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Interspecific divergence of ionoregulatory physiology in killifish: insight into adaptation and speciation

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Abstract

Adaptation to salinity is potentially a critical driving force of speciation in fishes. Here, we tested for differences in ion/osmoregulatory gene expression between two species of killifish *Lucania goodei* and *L. parva* that differ in salinity tolerance. Expression patterns of several genes encoding ion transport proteins were quantified for animals taken directly from populations that varied in salinity, as well as animals from a salinity transfer experiment. We found that *L. parva*, a euryhaline species, expressed higher levels of the genes involved in saltwater ion/osmoregulatory regulation than its stenohaline counterpart *L. goodei* (Na^+/K^+-ATPase 1a and 1b, Na^+-K^+-$2Cl^-$ cotransporter 1 and glucocorticoid receptor) when exposed to a change in salinity in the laboratory. However, both species expressed similar levels for two of the three genes involved in freshwater osmoregulation (14-3-3a and V-type H^+-ATPase). Surprisingly, we found little evidence for differential plasticity between *L. parva* and *L. goodei* in our salinity transfer experiment. *Lucania parva* expressed high levels of genes involved in both freshwater and saltwater ion/osmoregulation, while *L. goodei* only expressed high levels of genes involved in freshwater osmoregulation. These results indicate that *L. parva* may increase their transcript levels of osmoregulatory genes when faced with any type of salinity challenge. Thus, changes in ion/osmoregulatory physiology may be occurring post-transcriptionally via differential RNA processing or enzyme activity. These findings provide unique insight into the ion/osmoregulatory physiology that underlies species and population differences in salinity tolerance.

Introduction

Differential salinity tolerance often distinguishes sister species in teleost fishes and may be an important driving force in ecological speciation (Gabrielsen, Brochmann & Rueness, 2002; Hrbek & Meyer, 2003; Huyse, Van Houdt & Volckaert, 2004; Whitehead, 2010). Ecological speciation posits that selection on traits in contrasting environments leads to reproductive isolation (Schluter, 1996, 2001; Rundle & Nosil, 2005). Although the mechanisms of ion/osmoregulation in teleost fish are relatively well known (Wood & Marshall, 1994; Perry, 1997; Marshall, 2002; Evans, Piermarini & Choe, 2005; Hwang & Lee, 2007), little is known about how these mechanisms have diverged between species with differential salinity tolerance. Previous comparative studies on salinity tolerance mechanisms have focused on non-sister species or on differences within the species level (Able & Palmer, 1988; Singer *et al*., 2002; Scott *et al*., 2004*b*; Bystriansky *et al*., 2006; Kang *et al*., 2008). However, there has been less focus on evolutionary divergence in ion/osmoregulation between closely related species differing in salinity tolerance. Comparing closely related species allows for a delineation of which aspects of the ion/osmoregulatory machinery are most labile to evolutionary change and the way in which they undergo modification, providing critical insights into the physiological mechanisms underlying ecological speciation.

In teleost fish, ion excretion in seawater via gills is thought to involve a basolateral Na^+/K^+-ATPase as well as a basolateral Na^+-K^+-$2Cl^-$cotransporter, which help create an electrochemical gradient favoring ion movement (Evans *et al*., 2005). A chloride channel is also involved in apical chloride secretion and may be regulated by cortisol via a glucocorticoid receptor (GR) (Kiilerich, Kristiansen & Madsen, 2007). The exact mechanisms for freshwater ion/osmoregulation in teleosts are less well defined but are thought to involve an apical V-type H^+-ATPase, which may be coupled with a sodium channel to absorb sodium. Carbonic anhydrase (CA) produces H^+ ions for the ATPase (by catalyzing the reaction of water and CO_2) and a recently discovered 14-3-3 signaling protein may regulate these processes (Kultz, Chakravarty & Adilakshmi, 2001).

The studies that have investigated divergence in ion/osmoregulatory mechanisms have focused on non-sister

species or populations within species. These studies have identified which of the aforementioned genes differ in expression in fresh and saltwater. Within the medakas, a freshwater and a brackish water species differed in patterns of expression of Na^+/K^+-ATPase upon exposure to fresh, brackish and saltwater (Kang *et al*., 2008). The freshwater species had the highest expression in freshwater while the saltwater species had the highest expression in saltwater. Among three salmonid species, the one with the greatest capacity for salinity acclimation (*Salmo salar*) also had the highest Na^+/K^+-ATPase expression (Bystriansky *et al*., 2006). In the mummichug (*Fundulus heteroclitus*), northern populations exhibit much higher survival in freshwater than southern populations across multiple life stages (Able & Palmer, 1988; Scott *et al*., 2004*b*). Upon osmotic challenge, these two populations differ in gene expression (Na^+/K^+-ATPase, Na^+-K^+-$2Cl^-$), enzyme activity (Na^+/K^+-ATPase) and gill morphology. The northern population increased expression and activity of Na^+/K^+-ATPase to a greater extent than did the southern population. Similarly, gene expression of the cystic fibrosis transmembrane receptor, which is involved in chloride ion transport, differs between two strains of salmon smolts with differential salinity tolerance (Singer *et al*., 2002). These studies indicate which genes and pathways may be expected to change when species/populations diverge in salinity tolerance. However, cases where sister species are examined and salinity is known to have played a role in the speciation event still need to be examined.

