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THE COMPLEXITY OF COOPERATION IN ECOLOGICAL COMMUNITIES

by

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ABSTRACT OF THE DISSERTATION

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Positive interactions between species are one of the most common and important forces that structure, maintain, and protect natural systems. The environmental conditions that cause mutualisms (reciprocal positive species interactions) to proliferate are suggested by theory to be rare, though mutualisms have been found in virtually every ecosystem that scientists have examined and strongly influence all levels of biological organization. Explaining the ubiquity of mutualisms has proved to be a difficult yet fundamental problem. To address these issues, I have examined the impact of the biotic and abiotic environment on mutualism and the impact of mutualism on communities using models and experiments. First, I examined how mutualism impacts the stability of communities. I modeled 164 communities with mutualism ranging from simple single-trophic level systems to complex four-trophic level systems, and compared them to 55 reference communities. Models simulations yielded novel results demonstrating that mutualism is not predominantly unstable when embedded in communities. Instead, mutualism within trophic levels tends to increase community stability. Second, I tested an economic model of mutualism that predicts the advantage gained by mutualist partners should change as essential nutrients become more or less available. I subjected an endosymbiotic mutualism between two protists, *Paramecium bursaria* and *Chlorella*, to a gradient of low to high essential nutrients in aquatic microcosms. Mutualistic association did not change with nutrient availability in contrast with model predictions. Finally, I tested a conceptual model of mutualism that predicts mutualism should be more common, and, therefore, a more important determinant of community properties in both biotic and abiotically stressed systems. I tested the abiotic portion of this model by subjecting complex communities containing model mutualists, *Paramecium bursaria* and *Chlorella*, to a temperature-stress gradient. I show that in stressed communities individual species abundances were higher when mutualism was present. I tested the biotic portion of the conceptual model by subjecting the same model mutualism to predation. Results yielded qualified support for model predictions with only the predator of *P. bursaria* exhibiting measurable effects on the incidence of mutualism. This dissertation demonstrates the importance and complexity of cooperative interactions in natural systems.

PREFACE

Professor Kessler, a well-known Russian zoologist and Dean of St. Petersburg University, concluded his address to the St. Petersburg Society of Naturalists with these words in January 1880, a few months before his death (quoted in Peter Kropotkin's *"Mutual Aid"* 1903):

> *"I obviously do not deny the struggle for existence, but I maintain that the progressive development of the animal kingdom, and especially of mankind, is favoured much more by mutual support than by mutual struggle. All organic beings have two essential needs: that of nutrition, and that of propagating the species. The former brings them to a struggle and to mutual extermination, while the needs of maintaining the species bring them to approach one another and to support one another. But I am inclined to think that in the evolution of the organic world – in the progressive modification of organic beings – mutual support among individuals plays a much more important part than their mutual struggle."*

Though Kessler did not have access to the accumulated natural historical data ecologists can draw on today, he helped to frame questions we are still trying to answer: Why are there so many mutualistic interactions in nature? What causes reciprocal positive interactions to arise and persist? Selection favors mutualism because even small rewards when paid for with low cost goods increase fitness and promote the evolution of mutualistic interaction. Game theory shows that with repeated interaction complete strangers will cooperate (Axelrod and Hamilton 1981). This happens even in unlikely scenarios because the advantages gained from cooperative or mutualistic interactions often outweigh costs.

Antagonistic views of nature have ruled the study of ecology from Darwin until recently. It is only in the last two decades that the importance of positive interactions to the organization and structuring of communities has been embraced (Bruno *et al.* 2003).

Organisms are not merely fighting “tooth and nail” to survive, but are also using relative advantages they may have in acquiring and/or processing resources in order to cooperatively trade with other individuals for nutrition, protection, or aid in reproduction. This occurs on an individual level but the consequences extend to populations and communities. Some classic examples of this include mycorrhizae and plants, ant fungal farmers, pollination mutualisms, cleaner fish, clown fish and sea anemones, algal endosymbioses, and more. Mutualism is so pervasive that fully 80% of all plants have mycorrhizae and many plants cannot persist in natural systems without them. The structure you see when you walk into a forest is the result of the force of mutualism in interplay with other important ecological processes. I am continually fascinated by the ubiquity of cooperation between organisms. I thank my family, friends and colleagues for their patience and support throughout my doctoral dissertation.

P. Timon McPhearson

03 June 2004

DEDICATION

I dedicate this work to my parents,

Paul F. McPhearson and Rebecca D. Taylor.

Your support has been the greatest constant of my life.

ACKNOWLEDGEMENTS

I thank the many collaborators whom I benefited from during my dissertation research, including Aabir Banerji, Kyle Bennett, Timothy Casey, Robert Cox, Lin Jiang, Yoko Kato, Peter Morin, Mathew Palmer, Owen Petchey, Jen Price, and Jeffrey Townsend, and also the many professors and colleagues who profoundly influenced my life including but not limited to Jim Applegate, Mark Bertness, David Ehrenfeld, Joan Ehrenfeld, Michael Pace, Steward Pickett, Peter Smouse, Michael Sukhdeo, Robert Trivers, and Peter Vayda. This work was supported in part by a Graduate Research Fellowship from the National Science Foundation.

TABLE OF CONTENTS

Chapter 1:

Mutualism within trophic levels increases stability in model communities

Abstract

Mutualistic interactions are commonly viewed as inherently unstable and thus evolutionarily short-lived. This view is largely due to the dynamical instability of mutualism described by modified Lotka-Volterra equations. Mutualisms (reciprocal positive interactions), however, do not exist in isolation. They are interaction subsets of a larger community of interactions and, as I show, do not cause communities to be predominantly unstable and thus are not predicted to be evolutionarily short-lived. Here I report the community-level effects of mutualistic interaction in model communities ranging from two species on one trophic level to six species on four trophic levels. I compared 55 reference communities containing no mutualism to 164 communities where mutualistic interactions replaced competitive or predator-prey interactions. Inter-trophic mutualisms (mutualism that exists between trophic levels) caused stability to decrease in 86% of communities compared to reference antagonistic communities. Intra-trophic mutualisms (mutualism that exists within a given trophic level), however, either increased stability (49% of comparisons) or had no effect (41% of comparisons) compared to reference communities. These results were robust across a wide range of trophic levels, species richness, and food web connectance. Additionally, increasing the number of trophic levels in a community increased the likelihood that a community

would be stable. Mutualistic interactions when located either low or high in the food web had no differential effect on stability. These models suggest that mutualism is not generally destabilizing, and one common type of mutualism, intra-trophic mutualism, may play a significant and underappreciated role in maintaining stability in ecological communities. I propose that stabilizing effects of mutualistic interactions in ecological communities may be an overlooked yet important driver of biodiversity.

Introduction

Mutualisms are found everywhere in nature, are often ecologically dominant, and strongly influence all levels of biological organization (Bronstein 1994b; Herre *et al.* 1999). Historically mutualism has been overlooked in ecology (Boucher *et al.* 1982; Bertness and Callaway 1994; Menge 2000; Stachowicz 2001; Bruno *et al.* 2003), despite growing body evidence for the ecological importance of these interactions (Weber 1966; Boucher 1985; Beattie 1985; Clay 1990; Hacker and Bertness 1994; Bronstein 1994a; Conner 1995; Costanza *et. al.* 1997; van der Heijden *et al.* 1998; Krieger et. al. 2000; Christian 2001; Omacini *et al.* 2001; Cardinale *et al.* 2002; Schmitt and Holbrook 2003; Bruno *et al.* 2003). Even as interest in mutualism has increased, ecologists are only beginning to study mutualism in the context of complex communities (Bertness and Callaway 1994; Hacker and Bertness 1996; Menge 2000; Bruno *et al.* 2003). To accurately assess the ecological impact of mutualism on community and ecosystem processes, studies should consider systems composed of minimally more than two species (Menge 2000; Bronstein *et al.* 2003).

A central focus of community ecology concerns the processes and mechanisms that govern stability/diversity relationships. Because mutualisms are a process in the organization of ecosystems (Burns 1993), understanding the relationship between stability and diversity requires knowing how mutualisms impact stability. Here I counter the conventional wisdom that regards mutualism as inherently unstable. I present evidence that mutualisms within trophic levels (hereafter termed intra-trophic mutualisms) can stabilize otherwise unstable communities, countering the notion that mutualisms are necessarily unstable due to runaway positive feedback. More diverse systems should include a greater number and diversity of interaction types (Tilman 1996), including greater number of mutualistic interactions. I hypothesize that stabilizing effects of more diverse systems (Doak *et al.* 1998; Lehman and Tilman 2000) may be in part due to the presence of intra-trophic mutualism. The evidence suggests that incorporating mutualism into investigations of diversity/stability issues will demonstrate how interspecific interactions, such as mutualism, can cause compensatory changes in the abundances of species and stabilize community dynamics (Tilman *et al.* 1998).

Although many theoreticians have begun to incorporate positive interactions into ecological models (Travis and Post 1979; Wolin and Lawlor 1984; Post *et al.* 1993; He and Gopalsamy 1997), most theoretical investigations have been largely concerned with population dynamics (Heithaus *et al.* 1980; Dean 1983; Addicott and Freedman 1984; Bertness and Callaway 1994; Douglas 1999) and have not examined how the presence of mutualism may affect other trophic groups in a community (Hoeksema and Bruna 2000). The population approach to examining the stability of mutualism has bolstered the impression that mutualisms are inherently unstable (Douglas 1999) and thus destabilizing

(Ringel *et al*. 1996). Yet, mutualisms persist over long periods of time (Pirozynski and Malloch 1975; Stubblefield *et al*. 1987; Chapela et. al. 1994; Pellmyr and Huth 1994; Pellmyr and Leebens-Mack 1999) and occur in nearly every community ecologists have examined (Bronstein 2001). If positive interactions are destabilizing, how can they be so ubiquitous and persistent?

This approach differs from previous modeling efforts in that I explore how mutualisms influence the overall dynamics of more complex systems. I modeled simple and complex communities with and without mutualistic interaction to evaluate the impact of positive interactions in systems of differing complexity. I addressed the following questions in the models: Does community stability change when mutualism is within trophic levels versus among trophic levels? Does community stability change when the mutualism is low versus high in the food web? Does the community response to mutualism depend on community complexity (number of species, connections, trophic levels)?

I constructed 55 reference communities along an approximate gradient of species richness and trophic complexity (Figure 1), varying connectance, the sign of each interaction (+, –, 0), and number of trophic levels, attempting to create a broad spectrum of the possible food web interactions found in nature. I modeled 51 communities with intra-trophic mutualisms (mutualism within a trophic level) and 113 communities with inter-trophic mutualism (mutualism among trophic levels; inter-trophic mutualisms were more likely to exist given the vertical configuration of model communities) by replacing antagonistic interactions in reference communities with mutualism. I varied the position of the mutualism treatment from low to high in the food web. I then constructed Jacobian community matrices, explicitly manipulating the elements of the Jacobian to reflect the interactions (predator/prey, competition, mutualism) specified by the hypothetical food webs. Elements of the Jacobian were chosen from uniform random distributions to create 1000 replicates of each web. I followed Pimm and Lawton (1977, 1978) for assignment of interaction strength. Predator effects on prey were randomly drawn from -10.0 to 0.0, prey effects on predators from 0.0 to 0.1, reciprocal competitors from -1.0 to 0.0, and mutualism from 0.0 to 1.0. All species had intra-specific density dependence (parallel simulations with no density dependence yielded quantitatively similar results). Competition and mutualism were both reciprocal and symmetrical (though I recognize that asymmetries exist in nature). Jacobian matrices were then analyzed to determine their maximum eigenvalues and their stability.

I modeled 219 community configurations, 164 of which had mutualistic species and 55 of which did not. The 164 communities with mutualists had identical food web structure to the 55 reference antagonistic communities (Figure 1) and each community was constructed to approximate realistic sets of community interactions. I simulated each model community 1000 times using randomly selected parameter values for the elements of the Jacobian matrix corresponding to each community. In each simulation I extracted the eigenvalues of the 1000 Jacobians to generate a frequency distribution of the largest (negative or positive) eigenvalues (Figure 2). Negative eigenvalues corresponded to stable dynamics, while positive values represented unstable dynamics (Figure 2).

Comparing communities with and without mutualism, while holding all else constant, allowed us to attribute differential effects to mutualism. When I plotted the frequency distributions of eigenvalues I found that all 164 communities with mutualism had at least some stable outcomes (no distributions were completely positive). Nine of 55 reference communities exhibited complete quantitative stability (eigenvalues for all 1000 trials < 0; May 1973). However, 71 of the 164 communities with mutualism had >50% quantitatively stable outcomes (proportion of negative values in the frequency distribution; see Figure 2). This suggests that communities with some mutualistic interactions can be stable.

Position

Further examination revealed that stability critically depended on the position of the mutualistic interaction in the food web. Mutualisms occur on a number of trophic levels and even between trophic levels (pollination systems, mycorrhizal infections, ant fungal gardens). For example, in a simple three-species, two-trophic level food web with two basal species and one common predator, mutualism dramatically affected the stability of the community (Figure 2). When competition among basal species was replaced with mutualism, the likelihood of obtaining a stable community increased by approximately 34% ($p<0.0001$; Figure 2a,b). Conversely, when mutualism replaced a predator-prey interaction, the probability of obtaining a stable community decreased by approximately 37% ($p<0.0001$; Figure 2a,c). Obvious differences observed in Figure 2 were representative of statistically significant differences in all of the analyses (Figure 3).

Complexity

I specifically examined four characteristic food webs along a gradient from low to high community complexity (here as increasing species richness, connectance, and number of trophic levels; Figure 3). I used a Kolmogorov-Smirnov test (Sokal and Rohlf 1995) to determine whether the frequency distributions of eigenvalues from community simulations with intra-trophic mutualism, inter-trophic mutualism, or no mutualism were identical. Intra-trophic mutualism significantly increased ($p<0.0001$) the number of stable outcomes for each comparison, while inter-trophic mutualism significantly decreased ($p<0.0001$) the chance that a community would be stable for all comparisons (Figure 3). This analysis was repeated for 10 other community comparisons with identical results regardless of community complexity. Statistically significant differences were actually detectable when distributions only differed by 2.0%, which may not be a large enough difference to be ecologically meaningful. I chose a 5.0% difference as a cut-off point for ecologically significant differences across all 219 communities, though 10.0% difference yielded qualitatively similar results.

I classified the 164 communities containing intra-trophic and inter-trophic mutualism according to whether they increased or decreased the proportion of stable outcomes by at least 5.0% or had no effect (< 5.0% difference) compared to their paired reference communities. For example, inclusion of mutualisms in the community depicted in Figure 2b increased the proportion of stable outcomes from that of Figure 2a by greater than 5.0%. I summarized results in a 2x3 contingency table of intra- and inter-trophic categories crossed with either increased, decreased, or no effect of community stability (Table 1) and analyzed it using a Chi-Square frequency analysis (SAS 8.2). Generally, intra-trophic level mutualism increased the likelihood of stability in a given community or had no response, whereas inter-trophic level mutualisms were likely to decrease the chance that a community would be stable ($\chi^2 = 89.64$; $p<0.0001$). These results were consistent across multiple community configurations, across a gradient of species richness and connectance, and across a range of trophic positions from mutualisms low to high in the community. Intra-trophic level mutualism was, therefore, a stabilizing force, especially in communities that were particularly unstable without mutualists.

I further examined community stability across the complexity gradient by calculating mean percent change in stability for all 164 communities with mutualism compared to reference communities and analyzing for effects of position (intra-trophic or inter-trophic level mutualism). An analysis of variance confirmed that mutualisms within trophic levels increased the frequency of stable outcomes regardless of changes in number of trophic levels, number of species, and number of interactions (Figures 4a-c; X^2=36763.0045, F= 69.55, p<0.0001). The number of trophic levels in a community had a significant effect on community stability, causing stability to increase with increasing number of trophic levels (Figure 4c; X^2=4715.4716, F= 10.67, p<0.0001). Trophic complexity did not interact significantly with the position of mutualism in the community (position*trophic level; X^2=1044.0477, F= 2.36, p=0.0976), revealing a robust differential effect of the intra- and inter-trophic level mutualism treatment on community stability. Number of species (Figure 4a; X^2=630.6749, F=1.19, p=0.3187) and number of connections (Figure 4b; X^2=738.6895, F= 1.41, p=0.2150) in the community had no effect on community stability.

If mutualisms decrease the variance of the eigenvalues in systems containing many predator-prey interactions, they could stabilize communities (Jorgensen *et al.* 2000), although if there are few predator-prey interactions, they may destabilize communities. I have not analyzed the simulations to account for “many” or “few” predator-prey interactions, but I did not notice reduced variance in the eigenvalue distributions when mutualism was present. Adding reciprocal positive interactions to a community could increase the average interaction coefficient and thus decrease the variance by Harte's measure of stability (Harte 1979) but the analyses have not addressed this directly. The potential for decreased variance caused by reciprocal mutualistic interaction to drive stability in natural communities is potentially testable.

This theoretical approach differs from classic models of mutualism (Vandermeer and Boucher 1978; Goh 1979; Addicott 1981; Dean 1983; Addicott and Freedman 1984; Post *et al.* 1985; Kumar and Freedman 1989; Wright 1989; Zaghrout 1991) in that I do not take a specific population approach to modeling mutualism. Instead I explored how mutualism affects the overall dynamics of communities, which allowed us to reveal more general features of mutualism across a broad range of community configurations ranging from simple two-species systems to four-trophic level systems.

Real food webs are far more complex than nearly all model communities, including those analyzed here. Few past modeling efforts have been able to suggest that embedding mutualism in a community will stabilize the community, though empirical data show mutualisms to be globally common (Bronstein 2001). Ringel *et al.* (1996) modeled a pollination mutualism embedded in a community of other interactions and found that mutualisms can be stabilizing and enhance the chance of community persistence. However, the general applicability of this result is uncertain because of the limited number of community types included in their analysis. The results based on extensive modeling demonstrate that mutualisms do not necessarily destabilize communities and instead they often promote stability when they are intra-trophic.

Additionally, increasing the number of trophic levels increases stability in communities with mutualism, and changes in stability were not driven by other measures of community complexity (i.e. number of species, connectance).

Mutualisms often buffer individuals or communities when stressed (Bertness and Hacker 1994; Bertness and Leonard 1997; Callaway *et al.* 2003), influence biodiversity and ecosystem functioning (van der Heijden 1998; Cardinale *et al.* 2002), and may play an important role as "insurance" when communities experience changing environmental regimes (Mulder *et al.* 2001; Christian 2001). Understanding how important mutualisms are to the maintenance and function of communities requires empirically testing theoretical results such as those shown here. Identifying whether mutualisms are intra- or inter-trophic in real-world community interaction webs may be crucial for understanding how mutualistic interactions influence overall stability in natural systems.

I further hypothesize that more diverse communities should by chance harbor a greater number of mutualists. If so, according to these simulations, increased stability in more diverse systems (Tilman 1996; Lehman and Tilman 2000) may be driven in part by the presence of intra-trophic mutualists. Intra-trophic mutualism may be an overlooked yet critical component of diversity/stability issues in ecology and conserving these foundation species may be critical for effective biological conservation.

Methods

Simulation

I modeled 219 communities by manipulating the elements of Jacobian matrices with signed interactions that corresponded to hypothesized food webs (Figure 1). I controlled the strength and sign of the interaction, specifying each interaction for each species in the web. From a uniform distribution I randomly assigned values from -10.0 to 0.0 for the negative effect of predator on prey, from 0.0 to 0.1 for the slight positive effect of prey on predator, from -1.0 to 0.0 for competition, and 0.0 and 1.0 for mutualism for each simulation. I ran each simulation 1000 times. Eigenvalues were extracted from the matrices and the maximum real part of the eigenvalue was calculated as $M_t = max (Re(E_t))$, where t is an individual simulation from 0 to 999, E is the extracted eigenvalue for each simulation (scalar), Re is the real part, max is maximum, and where M is the vector created from replicate runs. Frequency distributions of the extracted eigenvalues showed proportion of stable outcomes of simulations (Figure 2). Negative eigenvalues represented stable communities, while positive eigenvalues represent unstable communities.

Analyses

Chi-square analysis was calculated (SAS v.8.2) to test for differences between effects of intra- and inter-trophic level mutualisms on communities in a 2x3 contingency table (Table 1). I rejected the null hypothesis in favor of significant differences between effects of the two different mutualism treatments (χ^2=89.64, $p<0.0001$).

I tested for differential effects of intra- and inter-trophic level mutualism on community stability using a Kolmogorov-Smirnov test (SAS v.8.2) in a paired 1-way nonparametric ANOVA, which tests for differences in dispersion, location, and skewness between two distributions. The Kolmogorov-Smirnov test measures differences along the entire distribution of the two samples and thus is less sensitive to differences in location only (Sokal and Rohlf 1995). Each test was done on the unsigned differences between the relative cumulative frequency distributions of a community with mutualism and a community without mutualism. In all statistical comparisons the distributions were significantly different at $p<0.0001$ (Figure 3).

Differential effects of intra- and inter-trophic level mutualism on mean percent change in community stability were determined using ANOVA (SAS v8.2). Mean change in percent stability was calculated as the difference in stability from individual communities with mutualism compared to paired reference communities. Individual one-way ANOVAs assessed the effect of trophic level, number of species, and number of connections in each community on community stability.

Acknowledgements

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Figure Legends

Figure 1

All 219 communities are simulated from these 55 food web structures. Communities with mutualism differ only in the sign of the interaction, and communities are designed to reflect broad patterns in realistic natural assemblages of species. Model communities span a gradient of species richness and trophic complexity where number of species, number of connections, and number of trophic levels generally increase from top left to bottom right of Figure 1. Circles correspond to species. Species above are feeding on species below and horizontal relationships are competitive when not explicitly mutualistic. Within these community types I manipulated the sign of the interaction, changing many competitive relationships to intra-trophic mutualistic relationships and many predator-prey relationships to inter-trophic mutualistic relationships. Jacobian matrices were analyzed by explicitly manipulating the elements of the Jacobian based on these reference communities. All 219 communities annotated with percent stability outcomes from simulations are available as supplementary information.

Figure 2a,b

Histograms reveal shifts in the distribution of eigenvalues when mutualism was added to a typical reference community. $\overline{X}$ is equal to the mean proportion of positive eigenvalues from 1000 simulations, which is equal to the proportion of unstable outcomes. Negative values indicate stable outcomes and positive values indicate unstable ones (May 1973). Communities with intra-trophic mutualism (Figure 2b) caused an increase in the proportion of stable outcomes, shifting the distribution left (more negative) compared to antagonistic reference food webs (Figure 2a). Inter-trophic level mutualism (Figure 2c) generally shifted the distribution to the right, resulting in a decrease in the proportion of stable returns.

Figure 3

I used a Kolmogorov-Smirnov nonparametric test to detect differences between distributions of eigenvalues for reference communities and communities with mutualistic interactions. Kolmogorov-Smirnov tests were performed separately for both intra- and inter-trophic mutualism. The null hypothesis, that the two communities are distributed identically, was rejected at $p<0.0001$ for every comparison. The four community types depicted are representative of the general gradient in community complexity and the magnitude of the differences in all other community comparisons. $\overline{X}$ is equal to the mean proportion of positive eigenvalues from 1000 simulations, which is equal to the proportion of unstable outcomes.

Figure 4

Summarized changes in stability of communities are shown with intra- and inter-trophic mutualism compared to reference communities across increasing number of trophic levels (4a), number of species (4b), and number of connections (4c). Line graphs display calculated difference in mean stability between reference communities and communities with mutualism for all comparisons with single standard errors across a gradient of community complexity measures. Open circles depict differences of mean % stability between intra-trophic mutualism and reference communities; closed circles depict differences between inter-trophic mutualism and reference communities. Single trophic level comparisons are omitted for simplicity. Effects of inter- and intra-trophic mutualism on community stability are significant across a broad range of trophic levels (a), number of species (b), and species connections (c). Additionally, communities with more trophic levels generally have higher stability (ANOVA, $p<0.0001$). There was no significant effect of number of species or connections on community stability.

Table 1

Contingency table (2x3) of summarized effects of intra- and inter-specific mutualism on community stability. Mutualism either increased, decreased, or had no significant effect on stability when added to a community compared to reference communities. Chi-square analysis revealed significant differences ($X^2 = 89.64$, $p< 0.0001$) between communities with inter-trophic mutualism and communities with intra-trophic mutualism across all configurations regardless of community complexity.

