Paralog switching facilitates euryhalinity: Ontogenetic, microevolutionary and macroevolutionary evidence

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Abstract

Euryhalinity is present in diverse aquatic taxa and requires flexible osmoregulation to field the challenges posed by differing salinities. Na^+, K^+-ATPase (NKA) is a ubiquitous ion pump in the gills of fishes and, for some species, paralogs of the catalytic α-subunit (NKA α1a and α1b) exhibit reciprocal expression between fresh- and seawater, termed paralog-switching. We investigated the expression and evolution of NKA paralogs in Alewife (*Alosa pseudoharengus*), a euryhaline and migratory fish. Comparisons between landlocked and diadromous life history forms and migrant and pre-migrant ontogenetic stages were used to study shifts in NKA paralog expression related to freshwater or seawater specialization. We exposed juvenile diadromous and landlocked alewives to freshwater (0 ppt) and seawater (30 ppt) for 2, 5, and 15 days. Additionally, we sampled migrant and pre-migrant alewives from the natal freshwater environment or after 24 hours in seawater. Diadromous Alewife exhibited salinity-dependent paralog switching, and the freshwater-specialized landlocked life history form showed greater upregulation of NKA α1b in seawater. Migrant Alewife showed a loss of freshwater readiness traded for seawater specialization through greater reliance (via upregulation) on NKA α1a in freshwater. Molecular phylogenies show Alewife NKA paralogs originated independently of paralogs in salmonids and other members of Euteleosteomorpha. This study demonstrated that NKA paralog switching is tied to halohabitat profile and that duplications of the ancestral NKA gene provided the substrate for multiple, independent molecular solutions for supporting a diadromous life history.

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ABSTRACT

Euryhalinity is present in diverse aquatic taxa and requires flexible osmoregulation to field the challenges posed by differing salinities. Na^+, K^+-ATPase (NKA) is a ubiquitous ion pump in the gills of fishes and, for some species, paralogs of the catalytic α-subunit (NKA α1a and α1b) exhibit reciprocal expression between fresh- and seawater, termed paralog-switching. We investigated the expression and evolution of NKA paralogs in Alewife (*Alosa pseudoharengus*), a euryhaline and migratory fish. Comparisons between landlocked and diadromous life history forms and migrant and pre-migrant ontogenetic stages were used to study shifts in NKA paralog expression related to freshwater or seawater specialization. We exposed juvenile diadromous and landlocked alewives to freshwater (0 ppt) and seawater (30 ppt) for 2, 5, and 15 days. Additionally, we sampled migrant and pre-migrant alewives from the natal freshwater environment or after 24 hours in seawater. Diadromous Alewife exhibited salinity-dependent paralog switching, and the freshwater-specialized landlocked life history form showed greater upregulation of NKA α1b in seawater. Migrant Alewife showed a loss of freshwater readiness traded for seawater specialization through greater reliance (via upregulation) on NKA α1a in freshwater. Molecular phylogenies show Alewife NKA paralogs originated independently of paralogs in salmonids and other members of Euteleosteomorpha. This study demonstrated that NKA paralog switching is tied to halohabitat profile and that duplications of the ancestral NKA gene provided the substrate for multiple, independent molecular solutions for supporting a diadromous life history.

KEY WORDS

NKA, paralog, euryhalinity, salinity, osmoregulation, diadromous

INTRODUCTION

Euryhalinity, the ability to tolerate a wide range of salinities, is a generalist physiological strategy that has facilitated the diversification of marine taxa into freshwater halohabitats, or habitats defined by salinity, over evolutionary time (Schultz and McCormick 2013). Euryhaline fishes possess the ability to maintain ion and water homeostasis (a capacity known as osmoregulation) in environments ranging from freshwater to seawater. This ability is particularly challenging given that the salt concentration of these environments varies dramatically (˜10 mOsm/0 ppt in freshwater vs. ˜1,050 mOsm/35 ppt in seawater). Such osmoregulatory flexibility requires that fish shift between absorbing ions in a dilute freshwater environment and secreting ions in a concentrated seawater environment. These functions are achieved by suites of ion pumps, channels, and transporters in specialized gill cells known as ionocytes (Evans et al. 2005; Marshall & Groswell 2006; Edwards & Marshall 2012). Perhaps because it is difficult to evolve the physiological machinery to accomplish these complex adjustments, there are relatively few fishes whose halohabitat includes both freshwater and seawater. Nonetheless, the capacity for euryhalinity has repeatedly permitted prominent bouts of adaptive radiation following colonization of freshwater by ancestrally marine taxa (Lee and Bell 1999; Betancur-R et al. 2012; Schultz and McCormick 2013). Hence, clarifying the proximate and ultimate underpinnings of euryhalinity deepens our understanding of how organisms adapt and diversify in divergent environments.

Differences in cellular requirements for osmoregulation in freshwater and seawater (as well as for various roles in other vertebrate tissues) have driven diversification of Na^+, K^+-ATPase (NKA) structure and function. Active transport by NKA creates electrochemical gradients that power transmembrane transport of ions, promoting ion absorption in hyper-osmoregulators and secretion in hypo-osmoregulators. The α-subunit of NKA is the catalytic subunit and therefore contains energetically important, and potentially functionally meaningful, binding sites (Lingrel and Kuntzweiler 1994). Multiple forms of the α-subunit are documented in teleost fishes and arose either early in the evolution of vertebrates as products of whole- or partial-

genome duplications, or from alternative gene splicing (Serluca et al. 2001; Sáez et al. 2009). Previous study of molecular diversity in the ion pump NKA in the gills of fishes have led to the hypothesis that salinity-dependent and reciprocal differential regulation between alternate forms of this enzyme, termed paralog-switching, has facilitated the evolution of euryhalinity (Dalziel et al. 2014). We refer to paralogs in a general sense to describe different forms of gene products, including those arising via post-transcriptional processing into splice variants that share one locus, and those that arise via transcription at duplicated genes (Koonin 2005). Two NKA α-subunit paralogs prominent in gill ionocytes of several euryhaline fishes, called NKA α1a and NKA α1b, which contain functionally important transmembrane region protein substitutions are upregulated upon exposure to freshwater and seawater, respectively (Salmonids: Richards et al. 2003, Shrimpton et al. 2005, Bystriansky et al. 2006, Nilsen et al. 2007, Jorgensen 2008, Larsen et al. 2008, Madsen et al. 2009, McCormick et al. 2009 & 2013, and Urbina et al. 2013; Cichlid: Tipsmark et al. 2011; Galaxiid: Dalziel et al. 2014).

The hypothesis that paralog-switching facilitates euryhalinity leads to an expectation that switching should be functionally important and more pronounced in taxa with diverse halohabitats than in taxa that are restricted to a narrow range of salinities. Alewives (*Alosa pseudoharengus*) present an exceptional opportunity to investigate the adaptive importance of NKA paralog-switching at a microevolutionary scale. Ancestrally, Alewife are diadromous – individuals hatch in freshwater, mature at sea, and complete spawning migrations back to freshwater. Multiple landlocked populations (i.e., those restricted to freshwater due to damming or other dispersal-preventing events) of Alewives occurring along the east coast of North America were independently derived from diadromous runs in the Holocene (Palkovacs et al. 2008). Physiological studies of landlocked Alewives have shown reduced tolerance to seawater and enhanced tolerance to freshwater compared to diadromous Alewives, underpinned by relaxed selection on seawater responsive gene expression in the landlocked form (Velotta et al. 2014, 2015, 2017). While differences in NKA enzyme activity exist between the diadromous and landlocked life history forms (LHFs), enzyme activity assays are not paralog-specific and functional studies are needed to investigate paralog-switching (Christensen et al. 2012; Velotta et al. 2015). On a within-population scale, diadromous Alewives have discrete ontogenetic stages with expected differences in seawater-readiness. During the juvenile stage, migratory behavior is presumably dictated by physiological preparedness for challenges like salinity transition and sustained swimming demands (Zydlewski & McCormick 1997a, b). Juveniles that have initiated migration, which we term migrants, are expected to be better prepared for seawater than pre-migrants that continue to hold in the natal freshwater environment. Because it is both energetically demanding and inextricably linked to euryhalinity, NKA activity is expected to be tightly regulated and subject to relatively strong selection (Evans et al. 2005; Lee et al. 2011; Schultz and McCormick 2013). Thus, we expect NKA gene expression differences between diadromous and landlocked Alewives to be pronounced and similar differences to exist between migratory stages of the diadromous form.