The current study examines differences in ion/osmoregulatory gene expression between two sister species with differential salinity tolerance. The Fundulidae family occupies diverse salinities and is a model system for salinity tolerance (Wood & Marshall, 1994; Burnett *et al*., 2007; Whitehead, 2010). The fundulids *Lucania goodei* and *L. parva* are particularly interesting because they are closely related sister species that have recently diverged (Duggins, Karlin & Relyea, 1983; Whitehead, 2010). Despite having 98% transcriptome sequence similarity (R. C. Fuller unpublished data), these two species differ radically in their salinity tolerance. *Lucania goodei* is found primarily in freshwater sites (restricted to Florida and southern Georgia) while *Lucania parva* is euryhaline and can be found in fresh, brackish and marine habitats (as far north as Massachusetts and as far west as central Mexico) (Lee, 1980). These differences in habitat correspond with differential adaptation to salinity at multiple life stages (Fuller, McGhee & Schrader, 2007; Fuller, 2008). Egg hatching success, larval survival and adult survival are reduced under high salinity conditions in *L. goodei* (Dunson & Travis, 1991; Fuller *et al*., 2007; Fuller, 2008). A breeding study between the two species has shown that these differences in salinity tolerance have led to extrinsic isolation (where the hybrids fail to fit into the parental niches) (Fuller, 2008).

The current study has two aims. First, we wanted to determine which ion/osmoregulatory genes (of the ones implicated in previous studies, see methods below) show differential expression between these two species. Second, we wanted to compare plasticity in gene expression between the two species. Since *L. parva* is euryhaline, we predicted that it should be more adept at adjusting to multiple environments than *L. goodei*, and should have greater phenotypic plasticity in gene expression. To meet these goals, we performed two studies. First, we collected fish directly from the field to determine whether gene expression from animals in natural populations varied between the two species and among populations within each of the species (field survey). For *L. parva*, we could compare gene expression between populations that were collected under different salinity conditions [freshwater ($n = 4$), brackish water ($n = 1$) and saltwater ($n = 1$)]. Second, we collected fish and exposed them to one of three salinity treatments (salinity challenge experiment). This allowed us to compare gene expression between species, populations within species, salinity treatments, and the interactions between salinity and species (salinity × species) and between populations nested within species and salinity [salinity × population (species)].

Materials and methods

Ion/osmoregulatory genes

Based on a review of the literature, we chose to measure expression of three genes involved in freshwater regulation and four genes involved in saltwater regulation. We chose the following freshwater genes: carbonic anhydrase 2 (CA2), V-type H^+-ATPase subunit A (H^+-ATPase) and 14-3-3a signaling protein (14-3-3). We chose the following saltwater genes: Na^+/K^+-ATPase α_{1a} (Na^+/K^+-ATPase 1a), Na^+/K^+-ATPase α_{1b} (Na^+/K^+-ATPase 1b), GR and Na^+-K^+-$2Cl^-$cotransporter 1 (NKCC1).

Field survey

The goal here was to compare gene expression between *L. goodei* and *L. parva*, as well as between *L. parva* populations that differed in salinity. Details on the sites can be found in Table 1. Fish were collected using dip nets and seines in July 2007. Salinity, in parts per thousand (ppt), was measured at each site using a YSI-63 meter (YSI Inc., Yellow Springs, OH). At each site, 2–3 animals from each species were euthanized with MS-222 (Argent Chemical Laboratories, Redmond, WA) immediately after collection. Their gills and opercula were removed and stored in RNAlater (QIAGEN, Valencia, CA). A literature survey failed to turn up any data on how MS-222 may affect expression. However, due to the immediate removal of the pertinent structures after death, any effect of MS-222 should be minimal. The samples were then transported back to the University of Illinois at Urbana-Champaign and stored at –20°C. We collected both the gills and operculum because prior research has shown that they are both involved in osmoregulation in fundulids (Scott *et al*., 2005).