Figure 1

Reference Model Communities

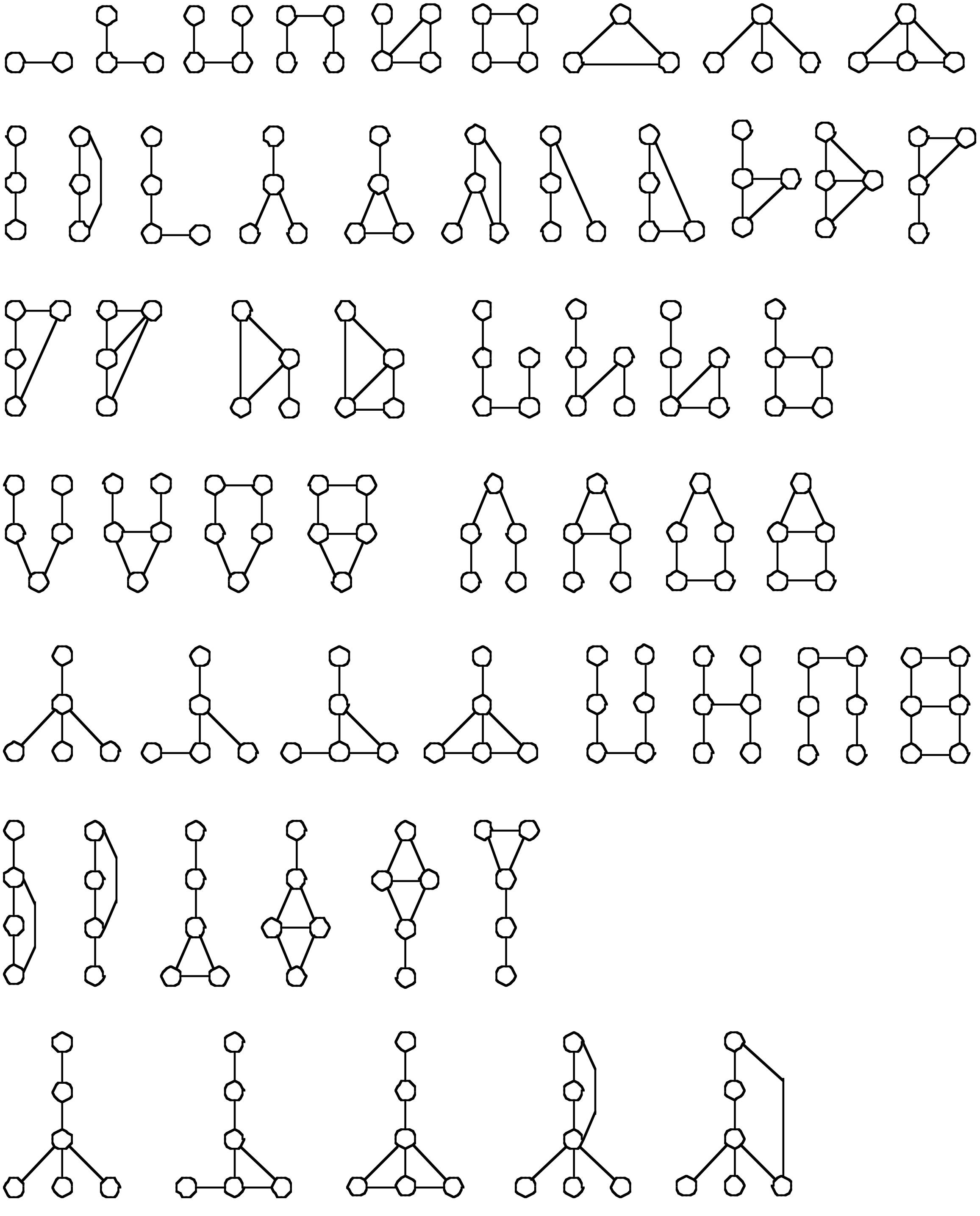


Figure 2

Eigenvalue Frequency Distribution

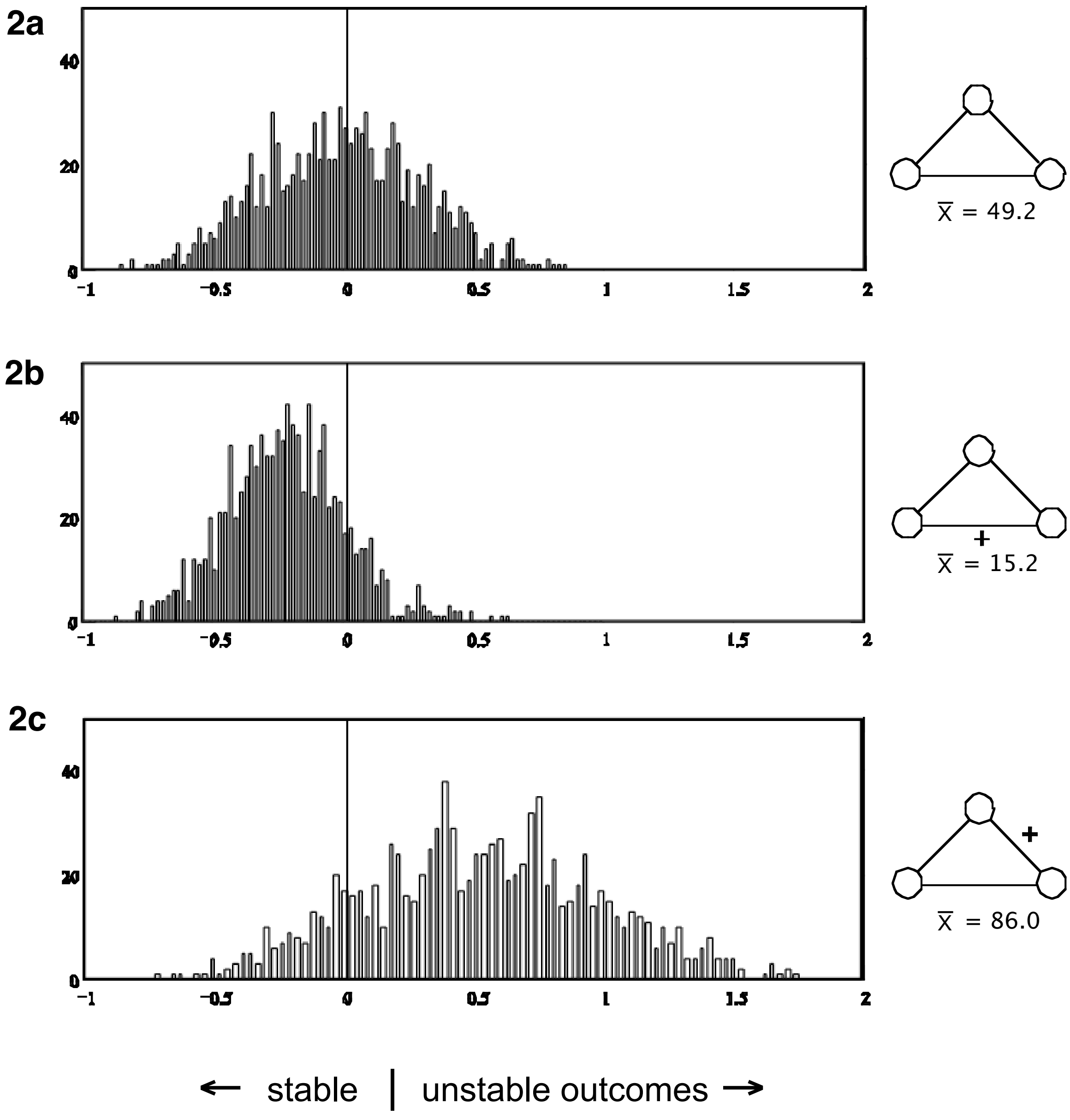
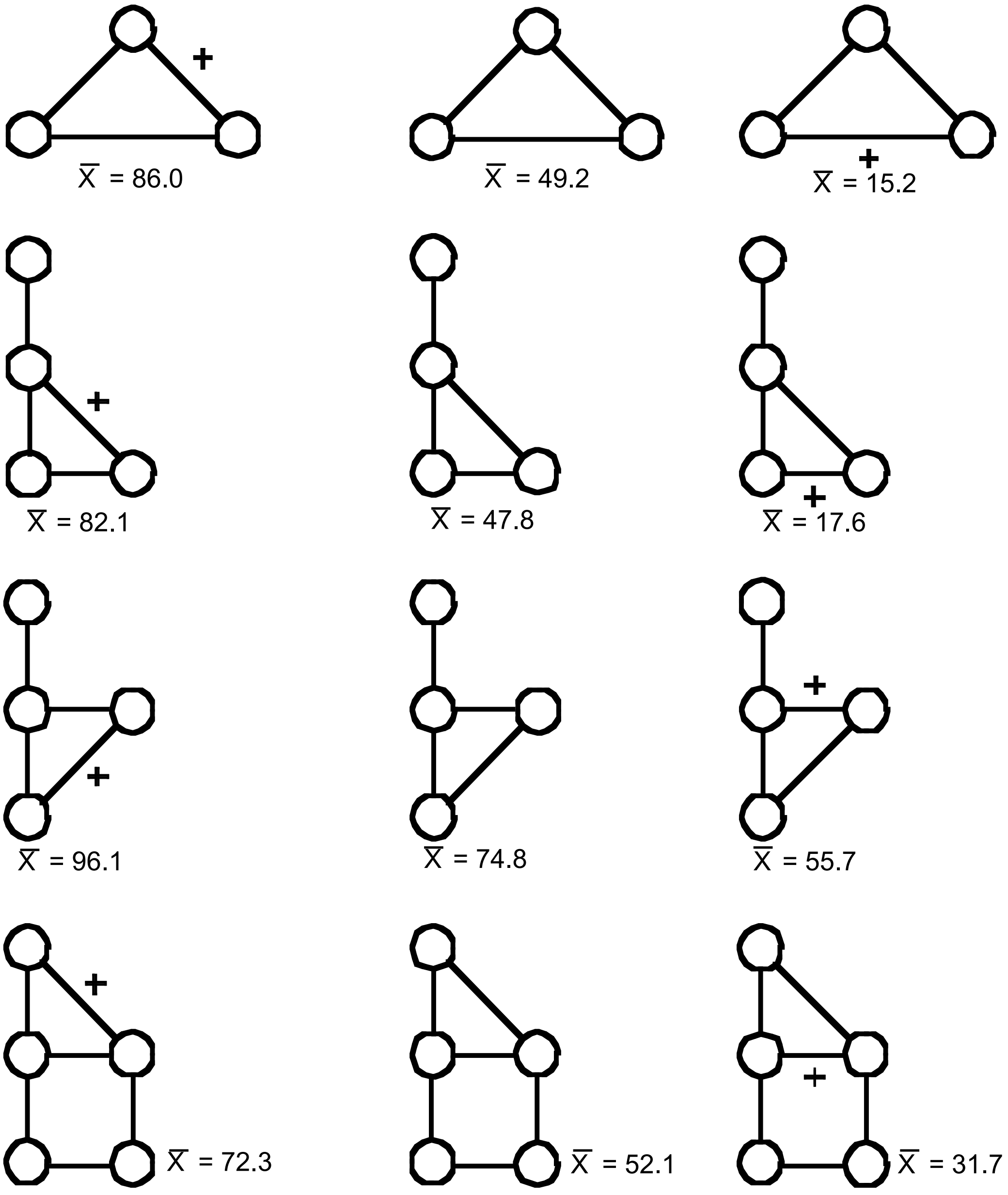


Figure 3

Kolmogorov-Smirnov Test for Difference Between Reference and Mutualism Communities



Inter-specific mutualism
decreases stability
$p < 0.0001$

reference
communities

Intra-specific mutualism
increases stability
$p < 0.0001$

Figure 4

Mean Change in Stability for Communities With Intra- and Inter-trophic Level Mutualism

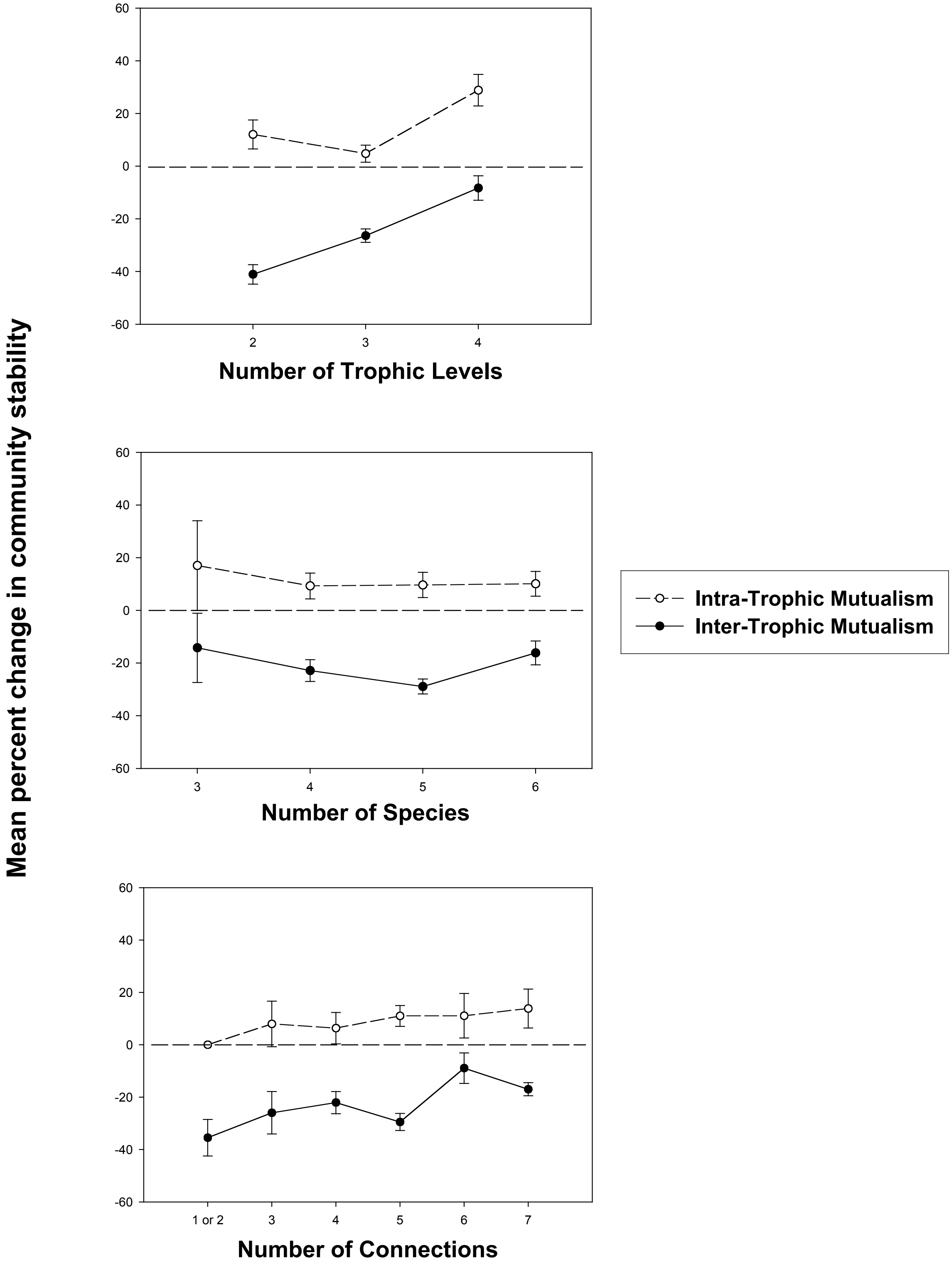


Table 1

Effects of Intra- and Inter-specific Mutualism

($\chi^2 = 89.64$; $p < 0.001$)

	Intra-specific	Inter-specific	Total
Increased proportion of stable outcomes	**25**	**13**	38
Decreased proportion of stable outcomes	**5**	**97**	102
No significant change	**21**	**3**	24
Totals	51	113	**164**

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Chapter 2:

Mutualism or slavery? Determining community dynamics in experimentally warmed food webs

Abstract

Mutualism is one of the most important forces structuring, maintaining, and protecting natural systems (Kropotkin 1903; Handel 1997; Callaway and Aschehoug 2000; Christian 2001; Callaway *et al*. 2002). Yet we still know little about how mutualism affects entire communities (Menge 2000; Callaway *et al*. 2002). With recent exceptions (Christian 2001; Stachowicz and Hay 1999; Bertness *et al*. 1999; Hacker and Bertness 1996), ecologists have not investigated the impact of mutualisms on complex systems, though theoretical implications for community-level effects have arisen from many studies (Bertness and Callaway 1994; Francis and Read 1995; Hacker and Gaines 1997; Clay and Holah 1999; Hoeksema and Bruna 2000; Stachowicz 2001; Choler *et al*. 2001; Cardinale *et al*. 2002; McPhearson and Jiang *in prep*). Here, I utilize a mutualism between two protists, *Paramecium bursaria* and *Chlorella*, to empirically test theory that says mutualism should be more common and, therefore, a more important determinant of community properties, in stressed systems. I show that in temperature stressed communities individual species abundances were higher when mutualism was present. I also show that mutualists may not exhibit simple reciprocally positive interactions as classically described (Smith and Douglas 1987; Wichterman 1986; Sleigh 1973). Instead, by assessing the effects on free-living and endosymbiotic (mutualistic) *Chlorella*,

I reveal an apparent case of host slavery, which determines major treatment effects in experimental communities. I thus demonstrate an often-overlooked conditionality inherent in many species interactions (Bronstein 1994a) by showing the community context that transforms mutualism into a host-slave interaction. This research highlights the need to define benefits to mutualistic partners at the individual, population, and community level.

Introduction

Though the ecological literature is replete with reports of positive associations between species (Darwin 1877; Kropotkin 1903; Boucher 1985; Bronstein 2001), only a handful of studies have demonstrated significant effects of mutualists, either positive or negative, on community and ecosystem-level properties (van der Heijden *et al*. 1998; Christian 2001; Cardinale *et al*. 2002). This is interesting given that observations of cooperative behavior date back at least to the historical writings of Herodotus, consequently influencing Aristotle to write about it in three different works (Egerton 1968; Boucher 1985). Here, I report how an endosymbiotic mutualism between *Paramecium bursaria* and its endosymbiotic alga, *Chlorella,* buffers other species in the community from negative effects of high temperature stress.

Single trophic-level studies have demonstrated that mutualists can have strong direct and indirect effects on other species in the community (Bronstein 1994b; Bertness and Hacker 1994; Callaway and Aschehoug 2000; Mulder *et al*. 2001; Callaway *et al*. 2002; Cardinale *et al*. 2002). To assess the potential impact of mutualism on communities with more than one trophic level, I constructed tri-trophic aquatic microbial communities with and without mutualists, and observed the responses of those communities over a temperature gradient that imposed different degrees of stress on the systems. I tested a prominent theory (Bertness and Callaway 1994; Hacker and Gaines 1997), which suggests that mutualism will have strong effects on communities under high environmental stress and weak effects under low environmental stress. I asked whether the existence of mutualism buffers mutualistic species from a general environmental stress. I also asked whether the presence of mutualism buffers responses of other species to stress that are not directly participating in mutualism. Finally, I asked whether effects of the mutualism are detectable for individuals, populations, and entire communities.

Microcosms have been used successfully to advance theory in a wide variety of fields in ecology (Gause 1934; Luckinbill 1973; McGrady-Steed *et al.* 1997; Kaunzinger and Morin 1998; Petchey *et al.* 1999; Naeem *et al.* 2000; Petchey *et al.* 2002). I used aquatic microcosms to build bacterial-based food webs with protists. Protists in this study have generation times that range from a few hours to one or two days and are easily cultured in 150ml glass bottles, which can house populations up to 10^7 individuals (Morin 1998). Given that many protists are very near the base of natural aquatic food webs, significant changes in productivity, resilience, structure, or diversity at this level may have profound effects on higher trophic levels.

Using the well-studied symbiotic relationship between *Paramecium bursaria* and its endosymbiotic alga, *Chlorella,* as the model mutualism, I built a series of similar communities with and without mutualism over a temperature stress gradient. Temperature was manipulated to alter the environmental conditions because organisms respond differently to temperature stress. The experiment was a 2x2x3 factorial design consisting of two community compositions (hereafter termed A or B), under two light regimes, (24hr dark or 12:12hr light:dark), incubated at three constant temperatures (22°C, 27°C, or 32°C) creating a benign, increased and severe warming environment, respectively. Treatments were replicated five times for a total of 60 microcosms. Temperature treatments provided a way to evaluate how systems with and without intact mutualisms respond to increasing thermal stress. The two communities (A and B) differ in whether there is an intact mutualism or not, and the light/dark treatment is a way to turn the mutualism on or off. Dark treatments controlled for mutualism presence in the light by eliminating *Chlorella* growth, essentially turning off the functioning of *Chlorella* and thus the mutualism. I also controlled for mutualism in Community B by replacing *P. bursaria* with a functionally similar species in Community A, *Paramecium tetraurelia,* which does not host endosymbiotic *Chlorella.* This treatment eliminated functioning mutualism in Community A. I considered *Paramecium bursaria* and *P. tetraurelia* functionally similar because they are 1) similar in body size and general feeding behavior, and results from previous monoculture experiments show that they have similar impacts on prey bacteria (R* values not significantly different, Tukey's test, p=0.05), 2) both *P. bursaria* and *P. tetraurelia* were the only bacterivores in the communities with some resistance to temperature stress (Figure 2), 3) both Paramecia were the competitively dominant species in their respective communities over the course of the experiment.

Community A was identical to Community B in light and dark treatments except for substituting *P. tetraurelia* for *P. bursaria* (Figure 1). I added free-living *Chlorella* to both Communities A and B (light and dark treatments) because, based on previous experience, I assumed that some *Chlorella* would be present in free-living form in systems with *P. bursaria,* and did not want to confound the presence or absence of autotrophs with the presence or absence of the mutualism. I monitored free-living *Chlorella* abundance throughout the experiment, though populations did not persist in dark treatments. Free-living *Chlorella* were cultured from the same strain of green *P. bursaria* used in the experiment and, therefore, all *Chlorella* in all treatments were an identical strain.

Densities of all protists were quantified twice weekly. Strong temperature treatment effects tended to accumulate over the course of the experiment. Given that questions were motivated by the effect of temperature stress on communities, I focused analyses on the final day (day 35) of the experiment to maximize the likelihood of observing organism responses to temperature stress.

Results

The complete absence of free-living *Chlorella* in Community B Light was a surprising result in the analyses (Figure 2). In this community, where free-living *Chlorella* might be at an advantage due to the specialized facultative mutualism with its host *P. bursaria*, free-living *Chlorella* were undetectable. Conversely, in Community A Light, which had no *P. bursaria*, free-living *Chlorella* dominated communities to such an extent that the medium was bright green with algal cells. Because I monitored *Chlorella* populations inside and outside of the host cell, I was able to quantify the effect of mutualism on both endosymbiotic and free-living *Chlorella*. I estimated the density of endosymbiotic *Chlorella* populations by multiplying cell counts of *Chlorella* living inside *P. bursaria* cells by the density of *P. bursaria* per ml. I found that total *Chlorella* populations in Community A were three to four orders of magnitude greater than *Chlorella* populations in Community B (Figure 3; ANOVA r^2=0.7838, X^2=25.0608, F=17.40, p<0.0001). This difference between communities with and without the putative mutualist *P. bursaria* demonstrates a striking cost in terms of total population size to *Chlorella* when living endosymbiotically within its host. This result suggests a novel example of host slavery that contradicts the assumed advantage of association in nearly all previous literature (Sleigh 1973; Wichterman 1986; Smith and Douglas 1987) on the *Chlorella*/*P. bursaria* mutualism.

Free-living *Chlorella*, in the absence of *P. bursaria*, were able to grow quickly and avoid significant predation by herbivores such as *Euplotes* (Community A Light). I found that free-living *Chlorella* were strongly correlated to the presence or absence of its host *P. bursaria*. When the mutualism between *Chlorella* and *P. bursaria* was functioning (Figure 2a, b; Community B Light), the growth of free-living *Chlorella* appeared to be completely suppressed, though endosymbiotic *Chlorella* populations remained abundant. Although the *Chlorella*/*P. bursaria* mutualism has been assumed to be somewhat facultative, since both species can grow in the absence of the other (Wichterman 1986; Smith and Douglas 1997), it now seems likely that *Chlorella* may not be able to free itself from its host even if the costs of association outweigh the benefits. Results suggest that *P. bursaria* may be effectively enslaving *Chlorella* cells, imposing a cumulative cost rather than benefit on *Chlorella* populations. With no other treatment differences between communities with or without *P. bursaria* to explain this pattern, I conjectured that *P. bursaria* was potentially controlling *Chlorella* growth via extracellular chemical inhibition.

To test this idea I performed a separate experiment where I cultured free-living *Chlorella* on sterile media, media inoculated with bacteria, media with bacteria and *P. bursaria*, and media conditioned by *P. bursaria* (*P. bursaria* grown to carrying capacity, then filtered out with 0.2 μm filter). Bacteria existed in all experimental treatments though bacterial levels in the media inoculated with bacteria treatment were experimentally elevated compared to other treatments in order to examine the response of *Chlorella* to increase bacterial densities. Two biotic factors directly affected *Chlorella* abundance: the presence of bacteria and the presence of *P. bursaria*. Bacteria and *Chlorella* appear to form a decomposer/producer positive feedback in the main experiment (Figure 2) and in the extract experiment (Figure 4). Free-living *Chlorella* grew twice as well on bacterized media than it did on all others, but grew only half as well on media with *P. bursaria* (Figure 4). I show that when *Chlorella* was inoculated on filtered media (all previously resident *P. bursaria* filtered out), the extract from *P. bursaria* cultures still managed to reduce *Chlorella* densities (Figure 4; Tukey's test, p=0.05). *Chlorella* populations growing with *P. bursaria* in monoculture were only able to establish at half the density (Figure 4) of cultures containing no *P. bursaria* (ANOVA r^2=0.4068, X^2=0.2277, F=3.23, p=0.0273), confirming that *P. bursaria* is involved in growth suppression of *Chlorella*.

Media filtered from *P. bursaria* cultures had bacteria in it and should have elicited a positive *Chlorella* growth response similar to the simple bacterized media (Figure 4). The significant difference between *Chlorella* growth on the extract media and the bacterized media suggest that *P. bursaria* are suppressing *Chlorella* growth via chemical inhibition. Additionally, bacteria contaminated sterilized media once *Chlorella* were added. Bacteria appear to benefit *Chlorella*, but only when experimentally elevated (Figure 4; bacteria treatment), thus adding confidence to the inhibitory effect of *P. bursaria* on *Chlorella*. I cautiously conclude that this mutualism is maintained by host control of the endosymbiont via extracellular chemical growth suppression, though clear evidence of a chemical mechanism was beyond the scope of this experiment. *Paramecium bursaria* regulation of algal population size is probably by host repression of symbiont growth and division rates (Wichterman 1986), though this has mostly been investigated in a *Chlorella* symbiosis with green hydra (McAuley 1985; Neckelmann and Muscatine 1983). The mechanism of host suppression is potentially achieved by restricting the supply of nitrogen to *Chlorella* (Rees 1986), though experiments were not able to assess *in situ* nitrogen concentration.

In the temperature stress experiment it may be important that bacterivores/herbivores were not able to significantly graze down free-living *Chlorella*. Since there was not a strong herbivore in the community, avoidance of significant herbivory may be of sufficient benefit to *Chlorella* to maintain its participation in the symbiosis in natural populations. I added *Euplotes* to all cultures as a strong herbivore specifically to control free-living *Chlorella* populations (Figure 1), since previous experiments showed that *Euplotes* could subsist on *Chlorella* alone (unpublished data). However, *Euplotes* did not manage to control free-living *Chlorella* populations in Community A Light though it was observed frequently ingesting *Chlorella* cells throughout the experiment. *Euplotes* was driven to very low densities at high temperatures (Figure 2), going extinct or nearly extinct (extinct in all replicates but one) in non-mutualism communities (Community A Light and Community B Dark, respectively). There was a trend toward increased persistence in communities with mutualism over communities without mutualism at 22° and 32°. Overall *Euplotes*' response was most strongly influenced by increased temperature (ANOVA $r^2=0.7760$, $X^2=8.7251$, $F=35.13$, $p<0.0001$). When analyzed for treatment effects, *Euplotes* showed a strong interaction between negative effects of temperature and Community type (A or B) (ANOVA temp*type, $r^2=0.7995$, $X^2=2.0741$, $F=10.05$, $p=0.0007$).

Paramecium caudatum was strongly negatively affected by increased temperatures, approaching extinction at 32°C (ANOVA r2=0.8133, $X^2=8.1454$, $F=50.48$, $p<0.0001$). However, *P. caudatum* was buffered from this negative effect of temperature in communities with mutualists (Community B), even though *P. caudatum* was not directly involved in the mutualism (Figure 2a). This positive effect is significant at all temperatures (ANOVA $p=0.0011$) and is consistent in light/dark comparisons in Community B (Figure 2a). In both Community B Light and Dark, free-living *Chlorella* populations are virtually extinct while *P. caudatum* persists (Figures 2a, b). The strong negative effect on *P. caudatum* abundance in Community B Light is likely due to dense algal growth. Previous experiments (Petchey *et al.* 1999 unpublished pilot data) have demonstrated protist susceptibility to algal blooms since bacterial decomposition of dead algae depletes dissolved oxygen and leads to the death of sensitive microbes. The presence of dense free-living *Chlorella* populations in Community A Light may have induced the decreased abundance of sensitive *P. caudatum* in Community A.

The lack of any differences between *P. caudatum* abundance in light/dark comparisons in Community B (Figure 2a) is interesting given the dramatic differences in *P. bursaria* growth. This implies that competition between *P. caudatum* and *P. bursaria* is of minor significance and that *P. caudatum* abundance is likely limited more by factors such as high temperature and the favorable effects of having *P. bursaria* in the web to potentially control free-living *Chlorella*. When the relationship between *P. bursaria* and *Chlorella* is experimentally severed (Figure 1; Community A Light and Community B Dark), the main effects on bacterivore abundance are caused by temperature and light. Temperature is the only factor affecting species abundance in Community B except for the strong light effect on *P. bursaria* abundance (ANOVA; r^2=4740, x^2=12.4843, F=16.67, p=0.0004). The positive light effect on *P. bursaria* abundance is predicted from the advantage it gained in association with autotrophic *Chlorella*, which may have caused it to be relatively unaffected by 32°C temperatures.

