2012-11-02

Osmoregulatory Physiology of Pupfish (Cyprinodon spp.) in Freshwater

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UNIVERSITY OF MIAMI

OSMOREGULATORY PHYSIOLOGY OF PUPFISH (*CYPRINODON* SPP.) IN FRESHWATER

By

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A DISSERTATION

Submitted to the Faculty
of the University of Miami
in partial fulfillment of the requirements for
the degree of Doctor of Philosophy

Coral Gables, Florida

December 2012

UNIVERSITY OF MIAMI

A dissertation submitted in partial fulfillment of
the requirements for the degree of
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BRIX, KEVIN V. (Ph.D., Marine Biology and Fisheries)
Osmoregulatory Physiology of Pupfish (*Cyprinodon* Spp.) in Freshwater (December 2012)

Abstract of a dissertation at the University of Miami.

Dissertation supervised by Professor Martin Grosell.
No. of pages in text. (148)

Active uptake of Na^+ at the gill by fish is fundamental to their osmoregulation and thus survival in freshwater environments. Studies to date have demonstrated several different mechanisms by which fish can accomplish this important physiological function. This dissertation provides a comparative characterization of Na^+ uptake in the coastal pupfish, *Cyprinodon variegatus variegatus* (*Cvv*), the closely related Lake Eustis pupfish, *C. v. hubbsi* (*Cvh*), and the endangered desert pupfish, *C. macularius* (*Cm*). When acclimated to slightly saline freshwater (7 mM Na^+) all three fish use a low affinity Na^+/H^+ exchanger (NHE) for apical Na^+ uptake. *Cvv* also uses a Na^+:K^+:2Cl^- co-transporter under these conditions, the first time this has been functionally demonstrated in a freshwater fish. When acclimated to 2 mM Na^+, all three fish strictly rely on a low affinity NHE for Na^+ uptake. Only *Cvh*, which naturally occurs in eight dilute freshwater lakes in central Florida, was able to acclimate to 0.1 mM Na^+ and under these conditions switches to a high affinity NHE for Na^+ uptake. In other fish studied to date that use a high affinity NHE in dilute freshwater, the NHE operates in a metabolon with an Rh glycoprotein (ammonia transporter) that provides H^+ for the operation of NHE in this thermodynamically constrained environment. This dissertation confirmed that *Cvh* does

not use an NHE-Rh metabolon, but instead acquires H^+ from carbonic anhydrase mediated hydration of CO_2 to allow for NHE operation in dilute freshwater. This is the first time this has been demonstrated in a fish exposed to low Na^+ water. Finally, because *Cvh* only occurs in eight lakes in central Florida, all of which are suffering from habitat loss and water quality degradation through urban development, this dissertation evaluated whether *Cvh* should be designated a separate species from *Cvv*, allowing for greater environmental protection. While there is no clear consensus on the definition of a species, within the regulatory framework of the Endangered Species Act, this dissertation demonstrated heritable physiological differences between *Cvv* and *Cvh*, partial pre- and post-zygotic isolation between the two subspecies, and likely monophyletic origin for *Cvh*, all of which support the designation of *Cvh* as an evolutionarily significant unit, and likely a separate species.

ACKNOWLEDGEMENTS

V. Matey, P. Blackwelder and H. Al Sayegh provided advice and assistance in preparing gills samples for SEM analysis. C. Wood provided valuable advice on experimental design and data interpretation for Chapter 3. K. Brix was supported by a University of Miami Maytag Fellowship and NSF Graduate Research Fellowship. M. Grosell was supported by NSF (IOB 0743903 and 1146695). *Cyprinodon variegatus variegatus* and *Cyprinodon variegatus hubbsi* were collected under Florida Fish and Wildlife Conservation Commission permit FNE-2010-09. *Cyprinodon macularius* were generously provided by Chris Ingersoll at the USGS Lab in Columbia, MO and held under USFWS Endangered Species Permit #TE2057312. Experiments were approved by the University of Miami animal care and use committee.

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Chapter 1:

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, Placoderms, Osteichthyans) 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 extracellular fluid osmolality of ~300-400 mOsm (Evans et al. 2005).

In teleost fish, extracelluar fluid osmolality is dominated by Na^+ and Cl^- with minor contributions from other ions (e.g., Ca^{2+}, K^+, and HCO_3^-). In order 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. This compensation occurs at the gill in specialized ionocytes often referred to as mitochondrial rich cells (MRCs) or historically chloride cells (Marshall and Grosell 2006). At these cells, ions must cross both apical and basolateral membranes to reach the fish plasma.

There has been considerable study of ion homeostasis at the whole animal, protein and gene expression levels of organization in several model freshwater fish (Evans et al. 2005; Marshall and Grosell 2006; Hwang et al. 2011). Active Na^+ uptake is fueled primarily by a basolateral Na^+/K^+-ATPase (NKA) in fish gill ionocyte (Karnaky et al.

1976). The importance of this protein in freshwater fish ionoregulation is well established although recent studies have demonstrated there are specific combinations of NKA subunit isoforms that allow for a diverse range of functionality in this protein across species and osmotic environments (Richards et al. 2003; Scott et al. 2005; Bystriansky et al. 2006; Jorgensen 2008).

In contrast, Na^+ uptake across the apical membrane of ionocytes appears much more variable and in some cases, controversial, with a number of different models having been developed over the past 80 years. Krogh (1938) was the first to suggest some sort of exchange between Na^+ uptake and NH_4^+ in freshwater fish and this model was supported later by additional studies (Maetz and Romeu 1964). However, studies over the next two decades suggested the correlation between Na^+ uptake and NH_4^+ excretion was poor (Maetz 1973). Studies on perfused fish heads and whole fish demonstrated that the more likely mechanism was Na^+/H^+ exchange (Cameron and Heisler 1983; Perry et al. 1985) and this has become an accepted model with numerous studies supporting the presence of NHE-2 and NHE-3 isoforms in the apical membrane of fish gills (Goss et al. 1992; Wilson et al. 1994; Perry et al. 2003; Edwards et al. 2005; Hwang et al. 2011).

Basic thermodynamic calculations suggest that NHEs cannot function in relatively low Na^+ (<2 mM) or low pH (<7.0) waters (Parks et al. 2008). However, there is evidence of a metabolon involving NHE and Rh glycoproteins which transport ammonia that could create a thermodynamically favorable environment for NHE function in low Na^+ and pH environments (Yan et al. 2007; Wu et al. 2010; Kumai and Perry 2011). Alternatively, the use of carbonic anhydrase (CA) to generate high intracellular H^+

concentrations could also create a thermodynamically favorable environment as has been observed in the Japanese dace (*Tribolodon hakonensis*) at low pH (Hirata et al. 2003).

Another mechanism for apical Na^+ uptake is a Na^+ channel associated with an H^+-ATPase, which hyperpolarizes the membrane creating a favorable electrochemical gradient allowing for Na^+ uptake in low Na^+ or acidic waters against the concentration gradient (Avella and Bornancin 1989). There is now evidence for this mechanism as demonstrated by inhibition of Na^+ uptake by pharmacological inhibitors of both the H^+-ATPase (Lin and Randall 1991; Lin and Randall 1993) and Na^+ channel (Bury and Wood 1999; Grosell and Wood 2002; Reid et al. 2003), as well as localization of the H^+-ATPase to the apical membrane (Lin et al. 1994). However, cloning of the putative Na^+ channel has not occurred in any fish species and the exact identity of this channel remains elusive.

Finally, in some freshwater fish, chloride-dependent uptake of Na^+ at the apical membrane has been postulated 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 immunohistochemical studies in tilapia support the presence, though not necessarily function of an apical NKCC (Hiroi et al. 2005) while studies in zebrafish support both the presence and function of NCC in high Na^+ (12 mM) freshwater (Esaki et al. 2007; Wang et al. 2009).

The vast majority of research on freshwater fish osmoregulation has been performed on a limited number of model species (*Danio rerio*, *Oncorhynchus mykiss*, *Fundulus heteroclitus*, and *Oreochromis niloticus*). What is clear from studies on these few species is that there is a diverse array of mechanisms for Na^+ uptake in freshwater teleosts and

that a given species may use different protein systems for acquiring Na^+ depending on the environmental conditions. The few studies that have investigated non-model species suggest there are likely additional undescribed mechanisms (Gonzalez et al. 2002; Hirata et al. 2003; Preest et al. 2005).

The main objective of this dissertation was to develop ion transport models for Na^+ uptake in the euryhaline pupfish *Cyprinodon variegatus*. *C. variegatus* is actually comprised of at least three subspecies. *C. v. variegatus* (*Cvv*) occurs along the Gulf and Atlantic coasts of North America up to North Carolina, with tidal backwaters and near freshwater environments high in estuaries being their primary habitat. *C. v. hubbsi* (*Cvh*) is limited to eight freshwater lakes in central Florida (Carr 1936) and is believed to have become isolated from *Cvv* ~250 kya (Guillory and Johnson 1986). A third subspecies which will not be considered here, *C. v. ovinus* (*Cvo*), occupies similar habitats to *Cvv* and ranges from Virginia up to Rhode Island (Haney et al. 2007).

Cvv is considered perhaps the most euryhaline fish in the world, tolerating salinities ranging from freshwater up to 167 g l^{-1} (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, *Cvv* does not survive, grow or reproduce at concentrations <2 mM Na^+ (Dunson et al. 1998). In contrast, the eight lakes in which *Cvh* occurs have ambient Na^+ concentrations of 0.4-1 mM. Given the relatively recent divergence of *Cvv* and *Cvh* and the apparent differences in ability to osmoregulate in dilute freshwater, I hypothesized 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 Chapter 2 of this dissertation was to provide an initial comparative characterization of Na^+ transport kinetics in freshwater acclimated *Cvv* and *Cvh*, and to begin to characterize the proteins that contribute to Na^+ uptake in these subspecies by manipulating environmental conditions (Na^+, Cl^-, pH) and using pharmacological inhibitors. In one of these experiments, exposure of fish to low pH, measurement of net acid flux was opposite of what was expected based on previous studies in the related euryhaline species, *Fundulus heteroclitus* (Patrick and Wood 1999). Careful analysis of these data revealed the discrepancy appeared to be the result of different methodologies for measuring titratable acid flux in fish. Chapter 3 focused on evaluating the cause of these disparate results between the two methodologies and attempting to resolve which method best characterizes net acid flux in fish under various environmental conditions.

Chapter 4 of the dissertation is also based on results from Chapter 2 which indicated that *Cvh* expresses a protein (or combination of proteins) at low Na^+ ($\leq$1 mM), which *Cvv* does not, that allows for Na^+ uptake in dilute freshwater. Based on inhibitor experiments, it appears that both subspecies lack a Na^+ channel/H^+-ATPase system and that the primary difference is *Cvh* utilizes a high affinity NHE in low Na^+ environments that is apparently not expressed in *Cvv*. The focus of Chapter 4 is on characterizing the mechanism(s) by which this high affinity NHE in *Cvh* functions. Specifically, experiments were designed to evaluate whether *Cvh* uses a NHE-Rh metabolon or the NHE-CA system to create a thermodynamically favorable environment for NHE function in low Na^+ water.

Chapter 5 provides an initial characterization of Na^+ transport in the endangered desert pupfish, *Cyprinodon macularius*. *C. macularius* populations are in decline as a

result of various man-made changes to water flow and salinity in the aquatic systems in which it naturally occurs. Hence, Chapter 5 provides useful information on the osmoregulatory capacity of this species for management and recovery of this endangered species. *C. macularius* is also a representative of the western clade of pupfish (~35 species) which is the group that *C. variegatus* diverged from ~3.5 mya. Hence, *C. macularius* may represent the basal Na^+ transport characteristics of *Cyprinodon*, providing a comparative context in which to evaluate observed differences in *Cvv* and *Cvh*.

The final research chapter (Chapter 6) considers whether *Cvh* should be designated a separate species from *Cvv*. This is important for *Cvh* conservation as all 8 lakes in which it occurs have undergone significant eutrophication in the past 25 years. Within the lakes, *Cvh* only occurs in a narrow niche of sandy shoreline and is competitively excluded from other habitats (Guillory and Johnson 1986). Eutrophication and urban development have led to loss of much of the sandy shoreline habitat. While a quantitative study has not been conducted, most of the collecting sites described by previous researchers (Johnson 1974; Guillory and Johnson 1986) are now overrun by aquatic grasses. Re-designation of *Cvh* to species status would provide a regulatory mechanism for quantitative population studies and subsequent protection if needed. To evaluate species designation for *Cvh*, a series of experiments were performed characterizing pre- and post-zygotic isolation between the two subspecies. Additionally, mitochondrial DNA was sequenced from three populations of *Cvh* and two populations of *Cvv*, which along with published data on other *Cvv* populations, was used to construct a phylogeny for the *Cvv*/*Cvh* species complex.

Chapter 2:

Comparative Characterization of Na^+ Transport in *Cyprinodon variegatus variegatus* and *Cyprinodon variegatus hubbsi*

Summary

The euryhaline fish *Cyprinodon variegatus variegatus* (*Cvv*) is capable of tolerating ambient salinities ranging from 0.3 to 167 g l^{-1}, but incapable of long-term survival in freshwater (<2 mM Na^+). A population of this species isolated in several freshwater (0.4-1 mM Na^+) lakes in central Florida for the past ~150 ky is now designated as a subspecies (*Cyprinodon variegatus hubbsi*; *Cvh*). We conducted a comparative study to characterize the Na^+ transport kinetics in these two populations when acclimated to different ambient Na^+ concentrations. Results reveal *Cvv* and *Cvh* have qualitatively similar low affinity Na^+ uptake kinetics (K_m = 7,000-38,000 μM) when acclimated to 2 or 7 mM Na^+, but *Cvh* switches to a high affinity system (K_m = 100-140 μM) in low Na^+ freshwater (≤1 mM Na^+). Inhibitor experiments indicate Na^+ uptake in both *Cvv* and *Cvh* is EIPA-sensitive, but this sensitivity decreases with increasing external Na^+. EIPA induced a 95% inhibition of Na^+ influx in *Cvh* acclimated to 0.1 mM Na^+ suggesting this subspecies is utilizing a NHE to take up Na^+ in low Na^+ environments despite theoretical thermodynamic constraints. Na^+ uptake in *Cvh* acclimated to 0.1 mM Na^+ is not bafilomycin sensitive but is phenamil sensitive leading to uncertainty about whether this subspecies also utilizes Na^+ channels for Na^+ uptake. Experiments with both subspecies acclimated to 7 mM Na^+ also indicate a Cl-dependent Na^+ uptake pathway is present. Additional inhibitor experiments reveal this pathway is not metolazone-sensitive (NCC

inhibitor) in either species but is bumetanide-sensitive in *Cvv*, but not *Cvh*, indicating an apical NKCC is increasingly involved with Na^+ uptake as external Na^+ increases from 2 to 7 mM in *Cvv*.

Finally, characterization of mitochondrial rich cell 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^+ in both subspecies. A linear relationship between the fractional area of emergent MRCs and Na^+ uptake rate was observed for both subspecies. However, *Cvv* have lower Na^+ uptake rates at a given MRC fractional area compared to *Cvh* indicating that the enhanced Na^+ uptake exhibited by *Cvh* 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 *Cvh* with the ability to osmoregulate in dilute freshwater.

Background

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, Placoderms, Osteichthyans) 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; Marshall and Grosell 2006; Hwang et al. 2011). 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.

In order 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 (NKA). Three mechanisms have been hypothesized to accomplish apical entry of Na^+ at the gill in dilute freshwater. A Na^+ channel associated with an 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 via one or more NHE isoforms. The functionality of an apical NHE in low Na^+ environments (e.g., <1 mM Na^+) is thermodynamically problematic (Parks et al. 2008), but there is increasing evidence of a metabolon involving NHE and Rh 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 while 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 mitochondrial rich cell (MRC) types and dynamic responses of these cell types to changes in external ion concentrations (Greco et al. 1996; Lee et al. 1996; Fernandes et al. 1998; Hirai et al. 1999; Chang et al. 2001). Several studies, particularly in tilapia, have also co-localized specific apical proteins to specific MRC types (Hiroi et al. 2005; Inokuchi et al. 2009) while 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* (*Cvv*) occurs along the Gulf and Atlantic coasts of North America and tolerates salinities ranging from near freshwater up to 167 g l^{-1} (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, *Cvv* does not survive, grow or reproduce at concentrations <2 mM Na^+ (Dunson et al. 1998). A freshwater pupfish population currently given subspecies status (*C. v. hubbsi*; *Cvh*) is found in eight lakes in central Florida with ambient Na^+ concentrations of 0.4-1 mM. *C. v. hubbsi* is estimated to have been isolated from coastal populations of *Cvv* for 100-250 ky (Darling 1976; Guillory and Johnson 1986).

Given the relatively recent divergence of *Cvv* and *Cvh* and the apparent differences in 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 current study was to provide an initial comparative characterization of Na^+ transport kinetics in freshwater acclimated *Cvv* and *Cvh*, 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.

Methods and Materials

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 with filtered natural seawater (35 g l^{-1}) from Bear Cut, FL. Adult fish were bred and F_1 offspring were hatched and raised in seawater until the late juvenile stage (~ 2 m 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).

F_1 fish were acclimated to near freshwater conditions (0.3 g l^{-1} salinity; 7 mM Na^+, pH 7.9 or 0.1 g^{-1}salinity; 2 mM Na^+, pH 7.9) for a minimum of 3 weeks prior to experimental use. 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. Preliminary experiments indicated 2 mM 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 d prior to experimental use.

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 F_1 offspring were hatched and raised in dechlorinated tapwater until the late juvenile stage (~ 2 m 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 *Cvh* refused to eat the flake food diet fed to *Cvv*. F_1 fish were acclimated to different Na^+ concentrations (0.1, 2, or 7 mM Na^+) for a minimum of 3 weeks prior to experimental use. Dechlorinated tapwater was diluted with nanopure water to create the 0.1 mM Na^+ treatment and had a pH of 6.8.

Characterization of Na^+ Uptake Kinetics and Efflux Rates

The Na^+ uptake kinetics of *Cvh* were determined for fish acclimated to 0.1, 1, 2 and 7 mM Na^+, while uptake kinetics for *Cvv* were determined for fish acclimated to 2 and 7 mM Na^+. For each experiment, Na^+ uptake rates were measured at between 7 and 10 different ambient Na^+ concentrations ranging from 0.014 to 36.5 mM Na^+ depending on the subspecies and Na^+ concentration to which they were acclimated. At each Na^+ concentration, 8 juvenile fish (50-300 mg) were placed in 50 ml of a defined media (480 µM $CaSO_4$, 150 µM $MgSO_4$, 100 µM $KHCO_3$, 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 media for 10

min after which the media 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 0.5-3 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 media, double rinsed in a 100 mM Na^+ solution to displace any loosely bound ^{22}Na, blotted dry, weighed to nearest 0.1 mg and then assayed individually for radioactivity.

Characterization of Na^+ efflux rates was accomplished using adult (1.0-1.6 g) *Cvh* and *Cvv* that had been acclimated for >3 weeks to 2 mM 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 provided with gentle aeration and allowed to acclimate for 1 h. After acclimation, the water was replaced with the previously described defined media without NaCl. A water sample was immediately collected for measurement of water Na^+. 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 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 μM as a result of Na^+ efflux from fish. This both minimized the

amount of waterborne Na^+ which the fish might take up and minimized changes in the diffusive Na^+ gradient over a given flux period.

Pharmacological Inhibitor Experiments

Juvenile *Cvh* and *Cvv* 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 *Cvh* exhibits a high affinity Na^+ transport system while *Cvv* exhibits a comparatively low affinity Na^+ transport system (see Results). It was hypothesized that *Cvh* utilized an H^+-ATPase/Na^+ channel system to take up Na^+ across the apical membrane while *Cvv* 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 *Cvh* acclimated to 0.1 and 2 mM Na^+ and *Cvv* acclimated to 2 mM 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}, 10^{-3} M) in an attempt to distinguish effects between these different pathways. For the control and amiloride treatments, 10 juvenile *Cvh* (8-43 mg) or *Cvv* (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} M amiloride and 0.1% DMSO, while 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 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. *Cvh* (9-36 mg) acclimated to 0.1 mM Na^+ were exposed to 10^{-6} M Bafilomycin A_1, an H^+-ATPase inhibitor (Bury and Wood 1999; Boisen et al. 2003). Due to the toxicity of this inhibitor, fish were only exposed for 11 min. In another experiment, *Cvh* (16-48 mg) acclimated to 0.1 mM Na^+ were exposed to 10^{-4} or 10^{-5} M phenamil, a potent Na^+ channel inhibitor with relatively low affinity for NHEs (Kleyman and Cragoe 1988). The final experiment exposed *Cvh* (16-132 mg) acclimated to 0.1 , 2 and 7 mM Na^+ as well as *Cvv* (31-175 mg) acclimated to 2 and 7 mM Na^+ to 5 x 10^{-5} M 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 *Cvh* (52-137 mg) were acclimated to 0.5, 2 or 7 mM Na^+ while juvenile *Cvv* (37-126 mg) were acclimated to 2 or 7 mM 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 *Cvh* and *Cvv*) 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 mM 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} M metolazone (NCC inhibitor) or 10^{-4} M bumetanide (NKCC inhibitor) in separate experiments.

Characterization of Gill Morphology at Different Ambient Na^+ Concentrations

A comparative analysis of apical crypt and emergent mitochondrial rich cell (MRC) density and size was undertaken for *Cvh* and *Cvv*. For these experiments, adult *Cvh* (0.3-1.0 g) were acclimated to 0.1, 2, or 7 mM Na^+ freshwater while adult *Cvv* (0.5-2 g) were acclimated to 2 or 7 mM Na^+ for at least 21 d. 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 then prepared for scanning electron microscopy. Gill arches were first placed in Karnovsky's solution (1% glutaraldehyde, 1% formaldehyde, 0.1 M phosphate buffer) overnight for fixation. The following day, gills were triple rinsed in 0.1 M phosphate buffer and then treated with a 1% OsO_4 solution in 0.1 M phosphate buffer for 1 h. After osmication, gills were dehydrated in an ethanol series (30, 50, 70, 80, 90, 100%) with two 10 min rinses at each concentration.

Subsequently, gills were rinse 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 and then digitally imaged in a Phillips XL-30 ESEM FEG set in SEM mode at 20 kV with a working distance of 15 mm. All images were taken at a fixed magnification of 2000x.

For each treatment, the second gill arch from 5 fish was sampled and 5 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 Image J (available at http://rsb.info.nih.gov/ij). A 2000-3000 uM 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 5 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, Connecticut). Rates of Na^{2+} 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).

All values are expressed as means ± SEM 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 K_m and 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 *Cvh* and *Cvv* acclimated to different ambient Na^+ concentrations (Fig. 2.1). For *Cvv*, estimates of K_m and V_{max} were extremely high indicating this subspecies possesses a very low affinity and high capacity Na^+ uptake system (Table 1). Both K_m and V_{max} in *Cvv* acclimated to 2 mM Na^+ were significantly lower (~50%) than estimated at 7 mM Na^+. For *Cvh*, the K_m for fish acclimated to 7 and 2 mM Na^+ were similar, while the V_{max} of fish acclimated to 2 mM Na^+ was significantly higher (~30%) than observed for 7 mM Na^+ acclimated fish. In contrast, the estimated K_m for *Cvh* acclimated to 0.1 and 1 was significantly lower and relatively invariate (104-110 μM; Table 2.1).

Measurement of Na^+ efflux rates over the course of a 48 hour exposure to "Na^+ free" water revealed that *Cvh* and *Cvv* 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}) (Figure 2.2). Efflux rates in *Cvh* were significantly lower ($p \leq 0.05$) than those of *Cvv* at 2 and 4 h after transfer to "Na^+ free" water indicating *Cvh* is able to reduce Na^+ efflux rates in dilute freshwater environments slightly faster than *Cvv*.

Table 2.1. Estimated K_m and V_{max} for *C. v. hubbsi* and *C. v. variegatus* acclimated to different external Na^+ concentrations. Different letters equal significantly different values for each parameter ($p \leq 0.05$).

Organism	Acclimation Water [Na^+] (μM)	K_m (μM)	V_{max} (nmol g^{-1} h^{-1})
Cyprinodon variegatus 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
Cyprinodon variegatus variegatus	2000	18509 ± 3342^C	18999 ± 1560^E
	7000	38271 ± 8321^D	30681 ± 3393^F

Pharmacological Inhibitor Experiments

Exposure of *Cvh* and *Cvv* acclimated to 0.1 or 2 mM Na^+ to increasing concentrations of amiloride resulted in sequentially increasing inhibition of Na^+ uptake (Fig. 2.3). *Cvh* acclimated to 0.1 mM Na^+ were the most sensitive with $K_{0.5} = 4.89 \times 10^{-6}$ M amiloride compared to fish acclimated to 2 mM Na^+ where $K_{0.5} = 3.57 \times 10^{-5}$ M amiloride. *Cvv* acclimated to 2 mM Na^+ were the least sensitive with $K_{0.5} = 2.96 \times 10^{-4}$ M amiloride.

Figure 2.1. Na^+ uptake rates (nmol g^{-1} h^{-1}) as a function of external Na^+ concentrations (μM) for A) *C. v. hubbsi* acclimated to 0.1, 1, and 2 mM Na^+ and B) *C. v. hubbsi* acclimated to 7 mM Na^+ and *C. v. variegatus* acclimated to 2 and 7 mM Na^+. Note significantly different x- and y-axis scales for Fig. 1A and 1B. Mean ± SEM (n=8). See Table 2 for estimates of K_m and V_{max} for each treatment.

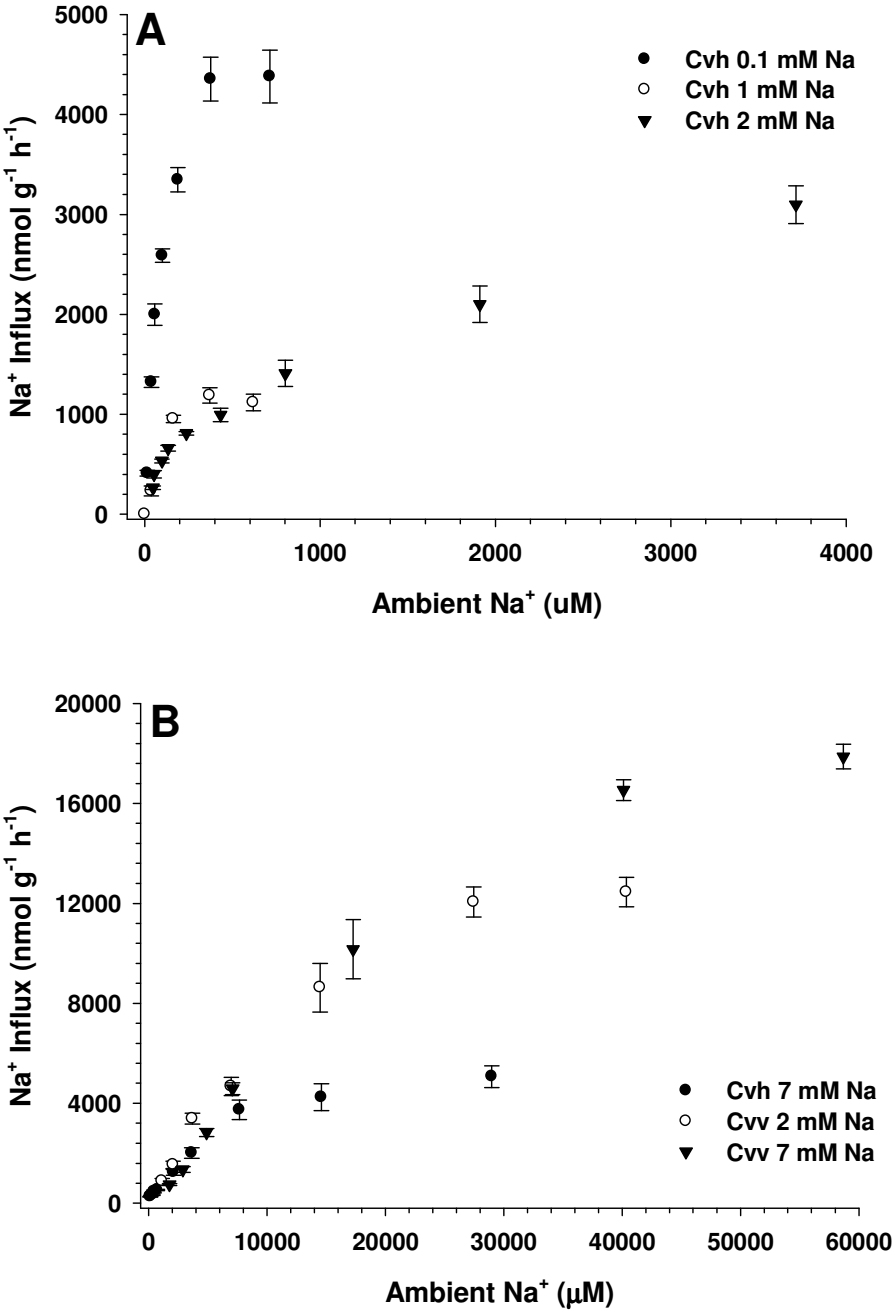
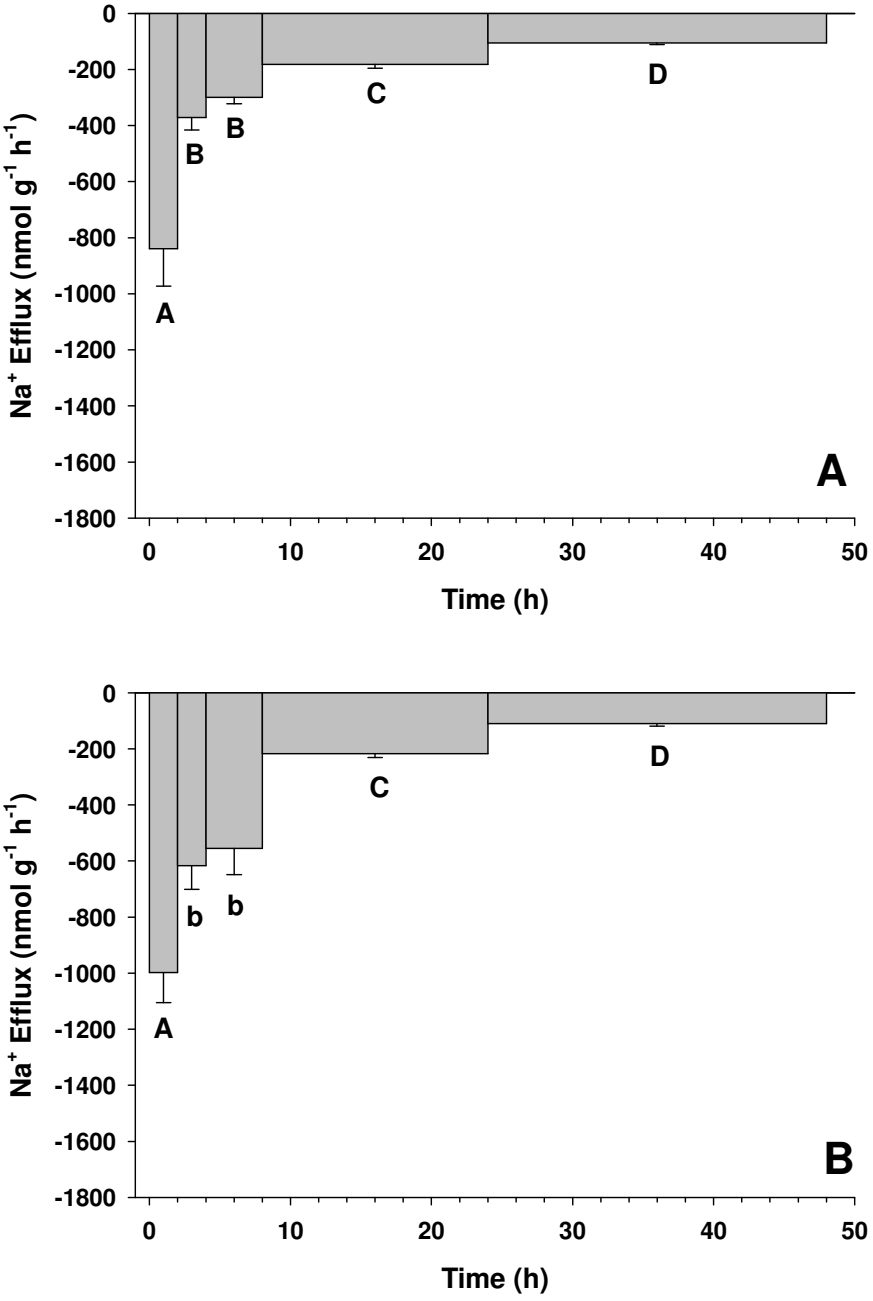


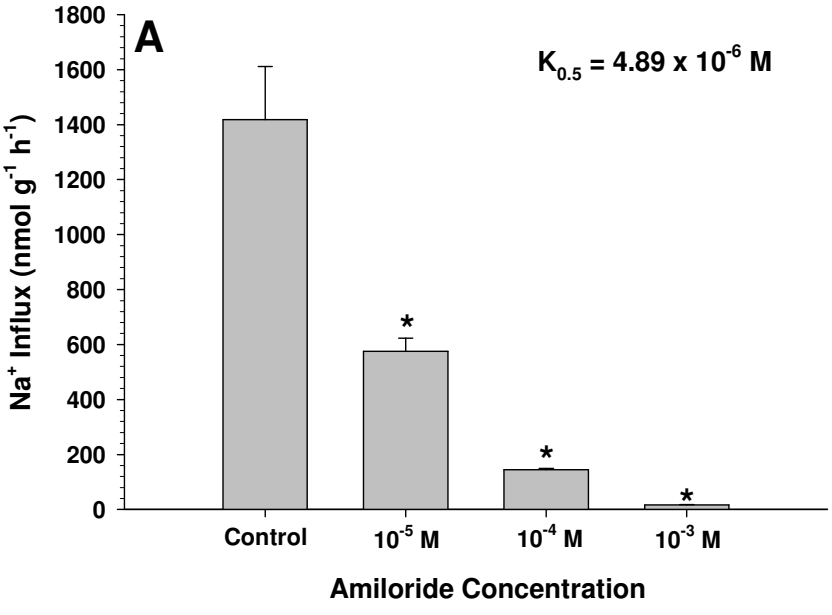
Figure 2.2. Na^+ efflux rates (nmol g^{-1} h^{-1}) over time after transfer from 2 mM Na^+ to NaCl "free" water. A) *C. v. hubbsi* and B) *C. v. variegatus*. Different widths of bars reflect different flux periods over the course of the 48 h experiment. Mean ± SEM (n=8). Different letters indicate significant differences ($p \leq 0.05$) between flux periods within a subspecies. Different cases indicate significant differences between subspecies within a given flux period.

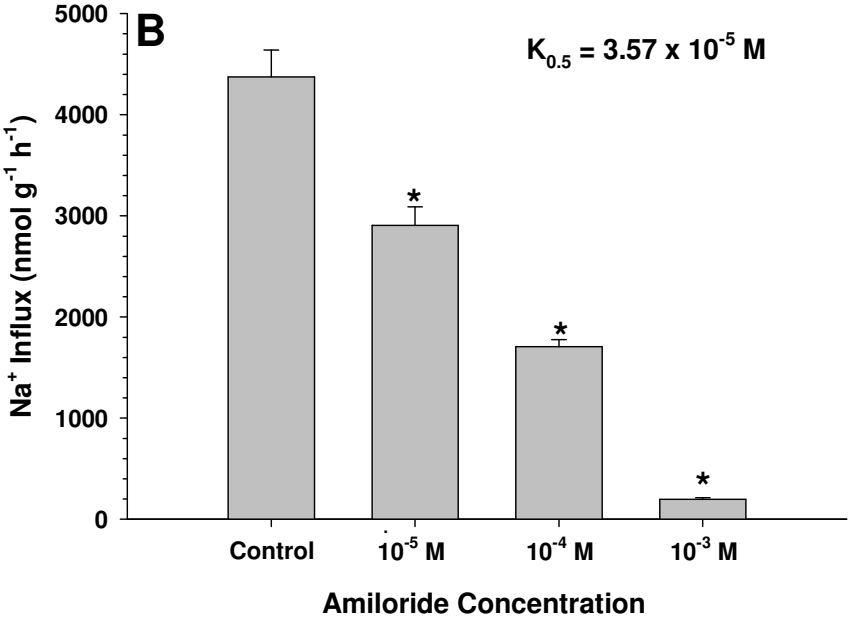


The next set of experiments involved using *Cvh* acclimated to 0.1 mM Na^+ and exposing them to bafilomycin A_1 (an H^+-ATPase inhibitor) and phenamil (a Na^+ channel blocker). Exposure to bafilomycin resulted in no significant inhibition of Na^+ uptake (Fig. 2.4a) while exposure to phenamil induced significant reductions in Na^+ uptake 71% and 25% at 10^{-4} M and 10^{-5} M phenamil (Fig. 2.4b).

