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Presence of a resident species aids invader evolution

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Abstract

Interactions between phytoplankton species shape their physiological and evolutionary responses. Yet, studies addressing the evolutionary responses of phytoplankton in changing environments often lack an explicit element of biotic interactions. Here, we ask (1) how the presence of a locally adapted phytoplankton species will affect an invading phytoplankton species' evolutionary response to a physiologically challenging environment; (2) whether this response is conserved across environments varying in quality; and (3) which traits are associated with being a successful invader under climate change scenarios. In a conceptual first step to disentangle these broad questions, we experimentally evolved populations of fresh- and seawater phytoplankton in a novel salinity (the freshwater green algae *Chlamydomonas* in salt water, and the marine *Ostreococcus* in freshwater), either as mono-cultures (colonizers) or as co-cultures (invaders: invading a novel salinity occupied by a resident species, for example, *Chlamydomonas* invading salt water occupied by resident *Ostreococcus*) for 200 generations. We superimposed a temperature treatment (control (22°C), mild warming (26°C), drastic warming (32°C), and fluctuating (22°C/32°C) warming) as a representative aspect of climate change with the potential to ameliorate or deteriorate existing environmental conditions. Invaders had systematically lower extinction rates and evolved overall higher growth rates, as well as broader salinity and temperature preferences than colonizers. The invading species' evolutionary responses differed from those of colonizers in a replicable way across environments of differing quality. The evolution of small cell size and high reactive oxygen species tolerance may explain the invaders' higher fitness under the scenarios tested here.

In a warming, changing world, invasion scenarios where phytoplankton will invade or be transported into novel environments occupied by resident species are likely to become more frequent (Walther et al. 2009; Mellin et al. 2016; Seebens et al. 2017), making it pertinent that we better understand how invaders evolve upon encountering new environments, and what types of phenotypes they will display. To date, it remains unclear to what degree the presence of a locally adapted species has the potential to alter the evolutionary response of the invading species, and whether there are traits or sets of traits that explain how invader fitness changes across environments.

Traits such as growth rates, which serve as a proxy for fitness, allow us to estimate in what kind of environments organisms can survive. Phenotypic traits other than growth rates, for example, cell size and photosynthesis rates, describe the roles of phytoplankton in the ecosystem in more detail and are also crucial components of ecosystem models (Dutkiewicz et al. 2020). As not all traits will follow the same reaction norms across, for example, temperature gradients or biotic interactions, extensive phenotyping is an important first step to search for patterns in how sets of phenotypic traits in phytoplankton behave across environments.

The responses of phytoplankton to aspects of climate change are now well understood across both short- (one to a few generations and based on the same genotype) and long-time scales (many generations, including some element of

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heritable genetic change). However, our understanding of the evolutionary response of phytoplankton is largely based on studies in single isolate experiments (Schaum and Collins 2014; Schluter et al. 2014; Listmann et al. 2016). Far fewer studies consider ecologically more complex environments, where multiple trophic levels (Yoshida et al. 2004; Becks and Hiltunen 2014; Frickel et al. 2016), or multiple phytoplankton species interact (Scheinin et al. 2015; Wolf et al. 2017, 2019). Studies that have explicitly tested evolution in action while species are interacting are often carried out using bacteria and in environments that are of low quality for the focal species (traditionally low-nutrient or toxic environments). Such environments lead to slower growth and a drastic and near-fatal decline in population size of the focal species (Rozen and Lenski 2000; Elena and Lenski 2003). It is unlikely however, that when a phytoplankton species invades—or gets transported into—a novel environment, it will always encounter an environment that is toxic or of extremely low quality. This makes it likely that phytoplankton interactions will go beyond competition, which is the most common type of interaction between phytoplankton if nutrients, light, or space are limiting (Tilman 1977; Ji et al. 2017; Burson et al. 2018). Interactions between microbes beyond competition can range from facilitation to mutualism to interdependence and combinations thereof (Hesse et al. 2017; Picoche and Barraquand 2020; Collins and Schaum 2021) depending on the quality of the environment, begging the question of whether patterns of invader evolution are repeatable across environments.

Here, we used an experimental evolution approach to conceptually investigate (1) how invader evolution in co-culture differs from that of a species colonizing the same environment in mono-culture (e.g., likelihood of survival, evolutionary trajectories); (2) whether differences between colonizers and invaders hold across environments of different qualities; and (iii) which traits characterize successful invaders, for example, whether evolution in a different biotic context leads to the development of different phenotypic traits, such as cell size, reactive oxygen species (ROS) or photosynthesis rates.

We chose salinity as our main environmental driver due to its potential to determine the distribution and phenotypic

