Effects of Community Richness and Competitive Asymmetry on Protozoa Evolution in *Sarracenia purpurea* Leaves

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**abstract:** Predicting evolution in natural systems will require understanding how selection operates in multispecies communities. We predicted that the amount that traits evolve in multispecies mixtures would be less than the amount that would be predicted from the additive contributions of the pairwise interactions and that subordinate species will be more likely to evolve in competitive systems than dominant species. We conducted an experimental test of these predictions using a guild of protozoans found in the water-filled leaves of the pitcher plant *Sarracenia purpurea*. The response to selection did not significantly change as we increased richness from monocultures to two- and four-species mixtures. In accordance with our second prediction, subordinate species demonstrated greater growth in competition after selection than before, while dominant species generally showed no response to selection. Monod-type experiments to determine minimum resource levels found that the dominant species had much higher resource requirements than the subordinate species and that the minimum resource requirements evolved to be higher in the subordinate species. Importantly, these results suggest that subordinate species evolve to become more similar to dominant species, which may involve resource use convergence. Our findings and other recent works suggest that community diversity can affect evolution in surprising ways that warrant further investigation.

**Keywords:** competition, convergence, resource use, diversity.

Introduction

Our understanding of how competing species evolve has been built from knowledge of genetic variation, selection, and trait change in single populations, usually in simple one- or two-species scenarios. The classic prediction from these simple scenarios is niche divergence, resulting in lower inter- than intraspecific competition for both species and promoting coexistence (e.g., Macarthur and Levins 1967), although convergence may also be expected under certain circumstances (Abrams 1987; terHorst et al. 2010). However, few studies have evaluated evolution with more than two competitors, and understanding evolution in a multispecies “community context” has become one of the next big questions in evolutionary ecology (Barraclough 2015; terHorst et al. 2018). Evolution in communities will be particularly difficult to understand because of two conditions inherent in multispecies systems: (1) the simultaneous action of multiple agents of selection and (2) the indirect effects or loops of interactions in complex systems.

We can use our current knowledge of simple community dynamics to make some first-order predictions about evolution in species mixtures. These predictions assume that the strengths of ecological effects will be roughly proportional to the evolutionary responses. For example, indirect effects among competitors are thought to often be positive, as strong direct competition can suppress other potential competitors (Yodzis 1988; Stone and Roberts 1991; Miller 1994). This might suggest that as more competitors are added to a system, the sum of the effects of competition will be less than additive, which could reduce the expected selection for traits associated with competition. As a result, individual species may respond to an amalgam of the effects of several other species rather than responding to the individual species. This has the potential to reduce overall interactions in a community relative to the sum of potential pairwise interactions (e.g., Holt 1977; Miller and Travis 1996; see review in terHorst et al. 2018).

Intraspecific interactions can also influence selection, but the strength of selection due to intra- and interspecific interactions may be correlated with relative abundances (e.g., Abrams 1980). For example, rare species may primarily
experience interspecific interactions, while dominant species may experience greater intraspecific interactions (Aarsen 1983; Abrams 1986). Miller et al. (2014) suggested that such frequency-dependent selection may explain why subordinate species in a protozoa community improved in interspecific competitive ability over successional time, while dominant species either did not change in competitive ability or became poorer interspecific competitors (see also Aarsen 1983). If subordinate species are more likely to improve their performance through evolution, it may also decrease their probability of going extinct and act to promote species diversity in communities. Similarly, if intra- and interspecific effects become more equal, then the reduced fitness differences will promote coexistence or reduce rates of competitive exclusion (Chesson 2000; Adler et al. 2007).

These simple predictions led us to two general hypotheses. First, selection in competitive environments will be less than the sum of the selection from individual competitors, as competitors interact to reduce net effects. Second, less abundant, potentially subordinate species might be expected to evolve greater interspecific competitive ability than more abundant, dominant species, as less abundant species may experience greater selective pressure to increase interspecific competitive ability.