An additional expectation arising from the 'euryhalinity via paralog-switching' hypothesis is that the patterns of evolutionary divergence between the NKA paralogs involved in switching should correspond with origins of euryhalinity; in cases where euryhalinity has been independently derived in phylogenetically-distinct lineages of bony fishes, specialization of NKA paralogs for freshwater versus seawater function should also be independently derived. NKA paralog-switching has been described previously in salmonids and cichlids, two taxa that independently evolved euryhalinity (Urbina et al. 2013, Dalziel et al. 2014). The euryhaline clade to which the Alewife belongs (Alosinae) arose within a clade of largely marine fishes (Li and Ortí 2007; Bloom and Lovejoy 2014). In this context, we predict that specialization of NKA paralogs in Alewife has been independent of specialization in these other teleosts.

The goals of the present study were to: (i) test for the presence of NKA paralog-switching in a euryhaline species of bony fish that is distantly related to taxa in which it has been previously documented; (ii) compare expression of paralogs within diadromous and landlocked populations as well as migrant and pre-migrant ontogenetic stages; and (iii) incorporate the Alewife paralogs into a phylogenetic analysis of NKA evolution. We predicted that: (1) NKA paralogs in diadromous Alewife exhibit paralog-switching in response to salinity challenge; (2a) landlocked Alewives exhibit freshwater specialization via dampened paralog-switching, espe-

cially through reduced upregulation of NKA α1b upon seawater challenge; (2b) diadromous migrant Alewives exhibit seawater preparedness compared to the pre-migrant stage via decreased NKA α1a expression in the natal, freshwater environment and/or greater upregulation of NKA α1b upon seawater challenge; and, (3) that Alewife paralogs originated independently of those in other bony fish groups.

METHODS

Field collection – We collected young-of-the-year diadromous Alewives from Bride Lake in East Lyme, Connecticut, USA (41.3271° N, 72.2379° W) in 2011 and 2018, and landlocked young-of-the-year Alewives from Rogers Lake in Old Lyme, Connecticut, USA (41.3637° N, 72.3000° W) in 2011. All fish caught in 2011 were captured via purse seine during nighttime hours (Velotta et al. 2015). Fish caught in 2018 represented two discrete ontogenetic stages and were either captured by weir trap at the Bride Lake outflow (migrants) or purse seine within the main body of the lake (pre-migrants). On each collection date, we euthanized a subset of fish and sampled *in situ* for total length and wet mass. Additionally, we sampled *in situ* for gill tissue in 2018. All fish were collected and handled in compliance with protocol A17-010 as approved by the University of Connecticut Institutional Animal Care and Use Committee.

Salinity trials – Following transportation to the Conte Anadromous Fish Research Center (Turners Falls, Massachusetts, USA) we transferred fish to 1,200 L recirculating tanks equipped with charcoal filters and maintained at 0.5 ppt salinity. Fish were acclimated to this common salinity in the lab for 4 weeks prior to salinity trials. For salinity trials in 2011, we adjusted tank salinities to 0 ppt (deionized freshwater) or 30 ppt (seawater) and sampled 6 individuals per tank at 2-, 5-, or 15-days post-transfer (experiment reported in Velotta et al. 2015). In 2018, we adjusted tank salinities to 30 ppt and sampled fish after 24 h. A salinity of 30 ppt was chosen because it approximates the strength of the Long Island Sound, into which both lakes historically flowed, and because previous studies showed that 30 ppt is sufficient to induce hypo-osmoregulation while leading to little mortality (Velotta et al. 2015). Each salinity treatment was performed in duplicate and, once treatments were completed, we euthanized Alewives, recorded total length and wet mass, and sampled gill tissue. All collected tissue was stored in RNAlater at -20 °C until RNA extraction.

Molecular assay preparation – We extracted RNA from homogenized gill tissue using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA), treated the extracts with DNase using the TURBO DNA-free kit (Life Technologies, Grand Island, NY, USA), and stored extracts at -80 °C for up to 6 months. We reverse transcribed samples using ˜500 ng of RNA with the High-Capacity cDNA Reverse Transcription Kit with RNase Inhibitor (Life Technologies, Grand Island, NY, USA) to obtain cDNA for quantitative analysis. A recently assembled Alewife transcriptome (Velotta et al. 2017) provided reference data for identification of candidate NKA paralogs. Using Primer3 (Koressaar and Remm 2007; Untergasser et al. 2012), we designed sequence-specific primers for Alewife transcripts that best matched known NKA α1a and α1b sequences according to NCBI BLAST (blast.ncbi.nlm.nih.gov). Prior to quantitative PCR, we performed a qualitative check for salinity-dependent expression in our chosen sequences via PCR and gel electrophoresis.

Quantitative PCR – We used real-time PCR (qPCR) to quantify expression through mRNA abundance of NKA paralogs. Primers suitable for qPCR (Supplemental Table 1) were designed for Alewife NKA paralogs, as well as EF1α, a common qPCR reference gene reported to be relatively unresponsive to variable environmental conditions (e.g., Ye et al. 2010; De Santis et al. 2011; Hu et al. 2014). We confirmed that amplification efficiency of primers fell within norms of standard qPCR methods. To confirm the validity of EF1α as a reference gene, we tested for salinity-invariance of EF1α. We found a slight salinity response in EF1α (Supplemental Table 2).

To perform qPCR, we ran samples in triplicate with Bio-Rad iTaq Universal SYBR Green Supermix using a Bio-Rad iCycler (Bio-Rad Laboratories, Hercules, CA, USA) under the following thermocycler conditions: 10 minutes at 95 °C, 45 cycles of 95 °C for 20 seconds and 60 °C for 50 seconds. We included triplicates of the appropriate standard on each qPCR plate to enable correction for potential plate-to-plate variance in analysis conditions. We quantified gene expression as (Pfaffl 2001):

$$\Delta\Delta\tilde{}_T \;=\; \frac{E_{\text{tar}}^{\,C_{\text{T}}\ \text{tar}\ (calibrator\ -\ test)}}{E_{\text{ref}}^{\,C_{\text{T}}\ \text{ref}\ (calibrator\ -\ test)}} \text{(Equation 1)}$$

in which E_{tar} is the amplification efficiency of the target NKA paralog primer, E_{ref} is the amplification efficiency of the EF1α primer, ΔC_T tar (target) is the cycle threshold value difference between calibrator and test sample for the target NKA paralog, and ΔC_T ref (reference) is the cycle threshold value difference between calibrator and test sample for EF1α.

To facilitate comparison of NKA paralog responses to salinity treatments, we quantified relative expression levels as a normalized paralog ratio as:

$$\Delta\Delta\tilde{}_{T,\ rel} \;=\; \frac{E_{\alpha 1b}^{\,C_{\text{T}}\ \alpha 1b\ (calibrator\ -\ test)}}{E_{\alpha 1a}^{\,C_{\text{T}}\ \alpha 1a\ (calibrator\ -\ test)}} \text{(Equation 2)}$$

in which $E_{\alpha 1\alpha}$ is the amplification efficiency of the NKA α1a primer, $E_{\alpha 1\beta}$ is the amplification efficiency of the NKA α1b primer, ΔC_T α1a is the cycle threshold value difference between calibrator and test sample for NKA α1a, and ΔC_T α1b is the cycle threshold value difference between calibrator and test sample for NKA α1b. This ratio represents the extent of the shift from expression of NKA α1a to NKA α1b.

