

Review

Genome architecture underlying salinity adaptation in the invasive copepod *Eurytemora affinis* species complex: A reviewCarol Eunmi Lee^{1,*}

SUMMARY

With climate change, habitat salinity is shifting rapidly throughout the globe. In addition, many destructive freshwater invaders are recent immigrants from saline habitats. Recently, populations of the copepod *Eurytemora affinis* species complex have invaded freshwater habitats multiple times independently from saline estuaries on three continents. This review discusses features of this species complex that could enhance their evolutionary potential during rapid environmental change. Remarkably, across independent freshwater invasions, natural selection has repeatedly favored the same alleles far more than expected. This high degree of parallelism is surprising, given the expectation of nonparallel evolution for polygenic adaptation. Factors such as population structure and the genome architecture underlying critical traits under selection might help drive rapid adaptation and parallel evolution. Given the preponderance of saline-to-freshwater invasions and climate-induced salinity change, the principles found here could provide invaluable insights into mechanisms operating in other systems and the potential for adaptation in a changing planet.

ADAPTATION DURING SALINITY SHIFTS IN A CHANGING WORLD

Natural habitats throughout the planet are being transformed at a rapid rate, due to factors such as habitat destruction, invasive species, and climate change.^{1,2} Much evidence indicates that the Sixth Mass Extinction is now underway, with devastating impacts on natural populations and communities.^{3,4} In response to such destructive human impacts, it is becoming increasingly critical to understand how populations are responding to these changes. Populations might survive under such challenges by potentially migrating to favorable locations, tolerating or acclimating to environmental stressors, or evolving through the action of natural selection.⁵ As the entire planet is now being impacted by anthropogenic stressors, migrating to more favorable conditions is becoming increasingly difficult.⁶

In the face of extraordinary environmental change, rapid acclimatory or evolutionary physiological responses are often required for populations to survive. Acclimation occurs through phenotypic plasticity at the individual organismal level. In contrast, adaptation occurs across generations through natural selection,^{7,8} which increases the frequency of beneficial alleles in a population. If the rate or extent of environmental change exceeds the threshold tolerance or plasticity of individuals within populations, the populations will need to adapt through natural selection in order to avoid extinctions.⁹

Physiological traits tend to be polygenic, that is, encoded by many genes, such that physiological adaptation would require natural selection to act on many genes simultaneously. However, we have a relatively poor understanding of the genomic architecture underlying most physiological traits. The increasing availability of genomic resources for a wide range of species, especially for non-model organisms, is allowing us to understand how polygenic traits evolve in response to various types of environmental change.^{10,11}

In aquatic habitats, salinity imposes a formidable biogeographic boundary that structures the distribution of most aquatic taxa.^{5,12,13} An intermediate salinity of 5 PSU tends to serve as a biogeographic and physiological barrier that separates saline and freshwater species.^{14,15} As life evolved in the sea, most marine organisms are osmoconformers and do not need to regulate the fluxes of water, ions, and osmolytes between their extracellular fluids and the environment.^{16,17} Over half of all animal phyla remain strictly marine, and the majority of animal taxa have not evolved the ability to tolerate lower salinities.^{18,19} Colonizations away from the sea into lower salinity habitats have required the evolution of body fluid regulation and mechanisms to maintain homeostasis.^{16,17} With increases in the ionic and osmotic gradients between the extracellular fluids and the surrounding environment, physiological mechanisms to perform ion uptake and prevent ionic losses became necessary.²⁰

With climate change, habitat salinity is changing rapidly throughout the globe.⁵ Drastic changes in the global water cycle are causing changes in patterns of evaporation and precipitation, resulting in rapid transformations in sea surface salinities, as well as large impacts on terrestrial biomes.^{21–26} In addition, massive volumes of ice melt are dumping into the seas.²⁷

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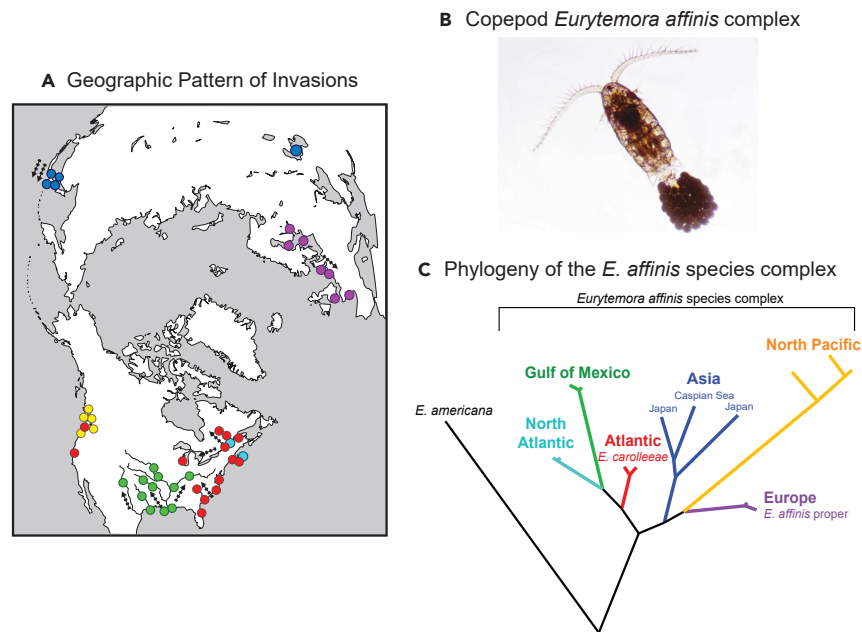


Figure 1. Geographic distribution and phylogeny of the *Eurytemora affinis* species complex

(A) Geographic distribution of genetically distinct clades of the *E. affinis* complex. Colored dots represent the clades shown in the phylogeny (in C). Dashed arrows represent recent independent saline to freshwater invasions. Based on data from Lee (1999).

(B) A photo of an adult ovigerous female *E. affinis* complex copepod from the Columbia River estuary, Oregon, USA (North Pacific clade). Photo by Carol Lee.

