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Evaluation of pre- and post-zygotic mating barriers, hybrid fitness and phylogenetic relationship between *Cyprinodon variegatus variegatus* and *Cyprinodon variegatus hubbsi* (Cyprinodontiformes, Teleostei)

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Abstract

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 a significantly higher affinity for Na^+ uptake suggesting that it has adapted to its dilute freshwater environment. We here 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 prezygotic 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 with con-specific embryos. Na^+ uptake kinetics in hybrids (both *Cvv* x *Cvh* and *Cvh* x *Cvv*) bred and raised under common garden conditions were 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, although phylogenetic analysis of *Cvv* and *Cvh* populations using mtDNA sequence for ND2 were unable to fully resolve a polytomy between *Cvh* and *Cvv* populations from northeastern Florida, these data do not falsify the hypothesis that *Cvh* is of monophyletic origin. Overall, the available data suggest that *Cvh* should be considered a separate species or at a minimum an evolutionarily significant unit.

Introduction

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 this region. Approximately 3 mya, *C. variegatus* (or its progenitor) entered the Gulf of Mexico and spread along the Gulf and Atlantic coastlines, as well as throughout the Greater Antilles, the Bahamas and Turks/Caicos Islands (Echelle *et al.*, 2005, 2006).

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The phylogeny of Caribbean populations is in disarray with several distinct species named, but recent molecular studies provide little support for these designations and suggest taxonomic revision is needed (Echelle *et al.*, 2006). 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 and Atlantic populations up to North Carolina and populations from North Carolina to Massachusetts which 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 form 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), whereas 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 & 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 (apparent affinity constant) = 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 ($\leq$ 1 mM Na^+) which is characteristic of its native habitat (Brix & 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 scouring 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 & 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.

Materials and methods

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 days. Dechlorinated City of Miami tapwater (~1.0 mM Na^+, 1.0 mM Cl^-, 0.5 mM Ca^{2+}, 0.2 mM Mg^{2+}, 0.5 mM SO_4^{2-}, 0.8 mM HCO_3^-, pH 7.9) was mixed with filtered natural seawater to achieve the desired salinity. After this holding period, fish were bred and offspring were hatched and raised to sexual maturity under the same near freshwater conditions. 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, Tetra, Inc., Blacksburg, VA, USA).

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 *Cvh* 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 followed previously described experimental designs (Crapon de Caprona & Ryan, 1990; Strecker & Kodric-Brown, 1999). Both experiments were performed in a 100-L aquarium (91 × 32 × 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 was placed in separate 7.5-L aquaria (26 × 17 × 17 cm) at opposite ends of the larger aquaria. Care was taken to size match males based on mass. Each male was provided with a 10 × 10 cm piece of blue bonded filter media (Marineland, Blacksburg, VA, USA) 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 preconditioned 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 within each of the three 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 the male was 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 five 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 = 14).

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. On the basis of these preliminary trials, we selected an experimental design in which three males from each subspecies were 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 where 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 were 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 (35 g l^{-1}) to evaluate the effects of salinity on *Cvv* reproduction as this subspecies is better adapted to reproduce in marine environments. In addition, 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 days and on the fourth day, egg breeding pads were collected. All eggs were carefully removed from the breeding pads and trans-

ferred to 100 mL of 7 mM Na^+ freshwater with gentle aeration. To prevent fungal growth on the eggs, methylene blue (10 mg l^{-1} final concentration) 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 days, 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 0.1, 0.25, 0.5, 1 or 2 mM 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 was characterized. In addition, results from the low salinity survival experiment demonstrated that ~70% of the *Cvv* 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 eight different ambient Na^+ concentrations ranging nominally from 0.1 to 8 mM Na^+. At each Na^+ concentration, eight 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 where 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 to 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 analysis of [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 acclimation 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 two locations for *Cvv* and three locations for *Cvh* (Table 1 and Fig. 1). Mitochondrial DNA (mtDNA) sequence data from these samples were supplemented with additional sequence data generated in previous studies (Duvernell & Turner, 1998; Echelle *et al.*, 2005, 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 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 & Gold, 2000) in 50 μL reactions using the following conditions: 94 °C for 3 min; 40 cycles of 94 °C for 30 s, 52 °C for 30 s, 72 °C for 90 s; 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.