Light/dark treatments significantly influenced the persistence of mutualists. *Paramecium bursaria* grew to higher densities in the Community B Light treatment with its endosymbiont *Chlorella* (ANOVA; r^2=0.4740, p=0.0004), than it did in the dark (Smith and Douglas 1987). Further analysis confirmed that this result was most pronounced at higher temperatures (27° and 32°C). *Paramecium bursaria* grew slowly and reached lower densities at low temperatures. Similarly, *Chlorella* grew best at higher temperatures and light levels (Figure 2). When these two mutualists occurred together, *P. bursaria* benefited dramatically. No *Chlorella* persisted in dark treatments, thus *P. bursaria* grew poorly in dark treatments, and only endosymbiotic *Chlorella* persisted in treatments with its host (Figures 2a, b; Community B Light). In Community B Dark, where no *Chlorella* persisted, *P. bursaria* was sensitive to elevated temperatures. The negative effect of temperature on *P. bursaria* was buffered by mutualistic association with *Chlorella* in Community B Light (Figures 2a, b).

Overall, *P. bursaria* and *P. tetraurelia* respond similarly to temperature stress. *Paramecium tetraurelia* grew best at higher temperatures (Figure 2a; 27°C, 32°C), similar to *P. bursaria*. These two species were the only organisms that were somewhat resistant to extreme temperature stress (32°C), demonstrating a higher thermal growth optimum than other protists in the system. This further supports the contention that these two species are functionally similar, with the exception of the tendency to associate with *Chlorella*. All other species in the communities were significantly negatively affected by elevated temperature.

The predator, *Actinosphaerium*, failed to establish in 41/60 microcosms. Therefore, observations of top-down predator effects were limited to the small set of communities where it became established (Figure 2). Analysis of variance revealed no effect of *Actinosphaerium* on any community, and regression analysis showed only *P. tetraurelia* experienced net negative effect on population growth when *Actinosphaerium* was present (Community A Light 22°C and 32°C, only). *Actinosphaerium* was therefore statistically controlled in subsequent analyses by treating its abundance as a covariate.

The most important determinant of species responses to treatments in the communities was the presence or absence of free-living *Chlorella* populations, which persisted in only one treatment, Community A Light (Figure 2b). Differences between Community A Light and Community B Light were dramatic in some cases. Bacterial species, however, appear to benefit from the presence of *Chlorella* (Figure 2b) with increased growth in communities with free-living *Chlorella*. Bacteria reached higher densities both in higher temperatures and in communities where dense *Chlorella* existed (ANOVA; r^2=0.5751, light effect p=0.0384, temperature effect p=0.0016, temp*light p=0.0551). I attribute the strong light effect on bacteria to increased growth of *Chlorella*, and the temperature effect was caused both by an increase in growth rate at higher temperatures and due to positive feedback with increased *Chlorella* growth at higher temperatures in Community A Light. These results demonstrate strong evidence of positive association between bacteria and *Chlorella*.

In addition, I found higher bacterial densities in communities with *Chlorella* than communities without *Chlorella* in the extract experiment, as well as higher *Chlorella* densities in communities with bacteria than without (Figure 4). These data support evidence (Naeem *et al*. 2000) of positive interaction between the decomposer/producer groups. This may be one of the strongest and most important structuring forces in model communities, as this basal portion in aquatic food webs governs many ecosystem properties (Naeem *et al*. 2000). The feedback allows *Chlorella* to further dominate Community A Light jars by increasing bacterial abundance, contributing to increased *Chlorella* abundance (Figure 3). Though all bacterivores were able to ingest free-living *Chlorella* cells (I observed every bacterivore species with *Chlorella* in their food vacuoles), free-living *Chlorella* seem to be associated with decreasing rather than increasing bacterivore abundance.

Conclusion

Since May's theoretical work on mutualisms (1976a,b; 1982), ecologists have progressed considerably towards developing an ecological framework that includes mutualistic interactions (Bruno *et al.* 2003). Though a handful of studies have begun to demonstrate community-level effects of mutualism, there is still little evidence to indicate the relative importance of mutualism compared with antagonistic interactions for determining community dynamics (Olofsson *et al.* 1999; Menge 2000). Results clearly show that the presence or absence of mutualism governs species abundance and persistence in this system. Though most species were directly competing, the presence or absence of the functioning *P. bursaria*/*Chlorella* mutualism was the most significant determinant of species abundance and persistence in microcosms.

Endosymbioses are often assumed to be mutualistic even though ecologists have not always measured benefits at the individual and population level for both host and endosymbiont (Douglas and Smith 1989). I show here that *P. bursaria* may act to control free-living *Chlorella* by inhibiting growth. This is a novel result illustrating that a presumed classical mutualism acts instead as a host/slavery interaction in communities. Results underscore theoretical work (Bronstein 1994b), showing the sign of many species interactions is conditional on the context of the surrounding community. These results demonstrate how conditionality in mutualistic interactions affects other trophic groups in the community.

Harsh physical conditions and/or intense consumer pressure are predicted to promote positive interactions, specifically neighbor amelioration of environmental stress and/or consumer pressure (Bertness and Callaway 1994). Conversely, where environmental stress or consumer pressure is benign, positive interactions should be rare and have less effect on community properties. Predictions for other (non-mutualistic) species in the community are not so clear; mutualism may have positive or negative direct or indirect effects on other species in the community. Though demonstrated in a number of studies (Hacker and Gaines 1997; Callaway *et al.* 2002), controversy still exists as to whether increased mutualism in stressed communities is a general property of ecological communities (Schwartz and Hoeksema 1998). These results support Bertness and Callaway's (1994) theory by demonstrating that positively interacting organisms are at an advantage when faced with environmental stressors. I extend this theory by linking the mutualists to the larger community, directly monitoring effects on producers, competitors, and predators.

It is well known that different environmental conditions are likely to change the strength and even the sign of species interactions (Bronstein 1994a), in this case changing reciprocal mutualism to host parasitism. However, though not explicitly tested for in the main experiment, under heavy predation by a strong herbivore, I suspect *Chlorella* populations may fare better within the host as a predator avoidance mechanism (unpublished data). The *Chlorella*/*P. bursaria* complex is potentially evolving into a tighter, more dependent association such as the fungal/algal association of lichens.

Communities are likely to have complex responses under high temperature stress (Petchey *et al.* 1999). Results show that species responses were relatively predictable with most species declining with severe temperature stress. However, communities showed more stable abundance of bacterivores when mutualists were present than when absent. Mutualism provided, through direct suppression of harmful *Chlorella* blooms, a mechanism for ameliorating an otherwise harsh environment in Community B Light created by the high temperature and light which fueled runaway *Chlorella* growth in Community A Light. The presence of mutualism positively affected the abundance of many species in model communities, including a species not directly involved, *P. caudatum*. *Paramecium bursaria* was directly buffered from high temperature stress by participating in mutualism with its endosymbiont *Chlorella*. I found evidence of multiple positive interactions, direct and indirect, of *Chlorella* on bacteria, of bacteria on *Chlorella*, of *P. bursaria* on bacterivores, and *Chlorella* on *P. bursaria*. If positive interactions are major structuring forces in communities (Bertness and Leonard 1997), then understanding and restoring natural systems requires a community-level perspective that more explicitly includes mutualism (Bruno *et al*. 2003).

Environmental warming is already causing shifts in species distributions, affecting the way species interact in communities (Harte and Shaw 1995; Parmesan *et al*. 1999; Root *et al*. 2003). The functional role of mutualism in warmed model communities was over-all positive, providing a habitat more tolerable for particular species. The potential for mutualism to buffer some species from harsh warming conditions provides a more optimistic view of predicted species extinctions (Walther *et al*. 2002) caused by a warmed earth.

Methods

Model mutualism

Chlorella is the dominant algal symbiont in freshwater hosts, including ciliated protozoa, sponges, coelenterates, neorhabdocoel turbellarians and bivalve mollusks (Smith and

Douglas 1987), and lives within the protoplasm of *P. bursaria* in modified food vacuoles. The model mutualism used here is an endosymbiotic relationship between *Chlorella* and *P. bursaria*. Endosymbioses are common and often crucial relationships in ecological systems. *Zooxanthellae*, the foundational dinoflagellates that fuel coral reef ecosystems, operate similarly to freshwater *Chlorella* (Douglas and Smith 1989). *Chlorella* receives nutritional benefit in the form of nitrogen and trace minerals as well as protection from potential predators (Douglas and Smith 1984), thus maintaining a population within *P. bursaria* hosts, using *P. bursaria* to mediate harsh environmental conditions. *Paramecium bursaria* receives benefit via excreted photosynthetic products from *Chlorella,* namely maltose, a sugar which allows *P. bursaria* to persist under low nutrient conditions and to thrive under high light conditions conducive to *Chlorella* growth (Weis 1974a, 1969; Pado 1965; Karakashian 1963; Allen 1988). *Paramecium bursaria* also has increased resistance to a common predator, *Didinium nasutum*, when involved in mutualism with *Chlorella* (Berger 1980).

Microcosms

Culture medium was an autoclaved mixture of 2.5g of soil, 0.55g of crushed protist pellet (Carolina Biological Supply), and 1500ml well water. Microcosms were 250ml bottles containing 100ml of growth medium and one wheat seed for additional nutrients (Lawlor and Morin 1993; McGrady-Steed *et al.* 1997). I established two controls for the mutualism treatment, a dark control for Community B Light and a biological control for Community B Light by substituting *P. tetraurelia* for *P. bursaria* in Community A Light. Analyzed treatments were a community with mutualism (Figure 1; Community B Light) and two communities without mutualism (Community B Dark; Community A Light). Communities were housed in Percival incubators under two light regimes 12:12hr light:dark cycle and 24hr dark cycle constituting two light treatments, Light and Dark, respectively. Communities A and B had identical species composition (bacteria, producers, primary consumers, and secondary consumers) except one contained *Paramecium tetraurelia* that lacked endosymbiotic *Chlorella* (Community A), and the other contained *Paramecium bursaria* and the algal endosymbiont, *Chlorella* (Community B). All communities were inoculated with identical initial densities of each species. Additions of bacteria (*Bacillus subtilis, B. cereus, Serratia marcescens,* and *Proteus vulgaris)* were lagged one day to bacterivore addition to ensure adequate prey for consumers. Media was partially replaced (7%/wk) to replenish nutrients and maintain volume. No significant contamination by eukaryotes occurred. A small number of microflagellates occurred in all cultures and were monitored, though they remained at low levels.

Free-living *Chlorella* cultured from burst *P. bursaria* were used to inoculate all communities maintained in the light. *Paramecium bursaria* cells containing endosymbiotic *Chlorella* were burst on glass plates with a fine sterile needle and rinsed with sterilized media into culture jars.

The predator, *Actinosphaerium*, was added after adequate prey established, but failed to persist in 41/60 bottles though it was added again in week two (and eventually factored out of subsequent analyses as a covariate).

In the second experiment a standardized amount of free-living *Chlorella* was added to each different media: media with bacteria, extract media from filtered *P.*

bursaria media with bacteria, media with *P. bursaria* and bacteria, and sterile media. *Chlorella* were counted once when populations had reached carrying capacity. Though endosymbiotic cell counts can vary from 0 to 600 cells per *P. bursaria* host, illuminated cultures averaged 104 cells/*P. bursaria* host (mean taken from 10 cells, ranging from 60 to 161 cells/*P. bursaria*) and dark cultures had 0 cells/host. When calculating endosymbiont population size, I used 100 cells/*P. bursaria* cell as the average, though if 400 cells/host is used, significant differences between free-living and endosymbiotic populations were unchanged.

Temperature stress and culturing

Stress was simulated by elevated temperature. I incubated each community type (A or B) at three constant temperatures (22°, 27°, and 32° C). Each temperature treatment was exposed to either 24hr dark or 12:12hr light:dark cycle. I cultured replicates of temperature and light/dark treatments in six incubators. A factorial design of 2x2x3 with five replicates totaled 60 bottles. Locations within incubators were randomized and changed twice weekly to minimize incubator effects.

Community monitoring

The abundance of all protists was determined twice weekly by direct microscopy of small samples, removed without replacement. Sample volumes were estimated by weight. Bacteria estimates were made on day 35 from plate counts, which provide repeatable estimates of total bacterial density. Density is reported as number cells/ml for each species. Only day 35 data is reported here to highlight cumulative effects of warmed treatments.

Data analyses

Effects of temperature and light/dark regimes on the abundance of species (*Actinosphaerium, P. bursaria, P. tetraurelia, P. caudatum, Euplotes, Chlorella,* and bacteria (grouped as single trophic group)) were determined with multiple comparisons for each community type (A or B). ANCOVA (PROC GLM; SAS v8.2) was used for analysis in Community A, as *Actinosphaerium* persisted in enough replicates to have top-down predator effects on replicates where it existed; however, *Actinosphaerium* persistence was idiosyncratic and was thus factored out of subsequent analyses as a covariate. ANOVA (PROC GLM; SAS v8.2) was used to test for differences between temperature treatments, light/dark treatments, and all interactions terms in Community A. Tukey's tests were used to provide conservative comparisons for treatment affects on abundance data where appropriate (p=0.05).

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Figure Legends

Figure 1

Communities were two types (A and B; Figure 1) with multiple functional groups similar to recent microcosm research (Kaunzinger and Morin 1998; Petchey *et al*. 1999; Petchey *et al*. 2002) differing only in the identity of a single species, *Paramecium tetraurelia* in Community A, *Paramecium bursaria* in Community B. Community A was a non-mutualist community inoculated with a functionally similar species, *P. tetraurelia*, *Paramecium caudatum* (bacterivore), *Euplotes* (bacterivore/herbivore), *Actinosphaerium* (predator), free-living *Chlorella*, and bacteria (*Bacillus subtilis, Bacillus cereus, Serratia marcescens,* and *Proteus vulgaris*) and microflagellates. Community B was identical to Community A except Community B was inoculated with *P. bursaria* (light treatment) harboring an average of 100 endosymbiotic *Chlorella* per *P. bursaria* cell, instead of *P. tetraurelia*, thus adding a mutualism to an otherwise antagonistic community. Community B Dark was identical to Community B Light except that I added bleached *P. bursaria* to Community B Dark. Bleached *P. bursaria* in Community B Dark had zero endosymbiotic *Chlorella* (identical strain of *P. bursaria* reared in the dark for two months prior to addition). All replicates were free of significant contamination during the entire experiment. No *Chlorella* persisted in any dark cultures.

Figure 2

Individual species plots by treatment show Community A and B Light treatments on the left and Community B Light and Dark on the right of each panel. Bars represent individual treatments (A or B is Community A or B; 22, 27 or 32 are temperature treatments; L/D correspond to Light/Dark treatments). Plots are logged mean protist and bacterial densities for each treatment from the end of the experiment (day 35) with single standard error bars. Community B Light plots in left and right panels are identical. Axes are identical on all panels though scales are only similar where possible.

Figure 3

Log plots of endosymbiotic *Chlorella* cells/ml in Community B Light (white bars) and free-living *Chlorella* in Community A Light (black bars). Endosymbiotic *Chlorella* were calculated from mean number *Chlorella* cells per *P. bursaria* cell. Counts were by microscopy from lysed *P. bursaria* cells. Endosymbiotic and free-living *Chlorella* are from identical lab strain. *Chlorella* in Community A Light were three to four orders of magnitude greater than the density of *Chlorella* in Community B Light.

Figure 4

Bar graph shows results of free-living *Chlorella* when cultured on different media: media with bacterial addition (Bacteria), media from filtered *P. bursaria,* media with resident bacteria (Extract), media with *P. bursaria* and resident bacteria (*P. bursaria*), and sterile media (Sterile). *Chlorella* abundance was assessed once when populations had reached carrying capacity. Asterisk shows significant difference between Bacteria treatment and all other treatments.

Figure 1

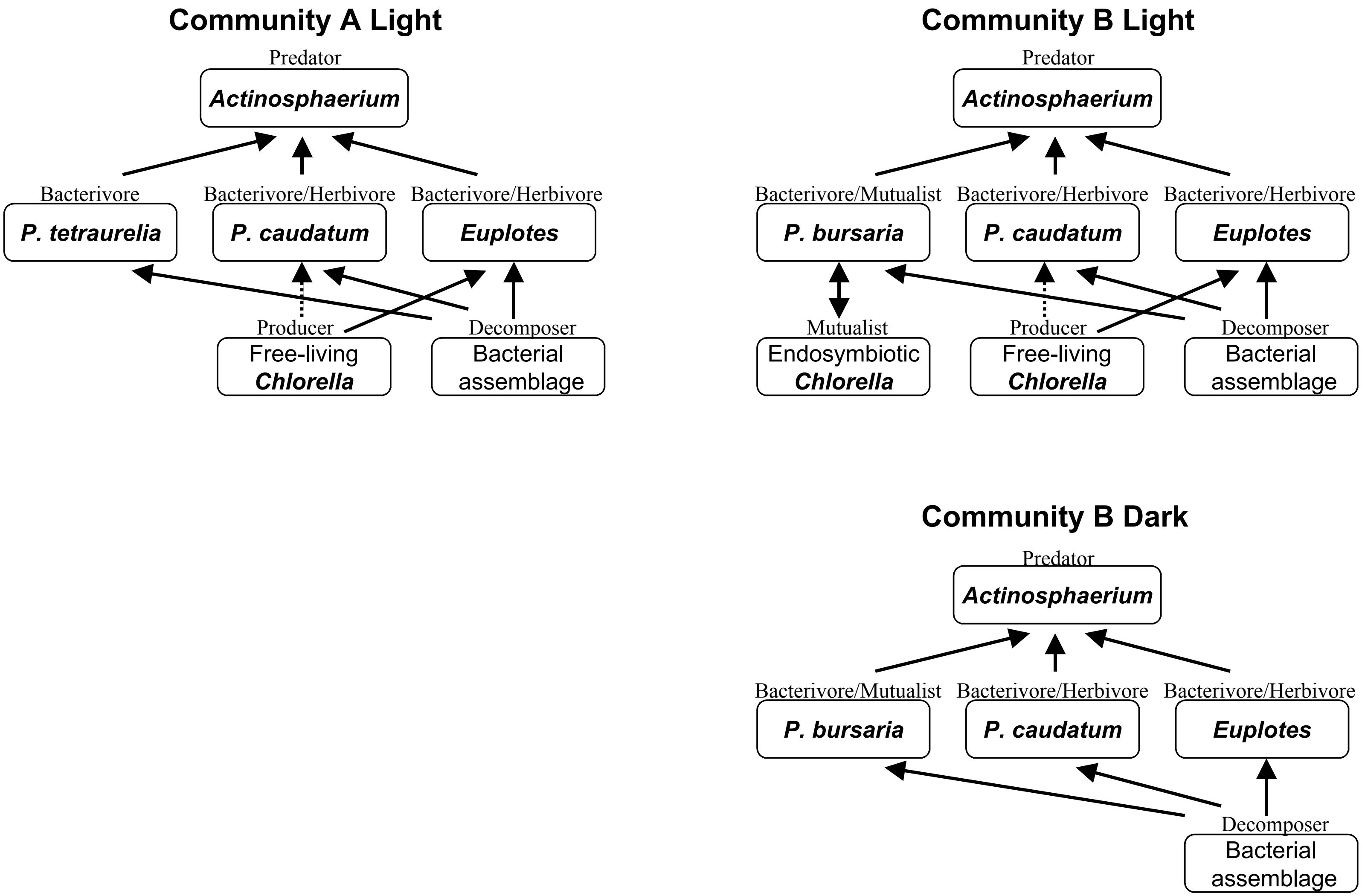
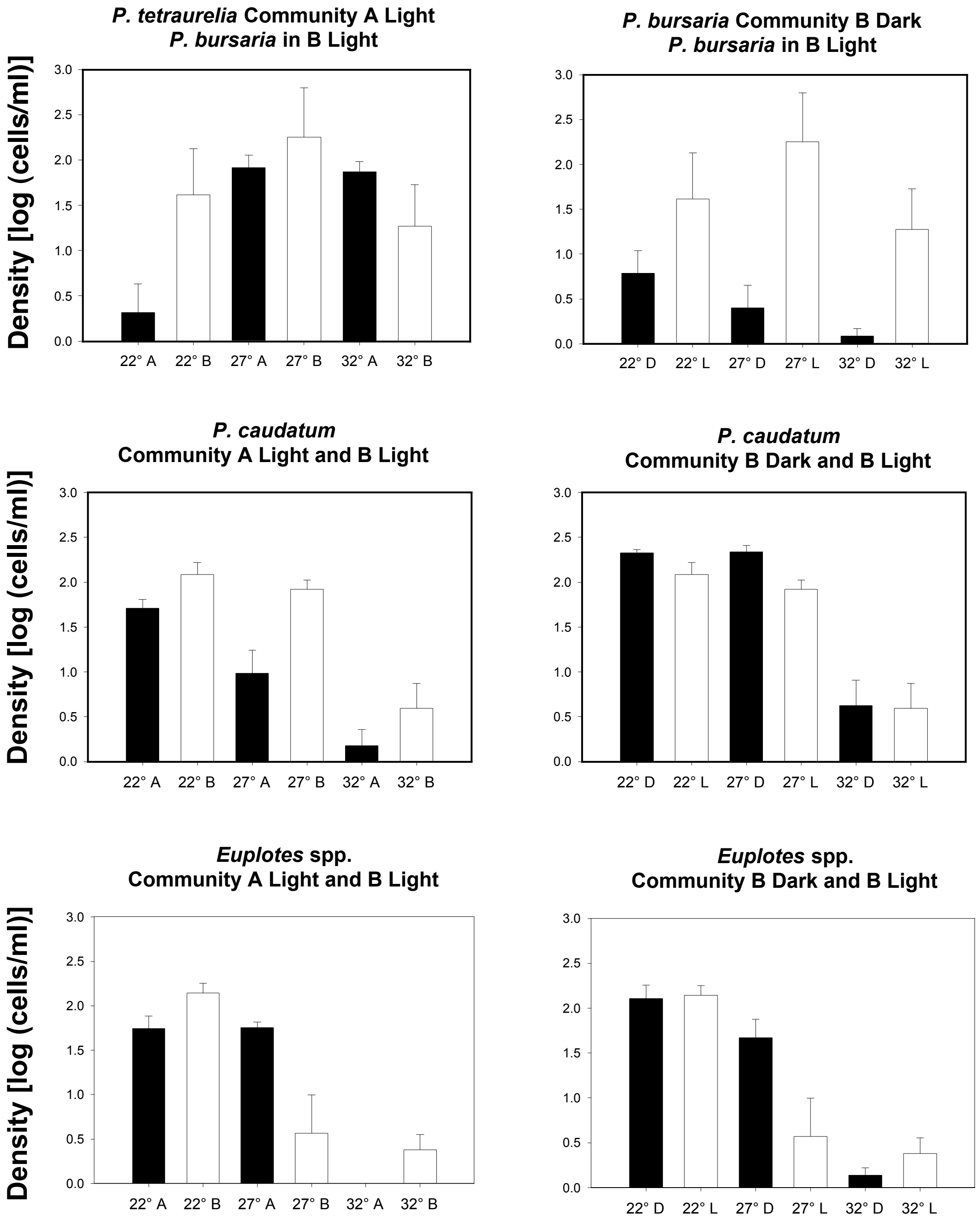

Community A Light
Predator
Actinosphaerium
Bacterivore
P. tetraurelia
Bacterivore/Herbivore
P. caudatum
Bacterivore/Herbivore
Euplotes
Producer
Free-living Chlorella
Decomposer
Bacterial assemblage
Community B Light
Predator
Actinosphaerium
Bacterivore/Mutualist
P. bursaria
Bacterivore/Herbivore
P. caudatum
Bacterivore/Herbivore
Euplotes
Mutualist
Endosymbiotic Chlorella
Producer
Free-living Chlorella
Decomposer
Bacterial assemblage
Community B Dark
Predator
Actinosphaerium
Bacterivore/Mutualist
P. bursaria
Bacterivore/Herbivore
P. caudatum
Bacterivore/Herbivore
Euplotes
Decomposer
Bacterial assemblage

Figure 2a



P. tetraurelia **Community A Light**
P. bursaria **in B Light**

P. bursaria **Community B Dark**
P. bursaria **in B Light**

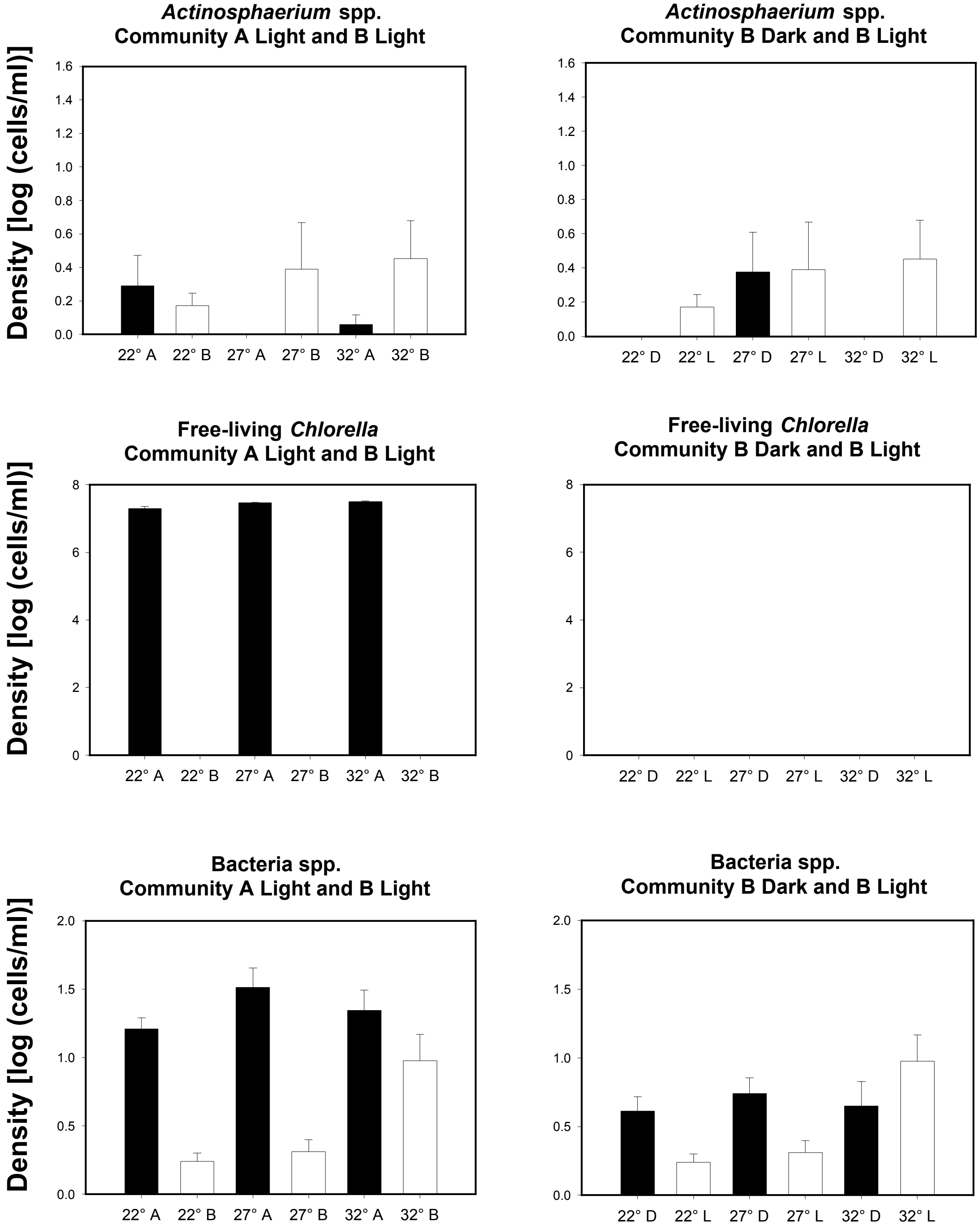
P. caudatum
Community A Light and B Light

