

Is salinity an obstacle for biological invasions?

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Abstract

Invasions of freshwater habitats by marine and brackish species have become more frequent in recent years with many of those species originating from the Ponto-Caspian region. Populations of Ponto-Caspian species have successfully established in the North and Baltic Seas and their adjoining rivers, as well as in the Great Lakes–St. Lawrence River region. To determine if Ponto-Caspian taxa more readily acclimatize to and colonize diverse salinity habitats than taxa from other regions, we conducted laboratory experiments on 22 populations of eight gammarid species native to the Ponto-Caspian, Northern European and Great Lakes–St. Lawrence River regions. In addition, we conducted a literature search to survey salinity ranges of these species worldwide. Finally, to explore evolutionary relationships among examined species and their populations, we sequenced the mitochondrial cytochrome *c* oxidase subunit I gene (COI) from individuals used for our experiments. Our study revealed that all tested populations tolerate wide ranges of salinity, however, different patterns arose among species from different regions. Ponto-Caspian taxa showed lower mortality in fresh water, while Northern European taxa showed lower mortality in fully marine conditions. Genetic analyses showed evolutionary divergence among species from different regions. Due to the geological history of the two regions, as well as high tolerance of Ponto-Caspian species to fresh water, whereas Northern European species are more tolerant of fully marine conditions, we suggest that species originating from the Ponto-Caspian and Northern European regions may be adapted to freshwater and marine environments, respectively. Consequently, the perception that Ponto-Caspian species are more successful colonizers might be biased by the fact that areas with highest introduction frequency of NIS (i.e., shipping ports) are environmentally variable habitats which often include freshwater conditions that cannot be tolerated by euryhaline taxa of marine origin.

KEYWORDS

freshwater origin, Gammaroidea, marine origin, nonindigenous species, Ponto-Caspian species, salinity tolerance

1 | INTRODUCTION

The anthropogenic movement of species is one of the leading threats to biodiversity resulting from globalization and rapid increases in trade and travel (Capinha, Essl, Seebens, Moser, & Pereira, 2015; Hulme, 2009; Kaluza, Kölzsch, Gastner, & Blasius, 2010; Ruiz, Fofonoff, Carlton, Wonham, & Hines, 2000). To date, thousands of non-indigenous species (NIS) have established in freshwater, brackish, and marine ecosystems facilitated by anthropogenic vectors, mainly shipping, aquaculture, and canal construction (Molnar, Gamboa, Revenga, & Spalding, 2008; Ricciardi, 2006; Ruiz, Rawlings, et al., 2000; Vitousek, D'Antonio, Loope, Rejmanek, & Westbrooks, 1997). To become established, at least a subset of a population has to be entrained into a transport vector, to survive transport, as well as the environment of the subsequent new habitat (Blackburn, Lockwood, & Cassey, 2009; Simberloff, 2009). Empirical and statistical evidence support propagule pressure, which is defined as the quantity, quality, and/or frequency of introduced individuals (Lockwood, Cassey, & Blackburn, 2005; Simberloff, 2009), as a main factor determining invasion success (Blackburn et al., 2009; Mack et al., 2000; Simberloff, 2009; Williamson & Fitter, 1996). Higher propagule pressure yields higher probability of success, yet many introduced populations seem to fail to establish in new habitats (Blackburn et al., 2011). Characteristics of individuals within the introduced population, such as phenotypic plasticity and preadaptation to variable environments, may facilitate survival during the invasion process, maintaining high propagule pressure leading to invasion success (Blackburn et al., 2009; Hoffmann & Hercus, 2000; Lande, 2015; Simberloff, 2009).

Salinity is considered one of the most significant factors limiting the distribution of species in aquatic environments (Grabowski, Bacela, & Konopacka, 2007; Ojaveer et al., 2010), however, the establishment of marine and brackish NIS in freshwater habitats has been frequently reported in recent years, with many of those species originating from the Ponto-Caspian region (i.e., Black, Azov, and Caspian Seas; Ruiz, Carlton, Grosholz, & Hines, 1997; Lee & Bell, 1999; Ricciardi & Maclsaac, 2000; Casties, Seebens, & Briski, 2016). On the contrary, only few freshwater NIS have been reported as established in brackish, but not marine, environments (Grigorovich, Pashkova, Gromova, & van Overdijk, 1998; Sylvester, Cataldo, Notaro, & Boltovskoy, 2013). There are 17 freshwater phyla, all of which originated from marine environments in evolutionary history (Briggs, 1995; Lee & Bell, 1999; Little, 1990). While marine organisms entering freshwater habitats must evolve to retain osmotic levels in body fluids, which requires high energetic costs, freshwater taxa entering marine environments must evolve to maintain lower body fluid concentrations relative to the highly concentrated environment (Łapucki & Normant, 2008; Morgan & Iwama, 1999; Schubart & Diesel, 1999). Consequently, one would assume that brackish or freshwater species would more readily invade marine environments than vice versa; but invasions of Northern Europe and the Laurentian Great Lakes by Ponto-Caspian species show the opposite pattern (Casties et al., 2016; Ricciardi & Maclsaac, 2000).

Ponto-Caspian species have established nonindigenous populations in both brackish and freshwater habitats of the North and Baltic Seas and their adjoining rivers, as well as in the Great Lakes–St. Lawrence River region (Bij de Vaate, Jażdżewski, Ketelaars, Gollasch, & Van der Velde, 2002; Casties et al., 2016; Reid & Orlova, 2002; Ricciardi & Maclsaac, 2000). Previous studies suggested that marine Ponto-Caspian taxa established in freshwater habitats might be of freshwater origin due to the geological history of the Ponto-Caspian region (Casties et al., 2016; Reid & Orlova, 2002). Ten million years ago during the Miocene, after the separation of the Sarmatian Lake from the Tethyan Ocean, the salinity of the enclosed lake started to decrease, being several times almost completely dry during Glacial Maxima (i.e., from 2.5 million years ago to 10,000 years ago) with freshwater flooding after ice melting at the end of each Glacial Maximum. During that period there were also few geological connections and disconnections of the region with the Mediterranean Sea causing several additional changes in salinity until finally the system was shaped as the Black, Azov, and Caspian Seas, with salinity ranging from freshwater to marine (i.e., 30 g/kg; Reid & Orlova, 2002; Zenkevitch, 1963). In contrast, taking into account almost two centuries of spread of Ponto-Caspian species through European freshwater canals, an alternative explanation may be that Ponto-Caspian taxa have marine origin but adapted to freshwater environments while moving northward.