Salinity challenge experiment

The goal here was to compare gene expression levels and phenotypic plasticity as a function of alterations in salinity

Table 1 Collection site information

Abbreviation	Site	Drainage	County	Site type	Type of fish collected	Salinity (ppt)	pH
3F[b]	3 Fingers	Wakulla River	Wakulla	Spring fed river	*L. parva* and *L. goodei*	0.2	8.2 (9.1 at time of lab animal collection)
BS	Blue Springs	Santa Fe River	Gilchrist	Spring fed river	*L. goodei*	0.2	8.1
DB	Delk's Bluff	St. John's River	Marion	Spring fed lake	*L. parva* and *L. goodei*	0.2	8.5
LB[b]	Lower Bridge	Wakulla River	Wakulla	Spring fed river	*L. parva* and *L. goodei*	0.2	8.1
LP[b]	Lighthouse Pond	Wakulla River	Wakulla	Coastal Brackish/Saltwater pond	*L. parva*	65 (25.8 at time of lab animal collection)[a]	8
MP	Mound's Pond	Wakulla River	Wakulla	Coastal Fresh/Brackish water pond	*L. parva*	7.2	Not recorded
UB[b]	Upper Bridge	Wakulla River	Wakulla	Spring fed river	*L. goodei*	0.1	8.1

[a]Lighthouse Pond was undergoing a dry down event when the salinity was 65 ppt (normal seawater is 34–34 ppt).
[b]Indicates sites where fish were collected both for the field survey and for the experiments. Sites whose abiotic conditions changed over time are denoted. Site values in parentheses reflect the conditions when experimental animals were collected.
ppt, parts per thousand.

between species, as well as between populations within species. Adult *L. parva* were collected from the following sites: Three Fingers (freshwater) and Lighthouse pond (saltwater). Adult *L. goodei* were collected from the Upper Bridge and Lower Bridge collecting sites (both freshwater). Animals were collected in March 2007. All sites were in Wakulla, Co., Florida. The fish were returned to Florida State University where they were sexed and measured. Afterward, they were immersed into one of three salinity treatments: 0, 10 and 20 ppt. There was one tank per population per salinity. Five fish were put into each tank with the exception of Lighthouse Pond fish where there were three fish per tank. Salinity treatments were created by filling 38 L aquaria with well water and adjusting the salinity with Instant Ocean® Sea Salt (Spectrum Brands, Atlanta, GA). Salinities were verified with a YSI-63 meter. Fish were fed daily with frozen brine shrimp, and lights were maintained on a 14L:10D cycle. Five days later, the surviving individuals were removed and euthanized with MS-222. Gills and opercula were removed and stored in RNAlater, and transported back to the University of Illinois at Urbana-Champaign. The samples were stored at –20°C.

Real-time polymerase chain reaction analysis of gene expression

We extracted mRNA from gills and opercula in a single reaction. Tissue was homogenized in 0.5 mL of TRIzol® (Invitrogen, Carlsbad, CA). Afterward, 200 μL of chloroform and 10 μL of glycogen (1 mg ml^{-1}) were added. After centrifugation, the clear aqueous layer was removed. Five hundred μL of isopropanol was added. The reaction was allowed to incubate on ice and then centrifuged. All liquid was removed, and 600 μL of 80% ethanol was added. The reaction was centrifuged, the ethanol was removed and the pellet allowed to dry. RNA concentration and integrity were verified using a spectrophotometer.

Total RNA was reverse transcribed into cDNA. The reaction contained 10 μL of RNA, 2.625 μL water and 2.5 μL oligo (dT)18 (Bioline, Taunton, MA). This reaction was heated to 65°C for 5 min then quenched on ice for 1 min. Five μL of 5X first strand buffer, 2.5 μL of 0.1 M DTT, 0.5 μL of 2 mM DNTP (Promega, Madison, WI), 0.625 μL of 40 U/μL RNase inhibitor (Fisher Scientific, Pittsburgh, PA), and 1.25 μL of 200 U/μL Superscript II were added. The reaction was then incubated at 25°C for 10 min, 42°C for 50 min, then 70°C for 15 min.

Quantitative real-time PCR (qRT-PCR) was performed for ion/osmoregulation genes. Primer sequences were taken from Scott *et al*., 2004*a* and Scott *et al*., 2004*b*, and can be found in Table 2.

Quantification of gene expression was performed with an ABI Prism 7900 sequence detector (Applied Biosystems, Foster City, CA). PCR reactions contained 5 μL SYBR® Green PCR master mix, 1.5 μL of 3 μM forward primer, 1.5 μL of 3 μM reverse primer and 2 μL cDNA. Three technical replicates of each cDNA sample were performed. Control reactions with no cDNA template were included to determine the level of background contamination. A 12-step

