CALIFORNIA STATE UNIVERSITY, NORTHRIDGE

Effects of Environmental Change on the Eco-Evolutionary Dynamics of Species in Natural Microcosm Communities

A thesis submitted in partial fulfillment of the requirements
For the degree of Masters of Science in Biology

by
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Abstract

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Natural communities are expected to undergo shifts in composition and species interactions as a result of environmental change, such as changes to regional temperature regimes and increased nutrient input into ecosystems. Predicting how communities will respond is complicated by three factors: non-additive effects of multiple stressors, differences in response among trophic levels, and trait evolution leading to adaptation. This study addressed all three of these factors using a natural microcosm community. The purple pitcher plant, *Sarracenia purpurea*, is a carnivorous plant that retains rainwater inside of its cup-shaped leaves. Within this water, an inquiline community of microbes and invertebrate larvae forms. I used a subset of this community consisting of a single protist species, *Colpidium* sp., and a community of bacteria to explore how the interaction between temperature and increased nutrient input affects different trophic levels ecologically and evolutionarily. I factorially manipulated temperature and nutrient input and maintained these conditions for 28 days (~150 protozoan generations). I then performed reciprocal transplants to each treatment, resulting in four common garden environments to test how evolutionary effects may depend upon ecological context.

I found that historic nutrient input levels affected two protist traits – cell size and peak density – but the magnitude of these effects depended on the contemporary

environment. The combined effects of historical temperature and nutrients did not differ significantly from the expected additive effect. Protist and bacterial abundances differed in their response to treatments, with protist abundance affected by both historical and contemporary environments while bacterial abundance was only affected by contemporary environment. Finally, bacterial community composition was affected by treatments both directly and indirectly, through their effects on protist traits and abundance. The results of this study show that the ways in which communities respond to environmental change can differ in light of evolutionary responses, the trophic levels being considered, and additivity of multiple stressors. The future success of management and conservation of natural systems rests upon the best possible understanding of not only the ecological implications but also, and perhaps even more importantly, the evolutionary consequences of a changing abiotic environment.

Introduction

Environmental changes caused by anthropogenic activities are likely to affect most natural communities (Vitousek *et al.* 1997). Two of the most important expected environmental changes are changes to regional temperature regimes and increased nutrient input into ecosystems because the interaction between these two stressors is expected to increase extinction risk for many organisms in marine, freshwater and terrestrial habitats (IPCC 2014). Populations and communities can respond via changes in phenology, range shifts, alterations to community composition and/or changes in species interactions (Walther *et al*. 2002). However, making predictions about how communities may be affected by global change can be complicated by three factors: non-additive effects of multiple stressors (Folt *et al*. 1999), differences in response among trophic levels (Petchy *et al*. 1999), and evolutionary changes in traits (Bradshaw and Holzapfel 2006).

Together, shifts in the multiple environmental factors contributing to global change may have non-additive effects on species that cannot be predicted based on their individual effects (Paine *et al*. 1998). These non-additive effects can result in responses that are either greater (synergistic) or less (antagonistic) than the additive effects one might expect based on the response to each factor individually. Many studies have investigated the combined effects of biotic stressors. For example, terHorst (2010) tested the effects of competitors and predators in protozoans and Ferguson and Stiling (1996) investigated how parasitoids and predators effected aphid populations. Others have looked at combinations of abiotic stressors, such as acidification and warming in boreal lakes (Schindler *et al*. 1996). A few have looked at combinations of both biotic and

abiotic stressors. Kneitel and Miller (2002) looked at community-level effects of resource availability and top predators in pitcher plants. Winsome and colleagues (2006) explored the ecological effects of competition and habitat quality in earthworms. Studies that incorporate multiple effects are vital to a comprehensive understanding of how ecosystems will respond to multiple stressors because in natural systems, stressors are rarely experienced in isolation.

Different trophic levels have been shown to vary in their response to ecological stressors, with sensitivity to climate change having a strong positive correlation with trophic level (Voigt *et* al. 2003). Warming alone can increase extinction frequencies of higher trophic levels more drastically than lower trophic levels (Petchy *et al*. 1999). In plant-based food webs, Petchy and colleagues (1999) found that higher trophic level organisms (top predators and herbivores) were more negatively affected by warming than lower trophic levels (autotrophs and bacterivores). Classic "bottom-up" food web dynamics suggest that both lower and higher trophic levels will benefit from nutrient enrichment (Power 1992). Contrary to this, Balčiūnas and Lawler (1995) found that gape-limited predatory protists went extinct in high nutrient environments because lower trophic level bacterivore protists increased cell size and were too large to be consumed. In some cases, trophic levels may not only differ in the magnitude but also the direction of their response. For example, producers may benefit from certain stressors while consumers are negatively affected (Beisner *et* al. 1997, Strecker *et al*. 2004, Vinebrooke *et al*. 2003). Beisner and colleagues (1997) investigated how increased temperature affected zooplankton (*Daphnia*) and phytoplankton (algae) biomass in a 50-day laboratory experiment. They reported that warmer conditions caused faster growth rates

in *Daphnia* populations, which led *Daphnia* to overexploit their food resources (algae) and ultimately drove these populations extinct. Following *Daphnia*'s extinction, phytoplankton biomass increased and mainly consisted of taxa that were resistant to grazing (Beisner *et al*. 1997). Environmental stressors may affect each trophic level directly and indirectly, through effects on other trophic levels (Gilman *et al.* 2010).

In addition to ecological responses, such as changes in abundance, species traits may also evolve. Trait evolution in response to anthropogenic effects has been reported in a variety of species (reviewed in Bradshaw and Holzapfel 2006) and can affect the ecology of the communities they are a part of (Thompson 1998, Palkovacs *et al*. 2012). Until recently, ecological theories developed to address issues of conservation and management in response to environmental change neglected to account for evolutionary changes, although evolution may alter the outcome of ecological experiments (Strauss *et al*. 2008). Traditionally, evolutionary effects were thought to occur over millions of years and be insignificant on the time scale of ecological processes (Slobodkin 1961; Hairston *et al*. 2005). However, recent experimental evidence demonstrates that rapid evolution occurs on ecologically relevant time-scales and can affect the ecological interactions that cause selection, resulting in eco-evolutionary feedbacks (Strauss *et al.* 2008, Palkovacs and Post 2008, Schoener 2011). When species rapidly evolve, aspect of their ecological dynamics can be difficult to detect unless evolution is taken into account. For example, by simply measuring patterns in abundance, the effect of predatory rotifers on the prey algae appeared to be weak. However, through mathematical modeling, Yoshida *et al*. (2007) found that the strength of this interaction was masked by rapid evolution of low-cost defenses in algal prey, which occurred along the same timescale as ecological

processes leading to changes in abundance. Similarly, terHorst and colleagues (2010) allowed the ciliate protist *Colpoda* sp. to evolve for a short period of time in the presence of the predatory mosquito larvae, *Wyeomyia smithii*. After this period of selection, predators showed no ecological effect on the abundance of protists, as they normally would if evolution had not occurred. This is a particularly interesting example because the predator population, itself, was responsible for selection driving protist evolution (terHorst *et al*. 2010).

These three factors – non-additive effects of multiple stressors, differences in response between trophic levels, and trait evolution leading to adaptation – are not mutually exclusive. It is possible that any combination of these processes, or all three, could contribute to the ways in which communities will respond to global change. Interactions between these factors would further complicate efforts to predict how changes to the environment affect these communities. Non-additive effects of multiple selective agents can change the way in which traits evolve (terHorst *et al*. in press). For example, plants can evolve different levels of resistance to one herbivore, like deer, depending on the presence or absence of another herbivore, such as insects (Stinchcombe and Rausher 2001). Trait evolution of a species at one trophic level may affect ecological interactions between that species and other trophic levels. Palkovacs and Post (2009) found that adaptive divergence in the traits of a predatory fish, alewife, alters the biomass, diversity, species richness, and average body size of their zooplankton prey community. Here I address how the interaction between warming and increased nutrient input affects different trophic levels both ecologically and evolutionarily.

Study System

In natural systems, evolution can occur in response to several factors simultaneously, making it difficult to partition the individual effect of each *in situ*. However, factorial manipulations of temperature and nutrient enrichment in microcosm communities can be used to experimentally test their individual and combined effects on eco-evolutionary processes. Purple pitcher plants (*Sarracenia purpurea*) are carnivorous plants native to bogs and wetlands of eastern North America. The plant's cup-shaped leaves retain rainwater to form a contained body of water called a phytotelma, which serves as a natural microcosm (Srivastava *et al*. 2004). The pitcher plant phytotelma, like many others, is habitat to a community of organisms known as the inquiline community. This community of aquatic invertebrates, single-celled protists, and bacteria provides an ideal model system for studying evolutionary responses (Srivastava *et al*. 2004). Natural microcosms are advantageous for evolutionary and ecological experiments because communities are easily replicated and motile organisms are constrained to naturally contained systems, eliminating the need for artifacts like fencing. The species in these systems also lend themselves to experimentation, due to their small size, the ease with which they can be directly manipulated, and their relatively short generation times (on the scale of hours for protists and bacteria).

The ecology of the purple pitcher plant inquiline community is well known and representative of other natural communities (Srivastava *et al*. 2004). Insect prey – primarily ants – are attracted to the plant and fall into the phytotelma where they are decomposed by bacteria. Protozoa consume the bacteria and are, in turn, consumed by the larvae of a specialist mosquito (*Wyeomyia smithii*). Trophic dynamics are influenced by

bottom-up effects from nutrient input (prey capture) and top-down effects from mosquito larvae (Kneitel and Miller 2002). The detritus-based food web of the *S. purpurea* inquiline community is limited primarily by organic carbon and, to a lesser degree, phosphorus (Gray *et al*. 2006). The amount of insect prey found in pitcher plants varies widely over the lifetime of each pitcher, with younger leaves having a greater number of trapped ants than older leaves (Miller and terHorst 2012).