P. caudatum
Community B Dark and B Light

Euplotes **spp.**
Community A Light and B Light

Euplotes **spp.**
Community B Dark and B Light

Figure 2b



Actinosphaerium spp.
Community A Light and B Light

Actinosphaerium spp.
Community B Dark and B Light

Free-living *Chlorella*
Community A Light and B Light

Free-living *Chlorella*
Community B Dark and B Light

Bacteria spp.
Community A Light and B Light

Bacteria spp.
Community B Dark and B Light

Figure 3

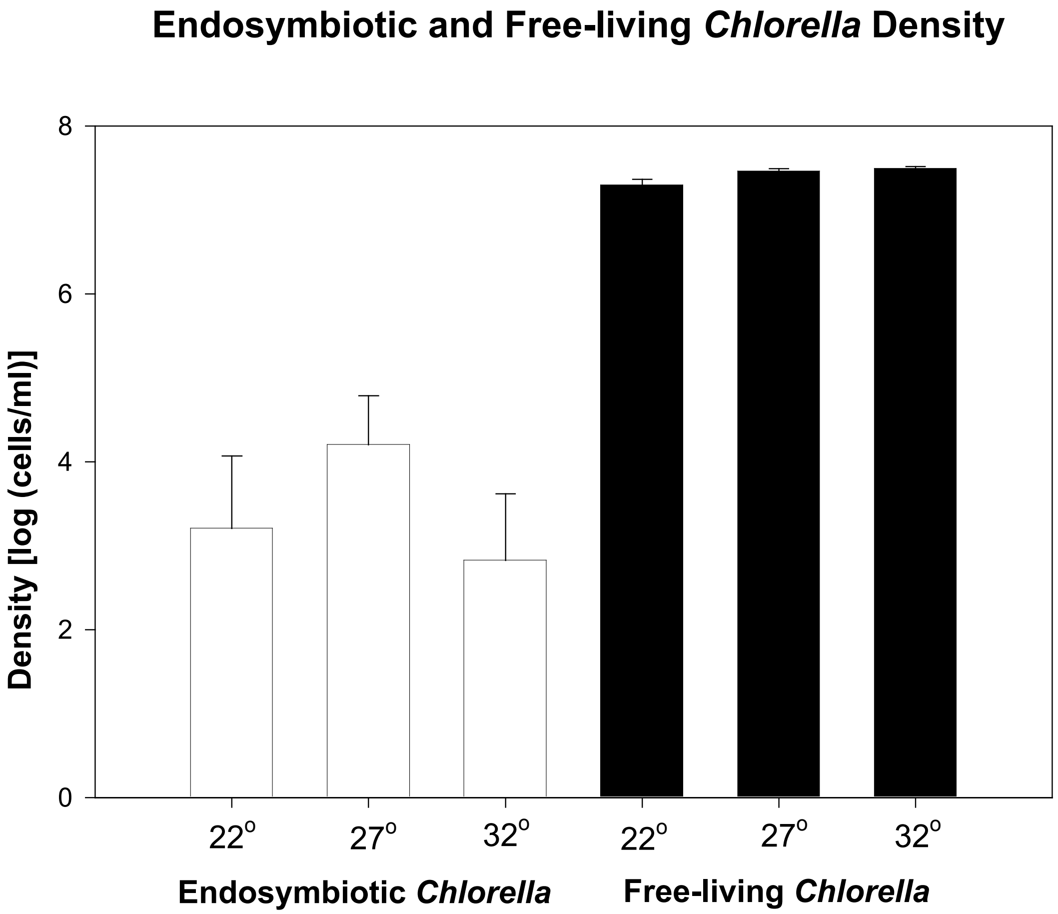
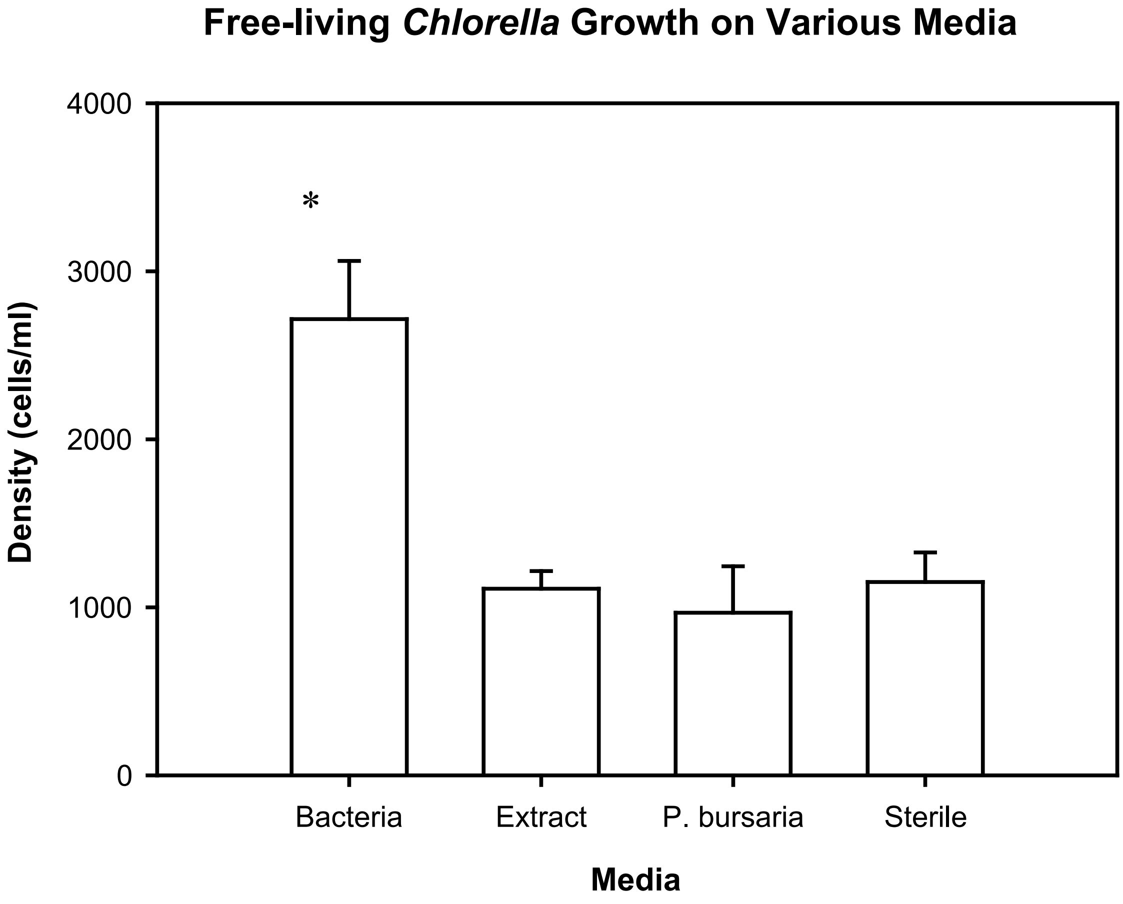

Endosymbiotic and Free-living Chlorella Density
Density [log (cells/ml)]
0
2
4
6
8
22°
27°
32°
22°
27°
32°
Endosymbiotic Chlorella
Free-living Chlorella

Figure 4


Free-living Chlorella Growth on Various Media
4000
3000
2000
1000
0
Density (cells/ml)
*
Bacteria
Extract
P. bursaria
Sterile
Media

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Chapter 3:

Testing an economic model of mutualism: Resource availability does not affect the incidence of mutualistic interaction in model communities

Abstract

The conditions that give rise to mutualism are suggested by theory to be rare, though empirical evidence confirms that mutualisms are common. Explaining the ubiquity of mutualisms has proved to be a difficult yet fundamental problem. Recently, an economic model of mutualism (hereafter EMM) was developed that predicts mutualism should be common as long as the cost of exchanging traded resources is low, the opportunity to ensure fair trade is high, or the cost of tolerating cheaters is low (Schwartz and Hoeksema 1998). The EMM predicts that the advantage gained by mutualist partners in a resource exchange mutualism should change as essential nutrients become more or less available to partners, so that at very low or very high nutrients partners will disengage from mutualism as cooperation becomes less advantageous. To test the EMM, I subjected a well-studied symbiotic mutualism between *Paramecium bursaria* and *Chlorella* to a gradient of low to high essential nutrients in aquatic microcosms. It was expected that at very low or high nutrients, partners would disengage from mutualism. Using population density data, I compared the effect of resource availability on the propensity for partners to associate. Though nutrient treatments produced significant effects on equilibrium population densities of both mutualist partners, there were no predicted changes in mutualistic association with changes in resource availability. The density of

endosymbiotic (mutualistic) *Chlorella* living inside *P. bursaria* did not change at any level of resources. Positive effects of mutualism were observed for *P. bursaria* but not for *Chlorella*. I conclude that though the EMM has intuitive appeal as a market model, the overall impact of resource availability does not appear to influence partners to alter the nature of their symbiotic mutualism. It is possible that non-symbiotic mutualisms may more closely fit model predictions than symbiotic mutualisms due to potential lack of flexibility in tight symbiotic associations. However, testing models such as the EMM can help ecologists understand the conditionality often observed in mutualisms while aiding efforts to develop a more comprehensive theory of mutualism. Further, I suggest that assessing benefits accrued to mutualistic partners requires measuring all putative effects on partners, both positive and negative, in order to properly calculate costs and benefits when applying market theory to communities with mutualism.

Introduction

Mutualistic interactions are commonplace in nature (Appendix 1). They underlie phenomena as diverse as the origin of eukaryotic cells, the radiation of angiosperms, and the diversity of coral reef systems (Bronstein 1994b; Douglas 1999; Palmer *et al.* 2003). Yet, ecologists know little about how changing environmental conditions affect mutualism. The lack of new theory and clear tests of existing theory contributes to confusion concerning the relative importance of mutualism compared with other interactions and processes that determine community patterns (Brooker and Callaghan 1998; Menge 2000; Callaway *et al.* 2002). Although there are relatively few empirical investigations of existing theories and models, efforts in the last ten years to erect synthetic conceptual frameworks (Boucher *et al.* 1982; Abrams 1987; Burns 1993; Kawanabe *et al.* 1993; Bertness and Callaway 1994; Conner 1995; Bruno *et al.* 2003) and theories (Trivers 1971; Axelrod and Hamilton 1981; Bronstein 1994b; Dugatkin 1997a, 1997b; Schwartz and Hoeksema 1998) of mutualism provide a starting point for empirical investigation. Clearly testing existing theory of mutualism is an essential building block towards developing a modern ecological theory that accurately includes mutualism as a core process (Bruno *et al.* 2003).

Empirical evidence of mutualism confirms that mutualisms are ubiquitous (Boucher *et al.* 1982; Bronstein 1994b), which is inconsistent with ecological theory that suggests mutualism should be rare or confined to high stress ecosystems (May 1973; Vandermeer and Boucher 1978; Axelrod and Hamilton 1981; Bertness and Callaway 1994; Callaway *et al.* 2002). Schwartz and Hoeksema (1998) have developed a model that suggests mutualism should be common. In their economic model of mutualism (EMM), mutualists engage in simple biological trading for limiting resources, where trade occurs whenever a species can specialize on obtaining one resource to trade for others, regardless of potential environmental stress. The EMM is based on Mill's (1877) and Ricardo's (1891) classical economics where resource specialization and trading is likely to occur whenever a nation or individual is more efficient at the production of one commodity than other nations or individuals are.

Schwartz and Hoeksema's (1998) biological market approach to modeling resource exchange between mutualists is based on studies of plants and mycorrhizal fungi. They use the theory of relative advantage to demonstrate the conditions under which species ought to specialize and trade for resources. The EMM is a two-resource model, which predicts that when costs of resource exchange are low, the opportunity to ensure fair trade is high, or the cost of cheating is low, species should engage in mutualistic interaction. Species ought to specialize in the acquisition of one resource and trade for a second resource as long as each species perceives different relative acquisition costs for two resources (Figure 1; Figure 2). When model assumptions are violated, species should disengage from mutualistic interaction, resorting instead to competitive, predatory, parasitic, or no interaction. Changing resource availability describes the conditions under which mutualistic partners will benefit or harm each other (Figure 1). At resource levels within predicted thresholds conducive to mutualism, mutualist partners should cooperate with the greatest frequency. This prediction also shows that specialization and trade confer an advantage even for species that are relatively poor resource competitors for both resources (Schwartz and Hoeksema 1998). Manipulating resource availability should affect the acquisition cost of a traded resource, thus changing the nature of the trading relationship between mutualistic partners (Figure 1). Therefore, mutualistic association should weaken if the cost of obtaining a limiting traded resource is either too high or too low.

The intuitive appeal of the EMM is readily apparent as it describes the basic dynamics of many mutualisms where partners trade quantifiable resources that impact individual fitness (e.g. ant-fungal (Mueller *et al.* 1998), ant-plant (Beattie 1985), *Yucca*-yucca moth (Pellmyr *et al.* 1996), plant-mycorrhizae (van der Heijden 1998), cooperative vertebrates (Clutton-Brock 2002), and ciliate-algal relationships (Loefer 1936)). The model also assumes tight feedback loops, which are inherent in symbiotic protist relationships, yielding ideal models for testing theory. The symbiotic relationship that exists between the ciliated protist, *Paramecium bursaria,* and its endosymbiotic alga, *Chlorella,* is well suited for model testing as the resource trade between host and symbiont closely approximates assumptions of this model. Maintaining protists in laboratory microcosms provides reliable control of environmental factors that potentially influence inter-specific relationships.

Recently, microcosm experiments have enabled ecologists to ask questions that have been intractable under normal field situations (Morin and Lawler 1995; Naeem and Li 1997; Kaunzinger and Morin 1998; Petchey et. al 1999; Naeem *et. al* 2000; Petchey *et al*. 2002; Morin and McGrady-Steed 2004). Studies of protists in microcosms have also made many important ecological contributions (Gause 1934; Huffaker 1958; Hairston *et al*. 1968; Luckingbill 1973; Addicott 1973; Tilman 1977; Lawler and Morin 1993; Lawler 1998), as they are particularly apt for experimental investigation of population to ecosystem level processes. This is due primarily to their short generation times that facilitate the collection of long-term population dynamic data essential for tests of theory (Gause and Witt 1935; Vandermeer 1969; Morin 1998). Protists have generation times that range from a few hours to one or two days and are easily cultured in small aquatic microcosms. With glass bottles containing from 50 to 100ml of culture medium, these systems can house populations from 10^5 to 10^7 individuals (Morin 1998). Here, I report a direct test of the EMM using the resource exchange mutualism between the ciliate, *Paramecium bursaria*, and its trading algal partner, *Chlorella,* in aquatic microcosms.

The Model System

The association between endosymbiotic *Chlorella* and its host, *Paramecium bursaria*, is a well-studied symbiotic mutualism commonly found in freshwater ponds, lakes, and streams (Reisser 1986; Wichterman 1986; Smith and Douglas 1987). These organisms are easily cultured under laboratory conditions and can be experimentally taken apart and reassembled (Jennings 1938a, 1938b; Karakashian *et al*. 1968; Reisser 1986, 1987; Gortz 1988; Hosoya *et al*. 1995; Nishihara *et al*. 1998). *Chlorella* is an endosymbiont of *P. bursaria*, but can also be cultured separately as free-living *Chlorella*. Similarly, *P. bursaria* can be bleached of its algae using a combination of light limitation and herbicide treatment, or other treatments (Wichterman 1986; Hosoya *et al*. 1995). Additionally, it is possible to reinfect bleached *P. bursaria* with free-living *Chlorella* cells, thus restoring the symbiosis. The flexibility inherent in this mutualism is particularly useful because clear experimental results require controls with mutualists growing together and alone.

Chlorella are spherical to broadly ellipsoidal in shape, measure 2-10μm in diameter, possess a large, single, parietal chloroplast, and have a rigid cell wall (Smith and Douglas 1987; Wichterman 1986). Many species of *Chlorella* are morphologically indistinguishable from one another, though physiological differences exist.[1] Endosymbiotic forms grow within a variety of freshwater hosts, including ciliated protozoa, sponges, coelenterates, neorhabdocoel turbellarians, bivalve mollusks, and platyhelminthes worms (Sleigh 1973; Finlay 1977; Smith and Douglas 1987) and are

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[1] There have been few attempts to identify *Chlorella* to the taxonomic level of species, though the common *Chlorella vulgaris* has been identified in some protozoa, hydra, and turbellaria (Smith and Douglas 1987).

considered the dominant algal symbiont in freshwater hosts (Douglas and Smith 1984; also see Appendix 2).

Paramecium bursaria are bactivorous ciliated protozoa, 85-150 μm in length, and possess a single, large compact micronucleus and two canal-fed contractile vacuoles (Wichterman 1986). They function well as hosts for *Chlorella* as the thin cell wall allows appreciable light penetration. Endosymbiotic *Chlorella* live intracellularly in *P. bursaria*, enclosed within individual perialgal vacuoles separated from the cytoplasm of the host cell by the host vacuole membrane. These vacuoles are usually very near the surface of *P. bursaria*'s cell wall where *Chlorella* can most effectively photosynthesize. The growth of *Chlorella*, in general, is synchronous with the growth of *P. bursaria* so that under normal conditions, neither partner outgrows the other (Karakashian 1963; Pado 1965; Weis 1976, 1977).

When *Chlorella* is endosymbiotic, it leaks a disaccharide sugar, maltose, to *P. bursaria*, providing a direct transfer of organic carbon to the host.[2] Maltose is a simple sugar produced by *Chlorella* during photosynthesis, but is also derived from algal polysaccharide reserves (Mews 1980). Because maltose is not strictly photosynthesis-dependent, it can be released continuously for several weeks in darkness (Smith and Douglas 1987; Mews 1980). Maltose release is unique to symbiotic *Chlorella* since strains of *Chlorella* that do not form symbioses do not release maltose (Smith and Douglas 1987). As a result, *P. bursaria* grow more rapidly in continuous or intermittent light compared with algal-bearing *P. bursaria* grown in the dark (Karakashian 1963;

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[2] The movement of organic photosynthate products from *Chlorella* to host has been traced using the radioactive isotope ^{14}C in the green hydra symbiosis (*Chlorella* are also endosymbionts of green hydra). More than 90% of the radioactive solutes released were in the form of the disaccharide maltose (Muscatine 1965; Cernichiari *et al*. 1969; Mews 1980).

Pado 1965; Weis 1969, 1974a; McPhearson 2003 unpublished data). Endosymbiotic *Chlorella* has also been shown to make *P. bursaria* distasteful to a common predator, *Didinium nasutum* (Berger 1980). However, the most significant benefit of mutualism for the host appears to be the additional carbon source from *Chlorella*, which can make *P. bursaria* partially or completely independent of external food for survival and growth (Reisser *et al.* 1983).

All nutrients received by endosymbiotic *Chlorella* are derived from or pass through the host cell. This direct transfer of nutrients from host to endosymbiont has been demonstrated by providing the host with food containing labeled isotopes ^{3}H and ^{14}C and subsequently recovered from the algal fraction (Thorington and Margulis 1981). Further evidence of transport of nutrients from the host to algal cells comes from a study of mutualistic *P. bursaria* cultured in the dark with continuous feeding. The algal symbionts continued to grow and divide for over a year in the dark (Douglas and Smith 1984). In a similar study, the growth rate of mutualistic *P. bursaria* in the dark also declined to rates lower than that of non-mutualist individuals (no algal symbiont), indicating that nutritional requirements of the algae represent a detectable cost to the host (Smith and Douglas 1987). *Chlorella* also benefit by gaining motility, which may be a significant benefit in association with mobile hosts (Young and Eaton 1975). Living inside host cells helps *Chlorella* avoid competition with other algal taxa, allowing symbiotic *Chlorella* to attain high population densities within the host (algal cell counts can reach >500 per *P. bursaria* cell; personal observation). Additionally, *Chlorella* are thought to benefit by avoiding predators when living inside host cells.

The direct trading of nitrogen and phosphorous by *P. bursaria* for organic carbon from *Chlorella* is similar to trade that occurs between mycorrhizae and plants, which the EMM is based on. The assumptions of nominal transportation costs fit the model mutualism since symbionts probably have the least transportation costs of any mutualism. Cheating should also be tolerated less in symbiotic relationships, as tight feedbacks inherent in symbiotic relationships are important for controlling cheaters.

Testing the EMM

The primary goal for conducting a resource gradient analysis of mutualism is to quantify predicted changes in mutualistic interaction with changes in resource availability. Predictions from the EMM (Schwartz and Hoeksema 1998) suggest that experimentally varying limiting resources from one-fourth to two times the normal concentration in laboratory protist cultures should alter the trading relationship between mutualist partners. The mutual advantage of trading for limiting resources is predicted to decrease as the availability of limiting resources increases or decreases beyond the threshold for optimal mutual resource exchange (Figure 1); this in turn should cause the incidence of mutualism to decrease at high or low resources. Therefore, the advantage for *Chlorella* to trade carbon products for limiting nitrogen and phosphorous from *P. bursaria* is expected to decrease as nutrients become more readily available in culture media. The EMM predicts that mutualist partner access to resources becomes less costly than trading for them as essential resources increase in culture medium (Figures 3a-d).

At low resource levels (1/4x, 1/2x treatments) mutualist partners are expected to form tighter linkages, characterized by increasing endosymbiotic *Chlorella* in *P. bursaria* cells and decreasing free-living *Chlorella* in *P. bursaria* communities (Figure 3b). *Paramecium bursaria* should likewise seek to increase association with *Chlorella* under low resource conditions as potential decreased bacterial production puts acquiring carbon from *Chlorella* at a premium. At high levels of nitrogen (2x), *Chlorella* and *P. bursaria* are predicted to disengage from each other, characterized by decreasing endosymbiotic *Chlorella* in *P. bursaria* cells and increasing free-living *Chlorella* in *P. bursaria* communities as trading for essential resources becomes more costly than obtaining resources directly from the aquatic environment (Figure 3b). Free-living *Chlorella* populations will be similar to free-living *Chlorella* populations growing alone (Figure 3c). Total *Chlorella* (endosymbiotic *Chlorella* plus free-living *Chlorella*) in mutualism cultures should increase with increasing resources, with advantages gained from mutualism decreasing at high resources so that total *Chlorella* abundance in mutualism is lower than *Chlorella* growing alone (Figure 3d).

Paramecium bursaria should also seek to purge *Chlorella* cells as increasing resources fuel increased bacterial production and allow *P. bursaria* to be less reliant on *Chlorella* for carbohydrates. *Paramecium bursaria* populations in mutualism cultures should increase, over and above *P. bursaria* alone populations (dark non-mutualism cultures), since mutualistic *P. bursaria* harboring any *Chlorella* will still be receiving maltose, which positively affects host abundance (Figure 3a). Both *P. bursaria* light and dark cultures should respond to increased resources with overall increased population size (Figure 3a).

Methods

I constructed aquatic communities in microcosms assembled from protists and bacteria with and without functioning mutualism across a gradient of essential resource (primarily nitrogen and phosphorous). The experiment consisted of four resource treatments (1/4x, 1/2x, 1x and 2x) crossed with three organism treatments (*P. bursaria* alone, *Chlorella* alone, *P. bursaria* and *Chlorella*) in a factorial design. Five replicates for each treatment (4 x 3 x 5= 60 microcosms) measured within treatment variation. Resource availability was manipulated using 1/4x, 1/2x, 1x, and 2x the normal nutrient concentration of standard lab protist pellet medium (normal lab PPM is equal to 0.75g protist pellet/1.350L water). Resources from 1/4x to 2x normal lab medium were chosen since previous lab experience demonstrated that concentrations above 2x cause osmotic difficulties for protists, limiting growth due to physiological stress, while below 1/4x nutrients are limiting for growth (Morin 2003, personal communication).

By simply growing *P. bursaria* and *Chlorella* in Light or Dark Treatments I was able to create communities with and without functioning mutualism. Dark cultures of *P. bursaria* contained only *P. bursaria* (*P. bursaria* alone; no functioning mutualism), light cultures of *P. bursaria* contained free-living and endosymbiotic *Chlorella* with *P. bursaria* (functioning mutualism), and light cultures of free-living *Chlorella* contained only free-living *Chlorella* (no functioning mutualism). All treatments had the same bacterial inoculum.

Culture methods were similar to Petchey *et al.* (1999, 2002) using 250ml covered glass bottles containing 100ml of growth media. Growth media was non-filtered de-ionized (DI) water with dissolved protist pellets and wheat seeds sterilized by autoclaving. Protist culture pellets (Carolina Biological Supply) were the primary nutrient source. Protist pellets are ground plant matter consisting primarily of nitrogen and phosphorous (2275.07mg and 91.26mg per gram protist pellet respectively), a small amount of organic carbon and other minor nutrients (Table 1). Two wheat seeds were added to each jar to provide a slow release carbon source to fuel bacterial production. De-ionized water was used to minimize background nutrients. Background major nutrient levels in DI water were 12.05mg total phosphorus/L and 148.84mg total nitrogen/L DI water.[3] The resource gradient was 1/4x, 1/2x, 1x, or 2x equal to 0.1875g, 0.3750g, 0.7500g, or 1.500g protist pellet per 1.375L DI water, respectively.

Following sterilization of media, all cultures were inoculated with a suite of bacterial species (*Bacillus subtillus, B. cereus, Serratia marcescens,* and *Proteus vulgaris*) to create an initial resource base for bactivorous *P. bursaria*, and allowed to grow for 24 hours before adding protists. *Paramecium bursaria* maintained in light incubators were added to the *P. bursaria/Chlorella* mutualism treatment. *Paramecium bursaria* from an identical strain yet previously housed in dark incubators were used to inoculate *P. bursaria* alone treatments. One hundred *P. bursaria* cells were added to each treatment jar. Endosymbiotic *Chlorella* densities averaged 298 cells/*P. bursaria* cell (mean of 10 direct counts; ranging from 209 to 409 cells/*P. bursaria*) in *P. bursaria* light cultures at the start of the experiment. Free-living *Chlorella* were diluted 1:28 to match densities of free-living *Chlorella* in *P. bursaria* cultures before addition to *Chlorella* alone treatment jars. Free-living *Chlorella* cultured from burst *P. bursaria*

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[3] Tara Darcy, 2002, Kellogg Biological Station, Michigan State University, performed DI water and additional protist pellet analyses. Total phosphorous was analyzed using the ammonium molybdate method following persulfate digestion, and total nitrogen was analyzed using second derivative scanning spectroscopy.

were used to inoculate all light communities, ensuring that free-living and endosymbiotic *Chlorella* were of an identical strain. This was accomplished by bursting *P. bursaria* cells containing endosymbiotic *Chlorella* on glass plates with a fine sterile needle and rinsing them with sterilized media into culture jars. Freed *Chlorella* were then allowed to grow to appreciable densities before addition to the *Chlorella* alone treatment. All cultures were maintained at 22°C for the duration of the experiment. Medium was refreshed at rate of five percent of total volume weekly (equal to 5ml fresh PPM/week) following all counts in order to prevent negative impacts of protist waste accumulation. No contamination by eukaryotes occurred.

