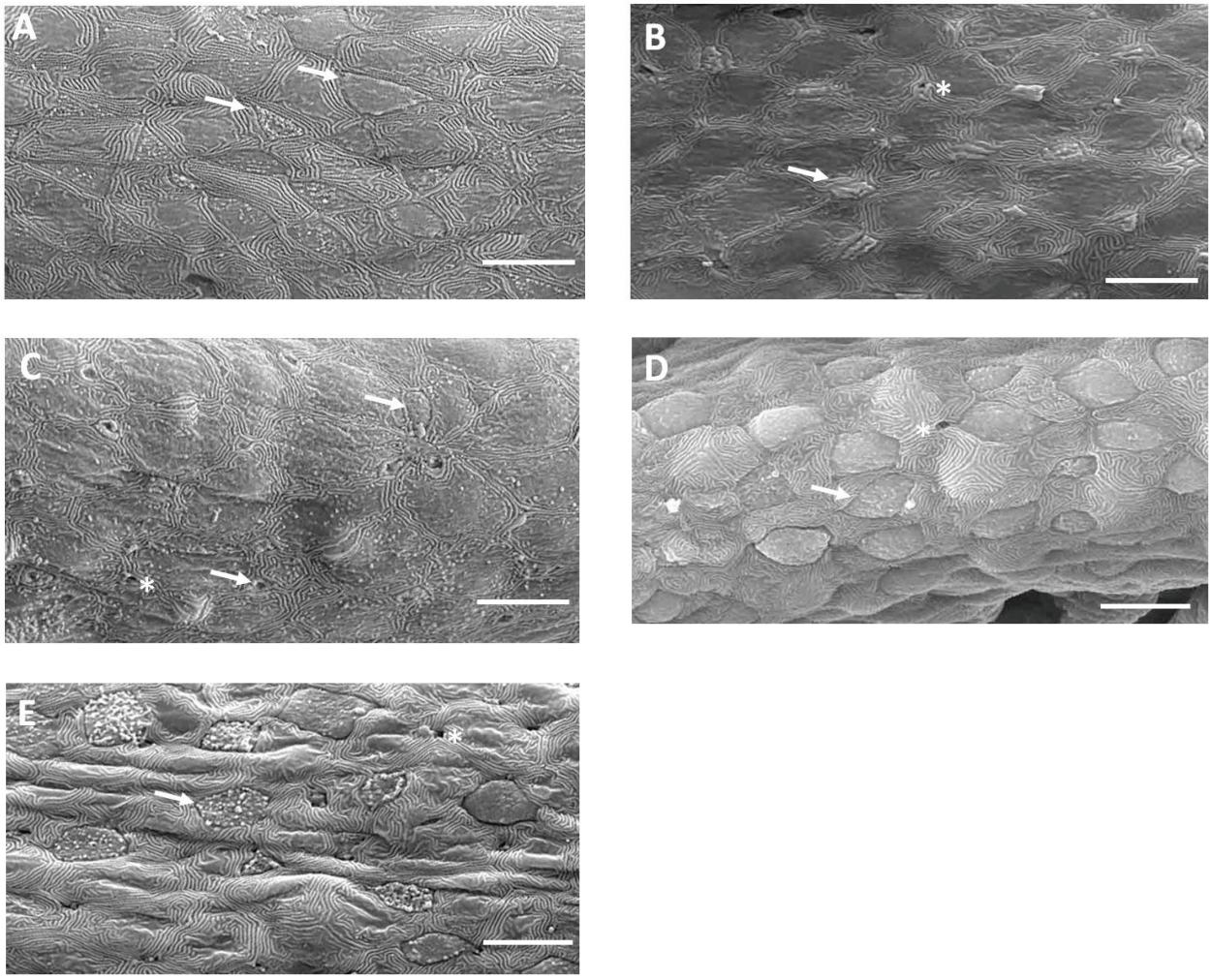

Fig. 8. Representative scanning electron micrographs of gill filament epithelia in *C. v. hubbsi* acclimated to (A) 0.1 mmol l^{-1} Na^+, (B) 2 mmol l^{-1} Na^+ and (C) 7 mmol l^{-1} Na^+, and *C. v. variegatus* acclimated to (D) 2 mmol l^{-1} Na^+ and (E) 7 mmol l^{-1} Na^+. All images at ×2000. Arrows indicate emergent mitochondria-rich cells (MRCs); asterisks indicate apical crypts. Scale bars, 10 μm.

transferred from 1 mmol l^{-1} Na^+ freshwater to 10 PSU (~150 mmol l^{-1} Na^+) brackish water (Scott et al., 2004). Although *C. v. hubbsi* and *C. v. variegatus* appear to exhibit characteristics within the range of responses observed in other euryhaline fish with respect to density of apical crypts and emergent MRCs, *C. v. hubbsi* did have significantly fewer emergent MRCs at 7 mmol l^{-1} Na^+ compared with *C. v. variegatus*, which may explain the lower Na^+ uptake capacity of *C. v. hubbsi* at this salinity.