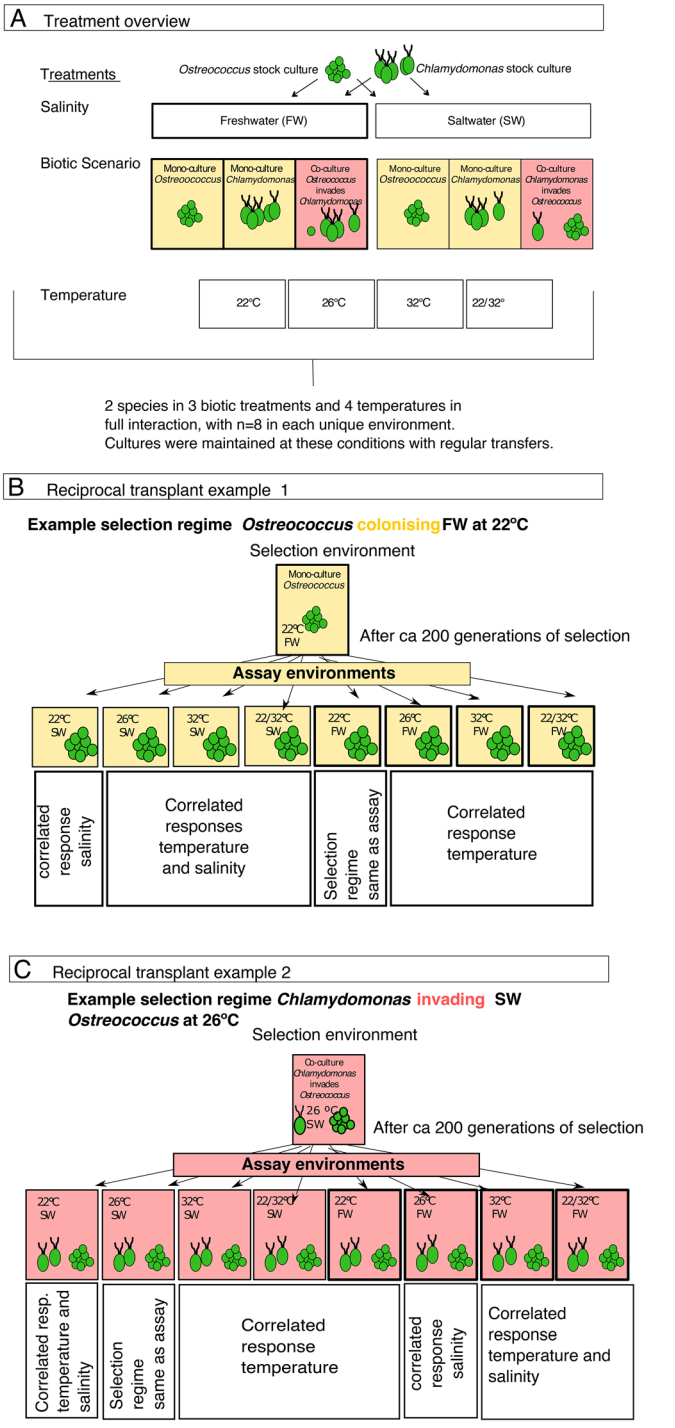

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Fig. 1. Experimental set-up. Throughout, color indicates the biotic scenario (yellow for colonization, red for invasion), and the thickness of frames, the salinity (thick for freshwater and thin for salt water) (**A**) treatment overview stock cultures of the marine *Ostreococcus* and the freshwater *Chlamydomonas* were used to inoculate two salinity regimes (a freshwater, FW, and a saltwater treatment, SW), crossed with three biotic treatments (*Ostreococcus* in mono-culture, *Chlamydomonas* in mono-culture, or co-culture with *Ostreococcus* invading *Chlamydomonas* and vice versa), and four temperature regimes (22°C as the control, 26°C as moderate warming, 32°C as extreme warming, and a variable environment where temperature cycled between 22°C and 32°C twice weekly), for a total of 24 unique selection environments. $N = 8$ for each unique combination of salinity, biotic, and temperature regime. Samples were propagated weekly by batch transfer for approximately 200 generations. Light yellow denotes *Ostreococcus* or *Chlamydomonas* in mono-culture, that is, the focal species is colonizing. Light red denotes samples grown in co-culture. There, the focal species is invading. (**B**, **C**) Example reciprocal assay: at the end of the experiment, all samples were assayed in all salinity and temperature treatments. This allows us to better estimate whether samples have evolved generally larger salinity and/or temperature tolerances, or become locally adapted to their novel environment.

characteristics of phytoplankton communities (Bertos-Fortis et al. 2016; Godhe et al. 2016; Mousing et al. 2016). We chose temperature as a second environmental driver as climate change is purported to modify both the temperature and salinity of aquatic environments in concert, due to changes in rainfall and evaporation patterns. We used two green algae, the freshwater phytoplankton *Chlamydomonas moewusii* and the marine picoplankton *Ostreococcus tauri*. Both genera of green algae are cosmopolitan in natural systems and established model organisms for long-term studies in experimental evolution (Lachapelle et al. 2015; Heath and Collins 2016; Brennan et al. 2017). We evolved all populations for *ca* 200 generations either in mono-culture ("colonizers") or in co-culture ("invaders" were added to locally adapted "residents," Fig. 1). To test whether differences between colonizers and invaders are repeatable across environments differing in quality and complexity, we superimposed a temperature treatment creating environments of varying quality.

Methods

Algae strains

The marine picoplankton *O. tauri* (clone of the original OTH95) and the freshwater alga *C. moewusii* (CCAP 11/5B) were sourced as non-axenic stock cultures from the Roscoff culture collection and the CCAP (Culture Collection of Algae and Protozoa) respectively. The fact that these two species do not usually co-occur in nature is not problematic here because species invasion is the result of a new species being introduced into a new environment with resident species it has not interacted with before.

Samples were maintained in semi-continuous batch culture (i.e., a fixed volume of exponentially growing cells was transferred into fresh medium at regular intervals, *see* Supporting Information Fig. S1) at 22°C, 100 μmol quanta s^{-1} m^{-2} under a 12 : 12 h light : dark cycle in INFORS multitron incubators with integrated shakers until use. *Ostreococcus* was grown at a salinity of 32 (PSU, roughly 30 g NaCl L^{-1}; referred to from now on as salt water or SW) in f/2 medium (Guillard 1975), *Chlamydomonas* in modified Bold's medium (roughly 0.025 g NaCl L^{-1}; referred to from now on as freshwater or FW). Concentrations of major nitrogen and phosphorus sources were approximately the same in the fresh- and saltwater media.

Selection experiment

We set up our experiment using two salinity regimes (salt water and freshwater, where we refer to the salinity that the focal species originated from as the "ancestral" salinity) and three biotic regimes (Fig. 1A; Table 1). Residents are species evolved in their ancestral salinity (i.e., *Chlamydomonas* in freshwater, *Ostreococcus* in salt water) either in mono-culture or in co-culture with an invading species. Invaders are species evolved in a novel salinity where the resident species is present (i.e., *Chlamydomonas* invading *Ostreococcus* in salt water, *Ostreococcus* invading *Chlamydomonas* in freshwater).

Table 1. Nomenclature for biotic scenarios used throughout.

Name	Salinity	Biotic scenario
Resident	Freshwater for *Chlamydomonas* Salt water for *Ostreococcus*	Can be either mono-culture or invaded by invader
Invader	Salt water for *Chlamydomonas* Freshwater for *Ostreococcus*	Invades resident species (i.e., is always in co-culture)
Colonizer	Salt water for *Chlamydomonas* Freshwater for *Ostreococcus*	Is always mono-culture

Finally, colonizers are species evolved in a novel salinity as a mono-culture (i.e., *Ostreococcus* in freshwater, and *Chlamydomonas* in salt water).