We present here an experimental test of these novel hypotheses, using a guild of four protozoans found in the water-filled leaves of the carnivorous plant *Sarracenia purpurea*. *Sarracenia purpurea* inquilines have been frequently used as a model system in ecology and evolution (e.g., Srivastava et al. 2004; Miller and Kneitel 2005; Canter et al. 2018; Miller et al. 2018). These leaves, while ephemeral, can provide relatively long-term habitats where competition (e.g., Miller et al. 2014) and predation (Kneitel and Miller 2002) have been shown to be important for relatively short-lived bacterivores, including protozoa. We conducted experimental evolution studies with four species of protozoa that we selected for growth in monocultures, two-species mixtures, and four-species mixtures, and we measured a variety of growth responses before and after selection (fig. 1). We made two specific predictions. First, selection will not increase in an additive manner as richness increases in mixtures. Second, poorer competitors in mixtures will have a greater evolved increase in interspecific competitive ability than the superior competitors. We believe that this is one of the first examples using experimental evolution in a natural community to understand evolution in multispecies communities.

**Methods**

**Overview**

At its core, the design is a typical selection experiment, measuring growth traits of species when grown in different competitive environments before selection (“preselection,” fig. 1.1), then allowing each of the species to undergo selection in different competitive environments to create selected lines (“selection,” fig. 1.2), then measuring the growth traits of each of these selected lines again in the same original environments (“postselection,” fig. 1.3). The experiment is necessarily complex because we want to compare the evolution of four species of protozoa when selected in five different environments for their growth in six different competitive environments (different species combinations), for a total of 120 final treatment groups (fig. 1). Finally, the entire experiment was replicated in five blocks conducted sequentially in time.

**Experimental Conditions**

We conducted all of the experiments in newly opened, sterile leaves of *Sarracenia purpurea* in a greenhouse unless otherwise noted. In local natural populations, these ecosystems depend on the insect prey captured by the leaf for nutrients and energy. The prey are broken down by a bacterial community (archaeal abundances in *S. purpurea* fluids are negligible; e.g., see Canter et al 2018), and this bacterial community is consumed by a suite of bacterivores, including several species of protozoa and rotifers. The bacterivores are in turn prey for filter-feeding larvae of an obligate mosquito, *Wyeomyia smithii*. These communities are relatively easy to manipulate and allow experiments with replicated entire ecosystems. For this study, we maintained plants in a temperature-controlled greenhouse; the five replicates of the experiment were blocked across time and varied somewhat in temperature and day length. The plants come from seeds obtained from the Apalachicola National Forest in 1998 that were germinated and grown in the greenhouse. Although there is airflow from outside the greenhouse via the evaporative cooling system, both novel prey and invasive protozoa were rarely found in greenhouse-raised plants. Because our work centers on protozoa competition, we created communities without predatory mosquito larvae.

**Microbial Sources for All Experiments (Fig. 1)**

We used a common bacterial broth for all of the experiments described in this study (see also Canter et al. 2018). Briefly, we obtained fluid from 50 leaves in each of four different natural populations of *S. purpurea* in the Apalachicola National Forest. We pooled the fluid, then passed it through a series of sterile filters with decreasing pore size, the last of which was 0.5 μm. We then divided the fluid into one hundred 1.5-mL microcentrifuge tubes and cryopreserved the bacteria using methods from Kerckhof et al. (2014), modified to use a lower concentration of 2% dimethyl sulfoxide.
Before we started any part of the experiment (fig. 1), a tube would be thawed and pelleted at 7,000 g for 15 min at 22°C to remove the DMSO. We then resuspended the pellet in 1,500 mL of sterile water mixed with 300 mg of dried ground ants (*Solenopsis invicta*), a common prey of *S. purpurea* leaves in north Florida, and then gently mixed for 24 h. This bacterial broth was used for all of the experiments shown in figure 1.

