Oecologia (2024) 205:571–586
https://doi.org/10.1007/s00442-024-05588-x

ORIGINAL RESEARCH

Paralog switching facilitates diadromy: ontogenetic, microevolutionary and macroevolutionary evidence

Rebecca S. Colby[1,2] · Stephen D. McCormick[3,4] · Jonathan P. Velotta[5] · Elizabeth Jockusch[1] · Eric T. Schultz[1]

Received: 9 December 2023 / Accepted: 1 July 2024 / Published online: 16 July 2024

Abstract

Identifying how the demands of migration are met at the level of gene expression is critical for understanding migratory physiology and can potentially reveal how migratory forms evolve from nonmigratory forms and vice versa. Among fishes, migration between freshwater and seawater (diadromy) requires considerable osmoregulatory adjustments, powered by the ion pump Na^+, K^+-ATPase (NKA) in the gills. Paralogs of the catalytic α-subunit of the pump (NKA α1a and α1b) are reciprocally upregulated in fresh- and seawater, a response known as paralog-switching, in gills of some diadromous species. We tested ontogenetic changes in NKA α-subunit paralog expression patterns, comparing pre-migrant and migrant alewife (*Alosa pseudoharengus*) sampled in their natal freshwater environment and after 24 h in seawater. In comparison to pre-migrants, juvenile out-migrants exhibited stronger paralog switching via greater downregulation of NKA α1a in seawater. We also tested microevolutionary changes in the response, exposing juvenile diadromous and landlocked alewife to freshwater (0 ppt) and seawater (30 ppt) for 2, 5, and 15 days. Diadromous and landlocked alewife exhibited salinity-dependent paralog switching, but levels of NKA α1b transcription were higher and the decrease in NKA α1a was greater after seawater exposure in diadromous alewife. Finally, we placed alewife α-subunit NKA paralogs in a macroevolutionary context. Molecular phylogenies show alewife paralogs originated independently of paralogs in salmonids and other teleosts. This study demonstrated that NKA paralog switching is tied to halohabitat profile and that duplications of the NKA gene provided the substrate for multiple, independent molecular solutions that support a diadromous life history.

Keywords NKA · Paralog · Euryhalinity · Salinity · Osmoregulation · Diadromous

Introduction

Migration is a critical life history strategy for many animal species. Demands of migration often require substantial physiological transformation, both in anticipation of and in response to new environments. Identifying how demand is met at the level of gene expression is critical for understanding migratory physiology and can potentially reveal how migratory forms evolve from nonmigratory forms and vice versa. Transcriptome studies have demonstrated upregulation of genes associated with reproductive physiology prior to and during spawning migration (Fudickar et al. 2017; Hagihara et al. 2020a; Hagihara et al. 2020b; Krause et al. 2022), changes in gene expression related to energetics (Demoranville et al. 2022; Drenner et al. 2018; Frias-Soler et al. 2021; Kendall et al. 2015; Liu et al. 2019; Maas et al. 2018; Twining et al. 2023), as well as tolerance or performance with respect to abiotic factors such as temperature, salinity (Breves et al. 2022; Ishikawa et al. 2016;

Communicated by Thomas Turner.

✉ Eric T. Schultz
eric.schultz@uconn.edu

[1] Department of Ecology and Evolutionary Biology, University of Connecticut, Storrs, CT, USA

[2] Institutional Research and Planning, Fitchburg State University, Fitchburg, MA, USA

[3] Department of Biology, University of Massachusetts, Amherst, MA, USA

[4] Department of Environmental Conservation, University of Massachusetts Amherst, Amherst, MA, USA

[5] Department of Biological Sciences, University of Denver, Denver, CO, USA

Kendall et al. 2015; Ma et al. 2022; Xu and Liu 2011), stress (Veldhoen et al. 2010), orientation and navigation (Bett et al. 2018; Freedman et al. 2018; Madsen et al. 2019), immune function (Drenner et al. 2018), and morphological change (Hagihara et al. 2020a; Kendall et al. 2015). Gene expression changes underlying physiological demands of migration have been interrogated via comparison of migratory and non-migratory states (i.e., ontogenetic differences; Frias-Soler et al. 2021; Hagihara et al. 2020b; Madsen et al. 2019; Sharma et al. 2022), Another approach has been comparative analysis of migratory and nonmigratory forms within or among populations (Boss et al. 2015; Fudickar et al. 2017; Kendall et al. 2015; Kimmitt et al. 2019; Lemmetyinen et al. 2013; Twining et al. 2023; Wynne et al. 2021). Differential expression of gene paralogs is a likely resource for physiological adjustment as animals migrate to a new environment. Shifts between forms of transporters functioning in osmoregulation (Bystriansky et al. 2007; Martinez et al. 2005; McCormick et al. 2013; Shrimpton et al. 2005), between isoforms of muscle constituents (Mänttäri et al. 2005), and between isoforms of retinal pigments for acclimating to changes in light environment (Schweikert and Grace 2017) have contributed to our understanding of migratory adjustments.

Among fishes, diadromy—characterized by migration between freshwater and seawater—has enhanced diversity of fishes in both environments (Corush 2019) and contributes to ecologically-significant nutrient transport (Kieran et al. 2021). Evolution of this remarkable migratory strategy necessarily involves changes to osmoregulation, the maintenance of ion and water homeostasis, that allow for wide salinity tolerance breadth (i.e., euryhalinity; Schultz and McCormick 2013). Across taxa, the capacity for euryhalinity has repeatedly permitted prominent bouts of adaptive radiation following colonization of freshwater by ancestrally marine lineages (Betancur-R et al. 2012; Lee and Bell 1999; Schultz and McCormick 2013). Hence, clarifying the proximate and ultimate underpinnings of strategies that promote euryhalinity and diadromy deepens our understanding of how organisms adapt and diversify in divergent environments.

Diadromy—and the euryhalinity it requires—is rare (Schultz and McCormick 2013), perhaps because it is difficult to evolve the physiological capacity to achieve such complex adjustments. Especially challenging are the dramatic differences between salt concentrations in differing salinity environments (~ 10 mOsm/0 ppt in freshwater vs. ~ 1050 mOsm/35 ppt in seawater). Such osmoregulatory pressure 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 (Edwards and Marshall 2013; Evans et al. 2005; Marshall and Grosell 2006).