We tested the effect of predictor variables on paralog expression via mixed-effects model analysis in R 1.4.1106 run through RStudio 1.4.1717 software (RStudio Team 2019; R Core Team 2020). To test the prediction that the NKA paralogs were differentially expressed in response to freshwater and seawater challenges (prediction 1), we tested for a salinity effect on $\Delta\Delta C_T$ of both paralogs, expecting $\Delta\Delta C_{T,\ rel} > 1$ in seawater and < 1 in freshwater. To test whether diadromous and landlocked Alewives differed in their response to freshwater and seawater challenges (prediction 2a), we combined diadromous and landlocked datasets and tested for an LHF effect. The prediction would be supported if there was an LHF-by-salinity interaction, the $\Delta\Delta C_{T,\ rel}$ was greater in the diadromous Alewives in seawater, and/or $\Delta\Delta C_{T,\ rel}$ was smaller in the landlocked Alewives in freshwater. We coded replicate tanks as random effects, included individual length as a continuous covariate, and main effects as categorical variables. Normalized estimates of gene expression $\Delta\Delta C_T$ and $\Delta\Delta C_{T,\ rel}$ were log transformed to eliminate mean-variance relationships. The full models included all interactions among main effects. We reduced models via backwards elimination in which the highest-order interactions remaining in the model were eliminated if they were not significant (i.e., $p > 0.05$). We further simplified models with multiple interactions by eliminating individual length when no effects including it were significant. Because LHF interactions were typically significant, and diadromous and landlocked Alewife populations are known to have different salinity tolerances, we analyzed the LHFs separately for target genes. Similarly, because time × salinity interactions were typically significant, we performed separate analyses for each timepoint of the 2011 dataset. To test prediction 2b, that migrant and pre-migrant Alewives differed in their response to *in situ* freshwater conditions and seawater challenge, we tested for an ontogenetic effect. The prediction would be supported if there was a group-by-salinity interaction (group referring to ontogenetic stage), the $\Delta\Delta C_T$ α1a was lower in migrants compared to pre-migrants in freshwater, and/or $\Delta\Delta C_T$ α1a was greater in migrants compared to pre-migrants in seawater. We included individual length as a continuous covariate and coded replicate tanks as random effects, main effects as categorical variables, and trial date as a random factor.

Molecular phylogenetics – To test whether Alewife paralogs originated independently from paralogs found in other bony fish groups (prediction 3), we conducted a molecular phylogenetic analysis of paralog relationships. Previously identified sequences obtained from NCBI GeneBank or Ensembl were compiled in Geneious 2021.1 software (Kearse et al. 2012; Supplemental Table 3). Nucleotide and amino acid alignments were made using the Translation Align and Geneious Alignment tools, respectively. We analyzed resulting alignments with PartitionFinder 2.1.1 (Stamatakis 2006; Lanfear et al. 2012, 2016) to identify the most appropriate evolutionary models for nucleotide and amino acid phylogenetic analyses. The selected model for nucleotide evolution was GTR+I+G (for all three codon positions, partitioning positions 1 and 2 together and codon 3 separately). The selected model for amino acids was WAG+I+G. Using the MrBayes and RAxML plug-ins for Geneious, we employed both Bayesian and Maximum Likelihood approaches for phylogenetic analysis (Stamatakis 2006). An NKA α1 sequence belonging to Spotted Gar (*Lepisosteus oculatus*), a non-teleost

bony fish, was used as an outgroup. Additionally, we compared 21 amino acid substitution sites of NKA α1a and α1b paralogs that have been identified as functionally important and/or under positive selection in salmonids (represented by Rainbow Trout, *Oncorhynchus mykiss* ; Jorgensen et al. 2008; Dalziel et al. 2014). All taxonomic classifications used in this analysis were derived from the most recent DeepFin phylogeny (Version 4; Betancur-R et al. 2017).

RESULTS

Expression of gill NKA α1a was upregulated in freshwater and down-regulated in seawater and expression of gill NKA α1b was upregulated in seawater (at least initially), supporting our predictions about paralog-switching in diadromous alewives (Figure 1a). However, contrary to our prediction, NKA α1b was not clearly downregulated in freshwater (Figure 1b). $\Delta\Delta C_T$ for α1b (Equation 1) exceeded $\Delta\Delta C_T$ for α1a by almost two orders of magnitude in seawater and, by day 15, was one fifth of $\Delta\Delta C_T$ for α1a in freshwater (Figure 1c). Regulation of NKA α1a and α1b was affected by both salinity treatment and time point (Supplemental Table 4), and interactions involving day and salinity were significant for both paralogs (mixed-effects model; diadromous LHF $\Delta\Delta C_{T,\ NKA\ \alpha1\alpha}$: salinity × day $F_{1,32} = 62.4$, $p < 0.001$, $\Delta\Delta C_T$ α1b: salinity × day $F_{1,32} = 13.3$, $p < 0.001$) and for the normalized paralog ratio (mixed-effects model; diadromous LHF $\Delta\Delta C_{T,\ rel}$: salinity × day $F_{1,32} = 44.1$,$p < 0.001$).

The prediction (2a) that landlocked individuals have dampened paralog-switching and reduced upregulation of NKA α1b in seawater was partially supported. Landlocked individuals retain salinity-dependent regulation of the NKA paralogs (Figure 1d & 1e). The $\Delta\Delta C_T$ of α1b exceeded that of α1a by only one order of magnitude in seawater and was one fifth of $\Delta\Delta C_T$ for α1a in freshwater by day 15 (Figure 1f). Our analysis of relative paralog expression showed a significant interaction involving salinity and LHF (mixed-effects model; $\Delta\Delta C_{T,\ rel}$: salinity × LHF $F_{1,16} = 19.4$, $p = 0.005$, all effects in Supplemental Table 5). Compared to the diadromous LHF, relative expression ($\Delta\Delta C_{T,\ rel}$) in the landlocked LHF was significantly muted in seawater, supporting our predicted dampening of paralog-switching (Table 1). Landlocked individuals also showed higher expression of α1a in seawater compared to the diadromous LHF, resulting from a decrease in downregulation. Contrary to our prediction, landlocked individuals had heightened $\Delta\Delta C_T$ for α1b in response to seawater.

Seawater readiness of Alewife migrants was present, as predicted (2b); however, it manifested differently than expected. Migrants showed greater upregulation of NKA α1a in natal freshwater conditions and no differences in $\Delta\Delta C_T$ for α1a or α1b in seawater (Figure 2a-c). The significant upregulation of NKA α1a in migrant individuals is also reflected in the lower $\Delta\Delta C_{T,\ rel}$ compared to pre-migratory individuals. Our analysis of the effects of salinity, ontogenetic stage, and length on the paralogs and normalized paralog ratio showed a stage effect on $\Delta\Delta C_T$ α1a but not $\Delta\Delta C_T$ α1b or $\Delta\Delta C_{T,\ rel}$ (mixed-effects model; diadromous LHF $\Delta\Delta C_T$ NKA α1a: group $F_{1,16} = 13.9$, $p = 0.002$, $\Delta\Delta C_T$ NKA α1b: group $F_{1,16} = 0.1$, $p = 0.718$, $\Delta\Delta C_{T,\ rel}$: group $F_{1,16} = 4.2$, $p = 0.054$; all effects in Supplemental Table 6).

Our phylogenetic analysis provides evidence for an independent origin of NKA paralogs in alewives. The Alewife NKA paralogs did not cluster with the paralogs from either salmonids or other teleosts (Figures 3 and 4; see supplemental figures 1 and 2 for corresponding maximum likelihood phylogenies). Instead, they were most closely related to each other, supporting an independent duplication event in the lineage leading to alewives. Salmonid NKA paralogs formed paralog-specific clades (Figures 3 and 4), as expected.