Significant differences were also observed both within and between species with respect to size of emergent MRCs. The general trend of increasing emergent MRC size with decreasing ambient Na^+ is again consistent with previous observations in other euryhaline and freshwater fish (Fernandes et al., 1998; Greco et al., 1996; King et al., 1989). In addition to this general trend, distinct differences in MRC size were observed between *C. v. hubbsi* and *C. v. variegatus* at a given salinity, with MRCs in *C. v. variegatus* significantly larger than those in *C. v. hubbsi* at the same Na^+ concentration. Previous studies have demonstrated a linear relationship between the MRCFA and Na^+ uptake in several different species of euryhaline and freshwater fish (Laurent and Perry, 1990; Perry et al., 1992). In evaluating this relationship across four species (*Anguilla anguilla*, *Ictalurus nebulosus*, *Onchorhynchus mykiss* and *O. mossambicus*), MRCFA explained 94% of the variance in Na^+ uptake (Perry et al., 1992). Similar linear relationships were also observed for *C. v. hubbsi* and *C. v. variegatus* (Fig. 10B). However, unlike the previous study (Perry et al., 1992), *C. v. variegatus* clearly have lower Na^+ uptake rates at a given MRCFA compared with *C. v. hubbsi*. This suggests that although there are distinct differences between *C. v. hubbsi* and *C. v. variegatus* with respect to MRCFA at a given ambient Na^+ concentration, the enhanced Na^+ uptake exhibited by *C. v. hubbsi* at low ambient Na^+ concentrations is not only a result of increased MRCFA. Rather, it is the differential expression of proteins or protein isoforms involved in Na^+ uptake that provides *C. v. hubbsi* with the ability to osmoregulate in dilute freshwater.

Potential mechanisms for apical Na^+ acquisition

Results from the pharmacology and Cl^--dependent Na^+ uptake experiments indicate a dynamic response of these two subspecies to relatively small changes in ambient Na^+ concentrations. The Cl^--dependent and bumetanide experiments demonstrate that *C. v. variegatus* utilizes NKCC for apical Na^+ uptake in brackish waters (≥7 mmol l^{-1} Na^+). For fish acclimated to 7 mmol l^{-1} Na^+, 50–70% of total Na^+ uptake occurred *via* this pathway (Figs 6, 7). Use of