To obtain protozoa, we surveyed samples taken from approximately 300 leaves from three natural populations of *S. purpurea* in the Apalachicola National Forest and ultimately selected five species for these experiments. These are *Colpoda cucullus* (CC), *Colpoda steinii* (CS), *Tetrahymena* sp. (TA), *Poterioochromonas* sp. (PS), and *Colpidium* sp. (CM). For each of these species, we created 8–10 stock cultures, each derived from a small initial number of cells (1–10) obtained from a unique leaf. We maintained these stock cultures in a growth chamber at 27°C with 12D:12L cycles, with ants as a food source for microbial prey; these stocks were the source lines for all of the experiments in this study.

One of the five species, CM, was used only as a non-evolving control. We grew CM in 100-mL cultures, then divided these into small aliquots of 1 mL, which were mixed with 0.05 mL of DMSO and stored at −80°C. When needed, we defrosted frozen aliquots in a 27°C water bath, pelleted at 7,000 g for 15 min at 22°C, then reconstituted the pellet in bacterial media to initiate fresh cultures. For each of the other protozoa species, to increase potential genetic diversity, we combined 0.1 mL from each of the 10 stock lines into a single monoculture to create a mixed-line culture at the beginning of each of the five blocks.

To initiate experiments in all portions of this project, we gently mixed appropriate protozoa monocultures, then directly counted moving cells in 0.1 mL in a Palmer counting...
levels as the lowest dilution that contained any living cells. We then placed from less than 10^2 cells/mL in the most diluted to approximately 10^10 cells/mL in the standard broth. We then serially diluted (1 to 10, this is the original used to create the broth. These are reported from the most dilution gradient of 10 levels of microbes and the ground ants mixture. In rare cases when cell densities were very high, we diluted cultures, then repeated the methods above to initiate the culture.

Quantifying Growth Before Selection (Fig. 1.1) and After Selection (Fig. 1.3)

We quantified population traits for each line of each species’ before selection and after selection in two ways: quantifying growth rates in different mixtures and performing experiments to determine minimum resource requirements. First, we grew all of the lines in monocultures, all of the two-species mixtures, and the four-species mixture by initiating cultures as described above in new S. purpurea leaves in the greenhouse (fig. 1.1). After 7 days, we estimated population abundances by gently mixing the contents of each leaf and counting 0.1-mL samples as described above. Seven days allows for approximately 21 generations; this period was deemed sufficient to quantify growth and competitive interactions based on our prior work showing that most populations reach maximum population sizes at around 4–5 days (Miller et al. 2014; Canter et al. 2018). In rare cases where cultures failed to establish, they were dropped from the analyses. We assumed that any evolution that occurred over these 7 days was minimal relative to the potential changes that might occur over the 12 weeks of selection.

Second, we conducted Monod-type experiments to quantify minimum resource requirements for growth of all preselection and postselection lines (similar to an R value). To initiate these experiments, we serially diluted the standard bacterial broth with sterile water nine times to create a dilution gradient of 10 levels of microbes and the ground ants used to create the broth. These are reported from the most diluted (1) to the most concentrated (10, this is the original standard broth). Flow cytometry confirms that this creates an exponentially increasing range of bacterial cell density from less than 10^6 cells/mL in the most diluted to approximately 10^9 cells/mL in the standard broth. We then placed 10 mL of all dilutions of in 50-mL microcentrifuge tubes, which we then inoculated with each of the pre- and postselection lines, again using an estimated 50 protozoan cells per tube and two replicates of each dilution and line. These cultures were placed in a growth chamber at 26°C with 12L:12D cycles for 4 days, then the protozoa in all tubes were censused as described above. We defined minimum resource levels as the lowest dilution that contained any living cells.

Preselection Methods (Fig. 1.1)

For each of the five blocks of the experiment, we identified 85 new leaves on separate S. purpurea in the greenhouse and randomly assigned leaves to 16 treatments with five nested replicates of each treatment in each block. Treatments were initiated with 10 mL of bacterial broth as described above and included all five species (including the control CM) grown in monoculture, all 10 possible two-species mixtures (again, including CM), and a four-species mixture (without CM). A small number (<5%) of nested replicates were lost when either inoculations failed to establish or leaves died or failed to hold their water. The protozoa grew for 7 days, then we gently mixed the content of each leaf, collected the fluid with a thin pipette, and determined the protozoa abundances as described above. The nested replicates of each treatment were averaged within blocks before analyses to calculate competitive effects and the selection response (SR) metrics (see below).