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 and functionally important binding sites (Lingrel and Kuntzweiler 1994). 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). 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 (Sáez et al. 2009; Serluca et al. 2001). Two NKA α-subunit paralogs prominent in gill ionocytes, called NKA α1a and NKA α1b, are upregulated upon exposure to freshwater and seawater, respectively, and are each downregulated by exposure to the alternate salinity; this has been demonstrated in Salmonidae (e.g., McCormick et al. 2013; Urbina et al. 2013), Cichlidae (Tipsmark et al. 2011), and Galaxiidae (Dalziel et al. 2014). These paralogs contain protein substitutions in the functionally important transmembrane region that can alter the transport characteristics of the enzyme in ways that may be adaptive for osmoregulation in freshwater and seawater (Jorgensen 2008). (We note that the NKA α1a and NKA α1b nomenclature is based on their response to salinity in each group, without implying a common homology of the paralogs among these families.) The repeated pattern in diverse taxa of salinity-dependent and reciprocal differential regulation between alternate forms of this enzyme, termed paralog-switching, has been hypothesized to facilitate the evolution of euryhalinity (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. Alewife (*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 alewife occurring along the east coast of North America were independently derived from diadromous runs in the Holocene (Palkovacs

et al. 2008). Physiological studies of the landlocked alewife life history form (LHF) have shown reduced tolerance to seawater and enhanced tolerance to freshwater compared to the diadromous alewife LHF (Velotta et al. 2014, 2015, 2017). Hence, paralog switching should be more pronounced in the diadromous LHF than the landlocked LHF.

Variability in paralog switching may also be evident with ontogeny. During the juvenile stage, migratory behavior is presumably dictated by physiological preparedness for challenges like salinity transition and sustained swimming demands (Zydlewski and McCormick 1997a, 1997b). Juveniles that have initiated migration to seawater, which we term migrants, are expected to be better prepared for seawater than pre-migrants that continue to hold in the natal freshwater environment. Hence, migrants should shift expression towards NKA α1b and away from NKA α1a, and paralog switching should be more evident in migrants than pre-migrants if both stages are tested in freshwater and seawater.

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 repeated origins of euryhalinity. Euryhalinity has been independently derived many times among teleosts, and in such cases any 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 (Dalziel et al. 2014; Urbina et al. 2013). The euryhaline clade to which alewife belongs (Alosidae) arose within a clade of largely marine fishes (Bloom and Lovejoy 2014; Li and Ortí 2007). In this context, we predict that specialization of NKA α1a and α1b paralogs in alewife has been independent of specialization in these other teleosts; hence, alewife paralogs should be more closely related to each other than either paralog is to its functional equivalent in salmonids and cichlids. Analysis of paralog relatedness should be conducted with respect to both amino acid and DNA sequences. Phylogenetic patterns of amino acid sequences may indicate a greater degree of shared ancestry between functionally equivalent paralogs than phylogenetic patterns based on DNA sequences, because of the potential for convergent evolution in amino acid differences.

The goals of the present study were to: (i) test for the presence of salinity-dependent 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 (Table 1): NKA paralogs in diadromous alewife exhibit paralog-switching in response to salinity challenge; diadromous out-migrant alewife juveniles exhibit increased paralog-switching via greater upregulation of NKA α1b upon seawater challenge; landlocked alewife exhibit dampened paralog-switching via reduced upregulation of NKA α1b upon seawater challenge; and, alewife paralogs originated independently of those in other bony fish groups, but show functional convergence between diadromous lineages. To test these predictions, we contrasted expression patterns of gill NKA paralogs between diadromous and landlocked LHFs, as well as between migrant and pre-migrant diadromous LHF life history stages, and we conducted molecular phylogenetic analyses to place alewife NKA paralogs in a broader evolutionary context.

Methods

Field collection

We collected young-of-the-year diadromous alewife from Bride Lake in East Lyme, Connecticut, USA (41.3271° N, 72.2379° W) in 2011 and 2018, and landlocked young-of-the-year alewife from Rogers Lake in Old Lyme, Connecticut, USA (41.3637° N, 72.3000° W) in 2011. Fish caught in 2011 were captured via purse seine during nighttime hours (Devine et al. 2018; 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 (juvenile out-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.

Salinity trials

Following transportation to the Conte Anadromous Fish Research Center (Turners Falls, Massachusetts, USA) we transferred fish to 1200 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-d 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 that of 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

Table 1 Summary of predictions, data, and findings

Comparison	Prediction	Data	Result
Paralogs	Expression shifts from NKA α1a to NKA α1b with change from freshwater to seawater	Anadromous alewife expression levels in different salinity levels	Supported
Ontogenetic	Juvenile out-migrants exhibit stronger paralog switching than pre-migrants via stronger upregulation of NKA α1b in seawater	Pre-migrant and migrant expression of paralogs in different salinity levels	Stronger paralog switching in migrants supported; but migrants more strongly downregulate expression of NKA α1a
Microevolutionary	Landlocked alewife exhibit weaker paralog switching, i.e. less upregulation of NKA α1b, than anadromous alewife upon exposure to seawater	Landlocked and anadromous alewife expression of paralogs in different salinity levels	Dampened paralog switching in landlocked alewife supported, but landlocked alewife upregulated NKA α1b more strongly than anadromous alewife in seawater
Macroevolutionary	Alewife paralogs diverged independently from those of other taxa	Phylogenetic reconstruction	Supported

mortality (Velotta et al. 2015). Each salinity treatment was performed in duplicate and, once treatments were completed, we euthanized subjects, recorded their 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 gill 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. We designed qPCR-suitable primers (Online Resource 1) for alewife NKA paralogs, as well as for EF1α, a common qPCR reference gene reported to be relatively unresponsive to variable environmental conditions (De Santis et al. 2011; Hu et al. 2014; Ye et al. 2010). We confirmed that amplification efficiency of primers fell within norms of standard qPCR methods (Online Resource 1). 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 (Online Resource 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 m at 95 °C, 45 cycles of 95 °C for 20 s and 60 °C for 50 s. We included triplicates of the appropriate standard (as well as controls) on each qPCR plate to enable correction for potential plate-to-plate variance in analysis conditions. We quantified expression of each NKA paralog as (Pfaffl 2001):

$$\Delta\Delta C_T = \frac{E_{tar}^{\Delta C_T \text{ tar (calibrator} - \text{test)}}}{E_{ref}^{\Delta C_T \text{ ref (calibrator} - \text{test)}}} \quad (1)$$

in which E_{tar} is the amplification efficiency of the target (NKA α1a or NKA α1b) paralog primer, E_{ref} is the amplification efficiency of the EF1α primer, ΔC_T tar (calibrator-test) is the cycle threshold value difference between calibrator and test sample for the target NKA paralog, and ΔC_T ref (calibrator-test) is the cycle threshold value difference between calibrator and test sample for EF1α. We will henceforth refer to $\Delta\Delta C_T$ values for NKA α1a or NKA α1b as paralog expression.