In pilot studies, we characterized temperature and salinity reaction curves for each species (Supporting Information Figs. S2, S3). Based on these pilot studies, the long-term experiment was replicated across four different temperature regimes, for a total of 24 unique treatments (×8 biological replicates = 192 cultures, Fig. 1A). The temperature regimes consisted of a fluctuating temperature treatment and three stable temperatures, encompassing a stable 22°C treatment (control), a stable 26°C treatment (mild warming, higher quality environment), and a stable 32°C treatment (drastic warming, low-quality environment). In the fluctuating temperature treatment, temperature was switched between 22°C and 32°C every three to five generations (fluctuating between control and lower quality environment).

All cultures started out as genetically diverse mono-cultures before invading species were added. Cultures were grown on 48-well plates with sterile, breathable membranes (Aeraseal, Sigma-Aldrich) to minimize uneven evaporation and air exchange across plates. Mono-cultures were initially inoculated with 100 cells of *Chlamydomonas* or 1000 cells of *Ostreococcus* to account for the difference in cell size. In co-cultures, the resident species were inoculated at 100-fold the biomass of the invading species, for an "invading from rare" scenario at the beginning of the experiment. The invasion event occurred only once at the beginning of the experiment, after which we tracked the fate of the invaders throughout the experiment. The 48-well plates were positioned randomly in the incubator, and their position was changed every other day to minimize location effects. Cultures were maintained in semi-continuous batch culture, where well-mixed samples of 200 μL were serially transferred into 1200 μL of new medium every 7–10 generations ("transfers"). At each transfer, cell count was determined using an Accuri c6 (BD Scientific) flow cytometer at a high flow rate. To test whether samples were in

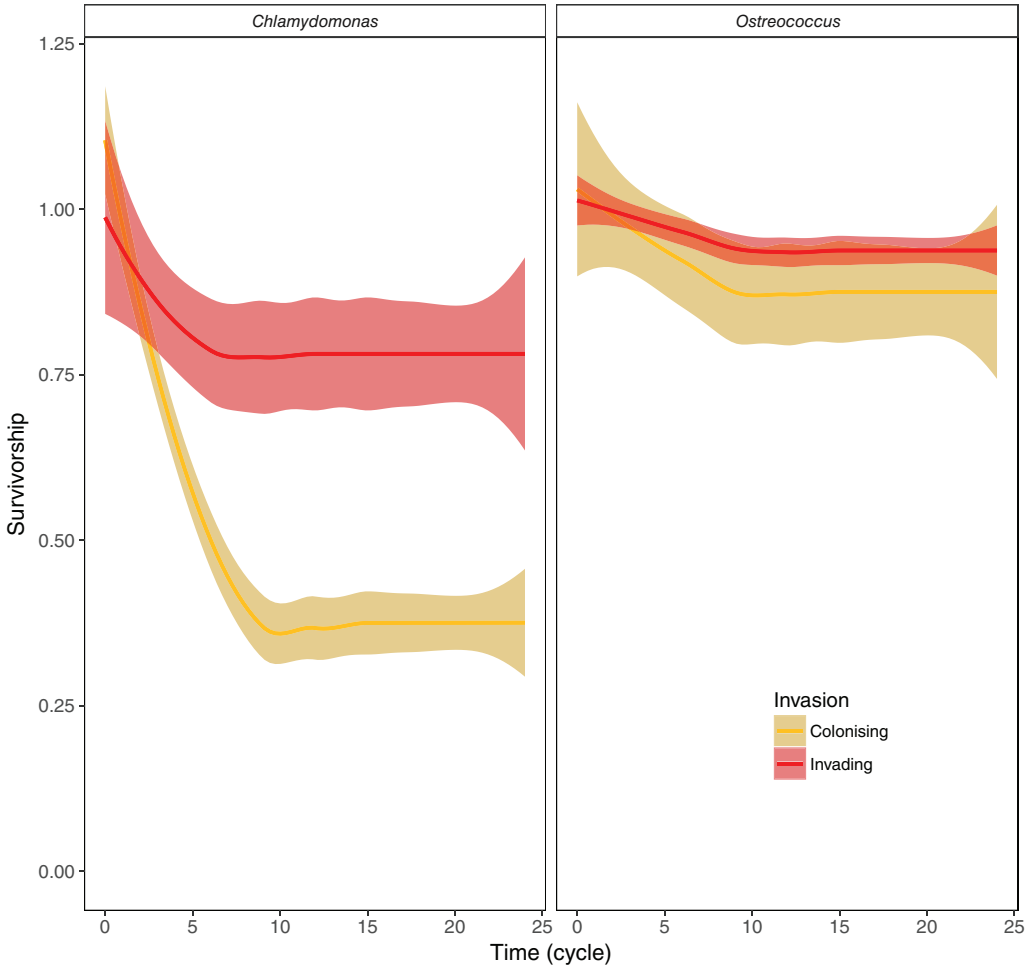

Fig. 2. Survival when invading into a novel salinity from rare is enhanced in the presence of a resident species across all selection regimes. Displayed is the mean survivorship for samples in all temperature regimes over time (where one cycle or transfer corresponds to 1 week), with 1.0 as 100% of populations surviving, and shaded areas denoting 95% confidence intervals. All treatment combinations started with $n = 8$. The proportion of populations and replicates surviving decreased rapidly at the beginning of the experiment but leveled off after about 10 transfers as extinctions stopped. The number of surviving biological replicates at the end of the experiment is shown in Supporting Information Table S1. Invader trajectories in light red, colonizer trajectories in yellow.

exponential growth, random samples were chosen to track growth throughout transfer cycles; however, this was not feasible for all samples and all transfers (Supporting Information Fig. S4). This allowed us to test whether samples were indeed in exponential growth and not limited by nutrients or light. Cells from the two species grown in co-culture could be distinguished based on the SSC (side scatter for granularity), FSC (forward scatter for cell size), and FL3 (red fluorescence for chlorophyll content) channels (Supporting Information Fig. S5), allowing for species growth curves to be tracked separately.

Cell counts at the beginning and end of each transfer cycle were used to calculate the rate of increase in cell numbers and approximate generation times. Rates of increase in cell number were determined assuming exponential growth, using the formula

$$\mu = (\ln(N_1) - \ln(N_0))/dt \quad (1)$$

where N_1 is the cell count at the end and N_0 at the start of the transfer, and dt is the length of the transfer cycle (7 days).

The experiment was carried out for approximately 200 generations.