Protists in the inquiline community are well suited for evolutionary studies because they reproduce asexually every 4-8 hours, providing the potential for protozoan traits to evolve on ecologically relevant time scales (about 4-6 weeks). Protists traits, such as cell size, have been shown to evolve rapidly in the presence of predators (terHorst *et al*. 2010), as well as competitors (terHorst 2011), and reduce their overall effect on protist populations. Large protist species are better interspecific competitors than small species (Kneitel 2002), although it is unclear how this trait contributes to intraspecific competitive ability. Body size is also one of the main parameters considered in the metabolic theory of ecology (Brown *et al*. 2004) and, therefore, may be affected by resource availability and temperature. Balčiūnas and Lawler (1995) found that nutrient enrichment results in larger *Colpidium* cells and greater abundance. Increase in abundance can be due to higher growth rates, greater carrying capacity or a combination of both. Peak density can be used as a proxy for resource use efficiency. Given the same amount of nutrients, populations with higher peak densities are thought to use those resources more efficiently. Laybourn and Stewart (1974) found that temperature is positively correlated with per-capita growth rates in *Colpidium*. In some ciliate species, there is an evolutionary trade-off between growth rate (r-selection) and carrying capacity

(K-selection), such that higher growth rates reduce carrying capacity and *vice versa* (Luckinbill 1979). To date, no studies have shown a cohesive picture of how temperature, nutrients and their interaction affect protist cell size, peak density and growth rates.

In contrast, the bacterial communities at the base of the inquiline food web have been explored relatively little. Initial work suggests that the composition of the bacterial community can vary greatly among and within sampling locations (Gray *et al*. 2012). The bacterial community of individual *Sarracenia* pitchers is not correlated with bacterial communities from the surrounding soil (Koopman *et al*. 2010) or adjacent pitchers (Peterson *et al*. 2008). Similarly, environmental predictors, such as pH and pitcher size, provide no explanation for variation in bacterial diversity among pitcher plant inquiline communities (Peterson *et al*., 2008). Carbon and phosphorus have been shown to increase bacterial abundance in pitcher plant inquiline communities, while nitrogen had little effect on this detritus-based system (Gray *et al.* 2006). In addition, bacterial diversity and richness increase in the presence of the mosquito larvae *Wyeomyia smithii* (Peterson *et al*. 2008) suggesting that trophic cascades play an important role in this system. It is unclear whether larvae affect bacterial diversity through larval feces contributing nutrients to the bottom of the food web or through suppression of bacterivorous protists. Here I clarified this pattern by testing the direct effects of nutrients on bacterial diversity in this system. To gain a full understanding of how evolution at one trophic level (protozoa) affects community dynamics at another trophic level (bacteria) in this system, I integrated techniques from evolutionary ecology and microbiology.

Objectives

I investigated how temperature and nutrients affect eco-evolutionary interactions among species. In order to fully address this complex idea, my project consisted of four major objectives. My first objective was to quantify how environmental factors affected evolution of protozoan traits in pitcher plant inquiline communities. More specifically, I assessed whether temperature and nutrient input affected two ecologically relevant traits, cell size and peak density, in the ciliate protozoan *Colpidium* sp. I also measured per-capita growth rates as a measure of fitness. Second, I determined whether the evolutionary effects of temperature and nutrient input on protist traits were non-additive, as predicted by global change literature (Folt, *et al*. 1999). In other systems, nutrient increase and warming have been found to have strong synergistic effects on biomass and decomposition (Greig *et* al. 2012). Third, I evaluated the effects of nutrients and temperature on protist and bacterial abundance separately to determine whether these factors vary in their ecological effects by trophic level. Finally, I evaluated if, and to what degree, evolutionary history affects species interactions. The ecological dynamics that I assessed included relative abundance of bacteria and *Colpidium* as well as bacterial community diversity and composition. This allowed me to determine whether the experimental treatments affected bacteria directly or indirectly, through effects on protist abundance and traits. In doing so, I was also able to evaluate the validity of a critical assumption of eco-evolutionary feedback, which states that evolutionary effects depend on ecological context.

Methods

Field Sampling & Microcosm Set-up

I sampled inquiline communities from naturally occurring *S. purpurea* in the Apalachicola National Forest in north Florida (Fig. 1) in May of 2014. I sampled pitcher plants by removing the majority of the liquid from inside of each pitcher with a sterile transfer pipet and placing it in a sterile 50 mL macrocentrifuge tube. I sampled ten pitcher plants at each of five separate sites (New River, Crystal Bog, Grass Field, Pleaphase Savanna and Naczi Bog) for a total of 50 pitchers. I took additional samples directly from one pitcher plant at three sites (New River, Grass Field and Naczi Bog), kept them on ice until I returned to the lab, and immediately passed them through a 0.22 micron vacuum filter that was later used for sequencing to determine bacterial diversity in naturally occurring pitcher plants. To gain insight into the temperatures experienced by the pitcher plant inquiline community, I placed remote data loggers inside of five pitcher plants at the Crystal Bog site and recorded hourly temperature data from May 19, 2014 through June 29, 2014. I used ambient temperature data collected at the same time points from a weather station near my sites (Weather Underground; www.wunderground.com) to compare temperatures experienced inside of pitcher plants to those of the surrounding air. These data were used as a baseline to understand the natural temperature regime and bacterial diversity of *S. purpurea* pitchers in the Apalachicola National Forest.

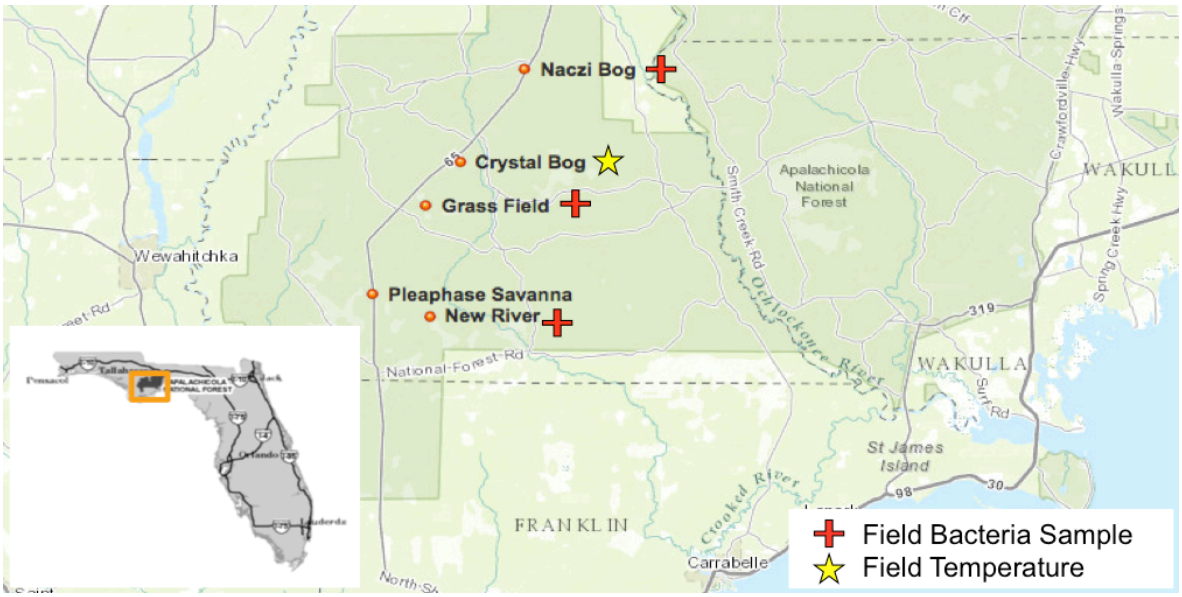

Figure 1 Sites in north Florida's Apalachicola National Forest used to sample naturally occurring inquiline communities within *S. purpurea* pitchers for this study. Map generated based on field coordinates using ArcGIS Online.

Through a series of dilutions, I was able to isolate cultures of the ciliate protozoan *Colpidium* sp. from four of these sites (Crystal Bog, Naczi Bog, Pleasphase Savanna and New River). I mixed these four cultures in order to maximize genetic diversity and used the resulting stock to inoculate all experimental microcosms. I passed field samples through a series of filters decreasing in size from 25 to 2.5 microns to exclude larger organisms and produced one mixed stock of bacteria. I used these samples to establish laboratory microcosms in 50mL macrocentrifuge tubes that were maintained at California State University, Northridge. Microcosms consisted of 25 mL of sterile water, 1 mL of *Colpidium* stock culture (approximately 582 cells/mL), and 4 mL of bacteria stock culture. Each microcosm also received 6 mg of fish food (freeze dried bloodworms), which mimics the insect-based detritus found in pitcher plant leaves and serves as a resource for bacteria (terHorst *et al*. 2010).

Experimental Design

Part I: Selection

I experimentally manipulated temperature and nutrient levels in laboratory microcosms in order to understand how these factors affect the eco-evolutionary dynamics of this system. Growth chambers were used to establish two temperature treatments, "ambient" and "warming". The ambient temperature treatment was held constant at 25°C. The warming temperature treatment was placed on a four hour daily temperature cycle that gradually increased from 24°C to 37°C and back to 24°C. On average, the warming treatment was 1°C warmer and also had a more variable temperature regime than the ambient treatment, which reflects how temperatures are expected to change in North America over the next century (IPCC 2014). I established two nutrient treatments using a 1:60 ratio of sodium phosphate as a source of phosphorus and a mixture of eight different carbon sources ranging in complexity (Gray *et al*. 2006), as well as a small amount of yeast extract as a source of micronutrients. I used glucose, xylose, cellobiose, glycine, N-acetylglucosamine, vanillin, lignin, and cellulose as carbon sources (Whitaker *et al*. 2014). To make the final nutrient mixture, I combined 1.0686 g of each carbon source (8.5488 g total), 0.14248 g of sodium phosphate and 0.075 g of yeast extract. I added 1 mg of this mixture to the low nutrient group and 10 mg to the high nutrient group every three days for the duration of the experiment. The level of nutrient input in the high nutrient treatment was designed to maintain eutrophic levels of enrichment (Nixon 1995). In order to partition the effects of protists and abiotic treatments on bacteria, I also manipulated the presence of *Colpidium.* I manipulated temperature, nutrients, and *Colpidium* in a full factorial design, resulting in eight

treatment groups. Five replicate microcosms were randomly assigned to each group for a total of 40 microcosms. The microcosms were maintained under these conditions for 28 days (~150 protozoan generations).