Another set of inhibitor experiments involved the NHE-specific inhibitor EIPA using *Cvh* acclimated to 0.1, 2, and 7 mM Na^+ and *Cvv* acclimated to 2 and 7 mM Na^+. In fish acclimated to 7 mM Na^+, a similar inhibition (29 and 21%, respectively) of Na^+ uptake relative to controls was observed ($p \leq 0.05$). In contrast, in 2 mM Na^+ acclimated fish there appeared to be differential sensitivity to EIPA, with *Cvv* Na^+ uptake inhibited by 51% and *Cvh* by 91%. In *Cvh* acclimated to 0.1 mM Na^+ inhibition of Na^+ uptake was 95% relative to control fish (Fig. 2.5).

Figure 2.3. Effect of increasing amiloride concentrations on Na^+ uptake rates (nmol g^{-1} h^{-1}) in *C. v. hubbsi* acclimated to A) 0.1 mM Na^+, B) 2 mM Na^+, and *C. v. variegatus* acclimated to C) 2 mM Na^+. Controls include DMSO carrier. Mean ± SEM (n=8). * = statistical difference compared to the control ($p \leq 0.05$).




B
$K_{0.5}$ = 3.57 x 10^{-5} M
Na^+ Influx (nmol g^{-1} h^{-1})
5000
4000
3000
2000
1000
0
*
*
*
Control
10^{-5} M
10^{-4} M
10^{-3} M
Amiloride Concentration

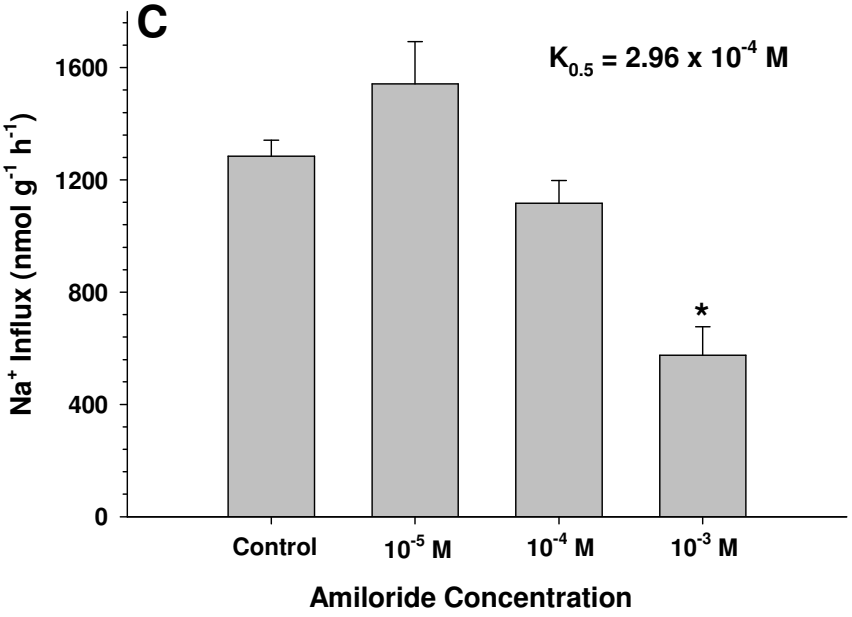

C
$K_{0.5}$ = 2.96 x 10^{-4} M
Na^+ Influx (nmol g^{-1} h^{-1})
1600
1200
800
400
0
*
Control
10^{-5} M
10^{-4} M
10^{-3} M
Amiloride Concentration

Figure 2.4. Effects of A) 1 x 10^{-6} M Bafilomycin and B) 1 x 10^{-4} M and 10^{-5} M Phenamil on Na^+ uptake rates (nmol g^{-1} h^{-1}) in *C. v. hubbsi* acclimated to 0.1 mM Na^+. Phenamil experiments were performed at different times with separate control for comparison. Controls include DMSO carrier. Mean ± SEM (n=10). * = statistical difference compared to the control ($p \leq 0.05$).

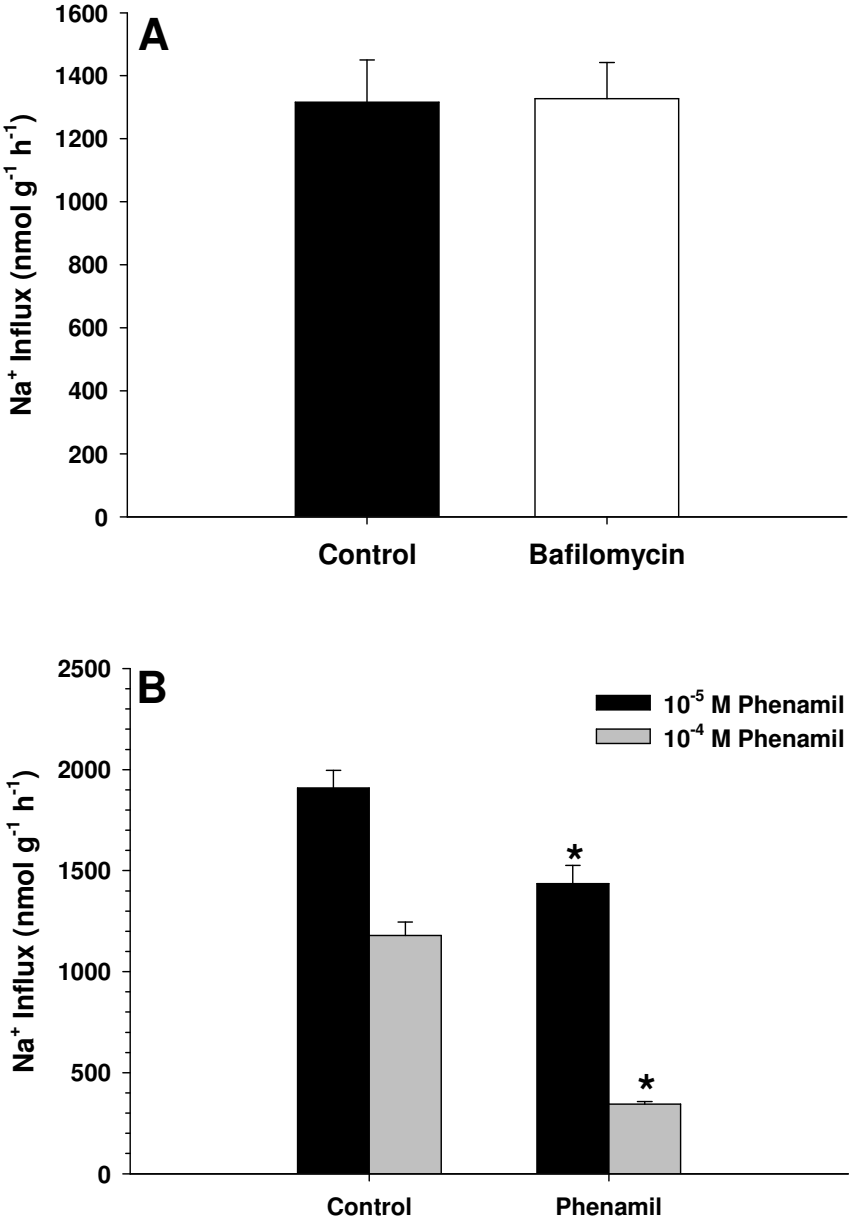
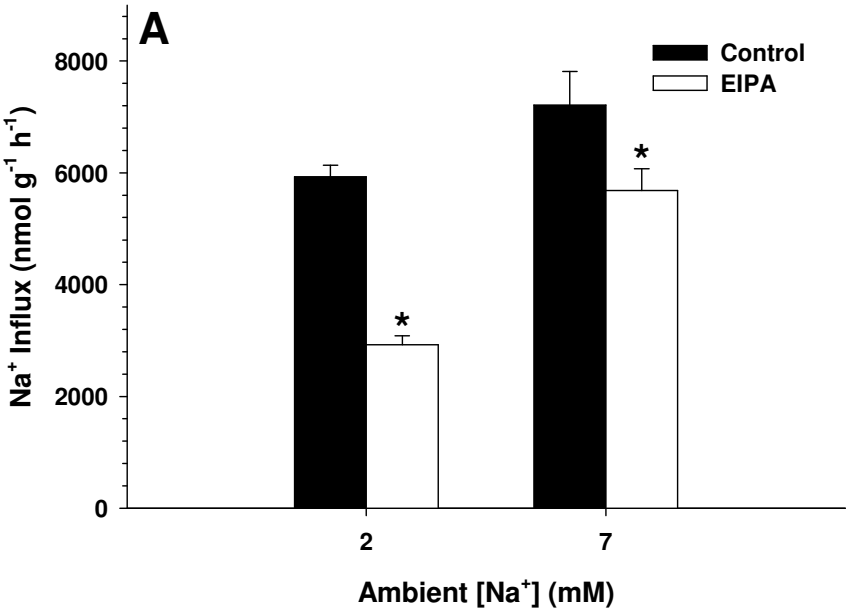
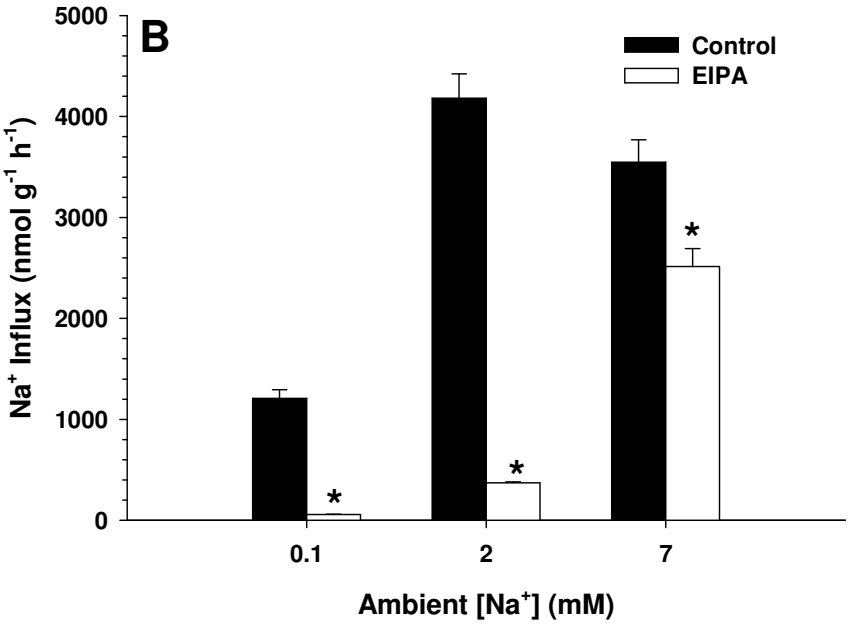


Figure 2.5. Effect of 5 x 10^{-5} M EIPA on Na^+ uptake rates (nmol g^{-1} h^{-1}) in A) *C. v. variegatus* and B) *C. v. hubbsi* acclimated to 0.1 (*Cvh* only), 2, and 7 mM Na^+. Controls include DMSO carrier. Mean ± SEM (n=10). * = statistical difference compared to the control ($p \leq 0.05$).





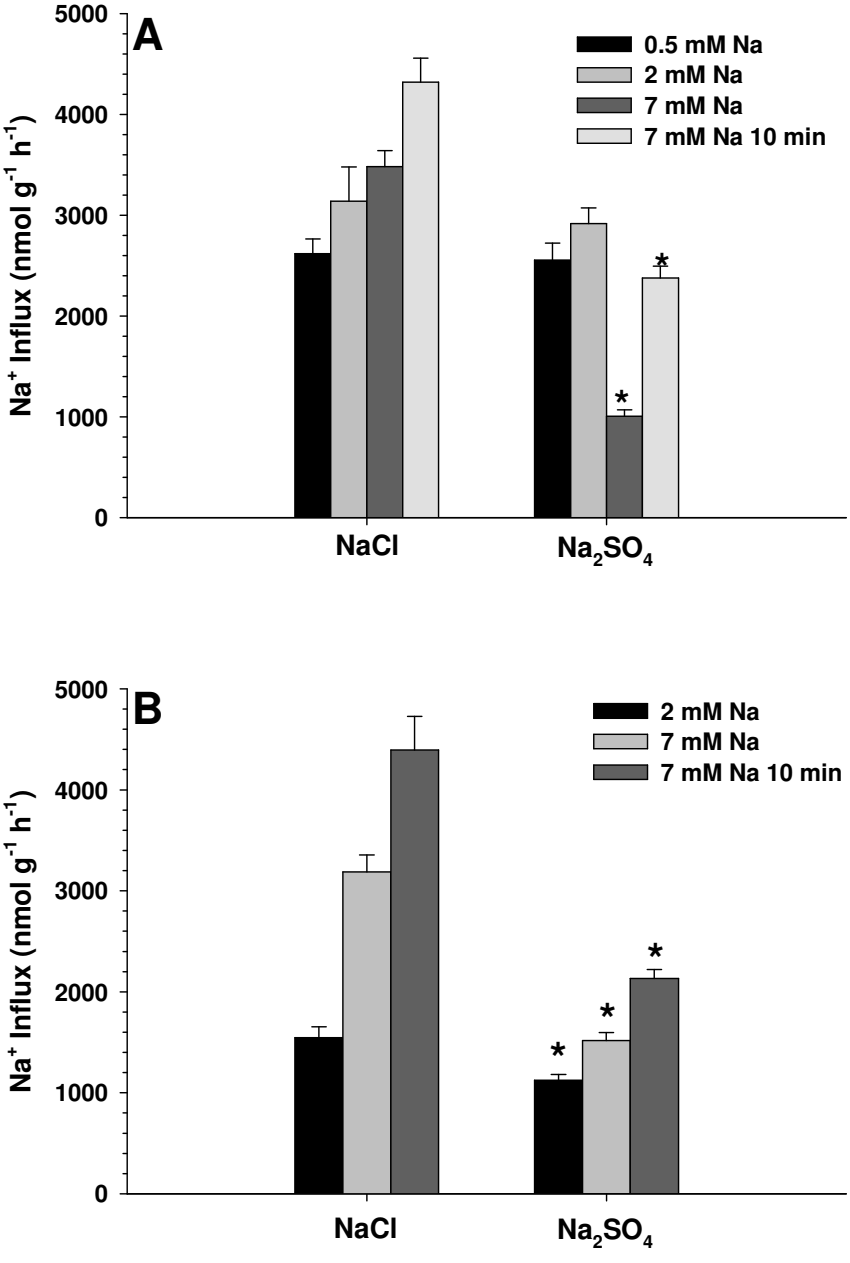
Chloride-Dependent Na^+ Uptake Experiment

Exposure of *Cvh* acclimated to 0.5 or 2 mM 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. 2.6). In contrast, *Cvh* acclimated to 7 mM Na^+ and *Cvv* acclimated to 2 or 7 mM Na^+ both exhibited significantly reduced Na^+ uptake after transfer to "Cl-free" water (Fig. 2.6). 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 to those 1.5 h in duration (Fig. 2.6). Additional experiments measuring Na^+ uptake in *Cvv* and *Cvh* acclimated to 7 mM Na^+ during exposure to metolazone (NCC inhibitor) or bumetanide (NKCC inhibitor) revealed no significant inhibition of Na^+ uptake in *Cvh* exposed to either drug or *Cvv* exposed to metolazone. However, for *Cvv* exposed to bumetanide, there was a significant (36%) inhibition of Na^+ uptake (Fig. 2.7).

Characterization of Gill Morphology at Different Ambient Na^+ Concentrations

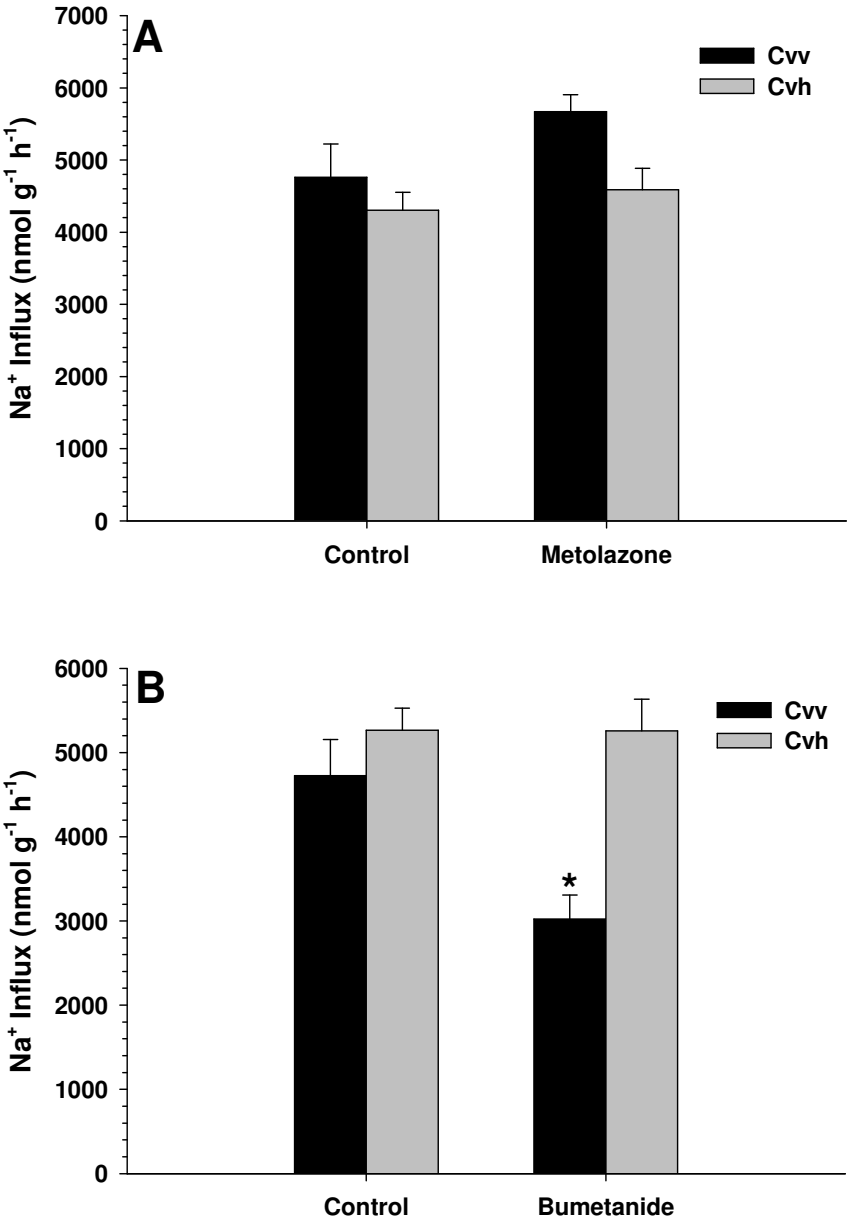
Typical of most fish, MRCs were only observed along the afferent edge of the gill filament with a smaller number of MRCs observed in the interlamellar spaces proximal to the afferent edge. No MRCs were observed on lamellae in any of the treatments. MRC distribution along the afferent edge appeared generally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

Figure 2.6. Na^+ uptake (nmol g^{-1} h^{-1}) in A) *C. v. hubbsi* acclimated to 0.5, 2, or 7 mM Na^+ and B) *C. v. variegatus* acclimated to 2 or 7 mM Na^+. All flux experiments were ~1.5 h in duration except the 7 mM 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. Mean ± SEM (n=10). * = statistical difference between the NaCl and Na_2SO_4 treatments ($p \leq 0.05$).



regardless of the ambient Na^+ concentration. This occurred even in *Cvh* exposed to 0.1 mM Na^+. Both of these regions were excluded from the analysis of changes in MRC density and type.

Figure 2.7. Effect of A) 1 x 10^{-5} M Metolazone and B) 1 x 10^{-4} M Bumetanide on Na^+ uptake rates (nmol g^{-1} h^{-1}) in *C. v. variegatus* and *C. v. hubbsi* acclimated to 7 mM Na^+. Controls include DMSO carrier. Mean ± SEM (n=8). * = statistical difference compared to the control ($p \leq 0.05$).



Representative scanning electron micrographs for each treatment are shown in Figure 2.8. 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 *Cvh* and *Cvv*. The number of apical crypts increased with increasing ambient Na^+ concentration ranging from ~500 mm^{-2} in *Cvh* acclimated to 0.1 mM Na^+ to ~3-4000 mm^{-2} in fish acclimated to 7 mM Na^+ (Fig. 2.9a). There were no significant differences in apical crypt density between subspecies in fish acclimated to 2 and 7 mM Na^+. As would be expected, the number of emergent MRCs increased with decreasing ambient Na^+ in both subspecies, with fish at 7 mM Na^+ having ~3-6000 emergent MRCs mm^{-2} reaching a maximum of ~11,000 emergent MRC mm^{-2} in *Cvh* acclimated to 0.1 mM Na^+ (Fig. 2.9b). *C. v. variegatus* had a significantly ($p \leq 0.05$) higher emergent MRC density than *Cvh* when both subspecies were acclimated to 7 mM Na^+, but emergent MRC densities were similar in fish acclimated to 2 mM Na^+ (Fig. 2.9b).

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 *Cvh* acclimated to 7 mM Na^+ to 16.6±2.1 μm^2 $cell^{-1}$ in *Cvv* acclimated to 2 mM Na^+ (Fig. 2.9c). Within a subspecies, emergent MRC size increased significantly with each reduction in ambient Na^+ concentration. For fish acclimated to 2 and 7 mM Na^+, emergent MRCs were significantly larger (approximately two-fold) in *Cvv*, and emergent MRCs trended toward being larger (not significantly, $p=0.16$) in *Cvv* acclimated to 2 mM Na^+ than *Cvh* acclimated to 0.1 mM 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. 2.10a). A plot of Na^+ uptake rates (using data from Fig. 2.1) as a function of MRCFA reveals strong linear relationships for both *Cvh* and *Cvv* with similar slopes but different intercepts (Fig. 2.10b).

Conclusions

Comparative Na^+ Transport in *Cvv* and *Cvh*

Characterization of Na^+ uptake and efflux in *Cvv* and *Cvh* reveals that changes in the mechanism of Na^+ uptake is the principle means by which *Cvh* has adapted to successfully regulate Na^+ in low Na^+ freshwater. Consistent with previous studies on tolerance to low Na^+ environments, *Cvv* exhibited a very low affinity, but high capacity Na^+ uptake system with estimated K_m values of 38,271 and 18,509 μM in fish acclimated to 7 and 2 mM Na^+, respectively (Table 2.1). This compares with K_m values of 1,723 and 8,000 μM for two other euryhaline fish, *Fundulus heteroclitus* and *Poecilia reticulata* acclimated to 1 mM Na^+ freshwater (Evans 1973; Patrick et al. 1997). In contrast, *Cvh* exhibited a somewhat higher affinity Na^+ uptake system at both 2 and 7 mM Na^+. Most interesting was the dramatic shift in K_m between fish acclimated to 2-7 and those acclimated to 0.1-1 mM Na^+. The K_m for *Cvh* does not change substantially in fish acclimated to Na^+ concentrations <1 mM Na^+ although there is a compensatory increase in V_{max}. The change in K_m between 2 and 1 mM Na^+ suggests *Cvh* utilize one or more

Figure 2.8. Representative scanning electron micrographs of gill filament epithelia in *C. v. hubbsi* acclimated to A) 0.1 mM Na^+, B) 2 mM Na^+, C) 7 mM Na^+ and *C. v. variegatus* acclimated to D) 2 mM Na^+ and E) 7 mM Na^+. All images at 2000x. Arrows indicate emergent MRCs, * indicate apical crypts. Bar =10 μM.

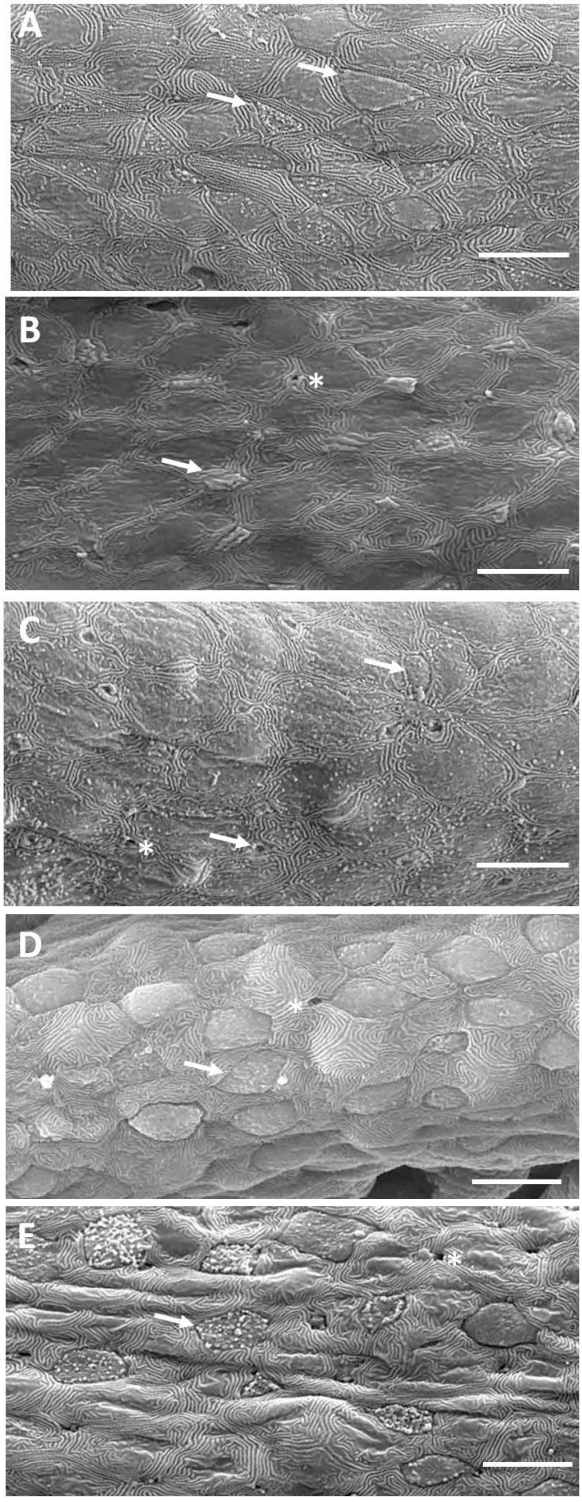
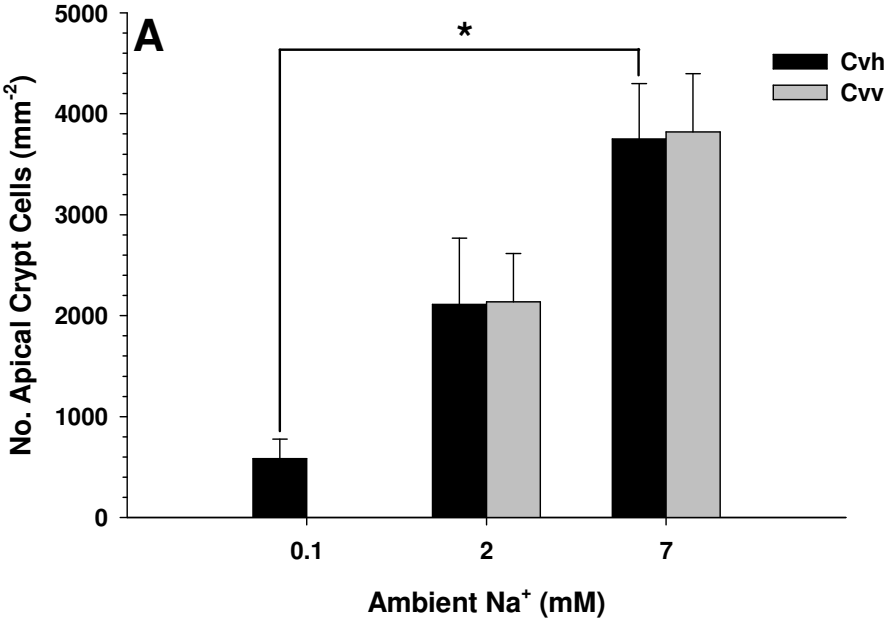
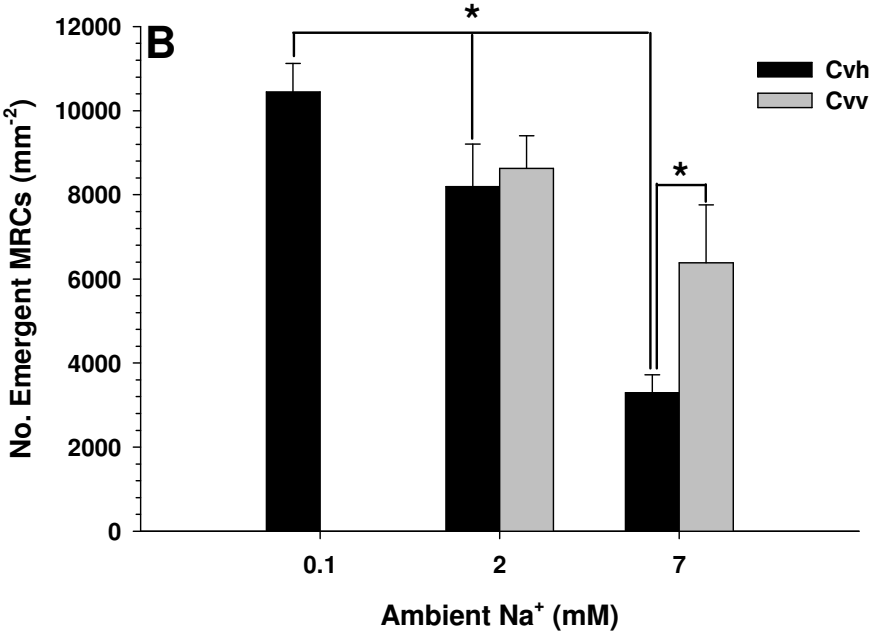


Figure 2.9. Effects of ambient Na^+ concentration on **A)** Apical crypt density, **B)** emergent MRC density, and **C)** size of emergent MRCs in *C. v. hubbsi* and *C. v. variegatus*, Mean ± SEM (n=5). In 9A and 9B * = significant differences ($p \leq 0.05$) between salinities within a subspecies. In 9C different letters indicate significant differences ($p \leq 0.05$) between salinities within a subspecies and different cases indicate significant differences between subspecies within a salinity.





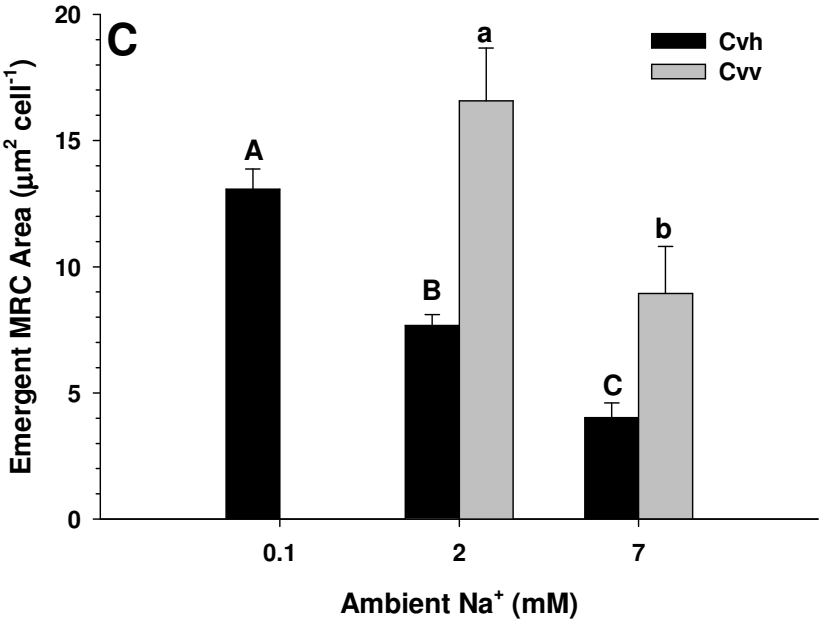
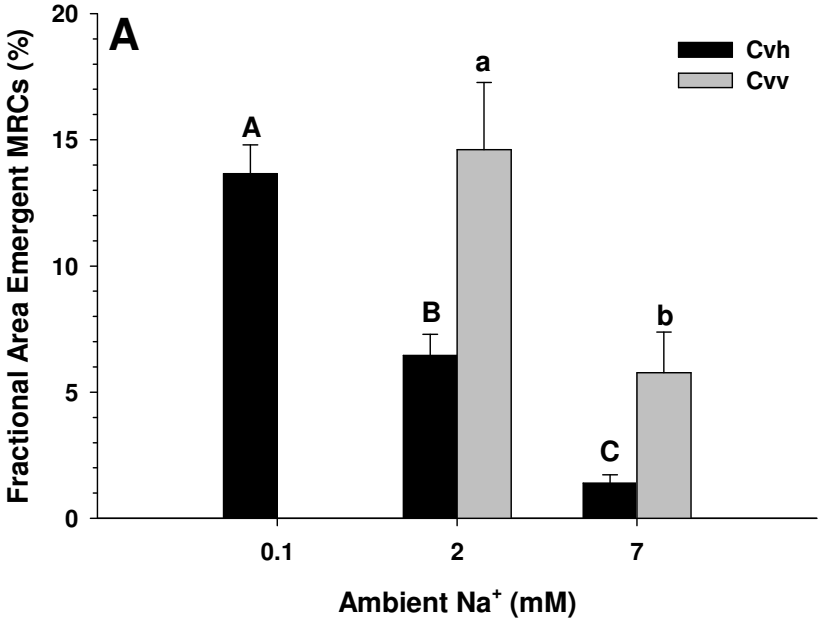
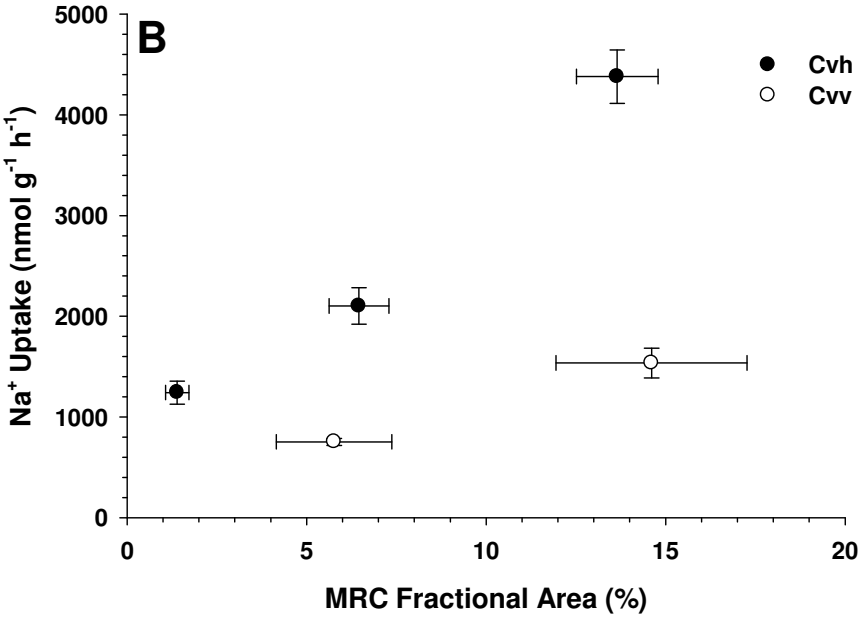


Figure 2.10. **A)** Effects of ambient Na^+ concentration on fractional area of emergent MRCs in *C. v. hubbsi* and *C. v. variegatus*. Different letters indicate significant differences ($p \leq 0.05$) between salinities within a subspecies. Different cases indicate significant differences between subspecies within a salinity. Mean ± SEM (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 mM ambient Na^+. Mean ± SEM (n=5 for fractional area and n=8 for Na^+ uptake rates).





different proteins to acquire Na^+ in dilute freshwater compared to more saline waters. The significant increase in Na^+ affinity at 1 mM Na^+ that occurs in *Cvh* but is lacking in *Cvv* appears to be the primary mechanism by which *Cvh* has evolved to survive in low Na^+ environments as efflux rates between the two subspecies were comparable (Fig. 2.2). Baseline Na^+ efflux rates were similar in the 2 subspecies when acclimated to 2 mM 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 most fish, including those adapted to low Na^+ waters. For example, at a comparable external Na^+ concentration to the current experiments, Gonzalez et al. (2002) measured efflux rates on the order of 300-400 nmol g^{-1} h^{-1} in two species of Amazonian fish (*Corydoras julii* and *Geophagus* sp.) adapted to low Na^+ environments (K_m = 56-112 μM in these species). Unlike these fish, C*vv* is unable to take up Na^+ in these environments (Table 2.1) 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. *C. v. 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 *Cvh* and *Cvv*. 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 mM) of Na^+ concentrations evaluated (Fig. 2.9b). The extent of emergent MRC proliferation observed in *Cvv* and *Cvh* is comparable to that observed in other euryhaline teleosts (Lee et al. 1996; Scott et al. 2004). The approximate 10-fold increase in apical crypt MRCs with increasing Na^+ (Fig. 2.9a) was also similar to that observed in *F. heteroclitus* when transferred from 1 mM Na^+ freshwater to 10 psu (~150 mM Na^+) brackish water (Scott et al. 2004). While *Cvh* and *Cvv* appear to exhibit characteristics within the range of responses observed in other euryhaline fish with respect to density of apical crypt and emergent MRCs, *Cvh* did have significantly fewer emergent MRCs at 7 mM Na^+ compared to *Cvv*, which may explain the lower Na^+ uptake capacity of *Cvh* 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 (King et al. 1989; Greco et al. 1996; Fernandes et al. 1998). In addition to this general trend, distinct differences in MRC size were observed

between *Cvh* and *Cvv* at a given salinity, with MRCs in *Cvv* significantly larger than those in *Cvh* at the same Na^+ concentration. Previous studies have demonstrated a linear relationship between the mitochondrial rich cell fraction area (MRCFA; the fraction of the total surface area occupied by MRCs) and Na^+ uptake in several different species of euryhaline and freshwater fish (Laurent and Perry 1990; Perry et al. 1992). Perry et al. (1992) demonstrated that this relationship could be applied across four species (*Anguilla anguilla*, *Ictalurus nebulosus*, *Onchorhynchus mykiss*, *Oreochromis mossambicus*) with MRCFA explaining 94% of the variance in Na^+ uptake in these species. Similar linear relationships were also observed for *Cvh* and *Cvv* (Fig. 2.10b). However, unlike the previous study by Perry et al., *Cvv* clearly have lower Na^+ uptake rates at a given MRCFA compared to *Cvh*. This suggests that although there are distinct differences between *Cvh* and *Cvv* with respect MRCFA at a given ambient Na^+ concentration, the enhanced Na^+ uptake exhibited by *Cvh* 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 provide *Cvh* 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 *Cvv* utilizes NKCC for apical Na^+ uptake in brackish waters ($\geq$7 mM Na^+). For *Cvv* acclimated to 7 mM Na^+, 50-70% of total Na^+ uptake occurred via this pathway (Figs. 2.6 and 2.7). Use of NKCC may explain the extremely high Na^+ uptake rates observed in

Cvv at high ambient Na^+ concentrations where V_{max} ranged from 19-31,000 nmol g^{-1} h^{-1} (Table 2.1). Although we did not investigate Cl^- uptake in this study, it is worth noting that the related euryhaline fish *Fundulus heteroclitus*, does not take up Cl^- and relies strictly on an NHE for Na^+ uptake under typical freshwater conditions (<2 mM NaCl). However, at higher ambient NaCl, significant Cl^- uptake does occur, suggesting the possibility that NKCC plays a similar role in this species (Patrick et al. 1997).