Table 2 Primers used in this study

Gene	Forward (5′–3′)	Reverse (5′–3′)
Na^+/K^+-ATPase 1a	AAG ATC ATG GAG TCC TTT AAG AAT CTG	CAC CTC CTC TGC ATT GAT GCT
Na^+/K^+-ATPase 1b	CAG TCA TGG GTC GGA TTG CT	TGG AGT GCG TCC AAC CTC TAG
NKCC1	CCC GCA GCC ACT GGT ATT	GCC ATC TGT GGG TCA GCA A
GR	GTA CCA AAA GAA GGC CTG AAG TG	CCT TGA TGT AAG TCA TCC TGA TCT CA
H^+-ATPase	TGA AGT TCA AGG ACC CGG TTA	CTG CGC GTA CTC GCC TTT
CA2	AGG GCT GAC GCT GAT TGG	CCT CTG CTG GAA AGC GTT ACC
14-3-3	CAA CGA GGA GCG CAA CCT	CGG GCA CCC ACA ACA TTC
EF1α	GGG AAA GGG CTC CTT CAA GT	ACG CTC GGC CTT CAG CTT

serial dilution standard curve was generated for each gene for each species five times using random cDNA samples. Results were then normalized to elongation factor 1α (EF1α), a common housekeeping gene. The absolute level of mRNA expression of each gene examined (X) was estimated using the following formula:

$$X = \text{efficiency}_{\text{focalgene}}{}^{-\text{ct}-\text{focalgene}} / \text{efficiency}_{\text{EF1}\alpha}{}^{-\text{ct}-\text{EF1}\alpha} \quad (1)$$

where efficiency is calculated from the slope of the standard curve after a log transformation, and ct corresponds to the threshold cycle number. Efficiency values for all primers can be found in supporting Table S1. Technical replicates were averaged giving one value per gene per biological sample.

Statistical analysis

The goal of the field survey was to determine whether there was variation within and among species in ion/osmoregulatory gene expression. General linear models were used to determine the effects of species and population on ln-transformed expression values. Because not all populations contained both species, we nested population within species. All main effects were fixed.

The goal of the salinity challenge experiment was to determine (1) whether there was variation within and among species, (2) whether there was overall plasticity as a function of salinity and (3) whether species or populations (nested within species) differed in their responses to salinity in ion/osmoregulatory gene expression. Hence, the analysis considered the effects of species, populations nested with species, salinity and all possible interactions. All main effects were modeled as fixed effects. We also performed a Tukey studentized range comparison to determine if individual species/salinity combinations differed from each other. Analyses were conducted using Proc Mixed and Proc GLM in SAS (Cary, NC).

Results

Overall, *L. parva* expressed higher levels of saltwater genes than did *L. goodei*, but this pattern was much stronger in the salinity challenge experiment than in the field survey. Field-caught *L. parva* had higher expression levels than *L. goodei* for two of the four genes involved in saltwater ion/osmoregulation (Fig. 1 and Table 3), while in the salinity challenge experiment, all four of the saltwater genes were expressed at higher levels in *L. parva* (Fig. 2 and Table 4). In contrast, there were no stark species differences in the overall expression of freshwater ion/osmoregulatory genes. (Figs 1,3, and S1; Tables 3 and 4). We examined expression of our control gene (EF1α) to determine if this was driving any patterns we saw. Expression levels of EF1α were not affected by any of our main effects for either our field or lab data. Below, we discuss the results for each of the genes. All of our results are shown in the tables, but only statistically significant (or nearly significant) results are displayed in figure form. For the salinity challenge, experiment Tukey comparisons supported the ANOVA results in all cases except for Na^+/K^+-ATPase 1b and GR. In these cases, although there were strong species differences across salinities, there were no differences within salinities.

Saltwater genes

Both Na^+/K^+-ATPase 1a and Na^+/K^+-ATPase 1b help create an electrochemical gradient favoring ion movement out of the cell in saltwater (Evans *et al*., 2005). *L. parva* had higher expression of Na^+/K^+-ATPase 1a than *L. goodei* in both the field survey (Fig. 1a, Table 3) and the salinity challenge experiment (Fig. 2a, Table 4). Na^+/K^+-ATPase 1b showed higher expression by *L. parva* in the salinity challenge experiment, but there was no significant species effect in the field survey (Tables 3 and 4, Figs 1b and 2b).

GR mediates cortisol levels, which in turn regulate chloride channels for the excretion of chloride (Kiilerich *et al*., 2007). Like all the other saltwater genes, *L. parva* also had higher expression of GR than *L. goodei* in the salinity challenge experiment (Fig. 2c, Table 4). While there was no species effect in the field survey, there was a marginally nonsignificant effect of population within species (Fig. 1c, Table 3).

NKCC1 helps create an electrochemical gradient favoring ion movement out of the cell in saltwater (Evans *et al*., 2005). *Lucania parva* had higher NKCC1 expression than *L. goodei* in both the field survey (Fig. 1d, Table 3) and the salinity challenge experiment (Fig. 2d, Table 4). NKCC1 was also one of two saltwater genes that varied with salinity in the salinity challenge experiment. NKCC1 expression increased with increasing salinity in both species (Fig. 2d).