Selection Methods (Fig. 1.2)

Using our four target protozoa species, we created all possible monocultures (4) and pairwise mixtures (6) and a four-species mixture, resulting in 11 selection treatments. Note that this results in five lines created for each of the four species: one from a monoculture, three from all of the possible pairwise mixtures, and one from the four-species mixture.

We identified 33 new leaves on separate plants in the greenhouse and randomly assigned one of the 11 selection treatments described above to each leaf, each treatment having three nested within-block replicates. We initiated these treatments as described above with the standardized bacterial broth and inoculates of an estimated 50 cells of each appropriate species. To account for evaporation and prey consumption, 1.5 mL of sterile water and two dead ants were added every 4 days. Over the next 12 weeks (more than 250 generations for protozoa), the water contents of the leaf were removed entirely every 2 weeks. We censused the protozoa microscopically as described above, adjusted the volume to 10 mL, and returned the sample to its original leaf. On rare occasions when leaves were found to be contaminated, one of the other two nested replicates was used to reinitiate the culture. If a leaf lost integrity and could no longer contain 10 mL, then the contents were placed in a new leaf.

Postselection Methods (Fig. 1.3)

For each block of the experiment, we combined the nested replicate leaves for each of the 11 treatments in the selection experiments to give a single replicate. We then used the combined sample to resolate each species from each treatment, with four species and five lines per species resulting in
20 different selected lines in each of the five blocks of the experiment (fig. 1.3). We grew each of these 20 lines for 7 days in all combinations of alone (20 cultures) and with the control (nonevolving) CM (20 cultures). We also grew each line in the four-species mixtures, where the other three species came from the four-species selection treatment (20 cultures). Finally, we grew each line in pairwise competition with the appropriate line with which it evolved in competition (6 cultures). For example, the CS line selected in competition with TA was now tested again against TA that was selected with CS. In sum this represents 66 competition trials, each of which was replicated twice within blocks. The postselection competition experiments were conducted with the same 7-day protocols used in the preselection experiments (fig. 1).

Analyses

Both the preselection and postselection experiments determined protozoa cell number per 0.1 mL at 7 days as an estimate of population growth rate. To quantify the preselection strength of competitive interactions, we expressed the preselection growth in mixtures of each species as a function of its growth in monoculture as in Miller et al. (2014):

\[
\ln(\text{no. mixture} + 1) - \ln(\text{no. monoculture} + 1) \\
\ln(\text{no. monoculture} + 1)
\]

If species are competing, this value goes from −1 (complete suppression) to 0 (no interaction). One-way t-tests were used to evaluate whether each measure was significantly less than zero, which would indicate significant competitive suppression.

To quantify differences between preselection and postselection abundances, we compared ln-transformed abundances for each species after selection, relative to its preselection abundance in the same environment:

\[
\text{SR} = \ln(\text{no. postselection} + 1) \\
- \ln(\text{no. preselection} + 1).
\]

Values not significantly different from zero indicate no effect of selection. Values greater than one suggest that populations evolved to grow faster after the 12 weeks of selection, while values less than one suggest evolution toward slower population growth.

We used the lines selected in different environments but grown in the same postselection environment to address two broad questions. First, within each species, did selection act to significantly increase or decrease growth, and was this response to selection different among selection environments? Second, did species differ in their general response to selection? We evaluated the first questions by determining confidence intervals for SR values within species, then determining whether these limits included zero. We evaluated the second question using analysis of variance (ANOVA). All analyses were conducted using the R environment (R Core Team 2019).