Despite a clear indication of Cl^--dependent Na^+ uptake in *Cvh* acclimated to 7 mM Na^+ (Fig. 2.6A) 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 *Cvh* acclimated to 7 mM Na^+ can be attributed to an NHE (Fig. 2.5). The majority (~70%) of this non-NHE Na^+ uptake appears to be Cl^--dependent. Given that *Cvh* is derived from *Cvv*, it is surprising that *Cvh* 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 mM 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 *Cvh* acclimated to 0.1 mM Na^+ provide potentially conflicting results. While bafilomycin had no effect on Na^+ uptake, phenamil caused a 65% inhibition of Na^+ uptake at 10^{-4} M and 25% inhibition at 10^{-5} M. 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 bafilomycin being 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 (~17x) and much less potent inhibitor of NHEs (~0.1x) than amiloride (Kleyman and Cragoe 1988; Wood et al. 2002). While 10^{-4} M is a relatively high concentration of phenamil, the results at 10^{-5} M 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 *Cvh* is sensitive to phenamil. It is also worth noting that the near complete (~95%) inhibition of Na^+ uptake by EIPA for *Cvh* acclimated to 0.1 mM 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 *Cvh* acclimated to 2 mM Na^+ and further reduction in $K_{0.5}$ for *Cvh* acclimated to 0.1 mM Na^+ to indicate the presence of two NHE isoforms in *Cvh* compared to the one expressed by *Cvv* (Fig. 2.3). The near complete (95%) inhibition of Na^+ uptake by EIPA in *Cvh* acclimated to 0.1 mM Na^+ suggests *Cvh* is only using an NHE for apical Na^+ uptake in low Na^+ water and that the primary adaptation of *Cvh* 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 (Yan et al. 2007; Inokuchi et al. 2009; Wu et al. 2010) while studies in the euryhaline *Fundulus* (also Cyprinodontiformes) have observed upregulation of NHE-2 and down regulation of NHE-3 in acute transfers from brackish water to 1 mM 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 while

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 relative putative NHE isoforms present in *Cvh* and *Cvv* and their relative sensitivity to amiloride.

Overall, results of the current study indicate that both *Cvv* and *Cvh* use several different proteins for apical Na^+ uptake of a relatively narrow range of ambient Na^+ concentrations. Additionally, this study indicates *Cvh* 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 *Cvh* 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, *Cvh* provides an excellent model organism for not only studying one mechanism by which estuarine fish can evolve to invade freshwater systems, but also a unique opportunity to study how NHEs can overcome apparent thermodynamic constraints to function in low Na^+ environments.

Chapter 3:

Measuring Titratable Alkalinity By Single Versus Double Endpoint Titration: An Evaluation in Two Cyprinodont Species and Implications for Characterizing Net H^+ Flux in Aquatic Organisms

Summary

In this study, Na^+ uptake and acid-base balance in the euryhaline pupfish *Cyprinodon variegatus variegatus* was characterized when fish were exposed to pH 4.5 freshwater (7 mM Na^+). Similar to the related cyprinodont, *Fundulus heteroclitus*, Na^+ uptake was significantly inhibited when exposed to low pH water. However, it initially appeared that *C. v. variegatus* increased apparent net acid excretion at low pH relative to circumneutral pH. This result is opposite to previous observations for *F. heteroclitus* under similar conditions where fish were observed to switch from apparent net H^+ excretion at circumneutral pH to apparent net H^+ uptake at low pH. Further investigation revealed disparate observations between these studies were the result of using double endpoint titrations to measure titratable alkalinity fluxes in the current study, while the earlier study utilized single endpoint titrations to measure these fluxes (i.e,. *Cyprinodon* acid-base transport is qualitatively similar to *Fundulus* when characterized using single endpoint titrations). This led to a comparative investigation of these two methods. We hypothesized that either the single endpoint methodology was being influenced by a change in the buffer capacity of the water (e.g., mucus being released by the fish) at low pH, or the double endpoint methodology was not properly accounting for ammonia flux by the fish. A series of follow-up experiments indicated that buffer capacity of the water

did not change significantly, that excretion of protein (a surrogate for mucus) was actually reduced at low pH, and that the double endpoint methodology does not properly account for NH_3 excretion by fish under low pH conditions. As a result, it overestimates net H^+ excretion during low pH exposure. After applying the maximum possible correction for this error (i.e., assuming that all ammonia is excreted as NH_3), the double endpoint methodology indicates that net H^+ transport was reduced to effectively zero in both species at pH 4.5. However, significant differences between the double endpoint (no net H^+ transport at low pH) and single endpoint titrations (net H^+ uptake at low pH) remain to be explained.

Background

In freshwater fish, the linkage between Na^+ uptake and exchange of acid equivalents at the gill apical membrane has been recognized for 85 years (Krogh 1937; Krogh 1939). Two mechanisms have been identified for Na^+ uptake at the apical membrane of the fish gill in dilute ($\leq$2 mM Na^+) freshwater. Na^+ is either directly exchanged for H^+ via a Na^+/H^+ exchanger (NHE) or indirectly via a H^+-ATPase linked to a putative Na^+ channel (Hwang et al. 2011). In perhaps the two most well-studied species, zebrafish (*Danio rerio*) and rainbow trout (*Oncorhynchus mykiss*), fish appear to use a combination of these mechanisms depending on the Na^+ concentration and pH of the ambient environment (Evans et al. 2005; Hwang 2009).

In contrast, the euryhaline mummichog (*Fundulus heteroclitus*), appears to rely exclusively on an NHE for Na^+ uptake in freshwater. Patrick and Wood (1999) characterized Na^+ transport and acid-base balance in this species in response to exposure

to amiloride and low pH (4.5). Exposure to amiloride inhibited both Na^+ uptake and net H^+ excretion. When fish were acutely exposed to pH 4.5 water, Na^+ uptake was strongly inhibited, with a corresponding reversal of apparent net H^+ transport from net secretion under control conditions to net uptake at pH 4.5. This effect was shown to be fully reversible upon return to control conditions. Subsequent gene expression studies support and refine these initial conclusions, indicating *F. heteroclitus* upregulates branchial expression of NHE-2 upon transfer from saline to freshwater, whereas branchial expression of V-type H^+-ATPase remains low and unchanged, and the already low branchial expression of NHE-3 decreases further (Scott et al. 2005).

More recently, studies on zebrafish and several other fish species (*Oryzias latipes*, *Oreochromis mossambicus*, *Tribolodon hakonensis*) have indicated that these species utilize an apical NHE-3/Rh glycoprotein metabolon for Na^+ uptake in freshwater (Hirata et al. 2003; Wu et al. 2010; Furukawa et al. 2011; Kumai et al. 2011; Kumai and Perry 2011; Shih et al. 2012). Unlike the response of *F. heteroclitus*, Na^+ uptake is enhanced when these species are exposed to low pH water. It is hypothesized that increased NH_3 excretion at low pH shifts the NH_3-NH_4^+ equilibrium toward the formation of NH_3 and H^+, providing a relatively high concentration of protons for the continued function of NHE-3 at low pH. Hence, measurement of Na^+ uptake at low pH potentially provides a diagnostic of the presence of NHE-2 versus NHE-3.

It is unclear whether lack of an H^+-ATPase/Na^+ channel or NHE-3/Rh system is characteristic of the euryhaline ecology of *F. heteroclitus* or related to evolutionary phylogeny. The limited number of species that have been characterized to any great extent limits our ability to infer the presence or absence of patterns in osmoregulatory

strategies between and within ecological and phylogenetic groups. In light of this, we selected another euryhaline species in the order Cyprinodontiformes for study.

The euryhaline pupfish *Cyprinodon variegatus variegatus* occurs along the Gulf and Atlantic coasts of North America and can tolerate salinities ranging from freshwater up to 167 g l^{-1} (Nordlie 2006). With respect to its ability to tolerate freshwater conditions, both growth of juveniles and reproduction are severely limited at ≤2 mM Na^+ (Dunson et al. 1998). We recently demonstrated that like *F. heteroclitus*, *C. v. variegatus* appears to rely predominantly on an NHE for apical Na^+ uptake in 2 mM Na^+ freshwater and appears to lack a H^+-ATPase/Na^+ channel system (Brix and Grosell 2012).

In the current study, we performed the same experiment used by Patrick and Wood (1999) to study *F. heteroclitus*, in which Na^+ uptake and net acid transport are simultaneously measured before and after acute transfer to pH of 4.5. We hypothesized that pH 4.5 would inhibit Na^+ transport associated with NHE-2, but not an NHE-3/Rh glycoprotein, and that there would be a concurrent reversal in net H^+ transport from excretion to uptake. With respect to Na^+ transport, observed results are consistent with the presence of NHE-2. However, we observed what appeared to be distinctly different results with respect to apparent net H^+ transport. A detailed comparison of our methods and results with those of Patrick and Wood (1999) suggested these discrepancies might be the result of methodological differences between the use of single endpoint titrations (used by Patrick and Wood 1999) and double endpoint titrations (used in the current study) to measure titratable alkalinity flux.

The measurement of whole fish acid-base flux is accomplished by measuring titratable alkalinity (base) and total ammonia flux, where influx (uptake) is typically

reported as positive and efflux (excretion) is reported as negative values. The titratable base flux (normally efflux) can also be considered titratable acid (TA) influx by changing the sign (e.g., a titratable base efflux of -100 units is a titratable acid (TA) influx of +100 units). The sum of the TA flux and total ammonia (NH_3 and NH_4^+) flux (normally efflux), taking into account signs, represents the net H^+ flux of the fish (McDonald and Wood 1981). Measurement of titratable alkalinity in physiological studies can be accomplished by either single or double endpoint titration and there are several variations of the method which was originally developed for measuring titratable acidity in blood and urine (Davies et al. 1920; Burton 1980). The double endpoint method specifically was developed for renal physiology studies to measure TA-HCO_3 in urine as a single value rather than measuring each parameter separately (Hills 1973). For both methods, a sample is initially sparged with N_2 to remove CO_2 from the system and the sample pH is recorded. In the single endpoint methodology, the sample is initially titrated to pH 4.2 with standardized HCl, sparged for 15 min to ensure removal of all CO_2 (derived from the conversion of HCO_3^- to CO_2 at this pH), and then titrated to pH 3.8/4.0. In the single endpoint methodology, the acid equivalents needed to make this titration to the fixed endpoint (pH 3.8/4.0) are equal to the titratable alkalinity of the sample. In the double endpoint methodology, the sample is titrated to pH 3.8/4.0 using standardized HCl, the sample is sparged with N_2 or CO_2-free air for 15 min to remove the CO_2 generated by the titration of HCO_3^- with HCl. The sample is then titrated back to the initial pH (pH_i) of the sample (after the initial sparge) using standardized NaOH. The titratable alkalinity of the sample is then calculated as the acid equivalents used to titrate to pH 3.8/4.0 minus the base equivalents used to titrate back to pH_i.

The single endpoint methodology assumes that samples analyzed from the beginning and end of a flux experiment have the same buffering capacity and that any measured change in alkalinity is strictly the result of net H^+ transport by the organism. In contrast, back titration of samples to pH_i in the double endpoint methodology provides an absolute measurement of alkalinity at each time, independent of water buffer capacity. Therefore the difference in titratable alkalinity (measured by the double endpoint method) from the beginning to the end sample should provide an accurate measurement of net H^+ transport by the organism, independent from any change in water buffering capacity that may result from substances released by an animal during a flux experiment (e.g., phosphate, mucus). Thus double endpoint titration should theoretically eliminate the potential error of the single endpoint method that may arise from changing buffer capacity of the water. Hence, it can be argued that it is important to always use the double endpoint methodology when determining net H^+ transport in aquatic organisms. However, conceptually, if the buffering capacity of the water is not changed during a flux experiment, the single and double endpoint titrations should produce the same answer. Under these conditions, use of single endpoint methodology is preferred given that it is considerably less time-intensive to perform.

With respect to the current experiment we hypothesized three possibilities could be causing the observed discrepancies. First, it was possible that when performing this type of experiment on fish at low pH (4.5), fish are releasing a buffer (e.g., mucus, phosphate) that leads to errors using the single endpoint methodology. Second, it was possible that at low pH, the double endpoint methodology was incorrectly accounting for the contribution of ammonia to net TA (see Discussion for detailed explanation). Finally, it

was possible that *C. variegatus* and *F. heteroclitus* simply respond differently with respect to net H^+ transport when exposed to low pH. The present paper describes a series of experiments to test these hypotheses and discusses the implications of experimental results for measuring titratable alkalinity flux, and therefore net H^+ transport in aquatic organisms.

Methods and Materials

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 12-39 g l^{-1}. Fish were held at the University of Miami in 110-L glass aquaria under flow-through conditions with filtered natural seawater from Bear Cut, FL. Adult fish were bred and F_1 offspring were hatched and raised in seawater until the late juvenile stage (~ 2 months old; 200-400 mg). Fish were fed *Artemia* nauplii for the first 2 weeks and then over a 1 week period gradually switched to flake food.

F_1 fish were then acclimated to near freshwater conditions (0.3 g l^{-1}; 7 mM Na^+) for a minimum of 2 weeks prior to testing. 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 (~7.0 mM Na^+, 8.0 mM Cl^-, 0.5 mM Ca^{2+}, 0.8 mM Mg^{2+}, 0.8 mM SO_4^{2-}, 0.8 mM HCO_3^-, pH 7.9). Preliminary experiments indicated this 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 d prior to experimental use.

Experiments on *F. heteroclitus* were performed on adult fish obtained from Aquatic Research Organisms Ltd. (Hampton, NH, USA). Fish were initially held at the University of Miami in 110-L glass aquaria under flow-through conditions with filtered natural seawater from Bear Cut, FL and fed flake food. One month prior to experimental use, fish were acclimated to City of Miami tapwater. Fish were not fed for 2 d prior to experimental use.

Low pH (4.5) Experiment with *C. v. variegatus*

For *C. v. variegatus*, juvenile fish (175-390 mg) were exposed for 3 h to control conditions (pH 7.9, 7 mM Na^+) followed by 3 h in water adjusted to pH 4.5 with 1 N H_2SO_4, and then another 3 h recovery period again under control conditions. Eight fish were used for each 3-h treatment. This experiment was performed twice, once to characterize Na^+ uptake and once to characterize acid-base balance. In each experimental run, fish were maintained individually in 29 ml of test solution.

For the Na^+ uptake component of the experiment, ^{22}Na was added only to the control treatment for the first 3 h sampling period. At the end of the control flux, control fish were terminally sampled. Water in the flux beakers was replaced with pH 4.5 water for the remaining two treatments (pH 4.5 and recovery), fish were allowed to settle for 5 min and then ^{22}Na was added to the pH 4.5 treatment. At the end of the 3-h exposure to pH 4.5, fish from this treatment were terminally sampled for analysis of ^{22}Na, while water in the recovery treatment was replaced with control water and ^{22}Na was added for the last flux period, at the end of which fish were terminally sampled. Water samples were collected at the beginning and end of each 3 h period for measurement of Na^+ and ^{22}Na in the treatment where ^{22}Na was added.

For the acid-base balance component of the experiment, the same 8 fish were used for the entire experiment. Water samples were collected at the beginning and end of each flux period for measurement of total ammonia and titratable alkalinity. Samples for total ammonia were frozen at -20 °C and analyzed within 1 week of collection. Titratable alkalinity samples were refrigerated at 4 °C and analyzed within 18 h of collection.

Evaluation of Single Versus Double Endpoint Titrations for Measuring Titratable Alkalinity

Given the disparate results between the single and double endpoint measurements for titratable alkalinity in the low pH experiment (see Results), we investigated potential reasons for observed differences. We hypothesized three possible reasons for the observed discrepancy between the two methods.

First, we hypothesized that when fish are exposed to low pH, they increased mucus production or they released some other buffer to the water. For example, urinary phosphate excretion has been shown to be stimulated in rainbow trout exposed to pH 4.2 water (McDonald and Wood 1981). The proton binding sites on fish mucus or an unidentified buffer added to the water by the fish during the flux period would consume additional acid during titrations and be interpreted as an increase in titratable alkalinity using the single endpoint methodology, but not by the double endpoint methodology. To test this hypothesis, we repeated the low pH experiment and collected water samples at the beginning and end of each 3-h flux period (control and low pH) for protein analysis which served as a surrogate for mucous production. Due to the small size of the fish, it was not practical to collect urine to evaluate its potential contribution to changing buffering capacity. Instead, we attempted to quantify any change in the buffering capacity of pH 4.5 water after a 3 h flux period as a way to determine whether an

unknown buffer was being released to the water. To accomplish this, double endpoint titratable alkalinity was measured at the beginning and end of a 3 h flux period with *C. v. variegatus* at pH 4.5 as previously described. However, in this analysis, during the back titration with NaOH, the pH was recorded after every 10 µl of 0.02 N NaOH addition and titration curves were developed based on these data.

A second possibility is that ammonia excreted by the fish was not being properly accounted for using the double endpoint method. This could be occurring either by the inadvertent titration of NH_4^+ by NaOH during back titration to pH_i or by not accounting for NH_3 excretion during titration of pH 4.5 water (see Discussion for detailed explanation). To test these possibilities, we conducted two experiments. First, ammonia (as NH_4Cl – i.e., as NH_4^+) was spiked into dechlorinated City of Miami tap water at nominal ammonia concentrations of 10, 50, and 100 µM. Samples were then analyzed using single and double endpoint methods (both titrated to pH 3.8) to evaluate the effects of increasing ammonia on titratable alkalinity measurements. Second, ammonia (as NH_4OH – i.e., as NH_3) was spiked into dechlorinated City of Miami tap water adjusted to pH 4.5 to see if it could be detected using either single or double endpoint techniques. For this experiment, the nominal NH_4OH concentration spiked was 10 µM which, in preliminary experiments, was the maximum concentration that could be added while maintaining pH <5.0.

A final possibility was that there are simply differences between *C. v. variegatus* and *F. heteroclitus* with respect to net H^+ transport and response to low pH water. To test this, we repeated part of the experiment by Patrick and Wood (1999) testing the effect of low pH exposure on acid-base flux. The experiment was performed using small adult *F.*

heteroclitus (1.00-1.82 g) in 139 ml of water dechlorinated City of Miami tapwater. Otherwise, the conditions and experimental procedure were the same as described above for *C. v. variegatus* except that Na^+ uptake was not characterized.

Analytical methods and calculations

Water pH was measured by a combination glass electrode (Radiometer pHC4000-8, Cedex, France) connected to a pH meter (Radiometer PHM201, Cedex, France). 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). Total ammonia (T_{amm}) in water was measured by a micro-modified colorimetric method (Verdouw et al. 1978). Proteins in water samples were concentrated 10x by vacuum centrifugation at room temperature and then analyzed for protein content by colorimetric assay (Micro BCA Protein Assay, Thermo Scientific, Rockford, Illinois).

Titratable alkalinity was measured by double endpoint titration to pH 3.8. Samples (10 ml volume) were sparged with N_2 for 30 min, initial pH recorded and the samples were titrated with standardized acid (HCl) to pH 3.8, sparged with N_2 for an additional 15 min and then titrated back to the initial pH with standardized base (NaOH). Thus the initial part of this protocol simultaneously provided a measurement of single endpoint alkalinity. Titration acid and base (0.02 N) was dispensed using 2 ml Gilson microburettes. Acid and base solutions were normalized against each other and all measurements corrected accordingly.

Rates of unidirectional Na^+ uptake, as measured by the appearance of ^{22}Na radioactivity in the fish (in nmol g^{-1} h^{-1}), were determined as described in Brix and Grosell (2012). Net titratable acid and ammonia transport were calculated as described in Patrick and Wood (1999).

Statistical analysis

All values are expressed as means ± SEM throughout. Comparison data were analyzed by two-tailed Student's t-test, except for the spiked ammonia experiments which used a paired Student's t-test. In cases of unequal variance a Mann-Whitney rank sum test was performed. Means were considered significantly different at $p<0.05$.

Results

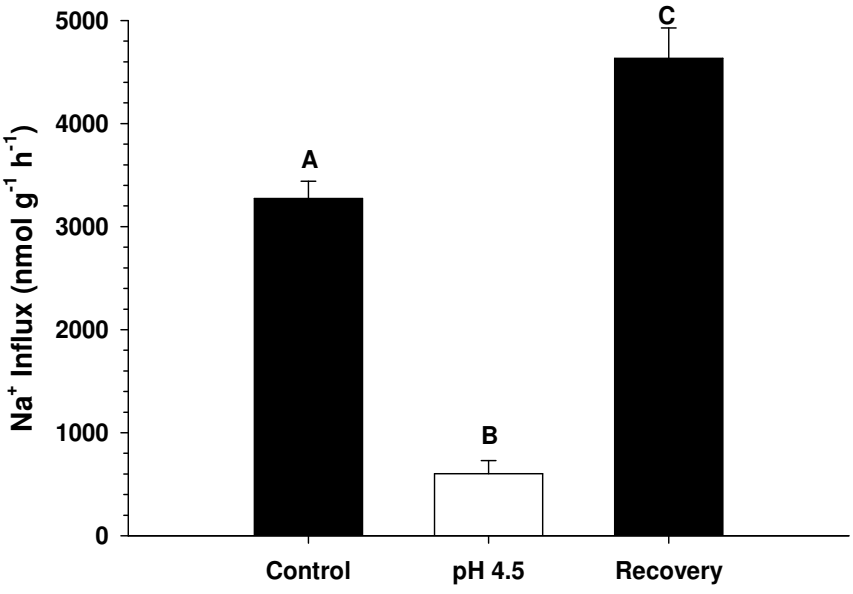
Low pH (4.5) Experiment

Exposing *C. v. variegatus* to low pH (4.5) for 3 h resulted in a significant and robust (82%) reduction in Na^+ uptake relative to the control. After fish were returned to control conditions, Na^+ uptake completely recovered and was significantly higher (41%) during the next 3 h than under initial control conditions (Fig. 3.1).

With respect to whole animal acid-base fluxes, as assessed by double endpoint titrations, exposure to low pH significantly inhibited apparent titratable acid uptake while T_{amm} excretion remained unchanged, resulting in a significant increase in calculated net H^+ excretion ($p<0.05$). Upon return to control conditions at the end of the 3 h acid exposure, both titratable acid uptake and net H^+ excretion returned to levels similar to the control (Fig 3.2a). The results for titratable acid uptake, as analyzed using the double endpoint titration method, are essentially the opposite of those reported by Patrick and

Wood (1999) for *F. heteroclitus*, using the single endpoint titration method. This observation prompted analysis of our data using the single endpoint method, data which are necessarily collected during double endpoint titrations. Analysis of single endpoint data resulted in observations qualitatively similar to those observed for *F. heteroclitus* by Patrick and Wood (1999). Titratable acid uptake (by single endpoint) was significantly increased upon exposure to low pH, and a corresponding reversal of the calculated net H^+ flux from net H^+ excretion during the control period to net H^+ uptake during the pH 4.5 exposure period occurred (Fig. 3.2b). Return to control conditions resulted in significant though incomplete recovery of titratable acid uptake at the end of 3 h, such that calculated net H^+ excretion was near zero.

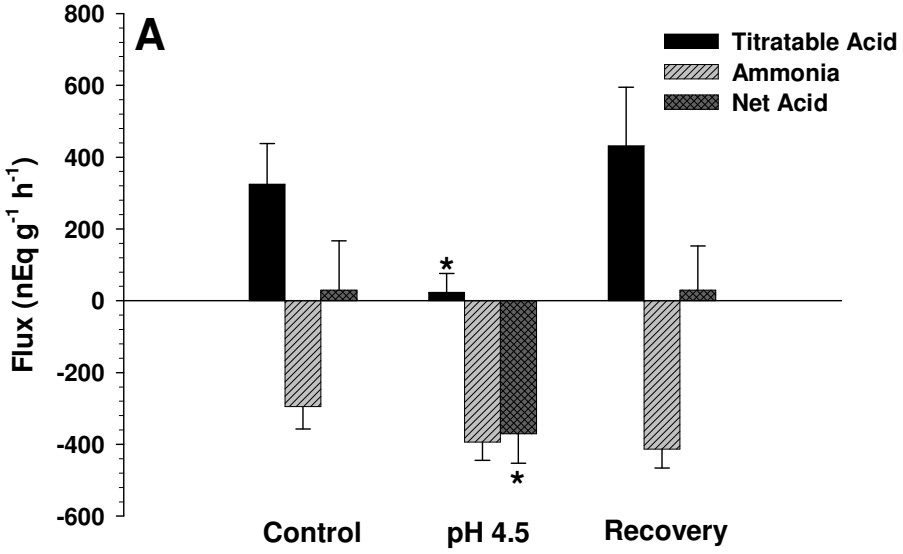
Figure 3.1. Effect of exposure to low pH (4.5) and return to control conditions on Na^+ uptake in *C. v. variegatus*. Mean ± SEM (n=8). Different letters indicate statistically significant differences between treatments ($p<0.05$).

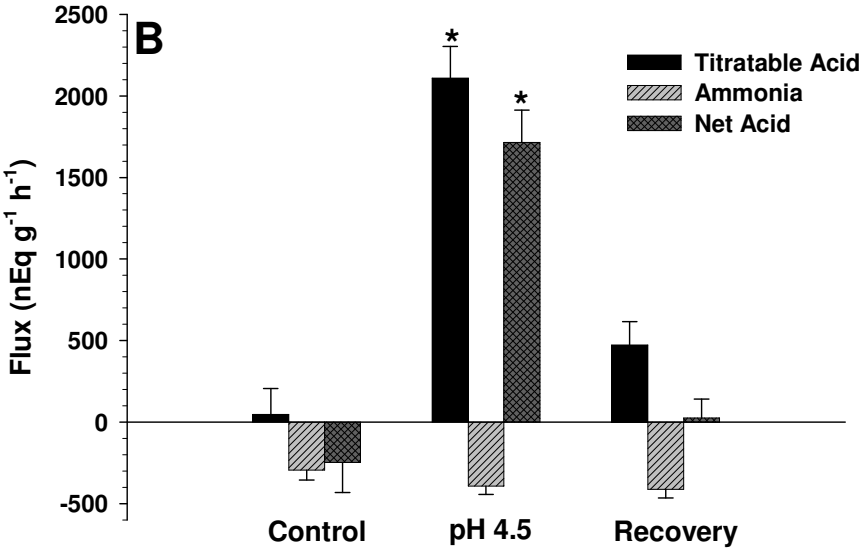


Our repetition of the experiment with *F. heteroclitus* produced the same pattern of responses just described for *C. v. variegatus* in terms of both response to the pH 4.5 challenge and discrepancy between single and double endpoint titration methodologies

(Fig. 3.3). By double endpoint titration, titratable acid uptake was reduced to zero, such that calculated net H^+ excretion significantly increased during exposure to pH 4.5, whereas by single endpoint titration, titratable acid uptake significantly increased, such that there was a calculated net H^+ uptake rather than excretion during this treatment. The results for *F. heteroclitus* were also qualitatively similar to those previously obtained by Patrick and Wood (1999). The only exception to this, both in comparison to *C. v. variegatus* and the earlier study on *F. heteroclitus*, was a significantly reduced T_{amm} efflux in pH 4.5 water relative to control conditions.

Figure 3.2. Effect of exposure to low pH (4.5) and return to control conditions on acid-base fluxes in *C. v. variegatus*. **A.** Results based on using double endpoint titrations to pH 3.8. **B.** Results based on using single endpoint titrations to pH 3.8. Mean ± SEM (n=8). * = statistical difference compared to the control.



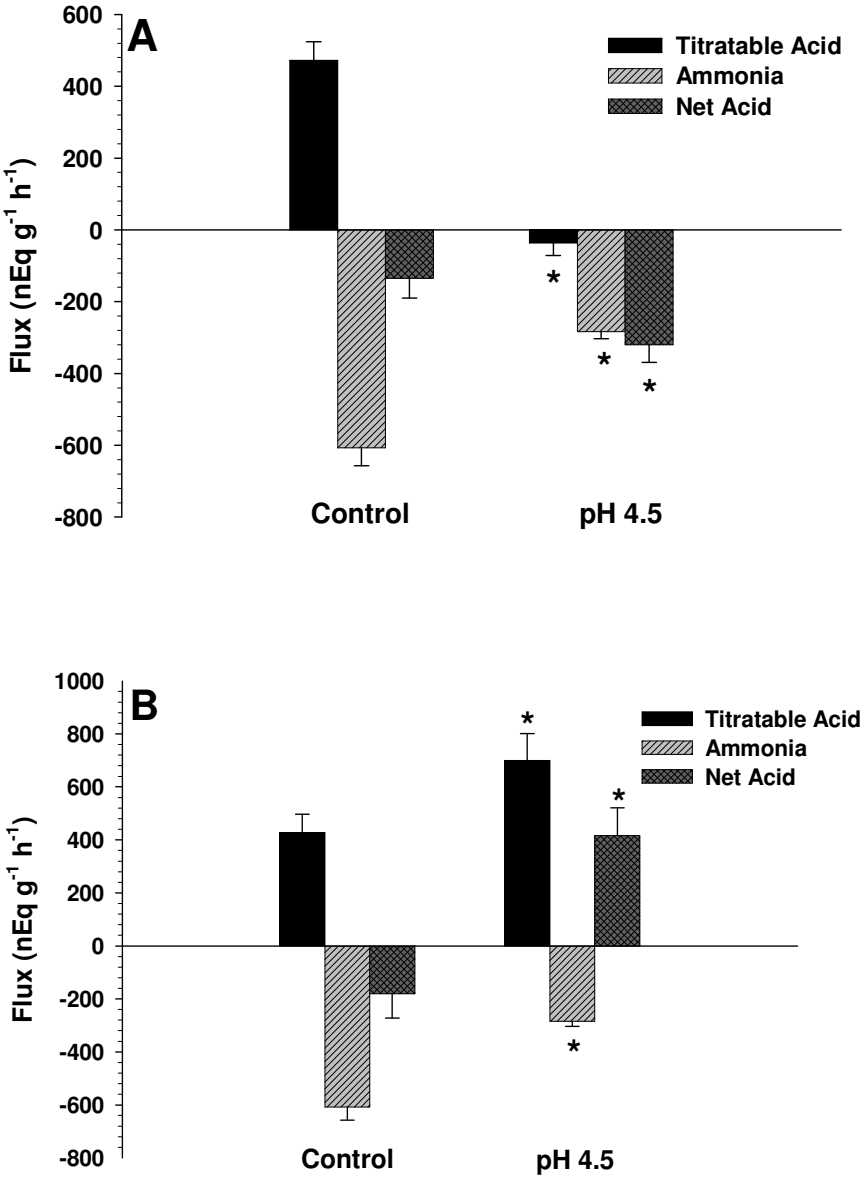


Evaluation of Single Versus Double Endpoint Titrations for Measuring Titratable Alkalinity

Protein secretion (measured as a surrogate for mucus secretion) rates in *C. v. variegatus* under control conditions and at pH 4.5 were significantly different. Under control conditions, fish excreted 19 μg protein g^{-1} h^{-1} while at pH 4.5, the excretion rate was significantly reduced to 9 μg g^{-1} h^{-1} (Fig. 3.4). The fine scale titration of pH 4.5 water before and after a 3 h flux with *C. v. variegatus* revealed no significant difference in the water buffering capacity although there was a trend of higher buffer capacity in the water at the end of the 3 h exposure to pH 4.5 over the pH range of 5.0 to 8.0 (Fig. 3.5).