Paramecium bursaria were counted twice weekly by microscopy (on Nikon SMZ-U) until reaching carrying capacity, then once weekly, with densities standardized by weight. Free-living *Chlorella* were counted on the same day as *P. bursaria* on a hemocytometer under a compound scope (Zeiss Lab 16). Endosymbiotic *Chlorella* densities were assessed once weekly. First day counts (Day 3) of endosymbiotic *Chlorella* were done by directly counting *Chlorella* on glass plates from burst *P. bursaria*. However, direct microscope counts are prone to error as bursting *P. bursaria* and then assessing endosymbiotic *Chlorella* is hampered by rapid movement of *Chlorella* cells even when performed using diluted methyl cellulose. On all subsequent sampling days endosymbiotic *Chlorella* were assessed using the sonication method. In this method, *Paramecium bursaria* cells were burst using a sonicator (Fisher Scientific Sonic Dismembrator - Model 60) where the intensity can be controlled so that *P. bursaria* cells burst while *Chlorella* cells remain intact. First, from a well-mixed jar, 1ml of *P. bursaria* (mutualism treatment) was sonicated (Level 20) for six seconds. Then, total *Chlorella* in the sonicated medium were counted on the hemocytometer, and previously counted free-living *Chlorella* densities were subtracted from total *Chlorella* density to give endosymbiotic *Chlorella* density for each replicate. Pilot sonication trials confirmed that *Chlorella* death caused by sonication was insignificant and sonication methods provided repeatable measures of endosymbiont densities. Densities are log transformed to facilitate comparisons with free-living and total *Chlorella*.

Statistical Analysis

All statistical analyses were carried out with Statistical Analysis System software (SAS v.8.2). I assumed differences in protist abundance (total population size) constituted real species responses to resource and mutualism treatments since all jars had identical starting densities of bacteria and protists (same number of *P. bursaria* and same number of free-living *Chlorella* in mutualism and non-mutualism communities).

Using each sampling day's species densities as independent variables, I performed individual repeated measures ANOVAs to examine the effect of time on abundance measures for each species across the resource gradient. The main effect used in repeated measures was the nutrient treatment (1/4x to 2x). Repeated measures ANOVAs were performed separately for free-living *Chlorella*, endosymbiotic *Chlorella*, and *P. bursaria*. Multiple species comparisons in analysis of variance were used to examine resource gradient treatment effects (main effect in analysis) on species abundances (procedure GLM) for the final day of the experiment (Day 28). Tukey's Studentized Range Test identified resource treatment differences on individual species abundances at $\alpha=0.05$. All analyses were done on log-transformed densities.

Results

Long-term dynamics

First day densities (Day 0) are inoculum densities and are identical for each species (Figure 4). *Paramecium bursaria* and free-living *Chlorella* grew significantly from inoculum density to equilibrium density (Figure 4). Repeated measures ANOVA revealed a significant effect of time (p<0.0001; except for endosymbiotic *Chlorella* per *P. bursaria* p=0.0148) for all species (Figures 4a-g). Initial low density and first counts (Day 3; Figure 4g) of endosymbiotic *Chlorella* per *P. bursaria* cell were due to different counting methods, as first counts of endosymbiotic *Chlorella* were direct plate counts and were thus factored out of repeated measures analysis

Total *Chlorella* in *P. bursaria* (mutualism) cultures (Figure 4f) was calculated by adding endosymbiotic and free-living *Chlorella* populations and is a measure of the total population size of *Chlorella* in the mutualism treatment. Endosymbiotic *Chlorella* were a smaller overall proportion of total *Chlorella* population than free-living *Chlorella* (Figure 4). Mean endosymbiotic *Chlorella* population (Figure 4c) increased with increasing *P. bursaria* (Figure 4a) while endosymbiotic *Chlorella* per *P. bursaria* cell (Figure 4g) remained fairly constant at all sampling times except Day 3. Free-living *Chlorella* growing alone (non-mutualism) also significantly increased over time (Figure 4e).

Mutualistic *P. bursaria* (light cultures) consistently reached higher overall densities than non-mutualistic *P. bursaria* (dark cultures) (Tukey's test, p=0.05), though both treatments increased over time (repeated measures ANOVA, Wilks' Lambda F=4256.57, p-value <0.0001) and equilibrated at carrying capacity (Figures 4a, 4b).

Resource gradient effects

Species responses to the resource gradient are evident in time series data (Figure 4), however, analyses of resource gradient affects focused on Day 28 of the experiment (Figure 5) so that conclusions could be drawn from equilibrium densities. Analysis of variance revealed strong effects of the resource gradient treatment.

Paramecium bursaria in mutualism cultures grew to higher density at higher resource levels (Figure 5a; overall ANOVA, $r^2=0.8735$, $F=36.84$, $p<0.0001$). However, *P. bursaria* in dark (non-mutualism) microcosms did not show an overall response to resources (Figure 5a; ANOVA, $r^2=0.1567$, $F=0.99$, $p=0.4299$) for any resource concentration comparisons (Tukey's, $p=0.05$).

Chlorella in mutualism cultures generally increased with increasing resource availability. Endosymbiotic *Chlorella* were affected by the resource treatment ($r^2=4690$, $F=4.71$, $p=0.0153$), but were only significantly different between 1/4x and 2x normal resource concentration (Tukey's, $p=0.05$). All other comparisons were not significant due to variability within treatments of endosymbiont *Chlorella* densities. Endosymbiotic *Chlorella* growth inside *P. bursaria* cells, showed no change regardless of resource levels (Figure 5e; ANOVA, $r^2=0.0428$, F-value=0.24, $p=0.8682$). Free-living *Chlorella* however, responded strongly to increasing resources (Figure 5b; ANOVA, $r^2=0.9552$, $F=113.77$, $p<0.0001$) with differences between every level of resource concentration (Tukey's $p=0.05$). Free-living *Chlorella* make-up the majority of the population in calculated total *Chlorella* density in mutualism cultures; therefore, total *Chlorella* mirrors free-living *Chlorella* results (Figure 5d; ANOVA, $r^2=0.9712$, $F=180.08$, $p<0.0001$) with significant differences between every level of resources (Tukey's, $p=0.05$).

Free-living *Chlorella* growing alone (non-mutualism) had a similar response to free-living *Chlorella* in mutualism cultures (Figure 5c). *Chlorella* alone generally grew to higher densities in higher resource concentrations (Figures 5c, 5d; ANOVA, r^2=0.6850, F=11.60, p=0.0003) with significant differences between 2x and 1/4x, 2x and 1/2x, and 1x and 1/4x (Tukey's p=0.05).

Species specific comparisons

Analyses from the final day when populations were near equilibrium show that the overall mean difference between *P. bursaria* light and *P. bursaria* dark abundance was statistically significant (Figure 5a; Tukey's results from two-way ANOVA, p=0.05). Most *Chlorella* in *P. bursaria* communities existed outside the host cell (Figure 5b; free-living *Chlorella* had higher mean densities than endosymbionts, Tukey's, p=0.05). Densities of free-living *Chlorella* in *P. bursaria* communities were lower than populations of free-living *Chlorella* growing alone (Figure 5c), however, when endosymbiotic *Chlorella* were added, the total *Chlorella* population size in mutualism cultures was not different than total free-living *Chlorella* growing alone (Figure 5d; Tukey's, p=0.05).

Discussion

Contrary to predictions from the EMM (Schwartz and Hoeksema 1998), the frequency of mutualistic interaction did not appear to change at any level of resource availability. There was a significant advantage to participating in mutualism for *P. bursaria*, with the advantage increasing at higher resources (Figure 5a). However, based on population densities at or near equilibrium, there appeared to be no fitness advantage for *Chlorella* at any level of resource availability (Figure 5d). Endosymbiotic *Chlorella* populations did not vary across treatments (Figure 5e). The lack of change in the association between mutualist partners is presumably because neither *Chlorella* nor *P. bursaria* sought to disengage from the mutualism. Increasing resource availability provided no overall measurable disadvantage for mutualist partners.

It is useful to consider mutualisms as biological markets in which species trade a product they either acquire or produce with relatively low cost in exchange for products from potential partners (Noe and Hammerstein 1994; Schwartz and Hoeksema 1998; Bronstein 2001). Mutualisms are usually described as reciprocally exploitative interactions that provide net benefits to the partner species (Thompson 1982; Herre *et al.* 1999; Janzen 1985; Connor 1995; Bronstein 2001). The net profit of the interaction of each participant is highest when it is able to maximize the benefit it receives for the least costly investment. Conflict often occurs between mutualist partners as each seeks to exploit maximum benefits from each other (Bronstein 2001). Though putative benefits accrued for a partner may be slight at a given level of resources, even immeasurably so (Schwartz and Hoeksema 1998), they can still be selected for even if costs outweigh benefits. In the case of *P. bursaria* and *Chlorella*, there is potential conflict between partners over the quantity and quality of photosynthate products *Chlorella* provides to the host and the quantity and quality of benefits *Chlorella* receives from the host, namely concentrated resources, such as nitrogen, in the host cell, increased motility, and protection from competitors and predators. Removal of a reward from a cooperative partner may cause the partner to disengage in mutualistic interaction. In the experimental microcosms, the mutualism has been removed from the more complex aquatic system in which it normally exists (ponds, lakes, and streams) to provide a controlled environment that more closely meets assumptions of the EMM and allows gathering of long-term population dynamics.

Though ecologists have now gathered long-term data for many mutualistic systems (Boucher *et al*. 1982; Bronstein 1994b), there has been little use of such data for mutualism model testing. It is important to differentiate transient from long-term responses to experimental treatments when quantifying variability in mutualistic interaction. In this system, resource treatments affects were variable until approximately Day 15 (equal to 15 to 30 protist generations) when populations began to stabilize (Figure 4). By Day 28 (~25 to 50 generations), populations of *P. bursaria* and *Chlorella* had adequately settled so that conclusions to the EMM could be drawn from long-term species responses.

The economic model of mutualism (EMM), using resource acquisition, relative advantage, and resource trade, gives specific predictions about the evolution and stability of mutualisms. Through specialization and trade, individuals may improve their net resource acquisition and increase individual fitness (Schwartz and Hoeksema 1998). Net benefits may be small, yet still be selected for. This model is valuable since it describes a way in which species may increase fitness through an interaction, and defines the boundaries under which these interactions continue to benefit both partners. Given the known context dependency often found among mutualist partners (Bronstein 1994a), benefits accrued to potential mutualist partners should vary with relevant changes in the biotic and abiotic environment. These predictions were not observed in the model mutualism tested here and may be attributable to a few potential ecological factors.

First, it is possible that *Chlorella* experiences less benefit when removed from the natural system where competition and predation are normally present. If benefits to *Chlorella* are comparatively less overall than when in more complex ecosystems, then *Chlorella* may already be relatively disengaged with highest densities in free-living populations (Figures 4e, 5b). Alternatively, the constant density observed in endosymbiotic *Chlorella* may suggest that there is a maximum number of *Chlorella* cells that can be housed in individual *P. bursaria* cells. The ability of endosymbiotic *Chlorella* to increase in *P. bursaria* cells is likely simply limited by *P. bursaria* cell size. This explanation, however, does not explain the lack of any decrease in endosymbiotic *Chlorella* with changes in resource levels predicted by the EMM (Figure 3b). Past research has shown that *Chlorella* has limited ability to disengage in mutualistic interaction (see Chapter 2).

Second, in the EMM, trade is voluntary and payment is obligatory. Our results suggest that trade may not be voluntary, and that *Chlorella* may in fact be obligated to pay in maltose even when benefits are theoretically less beneficial (e.g. the isolation cost ratio for acquiring resources is higher at low resources, increasing the trade cost ratio and causing the gain from trade to be lower). *Paramecium bursaria* appears to be able to control the growth rate of its algal endosymbionts either through nutrient limitation (Wichterman 1986; Reisser *et al.* 1983; Berk *et al.* 1991; Stabell *et al.* 2002) or by chemical suppression of growth (Smith and Douglas 1987; McPhearson *et al.* in prep), though this has only been investigated in the green hydra symbiosis (McAuley 1985;

Neckelmann and Muscatine 1983). Rees (1986) also found evidence that hosts may regulate *Chlorella* by restricting the supply of nitrogen. If endosymbiotic *Chlorella* is under host control and does not have the flexibility to disassociate from mutualism, then resource availability may have little to do with resource trade. Endosymbiotic *Chlorella* will be less able to respond at any resource concentration. *Chlorella* may not be able to escape its host when costs outweigh benefits, and is instead enslaved by the host. However, it is interesting to note that total *Chlorella* (endosymbiotic plus free-living) populations in mutualism treatments are not significantly lower than *Chlorella* in non-mutualism treatments (Figure 5d) and so total *Chlorella* populations in mutualism do not appear to suffer from mutualism.

Chlorella cells grow much more slowly in symbiosis than in culture (Jolley and Smith 1978; McPhearson 2002 unpublished data) and lose a significant amount of fixed carbon to their hosts (Smith and Douglas 1987), which explains the overall lower growth compared to free-living *Chlorella* growing alone, but fails to illustrate the advantages *Chlorella* is reported to receive from mutualism with *P. bursaria*. Though *Chlorella* is reported to benefit from increased motility, predator avoidance, and decreased competition with other algal species when endosymbiotic in host cells, these benefits are not observed in the experiment. It is remarkable that benefits to *Chlorella* are difficult to quantify (Smith and Douglas 1987) given its propensity to form facultative relationships with a variety of hosts (Appendix 2).

Additionally, there is potential inadequate flexibility among mutualist partners to respond to decreasing benefits. *Chlorella* grow and divide within their hosts and under normal laboratory conditions neither partner outgrows the other.[4] If *P. bursaria* lacks adequate means to expel *Chlorella* from its cytoplasm, even when benefits decrease below the predicted threshold for mutualistic interaction, then no change in mutualism would be observed with changes in resource concentration. Additionally, if symbiosis, due to the spatial closeness of the association, causes mutualism to be more difficult to disassociate from, then the threshold for switching from positive to neutral to negative interaction will be higher than for non-symbiotic mutualisms, and may be more difficult to observe. Adequate flexibility is an essential element of mutualisms that fluctuate with environmental change. In the EMM, species' flexibility can determine whether mutualism responds to resource augmentation (Schwartz and Hoeksema 1998).

Finally, it is possible that mutualism is not as costly (costs of resource transport and cheating) as modeled by the EMM. When interactions are tightly linked, slight changes in the community context can quantitatively or qualitatively alter the outcome of the association between any single pair of species (Bronstein *et al.* 2003). Advantages gained by being in a mutualism, only some of which are measured in this experiment (benefits to *Chlorella* of decreased competition and predation were not quantifiable), could nonetheless outweigh costs if total costs of resource trade are very low. Though proximate benefits of release from competition or predation for *Chlorella* could not be assessed in the scope of this experiment, it is assumed that more than twenty generations is adequate time for *Chlorella* and *P. bursaria* to perceive and respond to ecologically important factors. In a symbiotic mutualism, resource transport costs should be low

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[4] The proportion of algae to host rarely exceeds 1:10 either by volume or by biomass (Smith and Douglas 1987) though algal cell counts can reach above 500 individuals per *P. bursaria* cell (personal observation).

when compared to non-symbiotic mutualisms such as plant pollinators that expend energy traveling between plants. Costs of transport for symbiotic mutualisms may also be highly asymmetric, such as in ant-fungal farms where Attine ants experience the majority of transport costs. Ants gather leaves to cultivate fungi and harvest the fungal hyphae and gongylidia (Weber 1966, 1972; Chapela *et al.* 1994). Fungi in this case have relatively little resource transport costs compared to leafcutter ants. Additionally, symbiotic mutualisms are useful for testing models such as the EMM as symbiosis promotes increased control of cheating typified in most mutualisms (Bronstein 2001), since feedback in symbiotic relationships should occur over short time scales (Herre *et al.* 1999).

An alternate theory by Bertness and Callaway (1984) predicts that as stress in the system increases mutualistic interaction should increase. If low resources constitute stress, observed by decreased population density for both *Chlorella* and *P. bursaria* at low resource availability (Figures 4, 5), then I would expect increased mutualistic association in low resource treatments. Experimental results do not fully support this theory either, yet population densities reflect nutrient limitation on growth. Even though the incidence of mutualistic interaction should vary with the ecological context of the interaction (Bronstein 1994a; Bronstein 2001), costs, benefits, and flexibility may control the response of mutualist partners to environmental change (Schwartz and Hoeksema 1998).

The commonness of mutualism in nature remains a puzzle (Bronstein 2001). Though ecologists have historically been reluctant to incorporate positive interactions explicitly into experiments and theory (Kareiva and Bertness 1997; Bertness *et al.* 1999;

Bruno *et al.* 2003), recent studies suggest progress is underway (Bertness *et al.* 1999; Christian 2001; Callaway *et al.* 2002). The EMM is appealing as a market model, yet overall resource availability does not appear to influence partners to change the nature of their interaction in this symbiotic mutualism. The model mutualism used here meets requirements of the EMM such as low costs of resource transport and tight feedbacks on potential cheaters. In order to test the EMM in other mutualist systems, it is important to assess all putative effects on partners, both positive and negative, in order to accurately calculate costs and benefits central to market models. Testing models such as the EMM can help ecologists to understand the conditionality often observed in mutualisms while aiding efforts to develop a more comprehensive theory of mutualism.

Further illuminating the role of mutualism in community and ecosystem processes requires understanding how interspecific interactions are altered by changes in environmental conditions. Abiotic and biotic conditions are known to affect how mutualist partners interact (Barton 1986; Bronstein 1994a; Linsenmair *et al.* 2001; Holland *et al.* 2002). I suggest that integrating well-studied model mutualisms with relevant model testing, such as the gradient analysis used here, will further the development of ecological theory that includes mutualistic interaction as a core process (Bruno *et al.* 2003). Mutualism is clearly an important determinant of species abundance in this system, yet the impact of mutualism on species interactions is only beginning to be explored (Christian 2001, Callaway *et al.* 2002; Cardinale *et al.* 2002).

Figure Legends

Figure 1

The relationship between maximum resource acquisition rates and resource availability for two species. $R1_{max}$ is a linear function of the availability of a resource ($R1_{avail}$) and the ability of a species to capitalize on increasing resource availability. This function intersects the y-axis at X, the minimum amount of R1 required to sustain the species. The variability in the ability of species to respond to changing resource availability is defined as a flexibility coefficient (F_R), which is the slope of the line describing maximum resource acquisition across resource availability. In this example, species A is very flexible and therefore perceives the value of a resource to change based on its changing availability, while species B is not flexible and does not perceive the value of a resource to change with changing availability. A species' flexibility (F_R) is one factor that would determine whether resource augmentation changes the nature of a resource exchange mutualism (redrawn from Schwartz and Hoeksema 1998).

It is likely that the maximum amount of a resource a species can acquire depends to some extent on the amount available in the environment (Chapin 1980). Also, species vary in their ability to respond to changing resource availability (Chapin 1980; Tilman and Wedin 1991). In Figure 1, R_{max} is the maximum amount of one resource a species can acquire in a given time period (e.g. one year) as a function of the availability of the resource ($R1_{avail}$) and the ability of a species to actually capitalize on increasing resource availability. Therefore, the linear model is $R1_{max} = F_{r1} \times R1_{avail} + X_{R1}$, where X_{R1} is the baseline amount of R1 required to sustain species A, and F_{r1} is the flexibility of a species to respond to changing resource availability.

Figure 2

A diagram of a hypothetical relationship between the benefits received from trade and the trade cost ratio (*T*). For two species, A and B, engaging in a resource trade mutualism, the gain from trade (*G*) is lower for both species when the isolation cost ratio of species B (I_B) shifts toward that of species A (I_A). The solid line shows the function relating *G* to *T* for species B after I_B shifts to the right. T_1 and T_2 are the trade costs ratios before and after the shift of I_B, respectively. Notice that the gain from trade (*G*) for both species is lower at T_2 than at T_1 (redrawn from Schwartz and Hoeksema 1998). Trade will always be beneficial for both species if the trade cost ratio (T) remains somewhere between the isolation cost ratios of (I) of the two species. Also, the gain from trade (G) for one species increases the closer T is to the I of the other species. Thus, whether a change in I alters G depends on whether T also changes. This shift in T illustrates the prediction that fertilization may decrease the incidence of mutualism due to decreased gain (G) from trade.

Figures 3a-d

Figures 3a-d extend predictions of the EMM to predict abundance shifts for mutualistic species with and without mutualist partners across a gradient in resource availability. As resource availability increases, mutualistic *P. bursaria* abundance should increase in response to potential increased bacterial production and increased maltose production by endosymbiotic *Chlorella* (Figure 3a). Non-mutualistic *P. bursaria* are also expected to increase with changes in resources availability, but at a slower rate and stabilizing at a lower overall density since there are no *Chlorella* to facilitate increased utilization of available resources. Predictions to *P. bursaria* abundance will depend on responses of endosymbiotic *Chlorella* to resource augmentation, which could cause *P. bursaria* abundance gains to taper off at high resource levels.

As resource availability increases and *Chlorella*'s gain from trade decreases, endosymbiotic *Chlorella* should respond by decreasing association with *P. bursaria*, moving outside the host cell so that free-living *Chlorella* increase with increased resources and endosymbiotic *Chlorella* decrease (Figure 3b). Free-living *Chlorella* should increase in both mutualism (*P. bursaria* light) and non-mutualism (*Chlorella* alone) treatments in direct response to increased resources (Figure 3c). However, at high resource levels mutualism should be more costly than non-mutualism cultures, causing mutualism treatments to respond with overall lower densities than non-mutualism cultures (Figure 3c). Total *Chlorella* should mirror free-living *Chlorella* results if the mutualistic *Chlorella* populations exist primarily as free-living cells (Figure 3d). Overall total *Chlorella* densities (Figure 3d) should be slightly higher than free-living (Figure 3c), since total *Chlorella* includes endosymbiotic *Chlorella*.

Figures 4a-g

Plots of mean population densities are shown for each species over time with lines showing individual resource treatments (1/4x-2x). All data points are mean logged density with single standard error bars. Figure 4a is *P. bursaria* grown in light with its mutualist partner and Figure 4b is *P. bursaria* grown in dark without a mutualist partner. Mean endosymbiotic *Chlorella* density in mutualism treatment with host is depicted in Figure 4c while Figure 4g is mean number of endosymbiotic *Chlorella* cells calculated per *P. bursaria* cell. Figure 4e is free-living *Chlorella* in mutualism treatment. Total *Chlorella* equal to free-living *Chlorella* plus endosymbiotic *Chlorella* in *P. bursaria* cultures (mutualism treatment) are shown in Figure 4f. Mean density of free-living *Chlorella* in alone light treatment (no mutualism) is depicted in Figure 4d.

Figures 5a-e

Plots show comparisons of mutualism and non-mutualistic responses to the resource gradient. Data is taken from final day of the experiment (Day 28). Circles are logged mean species densities with single standard error bars. Resource gradient is 1/4x, 1/2x, 1x, and 2x normal nutrient concentration in laboratory culture medium (X-axis). *P. bursaria* cultures with *Chlorella* are mutualism cultures. *P. bursaria* alone is dark cultures with no *Chlorella*. *Chlorella* alone cultures are non-mutualism cultures in light with host absent.

Table 1

Rutgers University Soil Testing Lab results for Protozoan Pellet (Carolina Biological Supply 2003) nutrient contents. Ppm = mg/L.

Table 2

ANOVA table depicting responses to resource treatments. Results are from final day of the experiment (Day 28) when species responses had stabilized. Analyses show R-square, F-value, and P-value for each species or species group. Main effects in multiple species comparisons were nutrient treatments 1/4x to 2x. All analyses were done on logged densities.