We compared the minimum resource level experiments (Monod) among species before selection and for each selected line within species after selection. First, ANOVA was used to determine whether the species differed in preselection minimum resource levels, followed by a post hoc all-pairs comparison using Tukey’s honest significant difference (HSD) test. For the postselection lines within each species, 95% confidence intervals were used to determine whether each line differed from the preselection minimum resource level. We used a similar test with the means of each selected line within species to determine whether the minimum resource levels for all of the selected lines within a species differed from the preselection minimum resource levels. Data for this study have been deposited in the Dryad Digital Repository (https://doi.org/10.5061/dryad.bzk18990; Miller et al. 2022).

Results

The preselection (fig. 1.1) strength of interactions (table 1) shows that the four species had very different competitive effects and responses. The growth of CA and CC in mixtures was significantly less than their growth in monoculture in most combinations. Conversely, these two species generally had small competitive effects on other species. TA and PS, however, were much less affected by competition with other species while having generally strong effects on others (table 1). Similarly, the four-species mixtures over the 12 weeks of selection demonstrated clear differences in abundance of the four species, with PS dominating numerically (fig. 2). PS and TA were better pairwise competitors in preselection experiments and had high abundances when grown in mixtures during selection and will be referred to as dominant species. The other two species, CS and CC, were relatively poor competitors in preselection experiments and were several orders of magnitude less abundant in mixtures and will be referred to as subordinate species.

Evolution of Growth in Monoculture

We compared growth in monoculture before selection and after selection for different selected lines within each species. As a group, the CS lines had SRs significantly greater than zero (fig. 3A–3D), indicating greater population growth after selection. However, the individual lines were not necessarily greater than zero, and there were not any differences among the selected lines within a species. Similarly, as a group, SRs for the CC lines were also significantly greater than zero (fig. 3B), but with no significant
differences among lines. In particular, the lines selected in monoculture for either subordinate species did not exhibit higher SRs than lines in any other environment.

In contrast to the subordinate species, lines selected in monoculture for the two dominant species, TA and PS (fig. 3C and 3D, respectively), showed no evidence of any response to selection as a group or within selected lines. TA showed virtually no response to selection, while PS showed an nonsignificant trend to have lower growth after selection, as exhibited by SRs lower than zero.

**Evolution of Growth in Pairwise Mixtures with the Nonevolving Colpidium**

Because it is difficult to quantify pairwise evolution when both species may be evolving, lines were also grown in competition with the nonevolving control species, CM. The patterns within and among species were very similar to the studies in monocultures (fig. 3E–3H). Both of the subordinate species, CS and CC (fig. 3E, 3F), had significant positive SRs across all selected lines, with SRs generally greater than zero. However, again, there were no significant differences among any of the lines generated in different selection environments within species. The dominant species showed no significant group effect of the lines having a selective response greater than zero. However, in this case TA (fig. 3G) showed significant differences among lines, with generally higher SRs for the lines selected with CC and CS and lower growth in the lines selected with PS.

**Evolution of Growth in the Four-Species Mixtures**

All lines were also grown in the four-species mixtures before and after selection. In this case, note that all of the species

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Table 1: Effects of competition in different species mixtures expressed as a proportion reduction in monoculture growth

| Species responding | CA | CC | TA | PS | Four species |
|--------------------|----|----|----|----|--------------|
| CA                 | . . | -.236** | -.517** | -.150 | -.542* |
| CC                 | -.027 | . . | -.446** | -.446* | -.636* |
| TA                 | -.077** | -.058* | . . | -.040 | -.122* |
| PS                 | -.088 | -.068 | -.179 | . . | -.211* |

*Note: Values represent the effect of different species or species mixtures (columns) on each species (rows), expressed as a reduction in abundance of a species in competition relative to its abundance in monoculture using ln-transformed values. A value of −1 would indicate complete suppression (zero growth) in competition, and a value of 0 would indicate no effect of competition. CC = Colpoda cucullus; CS = Colpoda steinii; PS = Poterioochromonas sp.; TA = Tetrahymena sp.*

\* \( P < .05 \)

\** \( P < .01 \)