The inferred relationships of NKA paralogs in Galaxiiformes and Anabantiformes differed in trees inferred from amino acid and nucleotide data. Clustering by taxon in the nucleotide-based phylogeny suggests independent origins (Figure 3, while paralog-specific groupings with salmonids suggest a more ancient origin (Figure 4). Amino acid substitutions, some of which are functionally important for the direction of ion exchange and/or predicted to have arisen via positive selection, have been identified in salmonids (Jorgensen et al. 2008; Dalziel et al. 2014). Our results suggest that many of these changes have evolved convergently, and that this convergence is stronger than the signal of shared history for the paralogs. The salmonid paralogs show 35 conserved amino acid differences between paralogs. Alewife share 22 of these with salmonids in NKA

α1b and 10 in NKA α1a (Figure 5).

DISCUSSION

Our study provides evidence for multiple, independently-derived solutions to the physiological demands of euryhalinity. Key differences in NKA paralog sequences between species indicate that functional convergence of strategies for facilitating euryhalinity has occurred in the presence of molecular divergence. One pathway for the evolution of paralog-switching requires gene duplication, gene product diversification, and reciprocal differentiation in expression pattern – not a small or simple feat. Yet multiple, distantly related fishes (e.g., Alewife and salmonids) have converged on this similar, complex response enabling euryhalinity. Our findings also suggest that there are shared evolutionary pathways following landlocking in ancestrally euryhaline species, and paralog regulation at ontogenetic stages is related to halohabitat variability.

Origins of NKA paralogs – This study presents the first evidence that a clupeid species has evolved NKA paralog-switching, offering novel insight into the history of this osmoregulatory strategy. The first studies on the presence of NKA α1a and α1b paralogs were conducted on salmonids (Rainbow Trout, Richards et al. 2003; Sockeye Salmon, Shrimpton et al. 2005; Atlantic Salmon, Nilsen et al. 2007), and since then taxonomic coverage has broadened to include NKA α1-subunit paralogs in distantly-related species such as Mozambique Tilapia (*Oreochromis mossambicus* , Tipsmark et al. 2011), Inanga (*Galaxias maculatus* , Urbina et al. 2013), Rainbow Smelt (*Osmerus mordax* , Dalziel et al. 2014), and Sacramento splittail (*Pogonichthys macrolepidotus* , Mundy et al. 2020). Many, though not all, of these species exhibit paralog switching of NKA α1a and α1b, and the presence of such salinity-dependent expression is associated with euryhaline lifestyles, especially those involving movement between discrete salinities (i.e., migration). NKA paralog-switching likely represents an energetically efficient strategy for alternating between hypo- and hyper-osmoregulatory abilities (Jorgensen 2008).

The strong, salinity-driven differential expression of NKA α1a and α1b in Alewife (Figure 1a-c) resembles paralog switching dynamics in previously studied taxa. Consistently in these cases, NKA α1b is not up-regulated to the same magnitude or duration upon exposure to seawater as NKA α1a is upon exposure to freshwater. Expression of NKA α1a is elevated under hyper-osmoregulatory conditions for at least for 14-15 days in Alewife, similarly to Mozambique Tilapia and Atlantic Salmon (Bystrianski 2011; Tipsmark 2011). In contrast, expression of NKA α1b under hypo-osmoregulatory conditions exhibits a distinct 2- or 3-day peak followed by a decline in diadromous alewives, similarly to Rainbow Trout and Inanga (Richards et al. 2003; Urbina et al. 2012). NKA α1a may serve as a longer-term aid to freshwater osmoregulation than NKA α1b is to seawater osmoregulation. Perhaps NKA α1b is critical as an acute response for survival during initial transition while other mechanisms contribute to long term seawater tolerance and osmoregulation.

Evidence for functional significance of NKA paralogs: life-history form comparison – We found evidence for evolution in the landlocked LHF toward freshwater specialization through changes in NKA paralog expression. Paralog-switching was dramatically dampened in the landlocked LHF, in that relative expression measured as little as 3% of the corresponding diadromous values in seawater (Table 1). Hence there is much less difference between expression levels (less "switching") of NKA α1a and α1b in the landlocked compared to diadromous LHF in seawater, apparently compromising the effectiveness of the paralog-switching strategy. This is consistent with other evidence that landlocked Alewives have traded off reduced seawater performance (Velotta et al. 2014; Velotta et al. 2015). While we did not find evidence for LHF differences in NKA paralog expression under freshwater conditions, previous studies have shown that the landlocked LHF displays increased expression of ion transporters involved in freshwater osmoregulation. These include $Na^+/K^+/2Cl^-$cotransporter (NKCC), cystic fibrosis transmembrane regulator (CFTR), Na^+/H^+ exchanger 3 (NHE3), and V-type H^+ -ATPase (VHA) (Velotta et al. 2014; Velotta et al. 2015) as well as β-thymosin (Michalak et al. 2014), which has cytoskeleton organizing and tissue repair functions.

Differences in the magnitude of NKA α1b paralog response to seawater drove the majority of LHF differences in our study. The greatest difference in LHF paralog expression was seen after seawater exposure whereby landlocked NKA a1b peaked at more than three times the level of, and three days later than, the diadromous

LHF (Table 1). Additionally, landlocked Alewives may have lost mechanisms that support seawater tolerance in the diadromous LHF, especially those involving NKA transporters since NKA enzyme activity increased to a greater degree in seawater-challenged diadromous Alewives than in the landlocked LHF (Velotta et al. 2015).

Comparable intraspecies LHF differences in seawater NKA paralog expression have also been identified in some salmonids. Upregulation of NKA α1b upon seawater exposure was delayed but ultimately more pronounced in landlocked Atlantic Salmon compared to the diadromous LHF (Nilsen et al. 2007; McCormick et al. 2019). In contrast, in landlocked Arctic Char no upregulation of NKA α1b was detected even after seven days of seawater exposure (Bystriansky et al. 2006). Further investigation into species with migratory and landlocked LHFs may provide additional evidence for the consistency of this trend, especially since the loss of seawater halohabitat use is coupled with delayed and heightened NKA α1b expression in both Alewives and diadromous salmonids. Evaluating multiple species with varying degrees of euryhalinity is also recommended as Mozambique Tilapia, a euryhaline but mostly freshwater species, also shows a delayed peak (day 7) in α1b expression upon seawater exposure (Tipsmark et al. 2011).

Dampening of paralog-switching in the landlocked LHF is present and results from a loss of NKA α1a downregulation in seawater, which may impart energetic consequences. Following seawater exposure, the 1-, 2-, and 4-fold decrease seen in landlocked Alewife NKA α1a is trivial compared to the diadromous 8-, 350-, and 250-fold decrease over 2, 5, and 15 days, respectively (Fig. 3c, Table 1). In this case, freshwater specialization has resulted in the reduced downregulation of NKA α1a. Medaka, a euryhaline but mostly freshwater species, similarly maintains expression of NKA α1a in both freshwater and seawater and downregulates expression of NKA α1b in freshwater (Bollinger et al. 2016). This may represent a greater energetic cost for osmoregulation in seawater in Medaka and landlocked alewives relative to species that downregulate NKA α1a in seawater. This appears to be inconsistent with a previous finding that total NKA enzyme activity in the gills of seawater-challenged landlocked alewives is lower than that in the diadromous LHF (Velotta et al. 2015). This discrepancy may be due to NKA enzyme activity related to salinity tolerance mechanisms in other tissues (e.g., gut and kidney) that may be lost or suppressed in the landlocked LHF. Thus, increased seawater mortality of landlocked vs. diadromous alewives seen in previous studies is likely due to osmoregulatory failure rather than energetic exhaustion, especially considering that the bulk of mortality occurs within 24 hours of exposure (Velotta et al. 2015). Differing plasma osmolalities between the landlocked and diadromous LHFs in seawater further support this osmoregulatory failure hypothesis (Velotta et al. 2015).