(C) Phylogeny of the *E. affinis* species complex. Colored branches in the phylogeny correspond to the clades of the same color shown in the map (in A). Data from Lee (2000). The Atlantic clade has been named *E. carolleae*³⁹ and *E. affinis* proper was originally described based on populations in Europe.⁴⁰

Thus, at higher latitudes, the rates and extent of salinity change are projected to be more extensive than changes in temperature.²¹ For example, sea ice melt and advection are causing “Great Salinity Anomalies” near Greenland and Labrador, contributing to significant freshening of the Arctic and Northwest Atlantic oceans.^{28–30} Additionally, over the past ~60 years salinity in the Baltic Sea has already declined by up to 1.5 PSU in surface and bottom waters, in part due to increases in river runoff.^{31,32} Although, future salinity predictions are still unclear due to uncertainties in factors affecting the Baltic Sea water budget, such as future wind patterns, river runoff, and exchange between the Baltic and North Seas.³³

In contrast, sea surface salinities are expected to increase in many lower latitude regions.³⁴ For instance, the Mediterranean Sea is a hot spot for climate change, as a semi-enclosed marginal sea where rates of change in salinity and temperature are projected to be far greater than in the open seas.^{34,35} Some areas of the Mediterranean are projected to experience salinity increases by 2–4 PSU within 7 years, due to decreases in precipitation and reduced riverine flow from damming.³⁴ These increases in salinity and temperature are expected to have profound negative impacts on the productivity of estuaries and lagoons of the Mediterranean Sea.^{35–37}

In addition to salinity alterations induced by climate change, many invasive populations are experiencing salinity change during biological invasions³⁸ (e.g., Figure 1). Brackishwater species appear to be particularly successful as invaders in freshwater lakes and reservoirs.^{38,41–43} For instance, brackishwater invaders from the Black and Caspian Sea region are much more common in the Great Lakes than expected based on transport opportunity and abundance in the native ranges.⁴² These invaders that are crossing salinity boundaries include some of the most prolific invaders in aquatic habitats, such as zebra mussels, quagga mussels, the fishhook waterflea *Cercopagis pengoi*, and many species of amphipods.^{44–48,50} Understanding the evolutionary and physiological responses of invasive populations to salinity change is essential for understanding mechanisms of invasions into the Great Lakes and other freshwater habitats throughout the world.

Of aquatic invaders, planktonic crustaceans, such as copepods, amphipods, and branchiopods, are extremely pervasive.^{49–53} In particular, copepods form a huge biomass in aquatic ecosystems and serve essential roles as grazers of algae and food source for fisheries. Copepods constitute up to 90% of ballast water biomass and compose a large number of aquatic invaders throughout the world.^{54–57} For instance, many estuaries along the West Coast of North America are now dominated by copepods from East Asia.^{51,58–61} Copepod invasions can be highly problematic when introduced copepod populations compete with or displace native copepods that serve as food sources for local fisheries.^{58,62,63}

EURYTEMORA AFFINIS SPECIES COMPLEX AS A MODEL SYSTEM FOR STUDYING RAPID EVOLUTION

Of aquatic invertebrates, certain populations of the copepod *Eurytemora affinis* species complex are exceptionally successful as invaders into novel habitats, particularly into novel salinities. Over the past ~80 years, populations from this species complex have invaded freshwater

habitats multiple times independently from saline estuaries on three continents.^{64,65} These invasions occurred through human activity, such as via the dumping of ship ballast water, stocking of lakes with fish, and transformation of saline bays into freshwater lakes.^{41,64,66–69} During these saline to freshwater transitions, these copepod populations experienced evolutionary shifts in physiological tolerance and ion regulatory capacity.^{70–74} Notably, during these salinity transitions, ion transporter genes were repeatedly found to be under selection in both wild populations and in laboratory selection lines^{75–78} (see Section [genome architecture of the trait under selection during habitat transitions](#)).

Within the *E. affinis* complex, some clades appear to be more invasive than others based on geographic patterns of invasions and differences in physiological tolerances^{64,71} (Figure 1). Populations from the North American Atlantic clade (*E. carolleeae*³⁹; Figure 1, red clade) is particularly invasive and poised to displace *E. affinis* proper (Figure 1, purple clade) in several locations in Europe, such as in the Baltic Sea region.^{79,80} In contrast, there are no known cases of invasions from the North Atlantic (teal clade) and Pacific clades (yellow clade) of North America.^{64,81,82} The dearth of freshwater populations arising from these clades is unlikely due to lack of transport opportunity, as the noninvasive clades are sympatric with the invasive Atlantic clade in many estuaries.^{81,82} Physiological barriers are likely preventing some clades from invading freshwater habitats. For instance, clear physiological differences exist between sympatric populations from the invasive Atlantic (*E. carolleeae*) and noninvasive North Atlantic clades in the St. Lawrence estuary. Notably, an abundance of nutritious food (cryptophytes) can enhance the low-salinity tolerance of saline populations from the Atlantic clade, but not those from the North Atlantic clade.⁷¹

Intriguingly, this species complex is marked by high genetic divergence among clades, a considerable degree of morphological stasis, and idiosyncratic patterns of reproductive isolation between the clades. The timing of divergence among the clades is highly uncertain, due to the lack of fossil calibration of the molecular clock for this species complex.⁸¹ Subtle differences in morphology exist among the genetically distinct clades.⁸³ Interbreeding among the clades has revealed varying levels of reproductive isolation, often with asymmetric reproductive isolation between the reciprocal crosses.⁸¹

A surprising fact is that the genetically divergent clades of this species complex employ the same loci, and often the same SNPs (single nucleotide polymorphisms), during salinity adaptation. Parallel selection on the same alleles during salinity adaptation, shared among the genetically divergent Atlantic, Gulf, and Europe clades, suggests that the physiological mechanisms involved are quite ancient. This evolutionary capacity possibly originated prior to the formation of this species complex in the genus *Eurytemora*. This genus likely radiated in the subarctic region, including the coasts of Alaska, given that most of the described 21 species of *Eurytemora* have been found in the Cape Thompson to Kotzebue region of Alaska.^{84–88} This coastal region is dynamic, marked by sea level fluctuations and repeated marine incursions. Thousands of coastal pools of varying salinity house multiple sympatric species of *Eurytemora*.⁸⁵