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).

Table 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

Fig. 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 analysed by Student's *t*-test (Mann–Whitney *U*-test for nonnormal data) 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 (apparent affinity constant) and V_{max} (maximal transport rate) 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 & 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 & Nei, 1993) was determined to be the most appropriate substitution model based on Bayesian Information Criterion and Akaike Information Criterion (corrected) using MEGA v5.05. 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-neighbour-interchange method. For maximum parsimony analysis, the tree was inferred using the heuristic close-neighbour-interchange method with 10 initial trees used. Support for both analyses was evaluated using nonparametric bootstrap sampling (n = 500) (Felsenstein, 1985).

Results

Mate recognition experiments

No significant differences in time associated with visual (t_{22} = 0.642, P = 0.528 *Cvv* females; t_{22} = 0.982, P = 0.337 *Cvh* females) or chemical cues (t_{26} = 0.162, P = 0.873 *Cvv* females; t_{26} = −0.750, P = 0.460 *Cvh* females) were observed for any of the treatments (Fig. 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 (Mann–Whitney U = 180.0, n = 19, P = 1.00 *Cvv* males; Mann–Whitney U = 142.0, n = 19, P = 0.264 *Cvh* males) (Fig. 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 (Mann–Whitney U = 17.0, n = 10, P = 0.012) preference for con-specific mating by *Cvv* females was observed whereas *Cvh* females spawned with con- and hetero-specific males equally (Mann–Whitney U = 14.5, n = 10, P = 0.589) (Fig 3b). Across subspecies, *Cvv* males spawned more than twice as often (n = 74) as *Cvh* males (n = 31).

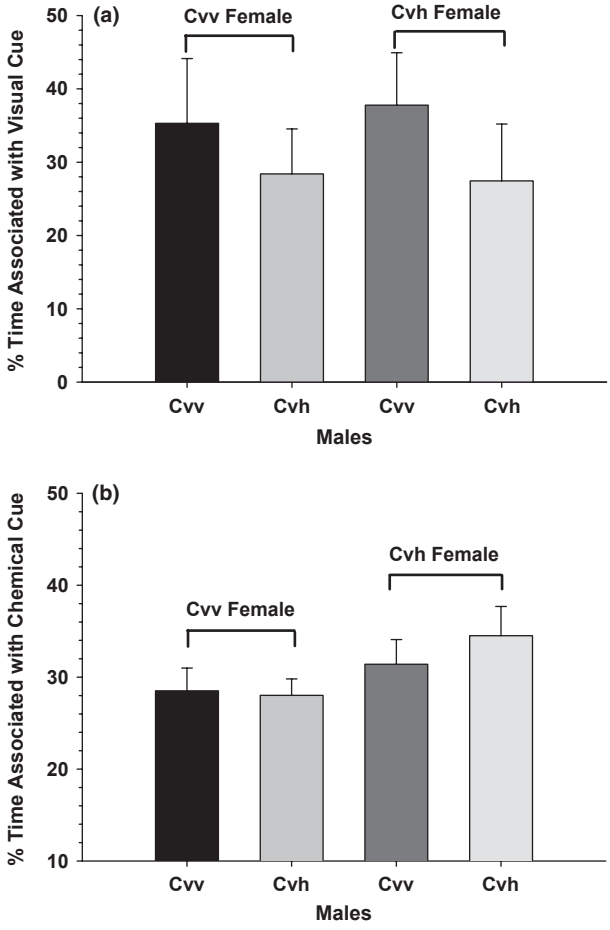


Fig. 2 Response of *Cvv* and *Cvh* to (**a**). Visual cues. (**b**) Chemical cues. Data reflects the fraction of time female fish associated with male visual or chemical cues in 30 min trials. Mean ± SEM (n = 12 for visual cues and n = 14 for chemical cues).