Part II: Reciprocal Transplant

After four weeks, I performed reciprocal transplants to and from each of the four abiotic environments, resulting in a total of 160 microcosms (n = 5). To do this, I added 1 mL from each of the 40 microcosms from the selection experiment ("historical samples") to each of four sterile 50 mL macrocentrifuge tubes with 29mL of sterile water and 6 mg of fish food. Within each historical treatment group, microcosms were randomly assigned to one of the four contemporary experimental groups (control, increased temperature, increased nutrients, or increased temperature and nutrients). This resulted in four common garden environments in which I compared the effects of evolutionary history (historical treatment group) to contemporary environment. Protists and bacteria were sampled after three days in their respective transplant environment. This period allowed enough time for ecological processes to occur, but minimized the time for further evolution.

Microcosm Sampling and Response Measurement

Protists were sampled from the original stock culture at the start of the selection experiment, from each microcosm at the end of the selection experiment, and again after reciprocal transplant experiment. I removed 1 mL of fluid from each replicate and fixed it using 20 μL of Lugol's iodine solution. Samples were gently centrifuged (300rmp for 1 minute) to concentrate cells at the bottom of the tube, after which, cells from the bottom 100 μL of each sample were counted using a Palmer counting cell.

Light microscopy was used to measure two protozoan traits (peak density and cell size) and abundance. Protist abundance at the start and end of each experiment was used to calculate per-capita growth rates as follows

$$Per - capita\ growth = \frac{(Abundance_{final} - Abundance_{initial})}{Abundance_{initial}}$$

which was used as a proxy for fitness. Digital images of cells were analyzed using Image J (National Institutes of Health) to determine cell size. Peak density was measured in three replicates from the *Colpidium* stock culture at the start of the experiment and at the end of the reciprocal transplant experiment. To do this, I transferred a 100 µL sample from each microcosm to a sterile macrocentrifuge tube with 20 mL of sterile water and 6 mg of fish food and stored them at room temperature (approximately 24ºC). I fixed 1 mL from each microcosm using 20 µL of Lugol's iodine every 24 hours for 6 days. I gently centrifuged (300 rmp for 1 minute) the fixed samples and counted cells from the bottom 100 µL using a Palmer counting cell. Over this 6-day period, the density of *Colpidium* increased and then decreased; I used the greatest density reached in this cycle as each microcosm's peak density.

Bacteria were sampled from the original bacterial and *Colpidium* stocks at the start of the experiment and then from each replicate microcosm at the end of the selection experiment, and the end of the reciprocal transplant experiment. I fixed 100 µL from each microcosm in 900 µL of a mixture of 10% formalin in phosphate buffered saline (PBS) solution to create a 10-fold dilution; 100 µL of this was added to another 900 µL of 10% formalin-PBS solution to make a 100-fold dilution. I used these diluted samples for direct cell counts to estimate total bacterial abundance. Total bacterial abundance was determined from direct cell counts using DAPI stain (Thermo Fisher Scientific, Inc.), a

fluorescent stain that binds to DNA, and fluorescent microscopy. I mixed 10 μL of DAPI stain, 100 μL of the diluted samples and 900 μL of PBS solution to stain cells then filtered them onto 0.22 micron black polycarbonate filters for visualization using fluorescent light microscopy.

All of the remaining volume from each microcosm was passed through a 0.22 micron vacuum filter and frozen to be used for sequencing. Bacterial community composition was analyzed in the original bacterial and *Colpidium* stock as well as each experimental microcosm using an Illumina MiSeq platform for paired-end 16s rRNA gene sequencing. Cultures taken directly from pitcher plants at three field sites were also sequenced in order to set a baseline for composition of naturally occurring communities in Apalachicola National Forest. I extracted DNA from the vacuum filters using MoBio RapidWater DNA Isolation Kits (Mobio Laboratories Inc.) following the manufacturer instructions. With this genomic DNA, I amplified approximately 300 base pairs of the V3-V4 region of the 16S rRNA gene using the archaeal and bacterial primers 515F and 806R and standard PCR protocols established by the Earth Microbiome Project (earthmicrobiome.org). I used a PicoGreen fluorescence assay to determine the molar concentration of dsDNA in each PCR product and used these data to pool samples at the same molar concentration. Pooled samples were cleaned up using a Wizard DNA Clean-Up System (Promega) and diluted to a molar concentration of 1.95 nM. Samples were sequenced on an Illumina MiSeq platform using an Illumina MiSeq v2 Reagent Kit (Illumina Inc.).

Raw FASTQ sequence files of 16S rRNA genes were processed with the UPARSE pipeline (Edgar 2013) using a modified version of the procedure described by

Ramirez and colleagues (2014). I used a custom Python script to demultiplex and prepare sequence files for paired-end assembly and clustering. Paired sequences were assembled using UPARSE according to the following parameters: fastq_truncqual 3, fastq_maxdiffs 1, fastq_minovlen 20, fastq_minmergegelen 200. Assembled sequences were then filtered at a maxee value of 0.5 (signifying that, on average, only one nucleotide in every two sequences is potentially incorrect) to remove low quality sequences. The remaining sequences were dereplicated and singletons were removed. Operational taxonomic units (OTUs) were assigned with UPARSE at a 97% sequence identity threshold. Taxonomy was assigned to sequences for each OTU using the RDP classifier (Wang *et al.* 2007) with a confidence threshold of 0.5 against the Greengenes 13_5 database (DeSantis *et al.* 2006, McDonald *et al.* 2012) as implemented by QIIME version 1.6.0 (Caporaso *et al.* 2010). I also used QIIME to generate phylogenetic tress using FastTree (Price *et al.* 2009), and to calculate alpha (species richness, phylogenetic diversity (Faith 1992), and Shannon-Weiner Index) and beta diversity (UniFrac (Lozupone and Knight 2005)) metrics. To eliminate any bias due to sequencing depth, all samples were rarefied to 25,000 sequences before calculating diversity metrics. I choose to use the UPARSE pipeline because it has been shown to reduce the number of spurious OTUs in comparison to other pipeline protocols (Edgar 2013).

Statistical Analysis

I compared protozoan traits and fitness – defined here as per-capita growth rates – across treatments to determine how historical and contemporary environments affect protozoan traits and whether these effects are dependent on each other. I compared protist cell size and peak density across treatment groups using a series of nested four-way

generalized linear models (GLMs). Each historical sample was nested within the interaction between historical temperature and historical nutrients. I also included each trait in the model as a random covariate of the other trait (i.e. cell size as a covariate of peak density and peak density as a covariate of cell size). Akaike Information Criterion (AIC) values were used for model comparison to determine the most suitable distribution and whether or not to include non-significant higher order terms in the final model.

A significant interaction between historical temperature and historical nutrients in the previous analyses would suggest non-additive evolutionary effects. To further address my second objective, I calculated the magnitude and direction of the combined effects of historical temperature and nutrients on protist traits. The sum of the individual effects of temperature and nutrient input were used to calculate an expected additive effect according to the following equation (Christensen *et al.* 2006):

$$Expected\ Additive\ Nutrient + Temperature\ Effect$$
$$= Control + (Temperature - Control) + (Nutrients - Control)$$

I then subtracted this expected additive value from the observed value for the trait in the historical increased temperature and nutrients treatment. I performed 10,000 bootstrap iterations of these calculations to produce a mean value for the difference between the observed trait values and the expected additive effect of temperature and nutrients as well as standard deviation. I repeated this process for each contemporary environment to see if additivity between historical temperature and nutrients depends upon contemporary context. I plotted the means and standard deviations of these differences to visually determine the magnitude and direction of any non-additive effects. If the difference was significantly greater than zero (i.e. SD bars did not overlap with zero) the effect was

defined as synergistic and if the difference was significantly less then zero, it was defined as antagonistic (Christensen *et al*. 2006). In cases where the difference did not differ significantly from zero, it was considered additive.

To compare how different trophic levels were affected by treatments, I used a nested four-way GLM to analyze effects of historical and current temperature and nutrient treatments on protozoan abundance, again with the historical sample nested within the interaction between historical temperature and nutrients. A similar model was used to assess bacterial abundance, however, this model also included a term for the presence or absence of *Colpidium*. I compared the results of these analyses to determine whether protists and bacteria differ in their response to treatments.

To address my final objective, I compared bacterial community composition using a five-way PERMANOVA, which included historical and contemporary temperature and nutrients as well as *Colpidium* as factors. I visualized these effects on bacterial beta diversity using the first three axes of a redundancy analysis, which is a constrained ordination technique. I also plotted species scores, which describe how taxa are correlated with each axis, from the redundancy analysis over the site scores to produce a biplot. This allowed me to visualize which taxa are strongly associated with my treatments.

It is important to note the response protozoan populations to temperature and nutrient regimes may impact the microbial communities, potentially confounding the measured responses of these communities to experimental treatments. To elucidate these complex interactions, I utilized structural equation modeling (Mitchell, 1992) with bacterial abundance and diversity measures acting as the response, experimental

treatments as main factors and protist traits, and abundance as mediating factors (Fig. 2). This method allowed me to quantify how much of the ecological response of bacteria was the direct result of the experimental treatment and how much was indirect, as a result of changes to protist traits and abundance. For these models, values inside of the boxes are R^2 values and those beside arrow are standardized coefficients from linear models. By standardizing these values, I was able to compare the strength of effect of multiple predictors on my response variables.