Additions of NH_4^+ (as NH_4Cl) resulted in measured T_{amm} concentrations of 2.4, 55, and 137 μM. When these samples were analyzed by single endpoint titration, a small (14 μM) but statistically significant reduction in titratable alkalinity was measured at the highest two ammonia concentrations (Fig. 3.6). This approximate 1% reduction in

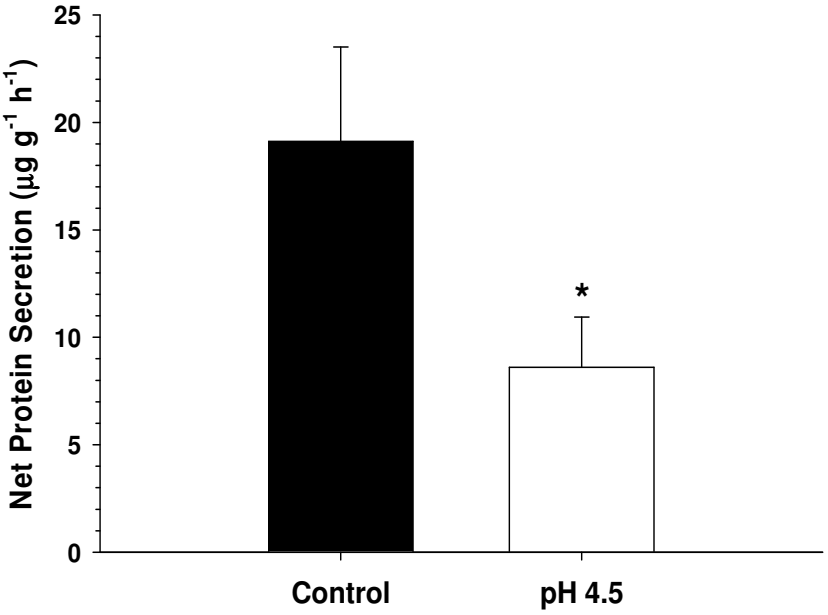
Figure 3.3. Effect of exposure to low pH (4.5) on acid-base fluxes in *F. heteroclitus*. **A.** Results based on using double endpoint titrations to pH 3.8. **B.** Results based on using single endpoint titrations to pH 3.8. Mean ± SEM (n=8). * = statistical difference compared to the control.



titratable alkalinity corresponds to the amount of dilution anticipated to result from spiking the solution with the ammonia stock solution. When the same samples were analyzed by double endpoint titration, the results were less consistent with a significant

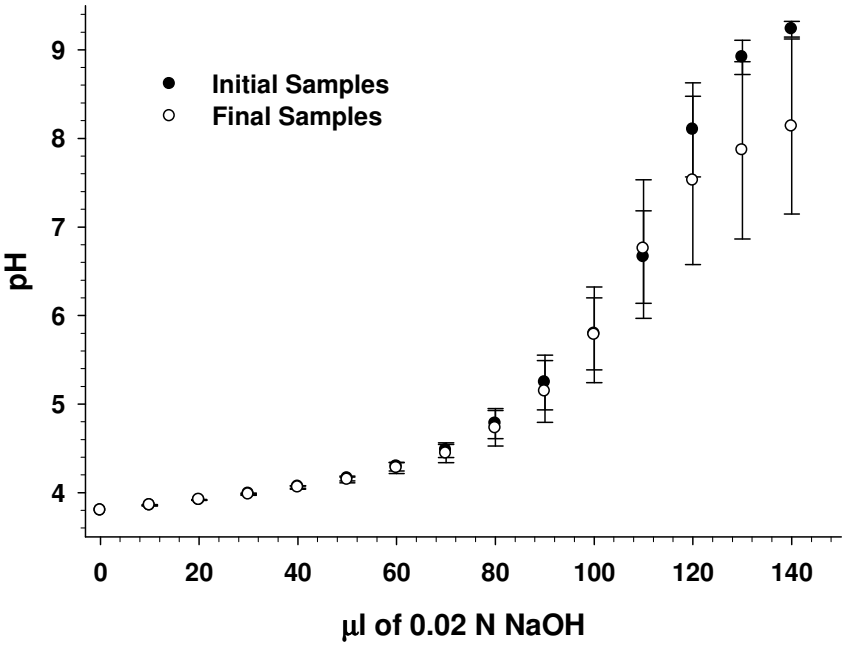
(14 μM) reduction in titratable alkalinity measured in the 55 μM NH_3 treatment, but not the 137 μM treatment. Overall, these changes are considered to be negligible.

Figure 3.4. Effect of exposure to low pH (4.5) on net protein secretion in *C. v. variegatus*. Mean ± SEM (n=8). * = statistical difference compared to the control ($p<0.05$).



Additions of NH_3 (as NH_4OH) to pH 4.5 dechlorinated tapwater resulted in a measured T_{amm} concentration of 10.5 μM. The double endpoint method did not detect the addition of NH_3, with a measured titratable alkalinity of 10.8±0.6 μM in unspiked water and 11.5±1.0 μM in NH_4OH spiked water. Using the single endpoint methodology, measured titratable alkalinity was 145.5±5.6 μM in unspiked water and 154.5±2.0 μM in NH_4OH spiked water (Fig. 3.7). Although the difference between unspiked and spiked waters using the single endpoint method closely approximated the expected difference based on the measured ammonia concentration, measured titratable alkalinity was not statistically different between the two waters.

Figure 3.5. The pH of water samples (10 ml) during back titration with 0.02 N NaOH at the beginning and end of a 3 h flux with *C. v. variegatus* in pH 4.5 water. Mean ± SEM (n=4).



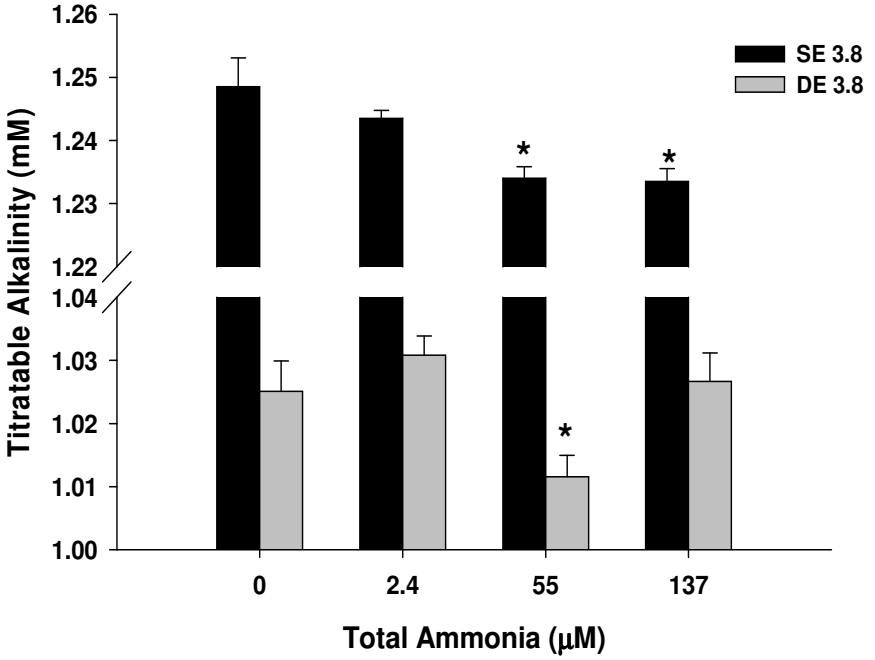
Conclusions

Effect of Low pH on Na^+ Uptake and Ammonia Excretion in *C. v. variegatus*

The initial objective of these experiments was to evaluate the effects of low pH on Na^+ uptake, ammonia excretion, and acid-base balance in *C. v. variegatus*. With respect to Na^+ uptake and ammonia excretion, our results indicate *C. v. variegatus* is similar to *F. heteroclitus*. Low pH water significantly reduced Na^+ uptake but had no significant effect on ammonia excretion (Fig. 3.2). Considering that we previously demonstrated that *C. v. variegatus*, like *F. heteroclitus*, lack an apical H^+ pump (Brix and Grosell 2012), these results, are consistent with NHE-2 being the NHE isoform involved in apical

Na^+ uptake for this species. This tentative conclusion awaits further confirmation via gene expression and/or immunohistochemistry studies.

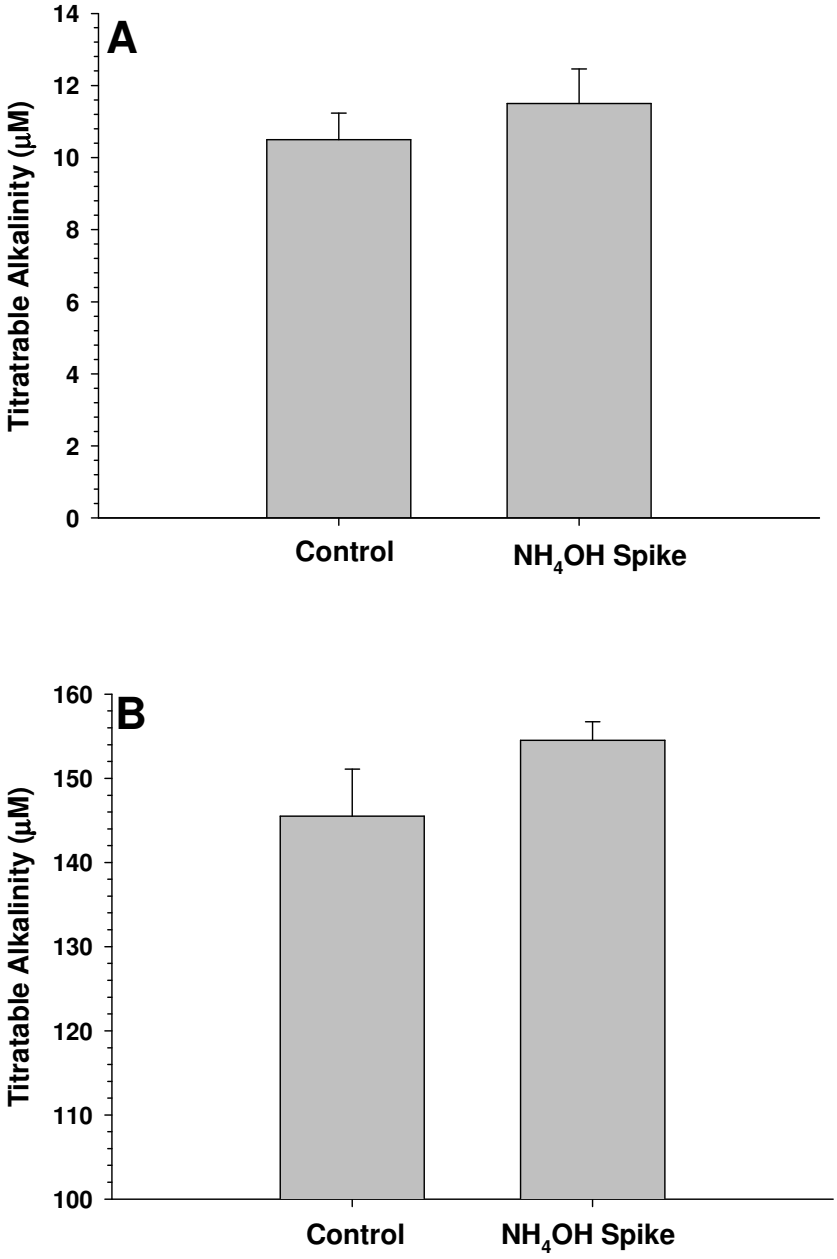
Figure 3.6. Effect of experimentally increasing ammonia NH_4^+ concentration (by addition of NH_4Cl to water samples) on single and double endpoint titration methods for quantifying titratable alkalinity. * = statistical difference compared to the 0 μM control ($p<0.05$). Mean ± SEM (n=4).



Single versus Double Endpoint Titrations for Measuring Titratable Alkalinity in Low pH Waters and Implications for Fish Net H^+ Transport

The analysis of net H^+ transport in *C. v. variegatus* and *F. heteroclitus* exposed to low pH raises an interesting methodological issue regarding the measurement of titratable acid flux in physiological studies. Based on single endpoint titrations, exposure to low pH water results in a reversal of the calculated net H^+ flux from excretion to uptake (Fig. 3.2b and 3.3b). This apparent reversal might be the result of a H^+ gradient that favors the influx of H^+ via an NHE (Parks et al. 2008). For *C. v. variegatus*, the presumed reversal of the NHE was much more pronounced than observed for *F. heteroclitus*. The apparent

Figure 3.7. Effects of experimentally increasing NH3 concentration (by addition of NH_4OH to water samples adjusted to pH 4.5 with H_2SO_4) on single and double endpoint titration methods for quantifying titratable alkalinity. **A.** Measured by double endpoint titration. **B.** Measured by single endpoint tritration. Mean ± SEM (n=8).



change in calculated net H^+ transport for *C. v. variegatus* is strictly a result of an apparent increase in titratable acid uptake at low pH, as ammonia efflux is unchanged by the low pH exposure. In contrast, our results indicate the change in calculated net H^+ transport in

F. heteroclitus is the combined result of reduced T_{amm} excretion and increased titratable acid uptake. The previous study by Patrick and Wood (1999) on *F. heteroclitus* did not observe a reduction in T_{amm} efflux at low pH.An alternative perspective, based on results using double endpoint titrations, is that when exposed to low pH, the net H^+ transport for *C. v. variegatus* changes from near zero to an excretion rate of 371 nEq g^{-1} h^{-1}. In this case, the calculated change in net H^+ transport is the result of a significant reduction in titratable acid uptake (or increased base excretion) from 325 to 23 nEq g^{-1} h^{-1} rather than the observed increase in the same parameter based on single endpoint titrations. For *F. heteroclitus*, the response is qualitatively similar, although the increase in net H^+ excretion is modulated somewhat by the observed reduction in T_{amm} efflux.

Which of these perspectives is correct? We postulated four possible confounding factors could contribute to the inconsistent results between the two methodologies. First, we hypothesized that observed differences were simply the result of inter-specific differences between *C. v. variegatus* and *F. heteroclitus* in response to low pH exposure. As we just described, although there do appear to be differences in the magnitude of change in net H^+ transport between the two species, qualitatively, they respond similarly based on both single and double endpoint methodologies.

A second hypothesis was that fish increased mucus excretion or excreted mucus of a different composition as a stress response and that the mucus increased the buffer capacity of the water during the titrations. This would be misinterpreted as an increase in titratable alkalinity when using the single endpoint methodology, but not when using the double endpoint methodology. Our results indicate this is probably not the case, as mucus production appears to be reduced in low pH water (Fig. 3.4). This of course does

not rule out the possibility that fish are secreting some other buffer into the water (e.g. phosphate via the urine), or that the mucus produced at low pH has a greater buffering capacity. However, careful titration of initial and final water samples after a 3 h flux at pH 4.5 for *C. v. variegatus* indicates there is no significant change in buffering capacity between the two sampling periods, although there is a trend of increasing buffering capacity at pH 7.5 and higher (Fig. 3.5). While this may suggest release of a buffer by the fish, the pK of this unknown substance appears to be relatively high and would not influence single versus double endpoint titrations in pH 4.5 water.

A third hypothesis was that ammonia secretion by fish, a large fraction of which would be present as NH_4^+ in the sample, was being titrated by NaOH during the double endpoint titration (Lemann and Lennon 1966). This could lead to an erroneously low measurement of titratable alkalinity at the end of the flux period, resulting in a correspondingly lower estimate of titratable acid uptake. However, both the single and double endpoint titration methods are relatively insensitive to experimental NH_4^+ additions (as NH_4Cl) to water samples even at concentrations well above those typically measured during a 3-h flux period. There was no significant increase in measured titratable alkalinity concentrations even in the highest ammonia treatment (137 μM) using the double endpoint method (Fig. 3.6). Given that the maximum measured ammonia concentration in our experiment was 22 μM, it is unlikely that titration of NH_4^+ explains the discrepancy between the two titration methods.

The final hypothesis was that at low pH, the double endpoint methodology would fail to properly account for ammonia excretion if it occurred in the form of NH_3. The theoretical basis for this hypothesis is as follows. In the calculation of net H^+ flux, H^+

and NH_4^+ excretion by the fish contribute to net H^+ transport, but only H^+ excretion is measured by the titration technique. Ammonia excretion as NH_3 is not H^+ excretion but is measured as titratable alkalinity by the titration technique. In the subsequent calculation of net H^+ transport, the fraction of T_{amm} excretion which is NH_3 is cancelled out by the measurement of NH_3 as titratable alkalinity.

Using the single endpoint methodology, initial and final samples collected from the flux experiment are titrated to pH = 3.8. The NH_3 excreted by the fish into the water in the final sample is accounted for by the titratable alkalinity measurement regardless of the pH range. If the flux is performed at circumneutral pH, NH_3 reacts with CO_2 and H_2O to form NH_4HCO_3. The HCO_3 is actually what is titrated. If the flux is performed at acidic pH, well below the pK (6.1) of the CO_2/HCO_3 equilibrium, then NH_4HCO_3 cannot form, instead NH_3 is simply protonated, removing an H^+ ion from the water and essentially forming NH_4OH. In this case, the OH^- is actually what is titrated. In either scenario, NH_3 excretion is measured as titratable alkalinity by the single endpoint method.

In contrast, using the double endpoint methodology, initial and final samples are titrated to pH 3.8, sparged with N_2 to remove CO_2 generated by the titration of HCO_3^- at low pH, and then titrated back to starting pH to measure any changes in water buffering capacity. If the flux is performed at circumneutral pH, NH_3 excreted by the fish is again measured as titratable alkalinity and properly accounted for in calculation of net H^+ excretion as described above. However, if the flux is performed at acidic pH, NH_4OH rather than NH_4HCO_3 is titrated. Because, OH^- will not be converted to CO_2 and

therefore not removed by N_2 sparging at pH 3.8, the NH_3 excreted by the fish will not be measured as titratable alkalinity using the double endpoint methodology.

We verified these theoretical differences between the two methodologies by measuring samples spiked with 10 μM NH_4OH into pH 4.5 water. As would be expected if the above hypothesis is correct, measurement of this water using the single endpoint method detected a 10 μM increase in titratable alkalinity, while the double endpoint method detected no change in alkalinity (Fig. 3.7).

Given the above, it is possible to correct for this problem with the double endpoint methodology, if one makes an assumption regarding the fraction of T_{amm} excretion that is NH_3. The maximum possible correction would be to assume 100% of T_{amm} excretion occurred as NH_3 at low pH and was therefore not detected by the double endpoint titration technique. This assumption is based on recent studies indicating that Rh glycoproteins are the principal mechanism of T_{amm} excretion in fish gills (Nawata et al. 2007) and that they transport ammonia as NH_3, not NH_4^+ (Nawata et al. 2010). If we make this assumption, the corrected double endpoint titration results and corresponding recalculated net H^+ transport estimates for *C. v. variegatus* (23 ± 53 nEq g^{-1} h^{-1}) and *F. heteroclitus* (-37 ± 35 nmol g^{-1} h^{-1}) are similar and indicate no significant net H^+ transport at pH 4.5.

This result still differs significantly from the single endpoint titration results (Figs. 3.2b and 3.3b), which indicate net H^+ excretion is not only inhibited, but is actually reversed to significant net H^+ uptake at low pH. There are two possible sources for this apparent net H^+ uptake. First, it is possible that NHE is operating in reverse, facilitating H^+ uptake (Parks et al. 2008). However, when one considers that operation of an apical

NHE in reverse would rapidly deplete intracellular Na^+, inhibition rather than reversal of the NHE seems more likely. Inhibition of NHE at low pH agrees with observations of net H^+ transport using the recalculated double endpoint titration results.

Alternatively, it is also possible that while NHE is not reversed, base (e.g. HCO_3^-) excretion is inhibited or down-regulated at low pH, which again would appear as net H^+ uptake. This scenario would be consistent with observations using the single endpoint methodology and indicate there is still an unidentified problem with the double endpoint methodology. While this is possible, we know that in *Fundulus*, branchial Cl^- uptake and Cl^-:HCO_3^- exchange are negligible in the ~1 mM NaCl water used in our experiment, but Cl^- uptake increases at >2 mM Cl^- in the water (Patrick et al. 1997; Patrick and Wood 1999). It is unknown whether *Cyprinodon* are capable of Cl^-:HCO_3^- exchange, although we do know that when water NaCl concentration is increased from 2 to 7 mM, an increasing fraction (from 10 to 40%) of Na^+ uptake appears to occur via an apical NKCC (Brix and Grosell 2012). This is at least suggestive that branchial Cl^-:HCO_3^- exchange is limited in both species.

Overall, this study demonstrated that when *C. v. variegatus* is exposed to low pH, Na^+ uptake is inhibited and ammonia excretion unchanged, suggesting this species is likely using NHE-2 to facilitate apical Na^+ uptake in freshwater. This tentative conclusion awaits confirmation through gene expression and/or immunohistochemistry. More importantly, our study revealed a significant discrepancy between the single and double endpoint titratable alkalinity methods when used to characterize net H^+ transport at low pH in both *C. v. variegatus* and *F. heteroclitus*. Part of this discrepancy can be accounted for by the inability of the double endpoint methodology to measure NH_3

excretion at low pH, but even after making the maximum possible correction for this error, a significant discrepancy remains between the two methods.

Historically, most studies have relied on either single endpoint titrations (McDonald and Wood 1981; Ultsch et al. 1981; Evans 1982; Evans 1984; Goss and Wood 1990; Patrick et al. 1997; Patrick and Wood 1999; Edwards et al. 2005; Georgalis et al. 2006) or double endpoint titrations (Taylor et al. 2007; Genz et al. 2008; Brix et al. 2011) to characterize net H^+ transport in aquatic organisms. To the best of our knowledge only two previous studies compared the two methods (Bucking and Wood 2008; Cooper and Wilson 2008) and both found congruence between the methods. Both of these studies were performed on rainbow trout in freshwater at circumneutral pH and after demonstrating close agreement between the two methods, used single endpoint titrations for their analyses.

We suggest that comparing results from single and double endpoint titrations may be a reasonable standard practice to adopt when conducting studies on a new species or under environmental conditions that depart significantly from previously validated conditions. Conditions of extreme pH and conditions where compounds with buffer capacity (e.g., fecal matter, regurgitated food, mucus, etc.) may be eliminated by the experimental animals call for a careful comparison of the two methods. With respect to fish exposed to acidic conditions, the methodological issues identified in the current study are problematic and unresolved. Additional research is needed to identify the most appropriate method for measuring TA flux in aquatic organisms at low pH before we can reliably characterize net H^+ transport under these conditions.

Chapter 4:

Evaluation of Mechanisms for NHE Function in *Cyprinodon variegatus variegatus* and *Cyprinodon variegatus hubbsi* in Low Na^+ Water

Summary

The function of Na^+/H^+ exchangers (NHEs) in the apical membrane of fish gills in low Na^+ freshwater environments is thermodynamically constrained and based on our understanding of intracellular Na^+ and H^+ concentrations should not function in the forward direction (Na^+ uptake) in neutral or acidic waters with $\leq$2 mM Na^+. To overcome this limitation, several fishes utilize an NHE-Rh metabolon and/or carbonic anhydrase (CA) to alter H^+ gradients in intra- or extracellular micro-domains that allow NHE to take up Na^+ against the chemical gradient of the bulk solutions. The euryhaline pupfish, *Cyprinodon variegatus variegatus* (*Cvv*), is capable of osmoregulating in near freshwater conditions (2 mM Na^+) and a freshwater subspecies (*C. v. hubbsi*; *Cvh*) can successfully osmoregulate at 0.1 mM Na^+. We previously demonstrated *Cvh* takes up Na^+ at 0.1 mM Na^+ strictly via an NHE. The present study comparatively investigated whether *Cvv* or *Cvh* use an NHE-Rh metabolon or CA to allow for NHE function in dilute freshwater. We accomplished this by simultaneously measuring Na^+ uptake and ammonia excretion while selectively manipulating Na^+, H^+, and ammonia gradients, using pharmacological inhibitors, and measuring CA activity. These experiments provided no support for the presence of an NHE-Rh metabolon in either subspecies under any salinity regime (0.1, 2 and 7 mM Na^+) evaluated. In contrast, CA appears to play an important role in Na^+ uptake in *Cvv*. CA activity was comparable in fish acclimated to 7

and 2 mM Na^+, but exposure to ethoxzolamide (CA inhibitor) stimulated Na^+ uptake by 91% and reduced Na^+ uptake by 58%, respectively. We hypothesize either H^+ or HCO_3^- produced by CA is downregulating the apical NKCC present in *Cvv* at 7 mM Na^+, but absent at 2 mM Na^+. When *Cvv* are acclimated to 2 mM Na^+, CA facilitates Na^+ uptake presumably through generation of H^+ via CO_2 hydration. In *Cvh*, which lacks an apical NKCC at 7 mm Na^+, CA plays a relatively minor role in NHE function with exposure to ethoxzolamide inhibiting Na^+ uptake by 19%. However, at 0.1 mM Na^+, CA activity increased by 75% compared to 7 mM Na^+ fish and Na^+ uptake was inhibited by 31% after treatment with ethoxzolamide. This suggests CA plays an important role in NHE function for *Cvh* in dilute freshwater but given the modest (31%) inhibition by ethoxzolamide does not rule out an additional unknown mechanism is contributing to NHE function.

Background

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 (Na^+, Cl^-) at the gill against their concentration gradients and efficiently reabsorb salts at the kidney producing a copious dilute urine (Marshall and Grosell 2006). Common to all fish, Na^+ uptake at the gill is largely driven by a basolateral Na^+/K^+-ATPase. A number of different transport proteins have been shown to 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 relevant for Na^+ uptake;

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).

Available evidence indicates both NCC and NKCC appear to operate in relatively saline freshwaters (≥7 mM Na^+), which is largely consistent with thermodynamic constraints (Wang et al. 2009; Brix and Grosell 2012). 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). Perhaps most interesting are the NHEs, of which two main isoforms (NHE-2 and NHE-3) have been identified as contributing to apical Na^+ uptake in freshwater fish. Both isoforms are found in a range of species, usually operating in conjunction with one or more of the other transport systems. Available data suggests with respect to Na^+ affinity, NHE-2 is a relatively low affinity isoform, while NHE-3 is a relatively high affinity isoform (Hwang et al. 2011). 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 (*Danio rerio*, *Tribolodon hakonensis*) (Hirata et al. 2003; Kumai and Perry 2011).

Based on current knowledge of intracellular Na^+ concentrations and pH, 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, NHE should operate in the reverse direction exporting Na^+ and taking up H^+. Fish have evolved 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 (CA) through 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).

More recently, in a series of elegant experiments, it was demonstrated that under low Na^+ or low pH conditions *D. rerio* and *O. latipes* accomplish Na^+ uptake via NHE-3 in conjunction with the Rh glycoprotein Rhcg-1, which is located in the apical membrane of mitochondrial rich cells (MRCs) (Wu et al. 2010; Kumai and Perry 2011). The exact mechanism by which this metabolon works is not currently clear. Ammonia is known to be transported from the blood into the MRC via the basolateral Rh protein Rhbg and through the apical Rhcg-1 in MRCs and Rhcg-2 in pavement cells (Nawata et al. 2007; Nakada et al. 2007a; Nakada et al. 2007b). 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 (i.e,. Na^+ inward, H^+ outward) operation of NHE in several possible ways. First, H^+ trapping by NH_3 outside the cell is likely to occur in the boundary layer providing localized alkalinization of the external environment, facilitating forward operation of NHE-3 along with providing a gradient for the continued diffusion of NH_3. 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 cause a localized acidification on the intracellular side of the apical membrane, again creating a favorable gradient for forward operation of NHE-3.

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

In a previous comparative study of Na^+ transport in the two subspecies, 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 = 7,000-38,000 μM) when acclimated to 2 or 7 mM Na^+, while *Cvh*, but not *Cvv*, 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^+. Na^+ uptake is not bafilomycin sensitive (H^+-ATPase inhibitor) in either subspecies under any condition, but Na^+ uptake in *Cvh* is phenamil sensitive leading to some uncertainty about the possible involvement of a Na^+ channel/H^+-ATPase system in this subspecies (Brix and Grosell 2012).

Given the above, we 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. The remainder of this chapter presents a series of experiments to test these hypotheses.

Methods and Materials

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. 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). 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. 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*.

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 ammonia (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 nearest 0.1 mg and then assayed individually for radioactivity.

The various treatments that departed from this general design were as follows: Fed – Fish were fed to satiation twice per day for 2 d, including a meal 3 h prior to experimentation; pH 4.5 – The exposure water pH was adjusted to pH 4.5 using HNO_3; HEA – Fish were exposed to high environmental ammonia (2 mM) as $(NH_4)_2SO_4$; MOPS – Fish were exposed to 5 mM of the buffer 3-(N-morpholino) propanesulfonic acid adjusted to pH 7.0 with KOH; EIPA – Fish were exposed to 5 $x10^{-5}$ M (5-(*N*-ethyl-*N*-isopropyl)-amiloride), which is potent NHE inhibitor with low affinity for Na^+ channels (Kleyman and Cragoe 1988). 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-hydroxpropyl)-β-cyclodextrin was found to be suitable for both solubilizing EIPA and performing the ammonia assay.

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 two weeks. After acclimation, fish were euthanized with an overdose of MS-222, and fish gills were perfused with a 150 mM NaCl heparinized saline to remove blood and the associated red blood cell form of CA from the gill tissue. 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). In brief, 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 x 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 levels as detected using the

Bradford assay (Sigma-Aldrich) with bovine serum albumin standards and measured using a plate spectrophotometer at 595 nm (Molecular Devices, Sunnyvale, CA).

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^{2+} 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. Comparison data were analyzed by Student's t-test or by ANOVA with a post-hoc analysis using the Holm-Sidak test, when multiple treatments were evaluated. All comparison analyses were performed using SigmaStat v3.5 (SPSS 2006).

Results

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, and HEA had no effect (Fig. 4.1). Corresponding measurements of T_{amm} excretion show feeding significantly increased T_{amm} excretion rates, while all other treatments were unchanged relative to the

control (Fig. 4.1). 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 (Fig. 4.2). 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, stimulated in the MOPS treatment, and feeding had no effect. Corresponding T_{amm} excretion rates were stimulated in the fed and pH 4.5 treatments, and unchanged in all other treatments (Fig. 4.3).

NHE-CA Experiments

Ethoxzolamide had varying results on *Cvv* acclimated to 2 or 7 mM Na^+. In 7 mM Na^+ acclimated fish, Na^+ was strongly (2-fold) stimulated while in 2 mM Na^+ acclimated fish it was strongly (58%) inhibited (Fig. 4.4A). In contrast, the response of *Cvh* to ethoxzolamide was only modestly different between acclimation salinities, with significant reductions in Na^+ uptake of 31% and 19% at 0.1 and 7 mM Na^+, respectively (Fig. 4.4B).

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^+ (Figure 4.5A). Carbonic anhydrase activity in *Cvh* acclimated to 0.1 mM Na^+ was significantly (75%) higher than observed in *Cvh* acclimated to 7 mM Na^+ (Fig. 4.5B).

Figure 4.1. 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). * = significant ($p<0.05$) difference relative to control or carrier control (EIPA treatment only).

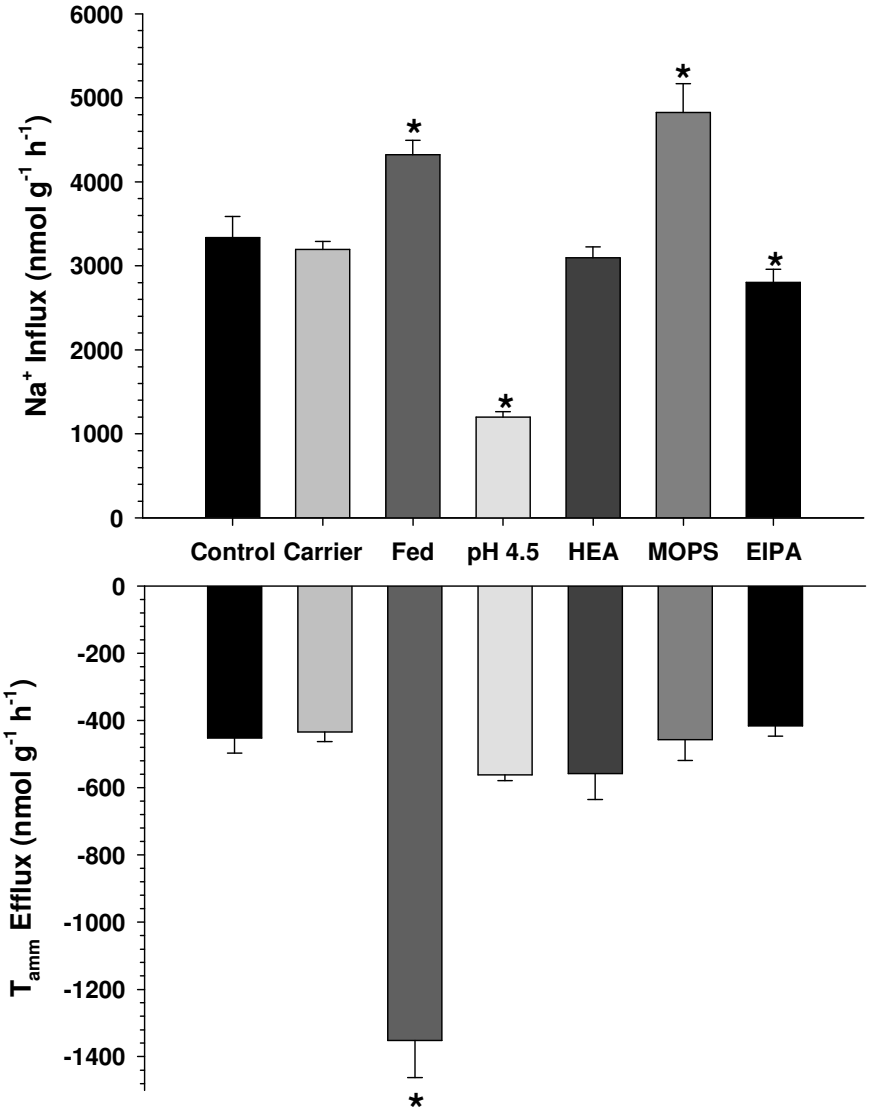


Figure 4.2. Effects of different treatments on concurrent Na^+ uptake and T_{amm} excretion rates (nmol g^{-1} h^{-1}) in *Cvh* acclimated to 7 mM Na^+. See Methods and Materials for description of each treatment. Mean ± SEM (n=8). * = significant ($p<0.05$) difference relative to control or carrier control (EIPA treatment only).

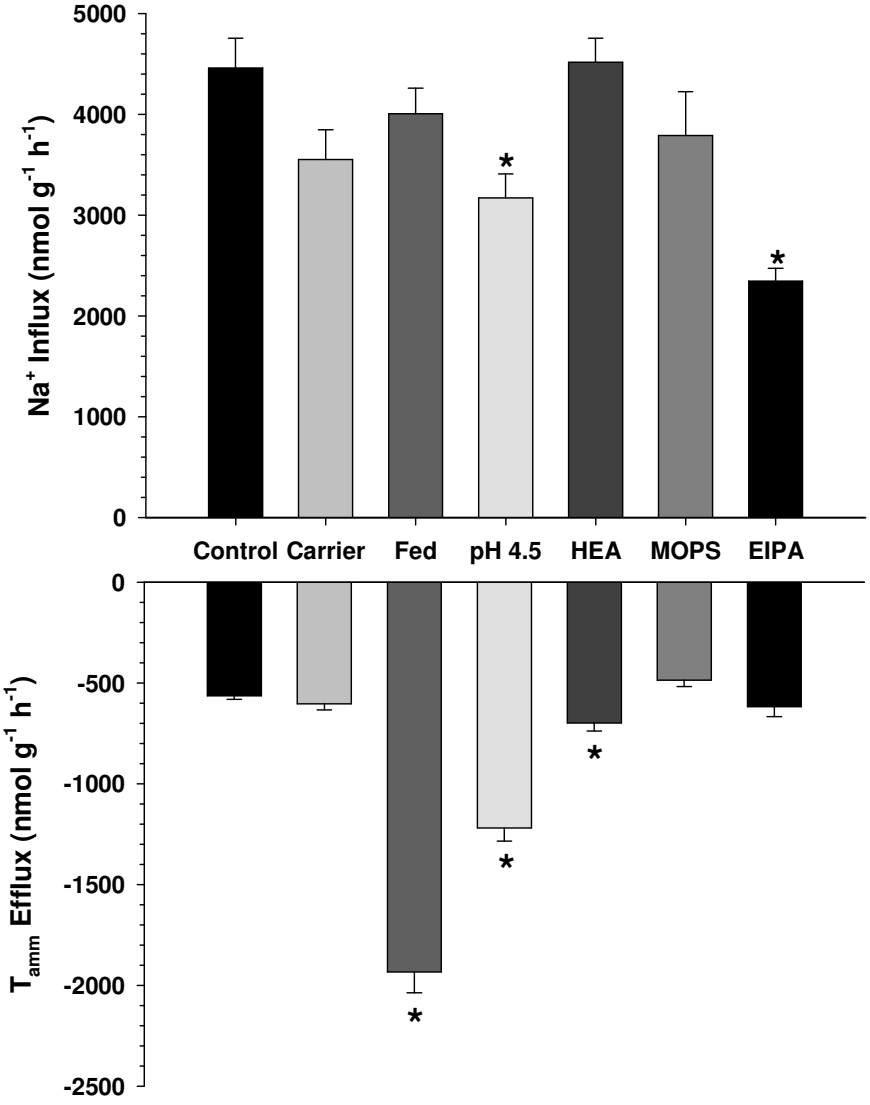


Figure 4.3. Effects of different treatments on concurrent Na^+ uptake and T_{amm} excretion rates (nmol g^{-1} h^{-1}) in *Cvh* acclimated to 0.1 mM Na^+. See Methods and Materials for description of each treatment. Mean ± SEM (n=8). * = significant ($p<0.05$) difference relative to control or carrier control (EIPA treatment only).