Reciprocal assays

Short-term responses, here, within the first few generations of organisms encountering a new environment, are largely mediated by physiological responses with no or little heritable change to the genome. Long-term responses are likely to include an element of evolution. We can infer the magnitude of evolutionary responses or whether or not samples have become locally adapted by using a reciprocal transplant experiment. After 27 transfers in their respective

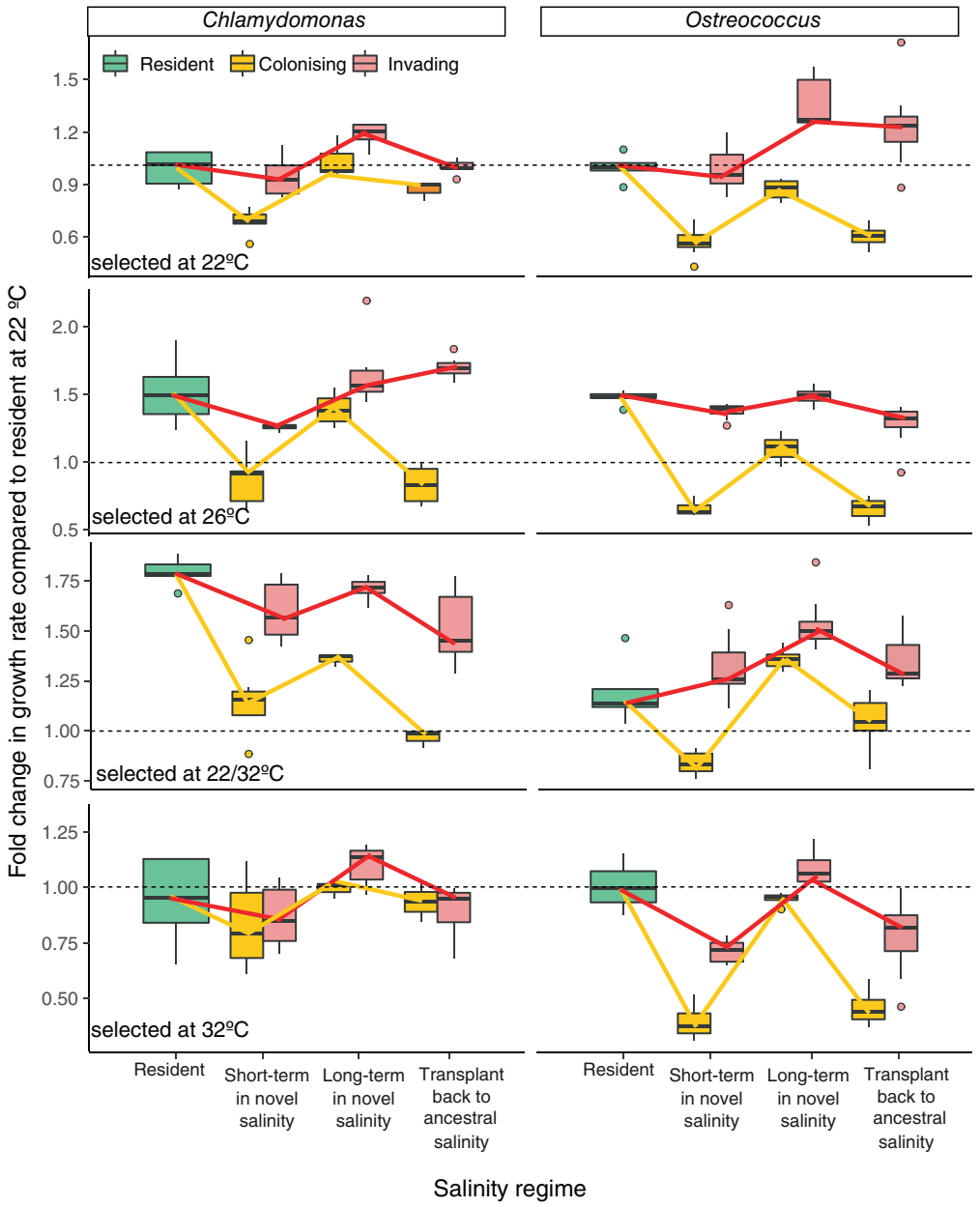

Fig. 3. Invaders and colonizers differ in their responses to salinity regimes across time scales. Salinity regime "resident" (in light green) denotes that the resident species was assayed in its selection salinity in mono-culture after 200 generations of selection. "Short-term in novel salinity" is for growth rates measured after the colonizer (yellow) or invader (light red) sample had spent two transfers in the novel salinity. "Long-term in novel salinity" is the growth rate of the colonizer (yellow) or invader (light red) sample after 200 generations in the formerly novel salinity that it was evolving in. "Transplant back to ancestral salinity" is for growth rates measured when samples were transferred back into the ancestral environment after 200 generations of evolution in the initially novel salinity. Throughout, colonizers were assayed in mono-culture, and invaders, in co-culture. We express changes in growth as compared to *Ostreococcus* or *Chlamydomonas* residents in their home salinity at 22°C in mono-culture, that is, values < 1 (below dotted line) indicate that a sample grew more slowly than the same species in mono-culture, in its home salinity at 22°C, and values > 1 (below dotted line) indicate that they grew faster. Each panel is for one selection temperature. Yellow boxplots are for colonizing species, and red, for invaders. *See* Supporting Information Table S2 for details on *n* per treatment. Boxplots are displayed as is standard, with the belt indicating the median. Fitted lines are for visualization only.

selection environments, all samples were subjected to a full reciprocal transplant assay in all salinity and temperature regimes to quantify the magnitude of the evolutionary responses, and to test whether the surviving colonizers and invaders had adapted to the novel salinity in each temperature regime (Fig. 1B,C, detailed methods for the reciprocal assays can be found in the Supporting Information S1, page 3).