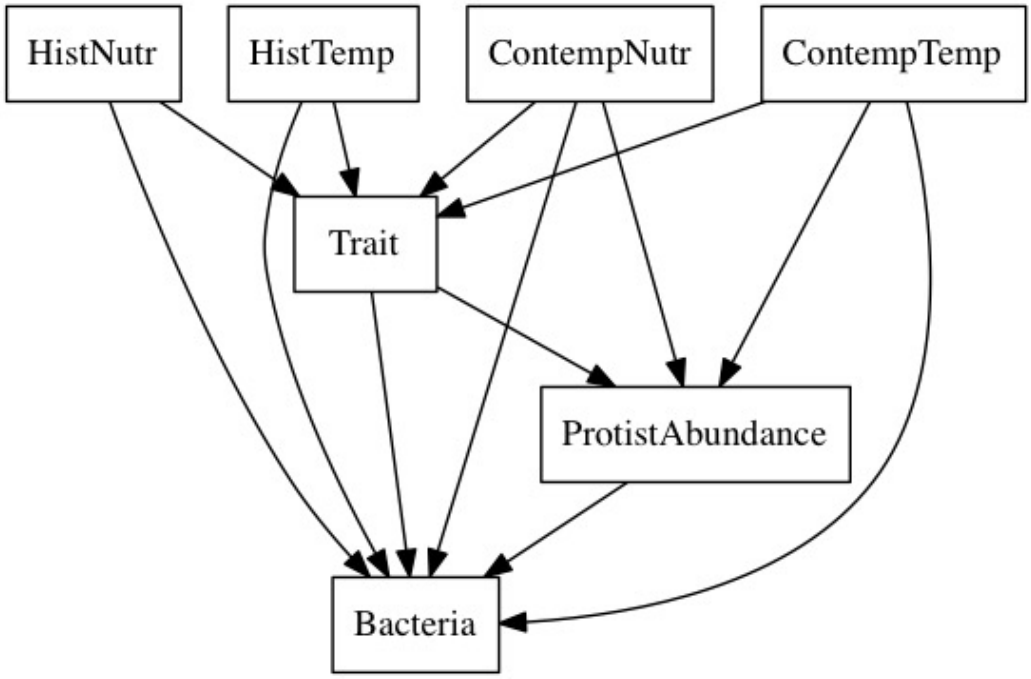

Figure 2 Example structural equation model showing theoretical causal relationships between variables.

Results

Field Samples

Temperatures experienced inside of pitcher plants were more variable than air temperatures. The average minimum temperature inside of pitcher plants (19.9°C) was 1.8°C cooler than the minimum air temperature and the average maximum temperature inside of pitcher plants (38.6°C) was 6.3°C warmer than the maximum air temperature (Fig. 3).

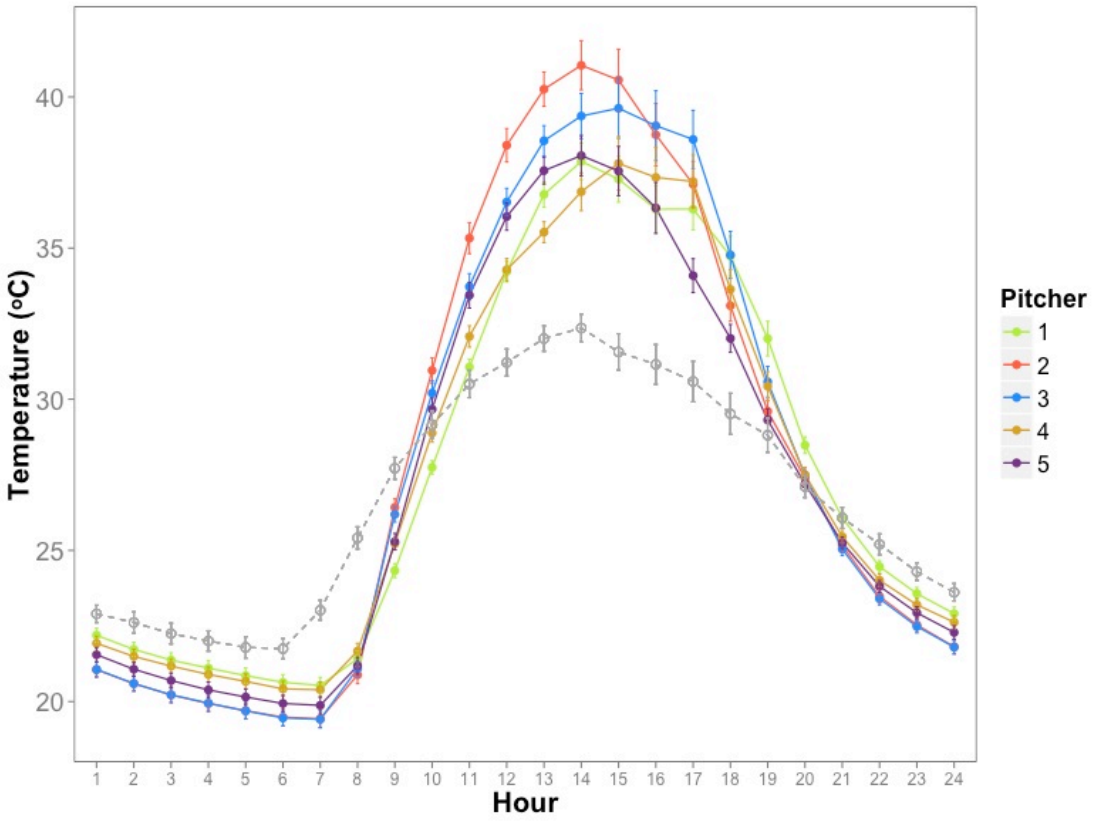

Figure 3 Average daily temperature cycles of five pitcher plants in Apalachicola National Forest, Florida. The dashed gray line shows average air temperature taken at the same time points.

Bacterial communities taken directly from naturally occurring pitcher plants had similar diversity (phylogenetic diversity and Shannon-Weiner Index) and species richness as initial laboratory stock cultures but show less species richness and phylogenetic

diversity than experimental microcosms (Table 1). The average species richness of naturally occurring pitchers and stocks were not significantly different while richness of experimental microcosms was far greater. The average phylogenetic diversity for field samples and stocks were not significantly different while experimental microcosms were more phylogenetically diverse. The average Shannon-Weiner Diversity index for field samples, stocks and experimental microcosms were not significantly different from one another (Table 1).

Metric	Mean (Standard Error)	Sample
Species Richness	193.6 (0.9)	Experimental
	121 (24)	Stocks
	137.7 (21)	Field
Phylogenetic Diversity	12.3 (0.06)	Experimental
	6.2 (1.24)	Stocks
	7.9 (1.24)	Field
Shannon-Weiner Index	3.6 (0.003)	Experimental
	3.9 (0.57)	Stocks
	3.3 (0.24)	Field
Pielou's Evenness	2.4 (0.03)	Experimental
	2.2 (0.33)	Stocks
	2.2 (0.33)	Field

Table 1 Alpha diversity metrics for all experimental microcosms (after the selection experiment and the reciprocal transplant), laboratory stocks (bacteria and *Colpidium*) and field samples.

Ecology and Evolution of Protist Traits and Fitness

I analyzed the effects of fixed factors (historical and contemporary temperature and nutrients) on protist per-capita growth rates using a nested four-way generalized linear model. Twelve samples of 80 were excluded from this analysis because either they or their parent sample persisted after being fixed in Lugol's iodine (n=68). There was a significant interaction between historical temperature, historical nutrients and contemporary nutrients (Table 3) on per-capita growth rates. *Colpidium* from a historical high nutrient environment had high growth rates when placed in a low nutrient environment. Historical temperature did not affect this interaction (Fig. 4). However, when protists from a high historical nutrient environment were placed in a contemporary high nutrient environment, increased growth rates were observed only when they were also from an ambient historical temperature (Fig. 4). Protists from a historical warming environment and contemporary high nutrient environment were not influenced by historical nutrient levels (Fig. 4).

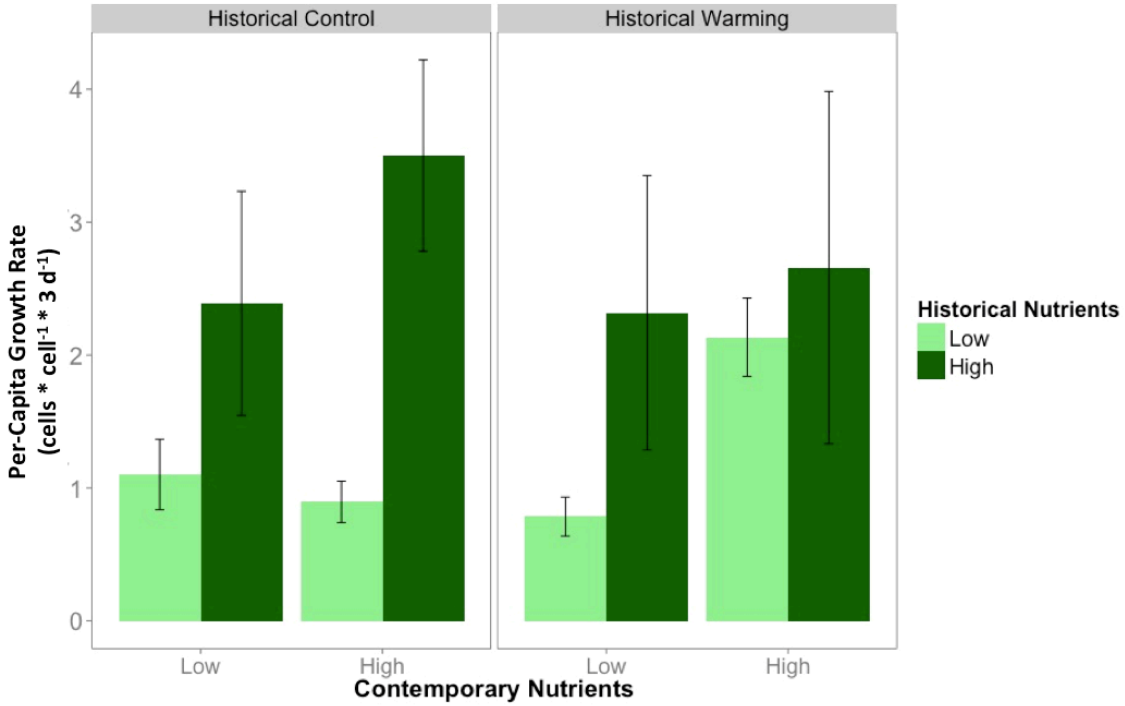

Figure 4 Effects of historical temperature, historical nutrients, and contemporary nutrients on *Colpidium* per-capita growth rates (means +/- SE). Contemporary temperature all other interactions were not significant so, the means represented were pooled across contemporary temperature treatments.