The significant differences in NKA α1a and α1b expression between Alewife LHFs underpin the evolutionary and functional importance of these paralogs to euryhalinity on a microevolutionary scale. The variability in paralog differential expression between euryhaline fish LHFs and across species with differing halohabitat breadths highlights that euryhalinity can be achieved through differing strategies and is strongly tied to halohabitat use and demands. In the case of alewives, maintenance of freshwater tolerance in the landlocked LHF and pre-migrant ontogenetic stage drives differences in osmoregulatory strategies (as opposed to expected changes in seawater tolerance). It should be noted that not all strategies for achieving euryhalinity may be dependent on or make use of NKA paralog switching (Wong et al. 2016).

Evidence for functional significance of NKA paralogs: ontogenetic stage comparison – NKA paralog expression is so strongly tied to halohabitat use that its variability can be driven by and seen in the need to tolerate seawater in different developmental or life stages within a single population. The degree of paralog-switching we found in migrant individuals is significantly more pronounced than that seen in the pre-migrant stage, indicating that paralog-switching is more pronounced in an ontogenetic stage that occupies a wider range of halohabitats (Fig 3c). Juvenile Alewife show physiological changes prior to initiating migration from natal freshwater habitats to the ocean, and the need to prepare for such a halohabitat transition drives preemptive changes in NKA paralog-switching (Colby 2022; Gahagan et al. 2010). The discrete migrant and pre-migrant stages of juvenile Alewife differ in body size and condition prior to emigration but show no physical changes as charismatic as smoltifying salmonids (Thorpe 1988). Though not as evident on the outside, Alewife still show changes "under the hood" (e.g., internally or at lower levels of organization) for which migratory status

has a significant effect on NKA paralog expression and seawater readiness (Supplemental Table 6).

Differences in NKA paralog regulation between migrant and pre-migrant Alewife indicate preparatory specialization for marine life through the loss of freshwater tolerance. The greater upregulation of NKA α1a in migrants compared to pre-migrants under freshwater conditions (Fig. 3a) suggests a loss of freshwater tolerance and greater need for this hyper-osmoregulatory tool as a tradeoff for seawater tolerance. Interestingly, ontogenetic changes of Atlantic Salmon show that NKA α1a protein abundance is higher in parr (stage most similar to pre-migrant) than smolt (stage most similar to migrant) during freshwater rearing (McCormick et al. 2013). While salmonids gain seawater tolerance late in juvenile development (during the smolt stage), Alewife possess seawater tolerance as early as the larval stage, explaining the similarity in NKA α1b expression between the ontogenetic stages (Fig. 3c), and show decreased survival in freshwater during juvenile development (DiMaggio et al. 2015; Yako 1998). These fish may be physiologically 'pushed' to sea by osmoregulatory demand if freshwater readiness is progressively lost. As mechanisms for freshwater tolerance are suppressed, migrant Alewife may use NKA α1a to compensate until they reach the ocean. Like Alewife, American Shad (*Alosa sapidissima*) possess seawater tolerance early in the juvenile stage and lose the ability to tolerate freshwater near the time of seaward migration (Zydlewski & McCormick 1997a, b). NKA α1a expression in American Shad could show a similar pattern prior to juvenile seaward migration, which would provide evolutionary insight into paralog expression for the *Alosa* genus. Additionally, a multi-month study could reveal a seasonal effect (i.e., early, middle, and late migration times) on differences between migratory stages like those seen between parr and smolt over multiple months of developmental time (McCormick et al. 2013). Seasonality of NKA paralog expression has also been shown in landlocked Atlantic Salmon (Nilsen et al. 2007; McCormick et al. 2019) and inclusion of an ontogenetic and/or LHF variable in a future multi-month Alewife study is recommended.

Molecular & evolutionary history of NKA paralogs– Molecular divergence within conserved regions of NKA paralog sequences co-exists with functional convergence between multiple fish lineages. Of the 10 amino acid substitutions that influence binding affinity for Na^+ and K^+ ions, 30% are mismatched in NKA α1b and 60% are mismatched in NKA α1a between salmonid and Alewife sequences (Figure 5; Jorgensen et al. 2008). In the 13 amino acid substitutions considered to be under positive selection in salmonids, over 30% are mismatched in NKA α1b and 70% are mismatched in NKA α1a between salmonid and Alewife sequences. Consistently, Alewife NKA α1a shows the greatest proportion of mismatches to its corresponding salmonid conserved substitutions, and both Alewife paralogs are more similar to salmonid NKA α1b than α1a. A similar pattern is seen in European Bass (Blondeau-Bidet et al. 2016). The prevalence of molecular divergence, especially in NKA α1a, across multiple fish taxa (Fig. 1 a & b) is striking considering the similarities in paralog-switching and paralog-specific expression patterns (Blondeau-Bidet et al. 2016; Dalziel et al. 2014). Despite molecular differences in the NKA α1a and α1b paralogs, which arose independently across euryhaline teleosts, their similar salinity-dependent regulation suggests functional convergence.

Our phylogenetic analysis supports the independent duplication of NKA α1a and α1b in Alewife and Euteleosteomorpha. Figure 3 shows that the derivation of NKA α1a and α1b in Alewife, a member of Clupeiformes, occurs as a branching unit separate from any members of Salmoniformes, Esociformes, or Galaxiiformes, indicating independent evolution of the Alewife paralogs. Similar to salmonid paralogs that originated from a small-scale duplication event preceding the divergence of Salmoniformes and Esociformes (Dalziel et al. 2014), Alewife NKA α1a and α1b likely arose from a small-scale gene duplication event accompanying the divergence of diadromous alosines and their sister taxon. There is now evidence for at least four cases of independent evolution of salinity responsive NKA paralogs, in the Salmoniformes, Galaxiiformes, Anabantiformes, and Clupeiformes. It is worth noting that only in Salmoniformes have multiple species been included. Expanding the phylogeny to include additional species for Clupeiformes, Galaxiiformes, and Anabantiformes is expected to result in multiple taxon-specific, paralog-specific clades like those seen within Salmoniformes.

Our phylogenetic analysis provides novel information regarding phylogenetic relationships of Euteleosteomorpha NKA paralog sequences. Consistent with previous nucleotide phylogenies, paralogs in taxa belonging

to Salmoniformes form paralog-specific clades, while paralogs found in members of Galaxiiformes and Anabantiformes group by taxon (Figures 3 & 4; Urbina et al. 2013; Dalziel et al. 2014; Blondeau-Bidet et al. 2016). The phylogenetic placement of paralogs is the same for salmonids whether based on nucleotide or amino acid sequence, but it differs for Galaxiiformes and Anabantiformes. No longer forming order-specific clades in the amino acid phylogeny, the galaxiid NKA α1a protein sequence is placed closer to the anabantid NKA α1a and α1b clade. The galaxiid NKA α1b protein sequence, on the other hand, is placed closer to the salmonid paralogs than to other galaxiid paralogs. These results may indicate that the galaxiid NKA α1b arose from the same or very similar product of a gene duplication event. While protein sequences are relatively stable compared to their nucleotide counterparts, expected convergence at the nucleotide level and predicted positive selection at multiple amino acid sites warrants further analysis of NKA paralog origins.

Future studies of duplication event products and functional divergence in NKA α1 genes should expand taxonomic coverage both within and beyond previously studied taxonomic orders to characterize the prevalence of independent origins in NKA paralogs. Further investigation of orders that already show taxon-specific grouping, Clupeiformes, Galaxiiformes, and Anabantiformes, would test whether Salmoniformes is the only taxon that shows within-order paralog-specific grouping. Additionally, expansion of the Esociformes or addition of Osmeriformes (e.g., Rainbow Smelt) might clarify the evolutionary relatedness of paralogs within Euteleosteomorpha (including Salmoniformes). Specifically, it could reveal whether paralogs of euteleosteomorph orders are derived from the same, similar, or different gene duplication events. Finally, strategic inclusion of representatives from additional orders would shed light on the broader evolutionary history of NKA α1 paralogs and potentially document additional incidences of independent evolution. Taxa that should be pursued are those that, like Alewife, are not members of Euteleosteomorpha (e.g., Acipenseriformes, Anguilliformes, Cypriniformes, or Siluriformes) or representatives of Neoteleostei that are not members of Percomorphaceae (e.g., Gadiformes like Atlantic Tomcod). The former would fill the evolutionary gap between the Salmoniformes (and relatives) and Anabantiformes.

