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Surprising competitive coexistence in a classic model system

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Abstract: Competing species often coexist, but the mechanisms allowing long-term coexistence are rarely tested via direct experimental manipulation. We experimentally tested the mechanisms of coexistence in a classic model system, laboratory microcosms in which two species of ciliate protists competed for bacteria. Previous work shows that the species used here compete for bacteria, but can coexist despite large differences in grazing ability. We tested three hypotheses that might explain this surprising coexistence: resource partitioning, chemically-mediated interference competition, and differential use of space. To test for resource partitioning, we conducted an experiment testing the effects of bacterial species richness and composition on the long-term outcome of competition. Manipulating bacterial diversity and composition alters the scope for resource partitioning. Despite strong evidence for differential resource use (e.g., the two ciliates shifted bacterial species composition in different ways), initial bacterial richness and composition did not affect the long-term outcome of competition. Remarkably, the competitive outcome was unchanged even when ciliates competed for a single bacterial species, indicating that the observed resource partitioning is irrelevant to the competitive outcome. In further experiments, we ruled out differential space use and chemically-mediated interference competition as explanations for this surprising coexistence. Coexistence of ciliates on a single bacterial species might reflect partitioning of intraspecific bacterial diversity, and/or osmotrophy or consumption of particulate detritus by the weaker competitor. The results show that this classic model system is not as well-understood as had been previously thought. More broadly, the results dramatically illustrate that merely observing "niche differences" between coexisting species is no evidence that those differences are either necessary or sufficient for long-term coexistence.

Introduction

Similar species often compete, and often coexist (Gurevitch et al. 1992, Gotelli and McCabe 2002), raising the question of how coexistence is achieved. This question is not directly addressed by most competition experiments, which typically comprise competitor removals that test the competitive outcome rather than the mechanisms of coexistence (Gurevitch et al. 1992). The most straightforward way to test a coexistence mechanism is to experimentally remove (or augment) the mechanism and observe the long-term effect on the competitive outcome. For instance, Shmida and Ellner (1984) manipulated immigration rates to show that local coexistence of competing plants was maintained by immigration. However, most competition experiments (including many manipulating the presence/absence of some putative coexistence mechanism) are short-term, and so are not easily extrapolated to identify long-term competitive outcomes (Gurevitch et al. 1992). Identification of the mechanisms of coexistence often relies on more indirect and necessarily less-definitive approaches (e.g., McKane et al. 1990). Sometimes, the mere observation of a putative "niche difference" among competitors is taken as sufficient to explain their coexistence, although observations alone cannot determine whether the observed "niche difference" is either necessary or sufficient for coexistence (Chesson 1990, Abrams 1998).

Protist microcosms are a classic model system for demonstrating the phenomenon of competition. Gause (1934) showed that the ciliate protist *Paramecium aurelia* competitively excludes *Paramecium caudatum* in laboratory cultures, and that the population dynamics of the

competitors are well-described by the then recently-proposed competition model of Lotka and Volterra. Many ecology textbooks use Gause's work to illustrate the phenomenon of competition, the competitive exclusion principle, and the applicability of the Lotka-Volterra competition equations to real organisms (e.g., Begon et al. 1996, Lampert and Sommer 1997). Many subsequent studies build on this work and use protist microcosms as a model system for examining how predation (Jiang and Morin 2004), resource enrichment (Fox 2002), environmental variation (Luckinbill and Fenton 1978, Orland 2003), assembly history (Fukami and Morin 2003), higher-order interactions (Vandermeer 1969), mortality rates (Steiner 2005), and other factors affect the outcome of intra- and interspecific competition.

Surprisingly, however, the mechanisms of competition and coexistence in protist microcosms are relatively little-studied. Fox (2002) found that ciliates able to graze bacteria to lower equilibrial densities were better competitors, implicating resource competition for bacteria as the competitive mechanism. Informal observations (e.g., Gause 1934) and basic biology (the ciliates used in most experiments are bacterivorous) also suggest that resource competition is the competitive mechanism. However, competitors with very different grazing abilities can coexist in a stable equilibrium (Fox 2002), and competitors with similar grazing abilities sometimes rapidly exclude one another (Fox and Smith 1996), indicating that the competitive mechanism is not simply competition for a single resource. Under competition for a single resource, the best competitor should exclude all others at equilibrium (Tilman 1982). Further, seemingly minor differences in culture conditions can change the outcome of competition between the same two species from exclusion to coexistence (compare Gause 1934 to Fox 2002 and Steiner 2005).

What additional mechanisms allow coexistence is unclear. Indirect evidence (Fox 2002, Jiang and Morin 2004, Steiner 2005), and one experiment (Hairston et al. 1968) suggest that bacteria are not a single homogeneous resource, and that resource partitioning might explain competitive coexistence. However, the experiment of Hairston et al. (1968) is not definitive. Hairston et al. (1968) manipulated the species richness and composition of bacteria available to three competing bacterivorous ciliates. Hairston et al. (1968) concluded that increasing resource diversity from one to three bacterial species weakened competition and promoted coexistence. Unfortunately, Hairston et al. (1968) did not quantify bacterial density or composition. Their microcosms probably included unquantified bacterial contaminants (J. W. Fox, personal observation), and so their results are somewhat difficult to interpret. Hairston et al. (1968) also restored the initial bacterial compositions on a daily basis. Their results therefore are not directly applicable to most protist microcosm experiments, in which protist grazing determines the long-term bacterial diversity and composition, and therefore the scope for resource partitioning (Abrams 1998, Jiang and Morin 2004, Steiner 2005). Other possible explanations for coexistence include differential use of space (Gause 1937), and chemical interference competition mediated by waste products (Gause et al. 1934, Kirk 1998).