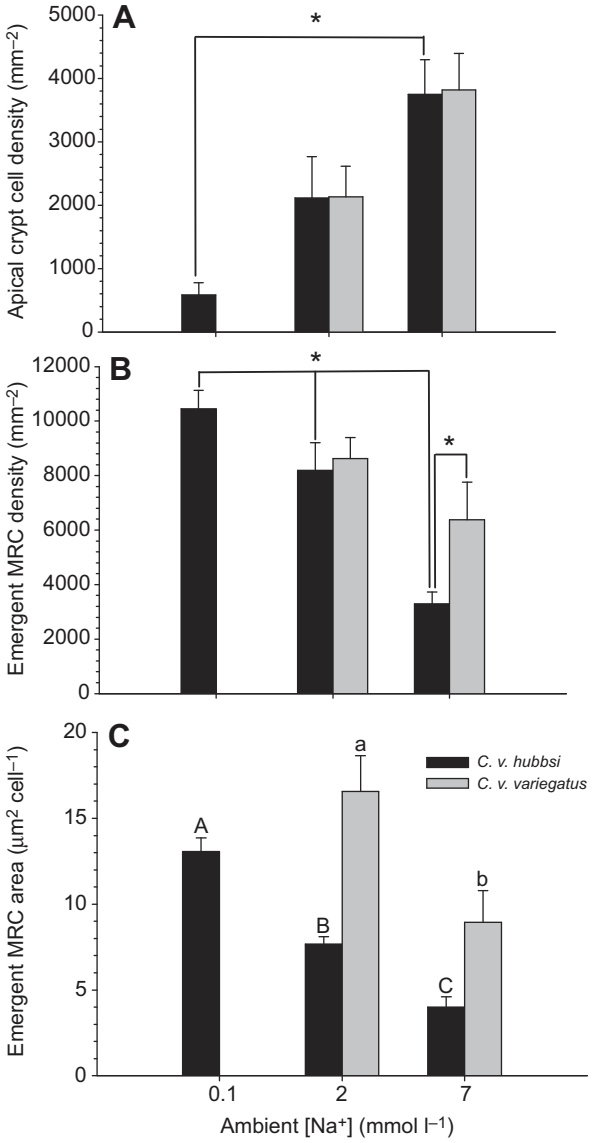

Fig. 9. Effects of ambient Na^+ concentration on (A) apical crypt density, (B) emergent MRC density and (C) size of emergent MRCs in *C. v. hubbsi* and *C. v. variegatus*. Data are means ± s.e.m.; *N*=5. In A and B, asterisks indicate significant differences (*P<0.05) between salinities within a subspecies. In C, different letters indicate significant differences (P<0.05) between salinities within a subspecies, and different cases indicate significant differences between subspecies within a salinity treatment.

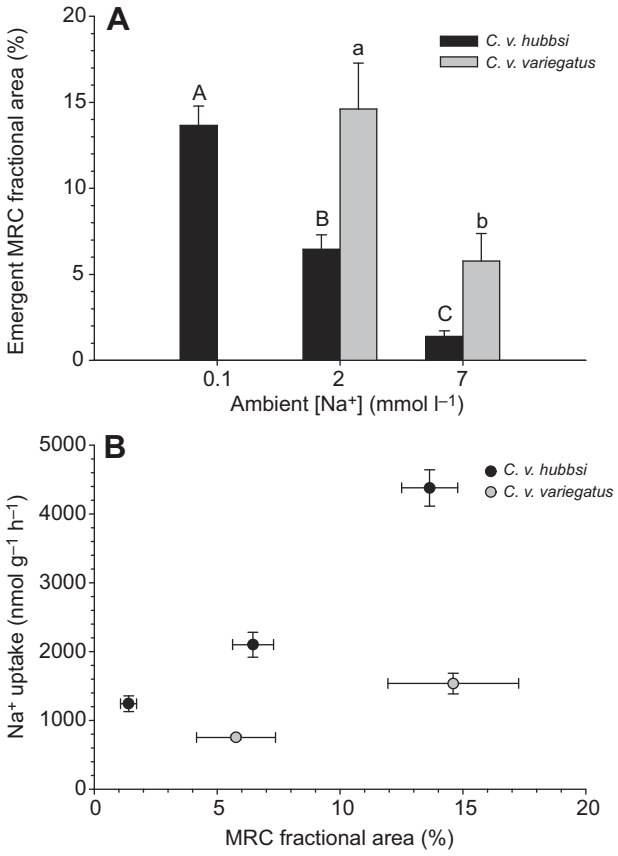

Fig. 10. (A) Effects of ambient Na^+ concentration on the fractional area of emergent MRCs in *C. v. hubbsi* and *C. v. variegatus*. Different letters indicate significant differences (P<0.05) between salinities within a subspecies, and different cases indicate significant differences between subspecies within a salinity treatment. Data are means ± s.e.m.; *N*=5. (B) Relationship between MRC fractional area and Na^+ uptake rate in *C. v. hubbsi* and *C. v. variegatus* acclimated to different Na^+ concentrations. All Na^+ uptake rates were determined at 2 mmol l^{-1} ambient Na^+. Data are means ± s.e.m.; *N*=5 for fractional area and *N*=8 for Na^+ uptake rates.

NKCC may explain the extremely high Na^+ uptake rates observed in *C. v. variegatus* at high ambient Na^+ concentrations where V_{max} ranged from 19,000 to 31,000 nmol g^{-1} h^{-1} (Table 1). Although we did not investigate Cl^- uptake in this study, it is worth noting that the related euryhaline fish *F. heteroclitus* does not take up Cl^- and relies strictly on an NHE for Na^+ uptake under typical freshwater conditions (<2 mmol l^{-1} NaCl). However, at higher ambient NaCl, significant Cl^- uptake does occur, suggesting that NKCC plays a similar role in this species (Patrick et al., 1997).

Despite a clear indication of Cl^--dependent Na^+ uptake in *C. v. hubbsi* acclimated to 7 mmol l^{-1} Na^+ (Fig. 6A), this subspecies does not appear to use either NCC or NKCC for apical Na^+ acquisition. As discussed below, results from experiments with EIPA indicate that only ~25% of Na^+ uptake by *C. v. hubbsi* acclimated to 7 mmol l^{-1} Na^+ can be attributed to an NHE (Fig. 5). The majority (~70%) of this non-NHE Na^+ uptake appears to be Cl^- dependent. Given that *C. v. hubbsi* is derived from *C. v. variegatus*, it is surprising that it does not also utilize apical NKCC. Further investigations into the proteins involved in this apparent Cl^--dependent Na^+ uptake are clearly needed.