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

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

in which $E_{\alpha 1a}$ is the amplification efficiency of the NKA α1a primer, $E_{\alpha 1b}$ is the amplification efficiency of the NKA α1b primer, ΔC_T α1a (calibrator-test) is the cycle threshold value difference between calibrator and test sample for NKA α1a, and ΔC_T α1b (calibrator-test) 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 will henceforth refer to $\Delta\Delta C_{T, rel}$ as relative expression of NKA α1b.

We tested the effect of predictor variables on paralog expression via mixed-effects model analysis in R 4.1.3 run through RStudio 1.4.1743 software (R Core Team 2020; RStudio Team 2019). To test the prediction that the NKA paralogs were differentially expressed in response to freshwater and seawater challenges in diadromous alewife (Table 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 the prediction that migrant and pre-migrant alewife differed in their response to in situ freshwater conditions and seawater challenge, we tested for an ontogenetic effect (Table 1). The prediction was supported if there was a ontogenetic stage-by-salinity interaction, 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. To test whether diadromous and landlocked LHFs differed in their response to freshwater and seawater challenges (Table 1), 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 alewife in seawater, and/or $\Delta\Delta C_{T, rel}$ was smaller in the landlocked alewife in freshwater. We coded replicate tanks as random effects, included individual length as a continuous covariate, and main effects as categorical variables. Estimates of paralog expression 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.

Molecular phylogenetics

To test whether alewife paralogs originated independently from paralogs found in other bony fish groups (Table 1), we conducted molecular phylogenetic analyses of both DNA and amino acid sequences. Previously identified sequences obtained from NCBI GeneBank or Ensembl were compiled in Geneious 2021.1 software (Kearse et al. 2012; Online Resource 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 (Lanfear et al. 2012, 2016; Stamatakis 2006) to identify the most appropriate evolutionary models and partitioning scheme. The selected partitioning scheme had two partitions: codon positions 1 and 2 together and codon position 3. The model for nucleotide evolution was GTR + I + G for each partition. Using the MrBayes and RAxML plug-ins for Geneious, we employed both Bayesian and Maximum Likelihood approaches (Stamatakis 2006). An NKA α1 sequence belonging to spotted gar (*Lepisosteus oculatus*), a non-teleost bony fish, was used as an outgroup. All analyses were run twice, and parameter estimates were essentially identical between runs. We confirmed that discarding the initial 10% of samples as burn-in was sufficient, based on examination of the parameter traces. Mixing was also sufficient: mean swap rate was 31.0–45.9% between neighboring chains in the nucleotide analysis and 25.6–37.7% in the amino acid analysis. Stationarity of all estimates was confirmed based on effective sample sizes as calculated in Tracer v1.7.2. For the nucleotide analysis, the effective sample size for all parameters was > 200 in each of the two runs, with the exception of rA-G, where it was 179 in run 1 and 367 in run 2. For the amino acid analysis, the

effective sample size for all parameters was > 1000 in each of the two runs. The means of the posterior were identical between runs, the traces were similar, and nucleotide composition was remarkably even; posterior means ranged from 0.233 (for T) to 0.273 (for C). Additionally, we tested for convergence of alewife and salmonid paralog differentiation, using amino acid differences between NKA α1a and NKA α1b of rainbow trout as reference. A portion of these amino acid substitutions have been identified as functionally important and/or under positive selection in salmonids (Dalziel et al. 2014; Jorgensen 2008); we tested whether differences between alewife NKA α1a and NKA α1b were more likely to be identical to the rainbow trout differences at these functionally important sites. All taxonomic classifications used in this analysis were derived from the most recent DeepFin phylogeny (Version 4; Betancur-R et al. 2017).

Results

Results supported the prediction that diadromous alewife exhibit paralog switching (Table 1). Expression of gill NKA α1a increased in freshwater and decreased in seawater (Fig. 1a); expression of gill NKA α1b increased in seawater (at least initially) but did not decrease in freshwater (Fig. 1b). Expression of NKA α1b exceeded that of NKA α1a (Eq. 1) by almost two orders of magnitude in seawater and, by day 15, was one fifth that of NKA α1a in freshwater (Fig. 1c). Expression of both paralogs varied with salinity and over time, and the effect of salinity varied over time (mixed-effects models; Online Resource 4). Similarly, relative expression of NKA α1b varied with salinity, time, and the effect of salinity varied over time (Online Resource 4).

Migrants and pre-migrants differed in paralog expression patterns, but in a fashion differing from that predicted