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Figure 2: Average abundances of the four protozoa species during the 12 weeks of selection in four-species mixtures in Sarracenia purpurea leaves. CC = Colpoda cucullus; CS = Colpoda steinii; PS = Poterioochromonas sp.; TA = Tetrahymena sp.
Figure 3: Selection responses for all of the lines within species when grown in different environments. Subordinate species (Colpoda cucullus [CS] and Colpoda cucullus [CC]) are on the left, and dominant species (Tetrahymena sp. [TA] and Poterioochromonas sp. [PS]) are on the right. The x-axis indicates the original selection environment for the focal species, with separate rows for each postselection growth condition. Boxes show the 50% confidence intervals of the data and the mean for lines when grown in monoculture (A–D), with the nonevolving competitor Colpidium (E–H), and in four-species mixtures (I–L); whiskers give the reasonable maximum and minimum values, excluding outliers (R Core Team 2019). Statistical comparisons are shown at the lower right in each graph. The first statistic asked whether the overall response of the species to selection was significantly different from zero, while the second asked whether there were significant differences among selected lines within a species (*P < .05; **P < .01).
had undergone selection, so it is difficult to distinguish the direct effects of evolution of one species from the indirect effects of evolution of others in the mix. However, we found no clear pattern (fig. 3L) in the effect of selection on protozoan growth in community context. Neither groups nor individual lines yielded SRs significantly different from zero, and there were no significant differences among lines.

**Evolution of Growth in Pairwise Mixtures**

The two-species treatments are different from experiments with the nonevolving CM, as both species in each pair may evolve. We ranked the species by their relative abundance in the four-species mixtures (fig. 2) as a measure of dominance (PS > TA > CC > CA), then compared the SRs of the dominant species with those of the subordinate species in all possible pairs (fig. 4). Figure 4 shows the combined response of both species in each pair; for example, if both species evolved to utilize different resources or to take up resources faster, then both would evolve to do better in competition and appear in the upper-right quadrant. This is seen with CS and CC, as both exhibited increased positive growth after selection in competition with each other, whereas the CC-PS pair shows that CC evolved to do better in competition, while PS had lower population growth following selection. Overall, this figure reveals a pattern that is similar to that seen in the monocultures and with CM (fig. 3). CS was the most subordinate species and always had larger populations sizes after selection (positive SR values), with CC, the other subordinate species, showing similar patterns (fig. 4). TA, a dominant species, mostly demonstrated no response to selection or a decreased growth following selection. The most dominant species, PS, demonstrated consistently poorer performance after selection. While these results are generally consistent with the other treatments, again note that within any pair of species, the apparent increased competitive performance of one species may reflect, in part, a decreased competitive performance of the other species.

**Evolution of Minimum Resource Use**

The preselection lines for each species and all of the postselection lines were each grown over a gradient of resource availability to determine the minimum resource levels that would support growth. Before selection, the

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**Figure 4:** Selection responses (SRs) from the evolution in species pairs. Each pair of crossed lines represents the SR values for two species, with the more subordinate (less abundant) species on the abscissa. Lines are determined by the first and third quartiles of the SRs (similar to boxplot hinges) for species when competing with the other species in the pair; the lines cross at the average values. Positive values correspond to increased population growth after selection. CC = *Colpoda cucullus*; CS = *Colpoda steinii*; PS = *Poterioochromonas* sp.; TA = *Tetrahymena* sp.
minimum resource levels were found to be significantly different among species ($F_{3,28} = 78.4, P < .001$), with CS surviving on very low resource levels (resource level 1.6), followed by the other subordinate species, CC (4.6). The two dominant species, TA and PS, required much higher resource levels to keep populations from going extinct (6.3 and 8.4, respectively). Post hoc tests showed that all of the species were significantly different from each other in minimum resource levels (Tukey’s HSD, all $P < .007$).

The minimum resource levels for each species were then compared before and after selection (fig. 5). Within each species, ANOVA found that there were no significant differences among lines from different selection environments. However, collectively all of the selected lines for CS and those for CC evolved to have greater minimum resource needs (fig. 5A, 5B), while TA and PS did not (fig. 5C, 5D), according to 95% confidence intervals. In particular, CS that evolved in monoculture and CC that evolved with TA both demonstrated significant increases in resource needs, while both CS and CC had significantly greater minimum resource requirements across all selected lines within a species.