The *E. affinis* complex in particular provides a remarkable model system for evolutionary, ecological, and physiological investigations due to the availability of genomic resources and the relative ease of conducting multi-generational laboratory experiments. The Lee Lab has generated chromosome-level high-coverage reference genomes for the *E. affinis* complex using sequences from inbred lines.⁸⁹ These inbred lines are critically important for assembling highly contiguous genomes, as the multiple alleles at loci in outbred lines are difficult to distinguish from gene paralogs during genome assembly. These inbred lines also provide useful tools for genetic association studies that link genotypes to phenotypes.^{90–92}

The short generation times of *E. affinis* complex populations (~20 days at 13°C) and ease of laboratory culturing enable experiments, such as common garden and laboratory evolution experiments, to dissect mechanisms of evolutionary change (see Section [genome architecture of the trait under selection during habitat transitions](#)). A common garden experiment involves rearing different populations under the same conditions for 1–2 generations to remove the effects of acclimation to previous environmental conditions so that the differences observed between populations represent heritable differences between the populations. Common garden experiments have uncovered heritable differences in physiological responses to salinity and temperature between *E. affinis* complex populations.^{70–74,93–98} Laboratory natural selection experiments, where environmental change is imposed on populations to determine their evolutionary responses, have revealed that *E. affinis* complex populations can evolve rapidly in response to environmental shifts, particularly in response to salinity^{70,73,76} and temperature.^{99,100}

POPULATION GENETIC PROPERTIES THAT PROMOTE RAPID ADAPTATION IN NOVEL HABITATS

So, what factors account for the ability of some populations to invade novel habitats, whereas most cannot? In terms of the success of invasive populations in novel ranges, ecological (extrinsic) factors are certainly important contributors, such as the presence or absence of predators, availability of food sources, and transport vectors and opportunity. However, the intrinsic properties of organisms and populations are also critically important factors that affect whether populations can survive, proliferate, and expand their ranges in the novel habitats.^{101,102} In particular, the capacity of introduced populations to tolerate, acclimate, and/or evolve (evolvability) in response to new habitat conditions is critically important for becoming established in the introduced range.

In terms of evolvability, or the capacity to evolve, the ability of a population to respond to natural selection impacts its capacity to adapt to novel environments.^{43,101,102} Factors that would affect the selection response of a population facing environmental change include allelic variation of the loci encoding the critical trait(s) under natural selection, genomic architecture underlying the trait(s) under selection (such as the number of loci, gene-gene interactions, etc.) (see next section), and constraints that prevent a trait from undergoing selection (such as pleiotropy).

Within populations, allelic variation encoding a trait is necessary for natural selection to act on the trait. This variation could arise from new mutations in microbial populations that have exceedingly short generation times and large effective population sizes.¹⁰³ However, metazoan populations with lower mutation rates must rely much more heavily on the presence of beneficial alleles in the starting population, even if at very low frequency. Balancing selection in the native source populations might play critically important roles in maintaining genetic variation

upon which selection could act during biological invasions or other forms of habitat change.⁴³ Balancing selection refers to any selective mechanism that maintains genetic variation in a population, such as fluctuating selection, overdominance, or negative frequency-dependent selection.⁴³

Most notably, in *E. affinis* complex populations, balancing selection in the native saline populations might be promoting parallel adaptation during freshwater invasions. Population genomic analyses revealed that a large proportion of the SNPs (single nucleotide polymorphisms) that are under parallel selection during three independent freshwater invasions also show signatures of balancing selection in the native range saline populations.⁷⁷ These results suggest that the maintenance of polymorphism in native range populations can serve as reservoirs for repeated selection favoring the same alleles during biological invasions.

Several features would promote the maintenance of genetic variation within *E. affinis* complex populations in many of their native saline habitats.⁷⁷ Saline populations of *E. affinis* complex tend to occur in estuaries and saltmarshes where salinity fluctuates widely on a seasonal basis.¹⁰⁴ With approximately six generations per year in many locations⁸² and 20–25 days generation times at 13°C,⁷⁴ seasonally fluctuating selection would favor different salinity tolerance alleles at different generations.⁴³ In addition, saline and freshwater tolerance are negatively genetically correlated in *E. affinis* complex populations,⁹³ such that selection favoring freshwater tolerance would select against saltwater tolerance and vice versa. Such a selection regime would lead to the extinction of maladapted alleles as environmental conditions change. However, the presence of diapause egg banks would preserve genetic variation from past generations within the populations.^{105–107} Populations of the *E. affinis* complex tend to produce diapause resting eggs that are stored in the bottom sediment during unfavorable seasons.

Moreover, beneficial reversal of dominance, which we found operating in *E. affinis* complex populations,⁹⁶ would act to protect maladaptive alleles against the action of negative selection.^{96,108,109} Beneficial reversal of dominance is the phenomenon in which the more fit alleles become dominant across different habitats. For instance, in heterozygotes, saline-adapted alleles are dominant in saline habitats, whereas freshwater-adapted alleles are dominant in freshwater habitats.⁹⁶ Under such conditions, the less fit alleles are recessive and masked from negative selection, such that they are preserved in the population. Thus, temporally varying selection, along with the mechanisms described above, would together act to preserve genetic variation within many *E. affinis* complex populations in their dynamic habitats.⁴³

GENOME ARCHITECTURE OF THE TRAIT UNDER SELECTION DURING HABITAT TRANSITIONS

The genome architecture underlying the traits under natural selection will profoundly impact the selection response and evolutionary potential of populations responding to environmental change.^{110,111} The genome architecture of a trait includes the numbers and genomic locations of genes that affect a trait, the magnitude of their effects, and the relative contributions of additive, dominant, and epistatic genetic effects. For instance, the number of genes encoding a trait will affect how readily the trait could evolve and the repeatability of evolutionary pathways during replicate adaptive events (parallelism).^{76,112} Specifically, as the number of loci encoding a trait increases, the degree of parallelism among replicate adaptive events is expected to go down.^{76,111,113}

In response to environmental change, the evolution of physiological traits would often be critical to enable survival and reproduction under novel conditions. As physiological traits are often polygenic, that is, encoded by many genes, the genome architecture of the traits would typically involve many genes and their interactions. According to theoretical predictions, as the number of loci encoding a trait increases, the extent of parallelism among replicated evolutionary events would be expected to go down.^{111,113,114} The reasoning is that as more loci contribute to a trait, the trait optimum could be achieved through multiple alternative evolutionary pathways, as many of the beneficial alleles would be redundant in function.^{112,115} As such, the null prediction is that the evolution of physiological responses to environmental change would be nonparallel (i.e., involve different loci and alternative evolutionary pathways) across replicate events.¹¹²