One protist population persisted after being fixed in Lugol's iodine and was therefore excluded from analyses of cell size (n = 79; peak density n = 80). The random nested factor, historical microcosm, was determined to be non-significant and a model that included the nested term had a higher AIC value ($dAIC_{34} = 8.5$) than a model without ($dAIC_{18} = 0.0$) so the nested term was dropped from the final model. There was a significant interaction between contemporary nutrients and contemporary temperature on protist cell size (Table 3). *Colpidium* that were grown in the low contemporary nutrient treatment had larger cells if they were grown in the contemporary ambient temperature treatment (Fig. 5A). This effect was opposite for *Colpidium* grown in high contemporary nutrients, where those grown in the contemporary warming treatment have larger cells on average than those grown in the contemporary ambient temperature treatment (Fig. 5A).

There was also a significant interaction between contemporary nutrients and historical nutrients (Table 3) on cell size. The average cell size of *Colpidium* from low historical nutrient and high historical nutrient environments did not differ significantly when grown in a high contemporary nutrient environment (Fig. 5B). However, in the low contemporary nutrient environment, *Colpidium* from the high historical nutrient environment had larger cells on average (Fig. 5B).

I found that cell size in the original stock was more variable than in the experimental microcosms across all treatments (Figs. 5A and 5B). Cell size in the *Colpidium* stock was not significantly different from cell size in any of the experiment treatments, although treatments did differ significantly from one another (Figs. 5A and 5B). This result shows that the effect of treatments on *Colpidium* cell size acted on standing variation found in the population before the experiment began and selection occurred in both directions, depending on the treatment.

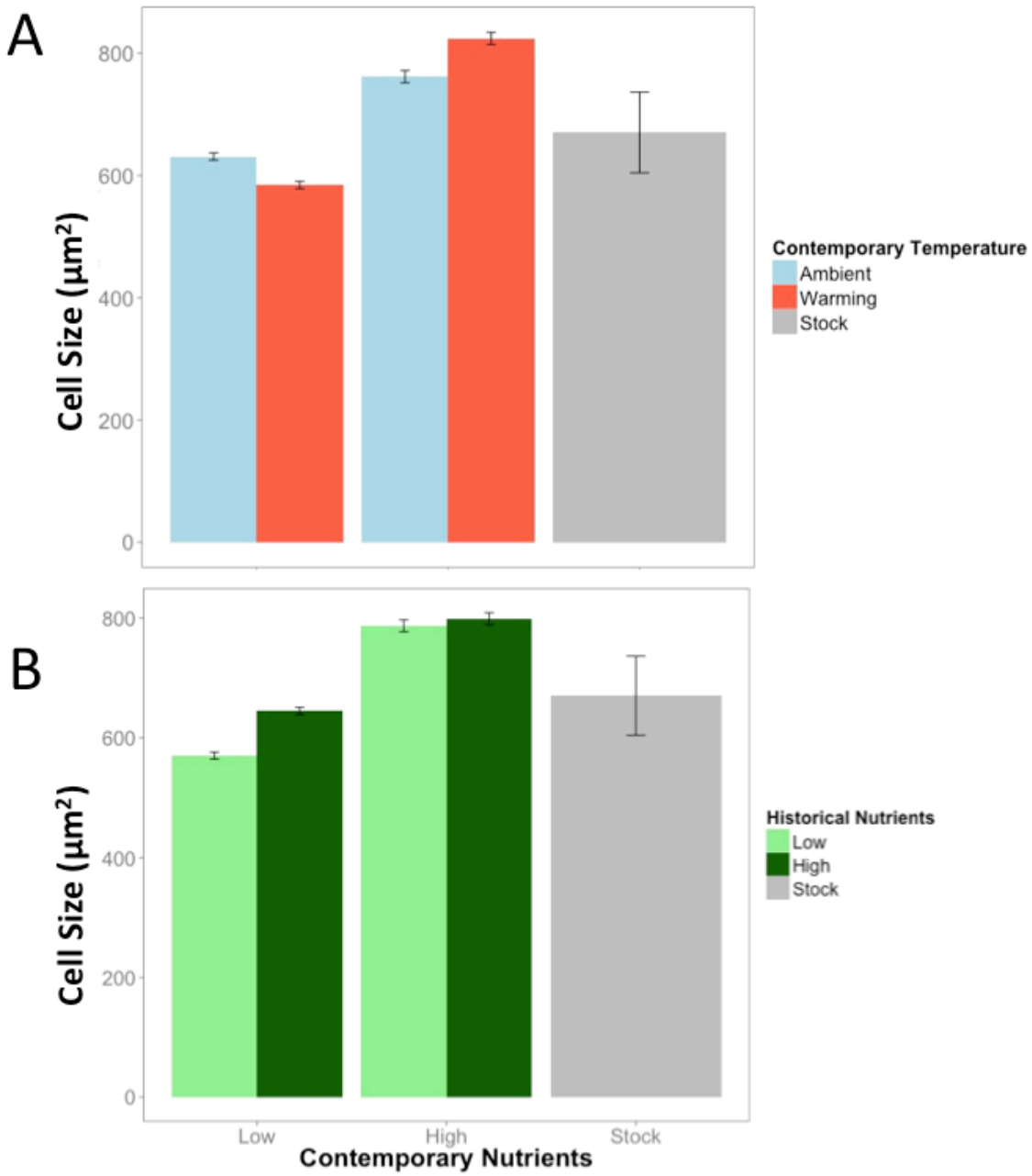

Figure 5 Effects of (A) contemporary nutrients and temperature and (B) contemporary nutrients and historical nutrients on *Colpidium* cell size (means +/- SE). Gray bars represent cell size in *Colpidium* stock used to inoculate experimental microcosms (mean +/- SE). All other effects and interactions were not significant so, the means represented were pooled across (A) historical treatments and (B) temperature treatments.

The nested factor, historical sample, was not significant and a model with the term had a greater AIC value ($dAIC_{34} = 4.4$) than a model without ($dAIC_{18} = 0.0$) so the nested term was dropped from the final model. The interaction between contemporary

and historical nutrients was significant (Table 3). *Colpidium* populations from the low historical nutrient environment had significantly higher peak densities when placed in a high contemporary nutrient environment (Fig. 6). However, protists in a low nutrient contemporary environment had similar peak densities regardless of historical nutrient levels (Fig. 6). Peak density of *Colpidium* from the initial stock culture was significantly higher than that of *Colpidium* in any of the experimental treatments at the end of the reciprocal transplant experiment (Fig. 6).

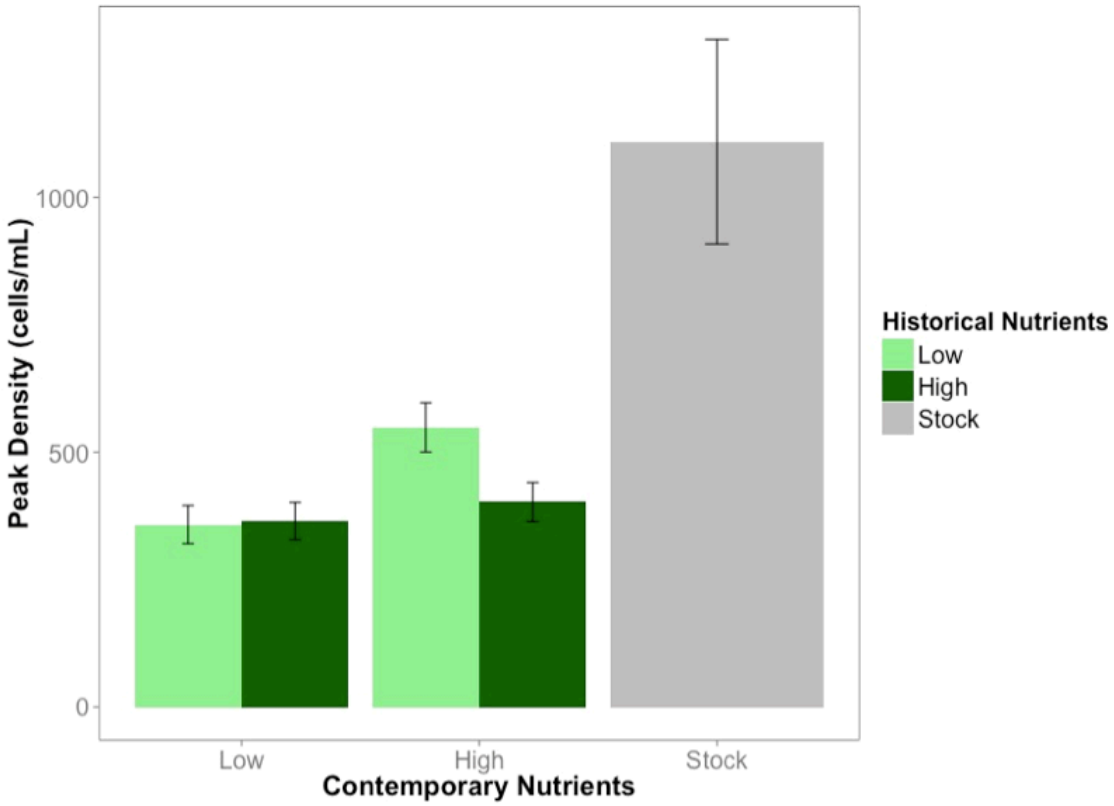

Figure 6 Effects of historical nutrients and contemporary nutrients on *Colpidium* peak density (means +/- SE). Historical and contemporary temperature and interactions were not significant so, the means represented were pooled across temperature treatments.