Discussion

These experiments allow us to test our two predictions. First, does selection in competitive environments appear to differ as the number of species increases? In particular, is there evidence that selection may not be “additive,” such that the response to selection either increases or differs in some way with an increased number of competitors? Second, do subordinate species evolve more quickly than dominant species? This is predicted if selection is frequency dependent, such that less abundant species may experience greater selective pressure to increase in interspecific competitive ability.

Our expectation for our first prediction is that interactions among shared competitors will reduce overall expected competitive effects (e.g., Stone and Roberts 1991; Miller 1994) and selection, leading to a diffuse and smaller response to selection as species number increased. We found that there were no significant differences among selected lines for any of our species, and there were not any notable trends in the data. This lack of a difference among responses in one-species, two-species, and four-species mixtures could be due to several factors. First, it is consistent with our prediction of reduced effects because it is less than would be expected from the additive effects of the individuals species: more species should have resulted in stronger selection and a greater evolutionary response. This could be due to diffuse interactions, where different competitors interfere with one another such that indirect effects reduced the expected direct effects (e.g., Meyer and Kassen 2007). It could also be due to shifts in the types of bacteria used by different protozoa species. For example, Canter et al. (2018) found that feeding by different protozoa species in *Sarracenia* results in different bacterial communities; interactions between the protozoa themselves could be driven by these changes in the bacterial identity. An alternative explanation is that the species were adapting to novel abiotic conditions created by our experiments, resulting in very similar responses to selection in all environments. We know that some of our treatments produced significant competition (e.g., table 1) and that these effects persisted throughout the 12 weeks of selection. But the 12 weeks of the selection period (fig. 1.2) could also have produced environmental conditions such as low oxygen or high pH that overwhelmed the effects of competition.

In accordance with our second prediction, we found strong and consistent differences among dominant and subordinate species, with subordinate species demonstrating a greater growth in competition after selection than before, while dominant species show no response to selection (figs. 3, 4). In fact the most dominant species, PS, showed a nonsignificant trend to have evolved lower growth in many competitive environments. This difference in evolutionary response between dominant and subordinate species was remarkably similar in monocultures (fig. 3A–3D) and two-species mixtures (fig. 4) and against a nonevolving control CM (fig. 3E–3H). However, no pattern was observed when the lines were each grown in four-species competition (fig. 3I–3L).

If this difference between dominant and subordinate species is related to density-dependent selection, then subordinate species that evolved in monoculture or with other subordinate species should show less of an evolutionary response in performance in interspecific competition than those same species when selected with dominant species. However, surprisingly, we found no evidence that the environment used for selection affected the evolutionary response within a species (figs. 3, 5). Regardless of whether the species was selected in monoculture, with dominant or subordinate competitors, or in four-species mixtures, the SRs were fairly consistent within species.

Related, it is surprising that there was no “home” effect of the selected environment on evolutionary response to different selection environments. For example, lines selected in monoculture or in four-species mixtures do not then do better than any other lines when grown back in these specific environments (fig. 3). We do not have an explanation for this result but recognize that it runs counter to our expectations of how intra- and interspecific selection should shape the evolutionary responses.