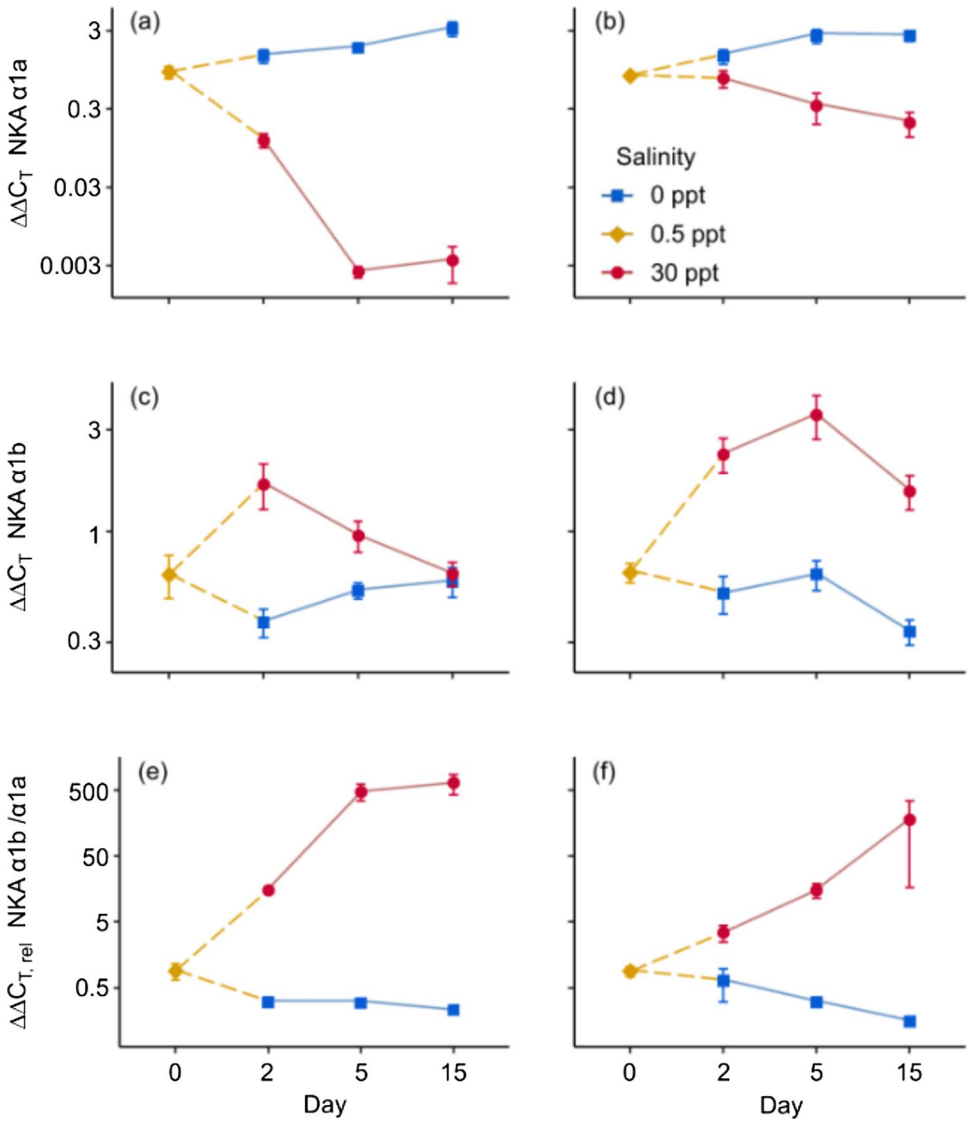

Fig. 1 NKA paralog expression in diadromous vs. landlocked alewife (*Alosa pseudoharengus*) gills. Note log scale used in y axes. N = 6 individuals of each life history form for each time point at both salinity levels). **a** mean (± SE) $\Delta\Delta C_T$ (mRNA abundance relative to EF1α) of NKA α1a among diadromous individuals. **b** mean (± SE) $\Delta\Delta C_T$ of NKA α1a among landlocked individuals. **c** mean $\Delta\Delta C_T$ (± SE) of NKA α1b among diadromous individuals. **d** mean (± SE) $\Delta\Delta C_T$ of NKA α1b among landlocked individuals. **e** mean (± SE) $\Delta\Delta C_{T, rel}$ (expression of NKA α1b relative to NKA α1a) among diadromous individuals. **f** mean (± SE) $\Delta\Delta C_{T, rel}$ (expression of NKA α1b relative to NKA α1a) among landlocked individuals

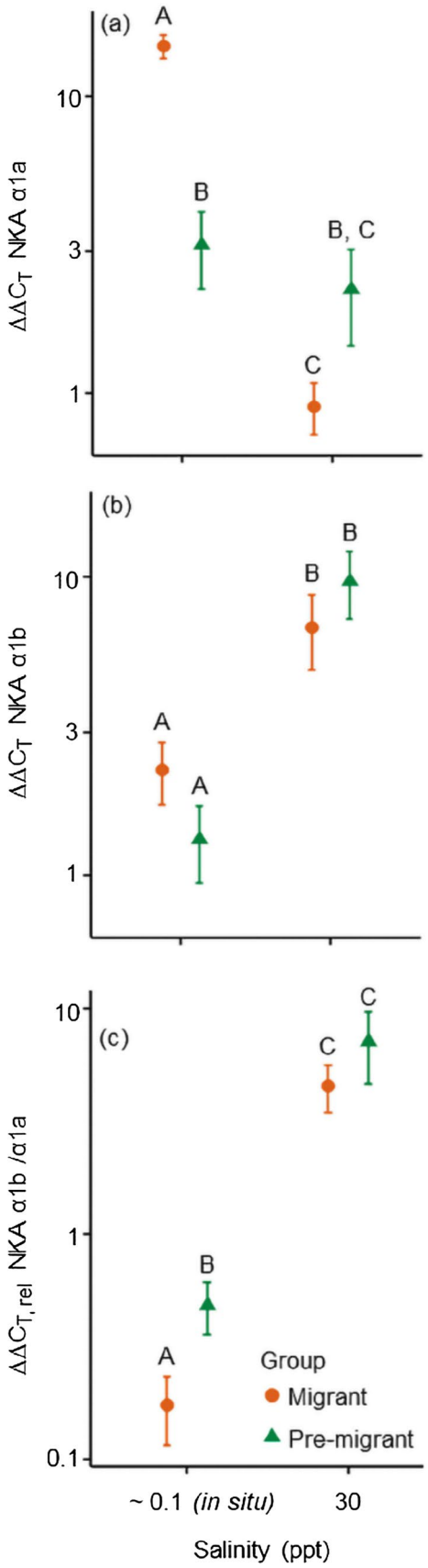

Fig. 2 Ontogenetic stage-specific NKA paralog expression in migrant ▶ and pre-migrant alewife gills in in situ (natal freshwater) and seawater (30 ppt) environments. Note log scale used in y axes. In situ N = 6 and 9 for pre-migrants and migrants, respectively; 30 ppt N = 5 for both pre-migrants and migrants). **a** mean (± SE) $\Delta\Delta C_T$ NKA α1a. **b** mean (± SE) $\Delta\Delta C_T$ NKA α1b. **c** mean (± SE) $\Delta\Delta C_{T,\,rel}$. Data points are annotated into groups defined by pairwise Wilcoxon p values

(Table 1). Migrants showed greater expression of NKA α1a in natal freshwater conditions (Fig. 2a) and no differences in expression of NKA α1a or NKA α1b in seawater (Fig. 2a, b). Relative expression of NKA α1b in migrants was lower than that of pre-migrants in freshwater and did not differ from that of pre-migrants in seawater (Fig. 2c, Online Resource 6).