CONCLUSIONS

Mechanisms involved in euryhalinity and the invasion of fishes into freshwater have great evolutionary importance as this ability led to significant diversification of bony fishes (Schultz and McCormick 2013). The instances of independent evolution of NKA α1a and α1b evidenced in this study indicate that multiple molecular solutions for tolerating varying salinities have arisen via diversification of the same gene (NKA). These independent molecular solutions have parallel functions useful for euryhalinity across bony fish taxa. Without salinity-specific forms of NKA α1a and α1b, some fishes may lack a critical mechanism for tolerating different salinity environments; reciprocal expression of NKA paralogs likely facilitate osmoregulatory flexibility that is necessary for euryhalinity. Consequently, fishes with diverse halohabitats are more likely to have evolved NKA α1a and α1b and to exhibit paralog-switching. The evolution of these paralogs, therefore, may have been important to the invasion of freshwater by fishes that has led to extensive vertebrate diversification. Additional work should be done to investigate the evolution of NKA paralogs across euryhaline species and create a more encompassing molecular phylogeny. The further study of NKA α1a and α1b may give important insight into the history and evolution of euryhalinity.

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DATA ACCESSIBILITY AND BENEFIT-SHARING

Data Access : Alewife (*Alosa pseudoharengus*) sequences used in this study are being accessioned in the NCBI GenBank public database as ON010522 & ON010523.

Benefits Generated : Benefits from this work include the sharing of novel sequence data via a public biological database and contribution to the investigation of a large-scale evolutionary question – the development of euryhalinity, leading to freshwater invasion and the radiation of vertebrate life.

AUTHOR CONTRIBUTIONS

Research Conception & Design – RC, ES, JV, SM

Funding & Materials Acquisition – RC, ES, JV, SM

Data Collection – RC, ES, JV, SM, EJ

Analysis & Interpretation – RC, ES, JV, SM, EJ

Writing of Original Draft – RC

Critical Manuscript Revision – RC, ES, JV, SM, EJ

Authors: Rebecca S. Colby (RC), Stephen D. McCormick (SM), Jonathan P. Velotta (JV), Elizabeth Jockusch (EJ), & Eric T. Schultz (ES)

TABLES AND FIGURES

Table 1 : Mean C_T values for NKA α1a, NKA α1b, and relative paralog expression. Life history form (LHF) is coded as D = diadromous or L = landlocked. Bolded values indicate significant differences between LHFs at the respective time points.

Salinity	LHF	[?][?]C_T NKA α1a Day 2	[?][?]C_T NKA α1a Day 5	[?][?]C_T NKA α1a Day 15	[?][?]C_T NKA α1b Day 2	[?][?]C_T NKA α1b Day 5
Freshwater	D	1.4	1.8	3.2	0.37	0.53
	L	1.4	2.6	2.6	0.51	0.63
Seawater	D	**0.12**	**0.00**	**0.00**	1.7	**0.95**
	L	**0.74**	**0.33**	**0.20**	2.3	**3.5**

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Figure 1 : NKA paralog mRNA abundance in *diadromous* vs.*landlocked* Alewife gills. Panels a-c show results for diadromous individuals and d-f show results for landlocked individuals that were transferred from the rearing salinity to FW or SW and sampled over 15 days. Panels a and d show mean values for C_T (mRNA abundance relative to EF1α) of NKA α1a, panels b and e show $C_{TT,\ rel}$ (relative paralog mRNA abundance). Error bars represent standard errors.

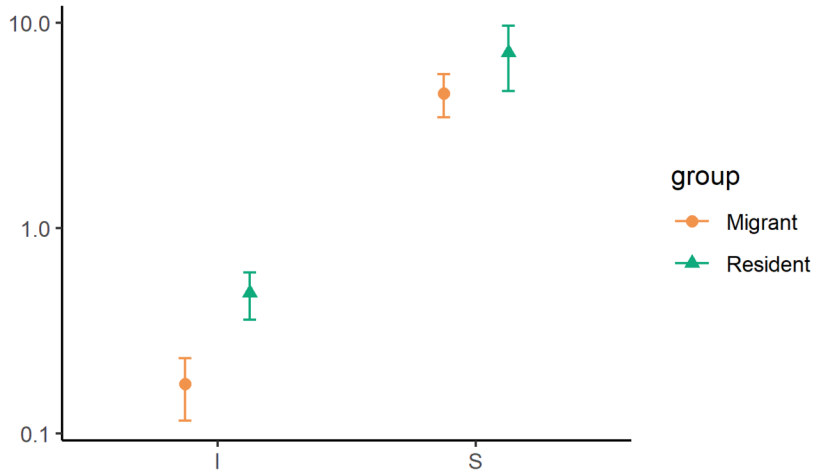

Figure 2 : Ontogenetic stage-specific mRNA abundance of NKA paralogs in*migrant* and *pre-migrant* Alewife gills. Panel (a) shows mean values for C_T NKA α1a, panel (b) shows $C_{TT,\ rel}$. Error bars represent standard errors and data points are annotated with Pairwise Wilcoxon test significances.

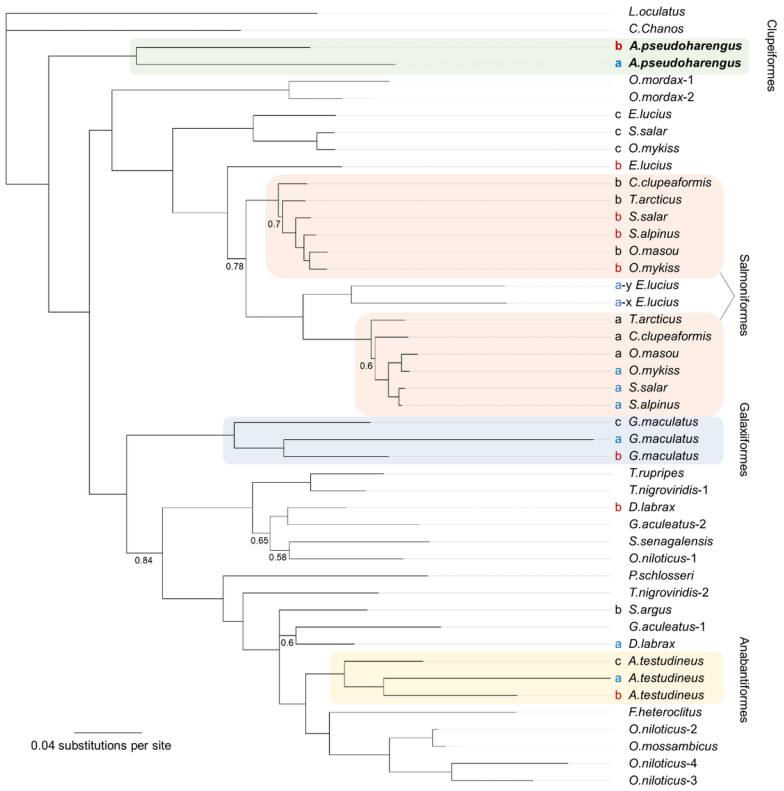

Figure 3 : Bayesian consensus tree for NKA α1 subunit genes and paralogs in euryhaline fishes inferred from nucleotide data (with GTR+I+G model of evolution). Taxon names preceded by a blue "a" are salinity responsive NKA α1a paralogs, and those preceded by a red "b" are salinity responsive NKA α1b paralogs. NKA α1a and α1b paralogs without color distinction are not known to be salinity responsive. Clades of interest are highlighted and labeled with their taxonomic order. Most internal branches were supported by a posterior probability of [?]0.95; only support values under 0.95 are shown.