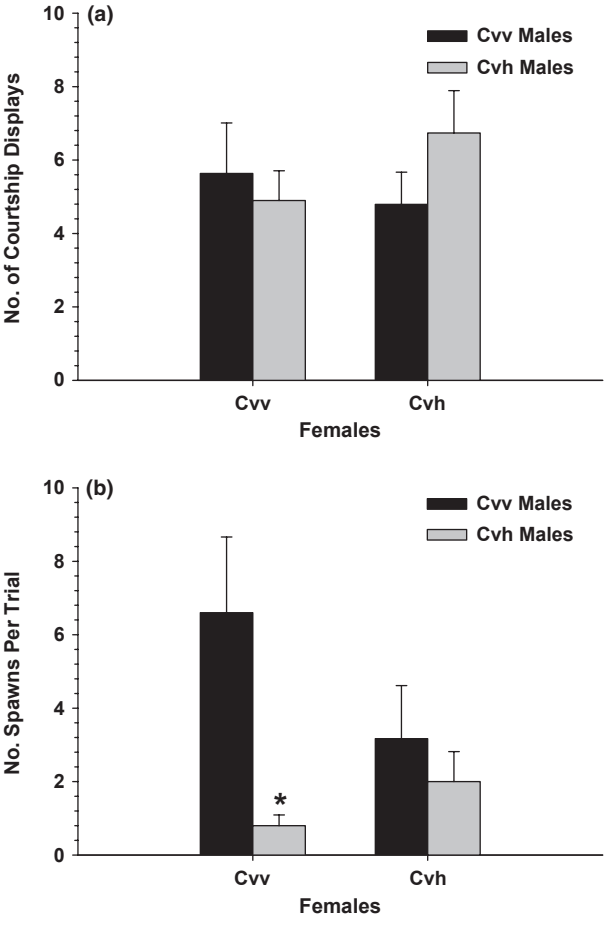


Fig. 3 Response of *Cvv* and *Cvh* in choice mating experiments. (**a**) Number of courtship displays by male fish with homo- and hetero-specific females. (**b**) Number of spawns per trial by female fish with homo- and hetero-specific males. Mean ± SEM (n = 19). Different letters indicate statistically significant difference ($P < 0.05$).

Reproduction and *H*ybrid 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. 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. Both self-crosses of F_1 hybrids resulted in significantly higher number of egg d^{-1} than original crosses (Kruskal–Wallis H = 37.5, d.f. = 6, $P < 0.001$). Hatching success ranged from 6 to 77%, with initial hybrid crosses having significantly lower hatching success rates than all other groups ($F_{6,63} = 68.486$, $P < 0.001$) (Fig. 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^+ FW, 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 days, with initial crosses and *Cvv* x *Cvv* in 7 mM Na^+ having significantly shorter hatching times than *Cvh* x *Cvh* and hybrid self-crosses (Kruskal–Wallis H = 49.558, d.f. = 6, $P < 0.001$) (Fig. 4c). Embryos from *Cvv* x *Cvv* in seawater took the longest to hatch.

Effects on larval survival through 21 days were relatively minor ranging from 83 to 96%, although *Cvh* x *Cvv* F_2 larval survival was significantly lower than for larvae from all other crosses (Kruskal–Wallis H = 17.981, d.f. = 6, $P = 0.006$) (Fig. 4d). Larval wet weight at the end of 21 days ranged from 29 to 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 (Kruskal–Wallis H = 32.779, d.f. = 6, $P < 0.001$) (Fig. 4e).