Figure 1

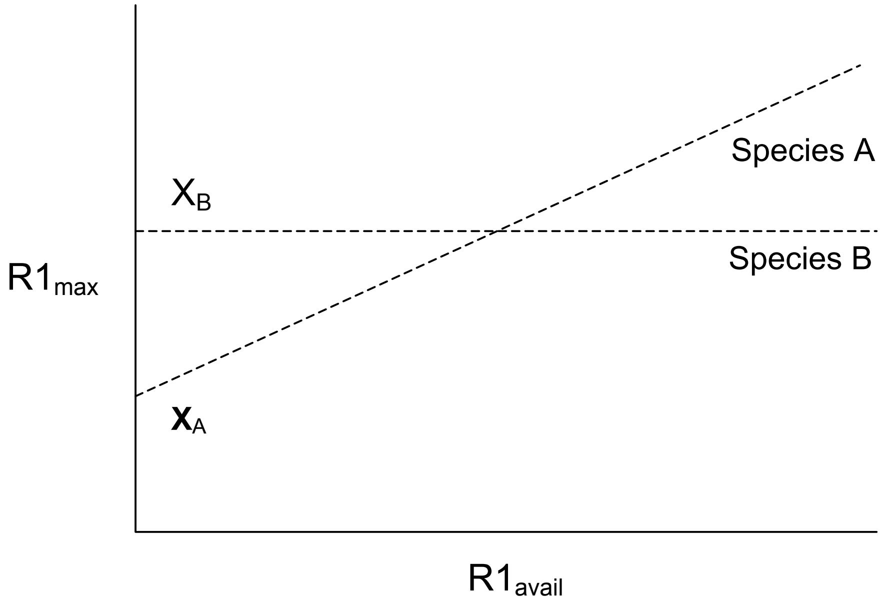


Figure 2

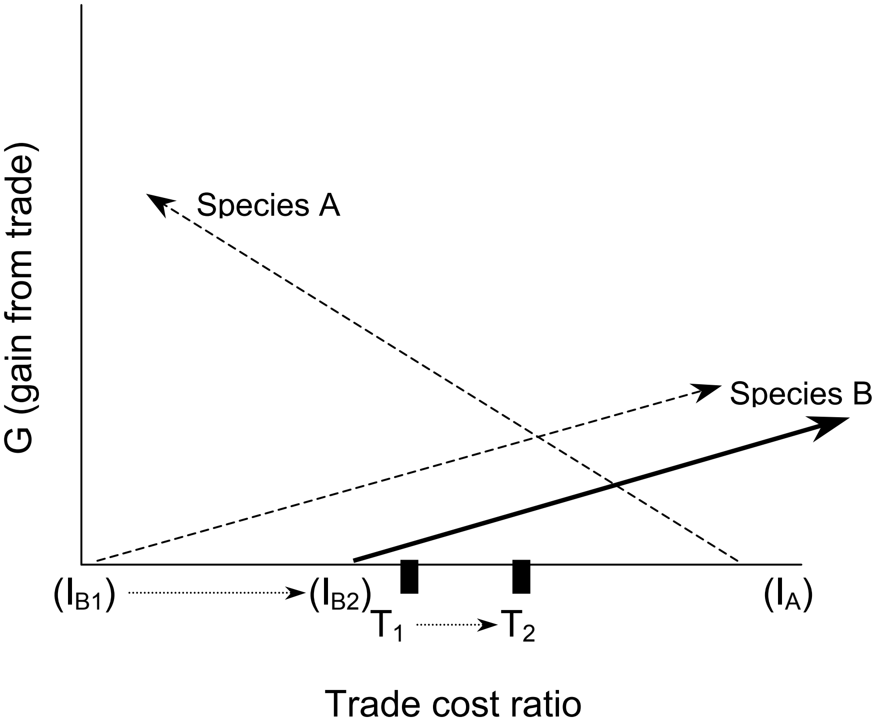
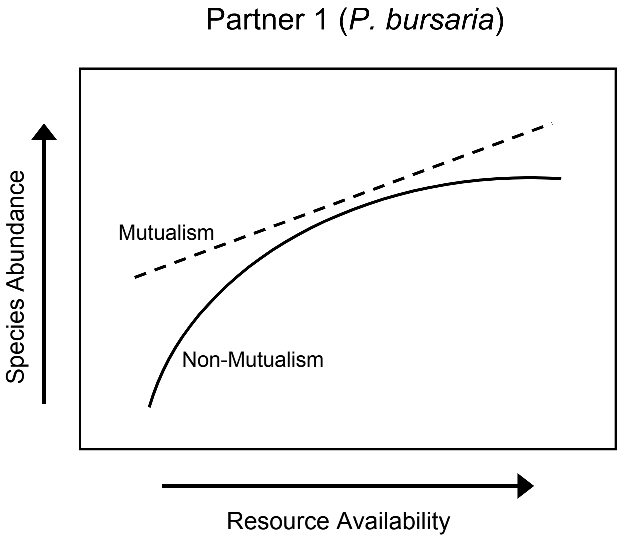
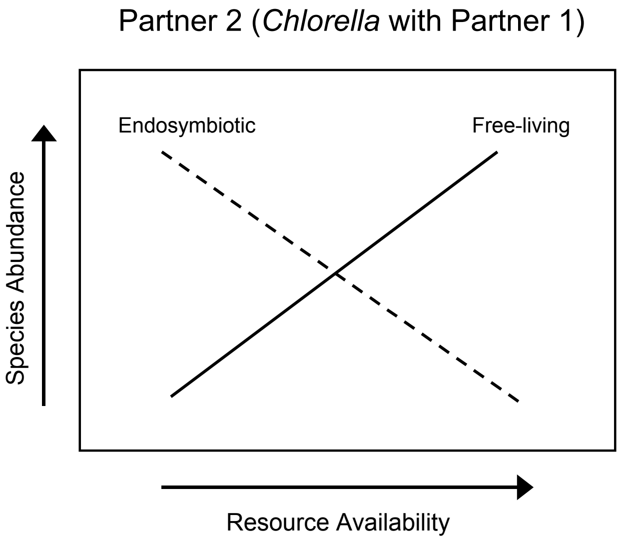


Figure 3a



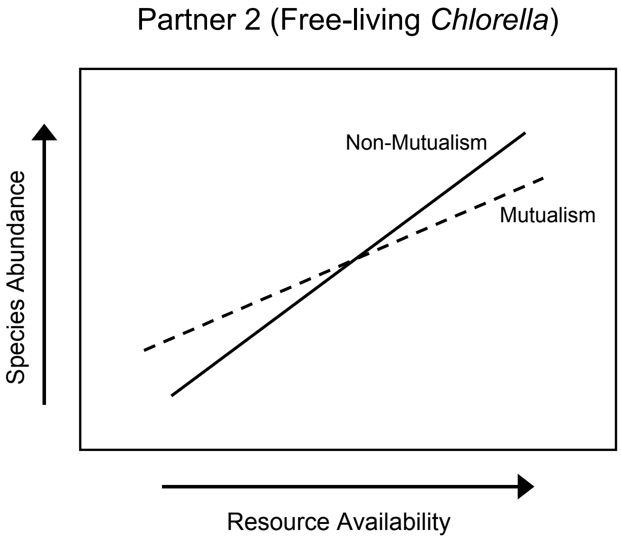
Partner 1 (*P. bursaria*)

Figure 3b



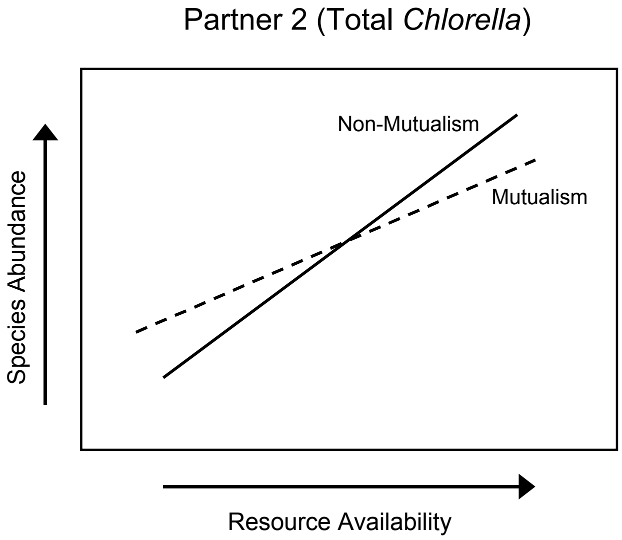
Partner 2 (*Chlorella* with Partner 1)

Figure 3c



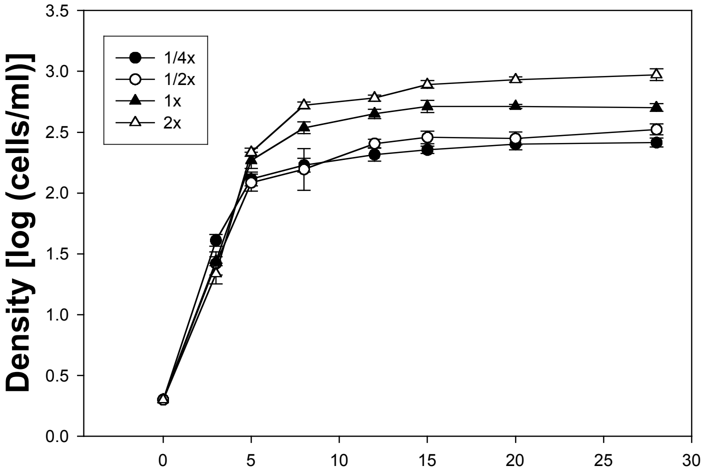
Partner 2 (Free-living *Chlorella*)

Figure 3d



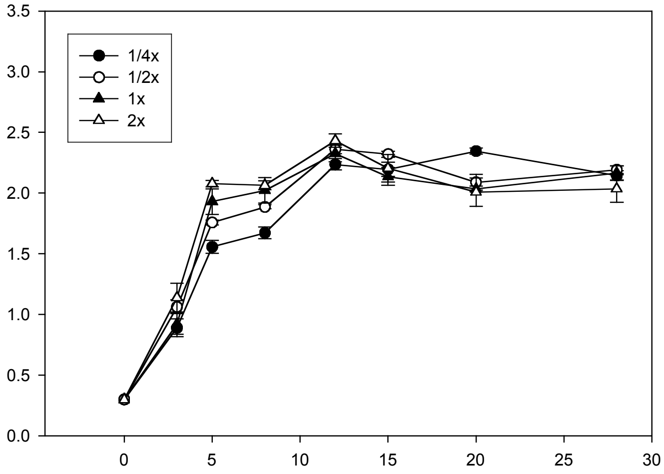
Partner 2 (Total *Chlorella*)

Figure 4a



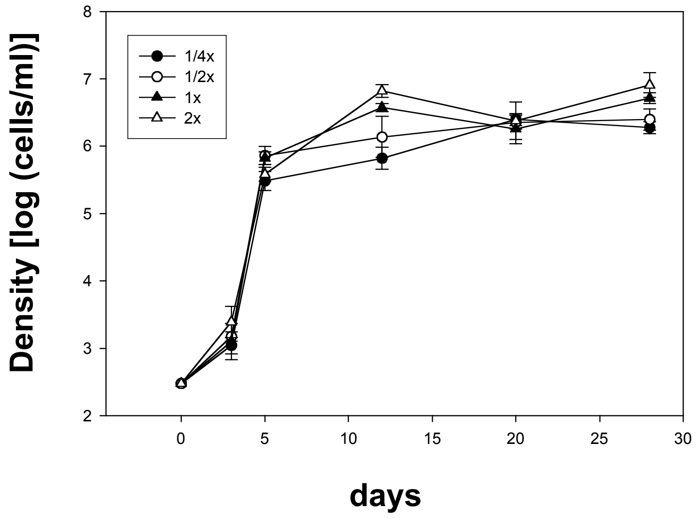
***P. bursaria* Light**

Figure 4b



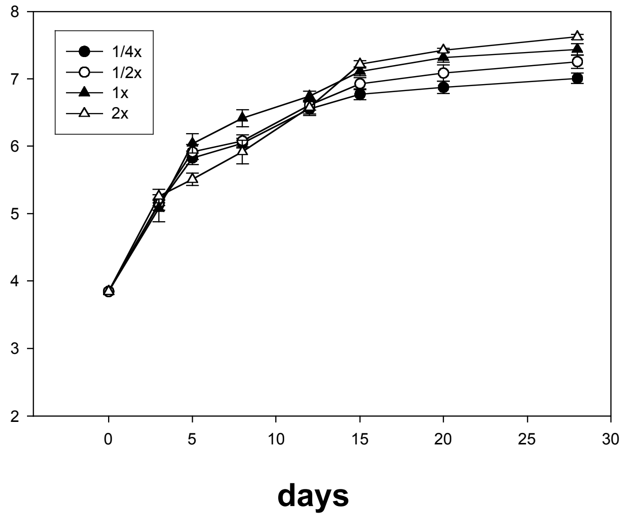
***P. bursaria* Dark**

Figure 4c



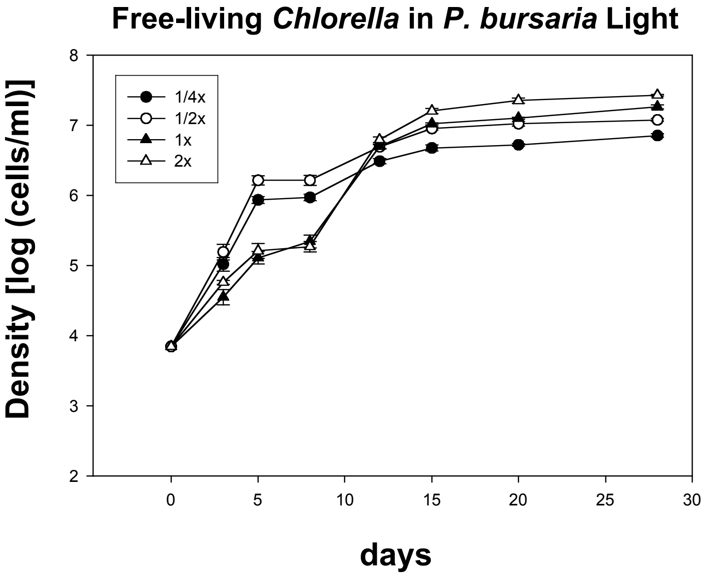
Endosymbiotic *Chlorella* in *P. bursaria* Light

Figure 4d



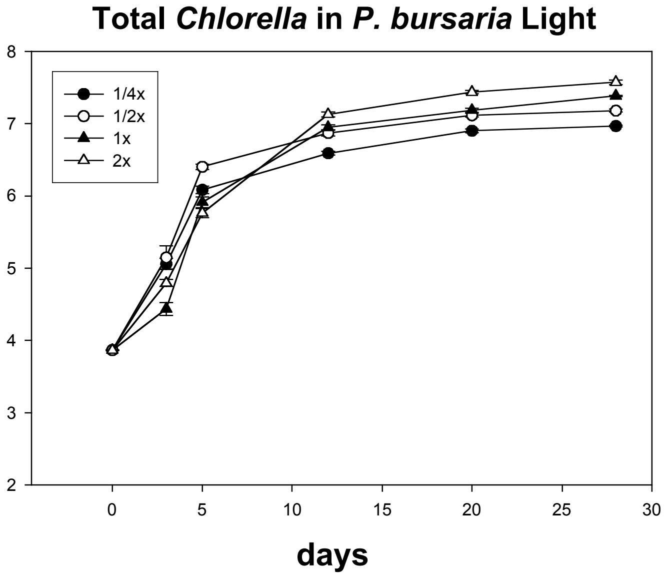
***Chlorella* alone**

Figure 4e



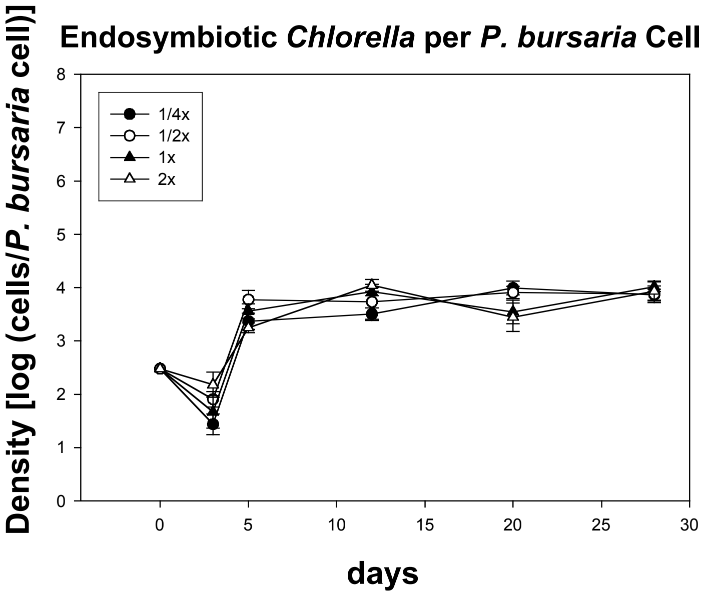
Free-living *Chlorella* in *P. bursaria* Light

Figure 4f



Total *Chlorella* in *P. bursaria* Light

Figure 4g



Endosymbiotic *Chlorella* per *P. bursaria* Cell

Figure 5a

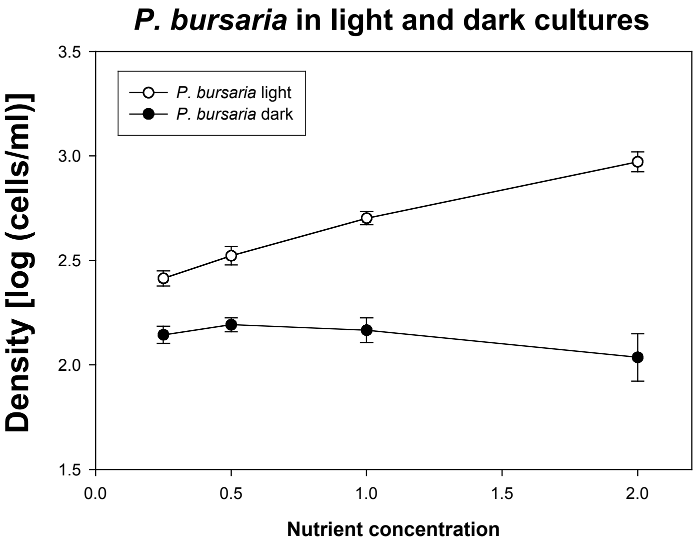


Figure 5b

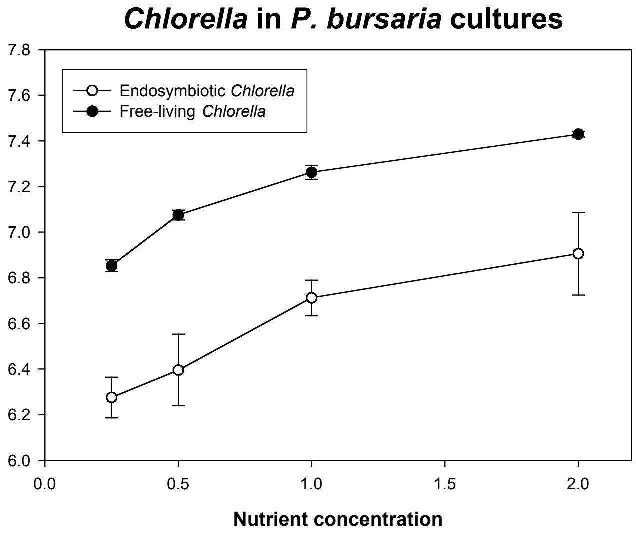


Figure 5c

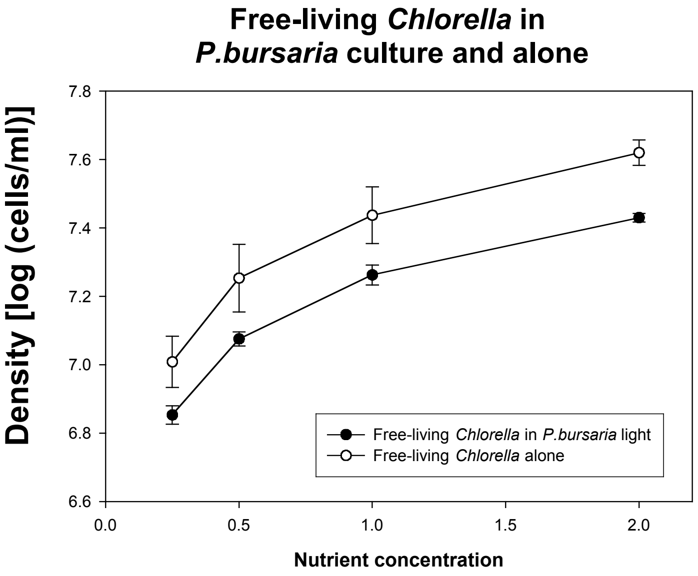


Figure 5d

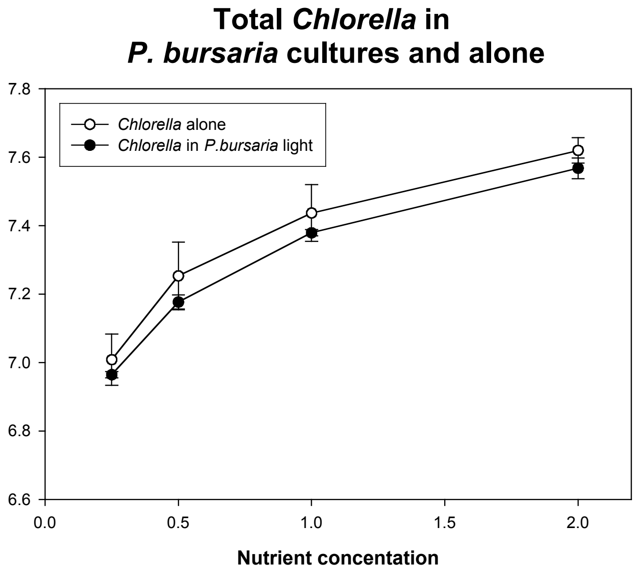


Figure 5e

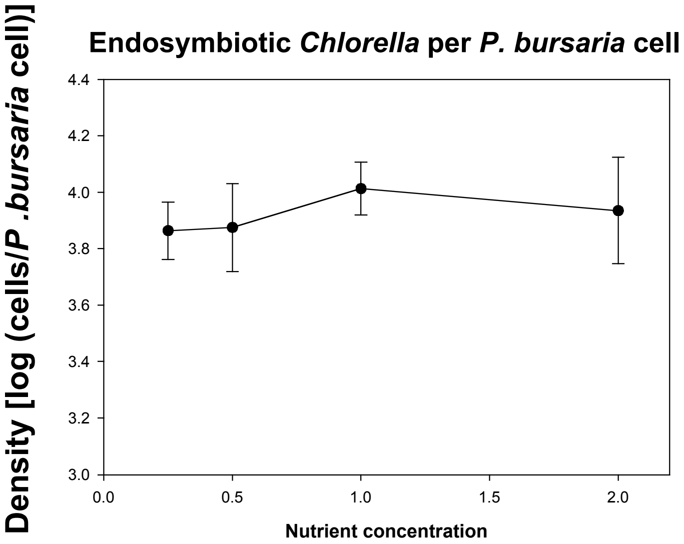

Endosymbiotic Chlorella per P. bursaria cell
Density [log (cells/P .bursaria cell)]
4.4
4.2
4.0
3.8
3.6
3.4
3.2
3.0
0.0
0.5
1.0
1.5
2.0
Nutrient concentration

Table 1

Protozoan Pellet Content	
Nutrients	Mean ppm
K	20.739
Mg	4.559
Ca	52.073
Mn	0.003
Zn	0.014
B	1.691

Table 2

ANOVA Table for Day 28 of Resource Concentration Treatments

Species Group	**R-square**	**F-value**	**p-value**
P. bursaria Light	0.8735	36.84	<0.0001
P. bursaria Dark	0.1567	0.99	0.4299
Endosymbiotic *Chlorella* per *P. bursaria* cell	0.0428	0.24	0.8682
Endosymbiotic *Chlorella* in *P. bursaria* Light	0.4690	4.71	0.0153
Free-living *Chlorella* in *P. bursaria* Light	0.9552	113.77	<0.0001
Total *Chlorella* in *P. bursaria* Light	0.9712	180.08	<0.0001
Free-living *Chlorella* alone	0.6850	11.60	0.0003

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Chapter 4:

Testing model predictions for predation on mutualist species

Abstract

Despite recent findings that mutualistic interactions between two species may be greatly affected by species external to the mutualism, the implications of such multi-species interactions for the population dynamics of the mutualists are virtually unexplored. Here, I asked how the mutualism between the ciliate host *Paramecium bursaria* and the endosymbiotic algae *Chlorella* is influenced by predation. In particular, I tested a hypothesis from a conceptual stress-dependent model of mutualism, which predicts that the incidence of mutualism should increase under significant predation and decrease when predators are absent. In contrast to model predictions, dynamics of mutualist partners were complex with only the predator of *P. bursaria* exhibiting measurable effects on the incidence of mutualism, while the predator of *Chlorella* did not affect mutualism. This study provides qualified support of the model and highlights the need to further explore mutualism in the presence of predators to more clearly understand the environmental conditions that select for mutualistic interaction.

Introduction

The importance of mutualisms to the organization and maintenance of natural communities has received renewed attention as widespread environmental change accelerates the need to understand the impact of mutualists on community dynamics

(Christian 2001; Bruno *et al.* 2003). The global decline of important mutualist species could have considerable impact on the organization of communities (Christian 2001). For example, worldwide decline of animal dispersers has heightened awareness of the importance of mutualism to plant communities (Cox *et al.* 1991; Chapman and Chapman 1995; Aparecida Lopez and Ferrari 2000; Christian 2001; Cordelro and Howe 2003). Predicting changes in communities when the availability of important mutualists is changing remains a difficult problem for ecologists.

Mutualisms exist in nearly every ecological system, within and between trophic levels, between species of all types. Positive species interactions range from the algae that help fuel reef-building corals, to symbionts that fix nitrogen, carbon, or defend hosts, to the myriad pollinators that assist in sexual reproduction in plants (Boucher *et al.* 1985; Douglas 1994; Pellmyr and Leebens-Mack 1999). Such mutualisms are often ecologically dominant and profoundly influential at all levels of biological organization (Herre *et al.* 1999). Although mutualisms can be defined as reciprocally beneficial relationships between organisms, they range from diffuse and indirect interactions to highly integrated and coevolved associations between species pairs. Mutualisms usually involve the direct exchange of goods and services (e.g. food, defense, or mobility) and often result in the acquisition of novel resources by at least one partner (Herre *et al.* 1999).

Mutualisms remain experimentally and theoretically under-studied though ecologists have accumulated extensive data on the biology of mutualistic organisms (Boucher 1985a-b; Bronstein 1994b). This is true since Darwin first broached the concept of mutualism (Darwin 1871) and van Beneden coined the term (van Beneden

1875). The evolutionary history of most known symbiotic mutualisms is long, up to 50-million years in ant-fungal relationships (Chapela *et al.* 1994; Currie *et al.* 2003), 40-million years in *Yucca*/Yucca moth mutualism (Pellmyr and Leebens-Mack 1999) or, in mycorrhizal relationships, as old as the origin of terrestrial plants (Pirozynski and Malloch 1975; Wagner and Taylor 1981; Stubblefield *et al.* 1987). Though theoretical work on mutualism has progressed, a central ecological and evolutionary question remains: What are the environmental conditions, both biotic and abiotic, that generate positive species interactions?

The dynamics of mutualist partners are affected by environmental conditions (Bronstein 1994a; Bertness and Hacker 1994; Greenlee and Callaway 1996; Callaway 1998; Brooker and Callaghan 1998; Callaway *et al.* 2002). Understanding these dynamics requires a multi-species perspective since mutualisms exist in a complex biotic environment including predators, competitors, parasites, and other mutualists (Bacher and Friedli 2002; Bronstein and Barbosa 2002; Stanton 2003; Bronstein *et al.* 2003). The impact of external species on mutualism is not fully understood, though "other" species may be very important. For example, in the widespread group of protection mutualisms, the benefits accrued to partners depends on the presence or abundance of predators (Agrawal and Fordyce 2000; Morales 2000; Fischer *et al.* 2001). The fact that two-species mutualistic systems can be strongly affected by species external to the relationship has recently garnered more attention (Bronstein *et al.* 2003; Palmer *et al.* 2003; Stanton 2003). Still, there are relatively few studies examining the impact of external species, and even fewer studies that integrate model testing with empirical results from multi-species experiments involving mutualism.

Theory

Recent research has demonstrated that the overall effects of species on each other may vary as the relative importance of competitive and facilitative mechanisms shift (Callaway 1995, 1997; Brooker and Callaghan 1998; Pugnaire and Luque 2000; Callaway *et al*. 2002). A conceptual model, first described by Bertness and Callaway (1994), addresses the fundamental question of how environmental conditions impact mutualism by proposing that the relative importance of positive interactions to the organization of communities increases with abiotic and biotic stress (see also Brooker and Callaghan 1998). This well-cited stress model of mutualism (hereafter SMM; Bertness and Callaway 1994) integrates both biotic interactions and abiotic factors into a single conceptual model. The SMM hypothesizes that the presence of consumers (predators) affects the advantages gained by potential mutualist partners and influences the probability of species forming mutualistic relationships.

The SMM is drawn from natural history accounts of mutualism as well as field observations and experiments in New England salt marshes demonstrating the significant role mutualism plays in determining community dynamics (Bertness and Leonard 1997). The model suggests that environmental conditions often dictate species interactions and mutualism tends to be driven by two major environmental stresses that species commonly encounter. First, predators constitute a biotic stress by consuming mutualists. Bertness and Callaway (1994) suggest that prey have an advantage when cooperating to avoid or otherwise defend against significant predation. Cooperation is unnecessary and thus unlikely when predation is low, leading to increased competitive interactions rather than mutualistic interaction in low stress environments (Figure 1). Second, abiotic stress is loosely defined as an environmental factor that creates physiological stress for organisms. Bertness and Callaway (1994) cite evidence that cooperation appears to be more common in physiologically stressful environments (e.g. saline aquatic or dry desert ecosystems) where positive interaction can facilitate amelioration of stressful habitat conditions. The SMM predicts that as environmental stress increases, mutualism should be selected for, resulting in increased mutualism and decreased competition in the community (Figure 1). By logical extension, the advantage that species gain by participating in mutualism depends on where organisms occur along a physiological/predation stress gradient (Bertness and Callaway 1994).

Many experimental studies generally support this model (Walker and Chapin 1987, Bertness and Shumway 1993, Bertness and Hacker 1994, Greenlee and Callaway 1996, Callaway 1998, Kitzberger *et al.* 2000) with some exceptions (Moen 1993, Olofsson *et al.* 1999). However, few studies have examined mutualisms among large numbers of species along gradients of consumer pressure, and there have been even fewer efforts to integrate experimental results from predation studies with tests of specific models of mutualism. In this paper, I describe the response of two mutualist partners to the presence or absence of predators, which constitute negative biotic pressure for individual mutualist species.