As both subspecies are transferred to lower Na^+ concentrations (2–0.1 mmol l^{-1} Na^+), they appear to increasingly rely on one or more NHE isoforms for apical Na^+ uptake, as evidenced by the significant inhibition of Na^+ uptake by both amiloride and EIPA. However, the experiments with bafilomycin and phenamil on *C. v. hubbsi* acclimated to 0.1 mmol l^{-1} Na^+ provide potentially conflicting results. Although bafilomycin had no effect on Na^+ uptake, phenamil caused a 65% inhibition of Na^+ uptake at 10^{-4} mol l^{-1} and 25% inhibition at 10^{-5} mol l^{-1}. Given that these inhibitors are targeting different components of the same system, these conflicting results are difficult to reconcile. We are not aware of any studies showing that bafilomycin is ineffective at reducing Na^+ influx in fish that express a Na^+ channel system. Phenamil, an amiloride derivative, is a much more potent inhibitor of Na^+ channels (~17×) and a much less potent inhibitor of NHEs (~0.1×) than amiloride (Kleyman and Cragoe, 1988; Wood et al., 2002). Although 10^{-4} mol l^{-1} is a

relatively high concentration of phenamil, the results at 10^{-5} mol l^{-1} phenamil would generally be considered indicative of a Na^+ channel. However, given the lack of testing of fish NHEs in an isolated expression system, we cannot rule out the possibility that the specific NHE isoform expressed in *C. v. hubbsi* is sensitive to phenamil. It is also worth noting that the near-complete (~95%) inhibition of Na^+ uptake by EIPA for *C. v. hubbsi* acclimated to 0.1 mmol l^{-1} Na^+ suggests that a 65% inhibition of Na^+ uptake by phenamil is not possible unless it is targeting an NHE.

Despite the uncertainties related to the phenamil experiments, we interpret the comparatively lower amiloride $K_{0.5}$ for *C. v. hubbsi* acclimated to 2 mmol l^{-1} Na^+ and further reduction in $K_{0.5}$ for *C. v. hubbsi* acclimated to 0.1 mmol l^{-1} Na^+ to indicate the presence of two NHE isoforms in *C. v. hubbsi* compared with the one expressed by *C. v. variegatus* (Fig. 3). The near-complete (95%) inhibition of Na^+ uptake by EIPA in *C. v. hubbsi* acclimated to 0.1 mmol l^{-1} Na^+ suggests that *C. v. hubbsi* is only using an NHE for apical Na^+ uptake in low-Na^+ water and that the primary adaptation of *C. v. hubbsi* to low-Na^+ water is the expression of this high-affinity NHE isoform. Studies in zebrafish, medaka and tilapia have demonstrated the upregulation of NHE-3 after acute transfer to low-Na^+ water (Inokuchi et al., 2009; Wu et al., 2010; Yan et al., 2007) whereas studies in the euryhaline *Fundulus* (also Cyprinodontiformes) have observed upregulation of NHE-2 and downregulation of NHE-3 in acute transfers from brackish water to 1 mmol l^{-1} Na^+ freshwater (Scott et al., 2005). Collectively, these data suggest that NHE-2 may provide a mechanism for Na^+ uptake in relatively high-Na^+ waters whereas NHE-3 is utilized in low-Na^+ waters. This trend in relative Na^+ affinities is supported by mammalian studies on Na^+ transport kinetics of these two isoforms (Orlowski, 1993; Yu et al., 1993). However, in mammalian systems, NHE-2 has a higher affinity for amiloride than NHE-3 (Orlowski, 1993; Yu et al., 1993), which, if similar in fish, would conflict with our observations regarding putative NHE isoforms present in *C. v. hubbsi* and *C. v. variegatus* and their relative sensitivity to amiloride.

CONCLUSIONS

Overall, results of the present study indicate that both *C. v. variegatus* and *C. v. hubbsi* use several different proteins for apical Na^+ uptake over a relatively narrow range of ambient Na^+ concentrations. Additionally, this study indicates that *C. v. hubbsi* has undergone several adaptations in order to successfully regulate Na^+ in freshwater environments. Most important of these is the differential expression of one or more proteins involved in Na^+ uptake. Most of the data support the hypothesis that *C. v. hubbsi* is utilizing a high-affinity NHE to take up Na^+ across the apical membrane although there is still some uncertainty with this conclusion. Future gene expression studies should help to resolve this uncertainty. If this hypothesis is validated, *C. v. hubbsi* provides an excellent model organism for studying not only one mechanism by which estuarine fish can evolve to invade freshwater systems, but also how NHEs can overcome apparent thermodynamic constraints to function in low-Na^+ environments.

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