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Ion Transporter Gene Families as Physiological Targets of Natural Selection During Salinity Transitions in a Copepod

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Salinity is a key factor that structures biodiversity on the planet. With anthropogenic change, such as climate change and species invasions, many populations are facing rapid and dramatic changes in salinity throughout the globe. Studies on the copepod *Eurytemora affinis* species complex have implicated ion transporter gene families as major loci contributing to salinity adaptation during freshwater invasions. Laboratory experiments and population genomic surveys of wild populations have revealed evolutionary shifts in genome-wide gene expression and parallel genomic signatures of natural selection during independent salinity transitions. Our results suggest that balancing selection in the native range and epistatic interactions among specific ion transporter paralogs could contribute to parallel freshwater adaptation. Overall, these studies provide unprecedented insights into evolutionary mechanisms underlying physiological adaptation during rapid salinity change.

ATPase; crustacean; ion transport; natural selection; osmoregulation; sodium antiporter

Introduction

Environmental salinity is a major variable that structures the distribution of taxa from all domains of life across the globe (1, 2). As such, salinity typically poses a formidable biogeographic barrier that most species cannot penetrate (2, 3). Over evolutionary time relatively few taxa have crossed this salinity barrier, such that the vast majority of animal diversity still remains in the sea (4, 5). However, in contemporary times, breaching this salinity barrier has become increasingly common due to human activity. On a global scale, many populations throughout the world are facing rapid transformations in environmental salinity (6–9). In particular, rapid ice melt and changing precipitation patterns are causing rapid and dramatic salinity declines in high latitude coastal habitats (6, 10–12).

In addition, populations that can breach this major salinity boundary comprise a disproportionately high number of successful contemporary invaders on earth (3, 13, 14). Saline immigrants are enriched among invaders into freshwater habitats, relative to expectations based on transport opportunity and propagule pressure (3, 15). In fact, many of the invaders originating from brackish environments include some of the most destructive invaders in freshwater ecosystems, such as zebra mussels, quagga mussels, and sea lamprey (16–18). Such rapid changes in salinity tend to impose great physiological challenges on organisms, often requiring rapid evolution of physiological tolerance and performance (19–26).

These recent freshwater invasions present intriguing questions regarding the physiological and evolutionary mechanisms underlying freshwater adaptation (27) (BOX 1). In particular, the common estuarine and saltmarsh copepod *Eurytemora affinis* species complex provides a powerful model system to investigate these mechanisms (FIGURE 1). Populations of this copepod serve as dominant grazers throughout the Northern Hemisphere and form an enormous biomass in estuaries and coastal habitats, with census sizes in the billions (37–42), providing a major food source for some of the world's most important fisheries, such as salmon, herring, and anchovy (43–48). Within the past ~80 yr, populations from the *E. affinis* complex have invaded freshwater habitats multiple times independently from genetically divergent clades (FIGURE 1A) (33, 34). These invasions occurred rapidly and repeatedly through human activity, due to increases in shipping and ballast water discharge into inland lakes. These multiple independent invasions enable the discovery of physiological and evolutionary mechanisms that are shared among these replicate invasions and are consistently associated with rapid adaptation and invasive success.

BOX 1. Definition of Terms and Concepts

Adaptation: The process by which organisms become better suited to their environment as a result of *Natural Selection* (specifically *Positive Selection*, see below). Thus adaptation occurs across multiple generations at the population level. Consequently, the frequency of beneficial alleles increases in the population, causing a shift in genetic composition of a population across generations. Thus adaptation requires genetic variation at the critical trait, upon which natural selection could act.

Natural Selection: The differential survival and reproduction of individuals due to variation in a heritable phenotypic trait, leading to proportional changes of that heritable trait in the population.

- **Positive Selection**: Natural selection favoring a beneficial allele, where individuals (with beneficial alleles) better suited to the environment survive and reproduce and poorly suited individuals die or fail to reproduce. As such, the beneficial allele increases in frequency in a population over generations.
- **Directional Selection**: Natural selection favoring an extreme phenotype, such that allele frequencies shift over time in the direction of that phenotype.
- **Balancing selection**: Any type of selection that maintains allelic variation in a population, such that there are two are more alleles at a locus in the population. Different types of selection could maintain allelic variation within a population, such as temporally varying selection, negative frequency-dependent selection, spatial heterogeneity, and overdominance.

Phenotypic Plasticity: The range of phenotypes that a genotype can express across environmental conditions. This term includes both developmental plasticity (occurring during development and generally irreversible, e.g., human height) and short-term plasticity (typically reversible, such as a suntan). Phenotypic plasticity results in ***Acclimation*** of an individual organism to an environmental condition. The curve on a graph showing the pattern of phenotypic plasticity of a given genotype is the ***Reaction Norm***.

Common-Garden Experiment: An experiment performed to distinguish between effects of adaptation versus acclimation. This approach involves rearing different populations under identical conditions for 1–2 generations to first remove the effects of developmental acclimation to native habitat conditions, so that the remaining differences between populations represent evolutionary differences in physiological performance. This step is necessary because rearing conditions during development significantly influence the physiological tolerances of adults (27).

Gene: DNA sequence on a chromosome that encodes a gene product (RNA and protein).

Allele: A DNA variant of a gene or locus (location in the genome). For a diploid organism (with two sets of chromosomes), there are two alleles at a locus for each individual. If the alleles at that locus are the same, the genotype is homozygous and if the two alleles are different, the genotype is heterozygous. There could be many alleles at a locus in a population.

Single Nucleotide Polymorphism (SNP): Variation at a single nucleotide position in a DNA sequence among individuals in a population.

Paralog: A gene copy that is created by a gene duplication event, followed by differentiation due to new mutations. Following gene duplication, a paralog sometimes gains a novel function that differentiates it from its ancestral gene (***Neofunctionalization***) or partitions the original function of the ancestral gene with another paralog, such that both paralogs are required to preserve the ancestral gene functions (***Subfunctionalization***). Paralogs are not the same as ***isoforms***, which are alternative splice variants transcribed from a given gene, resulting in different proteins encoded from the same gene.

Ionic Regulation: The regulation of the type and concentration of ions between the cell, body fluids (hemolymph), and environment. This regulation is achieved through the action of ion transporters.

Osmotic Regulation: The regulation of osmotic pressure between the cell and body fluids. This regulation is achieved through the production and destruction of osmolytes and the action of ion transporters.

Ion Transporter: A transmembrane protein that moves ions (or other small molecules) across a biological membrane to accomplish many different biological functions, such as ionic regulation, osmotic regulation, acid/base regulation, neuronal signaling, cellular communication, and energy production.

- **Primary Ion Transporter**: Primary transporters use energy, such as ATP, to power the transport of ions such as Na^+, K^+, H^+, and Ca^{+2} across a cell membrane against a concentration gradient. These transporters can generate electrical and chemical concentration gradients across the cell membrane. Examples include Na^+, K^+-ATPase and V-type H^+ ATPase.
- **Secondary Ion Transporter**: Secondary transporters transport ions (or small molecules) against the concentration gradient using the potential energy created by the primary transporters. Examples include Na^+/H^+ Antiporter and Na^+/H^+ Exchanger.

Parallel Evolution: Evolution of the same heritable trait or genetic property independently in different populations, lineages, or species. Parallel evolution might occur at the level of phenotype, genotype,

gene, allele, or SNP. It is important to specify the hierarchical level at which parallelism is occurring to avoid confusion. Parallel evolution differs from the term "convergent evolution," which refers to the independent evolution of the same trait among distantly related species, such that the given trait in the descendants converges to become much more similar to one another than in their ancestors. In the case of parallel evolution, the lineages change in a similar direction, such that the heritable trait is as similar to one another in the descendant lineages as in their ancestors. There is some confusion in the usage of these terms in the literature, such that "parallel" and "convergent" evolution are sometimes used interchangeably.