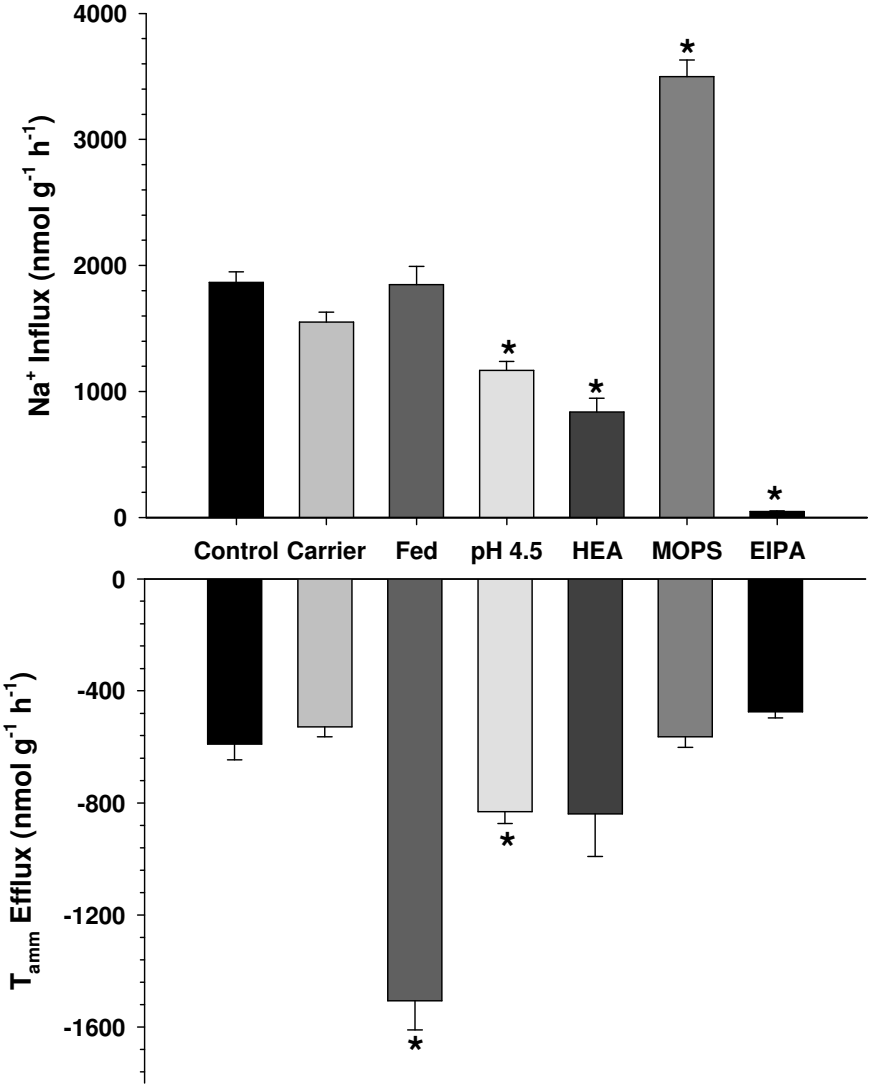


Figure 4.4. Effects of ethoxzolamide on Na^+ uptake rates (nmol g^{-1} h^{-1}) in **A.** *Cvv* acclimated to 2 or 7 mM Na^+; **B.** *Cvh* acclimated to 0.1 or 7 mM Na^+. Mean ± SEM (n=8). * = significant ($p<0.05$) difference relative to control.

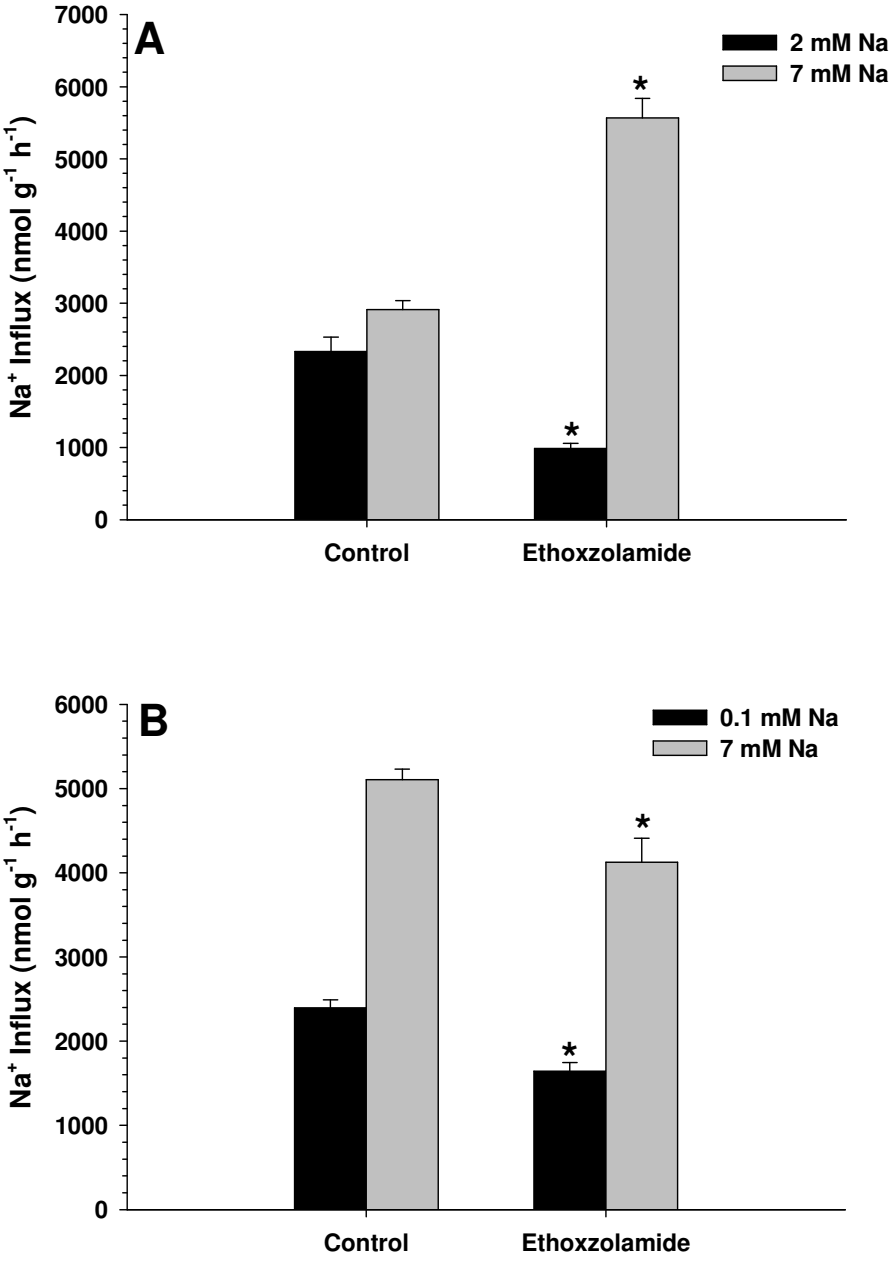
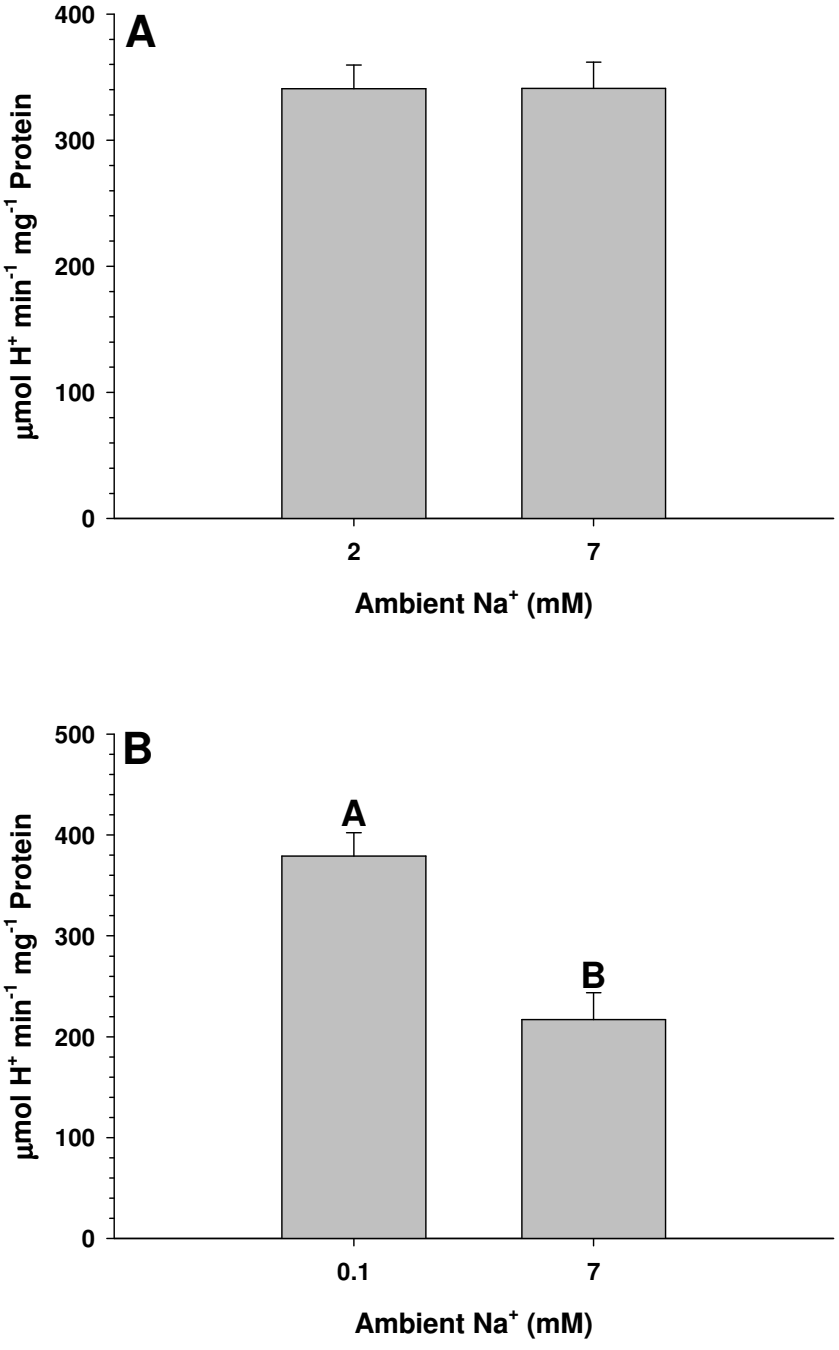


Figure 4.5. Carbonic anhydrase activity (μmol H^+ min^{-1} mg^{-1} protein) in **A.** *Cvv* acclimated to 2 or 7 mM Na^+ and **B.** *Cvh* acclimated to 0.1 or 7 mM Na^+. Mean ± SEM (n=8). Different letters indicate significant ($p<0.05$) difference between treatments.



Conclusions

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 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, increases in Na^+ influx was 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 (Fig. 4.1-4.3).

Results from this experiment differ significantly from similar experiments with *Danio rerio* and *Oryzias 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 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* is 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. 4.1-4.3). 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. 4.3).

Overall, results from these experiments demonstrate in a fairly conclusive manner that neither *Cvv* or *Cvh* is using an NHE-Rh metabolon under high or low Na^+ conditions. The observation supporting an NHE-Rh metabolon 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 increase in Na^+ uptake.

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^+ with ethoxzolamide, a 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 it would be higher in fish acclimated to 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) and a 75% increase in CA activity in fish acclimated to 0.1 mM Na^+ compared to 7 mM Na^+ (Fig. 5B). These results

support an important role for CA in NHE function in *Cvh* acclimated to low Na^+ water. Although we hypothesized that we would see a large increase in the effect of ethoxzolamide on Na^+ uptake at 0.1 mM Na^+, the relatively modest increase we observed might simply be the result of the large increase in CA activity. However, given these results, it is not clear that that H^+ generated via CA-mediated CO_2 hydration is the only 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. Equally interesting, was the nearly 2-fold stimulation of Na^+ uptake in CA-inhibited *Cvv* acclimated to 7 mM Na^+, a considerably different result that observed at 2 mM Na^+ despite fish having the same CA activity when acclimated to these different salinities (Fig. 4.4A and 4.5A). The stimulation in Na^+ uptake at 7 mM Na^+ is difficult to explain, but we hypothesize this response is related to the use of NKCC by *Cvv* (but not *Cvh*) at this salinity. This hypothesis is supported by the observation that in *C. macularius*, which also does not use NKCC for Na^+ uptake, ethoxzolamide inhibits Na^+ uptake in fish acclimated to 7 mM Na^+ (see Chapter 5). CA has been linked to stimulation of apical NKCC in 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 were operating in the *Cvv* gill, we would have expected a decrease rather than 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^- is yet to be described, but would be worth investigation in *Cvv* acclimated to 7 mM Na^+.

In conclusion, the experiments in this Chapter aimed to test whether a NHE-Rh metabolon and/or NHE-CA system explained the function of NHE in *Cvv* and *Cvh* in low Na^+ waters. Results provided strong evidence that an NHE-Rh metabolon is not operating in either subspecies. For *Cvv*, CA appears to play an important role in Na^+ uptake, downregulating uptake via a currently unknown signaling pathway at 7 mM Na^+ and contributing significant, 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^+ but a potentially 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 subspecies cannot be ruled out.

Chapter 5:

Characterization of Na^+ Uptake in the Endangered Desert Pupfish, *Cyprinodon macularius*

Summary

This study provided an initial characterization of Na^+ uptake in freshwater by the endangered pupfish *Cyprinodon macularius*. The lower acclimation limit of *C. macularius* in freshwater was found to be 2 mM Na^+. Fish acclimated to 2 or 7 mM Na^+ displayed similar Na^+ uptake kinetics with K_m values of 4321 and 3672 μM and V_{max} values of 4771 and 3602 nmol g^{-1} h^{-1}, respectively. A series of experiments using pharmacological inhibitors indicated Na^+ uptake in *C. macularius* was not sensitive to bumetanide, metolazone, or phenamil eliminating the Na^+:K^+:2Cl^- co-transporter, Na^+:Cl^- co-transporter, and the Na^+ channel/H^+-ATPase system as mechanisms for apical Na^+ uptake in fish acclimated to 2 or 7 mM Na^+. However, Na^+ uptake was sensitive to 1 x 10^{-3} M amiloride (not 1 x 10^{-4} or 10^{-5} M), EIPA and ethoxzolamide. These data suggest that *C. macularius* relies solely on a low affinity Na^+/H^+ exchanger for apical Na^+ uptake and that H^+ generated via carbonic anhydrase mediated CO_2 hydration are important for the regulation and function of this protein.

Background

The fish genus *Cyprinodon* (Cyprinodontiformes) is thought to have originated in the southwestern US or northern Mexico ~7-8 mya (Echelle et al. 2005). Today,

approximately 50 species of *Cyprinodon* have been described with ~40 of these species in the arid Southwest. The remaining species are spread along the Gulf of Mexico and Atlantic coastline from Venezuela to Massachusetts, as well as many of the Caribbean Islands. The majority of species live in relatively stable environments with respect to salinity ranging from slightly saline freshwater springs to athalassic lakes with salinities approximately twice that of seawater.

The desert pupfish, *Cyprinodon macularius*, historically ranged from the Gila River in Arizona to the Salton Sea in California and down into the Colorado Delta region of Sonora and Baja (Miller 1943). However, due to various water management projects (reservoirs, water diversion canals) and introduction of non-native species, *C. macularius* has disappeared from the Gila and lower Colorado rivers and populations have declined at other locations (Miller and Fuiman 1987; Dunham and Minckley 1998). Currently, there are only two populations of *C. macularius* located at the Salton Sea and the Colorado River delta, with a third population at Rio Sonoyta/Quitobaquito Springs now reclassified as *C. eremus* (Echelle et al. 2000; Loftis et al. 2009). In the United States, *C. macularius* is currently listed as an endangered species (Marsh and Sada 1993).

Despite the impact of various man-made alterations to water flow and salinity regimes in the region, the osmoregulatory capacity of *C. macularius* has received little study. Barlow (1958) concluded *C. macularius* had an upper salinity threshold of 90 g l^{-1} based on observations made in small hypersaline pools along the shoreline of the Salton Sea. Kinne (1960) demonstrated that larvae can survive and grow at salinities ranging from freshwater to 55 g l^{-1}, with an optimum salinity between 10 and 40 g l^{-1}.

Our interest was in the osmoregulatory capacity of *C. macularius* in freshwater conditions. We have previously characterized osmoregulation in the coastal pupfish *C. variegatus variegatus* as well as the subspecies *C. v. hubbsi* (Brix and Grosell 2012). *C. v. variegatus* occurs along the Gulf and Atlantic coasts of North America and tolerates salinities ranging from freshwater up to 167 g l^{-1} (Nordlie 2006). Previous studies indicate *C. v. variegatus* does not survive (long-term), grow or reproduce in freshwater with <2 mM Na^+ (Dunson et al. 1998). *C. v. hubbsi* only occurs in eight freshwater lakes in central Florida. These lakes have ambient Na^+ concentrations of 0.4-1.0 mM Na^+, below the level typically tolerated by *C. v. variegatus*, suggesting *Cvh* has adapted to this more dilute freshwater environment.

We demonstrated that *C. v. variegatus* and *C. v. hubbsi* bred and raised under common garden conditions (freshwater with 7 mM Na^+) have similar low affinity Na^+ uptake kinetics (K_m = 7,000-38,000 μM) when acclimated to 2 or 7 mM Na^+, while *C. v. hubbsi* 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 *C. v. variegatus* 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, *C. v. hubbsi* 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^+. Na^+ uptake is not bafilomycin sensitive (H^+-ATPase inhibitor) in either subspecies under any condition, but Na^+ uptake in *C. v. hubbsi* is phenamil sensitive leading to some uncertainty about the possible involvement of a Na^+ channel/H^+-ATPase system in this subspecies (Brix and Grosell 2012).

Given the above, we hypothesized that *C. macularius* would exhibit similar Na^+ transport kinetics and utilize the same Na^+ transport proteins as *C. v. variegatus*. In addition to providing useful information on this endangered species, if this hypothesis proved correct, it would provide weight of evidence regarding the basal Na^+ transport characteristics of *Cyprinodon* and a better context in which to evaluate the apparent adaptations in *C. v. hubbsi* to osmoregulate in dilute freshwater.

Methods and Materials

Animal Holding

Adult *C. macularius* were generously provided from an in-house culture maintained by the U.S. Geological Survey lab in Columbia, MO (U.S. Fish and Wildlife Service Endangered Species Permit #TE2057312). The USGS had collected their initial stock from the Salton Sea population of *C. macularius*. 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. 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). 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. 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, *C. macularius* 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).

Characterization of Na^+ Uptake Kinetics

The Na^+ uptake kinetics of *C. macularius* were determined in juvenile fish acclimated to 2 or 7 mM Na^+ for at least 3 weeks prior to experimentation. In a preliminary experiment, attempts to acclimate *C. macularius* to 1 mM Na^+ resulted in ~50% mortality within 96 h, so 2 mM Na^+ was considered the lower limit for this species. For each experiment, Na^+ uptake rates were measured at between 7 or 8 different ambient Na^+ concentrations ranging from 0.174 to 57.1 mM Na^+ depending on the Na^+ concentration to which they were acclimated. At each Na^+ concentration, 8 juvenile fish (26-240 mg) were placed in 50 ml of a defined media (480 μM $CaSO_4$, 150 μM $MgSO_4$, 100 μM $KHCO_3$, pH 7.0, 22-23 °C) 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 media for 10 min after which the media 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 0.9-2.3 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 media, double rinsed in a 100 mM Na^+ solution to displace any loosely bound

^{22}Na, blotted dry, weighed to nearest 0.1 mg and then assayed individually for radioactivity.

Pharmacological Inhibitor Experiments

Juvenile *C. macularius* were 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. Initial experiments were conducted using amiloride (N-amidino-3.5- diamino-6- chloropyrazinecarbromide), which 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}, 10^{-3} M) in an attempt to distinguish effects between these different pathways. For the control and amiloride treatments, 8 juvenile *C. macularius* were exposed in 48 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} M amiloride and 0.1% DMSO, while 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 exposed for 2 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 above.

Similar experimental designs were used in subsequent inhibitor experiments, again testing fish acclimated to 2 or 7 mM Na^+. *C. macularius* were exposed to 5 x 10^{-5} M EIPA (5-(*N*-ethyl-*N*-isopropyl)-amiloride), which is potent NHE inhibitor with low affinity for Na^+ channels and 1 x 10^{-5} M phenamil, a Na^+ channel inhibitor with relatively

low affinity for NHEs (Kleyman and Cragoe 1988). In order to investigate the presence/absence of a chloride-dependent Na^+ transporter, juvenile fish were exposed to h exposed to 10^{-4} M bumetanide (NKCC inhibitor) and 10^{-5} M metolazone (NCC inhibitor) in separate experiments. Finally, to evaluate the potential role of carbonic anhydrase (CA) in Na^+ uptake, fish were exposed to 1 x 10^{-4} M ethoxzolamide.

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^{2+} 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).

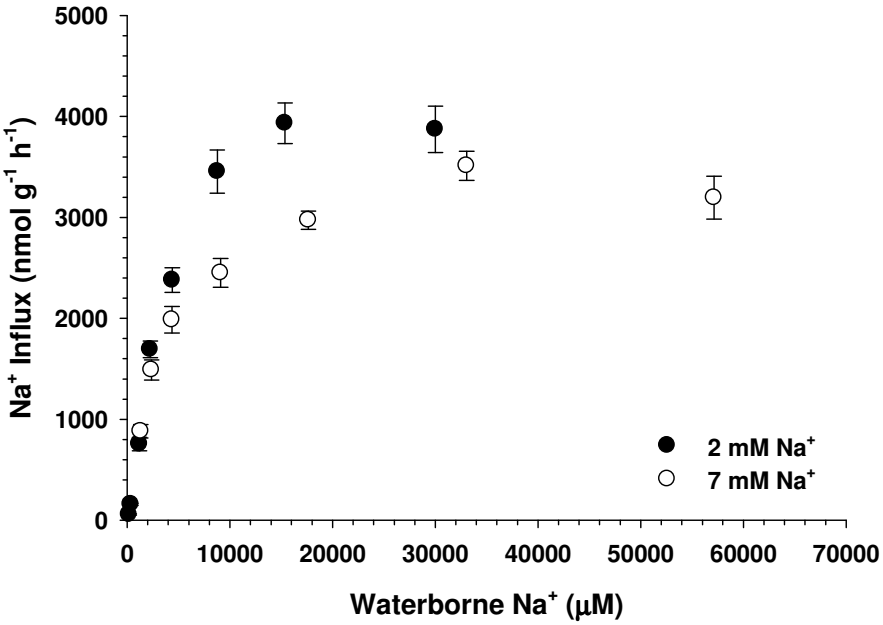
All values are expressed as means ± SEM throughout. Comparison data were analyzed by Student's t-test or by ANOVA when multiple treatments were evaluated. All comparison analyses were performed using SigmaStat v3.5 (SPSS 2006). Kinetic data were observed to fit a Michaelis-Menten function and estimates of K_m and 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^+ Uptake Kinetics

Sodium uptake rates increased with increasing ambient Na^+ concentrations and followed a hyperbolic curve that approximated Michaelis-Menten saturation kinetics for *C. macularius* acclimated to both 2 and 7 mM Na^+ (Fig. 5.1). The apparent K_m values were statistically similar between the two salinities while the estimated V_{max} for 2 mM Na^+ acclimated fish was significantly higher ($p<0.05$) than observed for 7 mM Na^+ acclimated fish (Table 5.1).

Figure 5.1. Na^+ uptake rates (nmol g^{-1} h^{-1}) as a function of external Na^+ concentrations (μM) for *C. macularius* acclimated to 2 or 7 mM Na^+. Mean ± SEM (n=8). See Table 1 for estimates of K_m and V_{max}.



Exposure of *C. macularius* acclimated to 2 and 7 mM Na^+ to amiloride resulted in progressively increasing inhibition of Na^+ uptake (Fig. 5.2). However, even at the highest amiloride concentrations tested (1 x 10^{-3} M), Na^+ uptake was only inhibited by 22-27%, precluding any estimates of $K_{0.5}$. The NHE-specific inhibitor EIPA also had a

very modest effect (10-12% inhibition) that was only significant in the 2 mM Na^+ acclimated fish (Fig. 5.3). No significant inhibition of Na^+ uptake was observed at either salinity in experiments with phenamil, bumetanide, and metolazone (Figs. 5.4-5.6). The final experiment with the CA-inhibitor ethoxzolamide resulted in 15% and 50% inhibition of Na^+ uptake in 2 and 7 mM Na^+ acclimated fish, respectively, with the latter being significantly different from the control (Fig. 5.7).

Figure 5.2. Effect of increasing amiloride concentrations on Na^+ uptake rates (nmol g^{-1} h^{-1}) in *C. macularius* acclimated to 2 or 7 mM Na^+. Controls include DMSO carrier. Mean ± SEM (n=8). * = statistical difference compared to the control ($p \leq 0.05$).

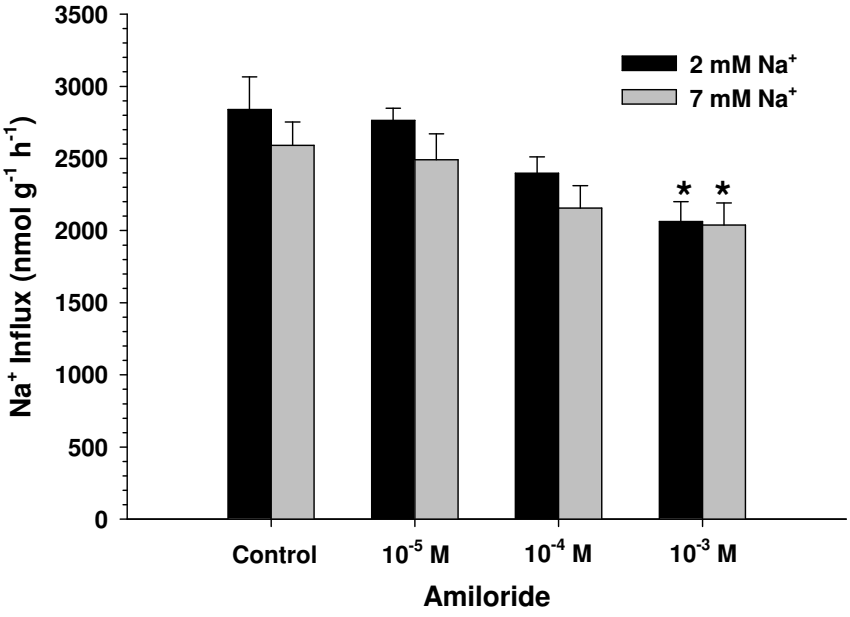


Table 5.1. Na^+ Uptake Kinetics in *Cyprinodon* spp.

Organism	Size (g)	Acclimation Water [Na^+] (μM)	K_m (μM)	V_{max} (nmol g^{-1} h^{-1})
C. macularius	0.03	2000	4321 ± 843	4771 ± 303
	0.10	7000	3672 ± 540	3602 ± 138
C.v. hubbsi	0.12	100	104 ± 14	5232 ± 234
	0.30	1200	110 ± 52	1437 ± 193
	0.23	2000	7464 ± 1615	10878 ± 904
	0.31	7000	6975 ± 996	6370 ± 348
C. v. variegatus	0.13	1000	2027 ± 175	8640 ± 360
	0.42	2000	18509 ± 3342	18999 ± 1560
	0.53	7000	38271 ± 8321	30681 ± 3393

Figure 5.3. Effect of 5 x 10^{-5} M EIPA on Na^+ uptake rates (nmol g^{-1} h^{-1}) in *C. macularius* acclimated to 2 or 7 mM Na^+. Controls include DMSO carrier. Mean ± SEM (n=8). * = statistical difference compared to the control ($p \leq 0.05$).

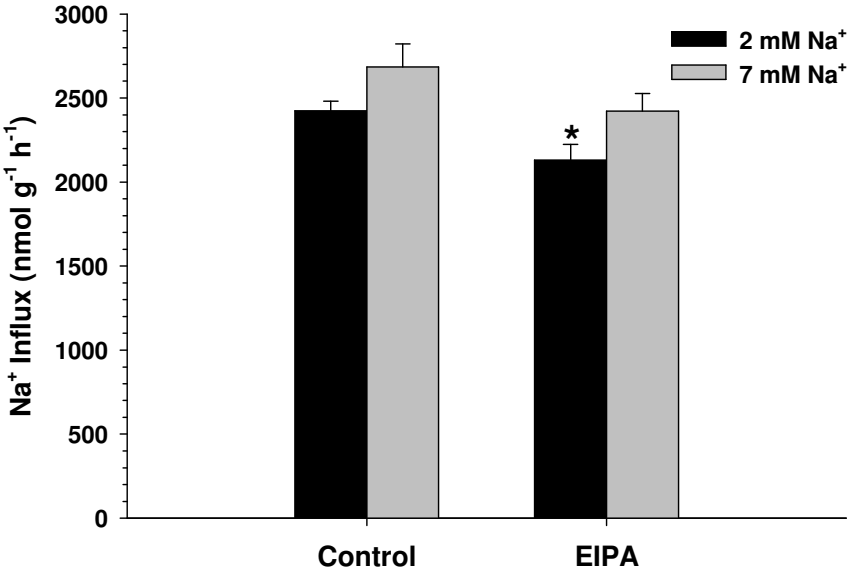


Figure 5.4. Effects 1 x 10^{-5} M phenamil on Na^+ uptake rates (nmol g^{-1} h^{-1}) in *C. macularius* acclimated to 2 or 7 mM Na^+. Controls include DMSO carrier. Mean ± SEM (n=8). * = statistical difference compared to the control ($p \leq 0.05$).

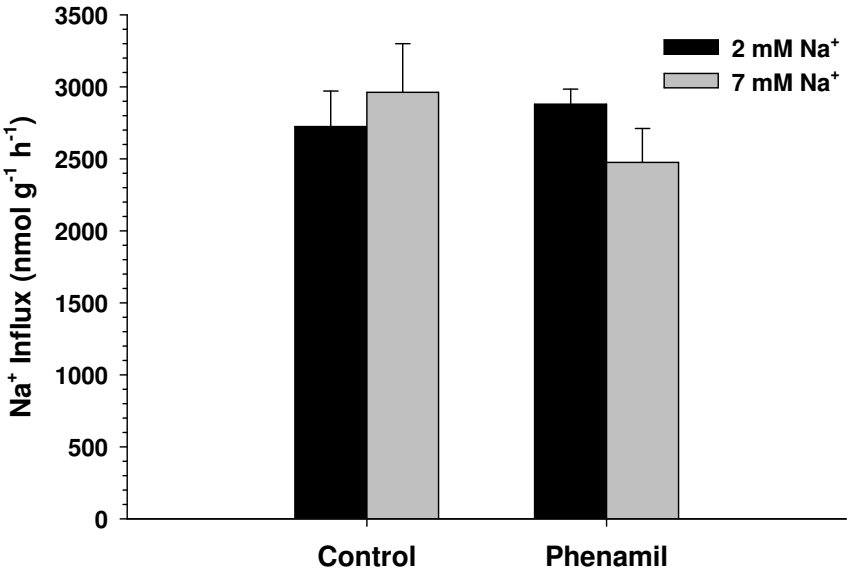


Figure 5.5. Effect of 1 x 10^{-4} M bumetanide on Na^+ uptake rates (nmol g^{-1} h^{-1}) in *C. macularius* acclimated to 2 or 7 mM Na^+. Controls include DMSO carrier. Mean ± SEM (n=8). * = statistical difference compared to the control ($p \leq 0.05$).

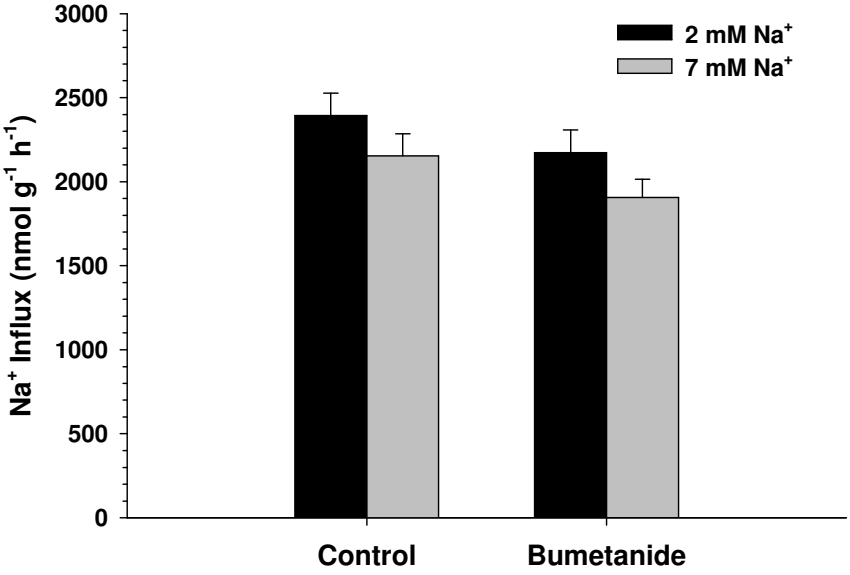


Figure 5.6. Effect of 1 x 10^{-5} M metolazone on Na^+ uptake rates (nmol g^{-1} h^{-1}) in *C. macularius* acclimated to 2 or 7 mM Na^+. Controls include DMSO carrier. Mean ± SEM (n=8). * = statistical difference compared to the control ($p \leq 0.05$).

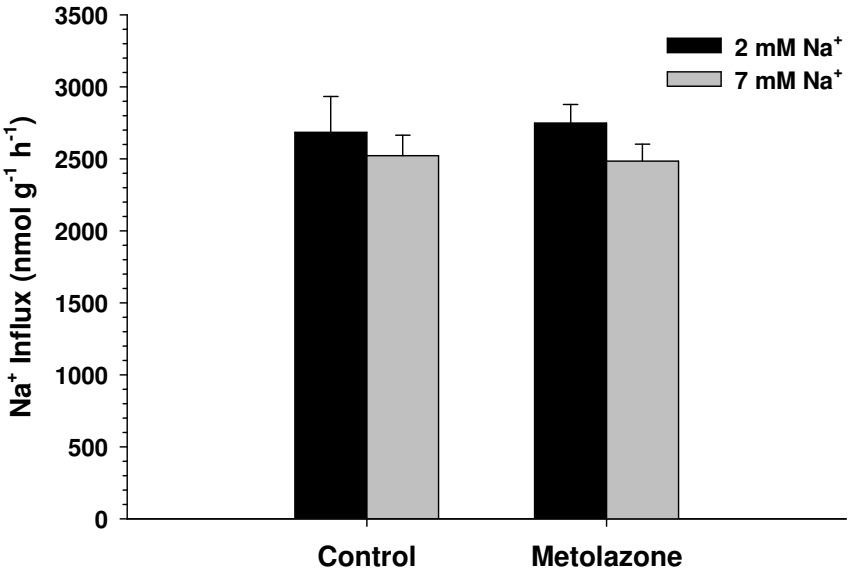
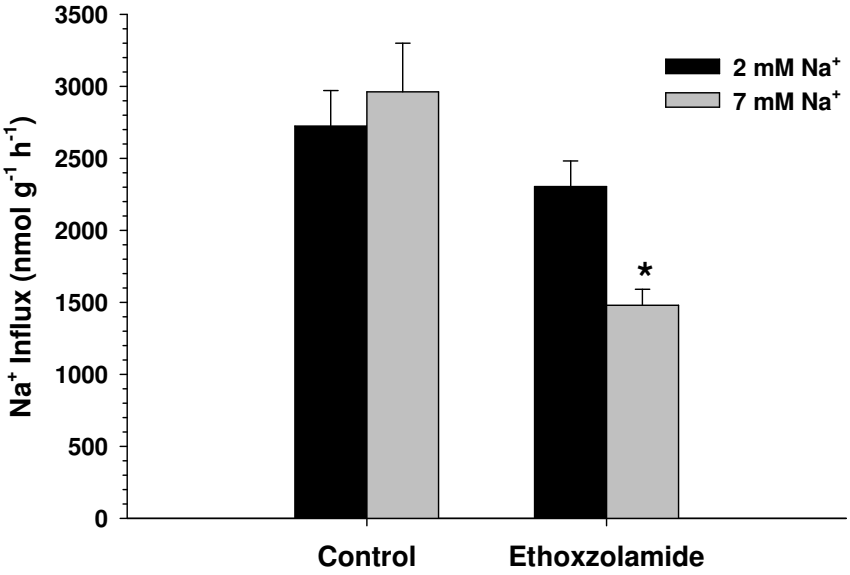


Figure 5.7. Effect of 1 x 10^{-4} M ethoxzolamide on Na^+ uptake rates (nmol g^{-1} h^{-1}) in *C. macularius* acclimated to 2 or 7 mM Na^+. Controls include DMSO carrier. Mean ± SEM (n=8). * = statistical difference compared to the control ($p \leq 0.05$).