The Monod-type experiments to determine minimum resource levels found that the dominant species had much higher resource requirements than the subordinate species. This suggested that the competitive environments in pitcher.
Figure 5: Change in minimum resource level for each selected line within each species from Monod trials. The dashed lines show the average preselection minimum resource level, while the bars indicate the postselection values. Asterisks above bars indicate where specific postselection lines are significantly different from preselection lines, while asterisks with the species name indicate whether collectively all of the selection lines are significantly different from zero. CC = Colpoda cucullus; CS = Colpoda steinii; PS = Poterioochromonas sp.; TA = Tetrahymena sp.
plant leaves in this experiment may not fit the R’ scenario in which the species that can survive at the lowest resource levels eliminates other competitors (e.g., Tilman 1988; Chase and Leibold 2003). In our experiment, we added new resources every 4 days. This may have allowed resource levels to stay sufficiently high to prevent the depletion of bacterial prey below minimum levels, even at relatively high protozoa abundance. If so, then dominance may still be exerted by higher resource uptake and growth when resources are available (a form of scramble competition; Nicholson 1954). This result is consistent with the fact that the subordinate species evolved to be more like the dominant species by requiring higher resource levels after selection. Better competitors in our leaves may need to take up resources more quickly rather than survive at low resource levels. Alternatively, dominance may occur through interference rather than exploitative or resource competition. This mechanism was suggested by Holdridge et al. (2016), who found interference competition at higher resource levels among pitcher plant protozoa.

We cannot determine the role that initial genetic variation may have played in our results. We assumed that our sampling method for our protozoa stocks reflects the natural variation in the species we used and that there were no significant differences in genetic variation among the species. But perhaps the CA and CC stocks were simply more genetically diverse and could thus respond more to selection. Unfortunately, little is known about genetic variation in natural populations of the five species used in this study or in fact most other protozoan species because of a variety of obstacles, including a lack of species definitions and scarce data on natural populations (Boenigk et al. 2012; Tarcz et al. 2018).

The results of our experiments are both confirming and surprising. They are consistent with previous work in this system (see Miller et al. 2014). The protozoans experience strong competition with significant variation among species in competitive performance. It appears that subordinate, less abundant species evolve in these environments to grow better in competition. Dominant species, however, do not appear to change in competitive performance when undergoing selection in these same environments. Furthermore, increased competitive ability and the evolution of increased competitive performance are both associated with higher minimum resource needs, which is a trait of the dominant species (fig. 5). The evolutionary responses in this study do result in a convergence in the competitive abilities of the four species used, as poor competitors improve relative to better competitors. Such convergence could be important for the maintenance of diversity in communities (Aarsen 1983; Chesson 2000; Hubbell 2001; Germain et al. 2020).

However, our results are in other ways surprising, especially the lack of within-species differences in evolution when selected in with different species or species mixtures. This clearly does not fit well with our simple theory about the relative importance of intra- and interspecific competition for dominant and subordinate species. Laboratory studies of selection have found that monocultures and mixtures lead to significantly different evolutionary responses. It is important to note that these studies have also led to other surprising results, such as selection for slower growth rates in mixtures (Lawrence et al. 2012; Fiegna et al. 2015) or lower rates of adaptive evolution with mixture diversity (de Mazancourt et al. 2008). Evolution studies in mixtures often produce unexpected results.

This is a very active time for theory on competition and the niche. The development of a modern coexistence theory (Chesson 2000; Adler et al. 2007) has ecologists thinking more about fitness or competitive asymmetries as well as niche differences in explaining coexistence. There is a recognition that competition can lead to niche and trait convergence (e.g., Fox and Vasseur 2008; terHorst et al. 2010), as well as the more classic niche divergence (e.g., Macarthur and Levins 1967). In fact, there are a variety of models that are now exploring the complexities that can occur when considering evolution within and among a suite of competitors (see Pastore et al. 2021). However, there are very few experimental studies of evolution in a community context (Pantel et al. 2015; terHorst et al. 2018), especially in natural systems. Despite a developing theory, our unexpected results and other recent works suggest that the balance between theory and experiments falls more heavily on a need for more work with experimental evolution in communities.

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Statement of Authorship

The study was designed by T.E.M. and O.U.M. Methods and experimental design were developed by T.E.M., O.U.M., A.I.P., and E.C. Data were collected by all authors and analyzed by T.E.M. and O.U.M. The original draft of the manuscript was written by T.E.M., with significant reviewing and editing from O.U.M. and C.G.-G. Funding was acquired by T.E.M., O.U.M., and A.I.P.
Data are available in the Dryad Digital Repository (https://doi.org/10.5061/dryad.bzkhl8990; Miller et al. 2022).

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