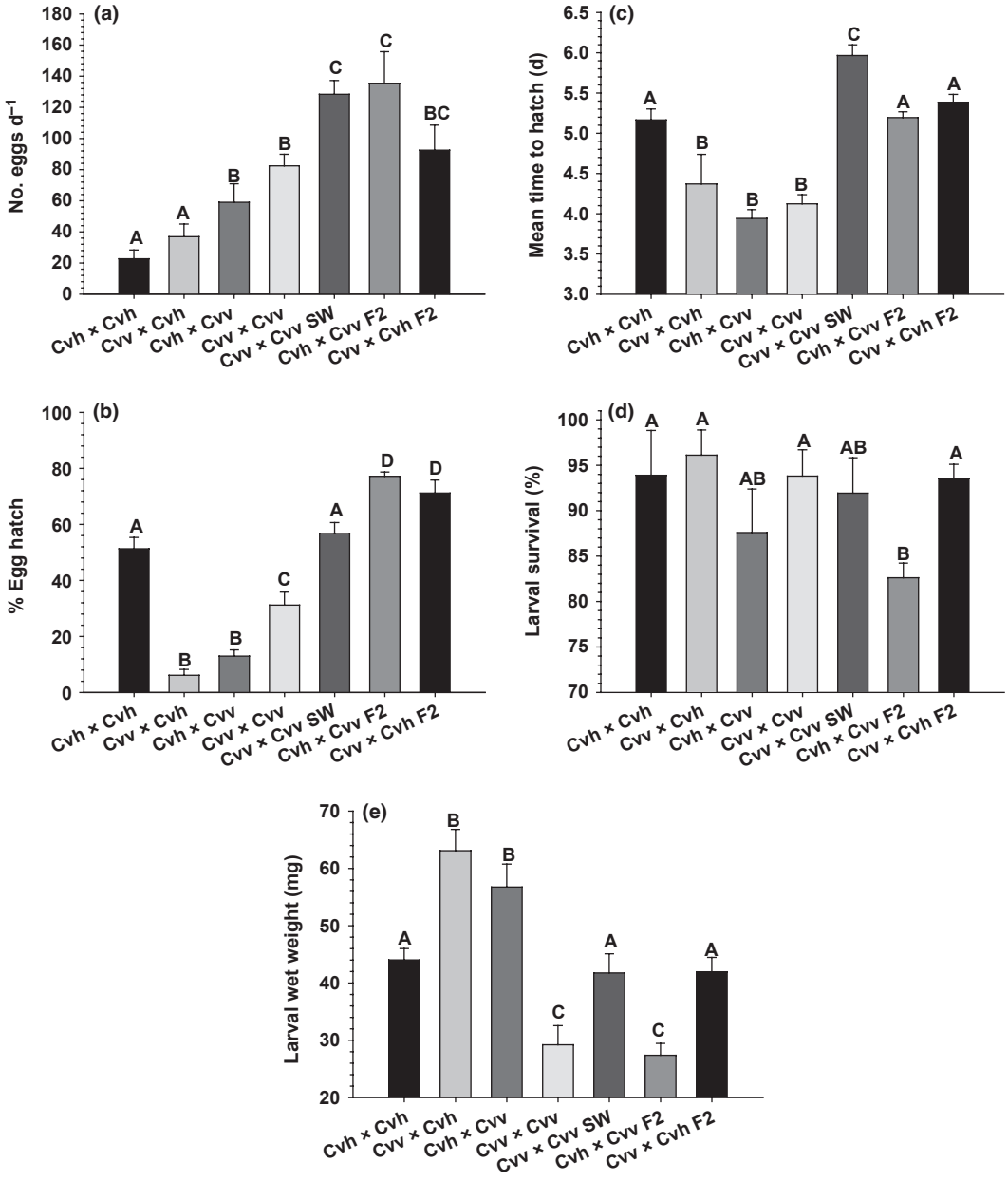


Fig. 4 Characterization of post-zygotic isolation in no choice mating experiments with *Cvv* and *Cvh*. All crosses presented as male x female. All mating experiments performed in freshwater with 7 mM Na^+ except *Cvv* x *Cvv* SW, which was performed in seawater. Note, F_2 crosses used surviving fish 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 days post-hatch. (**e**) Wet weight of larvae 21 days post-hatch. Mean ± SEM (n = 10 trials for each cross).

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 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^+.

Na^+ uptake kinetics and variability

Na^+ uptake kinetics for both hybrid crosses and *Cvv* exhibited Michaelis-Menten saturation kinetics (Fig. 6).