What are the physiological functions that would evolve in response to changes in environmental salinity? With salinity change, a great challenge for an invertebrate is to maintain ionic and osmotic homeostasis of its body fluids in the face of environmental change.^{16,17} Ionic versus osmotic regulation of the extra- and intracellular body fluids (e.g., hemolymph) are typically performed independently of each other. Across the cell membrane, between the intra- and extracellular fluids, an ionic concentration gradient must be maintained. In contrast, osmotic pressure must remain constant across the cell membrane. In terms of ionic regulation, when environmental salinities deviate from the isosmotic range for the organism, ion uptake or excretion is required. Constancy in osmotic pressure is maintained through the production, degradation, and transport of osmolytes.^{116,117}

Relative to their saline ancestors, freshwater populations of *E. affinis* complex display an evolutionary increase in hemolymph osmolality (body fluid concentration) under low salinity conditions.⁷² Freshwater populations from both the Atlantic (*E. carollaeae*) and Gulf clades display parallel shifts toward increases in hemolymph osmolality (by 16–31%) at low salinities (both 0 and 5 PSU), relative to their saline ancestors. This increase in body fluid regulation would require increases in ion uptake, increases in ion reabsorption from the urine, and/or reductions in ionic losses (such as through the reduction in integument permeability).

Indeed, we do find evidence supporting the evolution of increased ion uptake activity in freshwater *E. affinis* complex populations under freshwater conditions. Relative to their saline ancestors, freshwater populations from the Atlantic and Gulf clades exhibit the evolution of increased enzyme activity of V-type H⁺ ATPase (VHA) at 0 PSU.⁷⁰ The proton pump VHA is thought to be the major driver powering ion uptake under freshwater conditions.¹¹⁸ In addition, in the freshwater populations, we observe the evolution of increased gene expression of the ion transporter paralogs Na⁺/H⁺ antiporter paralog 7 (NHA-7), Na⁺/K⁺ ATPase α subunit, paralog 1 (NKA- α -1), and carbonic anhydrase paralog 9 (CA-9).⁹⁵ These genes likely encode ion transporters that are involved in ion uptake from freshwater conditions (Figures 2 and 3).

At the cellular level, the functions of ion uptake, ion reabsorption, and ion excretion are performed by ionocytes. Ionocytes are specialized cells that are rich in mitochondria and replete with ion transporters embedded in infolded epithelial membranes.¹¹⁸ Ionocytes are embedded

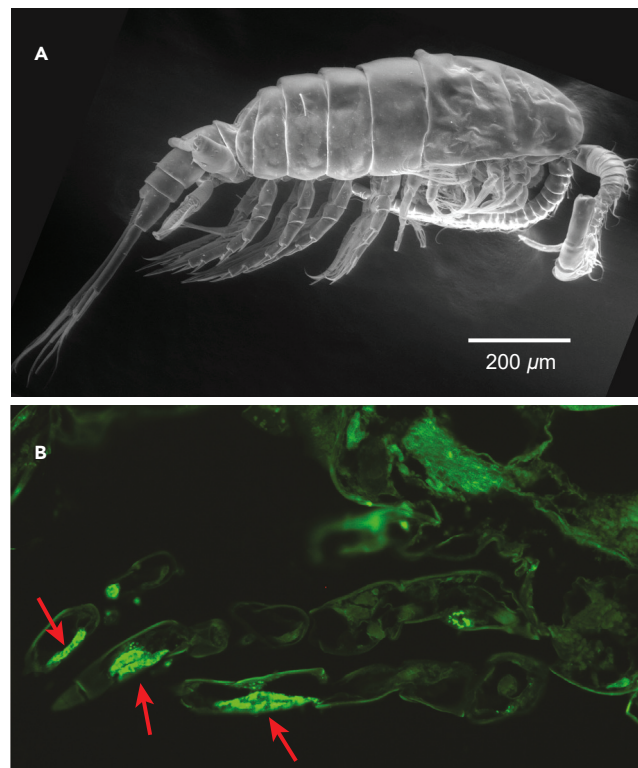


Figure 2. Image of copepod legs, showing the ion regulatory Crusalis organs

(A) SEM of the side view of an adult male copepod, showing the five pairs of swimming legs. Photo by Teresa E. Popp. Scale bar = 200 μm.

(B) Immunolocalization of Na⁺/K⁺ ATPase (NKA, bright green, with red arrows) in the swimming legs of an adult copepod from the St. Lawrence estuary (Baie de L'Isle Verte) at 15 PSU. Photo by Kelsey Johnson, from the study of Johnson et al. (2014).

within osmoregulatory organs, such as fish gills, crustacean gills, and epipodites, insect Malpighian tubules, and vertebrate kidneys. Ionocyte function is critical not only for ionic regulation but also for the regulation of pH, osmotic pressure, and nutrient uptake. As such, the evolution of ionocyte function is likely important for adapting to a variety of conditions (e.g., ocean acidification).

We localized ionocytes in the maxillary glands and swimming legs of adult copepods from *E. affinis* complex populations.^{119,120} Prior studies had hypothesized that the maxillary glands function as a kidney-like structure, where ion reabsorption from the urine takes place.^{121,122} On the other hand, based on *in situ* immunolocalization of ion transporter proteins VHA and NKA in the copepod swimming legs (Figure 2), we hypothesized that ion uptake is performed by organs within the legs that we named the “Crusalis organs”.^{119,120} The ion transporters VHA and NKA tend to energize ion uptake from the environment.^{78,118} Our localization of ion transporters in the legs of a copepod is interesting, given that the ion regulatory gills and epipodites of crustaceans are developmentally homologous to crustacean legs.¹²³

While mechanisms of ion uptake for most organisms are still uncertain and incompletely characterized,¹¹⁸ our results consistently implicate a set of ion transporters as involved in salinity adaptation.⁷⁸ Across salinity transitions in three genetically distinct clades (Figure 1, Atlantic, Gulf, Europe), ion transport-related genes form the largest functional (GO) categories showing evolutionary shifts in gene expression or signatures of selection in response to salinity change in both wild populations and in laboratory selection lines.^{70,72,75–78} Comparative genome-wide gene expression analysis revealed that under common garden salinities of 0 or 15 PSU, paralogs of *NHA*, *NKA*, *CA*, *Na⁺*, *K⁺*, *2Cl⁻* co-transporter (*NKCC*), and *Ammonia transporter* (*AMT*) show evolutionary shifts in gene expression between saline and freshwater populations (increased expression in either saline or freshwater populations).⁹⁵ In addition, across multiple independent salinity transitions in wild populations and in laboratory evolution experiments, paralogs of the ion transporter gene families *NHA*, *VHA*, *CA*, *NKA*, *NKCC*, and *Rh Protein* (*Rh*) tended to show allele (SNP) frequency shifts, consistent with signatures of selection.^{75–77} These results implicate a model of ion transport that utilizes VHA as the driver of ion uptake from the environment, with *NHA* as the likely secondary transporter that cooperates with VHA to import precious Na⁺ from the dilute environment (Figure 3).