Here, we experimentally investigate the mechanisms of competition between two bacterivorous ciliate protists (*Colpidium striatum* and *Tetrahymena thermophila*) in aquatic laboratory microcosms. Laboratory microcosms are a tractable model system that allowed us to determine the long-term (dozens of protist generations) outcome of competition under controlled conditions. These two species coexist stably under some culture conditions despite substantial variation in grazing ability (Fox 2002). We conducted a series of experiments testing three hypotheses to explain this surprising coexistence: resource partitioning, chemically-mediated interference competition, and differential use of space. These are the main hypotheses suggested in the literature to explain coexistence in this system (Gause et al. 1934, Gause 1937, Hairston et al. 1968, Fox 2002, Jiang and Morin 2004, Steiner 2005). The equilibrial dynamics observed in all protist competition experiments not subjected to large external perturbations rule out non-equilibrial coexistence mechanisms (Gause 1934, Hairston et al. 1968, Vandermeer 1969, Fox 2002). Few studies in any system systematically test for several coexistence mechanisms by experimentally manipulating the presence/absence of each candidate mechanism over many generations.

Methods

Resource partitioning experiment

This experiment tested the effect of initial bacterial diversity and composition on the long-term outcome of competition between *Colpidium striatum* and *Tetrahymena thermophila*. These two ciliates are bacterivorous, coexist in nature (Fenchel 1987), and coexist in the laboratory under the culture conditions used here, even though *Colpidium* is a far more effective bacterial grazer (Fox 2002). Both species reproduce asexually via binary fission, and unlike some other protists, neither species exhibits any inducible shifts in morphology in response to resource scarcity or the presence of other protists. Neither species can survive in the absence of bacteria in the me-

dium used here (J. W. Fox, personal observation; Fenchel 1987), although *Tetrahymena* can be grown axenically in other media, suggesting that neither obtains significant nutrition via osmotrophy or consumption of detritus. Neither species is cannibalistic, and neither can consume the other. Neither species encysts under the experimental conditions, and so neither can avoid competition via encystment.

Microcosms were 240 ml glass bottles, each containing 40 mL of nutrient medium (0.14 g Carolina Biological Supply protozoan pellets per l of distilled water) and one sterile wheat seed to provide additional slow-release nutrients. Bottles were loosely capped to permit gas exchange while minimizing airborne contamination. All materials were steam autoclaved before use.

The experiment was a 5×2×2 factorial design crossing five bacterial compositions with the presence or absence of each of the two ciliates. Bacterial compositions were monocultures of *Bacillus subtilis*, *Escherichia coli*, or *Micrococcus roseus*, a polyculture of all three monoculture species, and a polyculture of all three monoculture species plus an additional 10 species: *Micrococcus luteus*, *Pseudomonas fluorescens* SBW 25R, *Enterobacter aerogenes*, *Alcaligenes eutrophus*, *Staphylococcus epidermis*, *Chromobacterium lividum*, *Bacillus megaterium*, *Aquaspirillum serpens*, *Serratia marcescens*, and an unknown species isolated from a protist stock culture and hereafter referred to as "Unknown A." Each treatment combination was replicated 8 times for a total of 160 microcosms. However, 2 replicates of each treatment were intentionally sacrificed before the end of the experiment (see below), and so final analysis was based on 6 replicates/treatment.

The experimental design includes the largest number of treatments feasible, consistent with high replication. The design partly confounds bacterial diversity and composition, since the 13 species treatment includes 10 bacterial species not represented in the other treatments. We included the 13 species treatment so as to include a treatment with much higher resource diversity than in previous experiments (Hairston et al. 1968, Jiang and Morin 2004, Steiner 2005). However, we are not primarily concerned with separating the effects of bacterial diversity vs. composition on the outcome of competition, but rather with testing whether the initial presence of multiple bacterial taxa creates scope for resource partitioning and thereby allows the ciliates to coexist.

All 13 bacteria are (i) consumed by and support growth of both protists (see below), (ii) vary in ecologically-relevant traits (e.g., cell size, which affects vulnerability to bacterivorous protists, Jürgens and Matz 2002), (iii) grow in protozoan pellet medium and on liquid broth (LB) agar plates, and (iv) as far as possible, produce colony types that can be distinguished on LB agar plates. LB agar (10 g NaCl, 10 g Bacto-tryptone, 5 g yeast extract, and 20 g Bacto-agar in 1 l water, pH adjusted to 7.5 with 5 N NaOH) is a general-purpose agar which supports growth of many bacteria. In particular, the three bacterial monoculture species produce easily-distinguished colonies on LB agar. We obtained all organisms from Sciento (Manchester, UK), except for *P. fluorescens* (supplied by Dr. Richard Ellis, NERC Centre for Population Biology Ascot, UK), *S. marcescens* (Carolina Biological Supply, Burlington, NC, USA), and *Tetrahymena* (Culture Collection of Algae and Protozoa, Ambleside, UK).

To initiate the experiment, bacterial monocultures received 50 µL of bacteria and medium from high density (5 d old) stock cultures. Bacterial polycultures received 50 µL from a mix made up of equal volumes from 5 d old monoxenic stock cultures. Initial bacterial densities were similar in all bottles. We added protists 3 d after bacteria to allow bacteria to first reach high density. We added *Tetrahymena* as 100 µl aliquots drawn from 7 d old axenic stock cultures (proteose peptone medium). As *Colpidium* cannot be grown axenically, we added *Colpidium* to bacterial monocultures as 800 µl aliquots drawn from 7 d old monoxenic stock cultures of *Colpidium* growing on *B. subtilis*, *E. coli* or *M. roseus*. We added *Colpidium* to bacterial polycultures as 800 µl aliquots comprising equal parts of the three monoxenic stock cultures. We created monoxenic stock cultures by subjecting individual *Colpidium* cells to serial transfer in antibiotic solution (a mix of streptomycin, penicillin, neomycin and tetramycin), as in Kaunzinger and Morin (1998). We added 800 µl of *Colpidium*, rather than 100 µl as with *Tetrahymena*, to ensure approximately equal initial densities of each species (~500 individuals, <5% of carrying capacity in protozoan pellet medium). Different protist treatments received slightly different amounts and compositions of stock culture media, but these additions comprised <3% of the final culture volume, an amount unlikely to affect the results. Indeed, the results could not be related to the slight differences in initial medium composition in any obvious way.