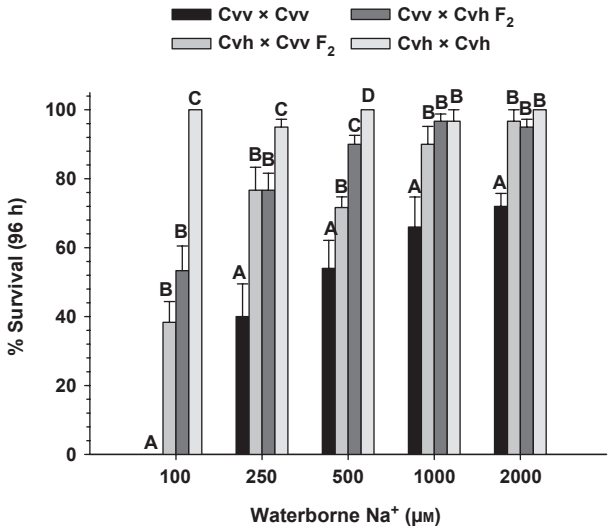


Fig. 5 Survival through 96 h of juvenile fish (n = 10 fish per replicate) after acute transfer from 7 mM Na^+. All crosses presented as male x female. Note, F_2 crosses used surviving fish from initial hybridizations. Mean ± SEM (n = 6). Different letters indicate statistically significant difference between populations within treatments ($P < 0.05$).

Estimated K_m (1257–1492 μM) and V_{max} (7745-8016 nmol g^{-1} h^{-1}) values were not statistically different between the hybrids ($F_{2,124} = 2.405$, $P = 0.094$), but both *Cvh* x *Cvv* F_2 ($F_{2,124} = 23.32$, $P < 0.0001$) and *Cvv* x *Cvh* F_2 ($F_{2,124} = 28.09$, $P < 0.0001$) had significantly lower K_m and V_{max} than observed for *Cvv* (K_m = 7993 μM; V_{max} = 19274 nmol g^{-1} h^{-1}) enabling the hybrids to maintain Na^+ homeostasis at lower ambient concentrations.

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. 7). As would be expected, *Cvh* had the highest mean Na^+ uptake, with the F_2 hybrids intermediate, and *Cvv* the lowest (Kruskal–Wallis H = 41.145, d.f. = 3, $P < 0.001$), 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.