Across independent saline to freshwater invasions in North America, we find a remarkably high degree of parallelism in the alleles under selection, with a high proportion of the selected sites occurring at ion transporter genes.⁷⁷ Based on whole-genome sequencing of 100 copepods per population, we found that across independent saline to freshwater invasions (four saline and five freshwater populations) natural selection repeatedly favored the same SNPs (single nucleotide polymorphisms) and genomic loci, to a much greater degree than expected by chance. Specifically, 42.5% of the SNPs (2970 SNPs) showed signatures of parallel selection across three independent invasions in the Atlantic and Gulf clades, out of 6,981 SNPs showing signatures of selection across any invasion. This result revealed parallel evolution even across

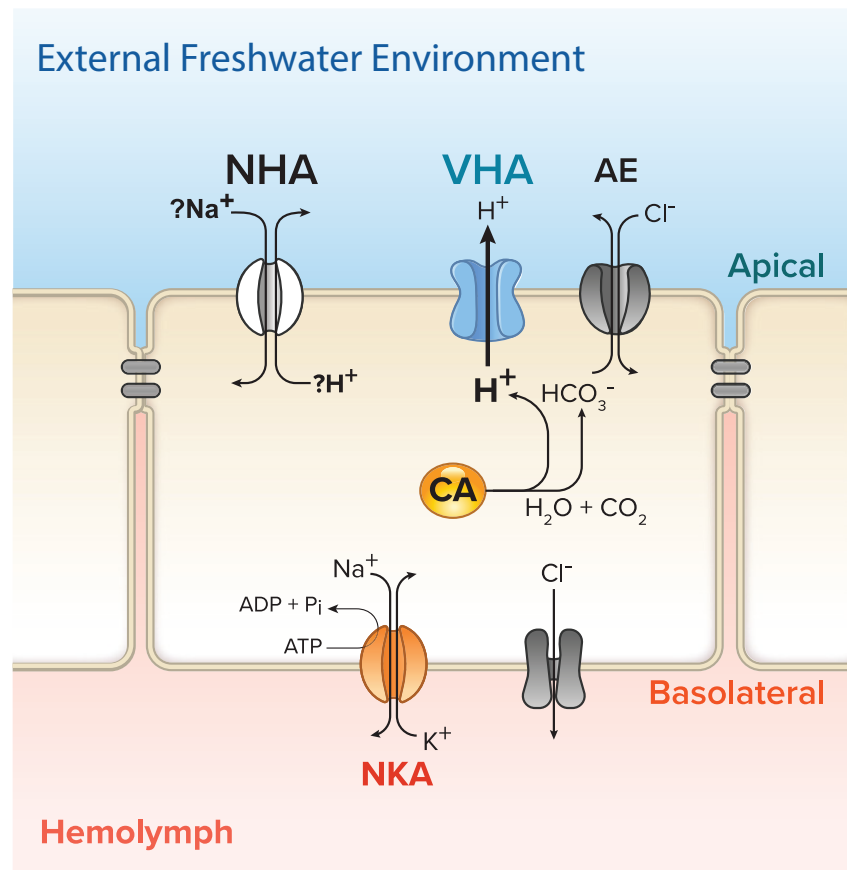


Figure 3. Model of ion uptake from the freshwater environment by ionocytes in *E. affinis* complex populations

Shown are primary active transporters for energizing ion transport (VHA, NKA) and a secondary transporter for sodium uptake (NHA). VHA (blue) pumps H^+ out of the cell to create a proton gradient, through which Na^+ is transported into the cell, likely by NHA. Na^+ is then transported to the hemolymph via NKA. Carbonic anhydrase (CA) supplies protons to VHA and HCO_3^- to anion exchanger (AE). NKCC might also play a role in ion uptake, but the localizations of NKCC paralogs are unknown for *E. affinis* complex. Alternative models have also been proposed and not all potentially relevant ion transporters are shown. Adapted from Stern and Lee (2020).

genetically divergent populations. The SNPs under selection during the salinity transitions tended to occur in genomic regions heavily enriched with ion transporter genes (with ion transporter genes comprising the largest gene ontology categories under selection).⁷⁷ In particular, we found that the highest density of SNPs under selection occurred in a genomic region containing seven tandem paralogs of the Na^+/H^+ antiporter (NHA) gene family. Of these paralogs, NHA paralog 7 (NHA-7) showed the strongest signal of parallel evolution across independent invasions in response to salinity change.

The Na^+/H^+ antiporter was discovered only recently in animals in 2005¹²⁴ and in humans in 2012.¹²⁵ With 8 distinct NHA paralogs in the *E. carollaeae* genome,^{77,89} these NHA paralogs might perform completely novel transport functions and modalities, including electrogenic cation/ H^+ exchange. Previous studies have detected physiological evidence for an electrogenic Na^+/H^+ exchanger (i.e., 2 Na^+ ions exchanged for 1 H^+) in crustacean epithelial cells, but to date, the molecular identity of this exchanger has remained elusive.^{126,127}