To understand the invasion pattern, a nearly unidirectional invasion of Ponto-Caspian species to other regions but not vice versa, we explored if Ponto-Caspian taxa are better able to acclimatize to and colonize habitats across a range of salinities than are taxa from other regions. We conducted laboratory experiments on 22 populations of eight gammarid species native to the Ponto-Caspian, Northern European, and Great Lakes–St. Lawrence River regions. The superfamily Gammaroidea was chosen due to its history of colonizing numerous geographic regions with different salinity patterns (Rewicz, Wattier, Grabowski, Rigaud, & Bączela-Spychalska, 2015; Szaniawska, Łapucki, & Normant, 2003). We tested the hypotheses that there is no difference in salinity tolerance among: (i) different populations of the same species; (ii) different species from the same region; and (iii) species from different regions. In addition, we conducted a literature search using Thomson's Institute for Science Information (ISI) Web of Knowledge to determine reported salinity ranges of indigenous and nonindigenous areas for each species. Finally, to explore evolutionary relationships among examined species and their populations, we sequenced the mitochondrial cytochrome c oxidase subunit I gene (COI) from individuals in all populations used for our experiments.

2 | MATERIALS AND METHODS

2.1 | Laboratory experiments

The salinity experiments were conducted from May to November 2015 and from April to June 2016. Eight species were tested: *Gam-*

marus locusta, *G. oceanicus*, *G. salinus*, and *G. zaddachi*—native to Northern Europe; *Pontogammarus maeoticus* and *Obesogammarus crassus*—native to the Black and Caspian Seas; *G. tigrinus*—native to eastern North America; and *G. fasciatus*—native to the Laurentian Great Lakes. At least two populations, preferably originating from different salinities, were tested for each species, except for *G. oceanicus* (one population tested). Populations of seven species were collected and tested in their native areas, whereas those of *G. tigrinus*, due to practicality and distance from available testing stations, were collected and tested in invaded regions (Appendix S1). All populations except *G. fasciatus* were collected from areas with annual salinity variations of a few g/kg; however, none of the collection sites have freshwater conditions except *G. fasciatus*.

After collection, individuals were transported in ambient water to the laboratories where each individual was morphologically identified according to Köhn and Gosselck (1989) for *G. locusta*, *G. salinus*, *G. zaddachi*, and *G. oceanicus*; Holsinger (1972) for *G. fasciatus*; Lincoln (1979) for *G. tigrinus*; and Birstein and Romanova (1968), Moiceiev and Filatova (1985), Sars (1896), Stock (1974), and Stock, Mirzajani, Vonk, Naderi, and Kiabi (1998) for *P. maeoticus* and *O. crassus*. Morphological identification was confirmed by randomly separating up to 30 morphologically identified individuals for DNA barcoding using COI in the case of seven species, while *G. fasciatus* was confirmed by 16S rDNA (protocol provided below).

Following 2 weeks of acclimatization to laboratory conditions at ambient salinity and temperature (same as the collection site), salinity tests were conducted using 10 mean-size adult individuals per replicate for tests with *G. locusta*, *G. salinus*, *G. zaddachi*, *G. oceanicus*, *G. tigrinus*, and *G. fasciatus*, while 30 individuals per replicate were used for *P. maeoticus* and *O. crassus*. Given that adults do not exhibit a distinct sexual dimorphism, except when females are ovigerous, sex was not specified. The experimental design consisted of three treatments: (i) control (except for *P. maeoticus* and *O. crassus*); (ii) increased salinity; and (iii) decreased salinity. Each treatment in the experiments was tested in triplicate. The water used for experiments was natural water collected locally near each institute, and filtered through a 20 µm filter. The salinity of the control treatments was identical to the ambient water of collection site. The increased and decreased salinity treatments began at the ambient salinity of the population collection site, which was then increased/decreased by 2 g/kg every 2 days, respectively (Delgado, Guerao, & Ribera, 2011). Increased salinity was achieved by adding artificial seawater (Instant Ocean®) to the local filtered water for all populations except for *P. maeoticus* and *O. crassus*; in the case of *P. maeoticus* and *O. crassus*, salinity was increased by evaporating the local filtered water. Decreased salinity was achieved by adding potable tap water to dilute the local filtered water. Mortality was checked daily throughout the experiments. When the values of 0 and 40 g/kg were reached, mortality was followed up for two more weeks, as well as the presence of offspring. As sex was not determined, we acknowledge that the ratio of male/female adults in each replicate could be inconsistent, although highly unlikely that all individuals in a replicate were of the same sex, leading to no offspring observation

for the replicate. The primary dataset containing experimental results is available at: <https://doi.org/10.1594/pangaea.884715>.

2.2 | Statistical analysis of salinity experiments

We tested for differences in the onset and rate of mortality between populations within species and between treatments within populations. To test for differences within species and within populations, we constructed a mortality curve for each treatment for each population, and for each population for each species, using pooled data from all replicates, described by the equation (Briski, Ghabooli, Bailey, & MacIsaac, 2011; Briski, VanStappen, Bossier, & Sorgeloos, 2008):

$$y = 100/1 + e^{-Z(s-Q)}, \quad (1)$$

where s is salinity change (i.e., change in g/kg), Z is the rate of mortality, and Q is the onset of mortality (i.e., percentage mortality). The model was expanded to compare the rate and the onset of mortality between two curves using the equation (Briski et al., 2008, 2011):

$$y = 100/1 + e^{-(Z_1+Z_2)(s-Q_1-Q_2)}, \quad (2)$$

where Z_1 and Z_2 are the rates of mortality, and Q_1 and Q_2 are the points of onset of mortality, for the first and second curves, respectively. All possible combinations of curve pairs were compared statistically by the Fit Nonlinear Model using Generalized Least Squares. Significance levels for statistical comparisons of estimated parameters Z_1 and Z_2 , and Q_1 and Q_2 , were adjusted for multiple pairwise comparisons by Bonferroni-type correction to guard against inflating the Type I error rate. The family-wise error rate of 0.05 was used. All tests were performed using S-Plus 6.1 (S-Plus® 6.1, 2002, Insightful Corp., Seattle, Wa, USA). Due to high variability across populations, our model did not allow comparisons among species.