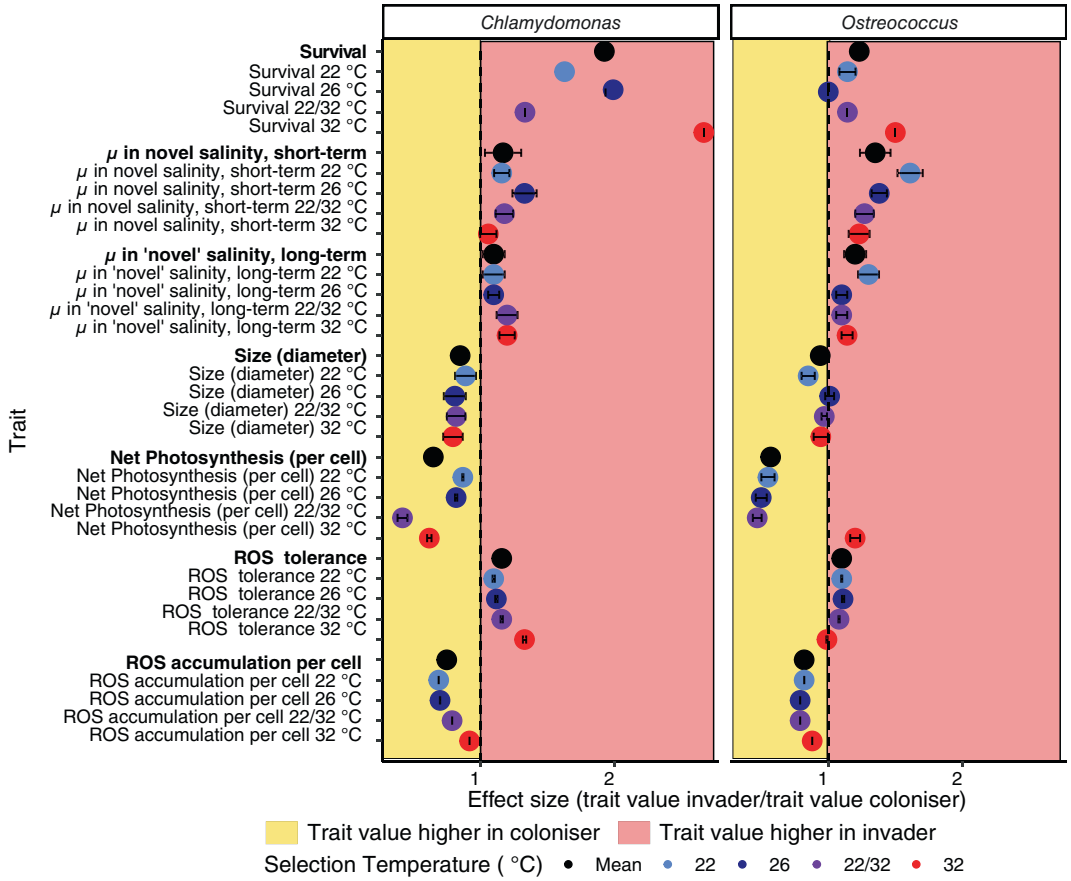

Fig. 4. Invaders differ from colonizers across environments varying in quality. In this visualization, we display an effect size of trait values in invaders vs. colonizers as a ratio. Values higher than 1 indicate higher trait values in invaders (red shaded areas). Values lower than 1 indicate higher traits values in colonizers (yellow shaded areas). For the all over mean (black), and trait values at 22°C (light blue), 26°C (dark blue), the fluctuating environment at 22°C/32°C (purple), and 32°C (red), we display means ± 1 SE. Within each regime, invaders tend to have higher growth rates, smaller cells, lower ROS accumulation per cell, and higher ROS tolerance. Net photosynthesis rates are higher in former colonizers, but less of it is channeled into growth (see also Fig. 5).

Experimental decomposition of populations grown and evolved in co-culture into mono-cultures

To test whether dependence on the species was established within very few generations, we re-created the starting conditions of the invasion experiment using mono-culture evolved samples. These samples were inoculated to recreate the invasion from rare scenario as described above. The new co-cultures were maintained for two cycles, and separated afterward ("decomposed") by filtration. We passed all co-cultured samples through a 5-μm nitrocellulose filter, allowing *Ostreococcus* cells to pass, while *Chlamydomonas* cells remained on the filter, from which they could be rinsed off. Samples were then inspected under the microscope and via flow cytometry to ensure that there were no visible remnants of the respective other species. We then measured growth rates and other phenotypic traits.

At the end of the selection experiment, we compared the growth rates of newly decomposed samples to the samples that had stayed in co-culture (Supporting Information Fig. S8). For logistic reasons, samples were only assayed (growth rates and traits measured) at the temperatures that they had evolved in and not across all temperatures. To assess whether invaders evolved in co-culture had adapted to the novel salinity, the presence of the resident species, or both, decomposed samples, as well as samples that had remained in co-culture were grown for two transfers in both their evolved and their ancestral salinity.

Characterization of phenotypic traits

To characterize phenotypes of invader and colonizer cultures, we studied whether evolved lineages differed in cell size, chlorophyll *a* content, relative quantities of intracellular polar and neutral lipids, capacity to detoxify harmful ROS, intracellular ROS levels, and net primary production.

Cell size and Chl *a* content, as well as relative quantities of intracellular polar and neural lipids using a Nile Red stain dye,