Conclusions

Our objective was to assess Na^+ transport characteristics in *C. macularius* and compare them to those previously observed in *C. v. variegatus* and *C. v. hubbsi*. We hypothesized that *C. macularius* would be similar to *C. v. variegatus* with previously observed differences between *C. v. variegatus* and *C. v. hubbsi* reflecting adaptations by the latter to its dilute freshwater environment.

With respect to Na^+ transport kinetics, *C. macularius* displayed a significantly lower K_m than either *Cyprindon variegatus* subspecies when acclimated to 7 mM Na^+, but was intermediate when acclimated to 2 mM Na^+ (Table 5.1). This reflects the lack of change in *C. macularius* K_m over this range, while both *C. variegatus* subspecies significantly increased their affinity for Na^+ with decreasing ambient Na^+ concentrations. The lack of flexibility in apparent K_m for *C. macularius* may reflect the relatively stable environment in which it has evolved as compared to *C. variegatus*.

Similar to *C. v. variegatus*, lack of phenamil sensitivity in *C. macularius* (Fig. 5.4) indicates that they do not utilize an apical Na^+ channel/H^+-ATPase system as has been observed for the majority of freshwater fish studied to date (Hwang et al. 2011). Interestingly, the only other species demonstrated to lack this system is *Fundulus heteroclitus* (Patrick and Wood 1999; Scott et al. 2005), also in the order Cyprindontiformes, suggesting this may be a well conserved trait across this group of fish.

C. macularius Na^+ uptake was also insensitive to both bumetanide and metolazone (Figs. 5.5 and 5.6) indicating this species does not use either NKCC or NCC for apical Na^+ acquisition. We previously hypothesized that the very high V_{max} for *C. v. variegatus*

was primarily due to the presence of NKCC in this species (Brix and Grosell 2012). The apparent lack of NKCC in *C. macularius* and correspondingly low V_{max}, provides further support for this hypothesis. We also hypothesized that the presence of an apical NKCC in *C. v. variegatus* was an evolved trait not basal to *Cyprinodon* sp. and may reflect the need for this species to rapidly take up large amounts of Na^+ when it occurs high in estuaries where >1 mM Na^+ concentrations may only occur for a few hours during high tide. The lack of NKCC in the more stenohaline *C. macularius*, as well as the loss of NKCC from the stenohaline *C. v. hubbsi* (Brix and Grosell 2012), supports this hypothesis.

C. macularius Na^+ uptake was sensitive to both amiloride, a Na^+ channel and NHE blocker, and EIPA, a NHE-specific inhibitor. Amiloride typically effects Na^+ channels at relatively low concentrations (10^{-5} M) while inhibiting NHE at higher concentrations (10^{-4} to 10^{-3} M) (Kleyman and Cragoe 1988). The lack of phenamil sensitivity, significant effect of amiloride only at 1 x 10^{-3} M (Fig. 5.2), and comparable effect of EIPA (Fig. 5.3), all suggest *C. macularius* is relying exclusively on a relatively low affinity NHE for apical Na^+ uptake. However, it is worth noting that the $K_{0.5}$ for C. macularius was >1 x10^{-3} M, significantly higher than that estimated for *C. v. variegatus* (3 x 10^{-4} M), raising the possibility that *C. macularius* is either using a different NHE isoform, or that there are substantial amino acid differences for the same protein.

The final experiment involved use of the CA inhibitor ethoxzolamide. In some fish species CA-mediated hydration of CO_2 provides intracellular H^+ for the function of NHE under thermodynamically unfavorable conditions (Hirata et al. 2003). We hypothesized inhibition of CA would have little or no effect on Na^+ uptake at 7 mM Na^+, as this

condition is favorable for NHE function, but would potentially effect Na^+ uptake at 2 mM Na^+, where the Na^+ gradient would be less thermodynamically favorable. Surprisingly, we observed ethoxzolamide had a significant effect on Na^+ uptake at both 2 and 7 mM Na^+, with the greater effect at 7 mM Na^+ (Fig. 5.7). These results contrast with *C. v. variegatus* where treatment with ethoxzolamide stimulated Na^+ uptake at 7 mM Na^+, but inhibited Na^+ uptake at 2 mM Na^+. The results for *C. macularius* are more similar to *C. v. hubbsi* where ethoxzolamide inhibited Na^+ uptake at both 2 and 7 mM Na^+, though to a lesser extent than observed for *C. macularius*.

We previously hypothesized that the stimulatory effect observed in *C. v. variegatus* was the result of an unknown signaling pathway activating NKCC, and the lack of stimulation in *C. macularius* is consistent with this hypothesis. The reason for the greater effect of ethoxzolamide in *C. macularius* at 7 mM Na^+ compared to 2 mM Na^+ is not clear. It suggests that the thermodynamic gradient is less favorable at 7 mM Na^+, which could only be the case if either intracellular Na^+ was higher or, in the absence of H^+ generated by CA, the H^+ concentration was lower in fish acclimated to 7 mM Na^+. One possibility worth investigating is that *C. macularius* switches Na^+/K^+-ATPase isoforms (Jorgensen 2008; Liao et al. 2009) between 7 and 2 mM Na^+, allowing for a lower intracellular Na^+ and therefore less reliance on CA-generated H^+ for NHE function. Regardless of the mechanism, the results for *C. macularius* and the two *C. variegatus* subspecies highlight the diverse and complex interplay of CA with proteins involved in Na^+ uptake in freshwater even in closely related species.

In conclusion, this study demonstrated that the endangered pupfish, *C. macularius*, appears to rely solely on a low affinity NHE for apical Na^+ uptake in freshwater. This

contrasts with two subspecies of *C. v. variegatus* that display a more dynamic response to varying Na^+ concentrations in freshwater, utilizing either a combination of NKCC and NHE or multiple NHE isoforms for apical Na^+ uptake. Similar to *C. variegatus*, H^+ generated by CA-mediated hydration of CO_2 plays an important role in regulating Na^+ uptake by *C. macularius* in freshwater.

Chapter 6:

An Evaluation of Pre- and Post-Zygotic Mating Barriers, and Hybrid Fitness between *Cyprinodon variegatus variegatus* and *Cyprinodon variegatus hubbsi* (Cyprinodontiformes, Teleostei)

Summary

The euryhaline fish *Cyprinodon variegatus variegatus* (*Cvv*) is capable of tolerating ambient salinities ranging from 0.3 to 167 g l^{-1}, but incapable of long-term survival in freshwater (<2 mM Na^+). However, a population of this species, now designated as a subspecies (*Cyprinodon variegatus hubbsi*; *Cvh*) has been isolated in several freshwater (0.4-1 mM Na^+) lakes in central Florida for the past ~150 ky. We previously demonstrated that *Cvh* has significantly higher affinity of Na^+ uptake suggest it has adapted to its dilute freshwater environment. Here, we evaluate whether *Cvh* should be considered a separate species by characterizing pre- and post-zygotic isolation, Na^+ transport characteristics of the two populations and their hybrids, and developing a molecular phylogeny of *Cvv* and *Cvh* populations in Florida using mtDNA sequence data. We found evidence of partial pre-zygotic isolation with *Cvv* females mating almost exclusively (89%) with con-specific males in choice mating experiments. Partial post-zygotic isolation was also observed with significant (59-89%) reductions in hatching success of hybrid embryos compared to con-specific embryos. Na^+ uptake kinetics in hybrids (both *Cvv* x *Cvh* and *Cvh* x *Cvv*) bred and raised under common garden conditions was intermediate to *Cvh* (high affinity) and *Cvv* (low affinity), indicating that

observed differences are genetically based. Similar observations were made with respect to short-term (96 h) survival of juveniles acutely transferred from 7 mM Na^+ to a range of more dilute (0.1-2 mM Na^+) freshwater. Finally, phylogenetic analysis of *Cvv* and *Cvh* populations using mtDNA sequence for ND2 indicates *Cvh* is of monophyletic origin although we were unable to fully resolve a polytomy between *Cvh* and *Cvv* populations from northeastern Florida. Overall, the available data suggests *Cvh* should be considered a separate species or at a minimum an evolutionarily significant unit.

Background

The euryhaline pupfish *Cyprinodon variegatus* occurs along the Gulf and Atlantic coasts of North America. The biogeography of *C. variegatus*, and the genus *Cyprinodon* in general, has been well studied through the use of mtDNA sequence and microsatellite data. *Cyprinodon* spp. is thought to have originated in the southwestern US or northern Mexico ~7-8 mya (Echelle et al. 2005). The genus has diversified considerably with ~40 species occurring in the southwestern US and northern Mexico. Approximately 3 mya, *C. variegatus* (or its progenitor) entered the Gulf of Mexico and spread throughout the Gulf and Atlantic coastlines. Coastal populations of *C. variegatus* have typically been classified into two subspecies which diverged ~2 mya, *C. v. variegatus* (*Cvv*) which includes all Gulf Coast populations and Atlantic populations up to North Carolina. Populations from North Carolina to Massachusetts are classified as *C. v. ovinus* (*Cvo*) (Echelle et al. 2006; Haney et al. 2007).

A third population of *C. variegatus*, is the Lake Eustis pupfish (*C. v. hubbsi; Cvh*), which only occurs in eight freshwater lakes in central Florida that are the headwaters of

the Oklawaha River system (St. John's river drainage). When first described, this population was given species status (*C. hubbsi*) based on morphological differences with *C. variegatus* (Carr 1936). However, later studies considered these morphological differences to be within the range of variation of *C. variegatus* (Johnson 1974), and allozyme data suggested a polyphyletic origin (Darling 1976; Duggins et al. 1983), such that the population was reclassified as a subspecies of *C. variegatus*.

We recently performed a comparative study on the osmoregulatory performance of *Cvv* and *Cvh* in freshwater. Prior to our study, it was known that *Cvv* tolerates salinities ranging from near freshwater up to 167 g l^{-1} (Nordlie 2006) and that *Cvh*, despite living exclusively in freshwater environments, retains the ability to tolerate salinities up to at least 90 g l^{-1} (Jordan et al. 1993). Previous studies indicated *Cvv* does not survive (long-term), grow or reproduce in freshwater with <2 mM Na^+ (Dunson et al. 1998), while the lakes in which *Cvh* naturally occurs have Na^+ concentrations of 0.4-1 mM.

Freshwater fish are hyper-osmotic relative to their environment. As a result, they suffer from diffusive loss of osmolytes (primarily Na^+ and Cl^-) and must compensate by active uptake of these ions at the gill (Marshall and Grosell 2006). Our comparative study 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 = 7,000-38,000 μM) when acclimated to 2 or 7 mM Na^+, but *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).

This demonstration of a genetically-based difference in osmoregulatory capacity between *Cvv* and *Cvh* prompted us to reconsider whether *Cvh* should be considered a

separate species. Within the eight lakes in central Florida, *Cvh* is restricted to a very specific habitat niche, occupying white sandy beaches within 1 m of the shoreline and avoiding areas of dense vegetation. Unfortunately, residential development around these lakes has reduced the availability of this habitat in the last 40 years. This is the result of both eutrophication and the construction of hundreds of private docks which break up shoreline wave action needed to maintain sandy beach habitat. The net result is that many of the locations identified as *Cvh* habitat during surveys in the 1970's and 1980's (Guillory and Johnson 1986) are now overgrown with aquatic grass (*Panicum* sp.). Hence, the designation of *Cvh* as either an evolutionarily significant unit or separate species has important implications for the conservation of this population (Waples 1991).

To further evaluate this issue, we performed a series of experiments to test whether pre- or post-zygotic isolation has developed between *Cvv* and *Cvh*. We further characterized the genetic basis for differences in osmoregulation by comparatively evaluating Na^+ uptake characteristic in *Cvv*, *Cvh*, and hybrids of the two populations. Finally, we re-evaluated the phylogeny of *Cvh* using mtDNA sequence data.

Methods and Materials

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 psu. Fish were held at the University of Miami in 110 L glass aquaria under flow-through conditions initially with filtered natural seawater (35 psu) from Bear Cut, FL. Fish were acclimated to near freshwater conditions (0.3 psu; 7 mM Na^+, pH 7.9) and held

for >30 d. After this holding period, fish were bred and offspring were hatched and raised to sexual maturity under the same near freshwater conditions (0.3 psu; 7 mM Na^+, pH 7.9). 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. These offspring represented the parental stock for all *Cvv* used 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*. These offspring represented the parental stock for all *Cvv* used 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*.

Mate Recognition – Visual and Chemical Cues

Assessment of the relative importance of visual and chemical cues in mate recognition by *Cvv* and *Cvh* generally following previously described experimental designs (Crapon de Caprona and Ryan 1990; Strecker and Kodric-Brown 1999). Both experiments were performed in a 100-L aquarium (91 x 32 x 37 cm) using the same 7

mM Na^+ freshwater in which fish were raised. In the visual cue experiments, a single adult male from each subspecies were placed in separate 7.5-L aquaria (26 x 17 x 17 cm) at opposite end of the larger aquaria. Care was taken to size match males based on mass. Each male was provided with a 10 x 10 cm piece of blue filter material weighted down with a small rock. These filter pads elicit a territorial response by males and are used as breeding substrate. Both male and females were pre-conditioned to these breeding pads during holding. The large aquarium was filled to a depth which prevented any water exchange with the smaller aquaria containing the males. The aquarium was divided into three equal sections using transparent tank dividers and a female fish from either subspecies was introduced to the middle section. Fish were allowed to acclimate to these conditions for 1 h after which the tank dividers were removed and the association of the female with in each of the 3 subsections of the tank was monitored for 30 min. The female fish was then removed and the following day a female from the alternate subspecies was tested with the same males. The experiment was then repeated with a separate set of males (n=12). The side on which males were placed and the introduction order of females was alternated between trials to eliminate potential biases.

In the chemical cues experiment, male fish were again held in a 7.5-L aquarium physically isolated from 5 conspecific females as described in the visual cues experiment. The fish were maintained under these conditions for 24 h to allow for the release of any reproduction related chemical cues. Water from the 7.5-L aquarium was then immediately used for the chemical cues experiment. For this experiment, a female fish was allowed to acclimate in the middle section of a 100-L aquarium for 1 h after which tank dividers were removed and water in which hetero-specific males had been held was

gravity fed into the distal ends of the aquaria at a rate of 3 ml min^{-1}. The time a female spent in each distal third of the tanks associated with a chemical cue was then recorded for 30 min. After each trial, the tanks were thoroughly cleaned with soap and bleach prior to initiating the next trial (n=28).

Mate Recognition – Choice Mating Experiments

Choice mating experiments were performed in the same tank as the visual and chemical cue experiments. A number of preliminary trials were performed to evaluate the most appropriate male:female ratio for these experiments. Based on these preliminary trials, we selected an experimental design in which 3 males from each subspecies was introduced to the tank. Males were carefully selected based on size so that there was a clearly dominant and two subordinate males from each subspecies. A breeding pad was placed in each end of the tank and a series of four plastic aquaria plants were placed along the midline of the tank to provide territorial boundaries for the dominant hetero-specific males. Males were allowed to acclimate overnight and the following morning fish were observed to ensure that a dominant male from each subspecies was actively defending its breeding territory. In cases where con-specific males established breeding territories (~10% of the time) on the two pads, all fish were removed and new fish were added, acclimated overnight and observed the following morning.

Once the desired hetero-specific breeding territories were established, four size-matched females (two from each subspecies) were introduced to the tank. Fish were then observed for 30 min. and all courtship displays and spawning events were recorded. At

the end of each trial, the tank was thoroughly cleaned with soap and bleach before initiating the next trial with new fish (n=19).

Reproductive Output and Hybrid Viability Experiments

Reproductive output and hybrid viability was assessed in no choice mating experiments. The same experimental design as described for the choice mating experiment was used except that all males were conspecific and all females were conspecific. All possible conspecific and heterospecific male x female crosses were evaluated in 7 mM Na^+ freshwater. A *Cvv* x *Cvv* cross was performed using fish acclimated for full strength seawater (34 psu) to evaluate the effects of salinity on *Cvv* reproduction as this subspecies is better adapted to reproduced in marine environments. Additionally, surviving F_1 *Cvh* x *Cvv* and *Cvv* x *Cvh* fish were raised to sexual maturity and self-crossed.

Preliminary experiments indicated that egg production during these mating experiments gradually increased during the first 3 days after fish were introduced to the breeding tank and then reached an asymptote. Therefore each trial consisted of introducing naïve fish to the breeding tank, allowing them to acclimate and reproduce for 3 d and on the fourth day, egg breeding pads were collected. All eggs were carefully removed from the breeding pads and transferred to 100 ml of 7 mM Na^+ freshwater with gentle aeration. To prevent fungal growth on the eggs, 1 ml 100 ml^{-1} of methylene blue was added to the hatching containers. A 60% water change was performed daily on the hatching containers (without addition of methylene blue) and time to hatch carefully monitored. All successfully hatched larvae were transferred to larger containers for growout. The volume of water in which the larvae were reared was normalized for the

number of larvae hatched out from a trial to avoid density-dependent growth effects. Larvae were feed brine shrimp nauplii twice daily to satiation and a 90% water change was performed daily to maintain water quality. After 21 d, the wet weight of surviving fish from each trial was determined. Ten trials were performed for each of the possible male x female crosses.

Low Na^+ Survival Experiments

To evaluate how hybridization affected tolerance to low Na^+ water, experiments were performed in which juvenile fish (100-200 mg) acclimated to 7 mM Na^+ freshwater were acutely transferred to either 100, 250, 500, 1000 or 2000 μM Na^+ freshwater and survival monitored for 96 h. For each experimental treatment, six replicate 1.5 L containers with 1 L of test solution were used, each containing ten fish. Containers were provided with gentle aeration and a 90% water change was performed daily to maintain water quality. Fish were not fed during the 96 h exposure period. Survival was monitored twice daily and dead fish were removed immediately. Experiments were performed using *Cvh*, *Cvv*, *Cvh* x *Cvv* F_2, and *Cvv* x *Cvh* F_2 fish.

Characterization of Na^+ Uptake Kinetics and Variability

The Na^+ uptake kinetics of *Cvh* x *Cvv* F_2 and *Cvv* x *Cvh* F_2 fish acclimated to 1 mM Na^+ for 3 weeks. Additionally, results from the low salinity survival experiment demonstrated that ~70% of the population is capable of acclimating to 1 mM Na^+. We therefore determined Na^+ uptake kinetics in *Cvv* acclimated to 1 mM Na^+ as well. For each experiment, Na^+ uptake rates were measured at 8 different ambient Na^+ concentrations ranging nominally from 0.1 to 8 mM Na^+. At each Na^+ concentration, 8 juvenile fish (50-250 mg) were placed in 50 ml of a defined media (480 μM $CaSO_4$, 150

μM $MgSO_4$, 100 μM $KHCO_3$, 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 media for 10 min after which the media was replaced and 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^+] and ^{22}Na activity. The total flux exposure period ranged from 0.5-2 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^+] and ^{22}Na activity 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 nearest 0.1 mg and then assayed individually for radioactivity.

In a second experimental series, the variation in Na^+ uptake rates was characterized in *Cvh*, *Cvv*, *Cvh* x *Cvv* F_2, and *Cvv* x *Cvh* F_2 fish. This involved the same experimental design just described except that after acclimated to 1 mM Na^+, Na^+ uptake rates of 50 fish were simultaneously determined in 400 ml of 0.1 mM Na^+ water.

Mitochondrial DNA Analysis

Fish were collected by either minnow trap or beach seine from 2 locations for *Cvv* and 3 locations for *Cvh* (Table 6.1 and Figure 6.1). Mitochondrial DNA (mtDNA) sequence data from these samples were supplemented with additional sequence data generated in previous studies (Duvernell 1998; Echelle et al. 2005; Echelle et al. 2006) from other locations in Florida, Mississippi, and Texas, as well as sequences for *C.*

dearborni and *C. tularosa*, which served as out groups for phylogenetic analysis (Table 6.1). For the new samples, DNA was extracted from either liver or muscle tissue using standard phenol/chloroform methods. The entire (1047 bp) NADH dehydrogenase subunit 2 (ND2) was sequenced using primers ND2B-L and ND2E-H (Broughton and Gold 2000) in 50 µl reactions using the following conditions: 94 °C for 3 min; 40 cycles of 94 °C for 30 sec, 52 °C for 30 sec, 72 °C for 90 sec; 72 °C for 15 min. PCR products were confirmed on a 1.3% agarose gel, gel extracted and then direct sequenced in the forward and reverse direction.

Table 6.1. Locations and sources of mtDNA sequence data

Population	Location	Source
C. v. hubbsi	Lake Weir, FL	This study
	Lake Eustis, FL	This study
	Lake Yale, FL	This study
C. v. variegatus	Plantation Key, FL	Echelle et al. 2006
	Vero Beach, FL	Echelle et al. 2005
	Flagler Beach, FL	Echelle et al. 2005
	Yankeetown, FL	Echelle et al. 2005
	Big Sabine Point, FL	Duvernell and Turner 1998
	Key Biscayne, FL	This study
	Jacksonville, FL	This study
	Biloxi River, MS	Echelle et al. 2005
	Edinburg, TX	Echelle et al. 2005
Cyprinodon dearborni	Canas, Bonaire	Echelle et al. 2005
Cyprinodon tularosa	Lost River, NM	Echelle et al. 2005

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^{2+} 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).

Figure 6.1. Map of sampling locations in Florida for phylogenetic analysis. BS= Big Sabine Point, YT=Yankeetown, PK=Plantation Key, KB=Key Biscayne, VB=Vero Beach, FB=Flagler Beach, JAX=Jacksonville, LW=Lake Weir, LE=Lake Eustis, LY=Lake Yale. Data for BS, YT, PK, VB, and FB from Echelle et al. (2005, 2006).

All values are expressed as means ± SEM throughout. Comparison data were analyzed by Student's t-test or by ANOVA when multiple treatments were evaluated. All comparison analyses were performed using SigmaStat v3.5 (SPSS 2006). Kinetic data

were observed to fit a Michaelis-Menten function and estimates of K_m and V_{max} were determined in GraphPad Prism v5.0 (GraphPad Software Inc. 2007). Differences in K_m and V_{max} estimates for fish were tested using an extra sum of squares F-test (Zar 2009).

Phylogenetic analyses were performed by first aligning sequences in Mesquite v2.73 (Maddison and Maddison 2010) and developing consensus sequences for each location. Consensus sequences were then imported into MEGA v5.05 (Tamura et al. 2011) for analysis. For this analysis the TN93 model (Tamura and Nei 1993) was determined to be the most appropriate substitution model based on Bayesian Information Criterion and Akaike Information Criterion (corrected). Phylogenies were developed using both maximum parsimony and maximum likelihood analysis. For maximum likelihood analysis, a uniform substitution rate was assumed and the tree was inferred using the heuristic nearest-neighbor-interchange method. For maximum parsimony analysis, the tree was inferred using the heuristic close-neighbor-interchange method with 10 initial trees. Support for both analyses was evaluated using non-parametric bootstrap sampling (n=500) (Felsenstein 1985).

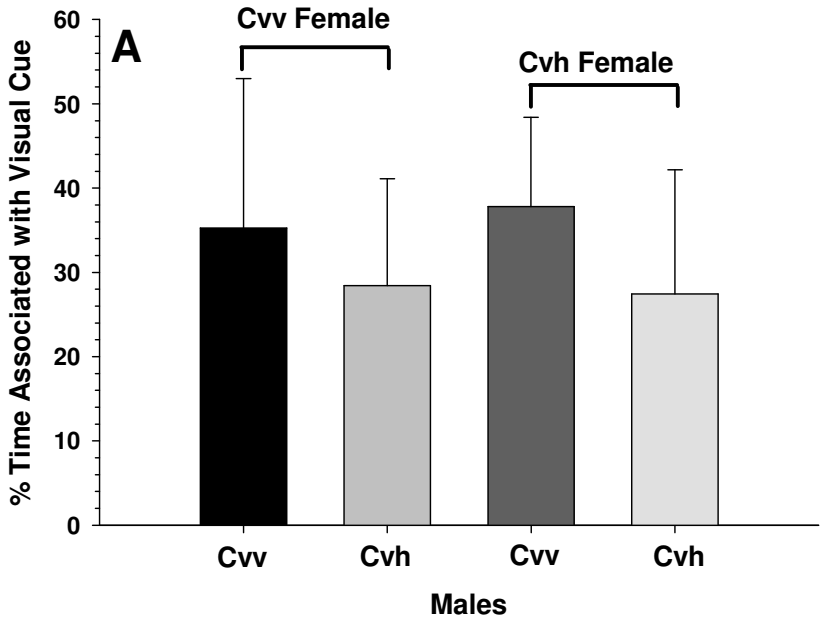
Results

Mate Recognition Experiments

No significant differences in time associated with visual and chemical cues were observed for any of the treatments (Fig. 6.2). In choice mating experiments, there were no significant differences in the number of courtship displays made by males to con- and hetero-specific females (Fig. 6.3A). Although we did not quantify it, qualitatively we observed the vast majority of courtship displays were made by subordinate males (male

choice). Subordinate males largely occupied the upper water column, well above the breeding pads that were aggressively defended by the dominant hetero-specific males. Actual spawning appeared to be a female choice in which females, which also predominantly occupied the upper water column, moved into the lower water column near one of the breeding pads. The dominant male would then quickly corral the female to the breeding pad where spawning would occur. A strong, statistically significant preference for con-specific mating by *Cvv* females was observed while *Cvh* females spawned with con- and hetero-specific males equally (Fig 6.3B). Across subspecies, *Cvv* males spawned more than twice as often (n=74) as *Cvh* males (n=31).

Figure 6.2. Response of *Cvv* and *Cvh* to **A.** Visual cues. **B.** Chemical cues. Data reflects the fraction of time female fish associated with male visual or chemical cues in 30 min trials. Mean ± SEM (n=12 for visual cues and n=28 for chemical cues).



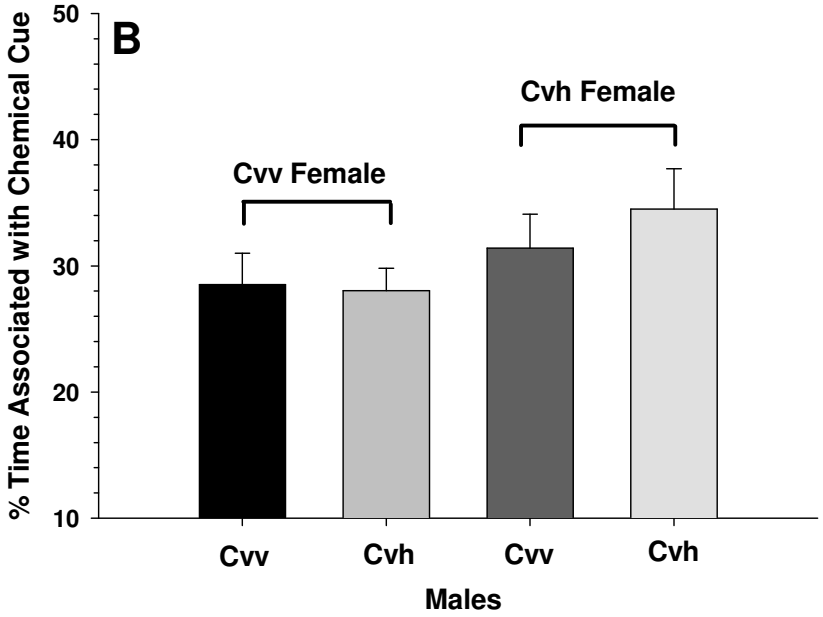
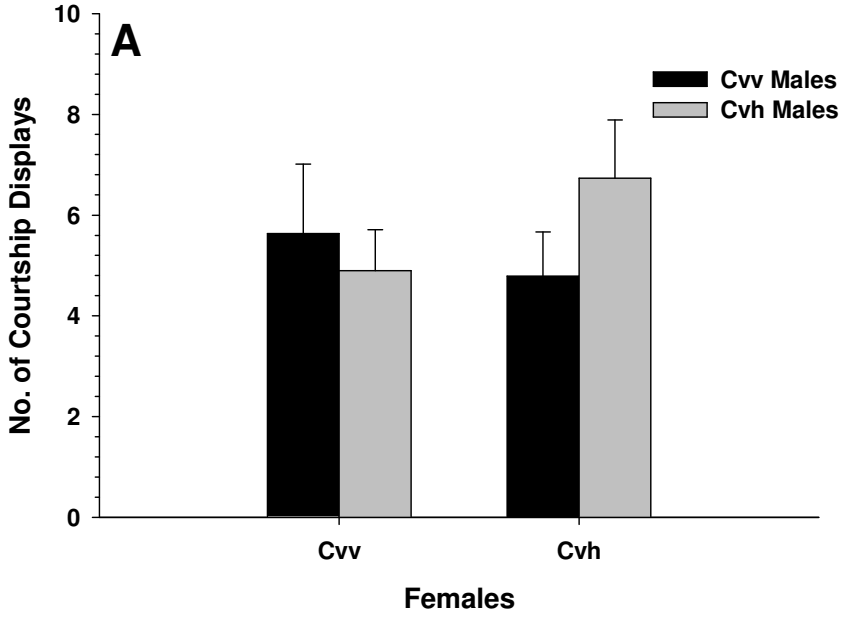
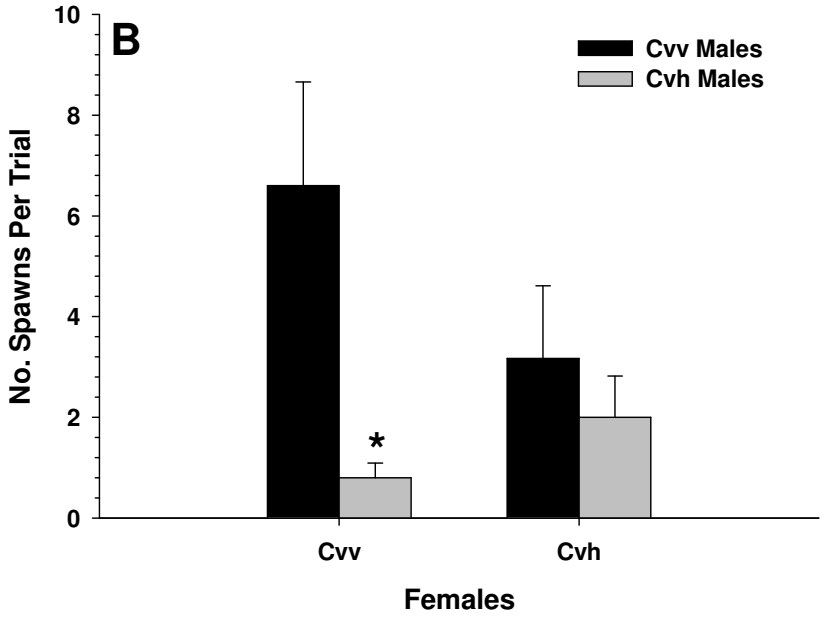


Figure 6.3. Response of *Cvv* and *Cvh* in choice mating experiments. **A.** Number of courtship displays by male fish with homo- and hetero-specific females. **B.** Number of spawns per trial by female fish with homo- and hetero-specific males. Mean ± SEM (n=19). Different letters indicate statistically significant difference ($p<0.05$).



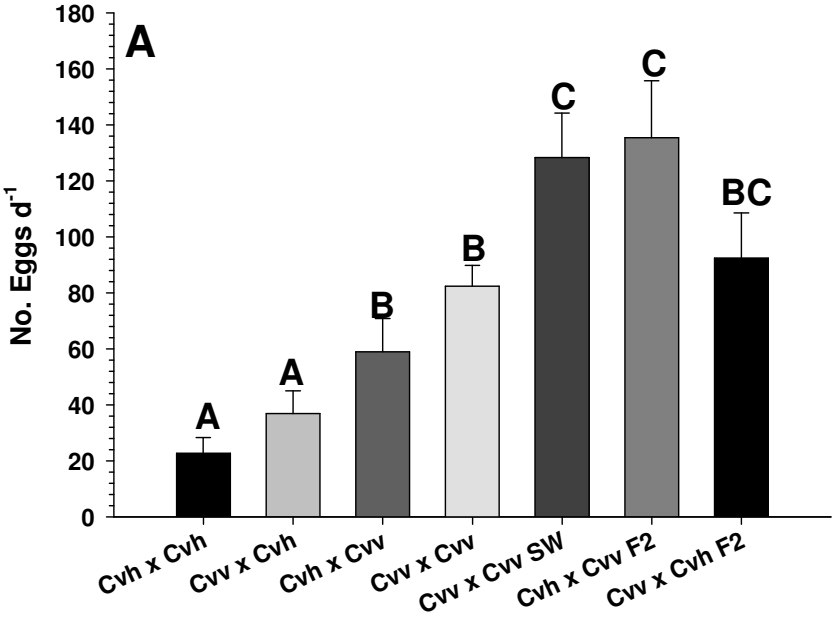


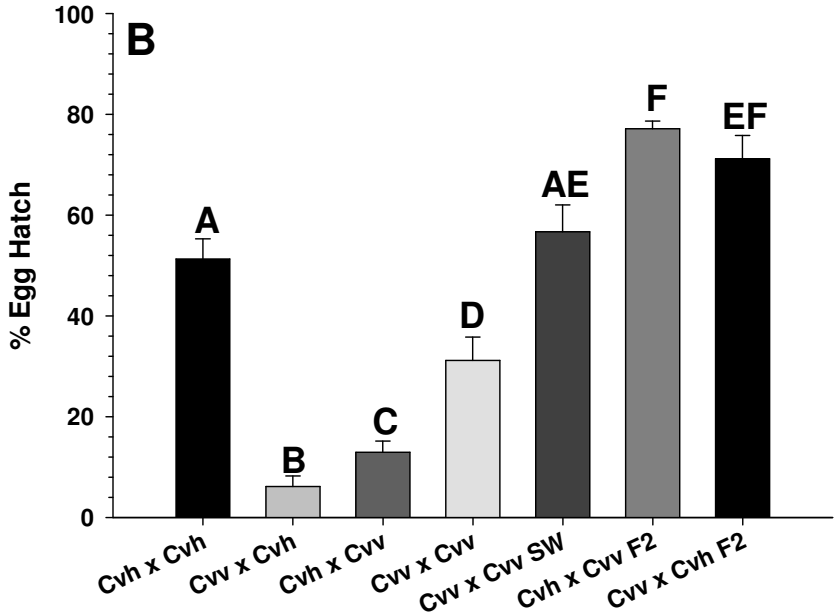
Reproduction and Hybrid Viability

In no-choice mating experiments, egg production ranged from 23 (*Cvh* x *Cvh*) to 136 (*Cvh* x *Cvv* F_2) eggs d^{-1} (Fig. 6.4A). *Cvv* x *Cvv* crosses produced significantly more eggs in seawater than 7 mM Na^+ FW, indicating there was a salinity effect overlying other observed effects. For both possibilities, self-crosses of F_1 hybrids resulted in significantly higher number of egg d^{-1} than original crosses. Hatching success ranged from 6 to 77%, with initial hybrid crosses having significantly lower hatching success rates than all other groups (Fig. 6.4B). Hatching success was comparable in *Cvh* x *Cvh* and *Cvv* x *Cvv* SW crosses, but significantly reduced in the *Cvv* x *Cvv* cross at 7 mM Na^+, again indicating a salinity effect. Self-crosses of F_1 hybrids had the highest hatching success rates. Mean time to hatch ranged from 3.9 to 5.9 d, with initial crosses and *Cvv* x *Cvv* in 7 mM Na^+ having significantly shorter hatching times than *Cvh* x *Cvh* and hybrid self-crosses (Fig. 6.4C). Embryos from *Cvv* x *Cvv* in seawater took the longest to hatch.

Effects on larval survival through 21 were relatively minor ranging from 83-96%, although *Cvh* x *Cvv* F_2 larval survival was significantly lower than for larvae from all other crosses (Fig. 6.4D). Larval wet weight at the end of 21 d ranged from 29-63 mg, with larvae from initial hetero-specific crosses significantly larger than all other crosses. *Cvv* x *Cvv* in 7 mM Na^+ and *Cvh* x *Cvv* F_2 larvae were significantly smaller than all other crosses (Fig. 6.4E).

Figure 6.4. Characterization of post-zygotic isolation in no choice mating experiments with *Cvv* and *Cvh*. All crosses presented as male x female. All mating experiments performed in freshwater with 7 mM Na^+ except *Cvv* x *Cvv* SW, which was performed in seawater. Note, F_2 crosses used surviving fish from initial hybridizations. **A.** Number of eggs per breeding trial. **B.** Percentage of eggs that successfully hatched. **C.** Mean time to hatch. **D.** Survival of larvae through 21 d post-hatch. **E.** Wet weight of larvae 21 d post-hatch. Mean ± SEM (n=10 trials for each cross).