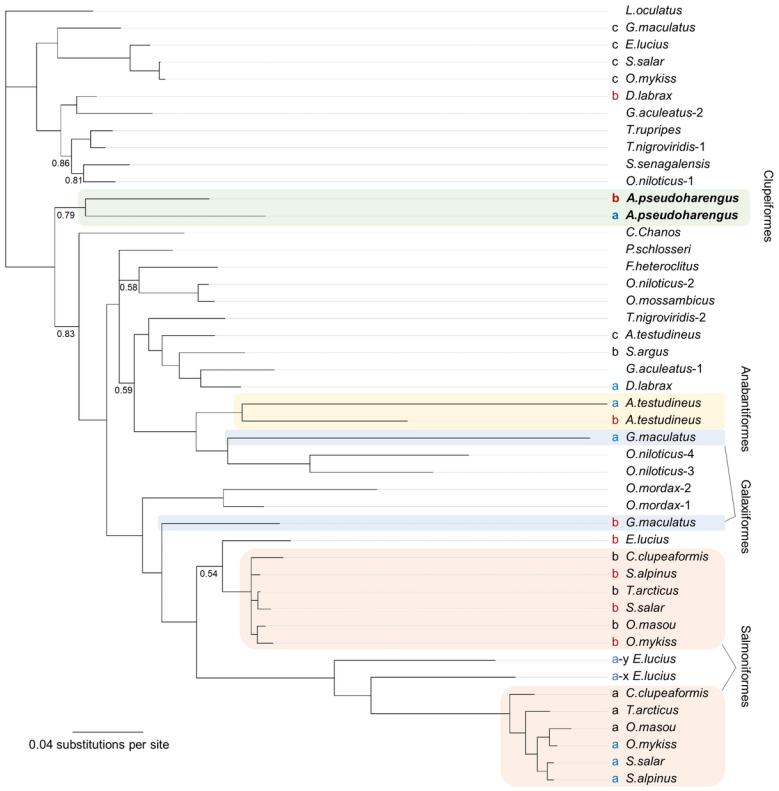

Figure 4 : Bayesian amino acid phylogeny (WAG+I+G) for NKA α1subunit genes and paralogs in euryhaline fishes. Sequences preceded by a blue "a" are salinity responsive NKA α1a paralogs, and those preceded by a red "b" are salinity responsive NKA α1b paralogs. NKA α1a and α1b paralogs without color distinction are not known to be salinity responsive. Clades of interest are highlighted and labeled with their taxonomic order. Most internal branches were supported by a posterior probability of >=0.95; only support values under 0.95 are shown.

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Figure 5 : Transmembrane region substitutions in NKA α1 paralogs with *O. mykiss O. mykiss* reference sequence. Bolded amino acids indicate shared paralog-specific substitutions between respective*A. pseudoharengus* (Alewife) and *O. mykiss* sequences. Amino acid sites are annotated to indicate those that are functionally critical (**O**), predicted to have evolved by positive selection (+), or both (; Dalziel et al. 2014).

SUPPLEMENTAL INFORMATION

Supplemental Table 1 : qPCR primers (F: forward; R: reverse) for alewife NKA α1a and α1b paralogs.

Paralog	Primer (5'-3')
NKA α1a	F: ggtggcagaggagcagtc

	R: cgacgcacagatccacag
NKA α1b	F: agacaggaaccgcttttgac
	R: tcttgaggatggggacattc

Supplemental Table 2 : Reduced mixed-effects models containing only two-way interactions for the effects on reference gene EF1α ($\Delta C_{T,\ ref}$ in Equation 1) expression. Our reference gene, EF1α, displayed salinity sensitivity and effects of day and identity (LHF or migratory stage). Notably, variability in reference gene expression was less than variability in NKA target gene expression under preferred salinities (variance among mean values for $\Delta C_T$of each gene were $\Delta C_{T,\ ref} = 0.08$, ΔC_Tα1a = 1.5, and ΔC_T α1b = 0.34 in 2011 and $\Delta C_{T,\ ref} = 0.02$, ΔC_T α1a = 2.7, and ΔC_T α1b = 2.8 in 2018).

Model with LHF Effect	Model with LHF F	Model with LHF F	Model with LHF P	Model with LHF P		Model with stage Effect	Model with stage Effect	Model with stage F	Model with stage F	Model with stage P	M w s F
Salinity (S)	Salinity (S)	9.2	9.2	**0.039**	**0.039**		Salinity (S)	Salinity (S)	5.9	5.9	**0**
Day (D)	Day (D)	9.2	9.2	**0.005**	**0.005**		Group (G)	Group (G)	3.3	3.3	0
Length (L)	Length (L)	1.6	1.6	0.216	0.216		Length (L)	Length (L)	0.6	0.6	0
LHF	LHF	2.9	2.9	0.166	0.166		S × G	S × G	3.6	3.6	0
S × D	S × D	13.1	13.1	**0.001**	**0.001**		S × L	S × L	2.3	2.3	0
S × L	S × L	1.2	1.2	0.280	0.280		G × L	G × L	8.6	8.6	**0**
S × LHF	S × LHF	0.01	0.01	0.922	0.922						
D × L	D × L	3.7	3.7	0.062	0.062						
D × LHF	D × LHF	5.6	5.6	**0.022**	**0.022**						
L × LHF	L × LHF	7.6	7.6	**0.009**	**0.009**						

Supplemental Table 3 : Previously identified NKA α1-subunit sequences used in this study for molecular phylogenetics.

Name in Phylogeny	Species	Genbank Accession or Ensembl ID
a *T.arcticus*	*Thymallus arcticus*	KJ175158
b *T.arcticus*	*Thymallus arcticus*	KJ175159
T.nigroviridis-1	*Tetraodon nigroviridis*	ENSTNIT00000009334
T.nigroviridis-2	*Tetraodon nigroviridis*	ENSTNIT00000009181
T.rubripes	*Takifugu rubripes*	ENSTRUT00000032672
a *S.alpinus*	*Salvelinus alpinus*	KJ175154
b *S.alpinus*	*Salvelinus alpinus*	KJ175155
a *S.salar*	*Salmo salar*	KJ175156
b *S.salar*	*Salmo salar*	BT058747.1
c *S.salar*	*Salmo salar*	KJ175157
O.mordax-1	*Osmerus mordax*	KJ175166
O.mordax-2	*Osmerus mordax*	KJ175167
O.niloticus-1	*Oreochromis niloticus*	ENSONIT00000015703
O.niloticus-2	*Oreochromis niloticus*	ENSONIT00000015672

O.niloticus-3	*Oreochromis niloticus*	ENSONIT00000015628
O.niloticus-4	*Oreochromis niloticus*	ENSONIT00000015603
O.mossambicus	*Oreochromis mossambicus*	U82549.2
a *O.mykiss*	*Oncorhynchus mykiss*	NM_001124461.1
b *O.mykiss*	*Oncorhynchus mykiss*	NM_001124460.1
c *O.mykiss*	*Oncorhynchus mykiss*	NM_001124459.1
a *O.masou*	*Oncorhynchus masou*	AB573640.1
b *O.masou*	*Oncorhynchus masou*	AB573639.1
G.aculeatus-1	*Gasterosteus aculeatus*	ENSGACT00000018945
G.aculeatus-2	*Gasterosteus aculeatus*	ENSGACT00000018961
a *G.maculatus*	*Galaxias maculatus*	JQ885968.1
b *G.maculatus*	*Galaxias maculatus*	JQ885969.1
c *G.maculatus*	*Galaxias maculatus*	JQ885967.1
F.heteroclitus	*Fundulus heteroclitus*	NM_001310013.1
a-x *E.lucius*	*Esox lucius*	KJ175162
a-y *E.lucius*	*Esox lucius*	KJ175163
b *E.lucius*	*Esox lucius*	KJ175164
c *E.lucius*	*Esox lucius*	KJ175165
a *C.clupeaformis*	*Coregonus clupeaformis*	KJ175160
b *C.clupeaformis*	*Coregonus clupeaformis*	KJ175161
a *A.testudineus*	*Anabas testudineus*	JN180940
b *A.testudineus*	*Anabas testudineus*	JN180941
c *A.testudineus*	*Anabas testudineus*	JN180942
L.oculatus	*Lepisosteus oculatus*	XM_006639371.2
C.chanos	*Chanos chanos*	DQ512799.1
a *D.labrax*	*Dicentrarchus labrax*	KP400258.1
b *D.labrax*	*Dicentrarchus labrax*	KP400259.1
P.schlosseri	*Periophthalmodon schlosseri*	KF410828.1
b *S.argus*	*Scatophagus argus*	KF649217.1
S.senegalensis	*Solea senegalensis*	AB759892.1

Supplemental Table 4 : Reduced mixed-effects models for the diadromous life history form containing two-way interactions for the effect of salinity (S), day (D), and length (L) on paralog-specific ($\Delta\Delta C_T$) and relative ($\Delta\Delta C_{T,\,rel}$) abundance (Equations 1 & 2).