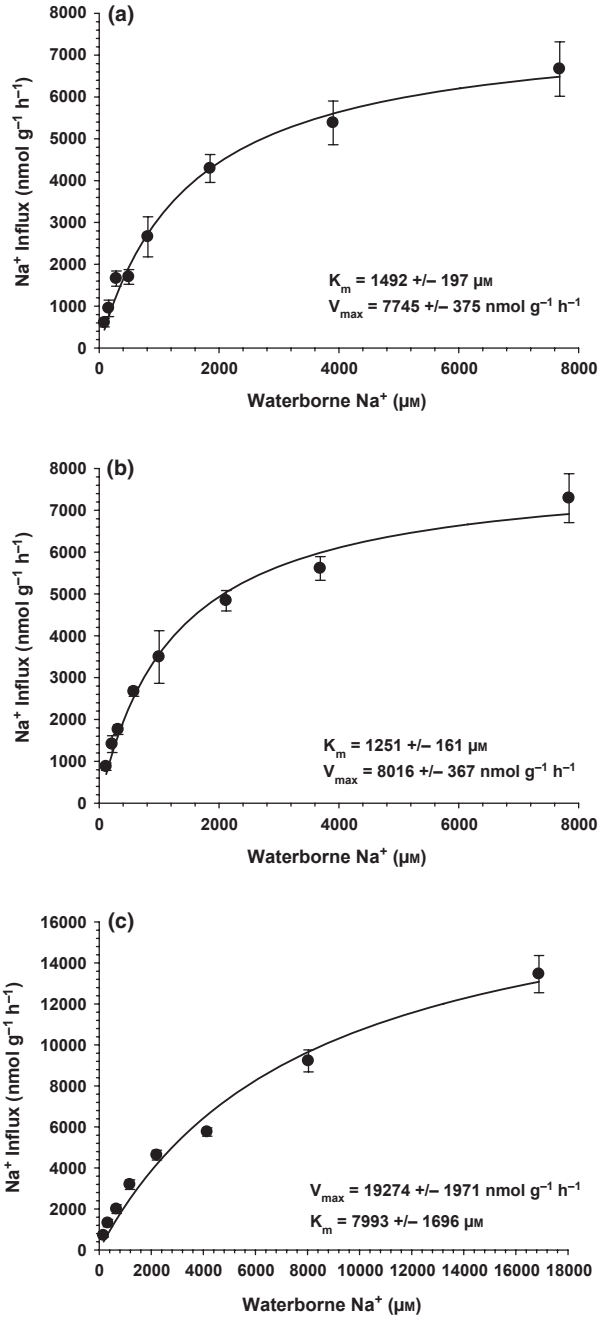


Fig. 6 Na^+ uptake rates (nmol g^{-1} h^{-1}) as a function of external Na^+ concentrations (μM). All fish acclimated to 1 mM Na^+. **(a)** *Cvh* x *Cvv* F_2. **(b)** *Cvv* x *Cvh* F_2. **(c)** *Cvv*. Mean ± SEM (n = 8).

Phylogeny of C. v. variegatus/hubbsi complex

All new sequences have been deposited in Genbank (JX856154-JX856168). 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. 8). Four clades were identified; Clade 1 included populations from Texas, Mississippi 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.

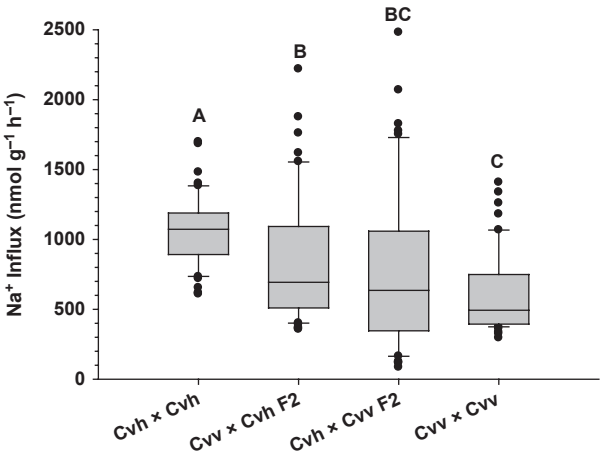
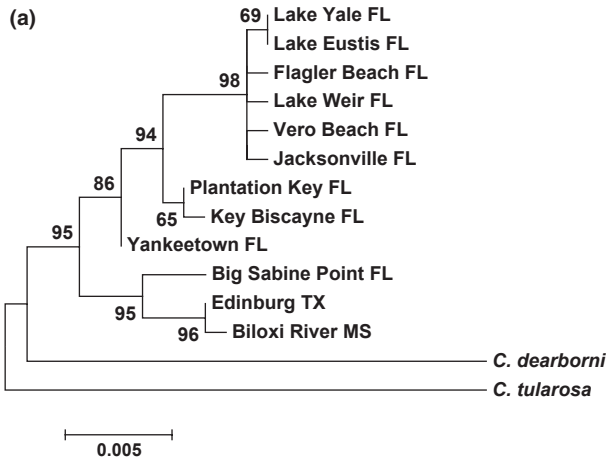


Fig. 7 Na^+ uptake rates (nmol g^{-1} h^{-1}) of fish exposed to 0.1 mM Na^+ after acclimation to 1 mM Na^+. Box whiskers represent 10th, 25th, 50th, 75th and 90th percentiles. Outliers represented by individual data points (n = 50 for each population). Different letters indicate statistically significant difference between populations within treatments ($P < 0.05$).



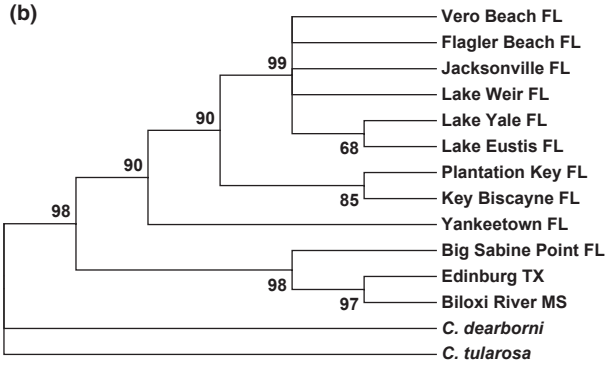


Fig. 8 Pupfish phylogency based on full length ND2 sequence. (a) Strict consensus maximum likelihood tree. Numbers at internal nodes represent bootstrap support (n = 500). (b) Maximum parsimony tree.