Bottles were maintained on a bench in a walk-in incubator (20°C). Bottles remained undisturbed until the end of the experiment. We did not periodically replace any medium or collect time series data, because the equipment and facilities available to us did not permit us to conduct the experiment under sterile conditions. Pilot experiments revealed that opening the bottles periodically for sam-

pling would have risked contamination by unwanted bacteria. We sampled the bottles 42 d after adding protists, sufficient time for dozens of generations of protist growth (Fenchel 1987). Four lines of evidence indicate that the bottles almost certainly reached or closely approached their asymptotic states after 42 d. First, Fox (2002) found that *Colpidium* and *Tetrahymena* reached a competitive equilibrium after ~40 d under the same culture conditions as used here (Fig. 3a in Fox 2002). Second, in a pilot experiment in which bottles were sampled periodically (results not shown), all treatments approached asymptotic states in <40 d. Third, numerous previous experiments in similar systems find that competing protists approach asymptotic states in ≤42 d (e.g., Gause 1934, Vandermeer 1969, Fox and Morin 2001, Fox 2002, Jiang and Morin 2004). While Weatherby et al. (1998) found that some protist species combinations did not approach their asymptotic states for >100 d, most species combinations they tested contained predators, in contrast to our study. Fourth, and most importantly, after 35 d we sacrificed two replicates of each treatment and sampled the protists. Densities after 35 d were statistically indistinguishable from densities after 42 d (results not shown), strongly suggesting that densities changed little after 35 d. These four lines of evidence together indicate that 42 d was almost certainly sufficient time for the experiment to attain quasi-equilibrium. Even in the unlikely event that 42 d was insufficient time for the treatments to approach their asymptotic states, results indicated that 42 d was sufficient time for differences among treatments to develop. These differences among treatments almost certainly resemble those that would have been observed had the experiment run longer (Gause 1934, Vandermeer 1969, Fox and Morin 2001, Fox 2002, Jiang and Morin 2004).

We sampled protists by withdrawing a sample of known volume (~0.3 ml) from each bottle and counting the protists under a dissecting microscope using a previously-published protocol (Fox 2002). We counted bacteria using plate counts on LB agar plates, distinguishing as many types of colonies (based on color and growth form) as possible. The usual concerns about failure of plate counts to detect unculturable bacteria in field samples do not apply here, because we conducted our experiment in a laboratory setting using bacterial species known to grow on LB agar. Plate counts and epifluorescence microscopy counts estimate similar bacterial densities in this system (compare Fox 2002 with Jiang and Morin 2004), demonstrating that plate counts accurately estimate bacterial density in this system. Our plating protocol was such that we would expect to detect any bacterial species present at a density ≥~500 ml^{-1}, a very low density in this system (Fox 2002, Jiang and Morin 2004).

We converted counts to densities (mL^{-1}), and applied a $\log_{10}(x+1)$ transformation to control heteroscedasticity. Although this transformation slightly biases low-density data, such data were rare, and results remained unchanged under alternative approaches to controlling heteroscedasticity (generalized linear models fitted by quasi-likelihood with a log link function). Inspection of residuals indicated conformity of transformed data with statistical assumptions.

We tested the effects of *Colpidium* presence/absence, *Tetrahymena* presence/absence, initial bacterial composition (*E. coli*, *B. subtilis*, *M. roseus*, three species, or 13 species), and their interactions, on total final bacterial density using ANOVA. We tested the effect of protist composition (none, *Colpidium*, *Tetrahymena*, or both species) on final bacterial composition using Mantel tests with 1000 randomizations. Mantel tests tested for a significant association between a data matrix (a matrix of dissimilarities in final bacterial composition among bottles) and a design matrix (a matrix of 0s and 1s, indicating pairs of bottles of the same or different protist compositions, respectively). We conducted separate Mantel tests for bottles with three and 13 bacterial species, and repeated the tests with two different measures of dissimilarity in final bacterial composition. Euclidean distance accounted for variation in densities (untransformed) of bacterial species, while binary distance accounted only for presence/absence of bacterial species. The binary distance between two bottles is $b/(a+b)$ where b is the number of bacterial species present in exactly one bottle, and a is the number of bacterial species present in both bottles.

Mantel tests considered as many species as we could reliably identify on plates, except that we lumped rare or hard-to-distinguish species into single categories. In the three species treatment we divided bacteria into four categories for purposes of analysis: *E. coli*, *B. subtilis*, *M. roseus*, and other (contaminant) species. In the 13 species treatment we divided bacteria into five categories for purposes of analysis: *E. coli*, *S. marcescens*, *B. subtilis*/*C. lividum*, *M. luteus*, and species producing smooth-edged round white colonies ("white species"). Note that *M. roseus*, *B. megaterium*, and "Unknown A" do not produce smooth-edged round white colonies, and were not observed on plates from the 13 species treatment.

For each protist species, we tested for effects of interspecific competition (present or absent), initial bacterial composition (*E. coli*, *B. subtilis*, *M. roseus*, three species, or 13 species), and their interaction, on final density using ANOVA. A significant interaction term indicates that the outcome of competition depends on initial bacterial com-

position. In bacterial monocultures the most competitive protist should exclude the other (Tilman 1982). We performed all statistical analyses using R 1.7.0 for Windows.

Follow-up to the resource partitioning experiment

To aid interpretation of the resource partitioning experiment, we conducted a follow-up experiment to estimate an index of ciliate feeding rates on each bacterial species. A consumer's per-capita feeding rates on the available resources together define its "resource utilization function", from which can be derived measures of overlap in resource use (MacArthur 1970, 1972, Chesson 1990, Abrams 1998). Competitive intensity is weakest and consumer coexistence most likely when consumers have very different utilization functions (= low overlap), assuming that consumer grazing does not lead to the exclusion of some resource species (MacArthur 1970, 1972, Chesson 1990, Abrams 1998). Per-capita feeding rates also will affect which bacteria, if any, are excluded in the long run (Abrams 1998).