Structural Mutation: Mutations that alter the amino acid composition, or secondary, tertiary, or quaternary structure of a protein. Structural mutations typically refer to changes in the coding sequence within a gene such that the gene product is altered, and a particular allele becomes a new allele.

Regulatory Mutation: Mutations that alter gene or protein expression or activity (e.g., allosteric control, conformational changes). Regulatory evolution does not change the identity of a gene or protein, but the amount or timing of gene or protein expression or its activity. **cis-*Regulatory Mutations*** occur near a gene, such as at a promoter or enhancer, so that expression of a nearby gene or set of genes is altered. In contrast, **trans-*Regulatory Mutations*** occur in a *trans*-acting factor (such as at a transcription factor, repressor, or microRNA) that can alter the regulation of expression of distant genes encoded somewhere else in the genome. Mutations in *trans*-acting factors will tend to alter the regulation of expression of many genes throughout the genome.

Epistasis: The interaction between two or more genetic loci where their joint effect on the phenotype differs from the additive effects of the loci (the sum of the independent effects of the loci). Epistatic genetic variance is the genetic variance attributable to the interaction of two or more loci.

Coadapted gene complex: Interacting genes within a population, where the functional interactions among the genes are critical for the fitness of individuals in a population. Coadapted genes often have compensatory mutations that have been favored by selection. Examples of coadapted gene complexes include enzymes in which their protein subunits must be coadapted to function together as a unit.

Genetic Assimilation: A process described by C. H. Waddington (28), where a phenotype is initially expressed as a plastic response to an environmental factor but then is favored by natural selection and becomes genetically "fixed" (constitutively expressed) in a population. As a result, this phenotype no longer relies on an environmental cue to be expressed.

Genetic Canalization: "Canalization" is the ability of a population to produce the same phenotype regardless of genetic or environmental perturbation (29–31). Variation in the phenotype is thus reduced in the population. "Genetic canalization" refers the capacity for an organism's phenotype to remain unchanged in spite of mutations.

Beneficial Reversal of Dominance (BRD): BRD with respect to salinity tolerance has been demonstrated in the *E. affinis* complex (32). BRD is the phenomenon in which alternate alleles are always dominant in the environment where they have higher fitness. For example, in the case of *E. affinis* complex populations, freshwater alleles are dominant in freshwater habitats and saltwater alleles are dominant in saltwater habitats. As such, the maladapted allele will be protected from negative selection in the heterozygous stage as environmental conditions change. Thus this mechanism could help promote the action of balancing selection in a population.

In our studies comparing ancestral saline and freshwater invading populations across multiple independent invasions (FIGURE 1A), we found evolutionary shifts in physiological and life history traits during these salinity transitions. These invasions were accompanied by increased freshwater tolerance and reduced saltwater tolerance in the freshwater invading populations, relative to their saline progenitors, with tradeoffs in performance between environments (19, 24, 49). The large shifts in salinity tolerance and performance during freshwater invasions could not be attributed to phenotypic plasticity (acclimation, BOX 1), as these shifts far exceed the degree of plasticity that is achievable by any individual (27). The transitions from saline to freshwater habitats do in fact require heritable changes in physiological tolerance and performance, resulting from natural selection favoring freshwater adapted alleles (50).

In response to strong selection during these salinity transitions, we have identified genome-wide targets of natural selection (next sections; FIGURES 1–3). Across multiple independent invasions, we found parallel evolution occurring at the levels of individual single nucleotide polymorphisms (SNPs) and genomic regions in genetically distinct wild populations (FIGURES 1, 3, AND 4; BOX 1), as well as in replicate lines during laboratory selection (FIGURE 4). Our genome-wide studies implicated ion transporter genes as a major category of candidate genes undergoing natural selection during salinity transformations (FIGURES 1–4; BOX 1) (35, 51, 52). Additionally, our results suggest that a set of ion transporter gene paralogs potentially form coadapted gene complexes that cooperate in function and evolve together in response to salinity change (see RAPID EVOLUTIONARY RESPONSES DURING LABORATORY SELECTION, THE GENOMIC

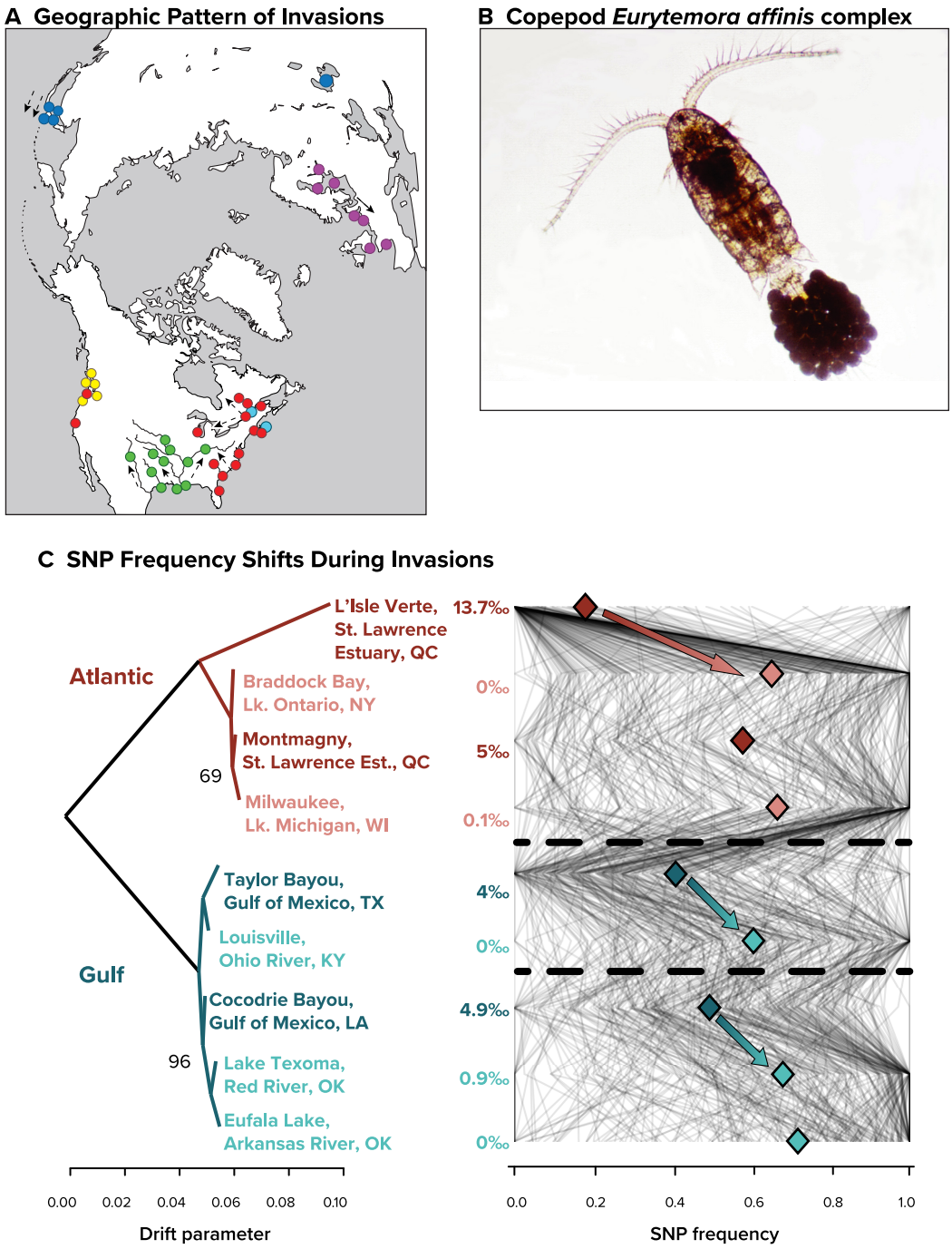
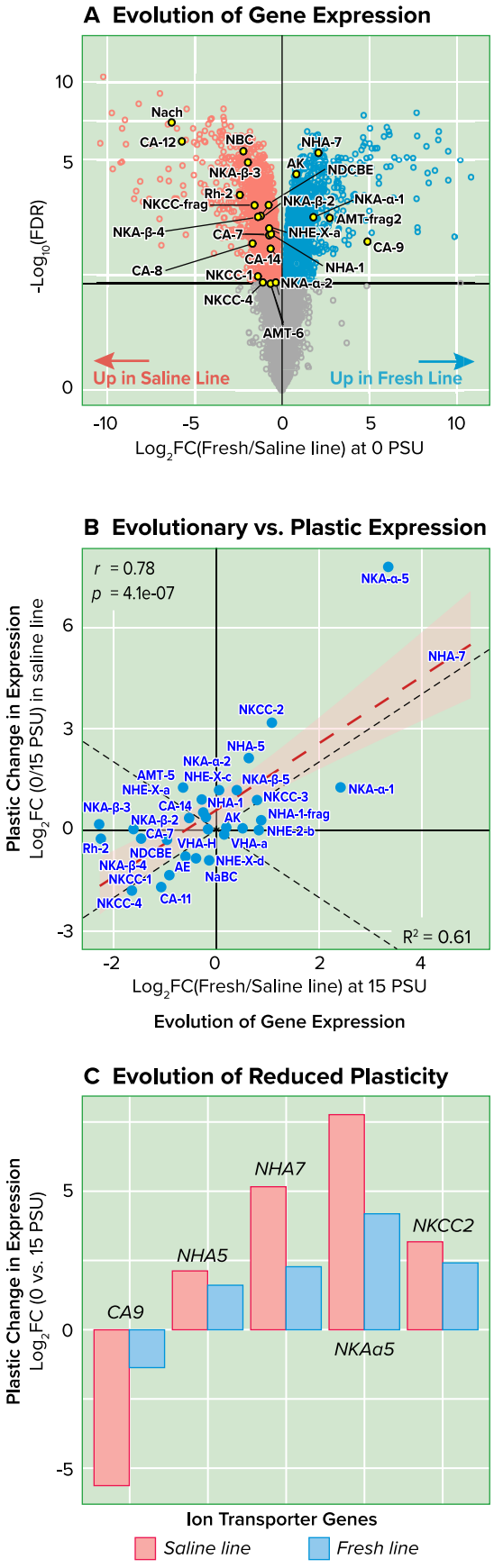