We performed a laboratory evolution experiment under declining salinity,⁷⁶ to determine whether changes in salinity alone in the laboratory could replicate the allele frequency shifts that we observed in the wild populations.⁷⁷ Starting with a saline Baltic Sea population (Europe clade) from Kiel, Germany (15 PSU), we reduced salinity down to 0 PSU (from a freshwater lake) over ten generations for 10 replicate selection lines, performing whole-genome sequencing (Pool-seq) at multiple time points.⁷⁶ We found largely parallel responses to selection that often acted on the same alleles at the same sets of loci, especially ion transporter paralogs, consistent with prior studies.^{77,95} Most notably, at the 10th generation in the experiment, selection favored the same alleles (SNPs) among the 10 replicate selection lines, much more than expected by chance. Specifically, at generation 10, we observed a surprisingly high degree of overlap in selected alleles (on average 79.5%) between replicate lines based on the Jaccard index.⁷⁶

This high degree of parallelism was consistent with positive synergistic epistasis among the selected alleles, where the effect of an allele is increased by the presence of other alleles. Using simulations of our experimental conditions, we found that only the simulations of positive epistasis could match the extent of parallelism observed among our replicate experimental lines.⁷⁶ The degree of parallelism that we found in

our data was far greater than simulations of the null multiplicative model where the effect of each allele is independent. In fact, the degree of parallelism among selection lines, for 4,977 SNPs (on 121 haplotype blocks) under selection, behaved like far fewer loci (20–30 loci) under selection. These results were consistent with positive epistasis acting among the loci, as functionally linked alleles would behave like a smaller number of alleles under selection. The potential role of positive epistasis is consistent with mechanisms of ion uptake, which require the action of multiple cooperating ion transporters to accomplish the role of ion uptake from the freshwater environment.

As other mechanisms could be driving parallel evolution in this system, we examined the roles of other factors using simulations of our experimental conditions.⁷⁶ For instance, we found that increasing physical linkage did not affect the degree of parallelism among replicate lines in our simulations. Increasing the starting frequency of beneficial alleles, to simulate selection from balanced polymorphisms, did increase parallelism among our simulated selection lines, especially in conjunction with positive epistasis. However, higher starting frequencies of beneficial alleles alone could not explain the high degree of parallelism that we observed among the replicated laboratory selection lines. Thus, our results support the role of positive epistasis in driving parallel adaptation, with selection from standing variation as a potential contributor. However, the importance of positive epistasis is still a hypothesis that should be tested with functional studies.

The high level of parallelism observed in this laboratory evolution experiment was greater than that found in experimental studies of polygenic adaptation in response to temperature change and acidification.^{115,128} For instance, in a laboratory natural selection experiment using *Drosophila simulans* in response to a new temperature (12 h cycles between 18°C and 28°C) across 60 generations, the 10 replicate lines showed heterogeneous responses.¹¹⁵ Only a single allele increased in frequency in all 10 replicates and on average 53 selected alleles were identified per replicate (out of 99 “alleles” or haplotype blocks, containing 23,835 SNPs).¹¹⁵ In the copepod *Acartia tonsa*, after 25 generations of laboratory evolution, of the 6,270 SNPs under selection in response to warming (22°C, 400 μ atm CO₂, pH ~8.2) 57% showed shared selection pressures across four replicate selection lines, whereas of 1,713 SNPs under selection in response to acidification (18°C, 2,000 μ atm CO₂, pH ~7.5) 20% were shared among the four replicates.¹²⁸

Interestingly, the SNPs that showed signatures of selection in our laboratory evolution experiment were found segregating in the wild Baltic Sea populations, with high variance in SNP frequencies (in terms of a high Q_x value).⁷⁶ Based on whole-genome sequencing of 11 populations in the Baltic and North Seas, we found population genomic signatures of selection associated with salinity and temperature gradients in both seas.⁷⁵ These results suggest that wild populations in the Baltic and North Seas might have the potential to evolve in response to future changes in salinity and temperature. Again, across the Baltic and North Sea populations, “ion transmembrane transport” was the most enriched gene ontology category showing signatures of selection.⁷⁵

Results across multiple studies indicate that selection on salinity tolerance proceeds in a repeatable and predictable manner, acting predominantly on the same complex of ion transporter genes across independent salinity transitions.^{70,75–78,95} The high degree of parallelism in SNPs under selection is surprising, given the polygenic nature of salinity adaptation. The theoretical expectation is that as the number of loci contributing to a polygenic trait increases, the degree of parallelism among replicate selection lines would be expected to decline.¹¹² There are likely a few thousand loci that contribute to salinity adaptation, given the ~600 ion transporter genes in the *E. affinis* complex genome⁸⁹ (Figure 4), as well as genes that affect integument permeability, water flux, and osmolyte regulation. Moreover, our studies indicate several thousand SNPs showing signatures of selection in response to salinity change.^{75–77}

Multiple factors might be contributing to the high degree of parallelism in the polygenic response to salinity change across the replicate populations and selection lines of the *E. affinis* complex. As mentioned previously, fluctuating salinity (resulting in balancing selection) in the native habitats could preserve alleles that are beneficial under different salinity conditions, making them available for selection during salinity change.^{43,77,96} Positive synergistic epistasis among cooperating beneficial alleles would help drive these alleles to increase in frequency during selection, especially if these alleles were at an elevated frequency in the starting population.⁷⁶ Moreover, it appears that certain loci are essential for salinity adaptation, such as *NHA-7*, which appears to be strongly associated with salinity adaptation across multiple studies.^{75–78,95} The presence of essential and non-redundant alleles that contribute to salinity adaptation would make alternative evolutionary pathways far less permissible and canalize the pathway for freshwater adaptation.

The peculiar genome architecture of this copepod likely contributes to its remarkable capacity to acclimate and evolve during salinity invasions. In terms of the genome architecture underlying salinity adaptation, the genome of *E. carolleeae*³⁹ (Atlantic clade of the *E. affinis* complex) exhibits massive expansions of ion transporter genes (Figure 4). Relative to 12 other arthropod genomes, 29.2% of the significantly enriched GO terms in the molecular function category are related to ion -transport activity.⁸⁹ Additionally, many of the ion transporter gene families show signatures of very recent duplications. Interestingly, ion transporter gene bodies exhibit extreme deficits of CpG sites,⁸⁹ likely arising from methylation events.^{129,130} Methylation of gene bodies increases expression of the genes and reduces variance in expression, suggesting tighter control of gene regulation.^{131,132} Another peculiar feature found in *E. carolleeae* is the occurrence of endopolyploidy in the majority of the somatic cell nuclei of adults, such that the DNA content is doubled.¹³³ Endopolyploidy could increase the amount and rate of gene expression within somatic tissues and could potentially be adaptive.¹³⁴ Together these genomic characteristics, particularly related to ion transporter genes, suggest the potential for high responsiveness to salinity change and salinity adaptation.