Unfortunately, per-capita feeding rates on each bacterial species are extremely difficult to measure *in situ* under the experimental conditions used here. However, we can measure each ciliate's intrinsic rate of increase r on each bacterial species; r should provide a reasonable index of per-capita feeding rate. For instance, the following simple model provides a reasonable description of the intrinsic rate of increase r_{ij} of ciliate i (i = 1,2) feeding on a monoculture of bacterial species j (j = 1,2,…,13) when the ciliate is at low density and the bacterial species is at high density:

$$r_{ij} = f_{ij}\, c_{ij}\, K_j - m_i, \qquad (1)$$

where K_j is the density (= carrying capacity) of bacterium j, f_{ij} is the per-capita feeding rate of i on j when j has density K_j, c_{ij} is the conversion efficiency of i when consuming j, and m_i is the per-unit density-independent loss rate of i (reflecting both metabolic losses and density-independent mortality) (MacArthur 1970, Chesson 1990, Fox 2002). The bacterium is assumed to be at its carrying capacity K because when the ciliate is rare the bacteria on which it feeds should be abundant. Equation (1) implies that intrinsic rate of increase r_{ij} is proportional to per-capita feeding rate f_{ij}, the maximum possible feeding rate of i on j (assuming that the density of bacterium j cannot exceed its carrying capacity). As long as the conversion efficiency c_{ij} of each ciliate does not depend too strongly on bacterial species identity, r_{ij} values should provide a reasonable index of f_{ij} values. Differential resource use is absent when the two ciliates exhibit proportional feeding rates; that is, when $f_{1j} = xf_{2j}$ for all j, where x is an arbitrary constant (Chesson 1990). Differential resource use is high when the association (coefficient of determination) between f_{1j} and f_{2j} is low (Chesson 1990).

We measured the intrinsic rate of increase of each ciliate on 12 of the 13 bacterial species. Stocks of the "Unknown A" bacterium were inadvertently discarded before the follow-up experiment began. The experimental units were circular 20 ml loosely-covered glass petri dishes containing 5 ml of the same medium as in the resource partitioning experiment, but no wheat seeds. We inoculated each dish with 10 µl of medium from monoxenic bacterial stock cultures, followed 3 d later by 10 individuals of one of the two ciliate species. We added ciliates individually via micropipette to avoid adding significant amounts of stock culture medium along with the ciliates. *Tetrahymena* individuals were drawn from an axenic stock culture, while *Colpidium* individuals were drawn from a stock culture grown on *B. subtilis* and washed several times in antibiotics before being added to the experiment. There were three replicates/treatment for a total of 72 dishes. The experiment was conducted at 20°C. After ~48 h we sampled protists and estimated r as $[\ln(N_t) - \ln(N_0)]/t$, where N_t is density after t hours and N_0 is initial density. Forty-eight h was sufficient time for measurable population growth, but insufficient time for the ciliates to become dense enough to experience detectable intraspecific competition. We calculated Pearson's correlation coefficient between the r values of the ciliates *Colpidium* and *Tetrahymena*; zero correlation suggests substantial resource use differentiation, while a correlation of 1 suggests no differentiation.

Differential space use experiment

A second possible explanation for long-term coexistence is spatial heterogeneity. The batch cultures described above, like those in previous experiments (e.g., Fox 2002), were not continuously mixed, so that the highest bacterial densities occurred in flocculent masses of bacteria attached to particulate detritus and wheat seeds at the bottom of the bottle (personal observation). Ciliates typically aggregate on the bottom of the bottle, particularly around the wheat seeds, although different species aggregate to different degrees (personal observation). Different areas of the bottle (e.g., bottom vs. water column) might therefore comprise different patches from the perspective of a ciliate (Gause 1937). If ciliate species differ sufficiently in their patch selection behavior, spatial heterogeneity might promote coexistence. One way to test this possibility would be to compete the ciliates in a well-mixed environment. Here we present results of such an

experiment. This experiment crossed three ciliate treatments (*Colpidium* and *Tetrahymena* alone, and both species together) with two mixing treatments (unmixed control, or continuous vigorous mixing on a bottle shaker). There were three replicates/treatment for a total of 18 bottles. Other experimental conditions were as described for the resource partitioning experiment, save that the initial bacterial composition was an undefined mixture of species. Once per week we withdrew 4 ml (=10%) of the medium (and any organisms in that medium), and replaced with 4.7 ml of fresh, sterile medium to replace medium lost during sampling. We sampled ciliate densities every 2-4 d for 42 d, using the same protocol as in the resource partitioning experiment. Sampling occurred before medium replacement on days when both were scheduled. Our response variables were the final of each ciliate (transformed as $\log_{10}((n/\text{ml}) + 1)$). For each species, we analyzed the effects of competition, mixing, and their interaction on final density using ANOVA. If spatial heterogeneity affects the outcome of competition, we would expect a significant interaction term in the ANOVA.

Chemical interference experiment

A third possible explanation for coexistence of competitors with different grazing abilities is that competition is not exclusively exploitative. For instance, build-up of waste products might lead to chemically-mediated intra- and interspecific interference competition (Kirk 1998). To test this possibility, we conducted an experiment in which we grew ciliates in nested pairs of bottles. The larger outer (glass) bottle contained the "recipient" ciliate population, while the smaller inner (plastic) bottle contained one of four "donor" treatments: sterile medium, medium bacterized with *Bacillus subtilis*, bacterized medium plus *Tetrahymena*, or bacterized medium plus *Colpidium*. The inner bottle had a 20 mm diameter circular hole, over which we glued a 25 mm diameter circular membrane filter with a pore size of 0.2 µm. The filter permitted exchange of dissolved chemicals between donor and recipient, while preventing exchange of bacteria, protists, and particulate matter. The two bottles contained 80 ml of medium (the same medium as in the resource partitioning experiment, inoculated with *B. subtilis*), with ~40 ml of this inside the inner bottle. The membrane filter was completely submerged. Each of the four donor treatments was replicated twice for each recipient ciliate species. Sampling occurred as in the other experiments. Once per week we briefly agitated each nested pair of bottles, withdrew 8 ml (=10%) of the medium (and any organisms in that medium), and replaced with 8.7 ml of fresh, sterile medium to cover medium lost during sampling. We withdrew medium from the recipient portion of each bottle. This was an arbitrary choice made for the sake of convenience, and cannot affect the results because the same procedure was used for all treatments. Our response variables were the final densities of each ciliate (transformed as $\log_{10}((n/\text{ml}) + 1)$). For each species, we analyzed the effect of the donor treatment using ANOVA. If chemical interference occurs, recipients should attain lower density when growing with ciliate donors than with other donor treatments.