FIGURE 1. **Rapid evolution during multiple independent invasions**
A: populations of the *Eurytemora affinis* species complex, showing independent invasions from saline to freshwater habitats. Colors of dots represent genetically distinct clades (33, 34). Dotted arrows represent direction of invasions, which occurred within the past ~80 yr (33). *B*: an adult female *Eurytemora affinis* complex individual from the Columbia River estuary (*A*, Pacific clade, yellow). Photo taken in 1994 by C. E. Lee. *C*: parallel allele frequency shifts, indicating signatures of natural selection, during repeated saline to freshwater invasions (35). Shown are SNP frequency shifts (arrows) from native saline (dark) to invasive freshwater (light) populations for the Atlantic (red) and Gulf (green) clades, with salinities shown to the *right* of each population (in parts per thousand). The grey lines show changes in single nucleotide polymorphism (SNP) frequencies for the 347 SNPs that show both significant signatures of directional selection (BayeScan 3) and association with salinity (BayPass). Diamonds represent mean candidate SNP frequencies of the corresponding population on the phylogeny. SNP frequencies are polarized to keep directionality consistent among SNPs. Dashed horizontal lines group genetically related invasive and native populations, signifying recent independent invasions. The population phylogeny was estimated from SNP frequency correlations of 366,781 SNPs shared between the clades using TreeMix v. 1.13. All nodes have bootstrap support of 100%, except for those shown. Only the Atlantic clade (red) has been named *E. carolleeae* (36), making the *E. affinis* species complex paraphyletic. Thus, the term "*Eurytemora affinis* complex" is used throughout the paper to include all the clades.



TARGETS OF NATURAL SELECTION, and EVOLUTIONARY MECHANISMS UNDERLYING RAPID PARALLEL ADAPTATION; BOX 1) (51, 52).

The following sections discuss the evolutionary physiological responses of this copepod to rapid changes in salinity, focusing on recent work from the Lee Laboratory. The terms "we" and "our" refer to the coauthors of the publications from the Lee Lab that are cited and discussed in the text. These studies provide novel insights into evolutionary mechanisms of rapid adaptation and the genetic targets of selection but also raise many questions that warrant further investigation. While these studies focus on evolutionary responses to rapid salinity change, many of the principles discussed here apply more generally to mechanisms underlying rapid and parallel adaptation in response to environmental change.

Physiological Evolution during Saline to Freshwater Invasions

To determine the traits and underlying loci that evolve in response to environmental change, we employed a "common-garden" experimental approach to distinguish between responses that are due to *acclimation* (phenotypic plasticity) versus *adaptation* (heritable changes resulting from positive selection) (see BOX 1). This approach revealed striking evolutionary changes in body fluid regulation during saline to freshwater invasions. Freshwater invasions were accompanied by the evolution of increased body fluid concentrations, with freshwater populations showing elevated

FIGURE 2. **Evolutionary shifts in genome-wide gene expression in the copepod *Eurytemora affinis* complex (Atlantic clade)**
Results from *experiment 1* in Posavi et al. are shown (51). *A*: evolutionary shifts in genome-wide gene expression between saline and freshwater inbred lines reared under common-garden conditions at 0 PSU. Genes potentially involved in ionic regulation are labeled with yellow dots. The horizontal axis indicates $\log_2$ fold changes in gene expression, with upregulation in fresh lines toward the right (blue dots, 1,716 genes) and upregulation in the saline lines toward the left (red dots, 2,087 genes). The vertical axis indicates the $-\log_{10}$ of false discovery rate (FDR) adjusted *P* value, with higher values indicating greater statistical significance. The horizontal line within the graph indicates the FDR = 0.05 significance threshold. *B*: correlation between evolutionary and plastic shifts in gene expression for 30 ionoregulatory genes. Correlation was highly significant between evolutionary shifts in gene expression (between saline and freshwater inbred lines at 15 PSU, horizontal axis) and plastic changes in expression (at 0 vs. 15 PSU for the saline line, vertical axis). The red dashed line represents the linear regression fit curve with the 95% confidence region shaded in pink. *r* = Pearson coefficient of correlation: R^2 = coefficient of determination indicating the effect size of correlation. *C*: evolution of reduced plasticity in gene expression in the freshwater (blue) relative to the ancestral saline (red) lines, showing the pattern for selected ion transporter genes. The *y*-axis shows plasticity in gene expression as the $\log_2$ fold change in expression between salinities 0 PSU vs. 15 PSU. See FIGURE 4 for full gene names.

hemolymph osmolalities under low-salinity conditions (below 15 PSU) relative to ancestral saline populations (25). The maintenance of elevated hemolymph osmolality in the freshwater populations is consistent with the prediction that increased body fluid regulation would evolve as organisms are further removed from the sea (53).