I tested predictions from the SMM on a model mutualism between the ciliate host *Paramecium bursaria* and the endosymbiotic algae *Chlorella*. The association between endosymbiotic *Chlorella* and its host, *P. bursaria*, is a mutualism commonly found in freshwater ponds, lakes, and streams (Reisser 1986; Wichterman 1986; Smith and Douglas 1987). These organisms are easily cultured under laboratory conditions and can be experimentally manipulated (Jennings 1938a, 1938b; Karakashian *et al*. 1968; Reisser 1986, 1987; Gortz 1988; Hosoya *et al*. 1995; Nishihara *et al*. 1998; Tanaka *et al*. 2002). *Chlorella* is an endosymbiont of *P. bursaria*, but can also be cultured separately as free-living *Chlorella.* Additionally, algal-free *P. bursaria* can be easily re-infected with free-living *Chlorella*, thus restoring the mutualism. The flexibility inherent in this mutualism is particularly useful since clear model testing benefits from controls with mutualists growing together and alone.

Experiments conducted in many terrestrial and aquatic systems are limited by the relatively slow population dynamics of focal organisms. The longest of these studies contain data that correspond to only a couple of generations of plants (e.g. Callaway *et al*. 2002), which raises concerns that observed relations could be driven by effects of initial experimental conditions or transient population dynamics. The short generation times of protists, hours to days, (Lawler and Morin 1993) make it possible to assemble communities from small initial populations (inocula) of organisms. Protists then rapidly grow to maximum densities where species compositions are set by system dynamics rather than initial experimental conditions (Petchey *et al*. 2002b; Morin and McGrady-Steed 2004). Protists microcosms make it possible to evaluate statistically significant long-term responses of component organisms to experimental treatments.

Methods

To test the effect of consumer stress on mutualisms, I constructed aquatic communities in microcosms assembled from protists and bacteria with and without mutualism. Experimental mutualism treatments were crossed with a predator/no predator treatment

(Table 1). Five replicates for each treatment (4x2x5= 40 microcosms) provided replication to control for within treatment variation. Communities without functioning mutualism between symbionts were used as controls to further examine how mutualism affects species responses to predators.

Strains of algal-free *P. bursaria* (MBW-1 strain) produced by a Paraquat herbicide method (Hosoya *et al*. 1995), and of free-living *Chlorella* (SA-2 strain) were provided by Hiroshi Hosoya (Hiroshima University). Algal-free strains of *P. bursaria* (MBW-1) were produced and confirmed by Hosoya, similar to methods used in Tanaka *et al*. (2002). Algal-free *P. bursaria* (MBW-1) were re-infected with free-living *Chlorella* (SA-2) to produce mutualistic *P. bursaria* (MB-1 strain) containing endosymbiotic *Chlorella*. Therefore, endosymbiotic *Chlorella* were genetically identical to free-living *Chlorella*.

Non-mutualism treatments were *Paramecium bursaria* alone containing only *P. bursaria*, and free-living *Chlorella* alone treatments containing only free-living *Chlorella*. Mutualism treatments were cultures of *P. bursaria* with *Chlorella* (functioning mutualism) that contained both free-living and endosymbiotic *Chlorella* (Table 1). All mutualism/non-mutualism treatments had the same bacterial inoculum (*Bacillus subtillus, B. cereus, Serratia marcescens,* and *Proteus vulgaris*) and were subjected to a predator/no predator treatment (Table 1). Starting densities of all species were identical and microcosms were housed under a 12:12hr light:dark regime in Percival incubators at 22°C for the duration of the experiment.

Microcosms were constructed as in previous experiments (Lawler and Morin 1993; Petchey *et al*. 1999; 2002a) using 250ml covered glass bottles containing 100ml of growth media. Growth media was protist pellet media (PPM) consisting of non-filtered de-ionized (DI) water with dissolved protist pellets and sterilized wheat seeds. Protist culture pellets (Carolina Biological Supply 2003), the primary nutrient source for cultures, consist of ground plant matter, which contains primarily nitrogen, phosphorous, and other minor nutrients. Two wheat seeds were also added to each jar to provide a slow release carbon source to fuel bacterial production.

Following sterilization of media, all cultures were inoculated with a suite of bacterial species (*Bacillus subtillus, B. cereus, Serratia marcescens,* and *Proteus vulgaris*) to create an initial resource base for bactivorous *P. bursaria.* Bacteria grew for 24 hours before protist addition. *Paramecium bursaria* and *Chlorella* were added on Day 0 of the experiment. Predators were not added until Day 7, when prey populations had become established. The predators were *Stentor sp*., a heterotrich predator that feeds on *P. bursaria*, and *Lepadella sp*., a rotifer that preys on *Chlorella sp*. but not *P. bursaria* (Table 1).

One hundred *P. bursaria* cells were added to each microcosm. Endosymbiotic *Chlorella* densities averaged 271 cells/*P. bursaria* cell (mean of 10 direct counts) in *P. bursaria* mutualism cultures at the initiation of the experiment. Similar densities of free-living *Chlorella* were added to both mutualism and non-mutualism treatments (*P. bursaria* with *Chlorella* and *Chlorella* alone). Medium was refreshed at rate of seven percent of total volume weekly (equal to 7ml fresh PPM/week) following weekly counts in order to limit negative impacts of waste accumulation. No contamination by eukaryotes occurred except in the *P. bursaria* alone treatment (no mutualism) with *Stentor*. Here, free-living *Chlorella* invaded and slowly established, undetectable until day 18, and may have contributed to increased variation of *P. bursaria* in treatment, though overall impact on experimental results was negligible.

Paramecium bursaria were counted twice weekly by microscopy (on Nikon SMZ-U) with densities standardized by sample volume, as determined by weight with an electronic balance. Free-living *Chlorella* were counted on the same schedule as *P. bursaria* using a hemacytometer. Endosymbiotic *Chlorella* densities were assessed once weekly using the sonication method (see Chapter 3). In this method, *Paramecium bursaria* cells were burst using a sonicator (Fisher Scientific Sonic Dismembrator - Model 60) where the time and intensity of disruption can be controlled so that *P. bursaria* cells burst while *Chlorella* cells remain intact. First, from a well-mixed jar, 1ml of *P. bursaria* (mutualism treatment) was sonicated at level 20 for six seconds. Then, total *Chlorella* in the sonicated medium were counted on the hemacytometer, and previously counted free-living *Chlorella* densities were subtracted from total *Chlorella* density to give endosymbiotic *Chlorella* density for each replicate. *Chlorella* death caused by sonication was insignificant and sonication methods provided repeatable measures of endosymbiont densities in previous trials and experiments (see Chapter 3). Densities are log-transformed to facilitate comparisons among organisms.

Statistical Analysis

All statistical analyses were carried out with Statistical Analysis System software (SAS v.8.2). Since all comparable replicates had identical starting densities of bacteria and protists (same number of *P. bursaria* and same number of free-living *Chlorella* in mutualism and non-mutualism communities), I assumed differences in mean protist abundance constituted real species responses to predator and mutualism treatments. Using each sampling day's species densities as independent variables, I performed individual repeated measures ANOVAs to examine the effect of treatments over time on abundance measures for each species. The main effect used in Repeated Measures was the presence or absence of predators. Multiple species comparisons in an analysis of variance were used to examine differences between organism responses to predator treatment effects (main effect in analysis) on species abundances (procedure GLM) and to identify statistically significant differences between mutualist and non-mutualist treatments on daily comparisons. Tukey's Studentized Range Test provided conservative comparisons between treatment differences on individual species abundances at $\alpha=0.05$. Tukey's tests were performed for all sampling dates and all analyses were done on log transformed densities.

Results

Multiple comparisons in an analysis of variance (ANOVA) revealed significant differences in species responses to predator and mutualism treatments. Individual species abundances over time are grouped by relevant treatment comparisons (Figures 2–6). *Lepadella*, which feeds on free-living *Chlorella*, attained high densities relatively quickly in both mutualism and non-mutualism treatments (Figure 6a). However, *Lepadella* abundance was significantly lower in communities with mutualism (repeated measures ANOVA; $X^2=2.7443$, $F=321.72$, $p<0.0001$). Additionally, densities of *Lepadella* were lower in mutualism treatments for all individual day comparisons except days 11 and 25 (Figure 6a; Tukey's $p=0.05$).

Lepadella decreased free-living *Chlorella* populations once it became established in the non-mutualism treatment, after day 7 (Figure 4c; Tukey's p=0.05), but this effect disappeared towards the end of the experiment. In mutualism cultures (free-living *Chlorella* with *P. bursaria*), free-living *Chlorella* had variable responses to predation (Figure 3b). Free-living *Chlorella* in the absence of *Lepadella* had significantly lower abundance than when in *Lepadella* treatments (Figure 3b; Tukey's, p=0.05, from days 18-28), with zero abundance on multiple days. In *Stentor* treatments, free-living *Chlorella* reached the highest overall densities by the end of the experiment, similar to densities in *Lepadella* treatments. Significant differences between *Lepadella* and *Stentor* treatments occurred only on days 18 and 25 (Tukey's, p=0.05). Interestingly, free-living *Chlorella* were most abundant when growing apart from its mutualist partner, *P. bursaria* (Figure 3a; X^2=413.1061, F=208.48, p<0.0001), with daily comparisons statistically different for all days except the last two (Tukey's, p=0.05).

Though endosymbiotic *Chlorella* populations appear to respond similarly to the treatments, differences were observed after day 14, once predators became established (Figure 5a). From day 18 through the end of the experiment, endosymbiotic *Chlorella* populations in *Lepadella* treatments were significantly lower than populations in *Stentor* treatments (Tukey's, p=0.05; Figure 5a). Endosymbiotic *Chlorella* populations in the non-predator treatment were variable between populations in *Lepadella* and *Stentor* treatments (Figure 5a). However, the mean number of endosymbiotic *Chlorella* per *P. bursaria* cell (Figure 5b) was not significantly affected by *Lepadella* (X^2=1.3497, F=1.21, p=0.1626). Though, *Stentor* did not have an overall significant affect on the mean number of endosymbiotic *Chlorella* per *P. bursaria* cell (repeated measures ANOVA,

X^2=2.5604, F=0.80, p=0.3979), populations in *Stentor* treatments were significantly different on days 18 and 21 in Tukey's comparisons (p=0.05). These two days (18 and 21) represent significant points in an overall trend for increased endosymbiotic *Chlorella* in *Stentor* treatments (Figure 5b). The proportion of mutualistic *Chlorella* in the total *Chlorella* population (Figure 5c) was analyzed for predator effects. The overall effect of predator treatments served to significantly decrease the proportion of endosymbiotic *Chlorella* (Figure 5c) in both *Stentor* (repeated measures ANOVA; X^2=0.0087, F=14.21, p=0.0055) and *Lepadella* treatments (X^2=0.0116, F=138.76, p<0.0001) compared to no predator controls where proportions were stable.

Total *Chlorella* includes endosymbiotic and free-living *Chlorella* populations. When examined for predator effects, highest densities were observed in *Stentor* treatments (Figure 4b, repeated measures ANOVA; X^2=2.1221, F=6.27, p<0.0367). Predation by *Lepadella* did not have an overall statistically significant effect (repeated measures ANOVA; p= 0.1960), though *Lepadella* did significantly reduce total *Chlorella* abundance for all days after day 14, except for day 25, compared to no predator controls (Figure 4b; Tukey's p=0.05). Significant differences in total *Chlorella* abundance observed following day 14 were similar to endosymbiotic *Chlorella* populations (Figures 5a, b). Total *Chlorella* populations were higher in *Stentor* treatments compared to *Lepadella* treatments for all days following *Stentor* establishment after day 14 (Tukey's, p=0.05). Total *Chlorella* with and without its mutualist host (Figure 4a) showed significant differences for all days until the last three sampling dates (Tukey's, p=0.05). After day 21, total *Chlorella* alone (non-mutualism) populations dropped with high variability eliminating any subsequent differences from populations in mutualism treatments (Figure 4a). When *Lepadella* was added to *Chlorella* alone populations (Figure 4c), the predator significantly reduced prey populations (repeated measures ANOVA, X^2=35.6077, F=14.30, p=0.0054).

To examine how mutualism affects total *Chlorella* grown with a predator, I plotted total *Chlorella* abundance cultured with *Lepadella*, with and without its mutualist host. Total *Chlorella* with *P. bursaria* (mutualism) were significantly higher than total *Chlorella* growing without *P. bursaria* (non-mutualism) when subjected to predation by *Lepadella* (Figure 4d; repeated measures ANOVA, X^2=19.6187, F=19.33, p=0.0023). The largest increase in free-living *Chlorella* occurred on day 25 (Figures 3a, b), which provides explanation for the transient lack of significant difference (Tukey's, p=0.05) on day 25 between total *Chlorella* measures (Figure 4d).

Stentor attained highest densities in mutualism cultures with *P. bursaria* and *Chlorella* (Figure 6b; repeated measures ANOVA X^2=7.6759, F=1202.71, p<0.0001). *Stentor* failed to establish at levels sufficient to impact prey in non-mutualism populations of *P. bursaria* (Figure 6b). Therefore, populations of *P. bursaria* in non-mutualism treatments were not consistently affected by the *Stentor* manipulation (Figure 2b, repeated measures ANOVA, X^2=0.00196, F=0.01, p=0.9058), though occasional differences did exist on days 7, 21, and 25 (Tukey's, p=0.05). Analysis of predation effects on *P. bursaria* in communities with *Chlorella* (Figure 2c) revealed strong effects of predation with significant declines in *P. bursaria* abundance after day 18, when *Stentor* reached appreciable densities (Tukey's, p=0.05). *Lepadella* had no effect on *P. bursaria* abundance compared to non-predator treatments (Figure 2c, repeated measures ANOVA, X^2=0.2571, F=4.83, p=0.0593). When in mutualism treatments with *Chlorella*

(Figure 2a), *P. bursaria* populations were significantly higher than when in non-mutualism treatments (repeated measures ANOVA, X^2=1.8261, F=43.94, p=0.0002), revealing further confirmation of the positive affect of mutualism on host abundance.

Discussion

Predator effects

The presence of predators caused predictable declines on prey abundance with variable effects on the incidence of mutualistic interaction between mutualist partners. The SMM (Bertness and Callaway 1994) predicts that mutualism should be favored under predation. This idea has been extrapolated to suggest that mutualist partners should increase their associations with each other under high stress, reducing them when benefits decrease under moderate or low stress. Mutualist partners in this study did not dramatically disengage from mutualism when predators were absent compared to predator addition treatments. However, by examining the relative changes in mutualistic *Chlorella* (endosymbiotic) inside host cells, it was clear predation influenced the mutualism between prey. Increases in the density of endosymbiotic *Chlorella* in the cytoplasm of *P. bursaria* were statistically significant on a number of sampling dates, indicating an overall trend towards increased incidence of mutualism with predation, as predicted by the SMM. *Chlorella*, however, did not increase the proportion of endosymbiotic cells involved in mutualism when its predator *Lepadella* was present. Therefore, mutualist partners exhibited more complex responses than predicted by the simple models.

For example, the proportion of endosymbiotic *Chlorella* from the total *Chlorella* population is a measure of the proportion of *Chlorella* cells involved in mutualism. This estimate of mutualistic interaction was affected by predator addition treatments once predators reached adequate densities to exert consumer pressure on prey. Responses of endosymbiotic *Chlorella* are a function of the response of its host, *P. bursaria*, since endosymbiotic *Chlorella* populations live inside host cells (Smith and Douglas 1987). As a result, the proportion of endosymbiotic *Chlorella*, though somewhat idiosyncratic, was overall lower in *Stentor* treatments where the host was preyed upon, compared to non-predator treatments. When free-living *Chlorella* are at very low densities, the proportion of endosymbiotic *Chlorella* will appear very high, as is the case in the non-predator treatment with *Chlorella's* host, *P. bursaria.*

Paramecium bursaria appear to repress growth of free-living *Chlorella* extracellularly (McAuley 1986; McPhearson *et al.* in prep), likely as a consequence of the need for *P. bursaria* to regulate *Chlorella* densities within the host cell to stabilize *Chlorella* growth rates to match its own (Smith and Douglas 1987). Benefits to *P. bursaria* come from photosynthate products produced by endosymbiotic *Chlorella*, however, free-living *Chlorella* can be harmful to the host if free-living *Chlorella* bloom, deplete dissolved oxygen outside the cell, and otherwise create physiologically stressful conditions for the host (McPhearson *et al.* in prep). Harmful effects of algal blooms appear to be over and above potential benefits host may receive from algal oxygen production within the host cell. In this study, free-living *Chlorella* populations in *P. bursaria* (mutualism) treatments were very low compared to densities in predator treatments. It appears that *P. bursaria* may have had a negative effect on free-living *Chlorella* abundance in this experiment similar to previous research (see Chapter 2). Because there were very low free-living *Chlorella* densities in *P. bursaria* cultures, endosymbiotic *Chlorella* make-up a significantly larger fraction of the total *Chlorella* population and consequently influence analytical results of all mutualistic *Chlorella* responses to treatments. This added complexity of potential inhibition of free-living *Chlorella* while in mutualism with endosymbiotic *Chlorella* demonstrates the complexity of even simple two-species mutualisms and the need to examine such mutualisms in multi-species assemblages to parse out factors important to the biology of mutualism.

The mean number of endosymbiotic *Chlorella* per *P. bursaria* cell and the overall population size of endosymbiotic *Chlorella* are important measures of the incidence of mutualism reflecting how mutualistic *Chlorella* are affected by environmental conditions such as predation. The abundance of endosymbiotic *Chlorella* is predicted by the SMM to be profoundly affected by host responses to environmental conditions. The addition of *Stentor* caused increases in populations of endosymbiotic *Chlorella* when compared to non-predator treatments for multiple sampling dates. These data show that when the host is experiencing predation pressure, it increases the association with its mutualist partner confirming predictions from the SMM (Bertness and Callaway 1994) and other studies demonstrating the effect of environmental stress on the incidence of mutualism (Bertness and Hacker 1994; Bertness and Leonard 1997; Callaway and Walker 1997; Callaway *et al*. 2002).

There was a strong trend towards increased mean number of endosymbiotic *Chlorella* cells per *P. bursaria* in *Stentor* treatments as well, though variability in *P. bursaria* populations caused this to only be significant on days 18 and 21. *Lepadella* does not affect mean number of endosymbiotic *Chlorella* compared to no predator treatments, contrary to predictions by the SMM. Accordingly, this study partially confirms predictions from the SMM but underlines the importance of complex life history details associated with even relatively simple microorganisms.

Mutualism effects

The benefits of positive interaction have been widely documented for a multitude of types of mutualism. In some cases species are protected from predators or herbivores by mutualist partners (Janzen 1966, 1985), in others, species receive nutrient support (van der Heijden *et al*. 1998; Weber 1966, 1972). In this experiment, the predator *Lepadella* reached higher densities in the non-mutualism treatment, causing total *Chlorella* populations to suffer more from predation when not in mutualism with its host, *P. bursaria*. This result confirms the suggestion of Smith and Douglas (1987) that *Chlorella* benefits from decreased predation by living inside host cells.

However, it is interesting that total *Chlorella* reach highest densities in *P. bursaria* cultures with *Stentor*. This corroborates suggestions from previous experiments that *P. bursaria* may depress free-living *Chlorella* abundance (McAuley 1986; Douglas and Smith 1989). When *P. bursaria* is preyed upon by *Stentor*, potential negative effects of *P. bursaria* on free-living *Chlorella* populations are weakened. Due to the dramatically low densities of free-living *Chlorella* in *P. bursaria* cultures, free-living *Chlorella* in predator treatments suffer very little effects of predation compared to non-predator treatments.

Chlorella may increase the ability of *P. bursaria* to resist predation by a common predator, *Didinium* (Berger 1980). In this experiment, effects of *Chlorella* on predator resistance were difficult to assess because *Stentor* failed to become established in non-mutualism *P. bursaria* cultures. Thus *Stentor* had little negative affect on *P. bursaria* alone cultures, though occasional significant differences did occur between predator and non-predator treatments in *P. bursaria* alone treatments (Days 7, 21, and 25). Negative effects of *Stentor* were easily observed in *P. bursaria* mutualism cultures. Overall, experimental results indicate predation pressure can be diminished by mutualistic association between partners.

Conclusion

Evaluating responses over relatively long time periods allows observation of the complexities involved in predicting responses to treatments that might otherwise appear simplistic if evaluated for only one or two generations. Small-scale microbial systems provide the opportunity to study the long-term consequences of environmental stress on mutualisms in systems that develop over many generations of the interacting organisms (Petchey *et al*. 2002b; Morin and McGrady-Steed 2004). When conducted over a large number of generations, such studies should display patterns reflecting the long-term impact of predators or physiological stress on species interactions and ecosystem processes, rather than revealing transient effects of initial species densities that complicate studies of longer-lived organisms. This makes aquatic microbial systems a powerful model system for experimental studies of mutualism. The mutualism between *P. bursaria* and *Chlorella* can be used as a model mutualism to test relevant theory of mutualism and aid understanding of the environmental conditions that give rise to mutualistic interaction.

Facilitation between species is thought to be a key mechanism in the relationship between biodiversity and ecosystem function (Cardinale *et al*. 2002). Realizing how environmental conditions impact mutualistic processes is crucial to understanding larger questions involving biodiversity and ecosystem functioning (Hacker and Gaines 1997; Stachowicz 2001; Bruno *et al*. 2003). This understanding will also become increasingly important as ecologists develop conservation priorities (Christian 2001) and apply principles of restoration ecology. The "keystone mutualist hypothesis" predicts that human-caused losses of important mutualists could trigger a cascade of linked extinctions throughout the community (Gilbert 1980). This view holds that mutualisms are crucial ecological interactions that maintain the structure and diversity of natural communities, which has only recently begun to be empirically demonstrated (Cardinale *et al*. 2002).

Strong results often come out of simplified systems. Model systems are highly abstracted, resembling mathematical models; however, they can show functional responses between model organisms. Empirically testing relevant models as described here is important if ecologists are going to elucidate why there are so many mutualisms and what environmental conditions encourage their proliferation. Although many studies suggest the importance of mutualism (Menge 2000; Bruno *et al*. 2003), empirical evidence for community-level impacts of mutualism remain scarce – specifically the roles played by predators in mediating dynamics between mutualist partners. This study tests the consumer stress axis of the SMM (Bertness and Callaway 1994) and provides support for predictions from the model while confirming the need to explore potential mediation of predation by mutualism.

Acknowledgements

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Figure Legends

Table 1

Experimental design is predator/no predator treatments crossed with organism treatments either with or without mutualism. Predators are *Stentor* and *Lepadella*. *Stentor* preys on the host *P. bursaria* and *Lepadella* preys on *Chlorella*. *Paramecium bursaria* alone (MB-1) are bleached cells with no endosymbiotic *Chlorella*, *Chlorella* alone (SA-2) treatments have only free-living *Chlorella*, and *P. bursaria* and *Chlorella* together (MB-1) treatments consists of both free-living and endosymbiotic *Chlorella*. All microcosms are replicated five times.

Figure 1

A conceptual model of positive interactions in natural communities. Positive interactions are predicted to be rare under mild physical conditions and low consumer pressure. Neighborhood amelioration of physical stress and associational defenses, however, are hypothesized to lead predictably to increased positive interactions under harsh physical conditions and intense consumer pressure, respectively (redrawn from Bertness and Callaway 1994).

Figure 2

Paramecium bursaria are shown with and without its mutualist partner (a), in a non-mutualism treatment with and without its predator (b), and in mutualism with *Chlorella* and no predator or predator (*Stentor* and *Lepadella*) treatments (c). Densities are log-transformed and calculated as cells/ml taken from direct counts over time with single standard error bars. Plots show effects of mutualism (a), predation without mutualism (b), and predation with mutualism (c).

Figure 3

Free-living *Chlorella* are depicted with and without mutualist host (a) and clearly show negative effect of host on free-living *Chlorella* populations. In Figure 3b, free-living *Chlorella* are plotted in mutualism treatments with and without predators and show complex results of predation treatments. *Chlorella* densities are log-transformed densities over time taken from direct counts with single standard error bars.

Figure 4

Total *Chlorella* are calculated as free-living plus endosymbiotic *Chlorella* to give total *Chlorella* populations in treatments bottles. Densities are log-transformed from direct counts over time with single standard error bars. Total *Chlorella* are plotted with and without mutualist host to demonstrate stability of population size through time (a). When in mutualism with *P. bursaria* (b), total *Chlorella* show positive increase through time with variable effects of predator treatments. When total *Chlorella* are separated from mutualism (c), predation initially reduces *Chlorella* abundance. Total *Chlorella* consist of free-living *Chlorella* only in alone treatments (c). Harsh effects of predation are mediated by mutualism in Figure 4d.

Figure 5

Endosymbiotic *Chlorella* are mutualistic *Chlorella* living inside *P. bursaria* cells. Endosymbiotic *Chlorella* populations tend to be higher in *Stentor* predation treatments (a) than non-predator or *Lepadella* treatments. Mean number of endosymbiotic *Chlorella* cells per *P. bursaria* cell (b) show similar results as in Figure 5a and are a direct measurement of how the incidence of mutualism changes within the host as a function of predation stress. Additional analyses involved calculating the proportion of endosymbiotic *Chlorella* (c) as a function of total *Chlorella* population size. Proportions have single standard error bars calculated from replicate means.

Figure 6

Predators were *Lepadella*, which preys on free-living *Chlorella* (a) and *Stentor* (b), a heterotrich ciliate that preys on *P. bursaria*. Densities are plotted as cells/ml over time with single standard error bars. *Stentor* reached highest densities in mutualism treatments while *Lepadella* reached highest densities in non-mutualism treatments.