Results

Resource partitioning experiment and follow-up experiment

Total final bacterial density varied significantly among initial bacterial compositions ($F_{4,95} = 25.56$, $P < 0.001$; Fig. 1). *Colpidium* significantly reduced final bacterial density ($F_{1,95} = 17.70$, $P < 0.001$; Fig. 1), but the

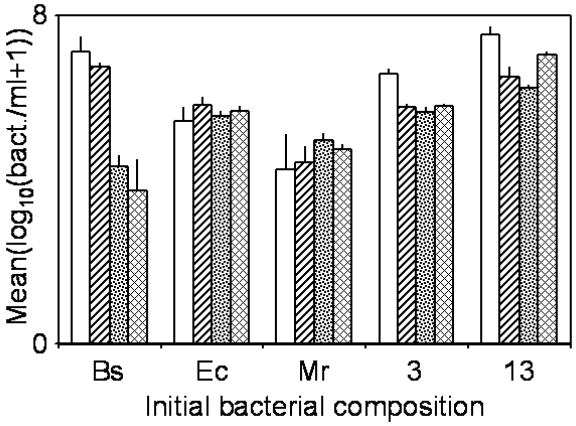

Figure 1. Effects of initial bacterial composition, protist composition, and their interaction on mean total bacterial density (+1 SE). Initial bacterial compositions are *B. subtilis* (BS), *E. coli* (EC), *M. roseus* (MR), three species (3), and thirteen species (13). Protist compositions are none (open bars), *Tetrahymena* (hatched bars), *Colpidium* (stippled bars), and both species (cross-hatched bars).

effect of *Colpidium* on final bacterial density varied significantly among initial bacterial compositions (two-way interaction term $F_{4,95} = 16.22$, $P < 0.001$). The three-way interaction of *Colpidium*, *Tetrahymena*, and initial bacterial composition was marginally non-significant ($F_{4,95} = 2.36$, $P = 0.059$; Fig. 1).

Protists significantly affected final bacterial composition in bottles with three bacterial species, whether bacterial composition was measured using densities or presence/absence (Mantel tests, $P < 0.001$ and $P = 0.014$, respectively; Fig. 2a). *E. coli* was always the most abundant bacterium, and was nearly monodominant in the absence of protists (Fig. 2a). *M. roseus* comprised a subdominant component of the bacterial fauna with *Tetrahymena* alone. *B. subtilis*, *M. roseus*, and other (contaminant) species together comprised a subdominant component of the bacterial fauna in bottles with *Colpidium* (Fig. 2a).

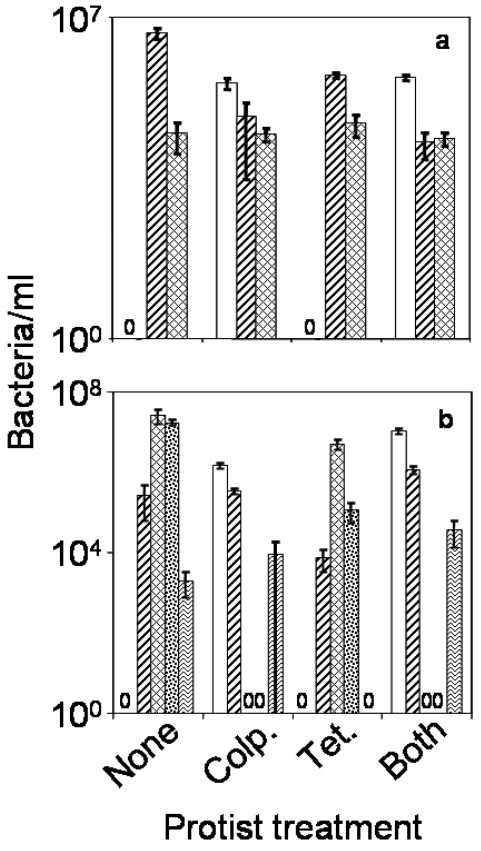

Figure 2. Mean densities (±1 SE) of different bacterial species, or categories of species, in different protist treatments, in bottles initially containing three (**a**) or 13 (**b**) bacterial species. In (**a**), open = *E. coli*, hatched = *B. subtilis*, cross-hatched = *M. roseus*. In (**b**), open = *E. coli*, hatched = *S. marcescens*, cross-hatched = *C. lividum*/*B. subtilis*, stippled = white spp., waves = *M. luteus*. In both panels, 0 indicates a species not found in a given treatment at the end of the experiment. Untransformed data are plotted to conform to the data used in the corresponding Mantel tests (see text for details), but data are plotted on a log scale to enable clear display of rare species; log scale plotting makes the error bars appear asymmetrical.

Protists significantly affected final bacterial composition (measured using densities, or presence/absence) in bottles with 13 bacterial species (Mantel test, $P < 0.001$ with either measure; Fig. 2b). White species and *B. subtilis*/*C. lividum* dominated the bacterial fauna in the absence of protists (Fig. 2b). *Tetrahymena* caused near-monodominance by *B. subtilis*/*C. lividum*, with *S. marcescens* and white species less abundant by 1-2 orders of magnitude (Fig. 2b). *Colpidium* caused dominance by *E. coli*, with *S. marcescens* subdominant and *M. luteus* present at low density, whether or not *Tetrahymena* was also present (Fig. 2b).

All bacteria supported growth of both protists in the follow-up experiment, indicating that all bacteria were edible to both protists (Fig. 3). There was a significant positive correlation between the intrinsic rates of increase achieved by *Colpidium* and *Tetrahymena*; bacteria supporting rapid growth of one species tended to support rapid growth of the other (Fig. 3). However, the correlation was far from perfect (coefficient of determination = $0.56^2 = 0.31$; Fig. 3). Some bacteria supported more rapid growth of *Colpidium* than *Tetrahymena*, while for others the reverse was true (Fig. 3).