To maintain this elevated hemolymph osmolality in the freshwater populations, physiological mechanisms would be required to either increase rates of ion uptake (21) or minimize ion losses (54). Consistent with the need for increased ion uptake rates in freshwater environments, we found the evolution of increased activity of a key primary ion transporter (BOX 1) that powers the uptake of cations from the environment. That is, under common-garden conditions, freshwater invading populations from both the Atlantic and Gulf clades of the *E. affinis* complex showed increased enzyme activity of the proton pump V-type H^+-ATPase (VHA) under freshwater conditions (0 PSU) but declines at higher salinities, relative to ancestral saline populations (21) (FIGURE 4C). This evolutionary shift was consistent with tradeoffs found between high- versus low-salinity tolerance (19, 24, 49). In contrast, activity of the primary transporter Na^+-K^+-ATPase (NKA) showed an evolutionary reduction in the freshwater populations across all salinities (21) (FIGURE 4C). However, individual NKA paralogs showed divergent evolutionary responses, suggesting functional differentiation among the paralogs (FIGURE 4A).

Evolutionary Shifts in Gene Expression During the Salinity Transition

To explore evolutionary genomic responses to salinity change, we examined genome-wide patterns of gene expression between ancestral saline and derived freshwater populations of the *Eurytemora affinis* species complex, reared under two different common-garden conditions (0 vs. 15 PSU) (51). Evolutionary

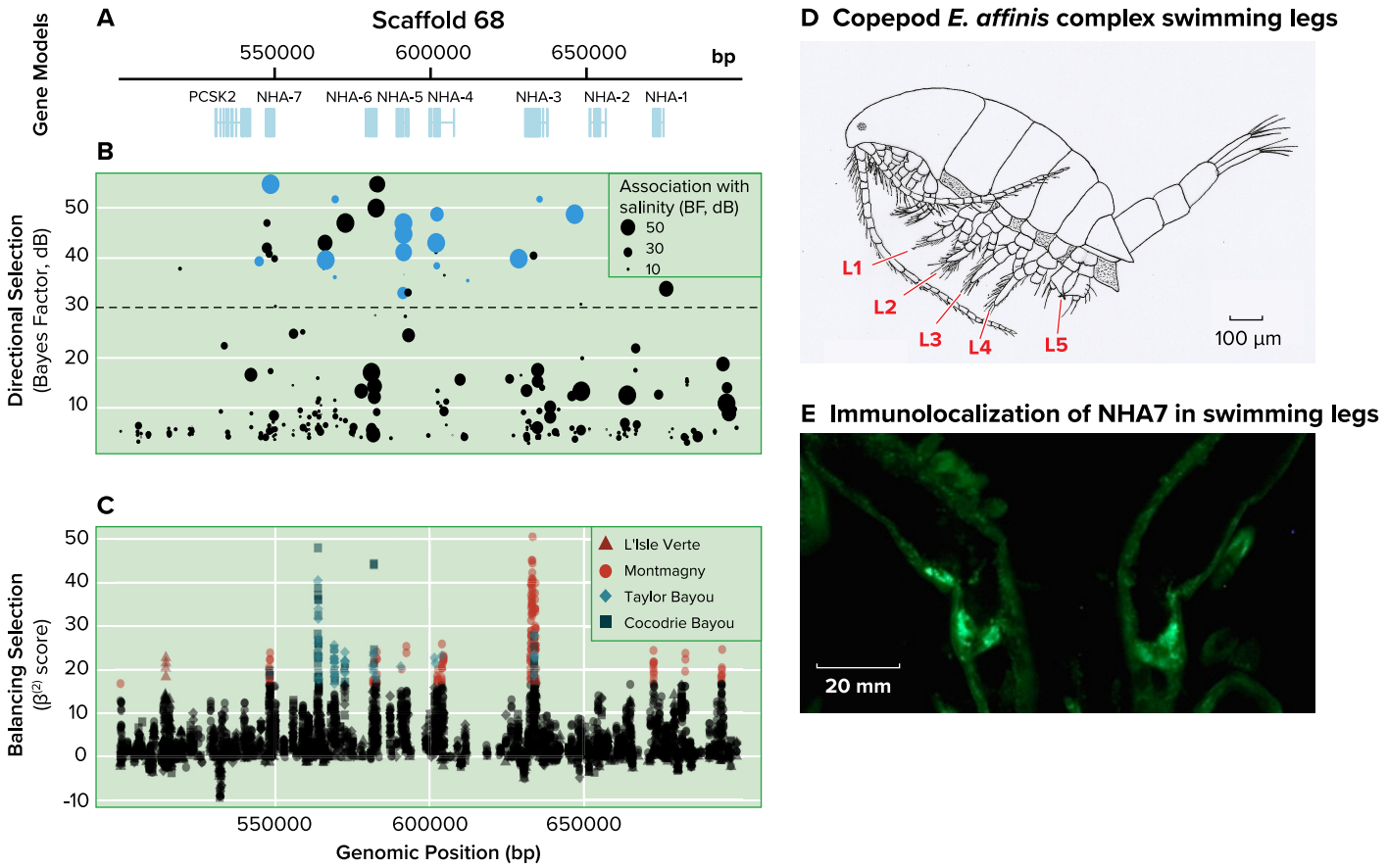


FIGURE 3. **The Na^+/H^+ Antiporter (NHA) gene family in the *Eurytemora affinis* complex**
A: Tandem paralogs of the *NHA* gene family on Scaffold 68 of the genome (35). *B*: support for directional selection between saline and freshwater populations (shown in FIGURE 1C) estimated with BayeScan 3 by comparing single nucleotide polymorphism (SNP) frequencies (35). The horizontal dotted line marks the significance threshold for directional selection. Blue dots correspond to SNPs with highest support for parallel directional selection (using BayeScan 3). Black dots above the dotted line correspond to SNPs with support for directional selection in one clade alone. Size of the dots corresponds to the level of support for association with salinity (Bayes Factor). *C*: signatures of balancing selection based on an excess of SNPs at similar frequencies [$\beta^{(2)}$ scores] in 4 native range saline populations from the Atlantic (red) and Gulf (green) clades (FIGURE 1C) (35). Colored data points represent SNPs in the top 1% of $\beta^{(2)}$ scores in each population. *D*: drawing of the copepod *E. affinis* complex, showing 5 pairs of swimming legs (L1–L5). *E*: immunolocalization of NHA-7 (bright green) in the swimming legs of an individual *E. affinis* complex copepod from a freshwater population. Photograph by Catherine Lorin-Nebel.

shifts in gene expression (between saline and freshwater inbred lines) showed far greater changes and were more widespread than acclimatory responses to salinity (0 vs. 15 PSU) (51).

Of the genes that were upregulated in the freshwater experimental lines, those related to ion transport function formed the largest gene ontology category. Specifically, in the freshwater populations (under freshwater conditions) we found the evolution of increased expression of several ion transporter paralogs, such as *Na^+/H^+ antiporter*, paralog #7 (*NHA-7*), *Na^+-K^+-ATPase* α subunit, paralog #1 (*NKA-α-1*), and *carbonic anhydrase* paralog #9 (*CA-9*) (FIGURES 2A AND 4A). Of these, NHA is of particular interest as the potential sodium transporter that might be cooperating with VHA to perform sodium uptake from freshwater environments (FIGURE 5A) (see THE GENOMIC TARGETS OF NATURAL SELECTION). Moreover, sets of ion transporter paralogs tended to show coordinated evolutionary shifts in gene expression