Discussion

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. 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 females (Fig. 3). Given the lack of response in visual and chemical cue 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, in a study of the *Cyprinodon* species flock in Lake Chicancanub (Mexico), Strecker & Kodric-Brown (1999) demonstrated strong con-specific mate selection by *C. maya* females in both visual and chemical cue experiments with *C. beltrani*, whereas *C. labiosus* females responded to chemical, but not visual cues, when paired against *C. maya* (Kodric-Brown & 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 & 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. 4a). This appears to be largely a maternal effect as *Cvv* x *Cvh* crosses were not significantly more fecund, whereas *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. 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 also appeared to be little effect of hybridization on 21-days larval survival (Fig. 4d). Although initial crosses of the two populations appears to elicit hybrid vigour in surviving larvae as evidenced by their significantly higher growth rates, this effect disappeared in the F_2 generation (Fig. 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. 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 days at 0.4 mM Na^+.

Na^+ uptake kinetics in the F_2 hybrids acclimated to 1 mM Na^+ were similar for the two crosses whereas *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 & 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 Na^+:H^+ exchanger (NHE) for Na^+ uptake at 2 mM Na^+ and a combination of NHE and an apical Na^+:K^+:2Cl^- cotransporter (NKCC) for Na^+ uptake in relatively high Na^+ waters ($\geq$ 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 ($\leq$ 1 mM Na^+), and a low affinity NHE in higher Na^+ waters ($\geq$ 2 mM Na^+), but lacks expression of NKCC for very high Na^+ uptake in more saline freshwater (Brix & 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. 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. 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. 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 geological 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 form the headwaters of the Oklawaha River system which drains north into the St. John's River, which discharges into the Atlantic Ocean at Jacksonville. These headwaters formed as marine depressions when the Penholoway terrace rose ~2 mya (Alt & Brooks, 1965). During the Aftonian interglacial (500–600 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 (200–400 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 have 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*, whereas 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 with these earlier studies, our analysis based on mtDNA sequence data for ND2, indicates 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 do not falsify 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. Alternatively, we cannot rule out the possibility of secondary contact between *Cvh* and northeast Florida *Cvv* populations. Hybridization of *Cyprinodon* populations during secondary contact is well documented (Childs *et al.*, 1996) and this study clearly demonstrates that *Cvh* and *Cvv* will hybridize if given the opportunity. A more comprehensive study using additional mitochondrial and nuclear genes, or the use of microsatellites, which have been developed for *Cyprinodon* (Burg *et al.*, 2002; Martin & Wilcox, 2004; Turner *et al.*, 2008), would likely resolve this uncertainty.

Are Cvv and Cvh different species?

Considering the foregoing data and analysis should *Cvv* and *Cvh* be considered separate species? Although 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. Within the context of species designation, it is important to note that many recognized species of *Cyprinodon* readily hybridize (Turner & 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, although 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 do argue for genetic differences. *Cvh* and *Cvv* raised in common garden conditions (7 mM Na^+ freshwater) have distinct Na^+ uptake characteristics with *Cvh* having distinctly higher affinity for Na^+ uptake, an adaptation necessary for survival in low Na^+ lakes. Furthermore, 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, most likely the result of differences in gene expression.

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 $\leq$ 1 mM Na^+. Although there are numerous populations of *Cvv* that occur either periodically or permanently in "freshwater" environments (McLane, 1955; Martin, 1968, 1972; Johnson, 1974; Dunson *et al.*, 1998; Lorenz & Serafy, 2006), all examples of *Cvv* permanently residing in freshwater are in environments with $\geq$ 5 mM Na^+. This 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*.

In addition, we observed that *Cvv* held at 1 mM Na^+ for ~150 days 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^+ reach reproductive maturity in < 80 days. 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 mitochondrial rich cell area in *Cvv* compared with *Cvh* at comparable salinities (Brix & 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. This fact alone, may justify designation of *Cvh* as a separate species.

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.

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