	[?][?]C_T NKA α1a	[?][?]C_T NKA α1a	[?][?]C_T NKA α1a	[?][?]C_T NKA α1a	[?][?]C_T NKA α1b	[?][?]C_T N
Effect		F	P			F
Salinity		3.1	0.178			13.4
Day		2.0	0.167			1.3
Length		0.1	0.918			0.3
S × D		62.4	**0.000**			13.3
S × L		0.2	0.670			0.1
D × L		0.1	0.795			0.1

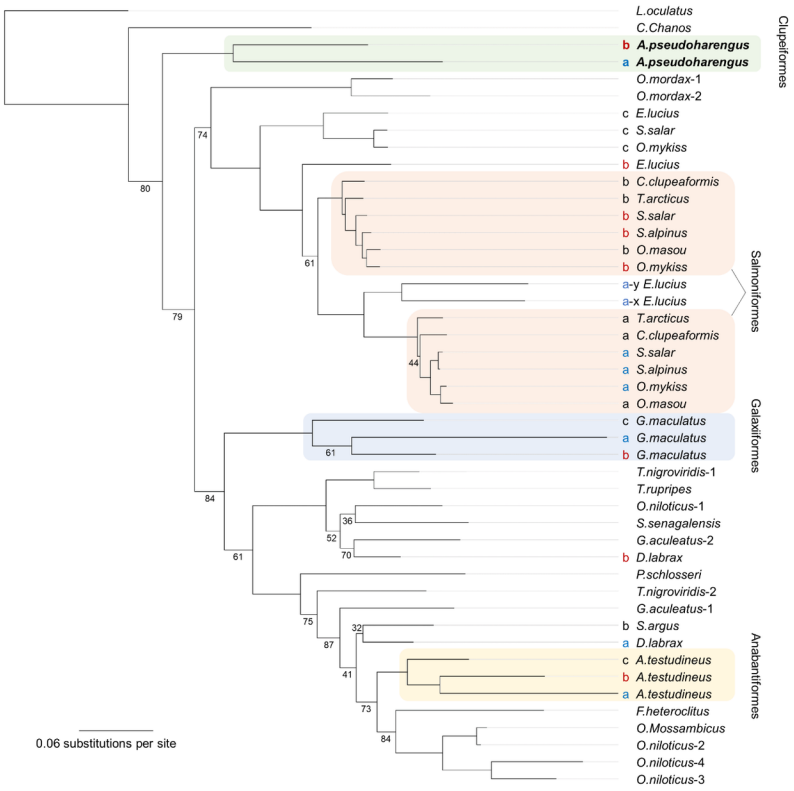
Supplemental Table 5 : Reduced mixed-effects models containing two-way interactions for the effect of salinity (S), day (D), life history form (LHF), and length (L) on relative NKA paralog abundance ($\Delta\Delta C_{T,\,rel}$; Equation 2).

	[?][?]$C_{T,\,rel}$	[?][?]$C_{T,\,rel}$	[?][?]$C_{T,\,rel}$
Effect	F	P	

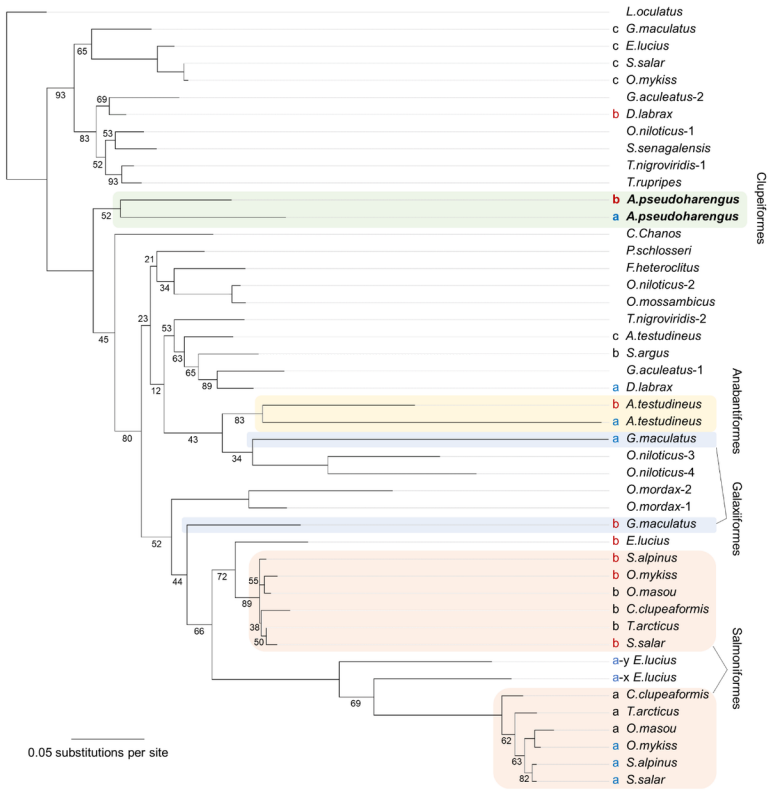
Salinity	0.2	0.663
Day	3.1	0.081
LHF	0.8	0.405
Length	0.1	0.805
S × D	61.6	**0.000**
S × LHF	19.4	**0.005**
S × L	1.9	0.169
D × LHF	1.2	0.273
D × L	0.1	0.796
LHF × L	0.1	0.861

Supplemental Table 6 : Reduced mixed-effects models, including migrant and pre-migrant stages, containing two-way interactions for the effect of salinity (S), ontogenetic stage (group, G), and length (L) on paralog-specific ($\Delta\Delta C_T$) and relative ($\Delta\Delta C_{T,\,rel}$) abundance (Equations 1 & 2).

	[?][?]C_T NKA α1a	[?][?]C_T NKA α1a	[?][?]C_T NKA α1a	[?][?]C_T NKA α1a	[?][?]C_T NKA α1b	[?][?]C_T N
Effect		F	P			F
Salinity		35.8	**0.000**			28.0
Group		13.9	**0.002**			0.1
Length		0.1	0.485			3.1
S × G		14.3	**0.002**			
S × L		2.1	0.170			
G × L		0.0	0.990			



Supplemental figure 1 : RAxML nucleotide phylogeny (GTR+I+G) for NKA α1 subunit genes and paralogs in euryhaline fishes. Sequences preceded by a blue "a" are salinity responsive NKA α1a paralogs, and those preceded by a red "b" are salinity responsive NKA α1b paralogs. NKA α1a and α1b paralogs without color distinction are not known to be salinity responsive. Clades of interest are highlighted and labeled with their taxonomic order. Statistical support for internal nodes were determined by bootstrap analysis. Only support values under 0.95 are shown.



Supplemental figure 2 : RAxML amino acid phylogeny (WAG+I+G) for NKA α1 subunit genes and paralogs in euryhaline fishes. Sequences preceded by a blue "a" are salinity responsive NKA α1a paralogs, and those preceded by a red "b" are salinity responsive NKA α1b paralogs. NKA α1a and α1b paralogs without color distinction are not known to be salinity responsive. Clades of interest are highlighted and labeled with their taxonomic order. Statistical support for internal nodes were determined by bootstrap analysis.