As predicted, landlocked individuals have dampened paralog-switching; this occurred despite greater upregulation of NKA α1b in seawater (Table 1). The landlocked LHF retains salinity-dependent expression of the NKA paralogs (Fig. 1d, e); landlocked individuals showed higher expression of NKA α1a in seawater compared to the diadromous LHF (i.e., downregulation of NKA α1a decreased in comparison to diadromous alewife). Contrary to our prediction, landlocked individuals also showed higher expression of NKA α1b in seawater. Relative expression of NKA α1b was muted in the landlocked LHF as predicted (Table 2); expression of α1b exceeded that of α1a by only one order of magnitude in seawater and was one fifth of NKA α1a expression in freshwater by day 15 (Fig. 1f, Online Resource 6).

Phylogenetic analysis supports the prediction that alewife NKA paralogs originated independently of those in other taxa (Table 1). Alewife NKA paralogs did not cluster with the paralogs from either salmonids or other teleosts (Figs. 3 and 4; see Online Resource 7 and 8 for corresponding maximum likelihood phylogenies). Instead, they were most closely related to each other, supporting an independent duplication event in the lineage leading to alewife. A similar pattern was observed for the two salinity-responsive paralogs in Anabantiformes. Salmonid NKA paralogs formed paralog-specific clades, which were closely related to each other, as expected. These results are consistent in the DNA-based and amino acid-based phylogenies (Figs. 3 and 4).

Inferred ancestry of NKA paralogs differed between DNA-based and amino acid-based phylogenies in some taxa, suggesting that there has been convergent evolution of functional paralog differences. The DNA-based phylogeny placed the paralogs in Galaxiiformes together (Fig. 3). In contrast, the amino acid-based phylogeny separated these paralogs; one copy appeared as a close relative of the paralog pair in Anabantiformes and the other copy as a close relative of the Salmoniformes NKA α clade (Fig. 4). Salmonid paralogs NKA α1a and NKA α1b, as represented by rainbow trout, have 34 amino acid differences (Fig. 5),

Table 2 Mean paralog expression ($\Delta\Delta C_T$) of NKA α1a, NKA α1b, and relative expression of NKA α1b ($\Delta\Delta C_{T, rel}$)

Salinity	LHF	$\Delta\Delta C_T$ NKA α1a			$\Delta\Delta C_T$ NKA α1b			$\Delta\Delta C_{T, rel}$		
		Day 2	Day 5	Day 15	Day 2	Day 5	Day 15	Day 2	Day 5	Day 15
Freshwater	D	1.4	1.8	3.2	0.37	0.53	0.58	0.30	0.29	0.23
	L	1.4	2.6	2.6	0.51	0.63	0.34	0.64	0.30	0.15
Seawater	D	**0.12**	**0.00**	**0.00**	1.7	**0.95**	**0.63**	**15**	**477**	**647**
	L	**0.74**	**0.33**	**0.20**	2.3	**3.5**	**1.5**	**3.5**	**15**	**180**

Bolded values indicate significant differences between LHFs at the respective time points

Life history form (*LHF*) is coded as *D* diadromous or *L* landlocked

of which 18 are considered functionally important or have arisen via positive selection (Jorgensen 2008; Dalziel et al. 2014). Alewife paralogs have differentiated at 14 of these 18 amino acid positions; five of these differences are identical to the rainbow trout differences and nine are different. Amino acid substitutions at functionally important positions are no more likely to be the same between alewife and rainbow trout than are those at other positions (exact hypergeometric probability = 0.42).

Discussion

Our study provides evidence for multiple, independently-derived solutions to the physiological demands of diadromy. One pathway for the evolution of paralog-switching requires gene duplication, gene product diversification, and reciprocal differentiation in expression pattern. Yet multiple, distantly related fishes (e.g., alewife and salmonids) have converged on this similar, complex response enabling diadromy. Our findings also suggest that landlocked and diadromous life history forms of multiple fish taxa have repeatedly and independently diverged in expression patterns in similar ways, and that paralog expression changes with ontogeny related to migratory readiness (summary of findings in Table 1).

NKA paralog switching in alewife

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 (Nilsen et al. 2007; Richards et al. 2003; Shrimpton et al. 2005), 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 euryhalinity, especially with diadromous 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 diadromous alewife (Fig. 1a–c) resembles paralog switching dynamics in previously studied taxa. Consistently in these cases, NKA α1a is expressed to a greater extent upon exposure to freshwater than NKA α1b is expressed upon exposure to seawater. Expression of NKA α1a is elevated under hyper-osmoregulatory conditions for at least for 14–15 d in alewife, similarly to Mozambique tilapia and Atlantic salmon (Bystriansky and Schulte 2011; Tipsmark et al. 2011). In contrast, expression of NKA α1b under hypo-osmoregulatory conditions exhibits a distinct 2- or 3-d peak followed by a decline in diadromous alewife, similarly to rainbow trout and inanga (Richards et al. 2003; Urbina et al. 2013). 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: 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 (Fig. 2c). Hence, among other physiological changes (Colby 2022; Gahagan et al. 2010), juvenile alewife make preemptive changes in the capacity for paralog-switching. Juvenile alewife exhibit no physical changes as charismatic as smoltifying salmonids (Thorpe 1988). Though not evident on the