Freshwater genes

CA is involved in multiple processes in the teleost gill, including gas exchange, acid-base balance and ion exchange (Sattin

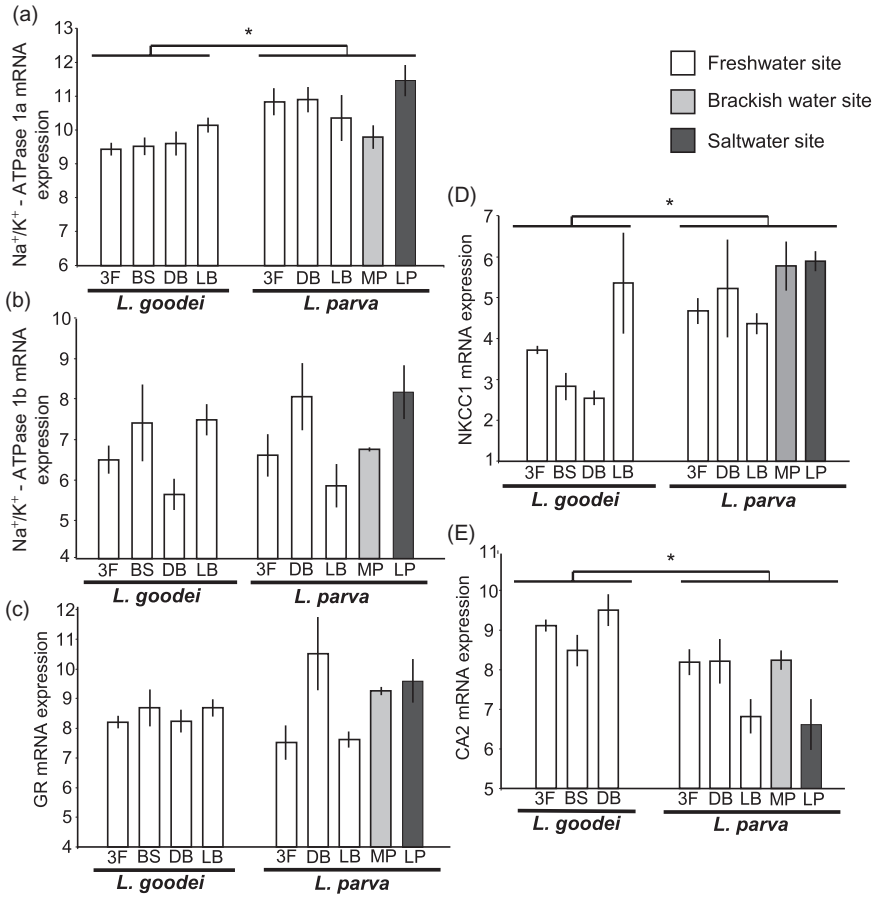

Figure 1 Population variation in field-caught animals. Expression of (a) Na^+/K^+-ATPase 1a, (b) Na^+/K^+-ATPase 1b, (c) GR, (d) NKCC1 and (e) CA2 normalized to EF1α then log transformed. Bars indicate ± SE. Note the change in *y*-axis scale between panels. Panels A–D represent saltwater genes, while panel E represents a freshwater gene. Asterisks denote statistically significant differences. 3F, 3 Fingers; DB, Delk's Bluff; LB, Lower Bridge; BS, Blue Springs; MP, Mound's Pond; LP, Lighthouse Pond.

Table 3 F- statistics for the field survey with *P*-values in parentheses. Significant values ($P < 0.05$) are in bold

Gene	Species	Population (species)
Saltwater genes		
Na^+/K^+-ATPase 1a	**$F_{1,15}$ = 16.3 (0.0011)**	$F_{7,15}$ = 2.25 (0.0883)
Na^+/K^+-ATPase 1b	$F_{1,16}$ = 0.67 (0.4265)	$F_{7,16}$ = 2.41 (0.0684)
GR	$F_{1,17}$ = 1.15 (0.2992)	$F_{7,17}$ = 2.49 (0.0590)
NKCC1	**$F_{1,17}$ = 5.04 (0.0383)**	$F_{7,17}$ = 1.07 (0.4207)
Freshwater genes		
CA2	**$F_{1,13}$ = 21.95 (0.0004)**	$F_{7,13}$ = 2.69 (0.0583)
H^+-ATPase	$F_{1,13}$ = 0.08 (0.7798)	$F_{7,13}$ = 1.92 (0.1462)
14-3-3	$F_{1,17}$ = 0.34 (0.5692)	$F_{7,17}$ = 1.28 (0.3183)

et al., 2010). CA plays a role in all three of these processes by catalyzing the reversible hydration/dehydration reactions of CO_2, producing equal amounts of H^+ and HCO_3^-. The field survey and the salinity challenge experiment indicated that *L. goodei* had higher CA2 expression than *L. parva* (Figs 1e and 3a). This was the only gene that we measured where *L. goodei* had higher gene expression than *L. parva*. Additionally, this was the only freshwater gene we examined where salinity altered expression. Unexpectedly, CA2 expression increased with salinity.