There was no obvious association between the intrinsic growth rates supported by different bacteria, and final

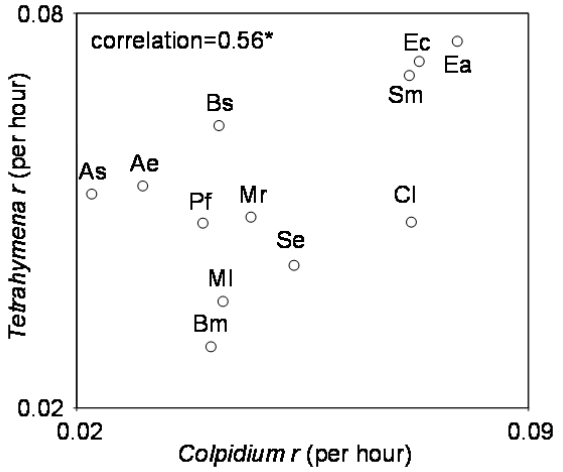

Figure 3. Intrinsic rate of increase *r* for *Colpidium* vs. *Tetrahymena*. Each point represents mean values for one bacterial taxon, labeled with the first letters of the genus and species (e.g., Ec = *E. coli*). Pearson's correlation coefficient is shown, $*P < 0.05$ (two-tailed test).

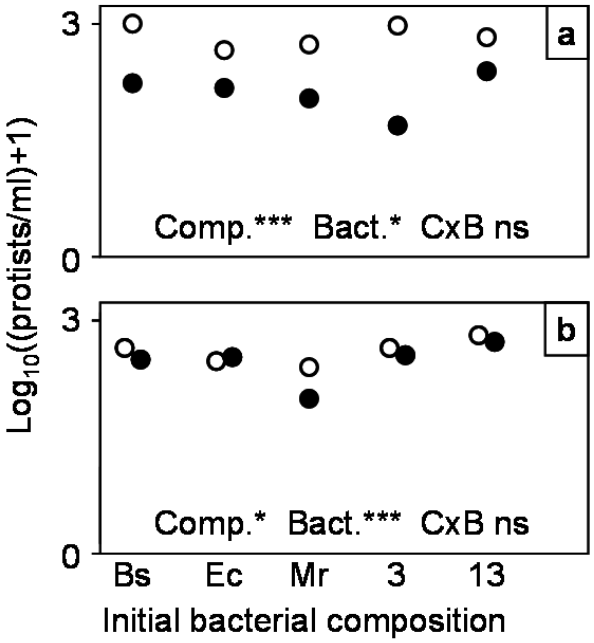

Figure 4. Effects of initial bacterial composition, competition, and their interaction on the mean final density of *Tetrahymena* (a) and *Colpidium* (b) in the resource partitioning experiment. Error bars (±1 SE) are too small to be visible. Bacterial compositions as in Fig. 1. Open circles, competitors absent; filled circles, competitors present. Within each panel, statistical significance of effects of intial bacterial composition (Bact.), competition (Comp.), and their interaction (C×B) in an ANOVA are indicated as $*0.01 < P < 0.05$, $***P < 0.001$, ns = not significant ($P > 0.2$ in both cases).

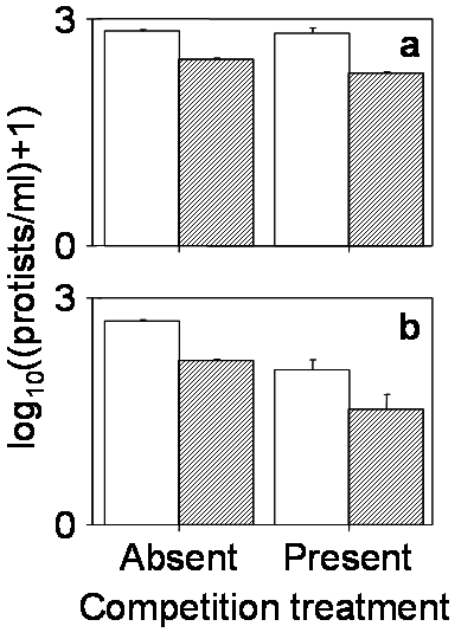

Figure 5. Effects of competition, shaking, and their interaction on mean final densities of *Colpidium* (a) and *Tetrahymena* (b) in the differential space use experiment. Open bars, unshaken; hatched bars, continuously shaken.

bacterial compositions in bottles with three or 13 bacterial species. Formal statistical tests for such an association would lack power, because of the small number of distinguishable bacterial species present at the end of the resource partitioning experiment (Fig. 2).

Competition significantly reduced final densities of both *Colpidium* and *Tetrahymena* in the resource partitioning experiment, although the latter was more strongly affected (Fig. 4). Final densities of both protists varied significantly among initial bacterial compositions (Fig. 4). Effects of competition on both species were independent of initial bacterial composition (i.e., non-significant interaction terms; Fig. 4).

Differential space use experiment

Both competition and shaking significantly reduced final *Colpidium* density (2-way ANOVA; competition: $F_{1,8} = 17.5$, $P = 0.003$; shaking: $F_{1,8} = 59.0$, $P < 0.001$), but the effect of competition was independent of shaking (interaction term: $F_{1,8} = 1.2$, $P = 0.312$) (Fig. 5a, 6). Both competition and shaking significantly reduced final *Tetrahymena* density (2-way ANOVA; competition: $F_{1,8} = 18.6$, $P = 0.003$; shaking: $F_{1,8} = 12.3$, $P = 0.008$), but the effect of competition was independent of shaking (interaction term: $F_{1,8} < 0.001$, $P = 0.989$) (Fig. 5b, 6).