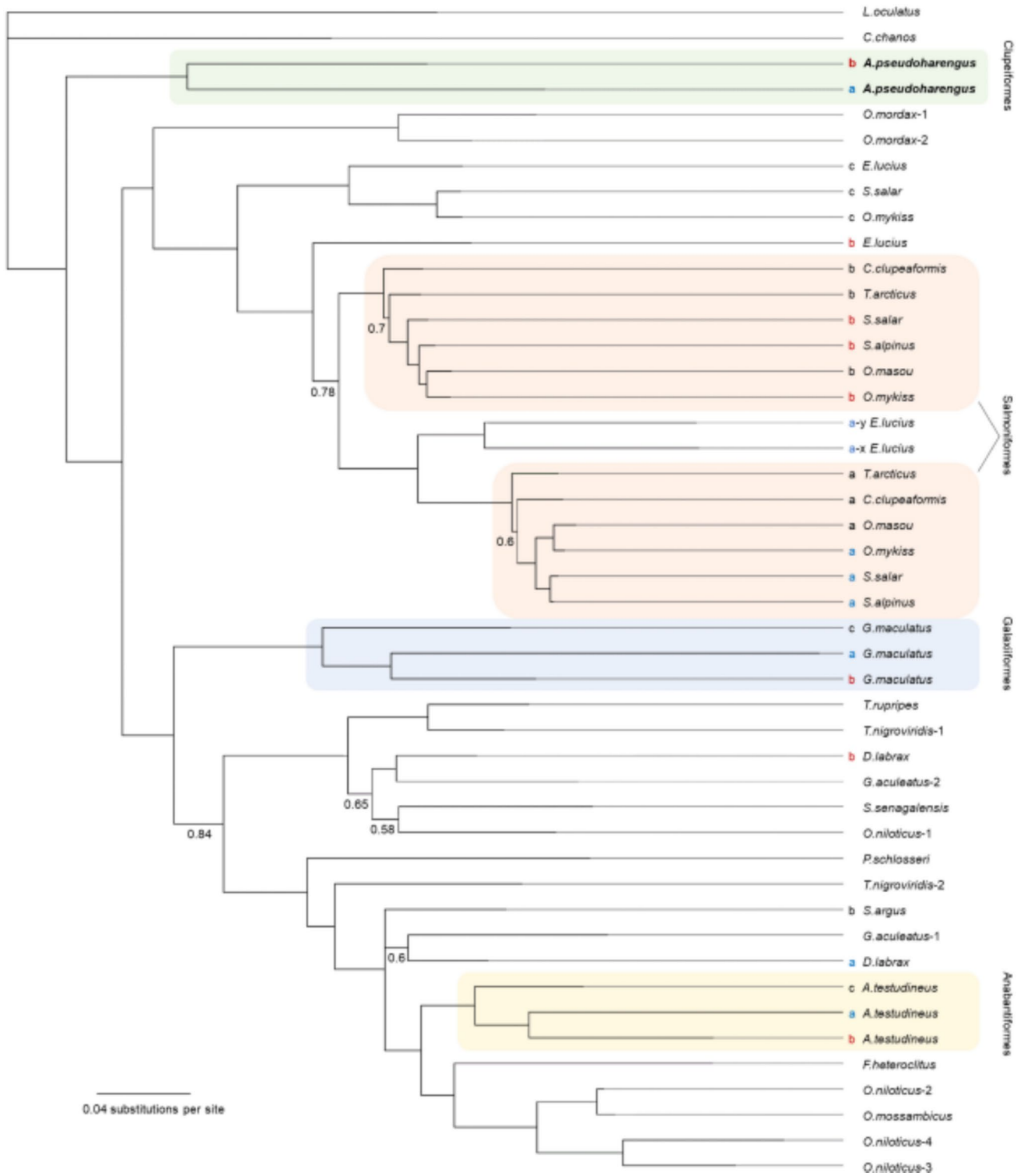

Fig. 3 Bayesian consensus tree for NKA α1 subunit genes and paralogs in euryhaline fishes inferred from nucleotide data (with GTR + I + G model of evolution). Taxon names preceded by a blue "a" are salinity responsive NKA α1a paralogs, and those preceded by a red "b" are salinity responsive NKA α1b paralogs. 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