Ion Transporter	#Paralogs	A Gene Expression[b]				B Signatures of Selection		C Enzyme Activity[a]
		Up in fresh population		Up in saline population		Within gene	Gene nearest to SNP under selection	
		at 0 PSU	at 15 PSU	at 0 PSU	at 15 PSU			
Na^+/H^+ antiporter (NHA)	8	*NHA-7*	*NHA-7* *NHA-1 frag*	*NHA-1, 5*		*NHA-1,2,4,5,7*[c] *NHA-4, 5, 7*[c]	*NHA-2, 3, 4, 6*[c] *NHA-2, 3*[c] *NHA-3, 7*[d] *NHA-6, 7*[e]	
V-type H^+ ATPase (VHA), 14 protein coding subunits	1 per subunit, except 2 per subunits G, a, e	*VHA* a subunit (microarray)[a]				*VHA* H subunit[e]	*VHA* A subunit[d] *VHA* A subunit[e] *VHA* H subunit[e] *VHA* C subunit[e]	Increase in fresh population at 0 PSU, for Atlantic and Gulf clades
Na^+/K^+-ATPase, subunit α (NKA-α)	8	*NKA-α-1*	*NKA-α-1, 5*	*NKA-α-2*		*NKA-α-2*[c] *NKA-α-2*[c] *NKA-α-2*[e]	*NKA-α-2, 4, 5*[c] *NKA-α-2, 6*[c] *NKA-α-2*[d] *NKA-α-2,5*[e]	Decline in fresh population at 0 and 15 PSU, for Atlantic and Gulf clades
Na^+/K^+-ATPase, subunit β (NKA-β)	7		*NKA-β-5*	*NKA-β-2, 3, 4*	*NKA-β-2, 3, 4*	*NKA-β-5*[c] *NKA-β-5*[c]	*NKA-β-5*[c] *NKA-β-3*[e]	
Carbonic anhydrase (CA)[†]	13 α-CA 1 β-CA	*CA-9*		*CA-7, 8, 11, 12, 14*	*CA-7, 8, 11, 12, 14*		*CA-1, 5, 12*[c] *CA-1*[c] *CA-12*[d] *CA-2,7,9,12,13*[e]	
Na^+,K^+,2Cl^- cotransporter (NKCC)	4		*NKCC-2, 3*	*NKCC-1, 4, frag*	*NKCC-1, 4, frag*	*NKCC-frag,2,3,4*[e]	*NKCC-3, 4*[c] *NKCC-4*[e]	
Rh protein (Rh)	4			*Rh-2*	*Rh-2*	*Rh-2*[c] *Rh-2*[c]		
Ammonia transporter (AMT)	7	*AMT-frag2*	*AMT-frag2*	*AMT-6*	*AMT-5*	*AMT-1*[e]	*AMT-3*[c] *AMT-5,6*[e]	
Na^+/H^+ exchanger (NHE)	8		*NHE-2-b*	*NHE-X-a, d*		*NHE-X-d*[e]	*NHE-X-e*[d] *NHE-X-e*[e] *NHE-X-b*[e] *NHE-2-a*[e]	
*Cl^-/HCO_3^- exchanger (Anion exchanger AE)**	?			*AE-?*	*AE-?*		*AE-?*[e]	
*Na^+/HCO_3^- cotransporter (NBC)**	?			*NBC-?*	*NBC-?*			
*Na^+-driven Cl^-/HCO_3^- exchanger (NDCBE)**	?			*NDCBE-?*	*NDCBE-?*		*NDCBE-?*[d] *NDCBE-?*[e]	
Na^+channel (Nach)	?			*Nach-?*	*Nach-?*		*Nach-?*[d] *Nach-?*[e]	

FIGURE 4. **Evolutionary shifts at ion transporter paralogs between saline and freshwater populations of the *Eurytemora affinis* species complex**

Shown are ion transporter paralogs that show: *A*: evolutionary shifts in gene expression based on RNA-Seq [except for V-type H^+-ATPase (VHA)]; *B*: signatures of selection at single nucleotide polymorphisms (SNPs) (based on Pool-seq), either within a gene or within 50 kb from an exon; and *C*: evolutionary shifts in enzyme activity based on enzyme kinetic assays. Number of paralogs found for each ion transporter gene family in the *E. affinis* complex genome are listed in *column 1*. Red, Atlantic clade (aka, *E. carolleeae*); green, Gulf clade; purple, Europe clade, Baltic Sea (Kiel) experimental evolution, parallel selection across 10 selection lines; magenta, Europe clade, signals of selection shared in both the Baltic and North Sea wild populations. Reference: [a]Lee et al. (21); [b]Posavi et al. (51); [c]Stern and Lee (35); [d]Stern et al. (52); [e]J. Diaz, D. B. Stern, and C. E. Lee, unpublished observations. *Members of the bicarbonate transporter gene family, such as Cl^-/HCO_3^--exchangers (AE), Na^+/HCO_3^--cotransporters (NBC), and Na^+-driven Cl^-/HCO_3^--exchanger (NDCBE), cluster together in a phylogeny (form a clade) and their exact identities are difficult to distinguish from one another. The question marks indicate either an unknown number of paralogs in the *E. affinis* complex genome or an unknown paralog identity. [†]Carbonic anhydrases in this table are all alpha-carbonic anhydrases.

between saline and freshwater populations (FIGURE 4A) (51). These results, along with those in the next section, suggest that particular sets of ion transporter paralogs form coadapted gene complexes that cooperate in function and evolve together in response to environmental change.

A notable feature of the *E. affinis* complex genome are the expansions of ion transporter gene families, relative to the lower number of paralogs found in insects (FIGURE 4). For example, the *E. affinis* complex genome contains eight paralogs of *NHA*, eight of *NKA-α*, and seven of *NKA-β*, whereas *Drosophila melanogaster* has only two to three paralogs of each. Most notably, patterns of gene expression evolution differ among ion transporter paralogs, with some showing opposite evolutionary responses to salinity (FIGURE 4A) (51). The different patterns suggest functional differentiation among the paralogs (neofunctionalization, BOX 1). Alternatively, different paralogs might have compensatory functions (subfunctionalization, BOX 1) or might display nuanced transcriptional regulation among paralogs with similar function.

Interestingly, we found that plastic and evolutionary responses in gene expression were highly correlated in magnitude and direction when examining thirty key ion regulatory genes (FIGURE 2B) (51). For instance, the ion transporter paralogs *NHA-7*, *NKA-α-5*, and *NKA-α-1* generally showed especially high correlation between acclimatory and evolutionary responses. These results indicate some degree of shared mechanisms of freshwater acclimation and adaptation.

In fact, the plastic response could potentially facilitate the adaptive response. A strong beneficial plastic response under novel conditions, such as increased expression of a critical ion transporter in fresh water, could enable a population to survive upon arrival in the novel habitat. Selection could then act to favor that expressed extreme phenotype, essentially selecting for the more plastic reaction norm (28, 55). Thus, in this manner, plasticity of a trait could enable natural selection to act on this trait in a novel environment and promote rapid adaptation.

Eventually, this plasticity would erode over time, as the trait that was beneficial in the original environment would no longer be required in the novel environment and might be costly. In addition, the extreme plasticity-induced trait would become genetically encoded, leading to "genetic assimilation" (BOX 1) (28, 55, 56). Thus, the general prediction is that canalization (BOX 1) would evolve over long periods of time following invasions into a stressful environment (28, 55, 56). Consistent with this prediction, we uncovered the evolution of reduced plasticity in populations following freshwater invasions, both genome-wide and for many of the same ion transporters showing evolutionary shifts in gene expression (FIGURE 2C) (51).

Genomic Signatures of Selection at Ion Transporter Genes during Freshwater Invasions

Adaptation of populations to environmental change often leaves characteristic imprints of natural selection within their genomes. In our comparison across multiple independent invasions, we discovered parallel genomic signatures of natural selection across replicate salinity clines in natural populations (35). Population genomic sequencing revealed that often the same single nucleotide polymorphisms (SNPs) and genomic loci were repeatedly favored by selection in freshwater invading populations, showing the same pattern of frequency shifts across three independent saline-to-freshwater habitat transitions (FIGURE 1C). Our data set contained 29 times the number of SNPs with signatures of parallel selection and association with salinity than expected under genetic drift alone. Specifically, of the 6,891 SNPs showing signatures of selection in at least 1 clade, 2,970 SNPs displayed signatures of parallel directional selection (BOX 1). Of these SNPs under parallel selection, 347 also showed significant association with salinity (FIGURE 1C) (35). These results indicate that the same alleles are repeatedly favored by natural selection across the salinity cline, even across genetically divergent populations.