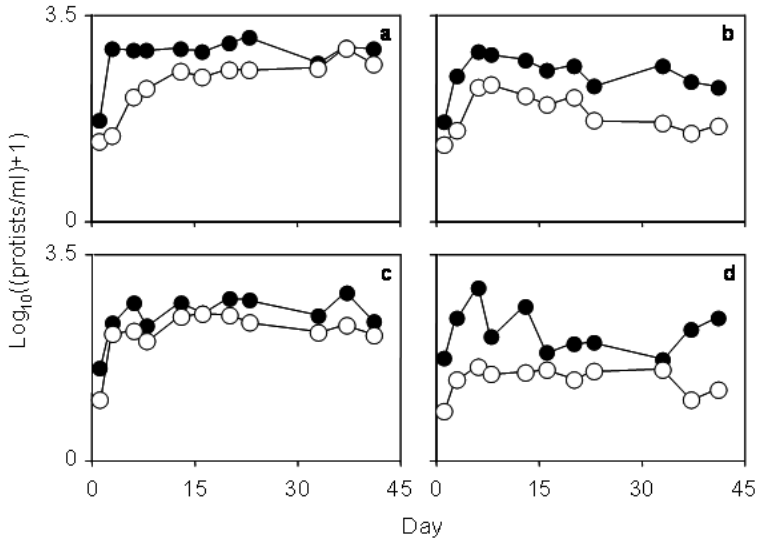

Figure 6. Representative population dynamics in the differential space use experiment. Each time series of population densities comes from a single population. Filled circles denote *Colpidium*, open circles denote *Tetrahymena*. **a.** Unshaken bottles, each species growing alone (i.e., *Colpidium* and *Tetrahymena* data come from two different bottles). **b.** Unshaken bottle, both species in competition. **c.** Shaken bottles, each species alone. **d.** Shaken bottle, both species in competition.

Chemical interference experiment

There was no significant effect of donor treatment on final density of either *Colpidium* or *Tetrahymena* (all $P > 0.2$).

Discussion

Our experiments reveal a remarkable result: none of the three mechanisms tested even partially explains coexistence. That differential use of space and chemical interference fail to explain coexistence is perhaps unsurprising, as the prior evidence for either of these mechanisms is relatively weak. However, several strong lines of indirect evidence implicate resource partitioning as a common mechanism of coexistence in this system (Fox 2002, Jiang and Morin 2004, Steiner 2005). Consistent with these lines of evidence, our results show that *Colpidium* and *Tetrahymena* exhibit substantial resource partitioning. However, this resource partitioning is irrelevant to the competitive outcome.

Bacterial composition differed in the presence vs. absence of protists, different bacterial compositions supported different protist densities, and different bacteria supported different protist growth rates, indicating that different bacterial species constituted different resources and were not all identical from the protists' perspective. Bacterial composition also differed in bottles with *Colpidium* alone vs. bottles with *Tetrahymena* alone, indicating that *Colpidium* and *Tetrahymena* differ in resource use. Some bacterial species supported more rapid growth of *Colpidium* than *Tetrahymena*, while for other bacterial species the reverse was true, also suggesting that each protist feeds more rapidly than the other on some of the bacteria. Jiang and Morin (2004) also found that different protists shift bacterial composition in different ways.

Colpidium was the more effective grazer overall, reducing bacterial density to a lower average level than *Tetrahymena*. Fox (2002) obtained a similar result. The level to which a protist is able to reduce total bacterial density is a good predictor of competitive success as long as differences among competitors are reasonably large (i.e., competitors differ by a factor of ~2; Fox 2002). However, the level to which *Colpidium* was able to reduce total bacterial density varied with initial bacterial composition. That the overall grazing effectiveness of *Colpidium* varies among bacterial compositions is consistent with the hypothesis that competitive outcomes will vary with bacterial composition.

However, competitive outcomes were independent of bacterial diversity and composition, and there was sufficient statistical power to detect any ecologically significant effect. Observed competitive outcomes are remarkably similar across different bacterial compositions. It is particularly surprising that both bacterivores coexisted for 42 d even when grown in bacterial monocultures, where opportunities for differential resource use should have been non-existent. Forty-two d almost certainly is sufficient time for the system to approach an asymptotic state

(e.g., Fig. 6), and in any case is a sufficiently long period of coexistence to require an explanation, given the large difference between *Colpidium* and *Tetrahymena* in overall grazing ability. But the explanation for coexistence cannot be differential grazing on different bacterial species.

The results of the resource partitioning experiment likely are not an artifact of errors in measuring bacterial composition. By lumping some plated bacteria into broad categories, we limited the scope for identification errors. The only possible identification errors would have occurred in the 13 species treatment. *S. marcescens* typically produces red colonies on LB agar, but sometimes produces white colonies and so might sometimes have been misclassified as a "white species". The observed effects of protists on bacterial composition in the 13 species treatment remain significant even if all "white" colonies are reclassified as *S. marcescens* (results not shown). Use of LB agar should have facilitated detection of many common laboratory contaminants, but we detected contaminants in very few bottles, and only in low densities. In particular, we detected no contaminants in monocultures of *E. coli* or *B. subtilis*, suggesting that bacterial contamination cannot explain protist coexistence in these treatments. Platable contaminants with a similar colony morphology to one of our 13 bacterial species would of course go undetected in any treatment containing that species. But any such contaminants would be readily detected in at least two of the three bacterial monocultures. The fact that no contaminants were detected in *E. coli* or *B. subtilis* monocultures suggests that platable contaminants resembling our treatment bacteria did not occur. Nor are unplatable contaminants likely to explain our results. Plate counts and epifluorescence microscopy estimate similar bacterial densities in this system (compare this study, Fox 2002, and Jiang and Morin 2004), indicating that unplatable bacteria likely did not comprise a significant part of the bacterial fauna.

Lack of an effect of bacterial diversity and composition on the outcome of competition might be taken to suggest that the ciliates were not competing for bacteria at all. However, several lines of evidence indicate that *Colpidium* and *Tetrahymena* were resource-limited and therefore engaged in resource competition. If protists compete for resources, the best competitor should be the most effective grazer (Fox 2002). *Colpidium* is the more effective grazer overall (i.e., it reduces bacteria to a lower average level than does *Tetrahymena*), and is less affected by competition, as expected under resource competition (see also Fox 2002). Increasing enrichment (protozoan pellet concentration) increases protist densities in this system, as expected if protists are resource-limited (Kaunzinger and Morin 1998, Fox 2002). Cell sizes of both protists in all experiments were much reduced after 42 d (J. W. Fox, personal observation), indicating resource limitation (Fenchel 1987). Finally, protist grazing shifted bacterial composition, strongly suggesting that protists were feeding on bacteria and not, e.g., exclusively on particulate detritus (Scherwass et al. 2005).