Table 1

Experimental Treatments	**Predator**	**No Predator**
P. *bursaria* alone (MBW-1)	*Stentor*, 5 reps	5 reps
Chlorella alone (SA-2)	*Lepadella*, 5 reps	5 reps
P. *bursaria* w/*Chlorella* (MB-1)	*Stentor*, 5 reps	5 reps
P. *bursaria* w/*Chlorella* (MB-1)	*Lepadella*, 5 reps	5 reps

Figure 1

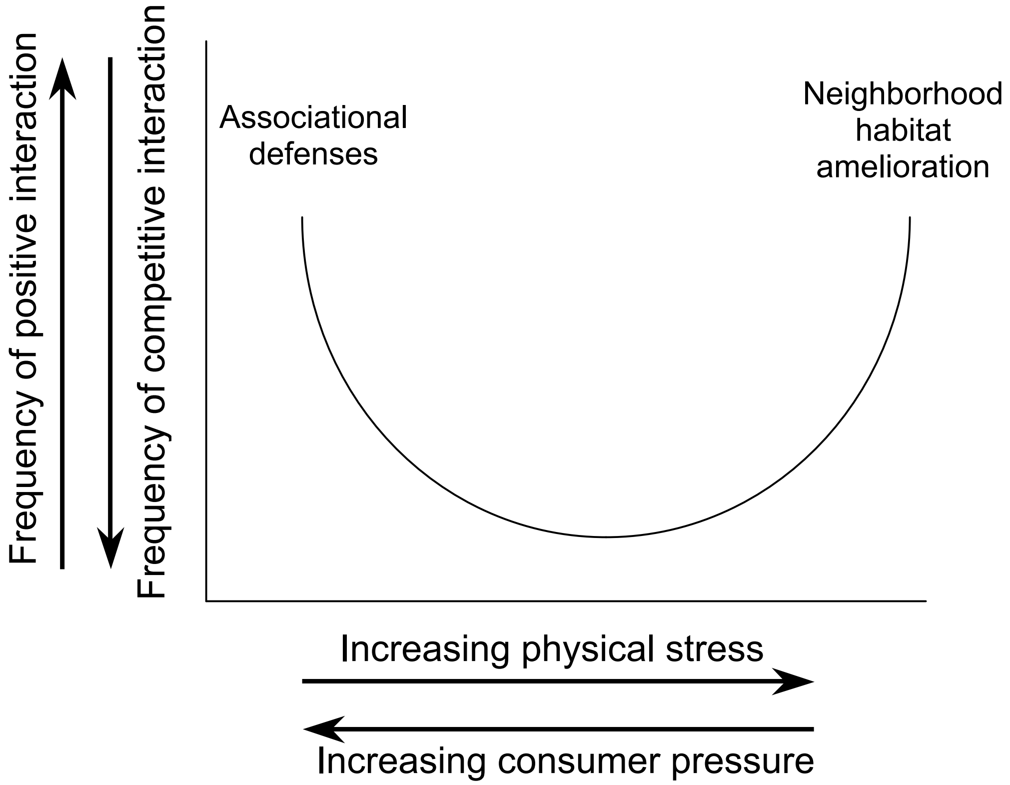

Frequency of positive interaction
Frequency of competitive interaction
Associational defenses
Neighborhood habitat amelioration
Increasing physical stress
Increasing consumer pressure

Figure 2a

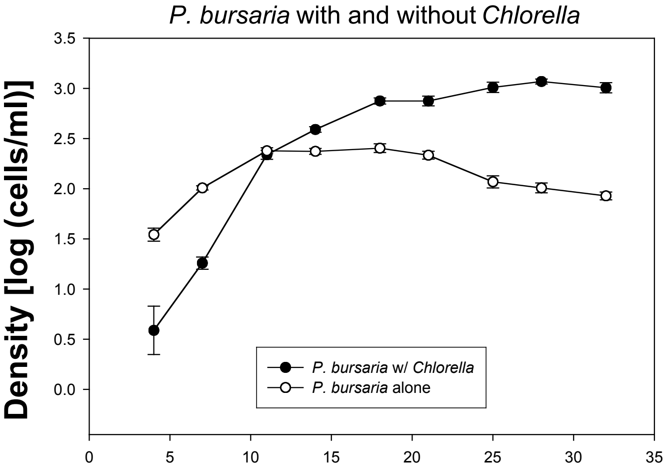


Figure 2b

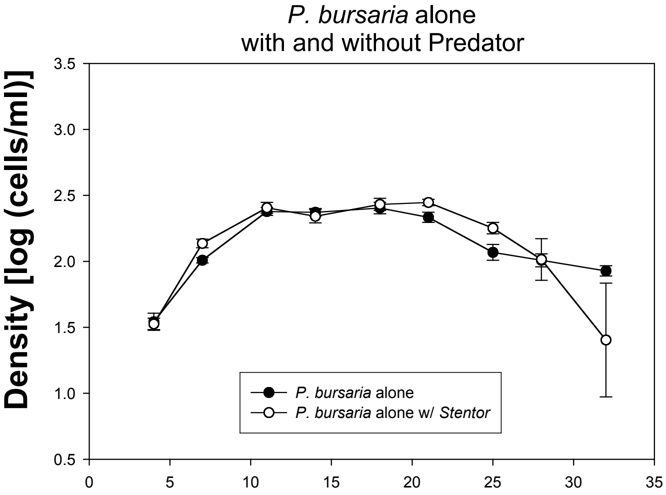


Figure 2c

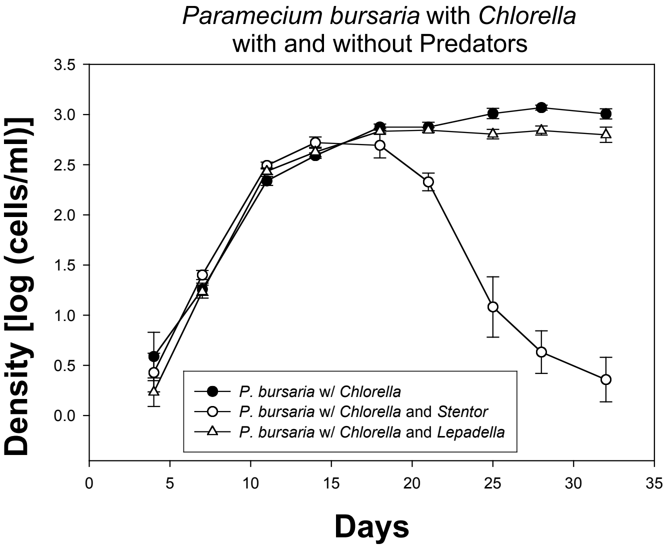


Figure 3a

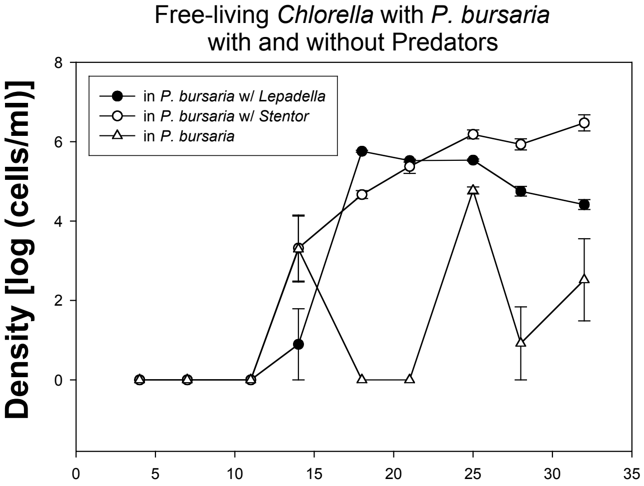


Figure 3b

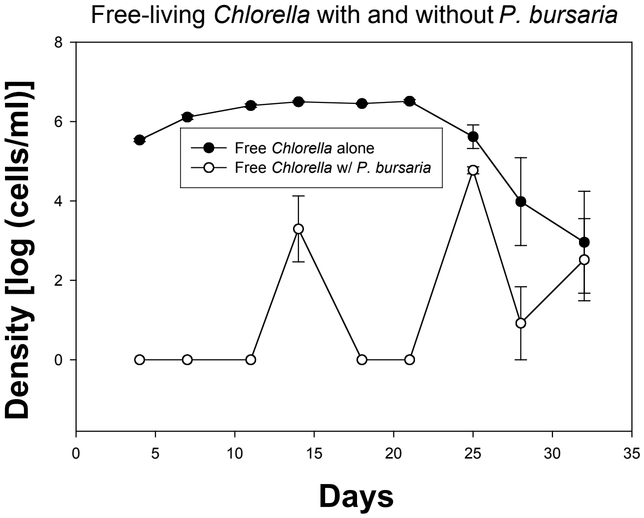


Figure 4a

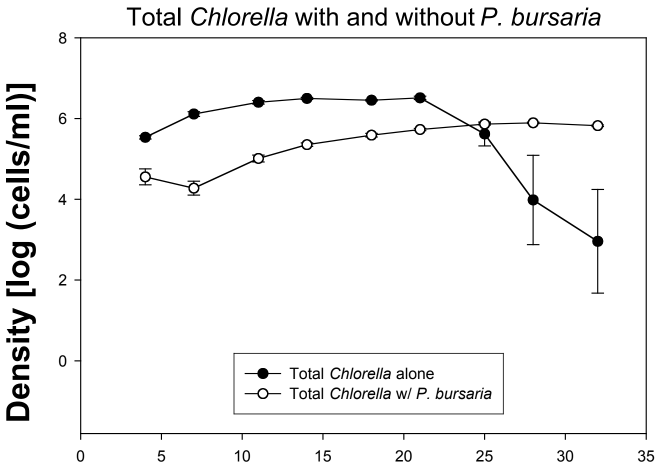

Total *Chlorella* with and without *P. bursaria*

Figure 4b

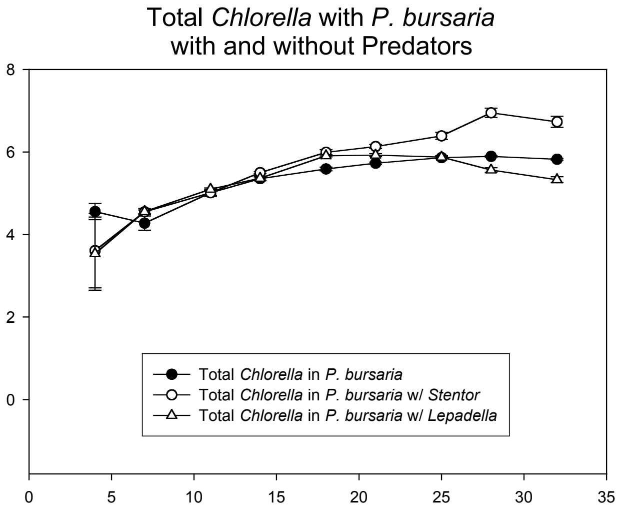

Total *Chlorella* with *P. bursaria* with and without Predators

Figure 4c

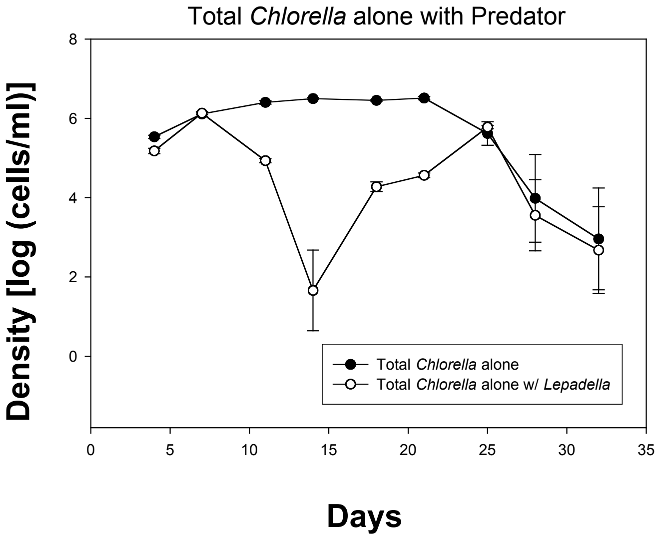

Total *Chlorella* alone with Predator

Figure 4d

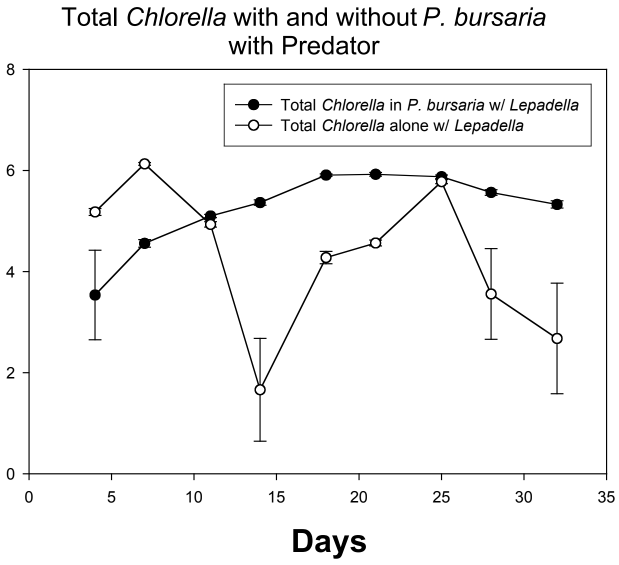

Total *Chlorella* with and without *P. bursaria* with Predator

Figure 5a

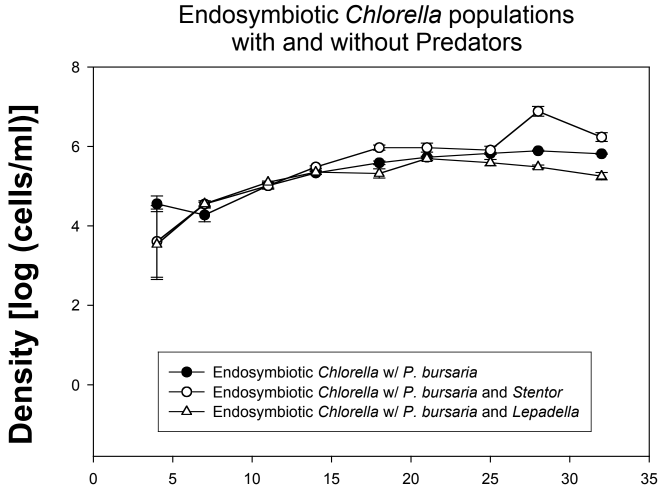

Endosymbiotic *Chlorella* populations with and without Predators

Figure 5b

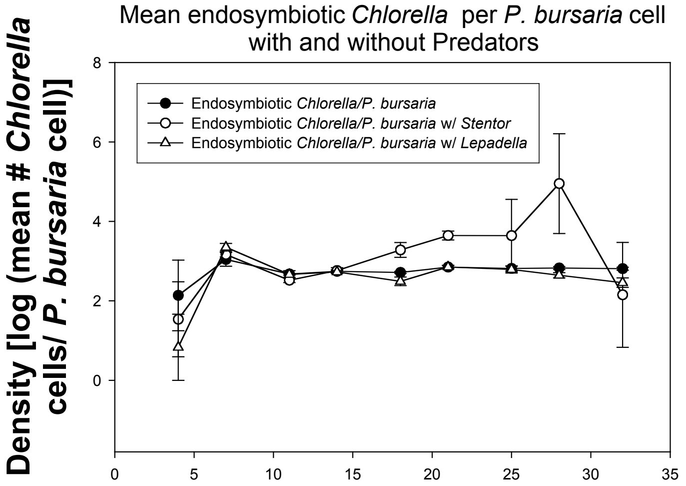

Mean endosymbiotic *Chlorella* per *P. bursaria* cell with and without Predators

Figure 5c

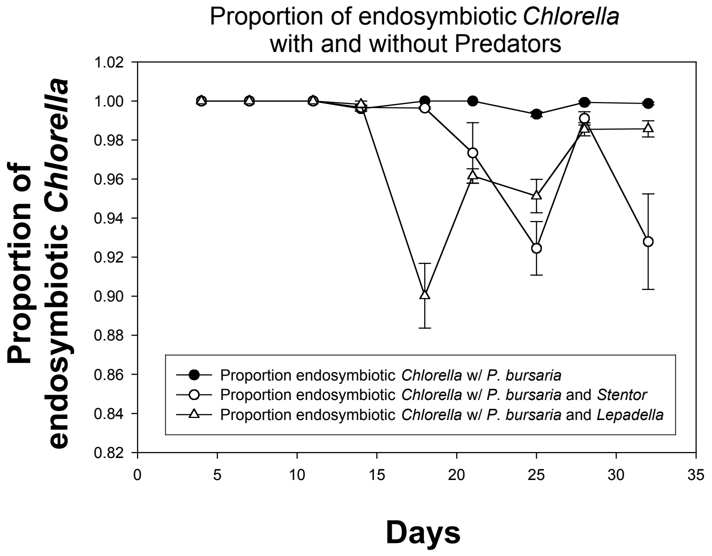

Proportion of endosymbiotic *Chlorella* with and without Predators

Figure 6a

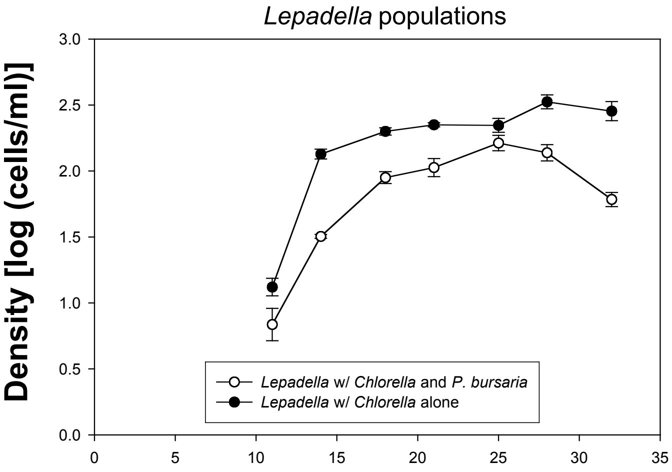
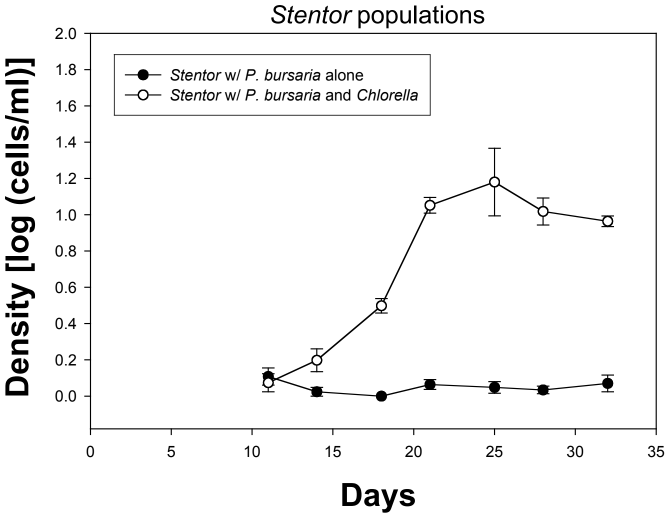


Figure 6b



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Appendix I: Abridged list of mutualisms found in nature and categorized by type

plant-plant interactions	Atsatt and O'Dowd 1976; Hay 1986; Bertness 1992; Bertness and Shumway 1993; Bertness and Hacker 1994; Callaway 1995; Hacker and Bertness 1996; Pugnaire and Haase 1996; Callaway and Walker 1997; Hacker and Gaines 1997; Holmgren *et al.* 1997; Callaway 1998b; Olofsson *et al.* 1999; Rousset and Lepart 2000; Callaway and Pennings 2000; Shumway 2000; Bruno 2000; Tewksbury and Lloyd 2001; Mulder *et al.* 2001; Choler *et al.* 2001; Jochen Schenk and Mahall 2002
plant-pollinator mutualisms	Englemann 1872; Darwin 1877; Janzen 1971; Aker and Udovic 1981; Schemske and Horvitz 1984; Bronstein 1988; Thompson and Pellmyr 1992; Pellmyr and Huth 1994; Pellmyr *et al.* 1996; Pellmyr and Huth 1996; Bronstein and Hossaert-McKey 1996; Patel 1996; Morris 1996; Kearns *et al.* 1998; Pellmyr and Leebens-Mack 1999; Pellmyr and Leebens-Mack 2000; Harrison 2000; Holland 2002; Parker and Haubensak 2002
plant-fungal associations	Clay 1988; Carroll 1988; Clay 1990; Clay and Holah 1999; Omacini 2001
plant-mycorrhizal associations	Law and Lewis 1983; Allen *et al.* 1989; Allen 1991; Allen 1992; Chapela *et al.* 1994; Francis

	and Read 1995; Simard *et al*. 1997; van der Heijden *et al*. 1998; Hogberg *et al*. 1999; Hartnett and Wilson 1999; Hoeksema 1999; Setala 2000; Hibbett *et al*. 2000; Lilleskov 2002
legume-rhizobial mutualisms	Parker 1999; Denison 2000
plant-decomposer relationships	Harte and Kinzig 1993; Naeem *et al*. 2000; Loreau 2001
plant-animal interactions	Abrahamson 1989; Handel 1997; de Mazancourt 2001
plant-insect mutualisms	Anderson and Midgely 2002; Kluth *et al*. 2002
ant-plant mutualisms	Janzen 1966; Bentley 1977; Handel 1978; Beattie 1985; Barton 1986; Hanzawa *et al*. 1988; Wilmer and Stone 1997; Heil 2001; Christian 2001
ant-fungal mutualisms	Weber 1966; Martin 1970; Weber 1972; Wetterer 1994; Fowler 1995; Mueller *et al*. 1998
insect-animal interactions	Haemig 1999
insect-insect interactions	Valerio 1975; Martinsen *et al*. 2000; Morales 2000; Hafernik and Saul-Gershenz 2000
insect-fungus mutualisms	Friedli and Bacher 2001
earthworms-fungi interactions	Darwin 1881

dispersal mutualisms	Amico and Aizen 2000
symbiosis and microorganisms	Loefer 1936; Siegel 1960; Buchner 1965; Henry 1966; Sleigh 1973; Goff 1983; Gortz 1988; Barbosa *et al.* 1991; Margulis and Fester 1991; Douglas 1994, 1995
coral-algal endosymbioses	Johnstone and Bshary 2002; Bshary 2002
cleaner mutualisms	Muscatine and Porter 1977; Chornesky 1991; Wulff 1997; Rowan 1998; Stachowicz and Hay 1999; Fagoonee *et al.* 1999
indirect mutualism	Davidson *et al.* 1984; Bertness *et al.* 1999
within-species mutualisms	Cockburn 1998; Bernasconi and Strassman 1999; Moore *et al.* 2002; Scantlebury 2002
cooperative vertebrates	Allee 1938; Clutton-Brock 2002; Clutton-Brock *et al.* 2002
human cooperation	Mace 2000; Wedekind and Milinski 2000; West *et al.* 2002; Fehr and Gachter 2002
evolution of cooperation	Hamilton 1963; Karakashian and Karakashian 1965; Trivers 1971; Axelrod and Hamilton 1981; Axelrod 1984; Howe 1984; Law and Koptur 1986; Axelrod and Dion 1988; Dugatkin 1997a,b; Axelrod 1997; Herre *et al.* 1999; Krieger *et al.* 2000; Nowak *et al.* 2000; Riolo *et al.* 2001;

	Hauert *et al*. 2002; Pepper and Smuts 2002; Nonacs 2002
game theory of cooperation	Connor 1986; Clements and Stephens 1995; Milinski 1996; Mesterton-Gibbons and Dugatkin 1997a; Roberts and Sherratt 1998; Dugatkin and Reeves 1998
evolutionary and community stability issues	May 1971; Goh 1977; Goh 1979; Ringel *et al*. 1996; Ferriere *et al*. 2002
population models of mutualism	Gause and Witt 1935; May 1973; Roughgarden 1975; May 1976a,b; Vandermeer and Boucher 1978; Travis and Post 1979; Heithaus *et al*. 1980; May 1982; Dean 1983; Wolin and Lawlor 1984; Addicott and Freedman 1984; Post *et al*. 1985; Wolin 1985; Addicott 1986; Wright 1989; Matsuda and Shimada 1993; Wilson and Nisbet 1997; He and Gopalsamy 1997; Abrams *et al*. 1998; Brooker and Callaghan 1998; Bever 1999; Hochberg *et al*. 2000
conceptual framing, synthesis, and comment	Kessler 1880; Pound 1893; Kropotkin 1903Nuttall 1923; Allee 1938; Nichols and Chambers 1946; Odum and Biever 1984; Janzen 1985; Abrams 1987; Oksanen 1988; Hunter and Aarssen 1988; Burns 1993; Kawanabe *et al*. 1993; Bond 1994; Conner 1995; Jones *et al*. 1997; Bertness and Leonard 1997; Callaway 1998a; Bertness 1998; Wall and Moore 1999; Hoeksema and Bruna 2000; Bronstein 2001; Bruno *et al*. 2003

Appendix 2: Freshwater hosts of endosymbiotic *Chlorella* (Sleigh 1973; Wichterman 1986; Smith and Douglas 1987)

Hydra sp.	Coelenterata
Aodonta cygnea	Mollusca
Unio sp.	Mollusca
Dalyellia viridis	Platyhelminthes
Typhlophlana viridata	Platyhelminthes
Phaenocora typhlops	Platyhelminthes
Castrada sp.	Platyhelminthes
Ephydatia fluviatilis	Porifera
Spongilla lacustris	Porifera
Paramecium bursaria	Protista
Stentor polymorphus	Protista
Vorticella sp.	Protista
Vorticella monilata	Protista
Climacostomum virens	Protista
Prorodon viridis	Protista
Frontonia vernalis	Protista
Frontonia leucas	Protista
Colpoda sp.	Protista
Acanthocystis turfacea	Sarcodia
Mayorella viridis	Sarcodia
Phaenocor typhlops	Turbellaria
Dalyellia viridis	Turbellaria
Typhloplana viridata	Turbellaria

Curriculum Vita

P. Timon McPhearson

EDUCATION

October 2004	Rutgers University Ph.D. in Ecology and Evolution Dissertation: *The Complexity of Cooperation in Ecological Communities*
May 1997	Taylor University B.S. *summa cum laude* Biology and Environmental Science; Minors in Philosophy and Fine Art

OTHER GRADUATE STUDIES

January 1999	Institute of Ecosystem Studies, New York Fundamentals of Ecosystems Ecology
Spring 1998	University of Costa Rica and Organization for Tropical Studies, Costa Rica Tropical Biodiversity
Fall 1998	Princeton University, New Jersey Ecosystems as Complex Adaptive Systems

PROFESSIONAL EXPERIENCE

National Science Foundation

1999 – 2002 Graduate Research Fellow

- Thesis: The Maintenance of Cooperation in Biological Systems

Rutgers University

1998 – 2003 Lecturer

- Conservation Ecology
- General Biology
- Plant Ecology
- Principles of Applied Ecology

1997 – 2004 Teaching Assistant

- General Biology
- Genetics
- Plant Ecology
- Principles of Applied Ecology

2000 – 2001 Supervisor: Undergraduate Research

- Conceived and advised student senior thesis: *Mutualism and Competition Dynamics in Ecological Communities*
- Trained student in laboratory methods, ecological theory and literature research

2000 – 2001 Graduate Student Representative of Ecology and Evolution Program

Northeast Ecology and Evolution Conference (NEEC)
2002 – 2003 Steering Committee, Advertising Chair, and Keynote Speaker Host

Princeton University / Rutgers University / University of Pennsylvania
1998 – 2000 Symposium Director

Taylor University
1996 Student Scientific Representative to Dagestan

ACADEMIC AWARDS

National Science Foundation Graduate Research Fellowship 1999–2002
Dean's Graduate Fellowship, Rutgers University 2002
Center for Biodiversity Fellowship, Rutgers University 1998
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CONFERENCES / WORKSHOPS

Ecological Society of America Annual Meeting: 1999, 2000, 2003
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Rutgers/Princeton Graduate Student Symposium 1997-2003

PROFESSIONAL MEMBERSHIPS

Ecological Society of America, 1997 – present
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Rutgers University Naturalists, 2000 – present
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The Nature Conservancy, 1996 – present

PUBLICATIONS (Papers)

McPhearson, P.T. and P.J. Morin. 2004. Review of "The Importance of Species", P. Karieva and S.A. Levin, eds. *Integrative and Comparative Biology* 43:603.

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McPhearson, P.T. and J.P. Townsend. 1999. Leaf-cutter ants harvest plant material comparable to a mass action model of harvesting behavior. *Proceedings of Ecological Society of America.*

E.R. Squiers, M. Bailey, P.T. McPhearson, and R. Mejeur. 1995. An analyses of the spatial distribution of the major legal, environmental, and social/political constraints to landfill siting in the east central Indiana solid waste district. *Proceedings of Indiana Academy of Sciences.*

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