2.3 | Molecular identification, GenBank sequences, and data analysis

Total genomic DNA was extracted from the telson of each individual with the innuPREP DNA kit (analytikjena, Jena, Germany) or Marine Animal DNA Kit (TIANGEN, Beijing, China) following manufacturers' instructions. A fragment of the COI gene was amplified using a few different pairs of primers: LCO1490 and HCO2198 (Folmer, Black, Hoeh, Lutz, & Vrijenhoek, 1994), UCOIF and UCOIR (Costa, Henzler, Lunt, Whiteley, & Rock, 2009), and *G. tigrinus* species-specific primers (Kelly, MacIsaac, & Heath, 2006). In the case of *G. fasciatus*, 16S primers and protocols following Zhan, Bailey, Heath, and MacIsaac (2014) were applied. PCR amplifications were carried out in 20 µl volume including 10× DreamTaq Buffer (containing MgCl₂), 100 mM dNTPs, 10 mM of each primer, 1–10 ng of genomic DNA, and 1 Unit of DreamTaq DNA polymerase (Thermo Fisher Scientific, Waltham, MA, USA) or 1 Unit of Taq DNA polymerase (Takara China; Dalian, China). The amplification protocol consisted of 5 min denaturation at 94°C, followed by 33 cycles of denaturation at 94°C for 35 s, annealing at 47°C for 45 s, extension at 69°C for 45 s, and

a final extension step of 69°C for 10 min. PCR products were cleaned with 0.4 U of FastAP Thermosensitive Alkaline Phosphatase (Thermo Fisher Scientific) and 4U of Exonuclease I (Thermo Fisher Scientific). Cleaned PCR products were prepared for sequencing using a BigDye Terminator v3.1 cycle sequencing kit (Thermo Fisher Scientific), purified with a BigDye XTerminator Purification Kit (Thermo Fisher Scientific) and sequenced on an automated ABI 3130XL capillary sequencer.

Evolutionary relationships among all species were inferred with phylogenetic trees. Two sequences were added to act as outgroups to the overall dataset (i.e., *Crangonix pseudogracilis*) and within genus (i.e., *Dikerogammarus villosus*; Hou & Sket, 2016). Accession numbers can be found in Appendix S1 (Baltazar-Soares, Paiva, Chen, Zhan, & Briski, 2017). Sequences were aligned and manually trimmed to a standard fragment size in BioEdit v7.0.4.1 (Hall, 1999). A nucleotide substitution model was estimated using a maximum-likelihood method allowing strong branch swapping. The best-fit model was chosen according to Bayesian inference criteria and was used in the construction of two phylogenetic trees: constructed with the Neighbor-joining method (Saitou & Nei, 1987) implemented in MEGA v6.0 (Tamura, Stecher, Peterson, Filipiński, & Kumar, 2013) and a coalescent-based Bayesian methodology (Kingman, 1982) in BEAST v1.8 (Drummond & Rambaut, 2007). For the Neighbor-joining method, we considered transitions/transversions and statistical support was inferred with 1,000 bootstrap iterations. For the Bayesian method, a molecular clock was set to a strict divergence rate of 2.2% per million years (Cristescu, 2015). All other parameters were retained as defaults. The Neighbor-joining tree was set to “coalescent constant size” and the Markov Chain Monte Carlo length to 1×10^8 ; convergence was inspected in Tracer v1.6 (Rambaut, Suchard, Xie, & Drummond, 2014). The Bayesian tree chosen for visual representation was selected through Maximum Clade Credibility, considering a posterior probability limit of 0.95 and 1,000 burn-in steps in TreeAnnotator v1.8.0, and drawn in FigTree v1.4.0 (Drummond & Rambaut, 2007).

2.4 | Salinity range of tested species

To compile the range of salinities of all habitats where the eight species occur in nature, we used Thomson's Institute for Science Information (ISI) Web of Knowledge 5.22.3 to search published data (search conducted between September 2015 and June 2016). The search was performed separately for each species using species name and “topic” as the search field through all available dates. Results were refined by subject area, including topics broadly relevant to ecology, comprising biodiversity conservation, biology, ecology, environmental sciences, freshwater biology, marine biology, oceanography, and zoology. We checked 441 studies recovered from ISI, and found 151 contained information on the salinity and/or coordinates of an occurrence location (Appendix S2). In cases where only coordinates were provided, salinity was determined for that location using the mean annual salinity from The World Ocean Atlas database (Antonov, Locarnini, Boyer, Mishonov, & Garcia, 2006).

3 | RESULTS

3.1 | Salinity experiments

In general, all species showed wide ranges of salinity tolerance. However, different patterns arose among species from different regions with those from Northern Europe better tolerating higher salinity and those from the Ponto-Caspian region better tolerating lower salinity (i.e., in the increased salinity and decreased salinity treatments); species from the Great Lakes–St. Lawrence River region did not perform well in laboratory settings—high mortality was observed in the control treatment as well (Table 1 and Appendix S3; Figures 1 and 2). The four Northern European species tested, collected from salinities ranging from 1 to 31 g/kg (Appendix S1), showed no difference in mortality rate among populations of the same species when salinity was increased to 40 g/kg (Table 2; Figure 1). The onset of mortality was different between two populations of *G. locusta* and *G. zaddachi* (i.e., Falckenstein and Helgoland, and Warnemünde and Kronenloch, respectively; Table 2; Figure 1). At 40 g/kg, mortality was lower than 50% and offspring were noted. In the decreased salinity treatment, Northern European species often showed differences among populations either in the onset, mortality rate, or both (Table 2; Figure 2). When salinity reached 0 g/kg mortality was again <50%. However, after 2 weeks at 0 g/kg, mortality increased above 50% for all species except *G. oceanicus* (Figure 2). The presence of offspring was recorded only for two populations of *G. salinus* (i.e., Helgoland and Kiel) but not for the other species.