Interestingly, despite differences in resource use, *Colpidium* and *Tetrahymena* together did not suppress bacteria to a lower level in the resource partitioning experiment than could either species alone. Increasing consumer diversity is widely believed to lead to greater resource suppression when consumers exhibit different, complementary patterns of resource use. However, the few other experiments testing the relationship between consumer diversity and resource suppression find mixed results (Cochran-Stafira and von Ende 1998, Norberg 2000, Sommer et al. 2001, Fox 2002, 2004a, Gamfeldt et al. 2005). This is perhaps unsurprising, as recent theory demonstrates that two consumers coexisting via differential resource use will not necessarily suppress resources to a lower level than could either one alone (Fox 2004b).

Coexistence does not reflect differential use of space or chemical interference. The outcome of competition is unaltered by continuous mixing, indicating that any differential space use is irrelevant to the competitive outcome. This result also implies that any other changes in culture conditions produced by mixing (e.g., changes in O_2 concentration) also are irrelevant to the competitive outcome. This result is unsurprising, since both species aggregate on the bottom of the bottle and so do not differ dramatically in their use of space, although *Colpidium* does tend to aggregate near wheat seeds while *Tetrahymena* does not (J. W. Fox, personal observation). *Colpidium* and *Tetrahymena* do not chemically interfere with one another, and since there is no other obvious means by which they could physically interfere with one another (particularly under continuous mixing), their coexistence apparently does not reflect a balance between interference and exploitative competition.

Coexistence almost certainly does not reflect non-equilibrial mechanisms of coexistence (e.g., Armstrong and McGhee 1980). Our results and those of many other experiments show that bacterivorous protists grown in unperturbed batch cultures do not exhibit the oscillatory dynamics characteristic of non-equilibrial coexistence (Gause 1934, Hairston et al. 1968, Vandermeer 1969, Luckinbill and Fenton 1978, Fox and Morin 2001, Fox 2002, Donahue et al. 2003, Orland 2003, Jiang and Morin 2004).

So how do competing protists coexist in this system? One possibility is that one or both protists obtains sufficient nutrition via osmotrophy (uptake of dissolved carbon and nutrients) and/or consumption of particulate detritus to avoid competitive exclusion, even though neither species can survive in the absence of bacteria in the medium used here. Scherwass et al. (2005) found that *Tetrahymena pyriformis* could attain positive intrinsic growth rates of 0.1-0.6 d^{-1} feeding exclusively on particulate detritus. While this mechanism might explain coexistence, indirect evidence suggests otherwise: *Tetrahymena* is rapidly excluded in more highly-enriched media (higher PP concentration with more wheat seeds) that contains equal or higher concentrations of dissolved resources and particulate detritus than the medium used here (Fox 2002). However, the present study illustrates the unreliability of indirect evidence for coexistence mechanisms. A second possibility is that even bacterial monocultures may harbor significant bacterial phenotypic diversity, perhaps generated during the experiment through genetic evolution and/or phenotypic plasticity. Bacteria can change cell size in response to grazing within 72 h via both phenotypic plasticity and adaptive evolution (Jürgens and Matz 2002). Protists might also vary in their abilities to graze on single bacterial cells vs. the loose bacterial aggregations which are common under the culture conditions used here (Liess and Diehl 2006). Testing these possibilities experimentally would be challenging. It would not be sufficient to simply demonstrate, e.g., that ciliates obtain some nutrition via osmotrophy, or that bacteria vary phenotypically. Testing whether these mechanisms generate coexistence would require elminating these mechanisms over a long-term experiment, to see whether long-term coexistence was eliminated. It would be difficult to experimentally eliminate genetic evolution and phenotypic plasticity in bacteria, or to prevent protists from obtaining any osmotic nutrition over the course of a long-term experiment.

Coexistence of competing protists is not the only surprising feature of the data. Protist grazing in some bacterial monocultures failed to reduce mean bacterial densities significantly below densities in protist-free controls (Fig. 1). A previous study in the same system (Fox 2002) found a similar result for some protists under some culture conditions. While it is tempting to conclude that protists were not consuming bacteria in these treatments, this conclusion merely begs the question of how protists that were not consuming bacteria could survive at all. It seems more likely that protists were consuming bacteria, but that there are poorly-understood complexities associated with the bacteria-protist interaction. Failure of protists to significantly reduce bacterial density in some treatments may reflect osmotrophy and/or detritus consumption by protists. If protists are partially supported by dissolved resources and/or detritus, they might not be expected to substantially reduce bacterial densities. However, the question is not straightforward, since protist consumption of alternative, non-bacterial resources should generate apparent competition between bacteria and the alternative resources (Holt 1977). Protists consuming dissolved resources and/or particulate detritus would also be competing with bacteria, and therefore would be expected to reduce bacterial density. Failure of protists to significantly reduce bacterial density in some bacterial monocultures might also reflect inducible defenses and or adaptive evolution of bacteria—the main effect of protist grazing might be to shift the phenotypic or genetic composition of the bacteria rather than reduce total bacterial density (Jürgens and Matz 2002, Liess and Diehl 2006). A complete understanding of how competing protists coexist, and of protist-bacterial interactions clearly will require much more detailed mechanistic studies of individual-level feeding, physiology, and growth. Unfortunately, the very features of protist microcosms that make them an excellent model system for community-level studies of the long-term outcome of competition make them challenging for individual-level studies of feeding and physiology. This is particularly true if the goal is to study feeding behavior, physiology, and growth under the culture conditions used in this and similar experiments. The culture conditions typical of protist microcosm experiments include several features (e.g., lack of continuous mixing, detritus particules of various irregular sizes and shapes, loose aggregations of bacteria) characteristic of many natural systems, but inconvenient for mechanistic studies of protist feeding and growth.

In conclusion, we suggest that our results are broadly relevant to many systems besides microcosms. In many systems similar competitors coexist, and some species are known (or strongly suspected) to be competitively superior to others. Conclusions as to how competitively-inferior species coexist often are based (out of necessity) on indirect evidence, sometimes much weaker than the indirect evidence that protists coexist via resource partitioning. Our results serve as a salutary reminder that determining the mechanisms of coexistence in any particular case is not so easy. Competing species often exhibit niche differences, and often coexist in the long run, but the former observation should not be taken to explain the latter in the absence of direct experimental evidence.

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