H^+-ATPase extricates hydrogen ions from gill cells in freshwater. This reaction is thought to be coupled with sodium absorption (Evans *et al*., 2005). There were no differences in H^+-ATPase expression between *L. parva* and *L. goodei* in either the field survey (Fig. S1, Table 3) or the salinity challenge experiment (Table 4, Fig 3b). However, there was a nonsignificant trend for H^+-ATPase expression to vary such that H^+-ATPase expression was highest in freshwater (0 ppt) and decreased with increasing salinity (Fig. 3b).

14-3-3 is a signaling protein thought to be involved in osmotic regulation (Kultz *et al*., 2001). There were no species effects in either the field survey or the salinity challenge experiment (Tables 3, 4, and S1, Fig. 3c).

Discussion

Our strongest result was that when exposed to a change in salinity, *L. parva* expressed higher levels of saltwater genes

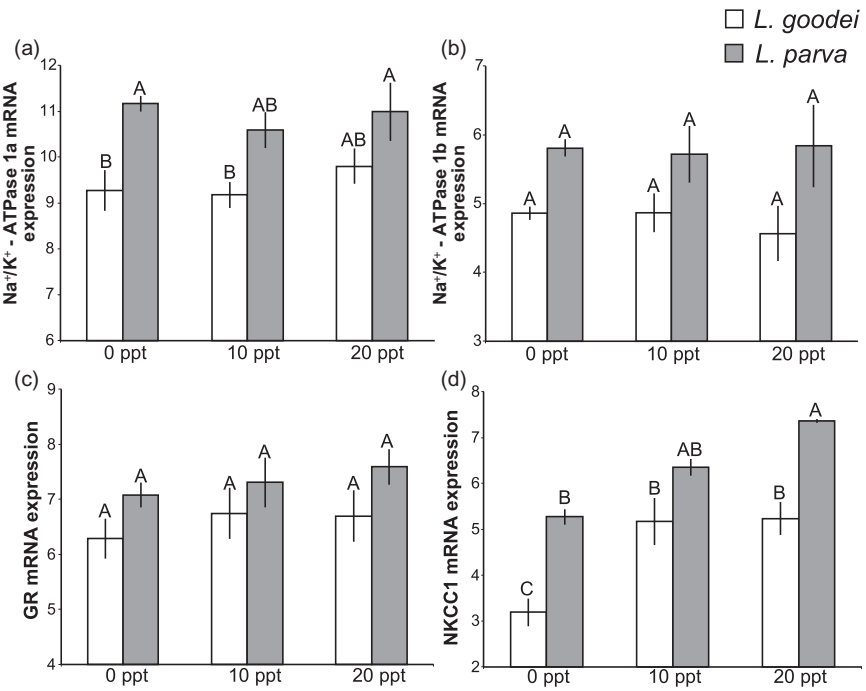

Figure 2 Expression of genes involved in saltwater osmoregulation in salinity challenge animals. Expression of (a) Na^+/K^+-ATPase 1a, (b) Na^+/K^+-ATPase 1b, (c) GR and (d) NKCC1 normalized to EF1α then log transformed. Species means averaged across populations are shown. Bars indicate ± SE. Note the change in *y*-axis scale between panels. Letters denote Tukey groups. Tukey groups with different letters are significantly different from each other. GR, glucocorticoid receptor; ppt, parts per thousand.

Table 4 F-Statistics for the salinity challenge experiment with *P*-values in parentheses. Significant values ($P < 0.05$) are in bold

Gene	Species	Population (species)	Salinity	Salinity × species	Salinity × population (species)
Saltwater genes					
Na^+/K^+-ATPase 1a	**$F_{1,18}$ = 40.44 (<.0001)**	$F_{2,18}$ = 0.05 (0.9546)	$F_{2,18}$ = 3.13 (0.0681)	$F_{2,18}$ = .35 (0.7114)	**$F_{4,18}$ = 5.38 (0.0050)**
Na^+/K^+-ATPase 1b	**$F_{1,18}$ = 21.68 (0.0002)**	$F_{2,18}$ = 1.23 (0.3163)	$F_{2,18}$ = 0.12 (0.8848)	$F_{2,18}$ = 1.40 (0.2720)	$F_{4,18}$ = 2.36 (0.0926)
GR	**$F_{1,16}$ = 8.19 (0.0113)**	$F_{2,16}$ = 0.26 (0.7744)	$F_{2,16}$ = 1.02 (0.3831)	$F_{2,16}$ = 0.38 (0.6895)	$F_{4,16}$ = 2.01 (0.1420)
NKCC1	**$F_{1,22}$ = 51.25 (<.0001)**	$F_{2,22}$ = 0.51 (0.6062)	**$F_{2,22}$ = 25.16 (<0.0001)**	$F_{2,22}$ = 1.46 (0.2528)	$F_{4,22}$ = 0.84 (0.5136)
Freshwater genes					
CA2	**$F_{1,20}$ = 13.10 (0.0017)**	$F_{2,20}$ = 3.28 (0.0584)	**$F_{2,20}$ = 9.30 (0.0014)**	$F_{2,20}$ = .77 (0.4753)	$F_{4,20}$ = 2.63 (0.0658)
H^+-ATPase	$F_{1,22}$ = 0.50 (0.2340)	$F_{2,22}$ = 1.46 (0.2539)	$F_{2,22}$ = 3.06 (0.0672)	$F_{2,22}$ = 0.40 0.6776	$F_{4,22}$ = 0.18 (0.9453)
14-3-3	$F_{1,20}$ = 0.01 (0.9229)	$F_{2,20}$ = 0.36 (0.7005)	$F_{2,20}$ = 1.20 (0.3228)	$F_{2,20}$ = 0.41 (0.6722)	$F_{4,20}$ = 1.20 (0.3417)