Ponto-Caspian species were collected from salinities ranging from 4 to <11 g/kg (Table 2 and Appendix S1; Figures 1 and 2). In the increased salinity treatment, mortality of all three *P. maeoticus* populations and one *O. crassus* population (i.e., Chaboksar) was 100% at 30 g/kg. Two populations of *O. crassus* reached 40 g/kg, however, mortality was more than 75% (Table 2; Figure 1). The onset and mortality rate often differed among *P. maeoticus* populations, while in the case of *O. crassus* there was a difference only in the onset of mortality between the Havigh population and the two other populations (Table 2; Figure 1). There were no offspring observed in the increased salinity treatment. In the decreased salinity treatment, there was a difference in the mortality rate between the Shafarud population and the two other populations of *P. maeoticus*, and the onset of mortality between Havigh and Chaboksar populations of *O. crassus* (Table 2; Figure 2). Mortality of all populations of both species was <30% at the end of the decreased salinity experiment (Figure 2), with offspring present.

The Great Lakes–St. Lawrence River region species *G. fasciatus* and *G. tigrinus*, collected from salinity <0.5 g/kg and from >4 to 10 g/kg, respectively, revealed high variability in both the onset and mortality rate among populations (Table 2; Figures 1 and 2). In general, both species did not perform well in laboratory settings, with high mortality in the control treatment (i.e., 94% and 74%, respectively). Mortality of *G. tigrinus* in the increased and decreased treatments was more than 60% and 50%, respectively (Figures 1 and 2), with no offspring observed. Interestingly, *G. fasciatus*, a freshwater

Species	Population	Experimental treatment	The onset of mortality (p-value)	The rate of mortality (p-value)
<i>Gammarus locusta</i>	Helgoland	Increase – decrease	<.0001	<.0001
	Falckenstein	Increase – decrease	<.0001	<.0001
	Warnemünde	Increase – decrease	<.0001	<.0001
<i>Gammarus oceanicus</i>	Kiel	Increase – decrease	.4188	.2585
<i>Gammarus salinus</i>	Helgoland	Increase – decrease	.5789	.5314
	Falckenstein	Increase – decrease	.0054	.0002
	Kiel	Increase – decrease	<.0001	<.0001
	Travemünde	Increase – decrease	<.0001	.3022
<i>Gammarus zaddachi</i>	Warnemünde	Increase – decrease	.0002	.0290
	Kronenloch	Increase – decrease	<.0001	<.0001
<i>Pontogammarus maoticus</i>	Jafrud	Increase – decrease	<.0001	<.0001
	Shafarud	Increase – decrease	<.0001	<.0001
	Bandar-e Anzali	Increase – decrease	.0037	.0001
<i>Obesogammarus crassus</i>	Havigh	Increase – decrease	<.0001	<.0001
	Gisom	Increase – decrease	<.0001	<.0001
	Chaboksar	Increase – decrease	<.0001	<.0001
<i>Gammarus tigrinus</i>	Liu	Increase – decrease	<.0001	.0004
	Pärnu	Increase – decrease	<.0001	.0002
	Travemünde	Increase – decrease	.2191	.1056

TABLE 1 Statistical comparison of parameters between pairs of fitted curves for the increased and decreased treatments, which showed significant difference in the onset, rate of mortality, or both

The increased treatment was compared to the decreased treatment of the same population to determine if the population equally tolerates higher and lower salinity stress. The *t* test incorporated in the Fit Nonlinear Model using Generalized Least Squares was used to test for differences between estimated parameters—rate of mortality (Z_1 and Z_2) and onset of mortality (Q_1 and Q_2). Significant *p*-values are presented in bold. Bonferroni-type protection to guard against inflating the Type I error rate and family-wise error rate of 0.05 were used for pairwise statistical comparisons.

species, survived increased salinity treatment up to 40 g/kg, although with mortality higher than 60% (Figure 1); offspring were observed below 27 g/kg.

3.2 | Nonindigenous status and salinity range of tested species

The literature search revealed that all four Northern European species and *G. fasciatus* from the Great Lakes–St. Lawrence River region have not spread outside their native ranges (Table 3). All species except *G. fasciatus* were reported from a broad range of salinity. While three Northern European species (i.e., *G. locusta*, *G. oceanicus*, and *G. salinus*) occupy habitats where salinity ranges from 5 to 30 g/kg, *G. zaddachi* inhabits both freshwater and marine environments (i.e., from <0.5 to 30 g/kg; Table 3). The Ponto-Caspian species, *P. maoticus*, has a broader salinity range in its indigenous (i.e., from 0.5 to 30 g/kg) compared to its nonindigenous region (i.e., from 17 to 18 g/kg), whereas *O. crassus* inhabits higher salinity in its indigenous (i.e., from 12 to 34 g/kg) than in its nonindigenous range (i.e., from <0.5 to 19 g/kg, Table 3). Finally, *G. tigrinus* inhabits both freshwater and marine habitats in both indigenous and nonindigenous regions (i.e., from <0.5 to 30 g/kg, Table 3), with numerous records in freshwater

environments in its nonindigenous region. *Gammarus fasciatus* has been recorded only from freshwater habitats (<0.5 g/kg).

3.3 | Evolutionary relationships

A total of 24 sequences (541 base pairs) used for the phylogenetic analyses included 10 species and 24 populations from different salinities. Both methods used to reconstruct phylogeny agreed for the majority of the species-specific clusters (Figure 3). Here, it is important to observe the strong bootstrap support obtained for the nodes of the Neighbor-joining tree that groups *G. tigrinus* and *G. fasciatus* (69%), and *G. zaddachi* and *G. salinus* (92%). These two clusters are also present in the Bayesian tree and supported by nonoverlapping 95% high probability density intervals in relation to each respective ancestral node.