The SNPs under selection during saline to freshwater transitions tended to occur in genomic regions heavily enriched with ion transporter genes (35). As in the gene expression analysis (previous section), gene ontology terms related to ion transport emerged as the dominant functional categories that were enriched with SNPs under parallel selection during freshwater invasions. Interestingly, many of the same ion transporter paralogs that showed evolutionary shifts in gene expression (previous section, FIGURE 4A) also showed genetic signatures of selection, such as *NHA-7, NKA-α-2, NKA-β-5*, and *Rh-2* (FIGURE 4A).

In particular, we found that the highest density of SNPs under selection occurred within a genomic region containing seven tandem paralogs (variable gene duplicates, BOX 1) of the Na^+/H^+ *antiporter* (*NHA*) gene family (FIGURE 3A). *NHA* paralog 7 (*NHA-7*) showed especially strong signatures of directional selection (FIGURE 3B, blue dots). We found that certain *NHA-7* alleles undergo parallel frequency shifts across *E. affinis* complex populations in North America (35) and in the Baltic Sea region, in both wild populations and during laboratory selection (next section) (FIGURE 4) (52). The high repeatability of selection acting on *NHA-7* alleles (and other ion transporter alleles, FIGURE 4) suggest that they contribute to physiological adaptation in response to changes in environmental salinity. Using in situ immunohistochemical staining, we localized the protein expression of three critical ion transporters (VHA, NKA, and NHA-7) within

the osmoregulatory "Crusalis organs" in the swimming legs of *E. affinis* complex copepods (FIGURE 3E) (57, 58). Overall, our results suggest that NHA might play an essential role in ion uptake in low-salinity habitats (see THE GENOMIC TARGETS OF NATURAL SELECTION, FIGURE 4). Although, we know nothing about the potentially divergent functions of the eight *NHA* paralogs within the *E. affinis* complex genome.

Interestingly, we found that for the genetic loci (SNPs) under parallel selection during invasions, a surprisingly high portion of genetic variation at these SNPs appears to be maintained by balancing selection in the native range populations (FIGURE 3C; BOX 1) (35). That is, of the loci under parallel selection during invasions, 15–47% exhibited significant signatures of balancing selection in four native range populations. These results suggest that balancing selection specifically promotes parallel evolution by maintaining shared adaptive variation in the native range.

Based on our results, freshwater adaptation appears to have arisen through selection on standing genetic variation in the native range (35). Native brackish habitats of *E. affinis* complex populations are marked by seasonal fluctuations in salinity, ranging from 5 to 40 PSU in some locations. In temporally varying habitats, genetic polymorphism could be maintained by selection favoring different alleles during different seasons. In addition, the presence of beneficial reversal of dominance (BOX 1) could preserve the maladapted alleles against negative selection as conditions change (32). Such mechanisms are likely to be operating to maintain genetic variation in salinity tolerance within native ranges of *E. affinis* complex populations, allowing rapid adaptation to salinity change during invasions into novel habitats.

Rapid Evolutionary Responses During Laboratory Selection

We performed a laboratory natural selection experiment (experimental evolution) under declining salinity, to determine whether changes in salinity alone in the laboratory could reproduce the allele frequency shifts that we found in the wild populations (FIGURE 1C) (52). Starting with a Baltic Sea saline population from Kiel, Germany (15 PSU), we reduced salinity down to 0 PSU (lake water) over ten generations, with ten replicate treatment lines and four control lines, performing whole-genome sequencing (Pool-seq) at multiple time points.

We found largely parallel responses to selection that often acted on the same alleles at the same sets of loci, especially for ion transporter paralogs (FIGURE 4B) (52), consistent with results for wild populations from genetically distinct clades (see previous section) (35). Our results indicated that selection tended to act on the exact same SNPs across 10 replicate laboratory lines in a manner that exceeded expectations given the number of loci involved in the selection response. That is, we found a greater degree of parallelism in our experiment than was expected from simulations of the same number of SNPs under selection.

Most notably, parallelism increased at later generations of selection, with the same alleles (SNPs) rising in frequency among the replicate selection lines, more than expected by chance (52). Our forward simulations indicated that positive epistasis could promote the observed pattern of parallel selection. This result suggests that alleles under selection potentially act in a nonadditive fashion such that synergistic epistasis drives the increase in parallelism with increasing generations of selection (see EVOLUTIONARY MECHANISMS UNDERLYING RAPID PARALLEL ADAPTATION; BOX 1). Positive synergistic epistasis would be consistent with the faster than expected convergence of allele frequency shifts, given that the positive interactions among alleles would tend to increase the rate of adaptation.

In addition, we found that across wild Baltic and North Sea populations, laboratory-selected SNPs were widely distributed and showed signatures consistent with balancing selection (52). These results were concordant with our findings for native range Atlantic and Gulf clade populations (FIGURE 3C). Thus selection has the potential to act on standing genetic variation in the Baltic Sea populations to enable rapid evolutionary responses to future salinity change.

The Genomic Targets of Natural Selection

Our results suggest that a set of cooperating ion transporters form a coadapted gene complex that undergoes natural selection as a unit. Repeatedly across our diverse studies, the same paralogs were implicated in low-salinity adaptation, such as *NHA-7*, *CA-9*, *NKA-α-1*, *NKA-α-2*, and *NKA-β-5* (FIGURE 4). We found that particular alleles of these ion transporter paralogs tend to rise in frequency in a coordinated manner in response to low salinity (35, 52) and show coordinated changes in gene expression (51) (see previous sections). These results are consistent with synergistic epistasis occurring between specific ion transporter alleles (BOX 1). Given that rates of reactions and ion fluxes must be tightly regulated among cooperating ion transporters (FIGURE 5) (59, 60), it is likely that activity of a suite of ion transporters must increase jointly to effectively increase rates of ion uptake from the environment. Thus, natural selection might favor a specific set of ion transporter and regulatory alleles to induce the evolution of ion uptake function for a set of cooperating ion transporter paralogs.

When we examined in detail the evolutionary shifts across salinities for the ion transporter paralogs (FIGURE 4), the patterns were consistent with hypothesized models of ion uptake from low-salinity habitats (FIGURE 5). According to several models of

ion uptake proposed for crustaceans (and other aquatic organisms), VHA is the primary ion transporter that powers the uptake of cations from freshwater habitats by generating a proton gradient across the apical membrane of ionocytes (FIGURE 5A) (59–65). The evolution of increased VHA enzyme activity under freshwater conditions that we found in both wild freshwater populations and laboratory selection lines (21) is consistent with this potential role. Due to thermodynamic constraints, NKA is considered insufficient as the sole driving force to power ion uptake below ~1.0 mM NaCl (66). While NKA likely helps power the uptake of ions in freshwater environments, it might be rate limiting for ion uptake under more saline conditions (67, 68).