THE GENOMIC ARCHITECTURE OF SALINITY ADAPTATION IN OTHER BIOLOGICAL SYSTEMS

Do the patterns and principles found in this copepod system apply to other biological systems? While salinity adaptation is polygenic, cases of parallel selection acting on the same loci might be more widespread than expected. Moreover, the factors that contribute to parallel adaptation in the *E. affinis* complex might be shared in other systems. Salinity adaptation might often require a set of specific loci to undergo selection in many organisms, especially with respect to ion transport mechanisms.¹¹⁸ The presence of essential and non-redundant alleles that

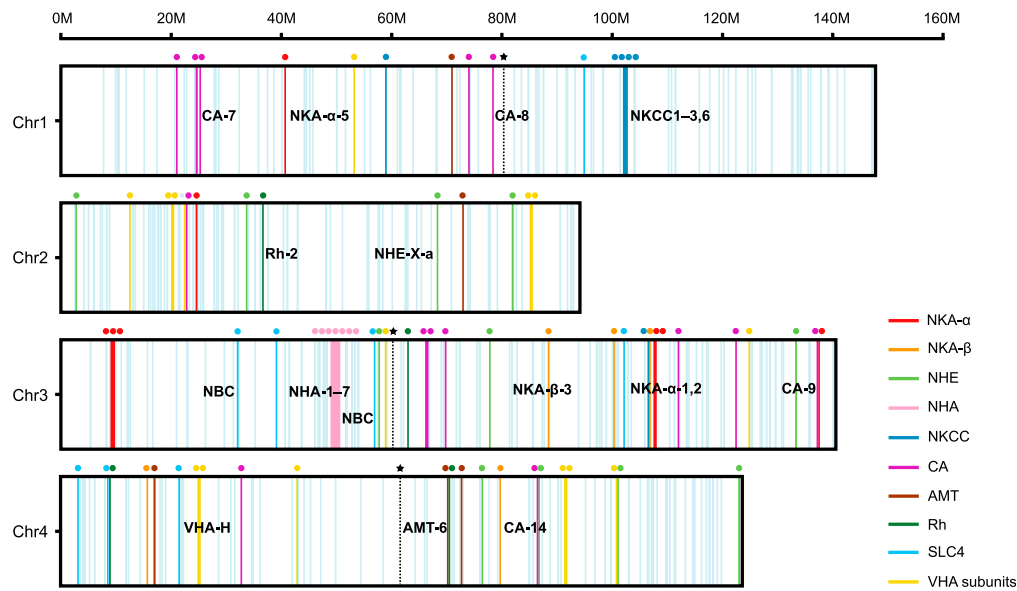


Figure 4. Localization of ion transporter gene paralogs on the four chromosomes of the genome of the copepod *Eurytemora carolleeae* (Atlantic clade of the *E. affinis* complex)

Vertical colored lines and dots indicate the 83 key ion transporter (and carbonic anhydrase) gene paralogs that showed evolutionary shifts in gene expression and/or signatures of selection between saline and freshwater populations.⁷⁸ Vertical light blue lines indicate 490 ion transporter genes that were identified from genome annotation. NKA- α = Na⁺/K⁺-ATPase α subunit, NKA- β = Na⁺/K⁺-ATPase β subunit, NHE = Na⁺/H⁺ exchanger, NHA = Na⁺/H⁺ antiporter, NKCC = Na⁺, K⁺, Cl⁻ cotransporter, CA = Carbonic anhydrase, AMT = Ammonia transporter, Rh = Rh protein, SLC4 = Bicarbonate transporters, including Cl⁻/HCO₃⁻ exchangers (AE) and Na⁺-coupled HCO₃⁻ transporters (NBC, NDCBE), VHA = V-type H⁺ ATPase. Figure from the supplementary file of Du et al. 2023.

contribute to salinity adaptation would make alternative evolutionary pathways far less permissible, as appears to be the case for *NHA-7* in *E. affinis* complex.^{76–78,95} In the case of ion transport, alleles that encode cooperating ion transporter loci might have positive epistatic interactions, which would help drive parallel adaptation.⁷⁶

Other than this copepod system, among the best studied systems examining adaptation during saline to freshwater transitions are cases of post-Pleistocene freshwater colonizations by fish populations. Freshwater colonizations by fish have typically occurred over longer time scales than the decadal *E. affinis* complex invasions; although, a few populations of the threespine stickleback have been introduced very recently (~10–40 years) into freshwater lakes.¹³⁵ Models of ion transport in fish are diverse among species and differ from invertebrates; but, they also involve multiple cooperating ion transporters.¹¹⁸

Among fish systems, saline to freshwater adaptation has been relatively well-studied in the killifish *Fundulus heteroclitus*.¹³⁶ Based on genomic scans using Restriction-site Associated DNA sequencing (RAD-seq) that identified 1,387 outlier loci between a pair of brackish and freshwater populations, several major ion transporters putatively involved in ion uptake in fresh water were detected as candidates under selection, such as *NBC*, *NKA*, *NHE3*, *Rh* protein, *CA*, and *aquaporin* (Supplementary Table S1 in ref¹³⁶). These candidate genes are consistent with ion transporters involved in models of ion uptake from fresh water by fish.¹¹⁸ For two population pairs of the rainwater killifish *Lucania parva*, an outlier SNP window analysis of transcriptomes revealed signatures of selection at ion transporter genes *electrogenic sodium bicarbonate cotransporter* (*NBC*) and *V-type H⁺ ATPase subunit S1* (*VHA-S1*).¹³⁷

Another system with extensive evolutionary studies on the transition from saline to freshwater habitats is the threespine stickleback *Gasterosteus aculeatus*. This system is notable for revealing striking cases of parallel evolution at loci related to morphological evolution (e.g., *Eda*, *Pitx1*).^{138–140}

In terms of dissecting the mechanisms of salinity adaptation, focusing on ion regulatory mechanisms, individual comparative studies on threespine stickleback populations have each uncovered a few ion transporters under selection that potentially comprise parts of an ion transport machinery. For instance, using 20 putatively adaptive microsatellite loci, threespine stickleback populations from 38 locations across natural salinity gradients in the Baltic Sea showed signatures of selection at ion transporter genes *NHE3* and *VHA* subunit A.¹⁴¹ Another study of 10 populations across the Baltic Sea, using >30,000 SNPs obtained with a pooled RAD-seq approach, found association with salinity at *carbonic anhydrase* “10a” (Supplementary Table S4 in ref¹⁴²).