Additivity of Temperature and Nutrients

The interaction between historical temperature and nutrients was not significant for protist cell size or peak density (Table 3) so I did not expect to find any non-additive

effects of these factors. Further investigation by comparing the expected additive values to observed values supported this finding. In all cases, the difference between the observed and expected additive values for the combined effects of historical nutrients and temperature on protist traits (+/- standard deviation) overlapped with the zero (Figs. 7A and 7B). This was true of all contemporary environments (Figs. 7A and 7B). I took this as evidence that the observed effects of historical temperature and nutrients on protist traits were additive.

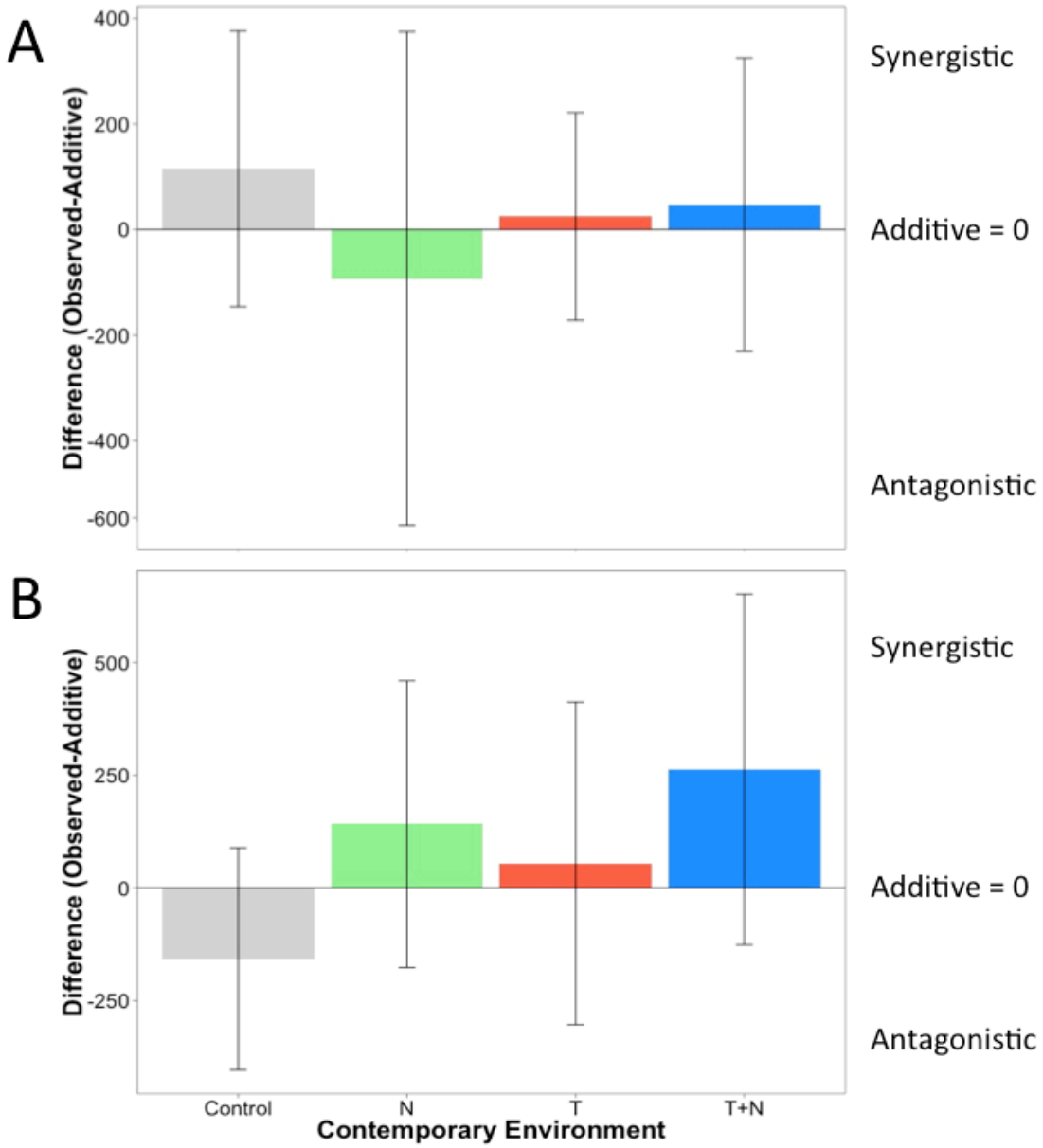

Figure 7 Difference between observed combined effects and bootstrapped additive expectation for historical temperature and historical nutrients on *Colpidium* (A) cell size and (B) peak density. Error bars are bootstrapped standard deviations of the means.

Differences Between Trophic Levels

After the reciprocal transplant experiment, the effects of the fixed factors historical and contemporary temperature and nutrients on protist abundance were modeled using a nested four-way generalized linear model. The nested factor, parent

sample from the selection experiment, was not significant and a model with the nested factor had a higher AIC value ($dAIC_{21} = 2.0$) than a model without ($dAIC_{17} = 0.0$) so the nested term was dropped from the final model. The three-way interaction between historical temperature, historical nutrients and contemporary nutrients was significant (Table 3). There was also a significant interaction between historical nutrients, contemporary nutrients and contemporary temperature (Table 3). All protist populations from a historical warming environment had similar abundances except populations transplanted from a low nutrient environment to a high nutrient environment. These had the greatest abundance of all the groups (Fig. 8A). Populations from a historical ambient temperature environment had similar abundances except the group that was transplanted from a high nutrient environment to a low nutrient environment, which had the lowest abundance of all the groups (Fig. 8A). The opposite was true with respect to contemporary temperature. *Colpidium* grown in the contemporary ambient temperature environment did not differ significantly in abundance except those that were transplanted from low historical to high contemporary nutrients, which had a significantly higher abundance than the other three groups in the contemporary ambient temperature treatment (Fig. 8B). Those grown in the contemporary warming treatment had similar abundance to one another except those transferred from high historical nutrients to low contemporary nutrients, which had significantly lower abundance than the other three groups (Fig. 8B).

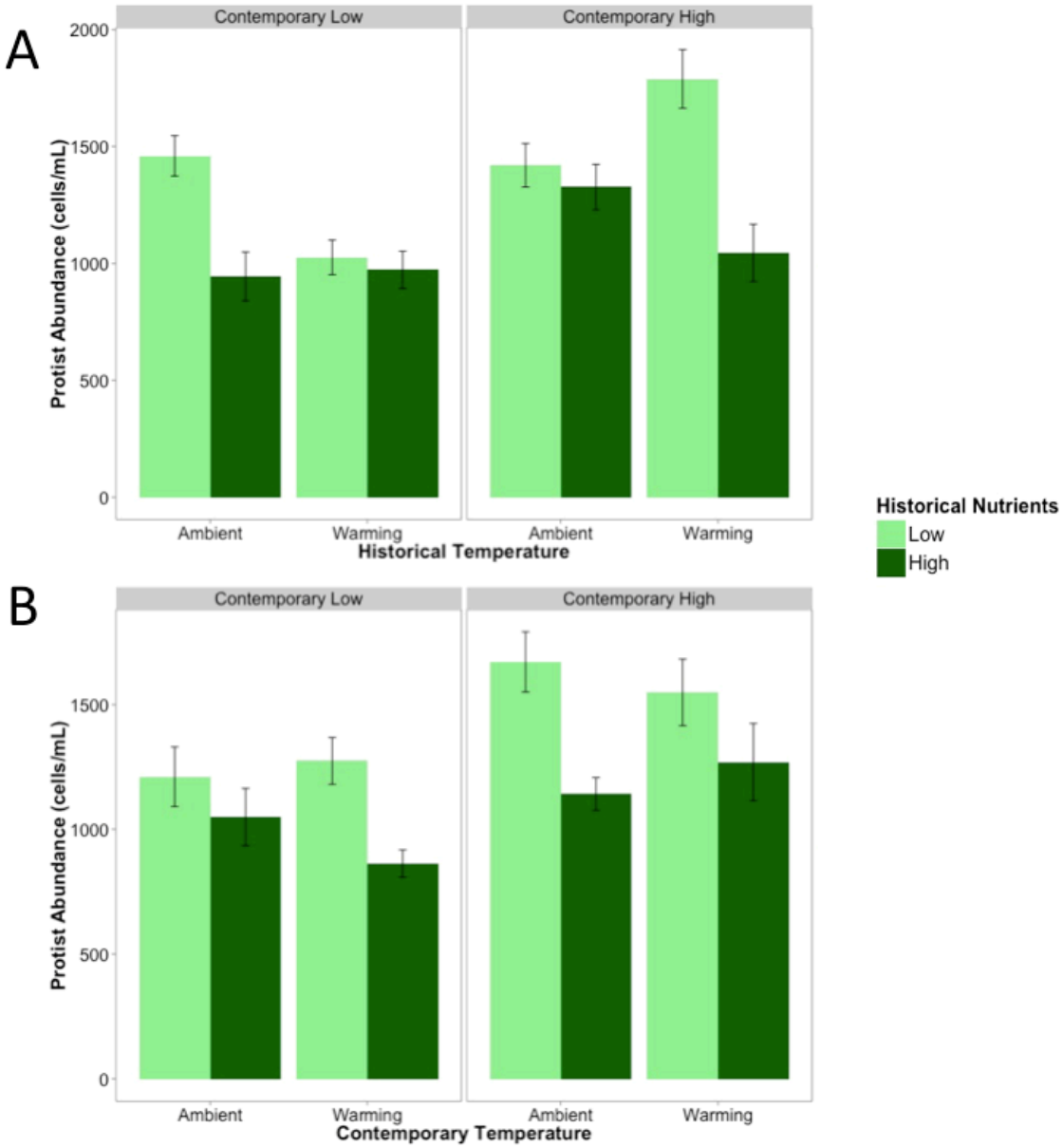

Figure 8 Effects of (A) contemporary nutrients, historical nutrients, and historical temperature and (B) contemporary nutrients, historical nutrients, and contemporary temperature on *Colpidium* abundance (means +/- SE) after the reciprocal transplant experiment. All other effects and interactions were not significant so, the means represented were pooled across (A) contemporary temperature and (B) historical temperature.