than did *L. goodei*. This occurred despite the fact that some of the *L. parva* populations inhabit freshwater. Conversely, there were few absolute differences between species in the expression of freshwater ion/osmoregulatory genes. Our observed species differences were robust to changes in salinity in our salinity challenge experiment, and we found little evidence for differential plasticity between *L. goodei* and *L. parva*. Below, we discuss these results in further detail.

Expression of genes involved in saltwater osmoregulation and ionoregulation

All four of the saltwater genes were expressed at higher levels in *L. parva* than *L. goodei* in the salinity challenge experiment (Fig. 2). However, in the field survey, only Na^+/K^+-ATPase 1a and NKCC1 were expressed at higher levels in *L. parva* compared with *L. goodei* (Fig. 1). Thus, *L. parva* does not have

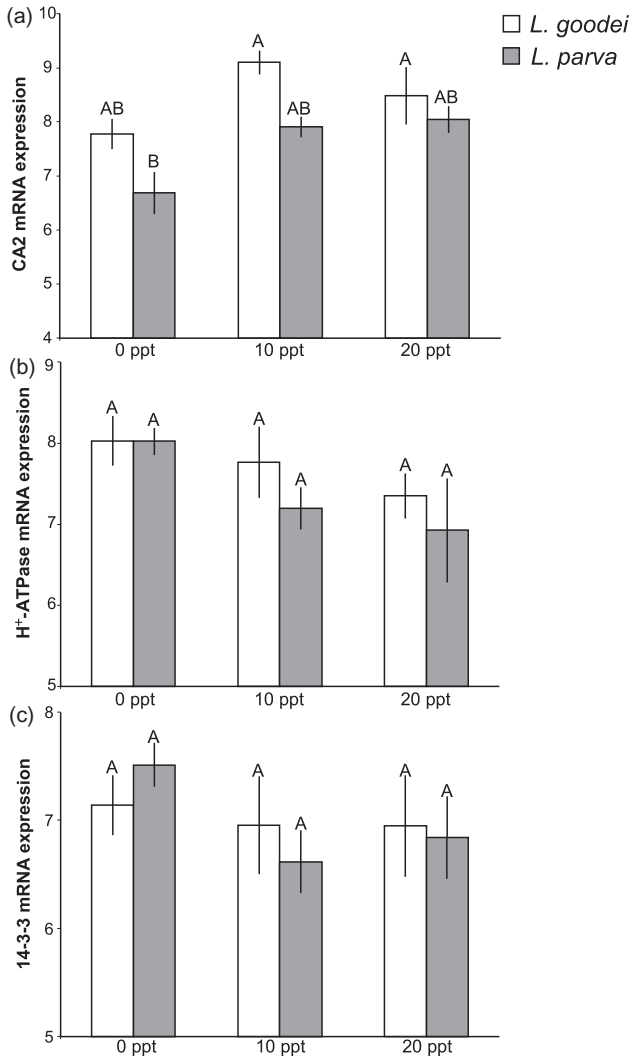

Figure 3 Expression of freshwater genes in salinity challenge animals. Expression of (a) CA2, (b) H^+-ATPase and (c) 14-3-3a normalized to EF1α then log transformed. Species means averaged across populations are shown. Bars indicate ± SE. Note the change in *y*-axis scale between panels. Letters denote Tukey groups. Tukey groups with different letters are significantly different from each other. ppt, parts per thousand.

high expression levels of these genes in the natural environment but increases expression of them to a greater extent than *L. goodei* upon entering a new environment. One possible explanation for this result is that *L. parva*, unlike *L. goodei*, is indeed able to mount a physiological response upon entering a new environment. Despite strong species differences, we observed a surprisingly small amount of overall phenotypic plasticity in the expression of saltwater genes between the different treatments. Only one of our four saltwater genes (NKCC1) showed an effect of salinity (Fig. 2). Additionally, our species specific differences remained strong across multiple salinity treatments. There are several physiological explanations for the lack of observed plasticity in *L. parva*. First, *L. parva* may react to all changes in salinity by increasing transcript levels with changes in ion/osmoregulatory physiology occurring post-transcriptionally via differential RNA processing or enzyme activity. Hence, the relationship between gene expression and the actual physiology may be weak. Second, we may have not measured the genes that are most vital to osmoregulation in our species. Several other enzymes, such as Na^+/H^+-exchanger and $Na^+/HCO3^-$ cotransporter, may be important and would have been missed by our experiment. Experimental design could also account low levels of observed plasticity. Phenotypic plasticity in gene expression may have been more transient than expected, and thus not captured by the time frame of this experiment. Scott *et al.* (2004*a*) have found that several genes in the closely related *F. heteroclitus* increase transiently over a 24 h period, and then return to normal levels by the fourth day. Because we assayed our fish on day five post-salinity transfer, we may have missed the period of greatest change and assayed the animals when the differences between salinities were less stark. Future studies would do well to examine gene expression on a finer temporal scale.