Using the proton gradient generated by VHA, cations are then transported into the cell via secondary transporters (BOX 1). However, the identity of the secondary transporter cooperating with VHA to perform Na^+ uptake has remained unclear and controversial. Based on stoichiometry, Wieczorek and colleagues (63, 69) had hypothesized that an electrogenic antiporter that exchanges cations with H^+ must be cooperating with VHA. This unknown Na^+ transporter that cooperates with VHA, later dubbed the "Wieczorek exchanger," has been hypothesized to be alternatively Na^+ channel (Nach), Na^+/H^+ exchanger (NHE), or Na^+/H^+ antiporter (NHA) (59, 60, 70–72). The *NHA* gene family (SLC9B, FIGURE 5A, white) was first discovered in animals only in 2005, and its functions are not fully understood (71–74). While NHA does seem to be critical for ion homeostasis and response to salt stress in insects, functional studies in animals have yielded divergent results, suggesting that functions might vary among *NHA* paralogs, cell types, tissues, and taxa (71, 72, 75).

Our results suggest that NHA might be serving as the Na^+ transporter cooperating with VHA to take up Na^+ from dilute media in freshwater environments. *NHA* paralog 7 showed evolutionary increases in expression in the freshwater inbred lines across salinities, relative to their saline ancestral lines (FIGURES 2A AND 4A) (51). Additionally, *NHA-7* showed strong correlations between acclimatory and evolutionary responses to salinity change (FIGURE 2B) (51), suggesting that its plastic response might potentially play an important role in rapid evolution during freshwater invasions (see EVOLUTIONARY SHIFTS IN GENE EXPRESSION DURING THE SALINITY TRANSITION). Moreover, *NHA* paralogs (especially *NHA-7*) showed significant signatures of selection associated with salinity change in wild populations from multiple clades (FIGURE 3) (35) (J. Diaz, D. B. Stern, C. E. Lee, unpublished observations) and during laboratory selection (FIGURE 4B) (52). Altogether, our results strongly suggest that *NHA* paralogs, especially *NHA-7*, might serve as important evolutionary targets during saline to freshwater invasions and likely contribute to freshwater adaptation.

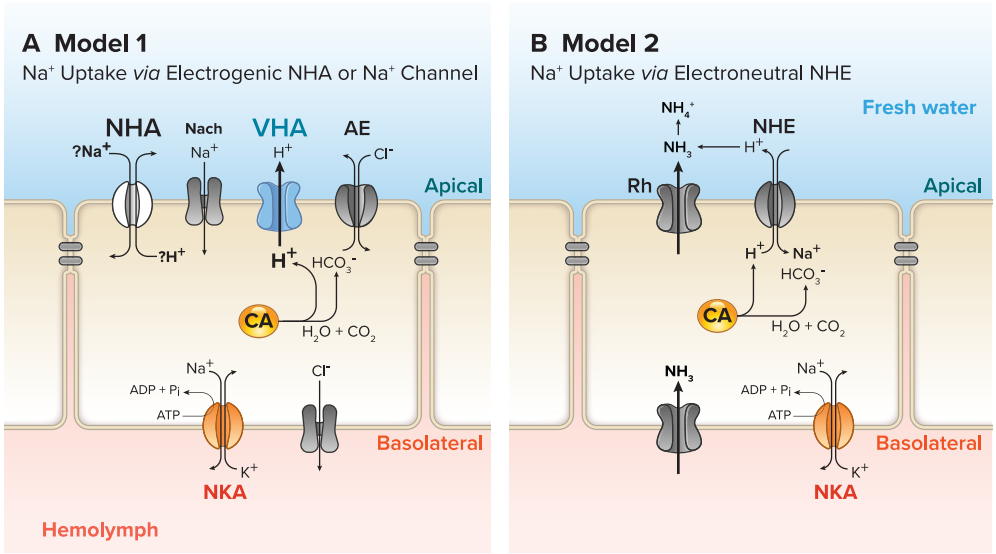


FIGURE 5. **Hypothetical models of ion uptake from fresh water by ionocytes in *Eurytemora affinis* complex populations**

Shown are primary transporters for energizing ion transport [V-type H^+-ATPase (VHA) and NKA] and hypothetical secondary transporters for sodium uptake (NHA, Na^+ channel [Nach], or NHE). *A*: *model 1* (Wieczorek's Model): VHA (blue) pumps H^+ out of the cell and creates a proton gradient, through which Na^+ is transported into the cell through an electrogenic NHA or Na^+ channel. The stoichiometry of ion transport for NHA is not fully known, even for *Drosophila*. *B*: *model 2*: ammonia is transported out of the cell by an ammonia transporter (Rh), which then drives electroneutral NHE to export H^+, and consequently import Na^+. In both models, Na^+ is transported to the hemolymph via NKA. Carbonic anhydrase (CA) supplies protons to VHA or NHE and HCO_3^- to anion exchanger (AE). The $Na^+/K^+/2Cl^-$ cotransporter (NKCC) might also play a role in ion uptake, but the localization of NKCC paralogs is unknown for *E. affinis* complex. In response to salinity change, certain paralogs of these ion transporters show evolutionary changes in gene expression and signatures of natural selection (see FIGURE 4). Alternative models have also been proposed and not all potentially relevant ion transporters are shown. Adapted from Stern and Lee (35).

Based on our results, Na^+ channel (Nach) and NHE are less likely to serve as the "Wieczorek exchanger" that cooperates with VHA to perform Na^+ uptake from freshwater habitats in *E. affinis* complex populations. *Nach* exhibited an evolutionary increase in expression in the saline lines across salinities (FIGURE 4A). The upregulation of both *NHE* and *Rh protein* in the saline lines at 0 PSU (FIGURE 4A) was consistent with the saline populations, but not the freshwater populations, using the mechanism of *model 2* for ion uptake under low-salinity conditions (FIGURE 5B).

The strong upregulation of a single paralog of *alpha-carbonic anhydrase* (*α-CA-9*) in the freshwater lines at 0 PSU (FIGURE 4A) was consistent with its importance in ion uptake under freshwater conditions (FIGURE 5). Carbonic anhydrase (CA) is an enzyme that reversibly catalyzes the chemical reaction $H_2O + CO_2 \rightleftarrows H^+ + HCO_3^-$ (76). The resulting protons H^+ are then supplied to VHA, whereas bicarbonate ions are supplied to Cl^-/HCO_3^--exchanger (anion exchanger) or some other bicarbonate transporter (e.g., NDCBE or NBC). Thus this enzyme is likely important for the functioning of ion transport by both NHA and NHE. The many *CA* paralogs (at least 14) in the *E. affinis* complex genome make it challenging to deduce their functions. In contrast to *α-CA-9*, five other *α-CA* paralogs showed evolutionary declines in expression in the freshwater lines (and increases in the saline lines, FIGURE 4A) (51). Given the various functions of carbonic anhydrase (e.g., ion uptake, acid-base regulation, respiration, excretion), different *α-CA* paralogs might be specialized to serve differing roles in different cell types and tissues.

Likewise, the primary transporter *Na^+-K^+-ATPase* (*NKA*) family includes multiple paralogs that display various evolutionary patterns of gene expression at different salinities. A few paralogs show striking increases in gene expression in the freshwater populations (*NKA-α-1* and *NKA-β-5*; FIGURE 4A). However, most of the paralogs showed declines in activity and expression across all salinities in the freshwater populations, relative to their saline ancestors (FIGURE 4A) (21, 51). This result suggests functional differentiation among *NKA* paralogs and the need to investigate their differing roles.

While our results suggest that *model 1* might serve as the dominant mechanism for ion uptake under freshwater conditions, *model 2* might still be operating under certain low-salinity conditions. At extremely low salinities, *model 1* would be more likely to operate, as the transmembrane proton gradient generated by VHA could enable cations to be taken up from extremely low ionic concentrations (FIGURE 5A). *Model 2* might be less likely to operate at extremely low salinities because NHE is thermodynamically constrained at low Na^+ concentrations (70). Thus, it is possible that each mechanism might perform ion uptake under different salinities and pH levels, with *model 1* favored under very low pH and salinities and *model 2* operating under brackishwater conditions (70). Far more studies are needed to dissect the specific roles of different ion transporters and paralogs in saline versus freshwater populations under differing conditions. Moreover, for the additional ion transporters under selection during freshwater invasions (FIGURE 4), such as *AMT*, *NKCC*, *AE*, *NBC*, *NDCBE*, and *Nach*, their functions in ion uptake from freshwater environments and their contributions to freshwater adaptation are poorly understood.