The effects of the fixed factors (historical and contemporary nutrients and temperature), as well as the fixed factor *Colpidium*, on bacterial abundance were tested using a nested five-way generalized linear model. The random nested factor, historical

microcosm, was not significant and a model with the nested factor had a higher AIC value ($dAIC_{41} = 7.9$) than a model without ($dAIC_{33} = 0.0$) so the nested term was dropped from the final model. The two-way interaction between contemporary nutrients and temperature was significant (Table 4) in this model. Microcosms in contemporary ambient temperature treatments had significantly higher bacterial abundance if they also were grown in high contemporary nutrients (Fig. 9A). In contrast, in the contemporary warming treatment, the low contemporary nutrient group has higher bacterial abundance (Fig. 9A). There was also a significant independent effect of *Colpidium* (Table 4) on bacterial abundance, such that bacteria were more abundant when *Colpidium* were present (Fig. 9B).

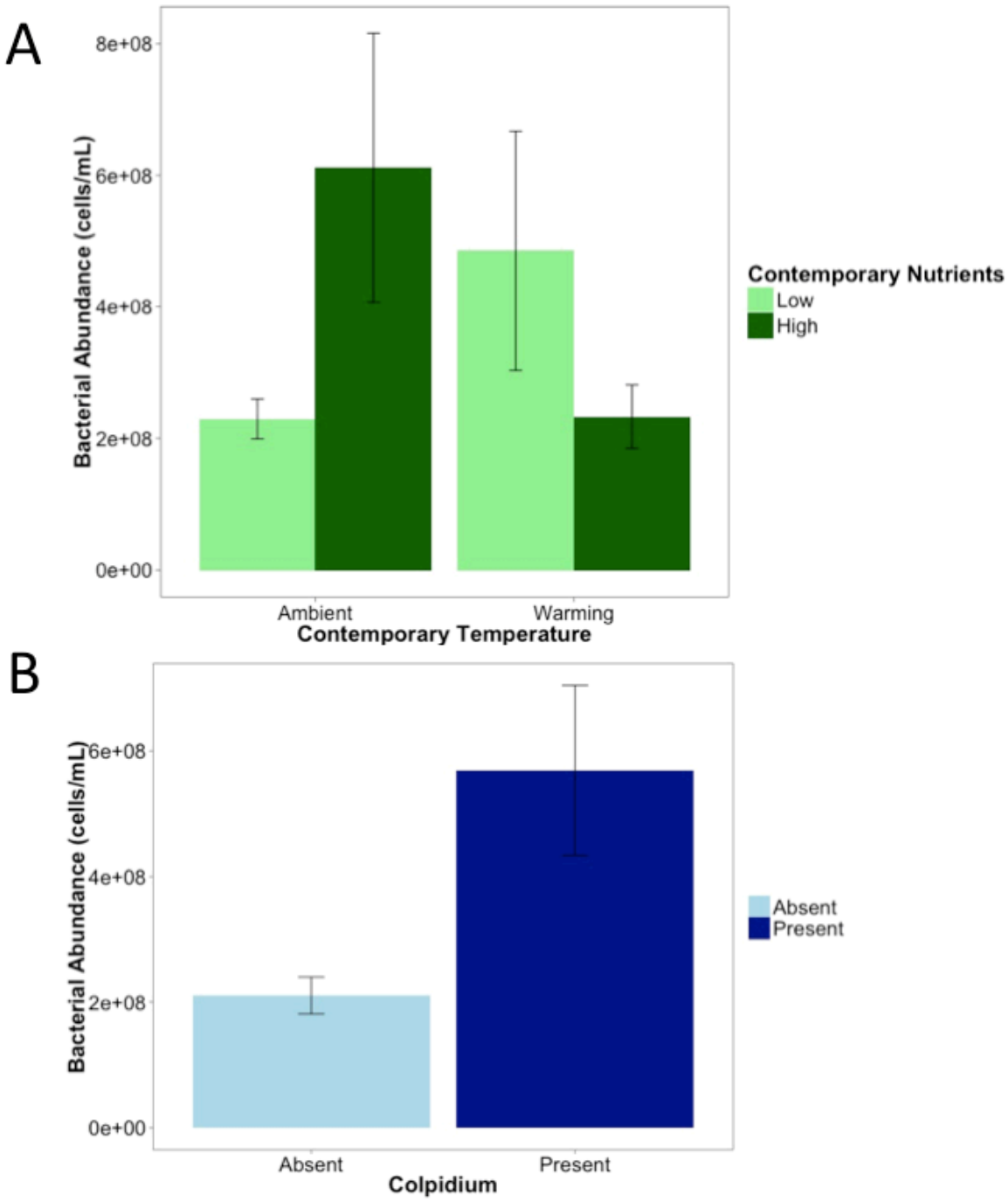

Figure 9 Effects of (A) temperature and nutrient in the contemporary environment and (B) *Colpidium* on bacterial abundance (means +/- SE) after the reciprocal transplant experiment. (A) The effects of historical temperature and nutrients and interactions were not significant so, means represented were pooled across historical treatments. (B) The independent effect of *Colpidium* was significant. However, no interactions were significant so, means represented were pooled across all abiotic treatments.

Evolutionary Effects and Ecological Interactions

I examined bacterial beta diversity (represented by UniFrac distance) using a nested five-way PERMANOVA that included historical and contemporary nutrients and temperature as well as *Colpidium* as fixed factors. Three separate three-way interactions were significant: between historical nutrients, contemporary nutrients, and *Colpidium*, between historical temperature, historical nutrients, and *Colpidium*, and between historical temperature, historical nutrients, and contemporary nutrients (Table 4). To determine which of these significant effects was most important, I referred to the eigenvalues and eigenvectors for the first five constrained axes of the redundancy analysis. The eigenvalue for each axis quantifies the amount of variation in the response variable that is captured by that axis. I calculated the proportion of the variance explained by each axis (Table 2) by dividing each eigenvalue by the sum of the eigenvalues for all constrained axes. In addition, each axis has an eigenvector that contains values for each predictor variable, with an absolute value ranging from zero to one. Predictors with a high absolute value (typically above 0.5) are considered factors that strongly structure community composition along that axis. The sign of the value indicates the direction of the predictor's effect on that axis. The first and second axes were largely explained by contemporary nutrients and *Colpidium* and the third axis was explained by historical nutrients (Table 2). Although historical temperature and contemporary temperature explained a great deal of variation captured by the fourth and fifth axes, respectively (Table 2), I reserved my interpretations here to the first three axes, which collectively explained over 92% of the variance in bacterial community composition.

	RDA1	RDA2	RDA3	RDA4	RDA5
Proportion Variance	0.691	0.133	0.100	0.066	0.009
Contemporary Nutrients	-0.703	-0.692	0.133	-0.092	-0.017
Contemporary Temperature	-0.031	0.002	-0.01	0.099	0.995
Colpidium	0.705	-0.641	0.291	-0.079	0.028
Historical Nutrients	0.107	-0.331	-0.868	0.353	-0.034
Historical Temperature	0.048	0.007	0.381	0.919	-0.084

Table 2 Eigenvectors for the first five axes of the redundancy analysis of bacterial community composition using a UniFrac distance matrix.

Bacterial communities were significantly different depending on their contemporary nutrient treatment (indicated by shape of points; Fig. 10A). Samples from the high contemporary nutrient treatment (triangles) showed tight clustering with one another but show little overlap with samples from the low contemporary nutrient treatments (circles). Presence of absence *Colpidium* significantly impacted microbial communities from a low contemporary nutrient environment (indicated by size of circles; Fig. 10A). Communities grown without *Colpidium* (small circles) cluster together and show no overlap with those with *Colpidium* (large circles). However, the presence or absence of *Colpidium* did not appear to impact communities from high contemporary nutrient environments as evidence by clustering of large and small triangles (Fig. 10A). As previously mentioned, historical nutrients explained variation in community composition in the third constrained axis of the ordination (indicated by open or solid points; Fig. 10B).