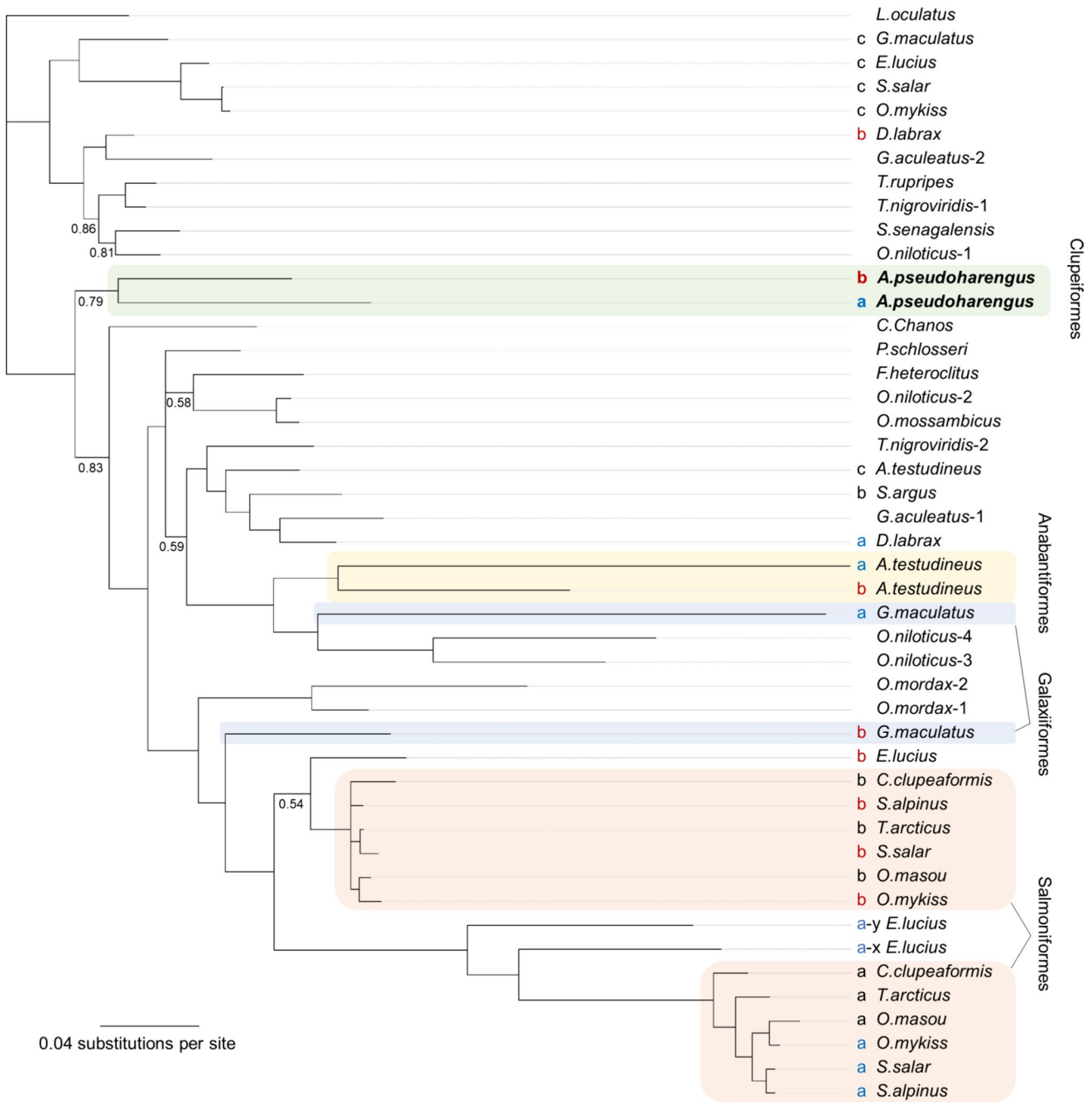

Fig. 4 Bayesian consensus tree for NKA α1subunit genes and paralogs in euryhaline fishes inferred from amino acid data (with WAG + I + G model of evolution). Sequences preceded by a blue "a" are salinity responsive NKA α1a paralogs, and those preceded by a red "b" are salinity responsive NKA α1b paralogs. 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

outside, alewife still show considerable internal changes associated with migratory status and seawater readiness.

The larger paralog switching response upon exposure to seawater that we observed among migrants than pre-migrants did not arise from a greater upregulation of NKA α1b, as we had expected, but instead from a sharper downregulation of NKA α1a expression. The comparable degree of NKA α1b upregulation among pre-migrants suggests that they are as prepared for seawater as migrants are. Indeed, diadromous alewife are tolerant of seawater as early as the larval stage (DiMaggio et al. 2015; Yako 1998). We hypothesize that the high level of expression of gill NKA α1a among migrants in

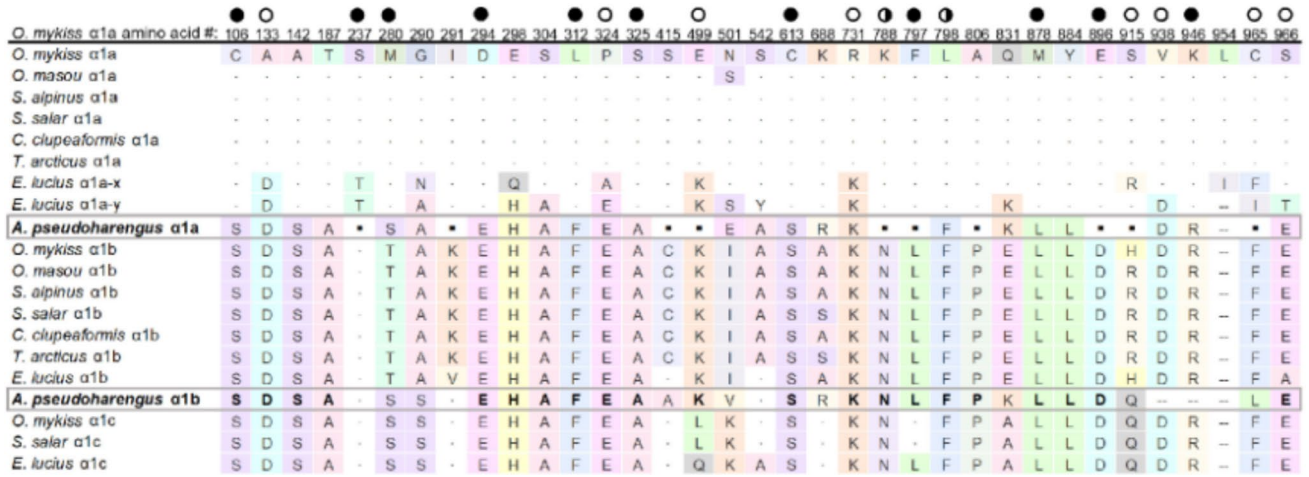

Fig. 5 Transmembrane region substitutions in NKA α1 paralogs with *O. mykiss* as a reference sequence. Cells with dots (·) indicate that the amino acid is identical to the *O. mykiss* reference sequence. Bolded amino acids indicate shared paralog-specific substitutions between respective *A. pseudoharengus* (alewife) and *O. mykiss* sequences. Amino acid sites are annotated to indicate those that are functionally critical (○; Jorgensen 2008), those predicted to have evolved by positive selection (●; Dalziel et al. 2014), or both (◑)

freshwater is a compensatory mechanism for loss of other hyperosmoregulatory processes. For example, adjustment to seawater among euryhaline fishes includes changes in claudin proteins that modulate intercellular permeability of the gill epithelium; tight junctions in the epithelium give way to more open junctions that permit paracellular secretion of Na^+ (Hwang et al. 2011). If these junctions are opening up prior to migration, the loss of Na^+ could place a greater demand on NKA ionocytes expressing NKA α1a to drive compensatory absorption of Na^+ at the apical membrane through a sodium hydrogen exchanger (e.g. NHE3, which has been observed in alewife Christensen et al. 2012).

Further comparative work on ontogenetic changes in paralog expression, paralog switching, and salinity tolerance is warranted. A contrast with American shad (*Alosa sapidissima*) will be instructive. Whereas juvenile alewife retain a lasting tolerance to freshwater, judging from the species' many landlocked populations and from our ability to rear fish in freshwater for months following their capture in October (Velotta et al. 2015), American shad lose the ability to tolerate freshwater near the time of seaward migration in autumn (Zydlewski and McCormick 1997a, 1997b). How the species differ in expression of NKA α1 paralogs and other osmoregulatory loci throughout their preparation for juvenile outmigration should be evaluated. No other species are known in which NKA α1a is elevated as juveniles prepare to out-migrate. In Atlantic salmon, NKA α1a transcription and protein abundance is higher in parr (stage most similar to alewife pre-migrant) than smolt (stage most similar to alewife migrant) during freshwater rearing (McCormick et al. 2013; Nilsen et al. 2007). Salmonids differ from alosids in that seawater tolerance develops later, during the smolt stage. Smolts do not evidently lose freshwater tolerance but some loss of spare hyperosmoregulatory capacity may occur: although plasma ions do not normally change in freshwater during smolt development ("smoltification") of Atlantic salmon, reductions in plasma ions following a stress event are greater in smolt relative to parr (Carey and McCormick 1998). The smoltification process in salmonids is modulated by seasonal cues (McCormick et al. 2013) and NKA paralog expression varies seasonally in Atlantic salmon (McCormick et al. 2019; Nilsen et al. 2007). It will be valuable to evaluate the roles of photoperiod and temperature in modulating changes in freshwater tolerance and NKA paralog expression in juvenile alewife.