4 | DISCUSSION

The present study compared the salinity tolerance of eight gammarid species with origin in three different regions—Northern Europe, Ponto-Caspian, and Great Lakes–St. Lawrence River regions—to

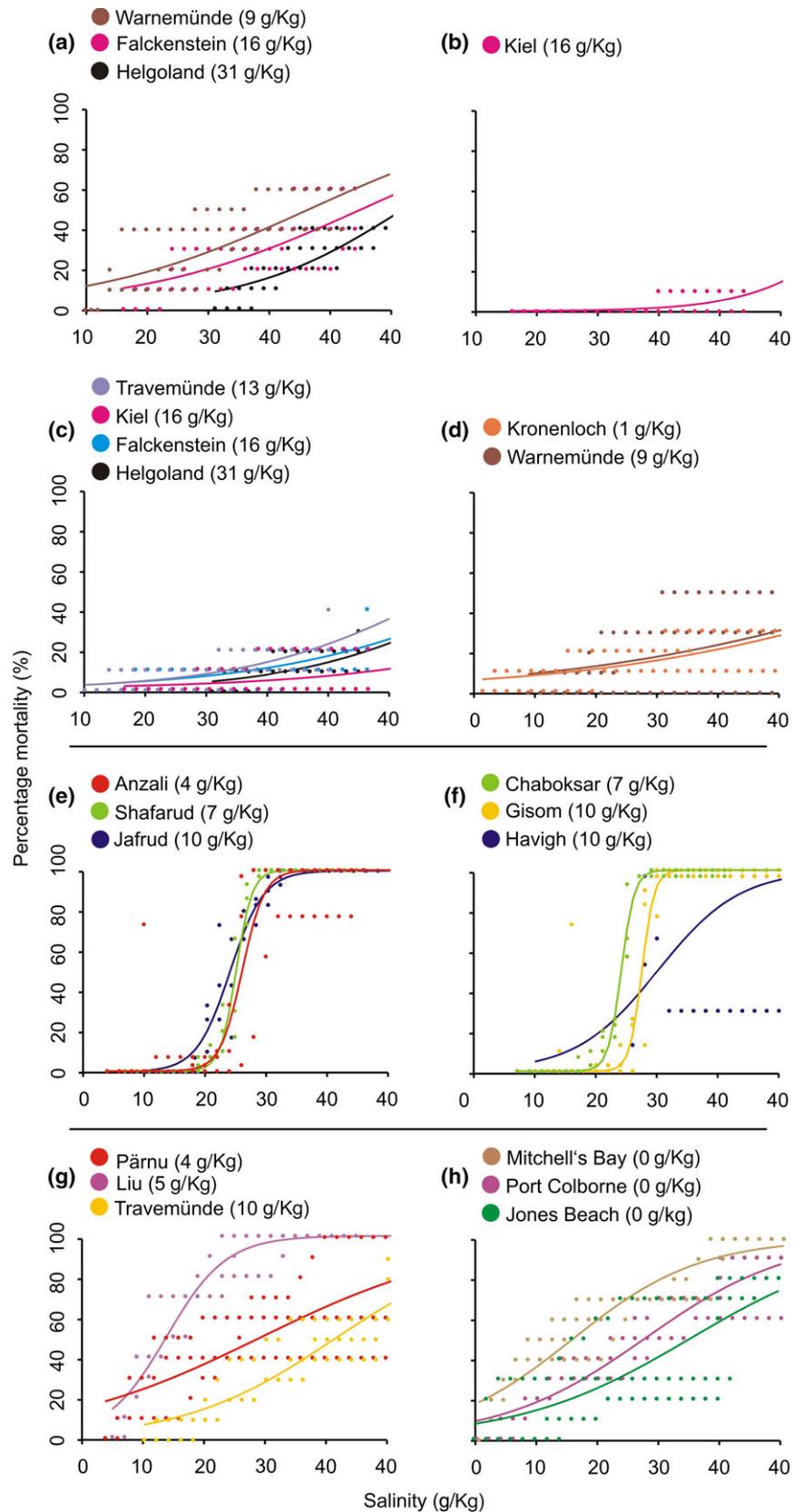


FIGURE 1 Mortality rates in the increased salinity treatment for Northern European species: (a) *Gammarus locusta*, (b) *G. oceanicus*, (c) *G. salinus*, and (d) *G. zaddachi*; Ponto-Caspian species: (e) *Pontogammarus maeoticus* and (f) *Obesogammarus crassus*; and Great Lakes–St. Lawrence River species: (g) *G. fasciatus* and (h) *G. tigrinus*. The curves were constructed using pooled data from three replicates. Note different scales used on x-axis across panels [Colour figure can be viewed at wileyonlinelibrary.com]

determine if Ponto-Caspian taxa more readily acclimatize to and colonize diverse salinity habitats than do taxa from other regions. Interestingly, although our study determined that species from all three

tested regions tolerate wide ranges of salinity, different patterns arose among species from different regions. Ponto-Caspian species had high survival in fresh water, while Northern European species