B
% Egg Hatch
100
80
60
40
20
0
A
B
C
D
AE
F
EF
Cvh x Cvh
Cvv x Cvh
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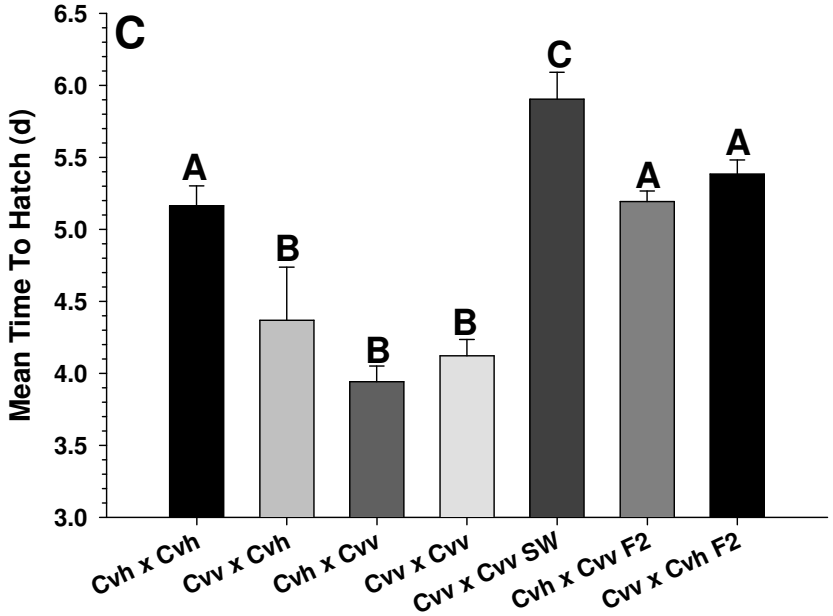
Cvh x Cvv F2
Cvv x Cvh F2


C
Mean Time To Hatch (d)
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
A
B
B
B
C
A
A
Cvh x Cvh
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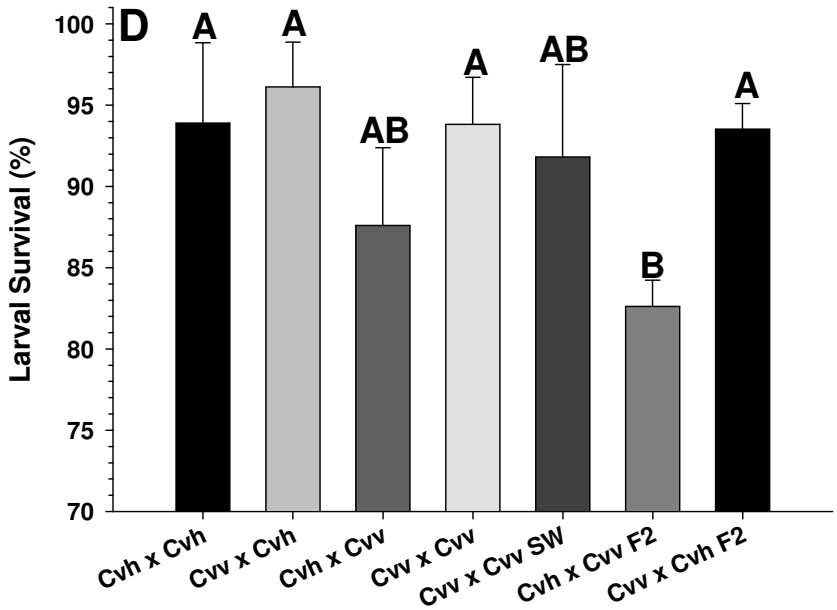
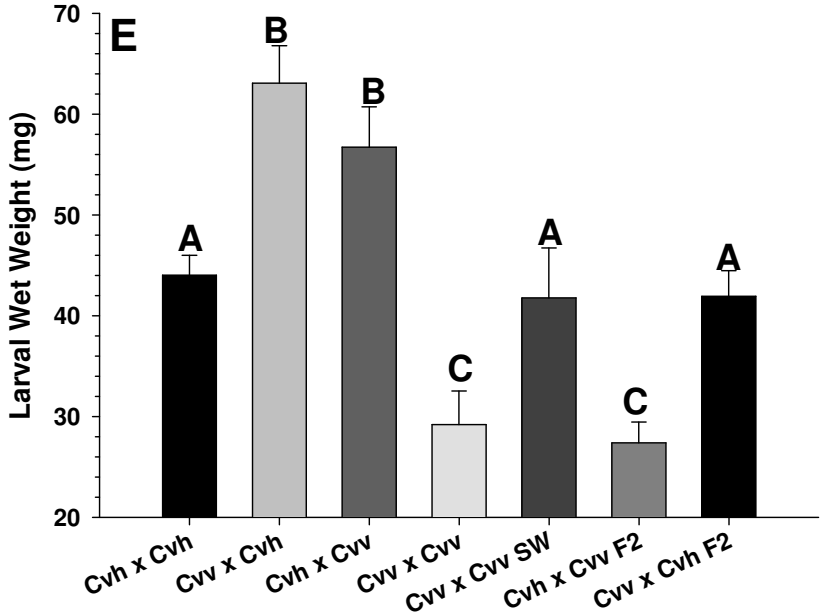
Cvh x Cvv F2
Cvv x Cvh F2



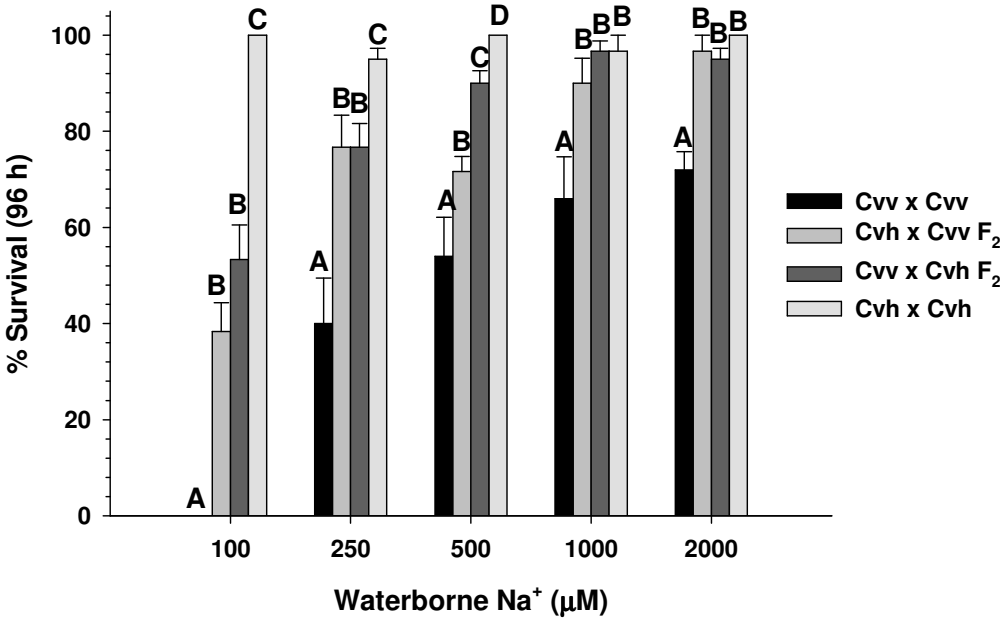


Low Salinity Survival Experiment

Acute transfer of juvenile fish from 7 mM Na^+ to lower Na^+ concentrations (0.1, 0.25, 0.5, 1, and 2 mM Na^+) for 96 h resulted in distinct survival patterns (Fig 6.5). *Cvh*

exhibited the best survival, ranging from 95 to 100% across all treatments. F_2 hybrids were intermediate with relatively low (38-53%) survival in 0.1 mM Na^+ progressively increasing to 95-97% at 2 mM Na^+. *Cvv* performed the poorest, with 0% survival at 0.1 mM Na^+ reaching a maximum of 72% survival at 2 mM Na^+.

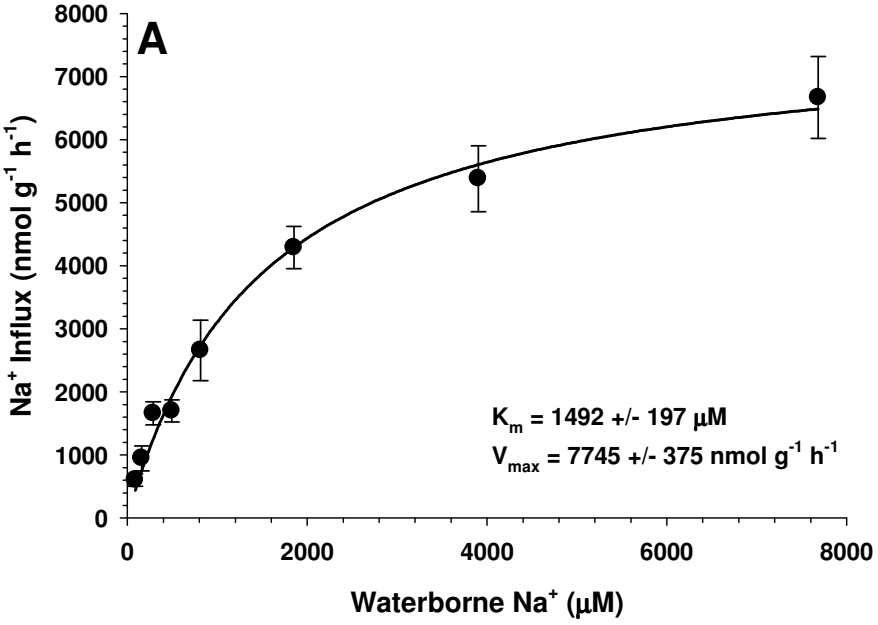
Figure 6.5. Survival through 96 h of juvenile fish (n=10 fish per replicate) after acute transfer from 7 mM Na^+. All crosses presented as male x female. Note, F_2 crosses used surviving fish from initial hybridizations. Mean ± SEM (n=6). Different letters indicate statistically significant difference between populations within treatments ($p<0.05$).

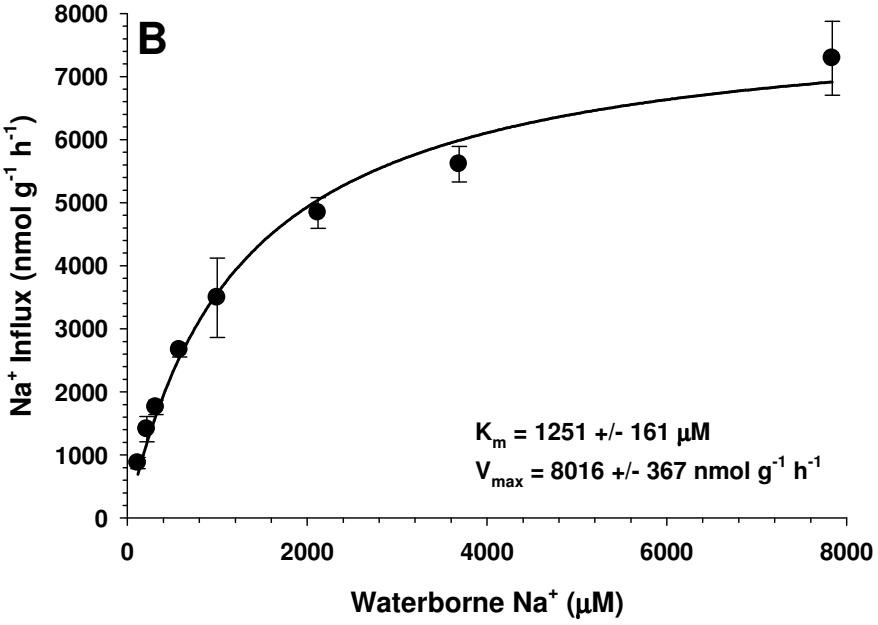


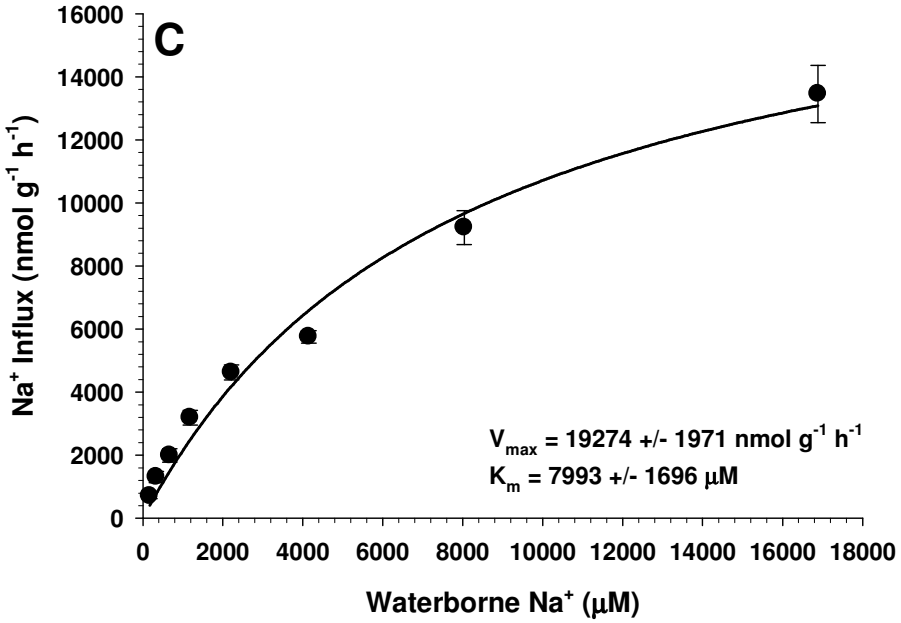
Na^+ Uptake Kinetics and Variability

Na^+ uptake kinetics for both hybrid crosses and *Cvv* exhibited Michaelis-Menten saturation kinetics (Fig. 6.6). Estimated K_m (1257-1492 µM) and V_{max} (7745-8016 nmol g^{-1} h^{-1}) values were not statistically different for the hybrids, but were significantly lower (both parameters) than observed for *Cvv* (K_m = 7993 µM; V_{max} = 19274 nmol g^{-1} h^{-1}).

Figure 6.6. Na^+ uptake rates (nmol g^{-1} h^{-1}) as a function of external Na^+ concentrations (μM). All fish acclimated to 1 mM Na^+. **A.** *Cvh* x *Cvv* F_2. **B.** *Cvv* x *Cvh* F_2. **C.** *Cvv*. Mean ± SEM (n=8).





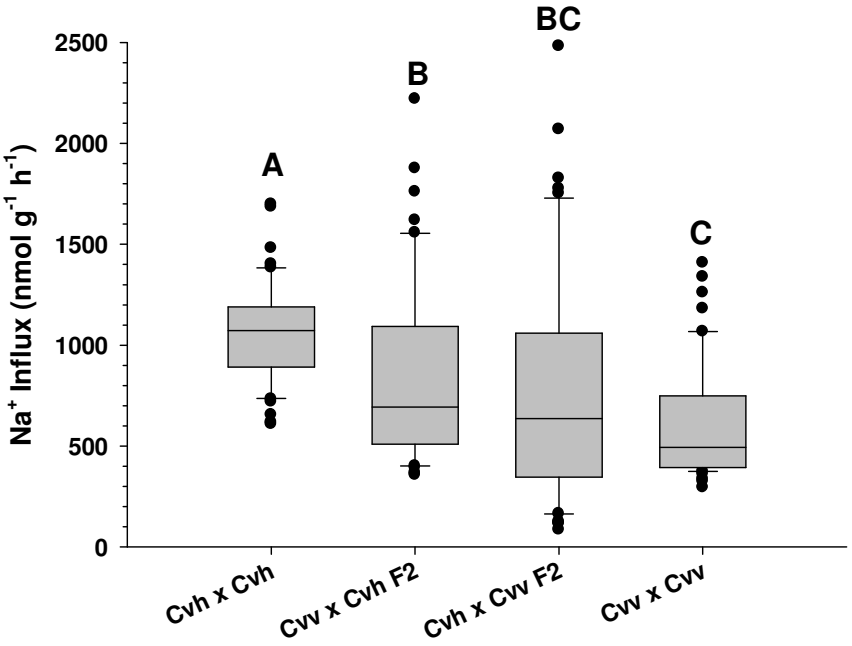


The evaluation of Na^+ uptake at 0.1 mM Na^+ for fish acclimated to 1 mM Na^+ revealed considerable variation both within and between populations (Fig. 6.7). As would be expected, *Cvh* had the highest mean Na^+ uptake, with the F_2 hybrids intermediate, and *Cvv* the lowest, with the range between the 25th and 75th quartiles being the greatest for the two F_2 hybrid populations. Surprisingly, despite the more than order of magnitude differences in apparent K_m between *Cvh* and the other populations, there was considerable overlap in the Na^+ uptake rates.

Phylogeny of *C. v. variegatus/hubbsi* Complex

All new sequences have been deposited in Genbank. No indels or gaps occurred in the ND2 sequence. Excluding outgroups (*C. tularosa* and *C. dearborni*), a total of 17 haplotypes and 28 parsimony informative characters were identified. Consensus maximum likelihood and maximum parsimony trees derived similar phylogenies (Fig. 6.8). Four clades were identified; Clade 1 included populations from Texas, Mississippi,

Figure 6.7. Na^+ uptake rates (nmol g^{-1} h^{-1}) of fish exposed to 0.1 mM Na^+ after acclimation to 1 mM Na^+. Box whiskers represent 10^{th}, 25^{th}, 50^{th}, 75^{th}, and 90^{th} percentiles. Outliers represented by individual data points (n=50 for each population). Different letters indicate statistically significant difference between populations within treatments ($p<0.05$).



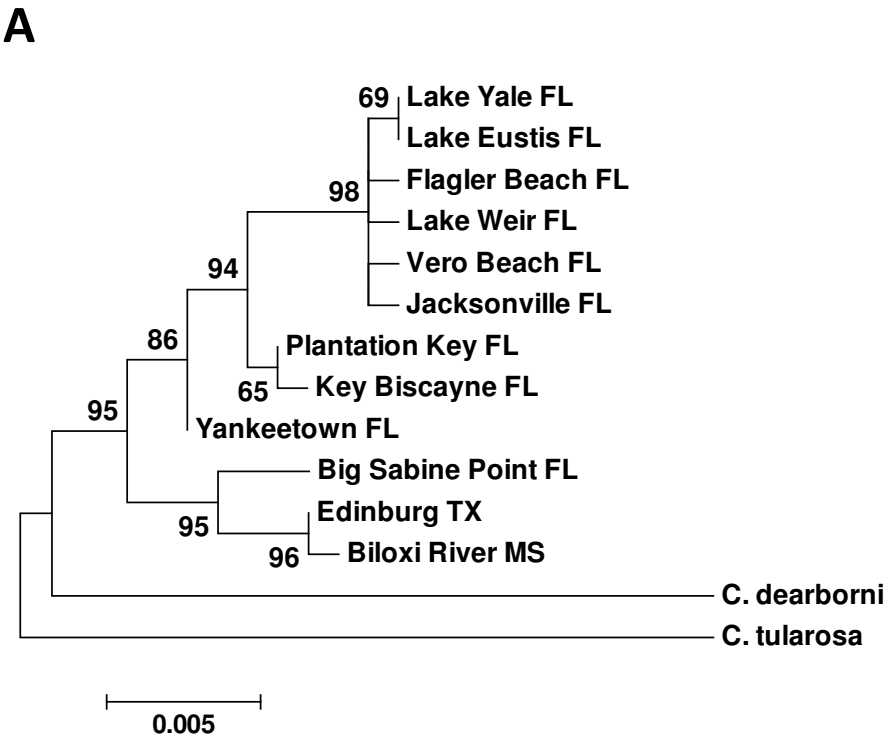
and the Florida panhandle; Clade 2 was represented by the single population from the western Florida Peninsula; Clade 3 comprised the Florida Keys; and Clade 4 comprised populations in northeastern Florida and the *Cvh* populations from central Florida in an unresolved polytomy.

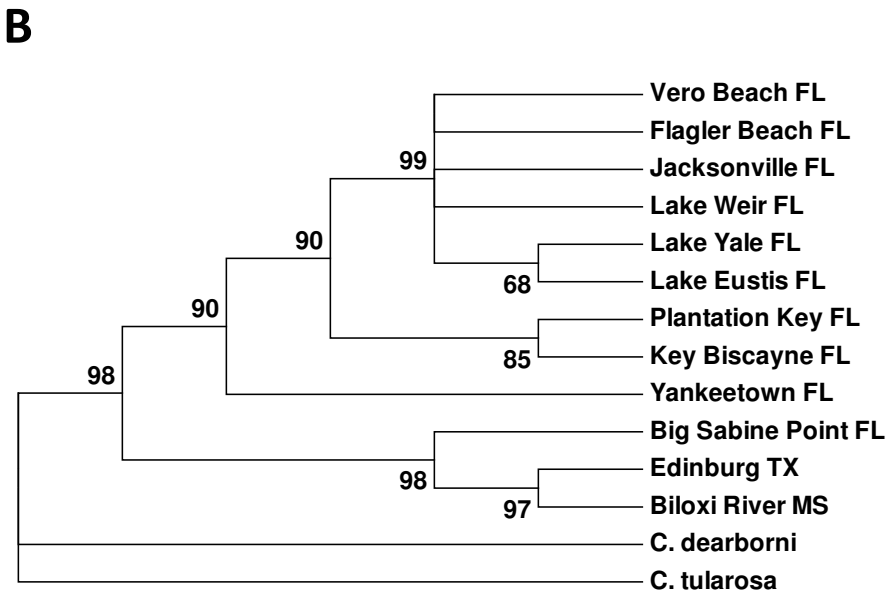
Conclusions

Pre- and Post-Zygotic Isolation of *Cvv* and *Cvh*

We observed no evidence of mate selection when evaluating chemical cues or visual cues in isolation (Fig. 6.2). In contrast, there was an asymmetric mate recognition in the mixed cues experiment, with *Cvv* females exhibiting a strong preference for mating with con-specific males (Fig. 6.3). Given the lack of response in visual and chemical cue

Figure 6.8. Pupfish phylogency based on full length ND2 sequence. Lakes Yale, Eustis, and Weir = *Cvh*, All other locations = *Cvv*. **A.** Strict consensus maximum likelihood tree. Numbers at internal nodes represent bootstrap support (n=500). **B.** Maximum parsimony tree.





experiments, the mechanism for con-specific mate selection by *Cvv* is unclear. However, this type of result has been observed in previous studies with *Cyprinodon* sp. For example, Strecker and Kodric-Brown (1999) demonstrated strong con-specific mate selection by *C. maya* females in both visual and chemical cue experiments with *C. beltrani*, while *C. labiosus* females responded to chemical, but not visual cues, when paired against *C. maya* (Kodric-Brown and Strecker 2001). In experiments with *C. beltrani* females, there was no evidence of visual or chemical cues when *C. maya* was the hetero-specific species. Despite this, and similar to our observations, in mixed cues experiments, *C. beltrani* females spawn almost exclusively with con-specific males (Strecker and Kodric-Brown 2000).

Several observations can be made from the post-zygotic isolation experiments. First, *Cvh* is significantly (~4-fold) less fecund than *Cvv* when held in the same 7 mM Na^+ freshwater (Fig. 6.4a). This appears to be largely a maternal effect as *Cvv* x *Cvh* crosses were not significantly more fecund, while *Cvh* x *Cvv* crosses had higher fecundity. There was also a clear salinity effect on fecundity for *Cvv*, and this effect was abolished in both hybrid crosses of surviving F_1 animals.

Perhaps most important was the reduction in hatching success of initial crosses between *Cvh* and *Cvv* (Fig. 6.4b). Both crosses provide evidence for significant, though incomplete, post-zygotic isolation. However, surviving fish from these initial crosses do not appear to suffer from further incompatabilities, as hatching success of F_2 fish is significantly higher than observed for con-specific crosses of the two populations. There appeared to be little effect of hybridization on 21-d larval survival (Fig. 6.4d). While initial crosses of the two populations appears to elicit hybrid vigor in surviving larvae as

evidenced by their significantly higher growth rates, this effect disappeared in the F_2 generation (Fig. 6.4e).

Survival in Low Na^+ Water and Na^+ Uptake Kinetics

The experiment in which juvenile fish were acutely transferred to low Na^+ water showed clear differences between *Cvh* and *Cvv*, with hybrids exhibiting intermediate tolerance (Fig. 6.5). Survival rates of *Cvv* in 0.25-1 mM Na^+ was entirely consistent with the previous study by Dunson et al. (1998) who observed 54% survival in *Cvv* after 52 d at 0.4 mM Na^+.

Na^+ uptake kinetics in the F_2 hybrids acclimated to 1 mM Na^+ were similar for the two crosses while *Cvv* had a significantly lower affinity and higher capacity for Na^+ than the hybrids. We previously characterized uptake kinetics in *Cvh* (K_m = 110 μM; V_{max} = 1437 nmol g^{-1} h^{-1}) acclimated to 1 mM Na^+ and *Cvv* (K_m = 18509 μM; V_{max} = 18999 nmol g^{-1} h^{-1}) acclimated to 2 mM Na^+ (Brix and Grosell 2012). Hence, F_2 hybrids appear intermediate to parental populations with respect to their Na^+ uptake characteristics. Previous experiments indicate *Cvv* utilizes a relatively low affinity NHE for Na^+ uptake at 2 mM Na^+ and a combination of NHE and an apical NKCC for Na^+ uptake in relatively high Na^+ waters (≥7 mM Na^+). It is NKCC that allows *Cvv* to take up Na^+ at very high rates in saline freshwater. In contrast, *Cvh* utilizes a high affinity NHE in relatively low Na waters (≤1 mM Na^+), and a low affinity NHE in higher Na^+ waters (≥2 mM Na^+), but lacks expression of NKCC for very high Na^+ uptake in more saline freshwater (Brix and Grosell 2012). It appears the F_2 hybrids also lack expression of NKCC. It is unclear whether the F_2 hybrids have a higher affinity for Na^+ than *Cvv* acclimated to 1 mM Na^+ or whether the higher apparent K_m for *Cvv* is an artifact of the high capacity of the

NKCC. In fact, re-evaluation of the Na^+ transport data for *Cvv* (Fig. 6.6c) using only data from treatments <5 mM Na^+ (where NKCC is not involved) results in a K_m of 2027 μM Na^+ and V_{max} of 8640 nmol g^{-1} h^{-1}, similar to the values for the F_2 hybrids.

Our assessment of variability in Na^+ uptake kinetics in *Cvh*, *Cvv* and hybrids shows that there is considerable standing variation (Fig. 6.7). It is important to keep in mind that for *Cvv*, our experiment underestimates standing variation as ~30% of the population did not survive acclimation to 1 mM Na^+ (Fig. 6.5), presumably due to the inability to take up sufficient Na^+. Interestingly, although Na^+ uptake rates were significantly different between *Cvh* and *Cvv*, there was overlap between the two species. The upper quartile of Na^+ uptake rates for *Cvv* were comparable to the interquartile Na^+ uptake rates in *Cvh*. It would be of considerable interest to isolate individuals from this upper quartile of the *Cvv* population and determine if their ability take up Na^+ was a result of differences at the protein level (i.e., expression of high vs. low affinity NHE).

Phylogeny of *Cvv*/*Cvh* Species Complex

The geologic history of the Florida peninsula provides an important backdrop for understanding the possible origins of *Cvh* in central Florida. The central Florida lakes in which *Cvh* resides are the headwaters of the Oklawaha River system which drains north into the St. John's River, which discharges into the Atlantic at Jacksonville. These headwaters formed as marine depressions when the Penholoway terrace rose ~2 mya (Alt and Brooks 1965). During the Aftonian interglacial (5-600 kya), the headwaters were likely connected to an estuary that drained to the Gulf Coast providing a potential western source for *Cvh*. By the time of the Yarmouth interglacial period (4-200 kya), the rise of the Pamlico terrace would have cut off connection to this estuary and a northward flow

toward the St. John's would be been established providing a potential eastern source for *Cvh*.

Previous studies concluded that *Cvh* had a Gulf Coast origin based on meristic and morphological characters, as well as allozyme data. Carr (1936) and Johnson (1974) noted several characteristics (humeral scale size, number of body scales) that made *Cvh* more similar to Gulf Coast *Cvv* rather than Atlantic *Cvv* populations. Similarly, Darling (1976) cited expression of the esterase-2 allele in *Cvh* (collected from Lakes Eustis, Dora, and Weir) and Gulf Coast *Cvv*, but lacking in Atlantic *Cvv*, as evidence for a Gulf Coast origin. However, a subsequent study comparing *Cvh* from individual lakes (Dora, Harris, Griffin, Eustis, and Weir) concluded that populations from Harris, Griffin and Eustis were more similar to Atlantic *Cvv*, while the Lake Weir population was more closely related to Gulf Coast *Cvv*, suggesting a polyphyletic origin for *Cvh*. The origin of the Lake Dora population of *Cvh* was unclear. This analysis was largely driven by differences in esterase-4 allelic expression, with esterase-2 being uninformative (Duggins et al. 1983).

In contrast to these earlier studies, our analysis based on mtDNA sequence data for ND2, indicate rather strongly that *Cvh* from Lakes Eustis, Yale, and Weir are most similar to *Cvv* from the northeastern coastline of Florida including a population at the mouth of the St. John's River (Jacksonville). Although we were unable to resolve the polytomy of *Cvh* and *Cvv* from northeastern Florida, these data suggest a monophyletic origin for *Cvh*, and the limited genetic distance to *Cvv* populations from northeastern Florida are consistent with the more recent connection of the Oklawaha headwaters with

this area. Additional sequence information on other mitochondrial or nuclear genes might help resolve this uncertainty.

Are *Cvv* and *Cvh* Different Species?

Considering the foregoing data and analysis should *Cvv* and *Cvh* be considered separate species? While there is no real consensus on the definition of a species, given the potential conservation issues associated with *Cvh*, we ask this question within the regulatory framework of the US Endangered Species Act (ESA). Under the ESA, three main criteria are used to define a species or evolutionarily significant unit (Waples 1991).

First, is the population reproductively isolated from other conspecifics? Given the location of *Cvh* populations, there are considerable geographic barriers to gene flow with *Cvv* populations, but there is at least the potential for connectivity via the St. John's River system. Biologically, we were able to demonstrate significant, but incomplete pre- and post-zygotic isolation mechanisms. With the context of species designation, it is important to note that many recognized species of *Cyprinodon* readily hybridize (Turner and Liu 1977) and so even partial post-zygotic isolation is quite significant within this genus. Results from our phylogenetic analysis were also inconclusive as we were unable to resolve the polytomy of *Cvh* and *Cvv* populations from northeastern Florida and our samples sizes were not large enough to evaluate the likelihood of gene flow between populations. Hence, while there are significant geographical and biological barriers to gene flow between *Cvh* and *Cvv*, we cannot rule out the possibility based on available data.

Second, is the population genetically distinct from con-specifics? As just discussed, the unresolved polytomy for *Cvh* and *Cvv* does not provide evidence for *Cvh* being

genetically distinct. However, the distinct differences in Na^+ uptake between the two populations does argue for genetic differences. *Cvv* and *Cvh* raised in common garden conditions (7 mM Na^+ freshwater) have distinct Na^+ uptake characteristics, with the latter having characteristics clearly adaptive to its more dilute freshwater environment. Further, hybrids of the two populations display Na^+ uptake characteristics intermediate to the two populations. Combined, these two observations strongly suggest a genetic basis for the difference between populations.

Third, does the population occupy unique habitat or show evidence of unique adaptation to its environment? To the best of our knowledge, *Cvh* is the only population of *C. variegatus* that continuously inhabits waters with ≤1 mM Na^+. While there are numerous populations of *Cvv* that occur either periodically or permanently in freshwater environments (McLane 1955; Martin 1968; Martin 1972; Johnson 1974; Dunson et al. 1998; Lorenz and Serafy 2006), all examples of *Cvv* permanently residing in freshwater are in environments with ≥5 mM Na^+. Chapters 2, 4, and 5 along with results from this Chapter provide clear evidence that *Cvh* is uniquely adapted to the relatively low Na^+ environment in which it resides. The current study did demonstrate that ~70% of the *Cvv* population is capable of acclimating to 1 mM Na^+. However, under these conditions, *Cvv* still has an order of magnitude lower affinity for Na^+ uptake than *Cvh*.

Additionally, we observed that *Cvv* held at 1mM Na^+ for ~150 d grew slowly and failed to reach reproductive maturity. In contrast, during the routine maintenance of in-house cultures we observed *Cvh* held at 1 mM Na^+ and *Cvv* held at 7 mM Na^+ in <80 d. These observations are consistent with previous studies that *Cvv* is not reproductively viable in low Na^+ freshwater (Dunson et al. 1998). The mechanisms underlying this low

growth and failure to reach reproductive maturity are unclear. It is possible that *Cvv* is suffering from an osmo-respiratory compromise (Randall et al. 1972; Nilsson 1986) as we have demonstrated a significantly greater increase in MRC area in *Cvv* compared to *Cvh* at comparable salinities (Brix and Grosell 2012). In support of this hypothesis, a 22% reduction in routine metabolism has been measured in freshwater acclimated *Cvv* relative to seawater acclimated *Cvv* (Nordlie et al. 1991). Regardless of the mechanism, it is clear that *Cvh* is uniquely adapted to low Na^+ freshwater relative to other populations of *C. variegatus* that have been studied to date.

In conclusion, from a conservation perspective, it is clear that loss of *Cvh* would represent a significant loss of genetic and physiological diversity. Considering all the factors discussed above, we suggest the weight of evidence supports designation of *Cvh* as a distinct species or at a minimum, an evolutionarily significant unit.

Chapter 7:

Discussion

This dissertation makes several contributions to our general understanding of teleost fish Na^+ regulation in freshwater environments and provides a relatively detailed characterization of these processes in several non-model species. Additionally, this dissertation demonstrates that *Cvh* should at a minimum be considered an evolutionarily significant unit (possibly a unique species) and should be afforded appropriate protection to ensure this population is not lost as a result of habitat and water quality degradation.

There are three major pathways by which freshwater teleosts accomplish Na^+ uptake: Na^+ channel/H^+-ATPase, Cl^--dependent pathways (NCC, NKCC), and NHE. The latter may operate independently or in conjunction with either CA or an Rh glycoprotein. There have only been a few systematic studies of how changing environmental Na^+ concentrations influence the mechanisms used by a species to acquire Na^+ and all of these previous studies have used only two treatments (e.g., high and low Na^+) in their experimental design (Boisen et al. 2003; Yan et al. 2007; Wu et al. 2010). This dissertation provides one of the first detailed studies of the influence of multiple (4) Na^+ concentrations on Na^+ uptake pathways within a given species.

Interestingly, we found that in the *Cvv*/*Cvh* species complex there at least 3 different mechanism for apical Na^+ uptake across the range of 0.1 to 7 mM Na^+. At high Na^+ concentrations, *Cvv* uses an apical NKCC, the first functional demonstration of this in a freshwater teleost, although there have been previous immunohistochemistry studies demonstrating its presence in the apical membrane of tilapia (Hiroi et al. 2005; Yang et al. 2011). Both subspecies also appear to use a low affinity/high capacity NHE at

intermediate and high Na^+ concentrations, while only *Cvh* can express a high affinity NHE at low Na^+ concentrations. This flexible approach to Na^+ uptake contrasts with what was observed in *C. macularius* which utilizes only a low affinity NHE, regardless of the environmental Na^+ concentration. We hypothesize the flexible strategy used by *Cvv* is a reflection of the euryhaline environments in which it occurs while the inflexibility of *C. macularius* is consistent with its comparatively stenohaline environment. While *Cvh*, using two different NHE isoforms might also be considered a flexible strategy despite living in a stenohaline environment, we contend that the high affinity NHE is simply an adaptation to its new dilute freshwater environment and that *Cvh* is actually losing flexibility (loss of apical NKCC, reduced ability to osmoregulate in hypersaline environments) over time.

The function of NHE in fish living in dilute and/or acidic freshwater environments is currently an area of intense research (Hirata et al. 2003; Yan et al. 2007; Wu et al. 2010; Kumai et al. 2011; Kumai and Perry 2011; Lin et al. 2012; Shih et al. 2012), with studies to date focused on two model species (zebrafish and Japanese medaka). For both species, NHE function in low Na^+ and low pH environments has been linked to an NHE-Rh metabolon. The only other species studied to date is the Japanese dace which is adapted to pH 3.5 water with 1 mM Na^+ and uses H^+ generated via CA to allow for NHE function in this acidic environment. The use of an NHE-Rh metabolon in this species cannot be ruled out as it has not been investigated.