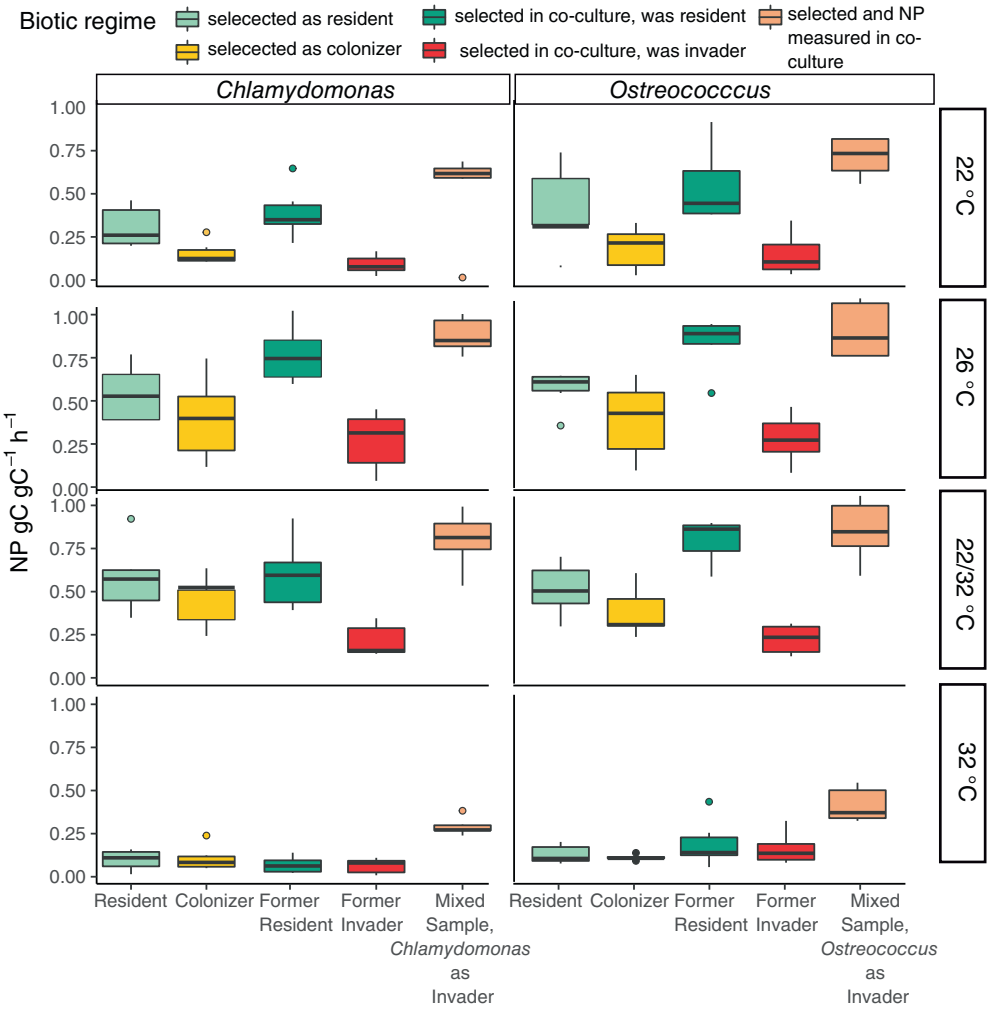

Fig. 5. Net photosynthesis of all evolved and decomposed (after 200 generations) samples at all selection temperatures at the focal species' (see panel header) selection salinity. Displayed is net primary production as gC per gC per hour for samples selected and assayed at. Residents (light green, evolved in mono-culture at their ancestral salinity) photosynthesized more than colonizers (yellow, evolved in mono-culture in an initially novel salinity). In samples that were deconstructed following 200 generations of selection in a mixed sample, former invaders (red) always photosynthesized less than the same species after evolution as a colonizer in mono-culture (yellow)—in line with reduced growth rates in these same samples. Former resident species (dark green) had higher net photosynthesis rates than the same species after evolution in mono-culture, but only for *Chlamydomonas* did this translate into higher growth rates (Supporting Information Fig. S8). Net photosynthesis per gC of the mixed-culture samples (orange, containing residents and invaders) was higher than that of single species isolates. Note that here we are displaying both species that made up the original mixed culture in the focal species' panel. Explicitly this means (using the*Chlamydomonas* panel as an example): The *Chlamydomonas* resident was selected and assayed in mono-culture, in freshwater (FW). The *Chlamydomonas* colonizer was selected and assayed in mono-culture, in salt water (SW). The mixed sample, with *Chlamydomonas* as an invader (in the *Ostreococcus* resident population) was selected and assayed in SW. The "former invader" in the *Chlamydomonas* panel is *Chlamydomonas* selected and assayed in SW. The "former resident in the *Chlamydomonas* panel is *Ostreococcus* selected and assayed in SW. We chose to display both species that made up the mixed culture in the focal species" panel to better show how the decomposed samples differed from the co-culture. Boxplots are displayed as is standard with the belt indicating the median. Number per treatment differed due to differences in extinctions at different selection salinities.

were measured using flow cytometry. ROS assays were performed with the protocols established by (Lindberg and Collins 2019; Malerba and Marshall 2019), allowing us to gain an estimate on whether samples under unfavorable conditions experience more stress, and are therefore producing more or being less able to tolerate ROS. Finally, net photosynthesis (NP) was assessed by measuring rates of oxygen production (in the light) and consumption (in the dark) using a 24-channel PreSens Sensor Dish Reader, and corrected per unit biomass (for phenotyping details, refer to Supporting Information S1, pages 4-5).

Statistical analyses

All data were analyzed in R version 3.3.3 (R Core Team 2022). We summarize packages used and model fitting below; detailed information on model selection, response variables,

and factor levels can be found in the Supporting Information S1, pages 6–8.

Statistical analysis of extinction rates

To determine the statistical significance of treatment effects on extinction rates, we first analyzed the extinction dynamics by performing a survival analysis using a Cox proportional hazards regression model with the R package "survival" (Supporting Information Tables S1, S2). The model included biotic regime, temperature regime, and species as fixed effects. Biological replicate strains (per species) were treated as random effects. We also included a censor variable for populations that had not gone extinct by the end of the experiment. Note that an extinction event here was defined as cell numbers of a population declining below the detection limit of the flow cytometer. We treat extinction as an event occurring on the replicate level in each individual treatment.

Statistical analysis of growth trajectories and local adaptation

We analyzed the growth of the surviving replicates as assayed at the end of the experiment in the reciprocal transplants using analyses of variance within a mixed effects model (package nlme, version 3.1-131). To specifically test whether samples had locally adapted to their selection temperature without risking over parameterizing the mixed model, we built a separate mixed effects model using data where the assay salinity was the same as the selection salinity, thus focusing on the temperature dependence of growth rates in the samples' selection salinity.

Statistical analysis of decomposed samples

To analyze whether invaders and residents developed a dependence on each other rapidly in the short term (2 weeks), we used the growth rate data of samples that had been grown in co-culture for 2 weeks and then decomposed, and compared these growth rates to those of the same species that had been in mono-culture for the same period of time, at the same temperature and salinity via a *t*-test.

For the long term-responses, we were interested in the fold-change in growth rate after samples evolved in co-cultures had been decomposed into mono-cultures, and therefore calculated a ratio of growth rate after decomposition over growth in co-culture. This ratio was then used as the response variable in a mixed model which also included species and temperature as fixed effects.

Statistical analysis of other phenotypic traits

In order to estimate the effect of the selection regimes (biotic scenarios, temperature, and salinity) on cell size and total biomass we fitted a mixed model with the full interaction of the parameters species (*Chlamydomonas* or *Ostreococcus*), assay salinity ("home" for the assay salinity being equal to the focal species' *selection* salinity, and "away" for assay salinity being different from the focal species' selection salinity), biotic regime (invading or colonizing), and selection temperature (22°C, 26°C, 32°C, fluctuating).