Gene expression of genes involved in freshwater osmoregulation and ionoregulation

CA is involved in multiple processes in the teleost gill, including gas exchange, acid-base balance and ion exchange (Sattin *et al.*, 2010). Although CA is present at high abundances in gill epithelial cells, studies examining the expression and activity in relation to salinity give contrasting results. Some studies show an increase in activity/expression of CA2 when fish are exposed to freshwater (Scott & Schulte, 2005), some show an increase when fish are exposed to saltwater or show higher CA2 levels for saltwater than freshwater populations (Kultz *et al.*, 1992), and other studies show no difference between populations or treatments in relation to salinity (Sender *et al.*, 1999; Tang & Lee, 2007).

We chose to categorize CA as a freshwater gene due to results in the closely related *F. heteroclitus*, where expression increased with decreasing salinity (Scott & Schulte, 2005). However, of our three freshwater genes, CA2 had the most complicated pattern. Two observations were consistent with CA2 playing a role in freshwater ion/osmoregulation. First, *L. goodei* had higher expression of CA2 than *L. parva* in both the field survey and the salinity challenge experiment (Figs 1e and 3a). Second, in the field survey, two of the three freshwater *L. parva* populations (3 Fingers and Delk's Bluff) had higher CA2 expression than the marine population (Lighthouse Pond) (Fig. 1e). These results suggest that animals from freshwater populations have higher CA2 expression than animals from saltwater populations. However, our salinity challenge experiment found an overall effect of salinity where CA2 expression increased with salinity (Fig. 3a). The discrepancies in our data may be due to the fact that we did not perfuse our gills with saline, and are thus measuring blood CA as well. However, other studies have found that perfusing has not changed overall expression levels (Perry, Beyers &

Johnson, 2000), and other studies have found strong effects of CA2 without perfusing (Scott *et al*., 2004*a*).

Our other two freshwater genes, H^+-ATPase and 14-3-3, showed no variation. Similarly, other studies have found no differences in H^+-ATPase expression between treatments or populations (Scott *et al*., 2004*a*; McCairns & Bernatchez, 2010). It is possible that in fundulids, H^+-ATPase is mostly used for acid-base regulation rather than osmoregulation due to its basolateral location. Another possibility is that H^+-ATPase plays a role in freshwater ion/osmoregulation in these species but is regulated primarily through post-transcriptional mechanisms. Reactivity of H^+-ATPase protein may increase in freshwater and decrease with increasing salinity (Katoh, Hyodo & Kaneko, 2003; Lee *et al*., 2011). 14-3-3 also showed no significant effects. Multiple studies in *F. heteroclitus* have found that 14-3-3 mRNA expression increased after transfer to freshwater (Kultz *et al*., 2001; Scott *et al*., 2005). The ion/osmoregulatory role of 14-3-3 has yet to be fully clarified, and 14-3-3 has been implicated in many pathways.

In conclusion, *L. parva*, the euryhaline species, consistently had higher expression of saltwater genes than *L. goodei*, the freshwater species. However, we found few overall differences between the two species in the expression of freshwater genes. When combined with previously published survival data (Dunson & Travis, 1991, R. C. Fuller, unpublished data), our results indicate that *L. goodei* may have lost the ability to deal with saltwater stressors without increasing their ability to deal with freshwater stressors. Our a *priori* expectation was that *L. parva* would show higher levels of phenotypic plasticity in gene expression in comparison to *L. goodei*. Instead, our study indicates that *L. parva* may maintain high transcript levels of osmoregulatory genes, allowing them to tolerate a broad range of salinities. These findings are consistent with the fact that salinity levels are much more variable for coastal *L. parva* populations than they are for freshwater *L. goodei* populations.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Expression of H^+ATPase and 14-3-3 from the field survey. Location variation in field-caught animals. RNA expression of (a) H^+ATPase and (b) 14-3-3 normalized to EF1α then log transformed. Bars indicate $\pm$SE. Note the change in y-axis scale between panels. 3F, 3 Fingers; DB, Delk's Bluff; LB, Lower Bridge; BS, Blue Springs; MP, Mound's Pond; and LP, Lighthouse Pond.

Table S1. Primer efficiencies.

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