Studies exploring parallel adaptation in threespine stickleback populations during saline to freshwater colonizations tended to yield weak signals of parallel evolution at ion regulatory genes, with differing targets of selection among studies. A study using outlier analysis with 50 microsatellite loci for paired saline and freshwater populations from six locations found no genes with parallel signatures of selection in all six locations.¹⁴³ Some transporters showed signatures of selection in three or four locations, namely *Aquaporin 3*, *NKA α subunit* (*NKA- α*), and

Na^+ , Cl^- cotransporter (NCC).¹⁴³ Another study using RAD-seq of ~45,000 SNPs found several ion transport-related genes within genomic regions associated with differences between two marine and three freshwater populations, namely VHA subunit A, VHA subunit a, NKA β subunit, carbonic anhydrase 4, Na^+/H^+ exchanger 6 (NHE6, SLC9A6), $\text{Cl}^-/\text{HCO}_3^-$ exchanger (AE, SLC26A3), and cation chloride cotransporter 6 (CCC6, SLC12A9) (Supplementary Table S2 in ref ¹⁴⁴). In another study using RAD-seq of 28,888 SNPs in two marine and seven freshwater populations in Denmark, parallel outlier genomic regions (found in at least two independent pairs of populations) contained the ion transporter genes NKA- α and cation chloride cotransporter 9 (CCC9, SLC12A8) (Supplementary Table S3 in ref ¹⁴⁵).

While these studies on the threespine stickleback are highly suggestive, there is insufficient information across these studies to construct a model of adaptation of ion transport mechanisms¹¹⁸ and inadequate replication to determine the extent of parallel adaptation across independent salinity transitions. Each of these studies sampled only part of the genome (using RAD-seq or microsatellite markers) and likely have not captured all the ion transporters under selection during the salinity transitions. Moreover, the ion transporter gene families appear to be incompletely annotated in the threespine stickleback genome(s), which contains an unknown number of unidentified gene paralogs. In addition, gene names are inconsistently used, such that the specific genes and paralogs under selection are difficult to identify across studies. As such, it is difficult to determine the homology of the gene paralogs among populations or with other taxa.

Across multiple studies on fish, we can begin piecing together mechanisms of salinity adaptation in fish populations.¹⁴⁶ For fish, relative to copepods, the long generation times (e.g., ~1 year for threespine stickleback versus ~20 days for *E. affinis* complex) make it challenging to perform laboratory natural selection experiments, to rigorously confirm the extent of parallel evolution among replicate lines. The long generation times also make it difficult to generate inbred lines, which would be useful for performing high-resolution genetic association studies to rigorously link genotypes with phenotypes (e.g., salinity tolerance, ion transporter function). Nevertheless, current fish studies could be vastly improved with more extensive and consistent annotation of genomes and more comprehensive whole-genome sequence coverage in comparative population genomic studies.

CONCLUDING REMARKS

While environmental conditions are changing rapidly across the globe, we still have a poor understanding of which populations will survive and which will go extinct. Salinity is arguably the strongest driver of species' distributions that structures biogeographic patterns in aquatic habitats.^{12,13,26,38} Therefore, as this critically important environmental variable changes, populations will be forced to adapt, migrate, or face extinction. Zooplankton populations, which constitute the largest animal biomass in aquatic habitats, may be particularly vulnerable to changes in salinity because they have limited capacity to migrate and often possess narrow salinity tolerance ranges.^{147–149} However, we lack a clear understanding of how populations will respond to rapid changes in salinity, as well as interactions between salinity and other variables, such as temperature.

Determining the evolutionary potential of populations will greatly aid our endeavors to gain insights into this problem. To achieve this goal, we need much greater knowledge regarding population genetic structure, the extent of gene flow between populations, the specific traits under selection during environmental change, and the genome architecture of the critical traits under natural selection.

Gaining this knowledge is difficult and painstaking, if not impossible, for most biological systems. However, we can obtain invaluable insights through the focused and comprehensive study of ecologically relevant model systems. With its ample genomic resources and short generation times, the copepod *E. affinis* complex can serve as such a model system. This copepod is a widespread and abundant species complex that serves critical ecosystem functions in many coastal habitats. Many insights gained from this system are likely applicable for understanding the physiological and evolutionary mechanisms that enable certain taxa to cross major habitat boundaries and thrive in response to global change.

In particular, the principles and mechanisms discovered in *E. affinis* complex are likely to be applicable to other invasive populations crossing habitat boundaries. For instance, many of the dominant invaders in the freshwater Great Lakes of North America originated from the brackish waters of Ponto-Caspian Seas, such as zebra and quagga mussels, the fishhook waterflea *Cercopagis pengoi*, and many amphipods.^{42,44–48} Given the salinity transitions from their native ranges into freshwater habitats, the evolution of ion uptake mechanisms is likely to be quite important for these invaders. For example, invasive Great Lakes populations of the zebra mussel *Dreissena polymorpha* and the amphipod *Corophium curvispinum* are inefficient osmoregulators, with higher rates of ion uptake, along with greater ionic losses, relative to native freshwater bivalves and amphipods.^{150–152} While physiological mechanisms of these invasive populations have likely evolved considerably since their emergence from their native range, it is quite likely that these populations are still evolving.

The increasing availability of genomic resources and the application of evolutionary and physiological analyses on these non-model invasive species will allow us to uncover general principles regarding their ability to become successful invaders in freshwater habitats. Given that salinity forms a formidable biogeographic barrier, it would be worth studying mechanisms of rapid physiological evolution during freshwater invasions for a wide range of taxa originating from saline habitats, such as the Black and Caspian Seas. Examining mechanisms that are shared across taxa versus those that are unique to each system will help uncover general principles that govern successful invasions that cross salinity boundaries. Understanding such general principles will give us some powers of prediction regarding which populations will likely have the capacity to survive, experience range expansions, or go extinct under global change in the future.

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DECLARATION OF INTERESTS

All other authors declare they have no competing interests.

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