This dissertation provides information on a fourth species (*Cvh*) which also uses NHE in low Na^+ freshwater. Unlike zebrafish and Japanese medaka, *Cvh* clearly does not utilize an NHE-Rh metabolon and appears to at least partly and perhaps wholly rely

on CA for facilitating NHE function. This represents the first example of this system being utilized by a fish in response to dilute freshwater (rather than low pH) and raises interesting questions regarding the evolutionary constraints that lead to the use of one approach over the other in a given species. Virtually nothing is known about the signaling pathways that regulate either system and this is clearly one possible way in which selection might preferentially act on one system or the other. The diverse variety of ways in which CA contributes to regulation of Na^+ uptake in *Cvv* and *Cvh* provide strong impetus to study these signaling pathways in detail.

Additionally, there is increasing evidence of variation between species with respect to MRC types that could influence this process. For example, in trout, NHE-2 and NHE-3 occur in one type of MRC cell (PNA^+), while the H^+-ATPase occurs in another type (PNA^-) (Galvez et al. 2002; Reid et al. 2003). This differs from zebrafish, where NHE-3 and H^+-ATPase co-occur in the same cell type (HR) (Hwang 2009) and again differs from Japanese medaka where NHE-3 and H^+-ATPase occur in different MRC types with the H^+-ATPase occurring basolaterally rather than apically (Lin et al. 2012). Of these three species, zebrafish and medaka utilize an NHE-Rh metabolon, while trout does not, although a H^+-ATPase/Rh metabolon is involved in ammonia excretion in trout pavement cells (Nawata et al. 2007). It would be of interest to characterize the MRC types used by *Cvv* and *Cvh* and identify which proteins are expressed in which cell types to determine whether any across species patterns emerge.

While the focus of this dissertation was on characterizing Na^+ uptake in *C. variegatus* and the possible adaptive mechanisms of *Cvh* to dilute freshwater, it is important to note that Cl^- is an equally important osmolyte in fish osmoregulation. We

were unable to study Cl^- ion transport due to the prohibitive costs of the ^{36}Cl isotope at this time. The related euryhaline fish, *F. heteroclitus* does not take up Cl^- in freshwater with <2 mM Cl^- (Patrick et al. 1997), above which uptake increases linearly, presumably via NKCC, and appears to lack the Cl^-/HCO_3^- exchange characteristic of most, but not all freshwater fish (Tomasso and Grosell 2005; Hwang et al. 2011). *F. heteroclitus* is capable of obtaining some Cl^- via the diet, but even with this is not capable of long-term survival in dilute (≤1 mM NaCl) freshwater (Wood et al. 2010). We hypothesize *Cvv* is similar to *F. heteroclitus* with respect to Cl^- regulation, but it seems unlikely that *Cvh* could survive long-term in dilute freshwater without having evolved some mechanism for Cl^- uptake at the gill. This would be an important area of future study regarding the adaptations of *Cvh* to freshwater.

The final chapter in this dissertation focused on whether *Cvh* should be designated a separate species from *Cvv*. This question is important from a conservation perspective because *Cvh* only occurs in eight lakes in central Florida, all of which are suffering from habitat loss and water quality degradation through urban development. While there is no clear consensus on the definition of a species, within the regulatory framework of the Endangered Species Act, our data on heritable physiological differences in Na^+ transport, partial pre- and post-zygotic isolation, and likely monophyletic origin, all support the designation of *Cvh* as an evolutionarily significant unit, and likely a separate species.

It is also worth pointing out that there is support for a number of other significant differences between *Cvv* and *Cvh* that were not studied in this dissertation. As just discussed, in addition to the clear differences in Na^+ regulation, there are likely differences in Cl^- regulation. Additionally, there are significant morphological

differences between *Cvv* and *Cvh*. *Cvh* lacks the extreme sexual dimorphism in body shape observed in *Cvv*, with both male and female *Cvh* having a more elongated dorso-ventrally compressed body than *Cvv*. These differences have been previously quantified (Johnson 1974; Darling 1976), but were attributed to environmental plasticity as reduced body depth (though not to the extent observed in *Cvh*) has been observed in several low salinity populations of *Cvv*. While this may be possible, we note that after breeding *Cvv* for 8 generations in 7 mM Na^+ over the course of this dissertation, we have observed no reduction in body depth in F_8 animals compared with wild caught specimens.

Finally, we point out that there are significant differences in diet with *Cvv* feeding generally on detrital plant matter while *Cvh* appears to feed exclusively on insect larvae in the wild (McLane 1955). These differences translated into the lab where adult *Cvv* readily consumed flake flood, but *Cvh* refused flake food to the point of starvation and would only accept *Chironomus* larvae (bloodworms) as a diet. These differences in diet potentially have significant implications for both digestive and osmoregulatory physiology that would be worth further study (Bucking and Wood 2006; Taylor et al. 2007).

In conclusion, this dissertation provided an initial comparative assessment of Na^+ transport processes in the *Cvv*/*Cvh* species complex. The data generated indicate this species complex will be a very useful comparative model for studying ionoregulatory physiology within the evolutionary context of teleost fish transitioning from euryhaline to freshwater environments.

References

Alt, D. and H. K. Brooks (1965). "Age of the Florida marine terraces." J. Geol. **73**(2): 406-411.

Avella, M. and M. Bornancin (1989). "A new analysis of ammonia and sodium transport through the gills of the freshwater rainbow trout (*Salmo gairdneri*)." J. Exp. Biol. **142**: 155-175.

Barlow, G. W. (1958). "High salinity mortality of desert pupfish, *Cyprinodon macularius*." Copeia **1958**(3): 231-232.

Boisen, A. M. Z., J. Amstrup, I. Novak and M. Grosell (2003). "Sodium and chloride transport in soft water and hard water acclimated zebrafish (*Danio rerio*)." Biochim. Biophys. Acta **1618**: 207-218.

Brix, K. V., A. J. Esbaugh and M. Grosell (2011). "The toxicity and physiological effects of copper on the freshwater pulmonate snail, *Lymnaea stagnalis*." Comp. Biochem. Physiol. **154C**: 261-267.

Brix, K. V. and M. Grosell (2012). "Comparative characterization of Na^+ transport in *Cyprinodon variegatus variegatus* and *Cyprinodon variegatus hubbsi*: A model species complex for studying teleost invasion of freshwater." J. Exp. Biol. **215**: 1199-1209.

Broughton, R. E. and J. R. Gold (2000). "Phylogenetic relationships in the North American cyprinid genus *Cyprinella* (Actinopterygii: Cyprinidae) based on sequences of the mitochondrial ND2 and ND4L genes." Copeia **2000**(1): 1-10.

Bucking, C. and C. M. Wood (2006). "Gastrointestinal processing of Na^+, Cl^-, and K^+ during digestion: implications for homeostatic balance in freshwater rainbow trout." Amer. J. Physiol. **291**: R1764-R1772.

Bucking, C. and C. M. Wood (2008). "The alkaline tide and ammonia excretion after voluntary feeding in freshwater rainbow trout." J. Exp. Biol. **211**: 2533-2541.

Burton, R. F. (1980). "Acid and base excretion: assessment and relationships to diet and urine composition." Comp. Biochem. Physiol. **66A**: 371-375.

Bury, N. R. and C. M. Wood (1999). "Mechanism of branchial apical silver uptake by rainbow trout is via the proton-coupled Na^+ channel." Am. J. Physiol. **46**: R1385-R1391.

Bystriansky, J. S., J. G. Richards, P. M. Schulte and J. S. Ballantyne (2006). "Recipricol expression of gill Na^+/K^+-ATPase α-subunit isoforms α1a and α1b during seawater acclimation of three salmonid fishes that vary in their salinity tolerance." J. Exp. Biol. **209**: 1848-1858.

Caceres, P. S., G. R. Ares and P. A. Oritz (2009). "cAMP stimulates apical exocytosis of the renal Na^+-K^+-$2Cl^-$ cotransporter NKCC2 in the thick ascending limb: role of protein kinase A." J. Biol. Chem. **284**(37): 24965-24971.

Cameron, J. N. and N. Heisler (1983). "Studies of ammonia in the rainbow trout: physio-chemical parameters, acid-base behaviour and respiratory clearance." J. Exp. Biol. **105**: 107-125.

Carr, A. F. (1936). "A new species of *Cyprinodon* from Lake Eustis, Florida." Copeia **1936**(3): 160-163.

Chang, I. C., T. H. Lee, C. H. Yang, Y. Y. Wei, F. I. Chou and P. P. Hwang (2001). "Morphology and function of gill mitochondria-rich cells in fish acclimated to different environments." Physiol. Biochem. Zool. **74**(1): 111-119.

Cooper, C. A. and R. W. Wilson (2008). "Post-prandial alkaline tide in freshwater rainbow trout: effects of meal anticipation on recovery from acid-base and ion regulatory disturbances." J. Exp. Biol. **211**: 2542-2550.

Crapon de Caprona, M. D. and M. J. Ryan (1990). "Conspecific mate recognition in swordtails, *Xiphphorus nigrensis* and *X. pygmaeus* (Poeciliidae): olfactory and visual cues." Anim. Behav. **39**: 290-296.

Darling, J. S. D. (1976). Electrophoretic variation in *Cyprinodon variegatus* and systematics of some fishes of the subfamily Cyprinodontinae. New Haven, Conneticutt, Yale University. **Ph.D.:** 160 pp.

Davies, H. W., J. B. S. Haldane and D. M. Kennaway (1920). "Experiments on the regulation of the blood's alkalinity." J. Physiol. Lond. **54**: 32-45.

Duggins, C. F., A. A. Karlin and K. G. Relyea (1983). "Electrophoretic comparison of *Cyprinodon variegatus* Lacepede and *Cyprinodon hubbsi* Carr, with comments on the genus *Cyprinodon* (Atheriniformes: Cyprinodontidae)." Northeast Gulf Sci. **6**(2): 99-107.

Dunham, J. B. and W. L. Minckley (1998). "Allozymic variation in desert pupfish from natural and artificial habitats: genetic conservation in fluctuating populations." Biol. Conser. **84**: 7-15.

Dunson, W. A., C. J. Paradise and D. B. Dunson (1998). "Inhibitory effect of low salinity on growth and reproduction of the estuarine sheepshead minnow, *Cyprinodon variegatus*." Copeia **1998**(1): 235-239.

Duvernell, D. D. (1998). Population genetics of Death Valley pupfishes (Cyprinodontidae: *Cyprinodon* Spp.) and the identification of a new retrotransposable element family. Biology. Blacksburg, Virginia, Virginia Polytechnic Institute. **Ph.D. Dissertation:** 107 pp.

Echelle, A. A., E. W. Carson, A. F. Echelle, R. A. Van Den Bussche, T. E. Dowling and A. Meyer (2005). "Historical biogeography of the new-world pupfish genus *Cyprinodon* (Teleostei: Cyprinodontidae)." Copeia **2005**(2): 320-339.

Echelle, A. A., L. Fuselier, R. A. Van Den Bussche, C. M. L. Rodriguez and M. L. Smith (2006). "Molecular systematics of Hispaniolan pupfishes (Cyprinodontidae: *Cyprinodon*): implications for the biogeography of insular Caribbean fishes." Mol. Phylogen. Evol. **39**: 855-864.

Echelle, A. A., R. A. Van Den Bussche, T. P. Malloy, M. L. Haynie and C. O. Minckley (2000). "Mitochondrial DNA variation in pupfishes assigned to the species *Cyprinodon macularius* (Atherinomorpha: Cyprinodontidae): Taxonomic implications and conservation genetics." Copeia **2000**(2): 353-364.

Edwards, S. L., B. P. Wall, A. I. Morrison-Shetlar, S. Sligh, J. C. Weakley and J. B. Claiborne (2005). "The effect of environmental hypercapnia and salinity on the expression of NHE-like isoforms in the gills of a euryhaline fish (*Fundulus heteroclitus*)." J. Exp. Zool. **303A**: 464-475.

Esaki, M., K. Hoshijima, S. Kobayashi, H. Fukuda, K. Kawakami and S. Hirose (2007). "Visualization in zebrafish larvae of Na^+ uptake in mitochondria-rich cells whose differentiation is dependent on *foxi3a*." Am. J. Physiol. Reg. Integr. Comp. Physiol. **292**: 470-480.

Evans, D. H. (1973). "Sodium uptake by the sailfin molly, *Poecilia latipinna*: kinetic analysis of a carrier system present in both fresh-water-acclimated and sea-water-acclimated individuals." Comp. Biochem. Physiol. **45A**: 843-850.

Evans, D. H. (1982). "Mechanisms of acid extrusion by two marine fishes: the teleost, *Opsanus beta*, and the elasmobranch, *Squalus acanthias*." J. Exp. Biol. **97**: 289-299.

Evans, D. H. (1984). "Gill Na^+/H^+ and Cl^-/HCO_3^- exchange systems evolved before the vertebrates entered freshwater." J. Exp. Biol. **113**: 465-469.

Evans, D. H., P. M. Piermarini and K. P. Choe (2005). "The multifunctional fish gill: dominant site of gas exchange, osmoregulation, acid-base regulation, and excretion of nitrogenous waste." Physiol. Rev. **85**: 97-177.

Felsenstein, J. (1985). "Confidence limits on phylogenies: an approach using the bootstrap." Evolution **39**(4): 783-791.

Fernandes, M. N., S. A. Perna and S. E. Moron (1998). "Chloride cell apical surface changes in gill epithelia of the armoured catfish *Hypostomus plecostomus* during exposure to distilled water." J. Fish Biol. **52**: 844-849.

Furukawa, F., S. Watanabe, M. Inokuchi and T. Kaneko (2011). "Responses of gill mitochondra-rich cells in Mozambique tilapia exposed to acidic environments (pH 4.0) in combination with different salinities." Comp. Biochem. Physiol. **158A**: 468-476.

Galvez, F., S. D. Reid, G. S. Hawkings and G. G. Goss (2002). "Isolation and characterization of mitochondria-rich cell types from the gill of freshwater rainbow trout." Am. J. Physiol. **282**: R658-R668.

Genz, J., J. R. Taylor and M. Grosell (2008). "Effects of salinity on intestinal bicarbonate secretion and compensatory regulation of acid-base balance in *Opsanus beta*." J. Exp. Biol. **211**: 2327-2335.

Georgalis, T., S. F. Perry and K. M. Gilmour (2006). "The role of branchial carbonic anhydrase in acid-base regulation in rainbow trout (*Oncorhynchus mykiss*)." J. Exp. Biol. **209**: 518-530.

Gonzalez, R. J., R. W. Wilson, C. M. Wood, M. L. Patrick and A. L. Val (2002). "Diverse strategies for ion regulation in fish collected from the ion-poor, acidic Rio Negro." Physiol. Biochem. Zool. **75**(1): 37-47.

Goss, G. G., S. F. Perry, C. M. Wood and P. Laurent (1992). "Mechanisms of ion and acid-base regulation at the gills of freshwater fish." J. Exp. Zool. **263**: 143-159.

Goss, G. G. and C. M. Wood (1990). "Na^+ and Cl^- uptake kinetics, diffusive effluxes and acidic equivalent fluxes across the gills of rainbow trout. I. Responses to environmental hyperoxia." J. Exp. Biol. **152**: 521-547.

GraphPad Software Inc. (2007). GraphPad Prism. La Jolla, California, GraphPad Software, Inc.

Greco, A. M., J. C. Fenwick and S. F. Perry (1996). "The effects of soft-water acclimation on gill structure in the rainbow trout *Oncorhynchus mykiss*." Cell Tissue Res. **285**: 75-82.

Grosell, M. and C. M. Wood (2002). "Copper uptake across rainbow trout gills: mechanisms of apical entry." J. Exp. Biol. **205**: 1179-1188.

Guillory, V. and W. E. Johnson (1986). "Habitat, conservation status, and zoogeography of the cyprinodont fish, *Cyprindon variegatus hubbsi* (Carr)." Southwest. Nat. **31**(1): 95-100.

Haney, R. A., B. R. Silliman, A. J. Fry, C. A. Layman and D. M. Rand (2007). "The Pleistocene history of the sheepshead minnow (*Cyprinodon variegatus*): Non-equilibrium evolutionary dynamics within a diversifying species complex." Mol. Phyl. Evol. **43**: 743-754.

Henry, R. P. (1991). Techniques for measuring carbonic anhydrase activity *in vitro*. Carbonic Anhydrases: Cellular Physiology and Molecular Genetics. S. J. Dodgson, R. E. Tashian, G. Gros and N. D. Carter. New York, Plenum**:** pp. 119-131.

Hills, A. G. (1973). Acid-base Balance: Chemistry, Physiology, Pathophysiology. Baltimore, Maryland, Wilkins and Wilkins.

Hirai, N., M. Tagawa, T. Kaneko, T. Seikai and M. Tanaka (1999). "Distributional changes in branchial chloride cells during freshwater adaptation in Japanese sea bass *Lateolabrax japonicus*." Zool. Sci. **16**: 43-49.

Hirata, T., T. Kaneko, T. Ono, T. Nakazato, N. Furukawa, S. Hasegawa, S. Wakabyashi, M. Shigekawa, M. H. Chang, M. F. Romero and S. Hirose (2003). "Mechanism of acid adaptation of a fish living in a pH 3.5 lake." Am. J. Physiol. Reg. Integr. Comp. Physiol. **284**: R1199-R1212.

Hiroi, J., S. D. McCormick, R. Ohtani-Kaneko and T. Kaneko (2005). "Functional classification of mitochondrion-rich cells in euryhaline Mozambique tilapia (*Oreochromis mossambicus*) embryos, by means of triple immunofluorescence staining for Na^+/K^+-ATPase, $Na^+/K^+/2Cl^-$ cotransporter and CFTR anion channel." J. Exp. Biol. **208**: 2023-2036.

Hwang, P. P. (2009). "Ion uptake and acid secretion in zebrafish (*Danio rerio*)." J. Exp. Biol. **212**: 1745-1752.

Hwang, P. P., T. H. Lee and L. Y. Lin (2011). "Ion regulation in fish gills: recent progress in the cellular and molecular mechanisms." Am. J. Physiol. **301**(R28-R47).

Inokuchi, M., J. Hiroi, S. Watanabe, P. P. Hwang and T. Kaneko (2009). "Morphological and functional classification of ion-absorbing mitochondria-rich cells in the gills of Mozambique tilapia." J. Exp. Biol. **212**: 1003-1010.

Johnson, W. E. (1974). Morphological variation and local distribution of *Cyprinodon variegatus* in Florida. Biological Sciences. Orlando, Florida, Florida Technical University. **M.S.:** 193 pp.

Jordan, F., D. C. Haney and F. G. Nordlie (1993). "Plasma osmotic regulation and routine metabolism in the Eustis pupfish, *Cyprinodon variegatus hubbsi* (Teleostei: Cyprinodontidae)." Copeia **1993**(3): 784-789.

Jorgensen, P. L. (2008). "Importance for absorption of Na^+ from freshwater of lysine, valine and serine substitutions in the α1a-isoform of Na,K-ATPase in the gills of rainbow trout (*Oncorhynchus mykiss*) and Atlantic salmon (*Salmo salar*)." J. Membrane Biol. **223**: 37-47.

Karnaky, K. J., S. A. Ernst and C. W. Philpott (1976). "Teleost chloride cell. I. Response of pupfish *Cyrpinodon variegatus* gill Na,K-ATPase and chloride cell fine structure to various high salinity environments." J. Cell Biol. **70**: 144-156.

King, J. A. C., D. C. Abel and D. R. DiBona (1989). "Effects of salinity on chloride cells in the euryhaline cyprinodontid fish *Rivulus marmoratus*." Cell Tissue Res. **257**: 367-377.

Kinne, O. (1960). "Growth, food intake, and food conversion in a euryplastic fish exposed to different temperature and salinities." Physiol. Zool. **33**: 288-317.

Kleyman, T. R. and E. J. Cragoe (1988). "Amiloride and its analogs as tools in the study of ion transport." J. Membrane Biol. **105**: 1-21.

Kodric-Brown, A. and U. Strecker (2001). "Responses of *Cyprinodon maya* and *C. labiousus* females to visual and olfactory cues of conspecific and heterospecific males." Biol. J. Linn. Soc. **74**: 541-548.

Krogh, A. (1937). "Osmotic regulation in freshwater fishes by active absorption of chloride ions." Z. Vergl. Physiol. **24**: 656-666.

Krogh, A. (1938). "The active absorption of ions in some freshwater animals." Z. Vergl. Physiol. **25**: 335-350.

Krogh, A. (1939). Osmotic Regulation in Aquatic Animals. Cambridge, University Press.

Kumai, Y., A. Bahubeshi, S. Steele and S. F. Perry (2011). "Strategies for maintaining Na^+ balance in zebrafish (*Danio rerio*) during prolonged exposure to acid water." Comp. Biochem. Physiol. **160A**: 52-62.

Kumai, Y. and S. F. Perry (2011). "Ammonia excretion via Rhcg1 facilitates Na^+ uptake in larval zebrafish, *Danio rerio*, in acidic water." Am. J. Physiol. **301**: R1517-R1528.

Laurent, P. and S. F. Perry (1990). "Effects of cortisol on gill chloride cell morphology and ionic uptake in the freshwater trout, *Salmo gairdneri*." Cell Tissue Res. **259**: 429-442.

Lee, T. H., P. P. Hwang, H. C. Lin and F. L. Huang (1996). "Mitochondrial-rich cells in the branchial epithelium of the teleost, *Oreochromis mossambicus*, acclimated to various hypotonic environments." Fish Physiol. Biochem. **15**(6): 513-523.

Lemann, J. and E. J. Lennon (1966). "A potential error in the measurement of urinary titratable acid." J. Lab. Clin. Med. **67**: 906-913.

Liao, B. K., R. D. CHen and P. P. Hwang (2009). "Expression regulation of Na^+-K^+-ATPase α1-subunit subtypes in zebrafish gill ionocytes." Am. J. Physiol. **296**: R1897-R1906.

Lin, C. C., L. Y. Lin, H. H. Hsu, V. Thermes, P. Prunet, J. L. Horng and P. P. Hwang (2012). "Acid secretion by mitochondria-rich cells of medaka (*Oryzias latipes*) acclimated to acidic water." Am. J. Physiol. **302**: R283-R291.

Lin, H., D. C. Pfeiffer, A. W. Vogl, J. Pan and D. G. Randall (1994). "Immunolocalization of H^+-ATPase in the gill epithelia of rainbow trout." J. Exp. Biol. **195**: 169-183.

Lin, H. and D. G. Randall (1991). "Evidence for the presence of an electrogenic proton pump on the trout gill epithelium." J. Exp. Biol. **161**: 119-134.

Lin, H. and D. G. Randall (1993). "H^+-ATPase activity in crude homogenates of fish gill tissue: inhibitor sensitivity and environmental and hormonal regulation." J. Exp. Biol. **180**: 163-174.

Loftis, D. G., A. A. Echelle, H. Koike, R. A. Van Den Bussche and C. O. Minckley (2009). "Genetic structure of wild populations of the endangered desert pupfish complex (Cyprinodontidae: *Cyprinodon*)." Conser. Gen. **10**: 453-463.

Long, J. A. (2011). The Rise of Fishes: 500 Million Years of Evolution. Baltimore, Maryland, Johns Hopkins University Press.

Lorenz, J. L. and J. E. Serafy (2006). "Subtropical wetland fish assemblages and changing salinity regimes: implications for everglades restoration." Hydrobiol. **569**: 401-422.

Maddison, W. P. and D. R. Maddison (2010). Mesquite: a modular system for evolutionary analysis.

Maetz, J. (1956). "Les echanges de sodium chez les poissons *Carassius auratus* L. Action d'un inhibiteur d l'anhydrase carbonique." J. Physiol. (Paris) **48**: 1085-1099.

Maetz, J. (1973). "Na^+/NH_4^+, Na^+/H^+ exchanges and the NH3 movement across the gill of *Carassius auratus*." J. Exp. Biol. **58**: 255-275.

Maetz, J. and F. G. Romeu (1964). "The mechanism of sodium and chloride uptake by the gills of a fresh-water fish, *Carassius auratus*. II. Evidence for NH_4^+/Na^+ and HCO_3^-/Cl^- exchanges." J. Gen. Physiol. **47**: 1209-1227.

Marsh, P. C. and D. W. Sada (1993). Desert pupfish (*Cyprinodon macularius*) recovery plan. U. S. F. a. W. Service. Albuquerque, New Mexico**:** 129 pp.

Marshall, W. S. and M. Grosell (2006). Ion transport, osmoregulation, and acid-base balance. The Physiology of Fishes. D. H. Evans and J. B. Claiborne. Boca Raton, Florida, Taylor and Francis**:** pp. 177-230.

Martin, F. D. (1968). "Intraspecific variation in osmotic abilitites of *Cyprinodon variegatus* Lacepede from the Texas coast." Ecology **49**(6): 1186-1188.

Martin, F. D. (1972). "Factors influencing local distribution of *Cyprinodon variegatus* (Pisces: Cyprinodontidae)." Trans. Amer. Fish. Soc. **101**(1): 89-93.

McDonald, D. G. and C. M. Wood (1981). "Branchial and renal acid and ion fluxes in the rainbow trout, *Salmo gairdneri*, at low environmental pH." J. Exp. Biol. **93**: 101-118.

McLane, W. M. (1955). The fishes of the St. Johns river system. Gainesville, Florida, University of Florida. **Ph.D.:** 362 pp.

Meade, P., R. S. Hoover, C. Plata, N. Vazquez, N. A. Bobadilla, G. Gamba and S. C. Herbert (2003). "cAMP-dependent activation of the renal-specific Na^+-K^+-$2Cl^-$ cotransporter is mediation by regulation of cotransporter trafficking." Amer. J. Bot. **284**: F1145-F1154.

Miller, R. R. (1943). The status of *Cyprinodon macularius* and *Cyprinodon nevadensis*, two desert fishes of western North America. Occasional Papers of the Museum of Zoology. Ann Arbor, Michigan, University of Michigan**:** 39 pp.

Miller, R. R. and L. A. Fuiman (1987). "Description and conservation status of *Cyprinodon macularius eremus*, a new subspeces of pupfish from Organ Pipe Cactus National Monument, Arizona." Copeia **1987**(3): 593-609.

Nakada, T., K. Hoshijima, M. Esaki, S. Nagayoshi, K. Kawakami and S. Hirose (2007a). "Localization of ammonia transporter Rhcg1 in mitochondrion-rich cells of yolk sac, gill, and kidney of zebrafish and its ionic strength-dependent expression." Am. J. Physiol. **293**: R1743-R1753.

Nakada, T., C. M. Westhoff, A. Kato and S. Hirose (2007b). "Ammonia secretion from fish gill depends on a set of Rh glycoproteins." FASEB J. **21**: 1067-1074.

Nawata, C. M., C. C. Y. Hung, T. K. N. Tsui, J. M. Wilson, P. A. Wright and C. M. Wood (2007). "Ammonia excretion in rainbow trout (*Oncorhynchus mykiss*): evidence for Rh glycoprotein and H^+-ATPase involvement." Physiol. Genom. **31**: 463-474.

Nawata, C. M., C. M. Wood and M. J. O'Donnell (2010). "Functional characterization of Rhesus glycoproteins from an ammoniotelic teleost, the rainbow trout, using oocyte expression and SIET analysis." J. Exp. Biol. **213**: 1049-1050.

Nilsson, S. (1986). Control of gill blood flow. Fish Physiology: Recent Advances. S. Nilsson and S. Holmgren. London, Croom Helm**:** pp. 87-101.

Nordlie, F. G. (2006). "Physiochemical environments and tolerances of cyprinodontoid fishes found in estuaries and salt marshes of eastern North America." Rev. Fish Biol. Fish. **16**: 51-106.

Nordlie, F. G., S. J. Walsh, D. C. Haney and T. F. Nordlie (1991). "The influence of ambient salinity on routine metabolism in the teleost *Cyprinodon variegatus* Lacepede." J. Fish Biol. **38**: 115-122.

Oritz, P. A. (2006). "cAMP increases surface expression of NKCC2 in rat thick ascending limbs: role of VAMP." Am. J. Physiol. **290**: F608-F616.

Orlowski, J. (1993). "Heterologous expression and functional properties of amiloride high affinity (NHE-1) and low affinity (NHE-3) isoforms of the rat Na/H exchanger." J. Biol. Chem. **268**(22): 16369-16377.

Parks, S. K., M. Tresguerres and G. G. Goss (2008). "Theoretical considerations underlying Na^+ uptake mechanisms in freshwater fishes." Comp. Biochem. Physiol. **148C**: 411-418.

Patrick, M. L., P. Part, W. S. Marshall and C. M. Wood (1997). "Characterization of ion and acid-base transport in the fresh water adapted mummichog (*Fundulus heteroclitus*)." J. Exp. Zool. **279**: 208-219.

Patrick, M. L. and C. M. Wood (1999). "Ion and acid-base regulation in the freshwater mummichog (*Fundulus heteroclitus*): a departure from the standard model for freshwater teleosts." Comp. Biochem. Physiol. **122A**: 445-456.

Perry, S. F., C. E. Booth and D. G. McDonald (1985). "Isolated perfused head of rainbow trout. II. Ionic fluxes." Am. J. Physiol. **249**: R255-R261.

Perry, S. F., G. G. Goss and P. Laurent (1992). "The interrelationships between gill chloride cell morphology and ionic uptake in four freshwater teleosts." Can. J. Zool. **70**: 1775-1786.

Perry, S. F., A. Shahsavarani, T. Georgalis, M. Bayaa, M. Furimsky and S. L. Y. Thomas (2003). "Channels, pumps, and exchangers in the gill and kidney of freshwater fishes: their role in ionic and acid-base regulation." J. Exp. Zool. **300A**: 53-62.

Preest, M. R., R. J. Gonzalez and R. W. Wilson (2005). "A pharmacological examination of Na^+ and Cl^- transport in two species of freshwater fish." Physiol. Biochem. Zool. **78**(2): 259-272.

Randall, D. J., D. Baumgarten and M. Malyusz (1972). "The relationship between gas an ion transfer across the gills of fishes." Comp. Biochem. Physiol. **41A**: 629-637.

Reid, S. D., G. S. Hawkings, F. Galvez and G. G. Goss (2003). "Localization and characterization of phenamil-sensitive Na^+ influx in isolated rainbow trout gill epithelial cells." J. Exp. Biol. **206**: 551-559.

Richards, J. G., J. W. Semple, J. S. Bystriansky and P. M. Schulte (2003). "Na^+/K^+-ATPase α-isoform switching in gills of rainbow trout (*Oncorhynchus mykiss*) during salinity transfer." J. Exp. Biol. **206**: 4475-4486.

Scott, G. R., J. B. Claiborne, S. L. Edwards, P. M. Schulte and C. M. Wood (2005). "Gene expression after freshwater transfer in gills and opercular epithelia of killifish: insight into divergent mechanisms of ion transport." J. Exp. Biol. **208**: 2719-2729.

Scott, G. R., J. T. Rogers, J. G. Richards, C. M. Wood and P. M. Schulte (2004). "Intraspecific divergence of ionoregulatory physiology in the euryhaline teleost *Fundulus heteroclitus*: possible mechanisms of freshwater adaptation." J. Exp. Biol. **207**: 3399-3410.

Shih, T. H., J. L. Horng, S. T. Liu, P. P. Hwang and L. Y. Lin (2012). "Rhcg1 and NHE3b are involved in ammonium-dependent sodium uptake by zebrafish larvae acclimated to low-sodium water." Amer. J. Physiol. **302**: R84-R93.

SPSS (2006). SigmaStat 3.5. Chicago, Ilinois, SPSS.

Strecker, U. and A. Kodric-Brown (1999). "Mate recognition systems in a species flock of Mexican pupfish." J. Evol. Biol. **12**: 927-935.

Strecker, U. and A. Kodric-Brown (2000). "Mating preferences in a species flock of Mexican pupfishes (*Cyprinodon*, Teleostei)." Biol. J. Linn. Soc. **71**: 677-687.

Tamura, K. and M. Nei (1993). "Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees." Mol. Biol. Evol. **10**(3): 512-526.

Tamura, K., D. Peterson, N. Peterson, G. Stecher, M. Nei and S. Kumar (2011). "MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods." Mol. Biol. Evol. **28**(10): 2731-2739.

Taylor, J. R., J. M. Whittamore, R. W. Wilson and M. Grosell (2007). "Postprandial acid-base balance and ion regulation in freshwater and seawater-acclimated European flounder, *Platichthys flesus*." J. Comp. Physiol. B **177**: 597-608.

Tomasso, J. R. and M. Grosell (2005). "Physiological basis for large differences in resistance to nitrite among freshwater and freshwater-acclimated euryhaline fishes." Environ. Sci. Tech. **39**: 98-102.

Tresguerres, M., L. R. Levin, J. Buck and M. Grosell (2010). "Modulation of NaCl absorption by [HCO_3^-] in the marine teleost intestine is mediated by soluble adenylyl cyclase." Am. J. Physiol. **299**: R62-R71.

Turner, B. J. and R. K. Liu (1977). "Extensive interspecific genetic compatability in the New World killifish genus *Cyprinodon*." Copeia **1977**(2): 259-269.

Ultsch, G. R., M. E. Ott and N. Heisler (1981). "Acid-base and electrolyte status in carp (*Cyprinus carpio*) exposed to low environmental pH." J. Exp. Biol. **93**: 65-80.

Verdouw, H., C. J. A. van Echteld and E. M. J. Dekker (1978). "Ammonia determination based on indophenol formation with sodium salicylate." Wat. Res. **12**: 399-402.

Wang, Y. F., Y. C. Tseng, J. J. Yan, J. Hiroi and P. P. Hwang (2009). "Role of SLC12A10.2, a Na-Cl cotransporter-like protein, in a Cl uptake mechanism in zebrafish (*Danio rerio*)." Am. J. Physiol. Reg. Integr. Comp. Physiol. **296**: R1650-R1660.

Waples, R. S. (1991). "Pacific salmon, *Oncorhynchus* spp., and the definition of "species" under the Endangered Species Act." Mar. Fish. Rev. **53**(3): 11-22.

Wilson, R. W., P. M. Wright, S. Munger and C. M. Wood (1994). "Ammonia excretion in freshwater rainbow trout (*Oncorhynchus mykiss*) and the importance of gill boundary layer acidification: lack of evidence for Na^+/NH_4^+ exchange." J. Exp. Biol. **191**: 37-58.

Wood, C. M., C. Bucking and M. Grosell (2010). "Acid-base responses to feeding and intestinal Cl- uptake in freshwater and seawater-acclimated killifish, *Fundulus heteroclitus*, a agastric euryhaline teleost." J. Exp. Biol. **213**: 2681-2692.

Wood, C. M., A. Y. O. Matsuo, R. J. Gonzalez, R. W. Wilson, M. L. Patrick and A. L. Val (2002). "Mechanisms of ion transport in *Potamotrygon*, a stenohaline freshwater elasmobranch native to the ion-poor blackwaters of the Rio Negro." J. Exp. Biol. **205**: 3039-3054.

Wu, S. C., J. L. Horng, S. T. Liu, P. P. Hwang, Z. H. Wen, C. S. Lin and L. Y. Lin (2010). "Ammonium-dependent sodium uptake in mitochondrion-rich cells of medaka (*Oryzias latipes*) larvae." Am. J. Physiol. **298**: C237-C250.

Yan, J. J., M. Y. Chou, T. Kaneko and P. P. Hwang (2007). "Gene expression of Na^+/H^+ exchanger in zebrafish H^+-ATPase-rich cells during acclimation to low-Na^+ and acidic environments." Am. J. Physiol. **293**: C1814-C1823.

Yang, W. K., C. K. Kang, T. Y. Chen, W. B. Chang and T. H. Lee (2011). "Salinity-dependent expression of the branchial $Na^+/K^+/2Cl^-$ cotransporter and Na^+/K^+-ATPase in the sailfin molly correlates with hypoosmoregulatory endurance." J. Comp. Physiol. B **181**: 953-964.

Yu, F. H., G. E. Shull and J. Orlowski (1993). "Functional properties of the rat Na/H exchanger NHE-2 isoform expressed in the Na/H exchanger-deficient Chinese hamster ovary cells." J. Biol. Chem. **268**(34): 25536-25541.

Zar, J. H. (2009). Biostatistical Analysis. Upper Saddle River, New Jersey, Prentice Hall.