Results

Invader evolutionary responses—reduced extinction rates, higher growth rates

Extinction rates in a new salinity were lower in invading than colonizing phytoplankton. *Chlamydomonas* invading the "salt water" (SW) environment were two-thirds less likely to go extinct than *Chlamydomonas* colonizing the same environment, though extinction was overall lower than reported for other species of *Chlamydomonas*. For *Ostreococcus* invading a freshwater (FW) environment, the likelihood of extinction was halved compared to colonizing *Ostreococcus* (survival analysis: $z = -2.90$, $p = 0.0037$). Extinction events occurred within the first 70 generations ($z = -4.13$, $p = 3.6 \times 10^{-5}$; Fig. 2; Supporting Information Fig. S7), with no further extinctions after 100 generations. After the first ~ 70 generations, population sizes in all surviving invader cultures also stabilized and were no longer statistically different from colonizers ($F_{1,2} = 1.72$, $p = 0.32$). Growth rates of the surviving cultures (Supporting Information Fig. S3) also stabilized within the first ~ 70 generations, were overall higher for invaders, and showed that the evolutionary strategies differed between colonizers and invaders.

Invader growth rates were marked by an initial increase followed by a decline and then a leveling out of growth rates. This is a common pattern in microalgae-facing environments that are not immediately toxic, such as mildly elevated CO_2 (Schaum and Collins 2014). Colonizer responses were more akin to those seen under evolutionary rescue, where growth first declined and then recovered, albeit never to levels seen in samples growing in their original salinity.

Extinction and environmental quality

Extinctions were overall lowest in the favorable environments, that is, under mild warming at 26°C, and in the fluctuating environment (survival analysis: $z = -1.22$, $p = 0.043$; Supporting Information Fig. S7). This is in line with theory predicting that evolutionary potential should be high in good-quality environments leading to increased or unchanged fitness, and that extinction risk should be low in environments that fluctuate predictably (Ashander et al. 2016).

Invaders evolve broader salinity and temperature tolerance

For this part of the experiment, colonizers were assayed in mono-culture. Invaders were assayed while in co-culture. These conditions reflect the biotic environment they were selected in. When colonizers were transferred back into their ancestral salinity, their growth rates were the same as or lower than they had been in that same salinity before evolution in a novel-salinity environment (Figs. 1, 3). A reduced ability to grow in the ancestral environment strongly suggests a degree of local adaptation to the selection environment. Growth rates

of invaders were overall higher and did not decrease significantly upon being transplanted back into their ancestral salinity. This would indicate a lower degree of local adaptation to the selection environment, and point toward the evolution of tolerance for a larger range of different salinities.

Invaders and colonizers also differed in their responses to temperature ($F_{9,62} = 18.8$, $p < 0.001$): invaders again outperformed colonizers. This pattern was exacerbated under mild warming (26°C), where invader growth rates were on average 1.3 times higher than colonizer growth rates (Tukey post hoc, $p < 0.001$), and under the fluctuating treatment, with an average fold increase of invaders vs. colonizers of 1.2 (Tukey post hoc, $p < 0.001$). Growth rates were lower under environmental deterioration at 32°C, but this was less pronounced in invading species (Fig. 4).

Phenotypic traits of colonizers and invaders

Once extinction is no longer one of the main mechanisms driving responses, the phenotypes of the surviving cells will dictate their growth rates and interactions with other species in a given environment. To integrate the responses into ecosystem (Dutkiewicz et al. 2015) and individual-based models (Beckmann et al. 2019), and to better understand the dynamics in laboratory experiments (Denman 2017), knowledge of (variance in) phenotypic traits and organismal biology is needed. Here, colonizers and invaders evolved strikingly different phenotypes.

Colonizers had overall higher intracellular ROS levels, and did n detoxify ROS less efficiently than invaders. In unfavorable environments with high temperature and changed salinity, intracellular ROS production was higher, and ROS tolerance was impeded (Fig. 4).

Cell size overall declined with selection temperature regardless of selection regime or species. *Ostreococcus* was more reactive to temperature than *Chlamydomonas* overall, and whether the species was invading or colonizing also had an impact on the focal species' cell size (Fig. 4), with smaller invaders than colonizers. The cell size of *Chlamydomonas* was more likely to change in response to a resident species than the cell size of *Ostreococcus*, with *Chlamydomonas* cells up to 1.43-fold smaller after evolution invading the marine species in salt water than after colonizing salt water on their own (Fig. 4).

While we cannot disentangle the relative contributions of the individual species to NP rates in the co-cultures (NP, i.e., rates of photosynthesis after respiration has been accounted for), NP per gram carbon of evolved co-cultured samples was on average 13% higher than expected from the NP of the same two species at the same salinity in mono-culture (Fig. 5), in line with over-yielding observed in other species (Bestion et al. 2020). The same pattern emerged when we assayed the same species at the same salinity after physically separating ("decomposing") a former mixed culture of *Chlamydomonas* residents and *Ostreococcus* invaders into mono-cultures (Supporting Information Tables S11–S14).

Experimental community decomposition

Experimentally separating ("decomposing") the evolved co-culture samples into mono-cultures yielded insights into how strongly the invaders had adapted to the presence of the resident species, and what effect the invader had on the growth of the resident. In samples that had only lived in co-culture for two transfers, or just below 20 generations, growth after decomposition was indistinguishable from growth in mono-cultures at the same salinity and temperature (Supporting Information Fig. S6; Supporting Information Table S15). In contrast, in samples that had lived in co-culture for ~ 200 generations (Supporting Information Fig. S8), growth of the invading species when assayed alone in the selection salinity was reduced by up to 30% compared to when assayed in co-culture. The growth of invaders was also lower than in the same species evolved in mono-culture throughout at the same salinity/temperature regime. Of the resident species, *Ostreococcus* selected in co-culture with *Chlamydomonas* showed evidence of a marked decrease in growth when the invading *Chlamydomonas* was removed. Resident *Chlamydomonas* grew faster when the invading *Ostreococcus* population was removed, with no significant effect of temperature on this pattern.