Evidence for functional significance of NKA paralogs: life-history form comparison

Evidence for evolution in the landlocked LHF toward freshwater specialization includes changes in NKA paralog expression. Paralog-switching was dramatically dampened in the landlocked LHF, in that relative expression NKA α1b was as little as 3% of the corresponding diadromous values in seawater (Table 2), apparently compromising the effectiveness of the paralog-switching strategy. Dampening of the response may contribute to reduced performance of landlocked alewife in seawater, observed as a lessened ability to maintain homeostasis in plasma osmolality (Velotta et al. 2014) and lower survival (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 is more tolerant of deionized freshwater (Velotta et al. 2015) and displays

higher expression of ion transporters involved in freshwater osmoregulation, including $Na^+/K^+/2Cl^-$ cotransporter, cystic fibrosis transmembrane regulator, $Na^{+/}H^+$ exchanger 3, and V-type H^+ -ATPase (Velotta et al. 2014, 2015) as well as β-thymosin (Michalak et al. 2014), which has cytoskeleton organizing and tissue repair functions.

LHFs differed in expression of both paralogs in response to seawater. In comparison to the downregulation of NKA a1a of diadromous alewife in seawater (8-, 350-, and 250-fold decrease over 2, 5, and 15 d, respectively), the response in landlocked alewife is modest (1-, 2-, and four-fold decrease). Expression of NKA a1b following seawater exposure differs between LHFs in the opposite way: landlocked NKA a1b expression peaked at more than three times the level of, and 3 days later than, the diadromous LHF. These differences suggest that landlocked alewife may bear a higher energetic cost upon seawater exposure than diadromous alewife. However, total NKA enzyme activity in the gills of seawater-challenged landlocked alewife is lower than that in the diadromous LHF (Velotta et al. 2015). In any case, reduced tolerance of seawater in landlocked alewife is likely due to osmoregulatory failure rather than energetic exhaustion, considering that the bulk of mortality occurs within 24 h of exposure and is associated with reduced ability to maintain homeostasis of plasma osmolality (Velotta et al. 2015).

Landlocked LHFs have altered or eliminated a response in NKA α1b following seawater challenge in other taxa. Upregulation of NKA α1b upon seawater exposure was delayed but ultimately more pronounced in landlocked Atlantic salmon compared to the diadromous LHF (McCormick et al. 2019; Nilsen et al. 2007). In contrast, no upregulation of NKA α1b was detected in landlocked Arctic char even after 7 days of seawater exposure (Bystriansky et al. 2006). Experiments on additional taxa with diadromous and landlocked LHFs will be fruitful, as will comparative studies of species with varying degrees of euryhalinity; 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). 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).

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 alewife, 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).

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 (Jorgensen 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 seabass (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 evolution of NKA α1a and α1b paralogs in alewife and Euteleosteomorpha. Figure 3 shows that the branch for NKA α1a and α1b in Clupeiformes, represented by alewife, is separate from the branch for paralogs in Salmoniformes, Esociformes (represented by *E. lucius*), or Galaxiiformes. 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 in Clupeiformes, Galaxiiformes, and Anabantiformes is expected to result in multiple taxon-specific, paralog-specific clades like those seen in Salmoniformes.

Our phylogenetic analysis provides novel information regarding relationships of Euteleosteomorpha NKA paralog sequences. Consistent with previous analyses (Blondeau-Bidet et al. 2016; Dalziel et al. 2014; Urbina et al. 2013), paralogs in Salmoniformes group by paralog, while paralogs in Galaxiiformes and Anabantiformes group by taxon in our nucleotide-based phylogeny, albeit with limited taxon sampling (Figs. 3 and 4). The phylogenetic placement of paralogs is the same for salmonids whether based on nucleotide or amino acid sequence, but it differs for Galaxiiformes. In the amino acid-based 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. 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. Resolution of when paralogs diverge is needed in multiple groups such as Clupeiformes, Galaxiiformes, and Anabantiformes. Additionally, expansion of sampling in 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 in 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 diadromy, euryhalinity, and the invasion of fishes into freshwater have great evolutionary importance as this ability led to significant diversification of bony fishes (Blondeau-Bidet et al. 2016; Dalziel et al. 2014; Schultz and McCormick 2013; Urbina et al. 2013). The instances of independent evolution of NKA α1a and α1b shown in this study indicate that multiple molecular solutions for the function of 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 diadromy. 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. Further study of NKA α1a and α1b may give important insight into the history and evolution of diadromy and, more broadly, euryhalinity.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00442-024-05588-x.

Acknowledgements The authors would like to thank Brandon Thai, Brennan Kane, Jessica Norstog, Daniel Hall, and Rachel O'Neill for lab and analysis assistance. Special thanks to Jeffrey Divino for insightful comments and review. Bo Reese and the Center for Applied Genomics and Technology provided sequencing and additional analysis support. Any use of trade, product, or firm names is for descriptive purposes only and does not imply endorsement by the U.S. Government.

Author contribution statement RC, ES, JV, and SM garnered the funding, acquired materials, conceived and designed the experiments. RC, ES, JV, SM, and EJ collected, analyzed and interpreted the data. RC wrote the initial draft and was assisted in revision by ES, JV, SM, and EJ.

Funding Funding was provided by the Society of Integrative and Comparative Biology (GIAR: G201903158723598), Connecticut Museum of Natural History, and University of Connecticut Department of Ecology and Evolutionary Biology.

Data availability Alewife (*Alosa pseudoharengus*) sequences used in this study are being accessioned in the NCBI GenBank public database as ON010522 & ON010523. Other datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All applicable institutional and/or national guidelines for the care and use of animals were followed. Fish were collected and handled in compliance with protocol A17-010 as approved by the University of Connecticut Institutional Animal Care and Use Committee.

Consent to participate Not applicable.

Consent for publication Not applicable.

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