Given that many of these ion transporters perform additional physiological functions, aside from ionic regulation, their evolutionary changes could result from responses to environmental variables other than salinity. For example, VHA, CA, and the bicarbonate transporters (e.g., AE, NBC, NDCBE) are typically involved in acid-base regulation (77), such that ionic regulation can occur concurrently with pH regulation. Thus, evolutionary changes at these gene families could potentially arise from changes in environmental pH during transitions from saline to freshwater habitats, given that lakes tend to exhibit higher variance in pH (pH 6.0–9.0) relative to marine and estuarine habitats (pH 8.0–8.3) (78, 79). However, the salinity transitions we studied tended to be associated with relatively little change in pH, making adaption to pH change less likely; though, we cannot rule out this possibility (80–83). It is worth noting, however, that evolutionary changes at VHA, CA, and the bicarbonate transporters are likely to be critical during lake and ocean acidification resulting from climate change.

At the molecular level, the evolutionary changes at the ion transporter genes include both structural (amino acid) and regulatory changes (FIGURE 4; BOX 1). That is, SNPs under selection are found both within genes and at locations near genes (FIGURE 4B), potentially with regulatory function. For instance, for *NHA-7*, SNPs under selection are found both within the gene and relatively proximate to the gene in the three genetically distinct clades (FIGURE 4B). In addition, *NHA-7* exhibits evolutionary increases in gene expression in the freshwater population, relative to the saline ancestral population (FIGURE 4A), indicating regulatory evolution. Thus, selection is likely acting both on alleles that encode amino acid differences and on nucleotide sites that alter gene expression.

Our results suggest that regulatory changes are more common than amino acid changes within ion transporter genes (FIGURE 4B). For some of the ion transporter paralogs, SNPs proximate to the genes suggest the presence of *cis*-regulatory changes (BOX 1). On the other hand, many of the evolutionary shifts in ion transporter gene expression likely arise from *trans*-regulatory changes, consistent with the fact that many transcription factor genes display evolutionary shifts in gene expression (51) and also signatures of selection (35). Such *trans*-regulatory changes would

be consistent with the coordinated evolution of gene expression for sets of ion transporter genes (51).

Further investigation is needed to dissect the consequences of structural and regulatory evolution of ion transporter paralogs and subunits. Additionally, we need to better understand how these evolutionary changes impact actual ion transporter enzyme activity and function. This need is especially pertinent for VHA, given its many subunits. Evolution of amino acid composition versus evolution of gene expression at individual enzyme subunits could have important consequences for the evolution of enzyme function.

Evolutionary Mechanisms Underlying Rapid Parallel Adaptation

The extent of parallel selection acting during freshwater invasions by *E. affinis* complex populations is extraordinary. For instance, selection acted repeatedly on the same paralogs in populations from divergent clades (FIGURE 4, Atlantic, Gulf, Europe clades), far greater than expected relative to neutral simulations (35, 52). Often, selection acted on the exact same SNP in genetically divergent wild populations and in replicate laboratory selection lines. These results were surprising given the moderately polygenic nature of freshwater adaptation. Given that most physiological traits are polygenic, encoded by many loci, parallel evolution at the level of genes and SNPs might not be expected. Under polygenic adaptation, genetic redundancy of interchangeable loci would be predicted to enable alternative evolutionary pathways and result in nonparallel evolution among replicate events (84, 85).

In addition, the high degree of parallel selection was surprising given the presence of multiple paralogs for key ion transporter genes in the *E. affinis* complex genome (4–8 paralogs, FIGURE 4). The multiple ion transporter paralogs might be expected to provide genetic redundancy, permitting replicate selection events to employ different paralogs and alternative evolutionary pathways. However, selection during freshwater adaptation tended to favor certain paralogs more than others (FIGURE 4), suggesting very specific roles and nonredundancy in paralog function.

Perhaps having multiple paralogs might release some of the ion transporter paralogs from pleiotropic constraint (86), such they could evolve specialized functions to particular environments. Such specialization might account for the differences in evolutionary responses to salinity among the paralogs, with some paralogs of the same ion transporter being upregulated in the freshwater population under freshwater conditions, but others downregulated (FIGURE 4A). The repeated parallel selection on specific paralogs is consistent with specialization among paralogs. Functional studies would be required to investigate this possibility (87–89).

A notable exception to the observed parallelism was the case of the proton pump V-type H^+ ATPase (VHA). We typically did not see parallel evolution for *VHA* subunits, except in a few cases (FIGURE 4). VHA is a complex molecular machine, comprised of many subunits encoded by 14 protein coding genes in the *E. affinis* complex genome. It is possible that these multiple subunits provide some genetic redundancy, allowing selection at different subunits to accomplish the evolution of increased enzyme activity (21).

Several factors could promote the prevalence of parallel directional selection favoring the same alleles across independent evolutionary events. Our results suggest that positive synergistic epistasis among beneficial alleles at different loci is helping drive the pattern of parallel polygenic adaptation that we observe among our replicate selection lines (see RAPID EVOLUTIONARY RESPONSES DURING LABORATORY SELECTION) (52). Synergistic effects might arise among alleles in cases where coadapted gene complexes underlie physiological phenotypes (90). For example, ion transport is achieved through a suite of cooperating ion transporter proteins (FIGURE 5). The role of epistasis has been largely overlooked in genomic studies (91–93), but our recent study provides empirical support for its role in promoting parallel polygenic adaptation (52).

In addition, balancing selection might also serve as an important mechanism driving parallel selection (13, 32, 35, 52). Selection could repeatedly favor the same alleles in multiple populations if the beneficial alleles are maintained at intermediate and high frequencies, increasing the probability of selection favoring the same alleles in different populations (84, 94). However, balancing selection had long been thought to make limited contributions to the maintenance of genetic variation (95, 96) and its contribution to adaptation had remained unresolved (97, 98). Our studies are notable in being the first to show that specific SNPs under parallel selection in invading populations are enriched for same SNPs that are under balancing selection in the native range populations (35).

Concluding Remarks

The research summarized here is noteworthy for identifying the genetic architecture underlying rapid adaptation during environmental change. Across multiple studies, we have uncovered the evolutionary and physiological mechanisms of niche expansions to an unprecedented level of detail. We determined the specific loci under selection to the level of genes, paralogs, subunits, and SNPs. Moreover, our studies have found that the evolutionary response to salinity decline, while polygenic, appears to repeatedly involve a set of loci with synergistic epistatic interactions. This pattern of selection response was replicated across laboratory selection lines, multiple independent invasions, and environmental gradients in nature, encompassing populations from genetically

divergent clades (FIGURE 1). Thus, we witnessed parallel evolution due to relatively canalized evolutionary pathways utilizing the same genetic tool kit.

Our studies explored the genetic targets of natural selection during salinity change, and pinpointed ion transporter alleles as the main physiological targets under selection. A major discovery is that the ion transporter paralogs differ in their evolutionary responses and specific sets of paralogs are implicated in freshwater adaptation. Additional studies are needed to dissect the specific physiological functions of the different ion transporter paralogs and the extent to which they have undergone neofunctionalization or subfunctionalization in response to freshwater adaptation on multiple time scales. Moreover, it would be especially useful to determine exactly how the beneficial mutations favored by selection during salinity adaptation are altering ion transporter function to enable survival into novel freshwater habitats. ■

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