In the decomposed samples, patterns in net primary production of former invaders mirrored the patterns found in growth rates: invaders always photosynthesized less after decomposition than the same species evolved in mono-culture in the same selection salinity. High photosynthesis rates in formerly invaded *Chlamydomonas* were in line with higher growth rates in formerly decomposed *Chlamydomonas* (Fig. 5; Supporting Information Tables S11–S14). The resident species *Ostreococcus* photosynthesized more after decomposition than when evolved in mono-culture—but grew more slowly. The higher NP rates were, at least for the duration of the assay, not directly channeled into growth, indicating that the presence of other species may explain hitherto often observed but poorly explained variations in growth rates in more complex systems (Wolf et al. 2019; Collins and Schaum 2021). We found that samples with the highest surplus NP or least increase in growth had a tendency to have higher lipid storage (approximated by Nile Red dye fluorescence, Supporting Information Fig. S9). Similar responses including high rates of NP but suppressed growth can be achieved by merely spiking *Ostreococcus* and *Chlamydomonas* cultures with water conditioned by the other species (Supporting Information Fig. S10).

Discussion

Rapid adaptation to a novel salinity or the evolution of salinity tolerance are major driving forces in determining the distribution and phenotypic characteristics of phytoplankton communities (Larson and Belovsky 2013; Rengefors et al. 2014; Godhe et al. 2016). Changes in salinity, particularly in combination with elevated temperatures, have the

potential to impact the phenotypic characteristics of phytoplankton species, the communities they populate, and the role of phytoplankton species on aquatic food webs and global nutrient cycles (Rengefors et al. 2014; Godhe et al. 2016; Godhe and Rynearson 2017). We studied whether the biotic context, that is, the presence of a resident species, affected evolution to a novel salinity in phytoplankton, and whether such effects depended on the thermal quality of the environment. In particular, we studied extinction rates, adaptation to novel environments through changes in growth rate, and the traits potentially related to this adaptation (i.e., cell size, NP, ROS tolerance, and production). We found that invaders had strikingly different evolutionary response to a novel salinity than colonizers, with lower extinction rates, and shallower decline in growth rates in the surviving samples. While it was outside the scope of our study to test whether this holds for species pairs other than the one chosen here, we found that the responses were strikingly similar for the species tested and were conserved across environments differing in their quality. Specific traits were associated with invader success, although it remains to be seen whether those traits made the invaders more successful or evolved as a consequence of the selection regime.

We propose that ROS tolerance and production might be one key mechanism explaining differences in colonizing vs. invading species. Rapid evolution to a novel salinity has been proven before in species colonizing a novel salinity on their own (Latta et al. 2012; Lachapelle et al. 2015; Rescan et al. 2020), but evolution as a single species (or clone thereof) might not be a common ecological scenario, as species are not likely to arrive in a new environment and find it unoccupied. Here, species *invading* a new salinity were less likely to go extinct and evolved high tolerance to both fresh and salt water, especially under mildly elevated or rapidly fluctuating temperatures. Invaders had higher survival and growth rates than colonizers, and were also characterized by overall smaller cell size, a tendency to store more lipids, and, prominently, lower ROS production, as well as higher ROS tolerance. ROS are a natural by-product of cellular metabolism, but can damage the cell in high quantities (Shuryak and Brenner 2009). Therefore, higher tolerance toward or lower quantities of ROS may infer a fitness benefit (Lindberg and Collins 2019). Our results add to the body of literature suggesting that under warming and increased climate variability, invasions through small, warm-adapted, more generalist (Mellin et al. 2016; Sriswasdi et al. 2017) taxa with intrinsically elevated metabolic and growth rates may become more frequent, but it remains an open question whether generalist traits enable successful invasion or whether organisms evolve to have more generalist traits as a consequence of invasions. Regardless of the underlying mechanism, this "tropicalization" (Vergés et al. 2014, 2016) may have nigh-unpredictable knock-on effects on aquatic ecosystems as a whole. As changes in fitness, cell size, and metabolic activity are often linked (Brown et al. 2004; Marañón 2015), it stands to reason that one possible mechanism for higher invader fitness in our invader samples lies in their ability to rapidly down-regulate cell size (Key et al. 2010; Malerba et al. 2018; Malerba and Marshall 2019). This in turn might be what is giving rise to their ability to better handle ROS (Lindberg and Collins 2019; Malerba and Marshall 2019)—the smallest cells had the highest fitness and were better able to detoxify ROS.

Strategies that increase fitness can vary over time (Lenski 2017*b*) and when there are multiple genotypes in a population, evolutionary trajectories, as well as the traits evolved will depend on the environment as well as the genotype (Lenski 2017*a*). Still, *Ostreococcus* selected in co-culture with *Chlamydomonas* showed evidence of a marked decrease in growth when the invading *Chlamydomonas* was removed, suggesting that interactions established rapidly and were mutualistic or facilitating in nature. *Chlamydomonas*, when *Ostreococcus* were removed, did *not* show a marked decrease in growth, making it seem likely that the fact of being an invader had direct phenotypic and fitness consequences regardless of the nature of the interaction. With just one pair of species tested, we cannot say whether these differences are species-specific or whether the "easier" invasion (from marine to freshwater) is more likely to be based on facilitative strategies than the invasion from freshwater into a marine salinity. While not investigated here, it is possible that other, indirect, interactions also had an impact on the way in which resident and invader species influenced each other. For example, some brackish and marine phytoplankton species can locally engineer the salinity of the surrounding medium (Figler et al. 2019). Our samples were well-mixed and bulk salinity as measured in representative samples did not change significantly, but small-scale changes of salinity in the presence of *Ostreococcus* may have allowed greater survival of the invading *Chlamydomonas*.

However, this would not explain the better performance of invading compared to colonizing *Ostreococcus*. Potentially, invaders were benefitting from the residents' bacteria and thereby increased micronutrient and vitamin availability. *Ostreococcus*, in particular, can easily take up organic carbon sources to supplement growth (Listmann 2021). There might be a greater variety of carbon sources if there is not just one, but two sets of associated bacteria remineralizing nutrients in the populations.

Fluctuating selection regimes and biotic interactions impose complex selection pressures and barring further analyses—for example, on the level of the transcriptome—we cannot with certainty elucidate the exact mechanism that allows for the evolution of these strikingly different phenotypes in invasion vs. colonization scenarios. Understanding the impacts of environmental change over evolutionary timescales will require that we experimentally investigate the mechanisms underlying the differences between colonizers and invaders, the direct effects of rising temperatures on

species interactions, and the indirect reciprocal feedbacks between ecological and evolutionary dynamics (Gravel et al. 2010; Urban 2013; Fussmann et al. 2014).

Data availability statement

All data and R code are available on Zenodo at 10.5281/zenodo.6884040.

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Conflict of Interest

None declared.

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