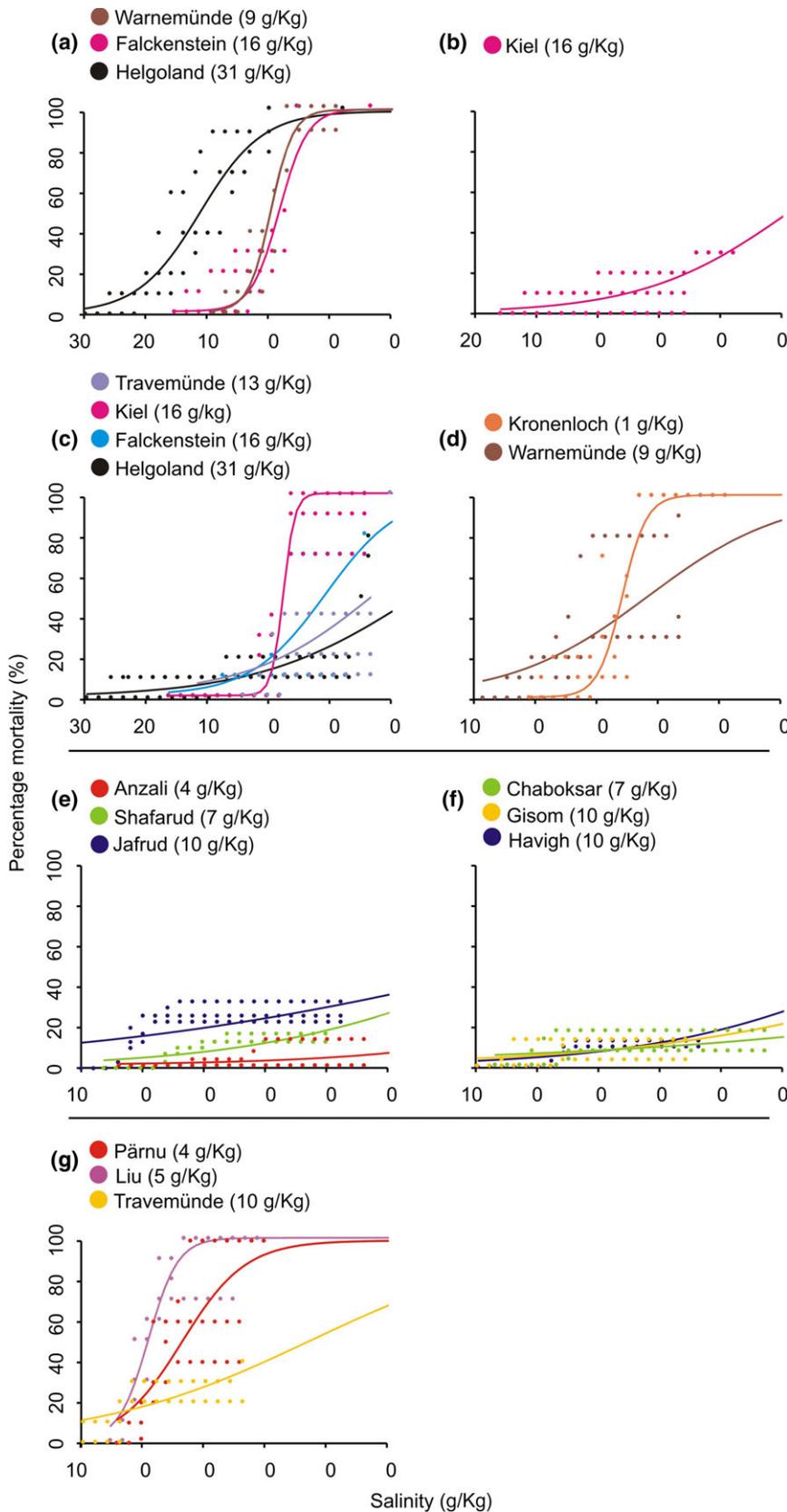


FIGURE 2 Mortality rates in the decreased salinity treatment for Northern European species: (a) *Gammarus locusta*, (b) *G. oceanicus*, (c) *G. salinus*, and (d) *G. zaddachi*; Ponto-Caspian species: (e) *Pontogammarus maeoticus* and (f) *Obesogammarus crassus*; and Great Lakes–St. Lawrence River species: (g) *G. tigrinus*. The curves were constructed using pooled data from three replicates. Note different scales used on x-axis across panels [Colour figure can be viewed at wileyonlinelibrary.com]

had high survival in fully marine conditions. Genetic analyses that determined the evolutionary distance among species from different regions, particularly of those from the Ponto-Caspian and the two

other regions, aligned with our experimental results. Due to the high tolerance of Ponto-Caspian taxa to freshwater conditions and the evolutionary relationships, in addition to the geological history of the

TABLE 2 Statistical comparisons of parameters between pairs of fitted curves for the populations, which showed significant difference in the onset, rate of mortality, or both

Species	Population compared	Experimental treatment	The onset of mortality (p-value)	The rate of mortality (p-value)
<i>Gammarus locusta</i>	Falckenstein–Helgoland	Increase	.0099	.1220
	Falckenstein–Warnemünde	Increase	.2415	.8315
	Helgoland–Warnemünde	Increase	.0690	.1349
	Falckenstein–Helgoland	Decrease	.0300	.0041
	Falckenstein–Warnemünde	Decrease	<.0001	.3267
	Helgoland–Warnemünde	Decrease	<.0001	<.0001
<i>Gammarus salinus</i>	Falckenstein–Helgoland	Increase	.0772	.5347
	Helgoland–Kiel	Increase	.1368	.5554
	Helgoland–Travemünde	Increase	.1710	.9661
	Falckenstein–Kiel	Increase	.3682	.8287
	Falckenstein–Travemünde	Increase	.4914	.4290
	Kiel–Travemünde	Increase	.2689	.5177
	Falckenstein–Helgoland	Decrease	<.0001	.0019
	Helgoland–Kiel	Decrease	<.0001	<.0001
	Helgoland–Travemünde	Decrease	<.0001	.3401
	Falckenstein–Kiel	Decrease	<.0001	.001
	Falckenstein–Travemünde	Decrease	.0908	.4914
	Kiel–Travemünde	Decrease	<.0001	.0134
<i>Gammarus zaddachi</i>	Warnemünde–Kronenloch	Increase	.0002	.1774
	Warnemünde–Kronenloch	Decrease	<.0001	.0003
<i>Pontogammarus maeoticus</i>	Jafrud–Shafarud	Increase	<.0001	<.0001
	Jafrud–Bandar-e Anzali	Increase	<.0001	.0960
	Shafarud–Bandar-e Anzali	Increase	<.0001	.0102
	Jafrud–Shafarud	Decrease	.7971	.0333
	Jafrud–Bandar-e Anzali	Decrease	.4479	.5337
	Shafarud–Bandar-e Anzali	Decrease	.1717	.0100
<i>Obesogammarus crassus</i>	Havigh–Gisom	Increase	<.0001	.5720
	Havigh–Chaboksar	Increase	<.0001	.7057
	Gisom–Chaboksar	Increase	.6910	.0579
	Havigh–Gisom	Decrease	.3907	.4088
	Havigh–Chaboksar	Decrease	.0352	.0511
	Gisom–Chaboksar	Decrease	.2362	.2711
<i>Gammarus tigrinus</i>	Liu–Pärnu	Increase	<.0001	<.0001
	Liu–Travemünde	Increase	<.0001	<.0001
	Pärnu–Travemünde	Increase	.0056	.0902
	Liu–Pärnu	Decrease	<.0001	.0026
	Liu–Travemünde	Decrease	<.0001	<.0001
	Pärnu–Travemünde	Decrease	<.0001	.0006
<i>Gammarus fasciatus</i>	Mitchell's bay–Port Colborne	Increase	<.0001	.0003
	Mitchell's bay–Jones Beach	Increase	<.0001	.0573
	Port Colborne–Jones Beach	Increase	<.0001	.0138

The *t* test incorporated in the Fit Nonlinear Model using Generalized Least Squares was used to test for differences between estimated parameters—rate of mortality (Z_1 and Z_2) and onset of mortality (Q_1 and Q_2). Significant *p*-values are presented in bold. Bonferroni-type protection to guard against inflating the Type I error rate and family-wise error rate of 0.05 were used for pairwise statistical comparisons.

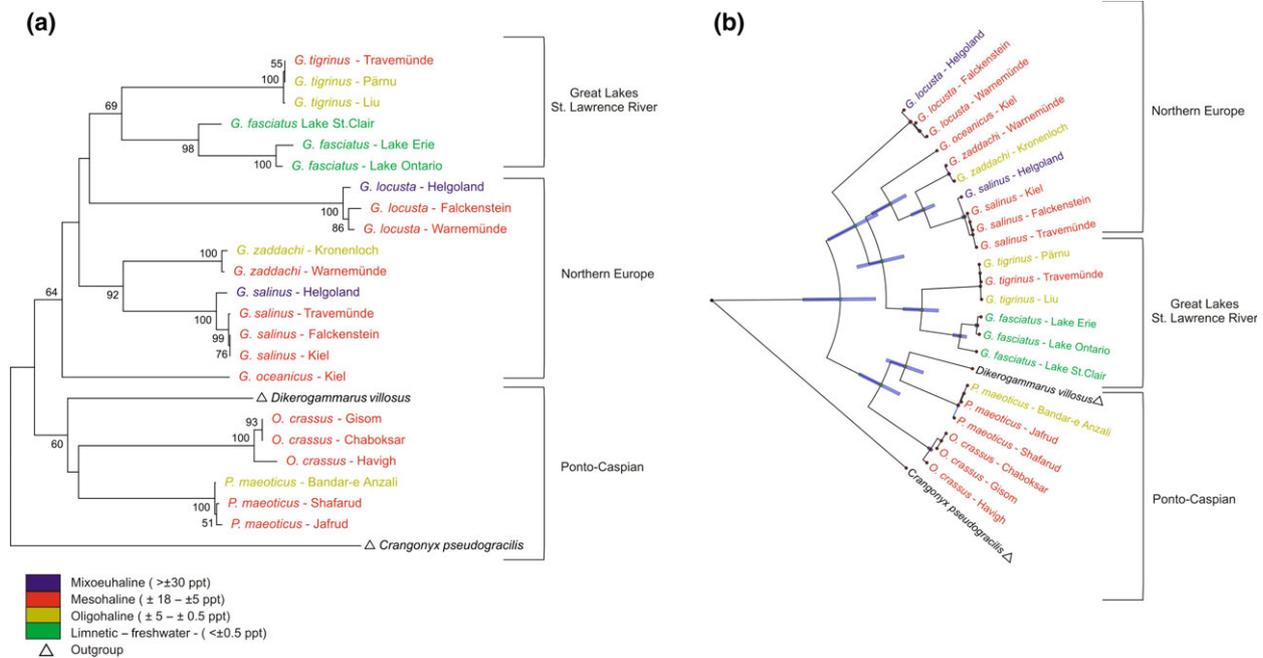


FIGURE 3 Evolutionary relationships of taxa: (a) Evolutionary history inferred using the Neighbor-Joining method (Saitou & Nei, 1987). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (10,000 replicates) is shown next to the branches (Felsenstein, 1985). Evolutionary distances were computed using the Tamura 3-parameter method (Tamura, 1992); (b) Evolutionary history inferred through a coalescent-based Bayesian approach. High probability density intervals (HPD) are depicted for each node height. Salinity assignment was based on the Venice System (1958) [Colour figure can be viewed at wileyonlinelibrary.com]

TABLE 3 Salinity range of the eight tested species based on occurrences in their indigenous and nonindigenous regions

Species	Salinity range g/kg (indigenous areas)	Salinity range g/kg (nonindigenous areas)
<i>Gammarus locusta</i>	5 to >30	No invasion range recorded
<i>Gammarus oceanicus</i>	5 to >30	No invasion range recorded
<i>Gammarus salinus</i>	5 to >30	No invasion range recorded
<i>Gammarus zaddachi</i>	<0.5 to >30	No invasion range recorded
<i>Pontogammarus maeoticus</i>	0.5–30	17–18
<i>Obesogammarus crassus</i>	12–34	<0.5 to 19
<i>Gammarus tigrinus</i>	<0.5 to 30	<0.5 to >30
<i>Gammarus fasciatus</i>	<0.5	No invasion range recorded

region, we suggest that Ponto-Caspian species are of freshwater origin.

Nowadays, Ponto-Caspian species thrive in low salinities of Northern Europe and in the Great Lakes–St. Lawrence River region (Bij de Vaate et al., 2002; Casties et al., 2016; Reid & Orlova, 2002; Ricciardi & Maclsaac, 2000). Geologically, the Ponto-Caspian region has been subject to a number of salinity changes that significantly influenced the biological and physical characteristics of the basins (Zenkevitch, 1963). The species that have persisted and thrived despite these complex changes and successions of low and high

salinity levels over millions of years are mostly settled in estuaries, lagoons, and the lower courses of rivers (Mordukhay-Boltovskoy, 1964; Zenkevitch, 1963). From those habitats they might “naturally” migrate northward through rivers/canals to the Baltic Sea, although some of them were intentionally introduced to reservoirs in Russia, Lithuania, and Ukraine (Arbačiauskas, Rakaukas, & Virbickas, 2010; Casties et al., 2016; Jazdzewski, Konopacka, & Grabowski, 2002; Ketelaars, Lambregts-van de Clundert, Carpentier, Wagenvoort, & Hoogenboezem, 1999). Nevertheless, Ponto-Caspian NIS did not spread to more saline habitats of the Black and North Seas, or to the Mediterranean Sea (Paavola, Olenin, & Leppäkoski, 2005; Shiganova, 2010). In contrast, Northern European species tested in our study performed better under marine conditions, confirming their marine origin, which is consistent with the Baltic Sea geological history; the current ecosystem is as recent as the Last Glacial Maximum, that is, <12,000 years ago, and inhabited by Atlantic species (Leppäkoski et al., 2002; Reid & Orlova, 2002). The phylogenetic analyses conducted in our study further supported that evolutionary relationships of these species can be related to their biogeographic regions, as in a fully justified phylogeographic scenario. Fascinatingly, despite the wide salinity tolerance and great number of shipping routes between Northern Europe and other world ports, none of the tested Northern European species have an invasion record. Due to the high tolerance of Ponto-Caspian species to freshwater conditions and Northern European species to high salinity, in addition to the geological history of the two regions, we hypothesize that these species are of freshwater and marine origins, respectively. Therefore,

the establishment of Ponto-Caspian species in the Great Lakes and other freshwater habitats should not be a surprise. However, we advise that investigating the putative molecular basis of adaptation that permits Ponto-Caspian taxa to thrive in fresh water should be done with state-of-the-art techniques, such as high-throughput sequencing, particularly for functional genes responding to salinity conditions.

Species from the same region tested for salinity tolerance demonstrated similar patterns on a large scale, yet they also demonstrated differences among populations on a fine scale. For instance, current literature states that *G. zaddachi* naturally occurs in both freshwater and marine habitats; however, the two populations tested in our study were unable to cope with freshwater conditions even though one of them was collected from salinity of 1 g/kg. Moreover, none of our tested populations of *G. tigrinus*, which has invaded diverse regions from freshwater to fully marine habitats (Table 3; Kelly et al., 2006), perform well either under freshwater or fully marine conditions. This may be the result of different populations of a species adapting to varying local environmental conditions. Several previous studies have indicated that populations may differ significantly in their growth responses to climate change (O'Neill, Hamann, & Wang, 2008), have different tolerance to cold temperatures due to local adaptation (Preisser, Elkinton, & Abell, 2008), and have different upper thermal limits across different latitudes (Kuo & Sanford, 2009). We emphasize that predictions of establishment success, particularly considering future climate change scenarios, might fail as a species is often considered a single homogeneous unit in those predictions instead of diverse locally adapted populations (Crickenberger, Walther, & Moran, 2017; Laurel, Copeman, Spencer, & Iseri, 2017; Parker et al., 2017). While many studies determining invasion success of NIS ask the question: "which traits enable a species to invade a new habitat?" (Alpert, Bone, & Holzapfel, 2000; Marco, Páez, & Cannas, 2002), we strongly suggest that future studies in both invasion ecology and global change ecology take into consideration spatially varying selection among populations in order to better predict future scenarios.

Both species originating from the Great Lakes–St Lawrence River region did not perform well in our laboratory experiments, often having higher mortality in the controls than in the stressed treatments (Appendix S3). As we regularly observed dark spots on tested animals from these regions, we suspect that the populations were infected with a parasite, most likely oomycetes, also known as water molds. Kestrup, Thomas, van Rensburg, Ricciardi, and Duffy (2011) identified a specific parasitic oomycete as a cause of high mortality of *G. fasciatus* in its native area of the St. Lawrence River. Interestingly, as the salinity of our experiments was increased, the stressed animals showed lower mortality than those in the control, indicating that the parasite may not have been able to cope with salinity changes. Consequently, if parasites or other disease agents are not able to cope with environmental conditions in a new habitat while the host species is, the effects of the parasite or disease would be reduced in a new region, giving the host a fitness advantage

compared to the native region. This parasite or disease loss has been described as the enemy release hypothesis in invasion ecology (Keane & Crawley, 2002). As *G. tigrinus* successfully invaded numerous habitats having a range of salinity and often freshwater areas, one possible explanation for its success may be release from parasites when invading habitats with a different salinity than that of its indigenous area. This enemy release might be short-lived as parasites also might adapt and evolve with time, however, short-term release during the early establishment phase might give enough advantage to facilitate the establishment of the species in a new habitat.

Numerous regions which are geographically far apart have become more similar biologically over recent centuries due to a high exchange of species (Capinha et al., 2015; Hulme, 2009). As Ponto-Caspian species have successfully established in a number of brackish and freshwater habitats in Northern Europe and the Great Lakes–St. Lawrence River region, respectively, but not vice versa, it has been hypothesized that Ponto-Caspian species may have inherent advantages over other species in colonizing new habitats (Casties et al., 2016; Leppäkoski et al., 2002; Ricciardi & MacIsaac, 2000). A major vector for introduction of NIS in aquatic habitats is commercial shipping (Molnar et al., 2008; Ricciardi, 2006), with many shipping ports being located in river mouths and estuaries characterized by broad temporal salinity changes that frequently include freshwater conditions (Keller, Drake, Drew, & Lodge, 2011). As a result, euryhaline taxa might have a great advantage when introduced to those fluctuating environments. Our study suggests that although both Ponto-Caspian and Northern European species tested have quite broad salinity tolerance, possible freshwater origin of relict Ponto-Caspian species might increase establishment success of these taxa due to their tolerance to freshwater conditions. Consequently, Ponto-Caspian species might not be inherently better colonizers, rather, their intrinsic advantage may simply correlate with the fact that areas with greatest introduction frequency of NIS (i.e., shipping ports; Seebens, Gastner, & Blasius, 2013) are environmentally variable habitats which often include freshwater conditions intolerable to euryhaline marine taxa. Finally, although Ponto-Caspian NIS are currently not established in higher salinity areas of the North and Baltic Seas (Casties et al., 2016; Paavola et al., 2005), a future scenario modeled by Meier et al. (2012) predicts a decline in salinity across the Baltic Sea by the end of the twenty-first century, therefore, it may be expected that Ponto-Caspian species will spread further in the system, as well as in other areas with future salinity declines.

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SUPPORTING INFORMATION

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