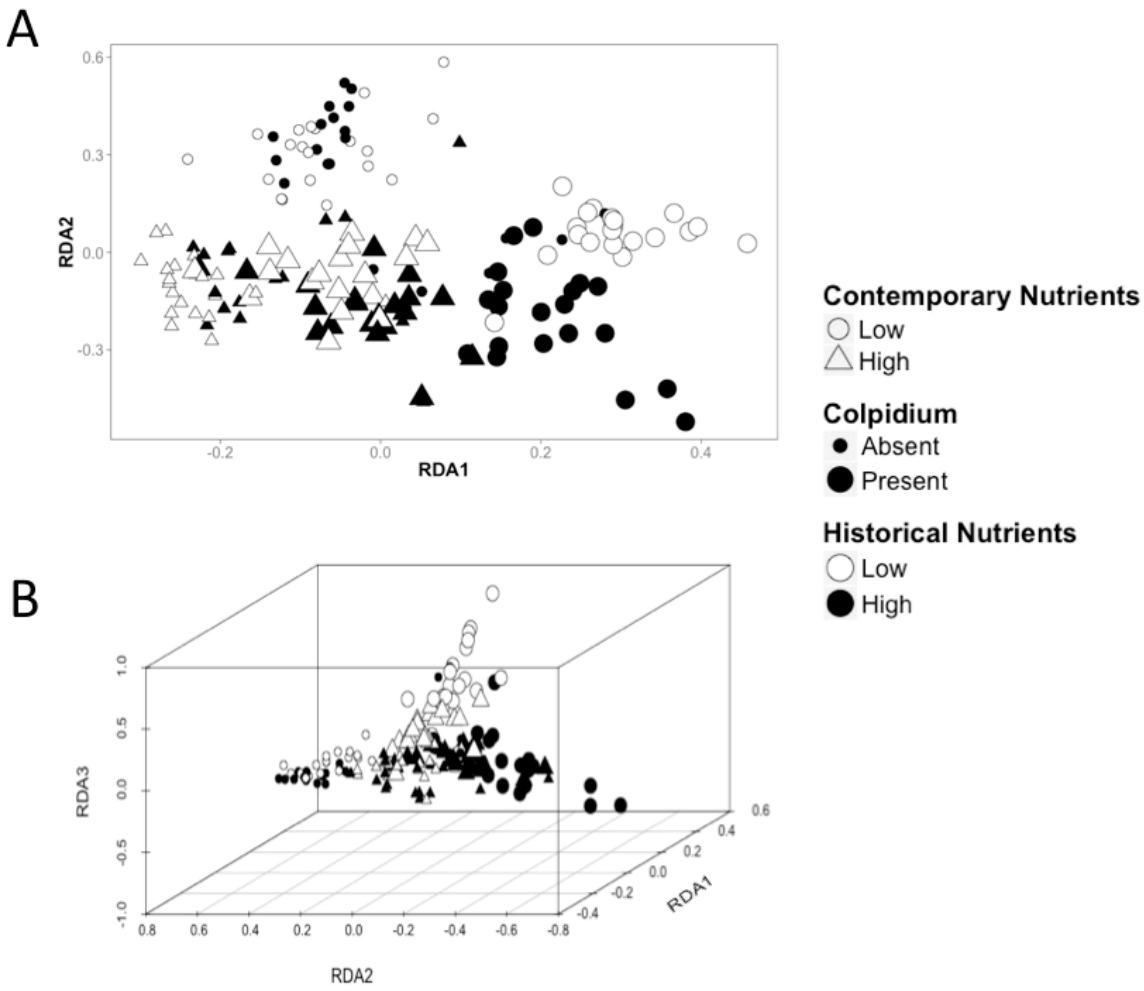

Figure 10 Ordination plot of bacterial community beta diversity based on UniFrac distances. Each point represent one replicate and those closer together have a more similar bacterial community. Shown are (A) the first two and (B) first three axes of site scores from the redundancy analysis.

Through visualization of species scores on a biplot, I found that samples with low first axis values were dominated by *Enterobacter* and those with high first axis values were abundant in *Azospirillum* (Fig. 11). *Enterobacter* was most abundant in eutrophic environments (triangles) while *Azospirillum* thrived best in oligotrophic environments (circles). More specifically, *Azospirillum* was the dominant taxon found in samples grown in low historical and contemporary nutrient environments with *Colpidium* (large open circles). This is not surprising because organisms within the genus *Azospirillum* are

known for being oligotrophic denitrifiers (Ishii *et al.* 2011) that play an important role in the nitrogen cycle by reducing nitrate and nitrite into nitric oxide, nitrous oxide, and finally nitrogen gas, which can then be fixed by other organisms (for example, the numerous Rhizobiales present in the community) into ammonia for use by the plant. Samples with high values for the second ordination axis were enriched in bacteria of the family Chitinophagaceae (Fig. 11), which is known for being responsible for chitin hydrolysis (Kämpfer *et al*. 2011). Samples in the region of the ordination plot associated with chitinolytic taxa were those grown in low contemporary nutrients without *Colpidium* (Fig. 11). Taxa that have low values for the second ordination axis, such as *Burkholderia*, were abundant in samples when *Colpidium* is present (large circles and triangles) and are likely key community members when protist abundance is high in *S. purpurea*.

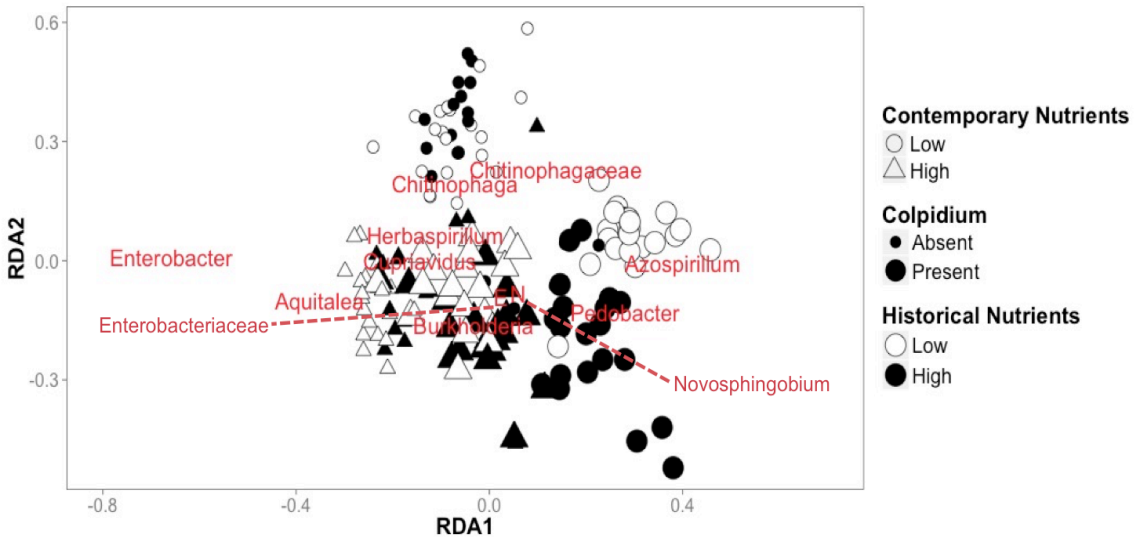

Figure 11 Biplot of bacterial community beta diversity based on UniFrac distances. Each point represent one replicate and those closer together have a more similar bacterial community. The positions of red text represent taxa scores for those taxa taken from the redundancy analysis.

In the model that included *Colpidium* cell size, there was a negative indirect effect of contemporary nutrient environment on bacterial community phylogenetic diversity

mediated by *Colpidium* abundance (Fig. 12). Increase in contemporary nutrients resulted in a significant ($P < 0.001$) increase in protist abundance, which in turn significantly ($P = 0.031$) decreased bacterial phylogenetic diversity. Protist abundance and historical temperature environment had negative effects on bacterial phylogenetic diversity that were similar in magnitude (Fig. 12). When peak density of *Colpidium* was considered in the model, the negative direct effect of historical temperature on bacterial phylogenetic diversity remained (Fig. 13), but the negative effect of *Copidium* abundance on bacterial phylogenetic diversity was only marginally significant ($P = 0.067$) and weak in magnitude relative to the effect of historical temperature. Historical nutrients negatively affected *Colpidium* peak density while contemporary nutrients had a stronger positive effect on peak density (Fig. 13). *Colpidium* abundance increased directly in response to both higher contemporary nutrients and higher peak density (Fig. 13). Increased historical nutrients had an indirect negative effect on *Colpidium* abundance, mediated by its negative effect on peak density. However, increased contemporary nutrients had both direct and indirect positive effects on *Colpidium* abundance, which were stronger in magnitude than the negative indirect effect of historical nutrients.

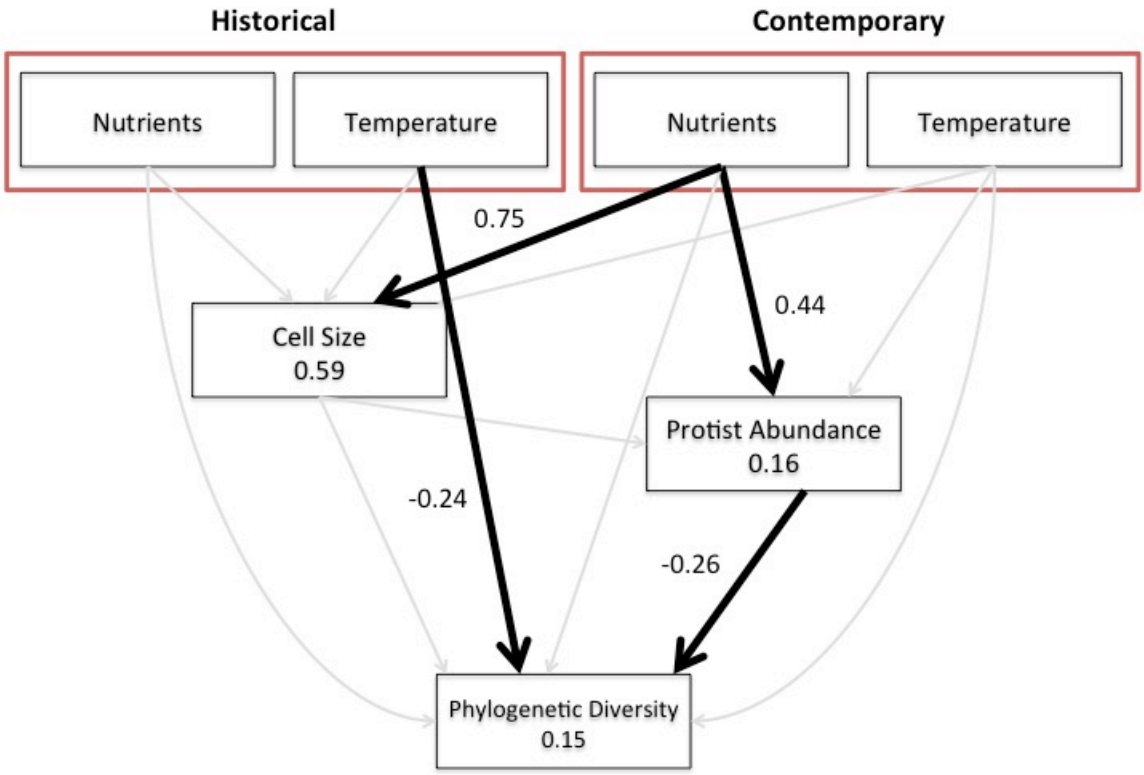

Figure 12 Structural equation model showing the causal effects of treatments and protist cell size and abundance on bacterial alpha diversity (phylogenetic diversity). Values inside of boxes are R^2 values and those beside arrows are standardized coefficients from linear models. Bolded arrows show significant effects while gray arrows are non-significant.

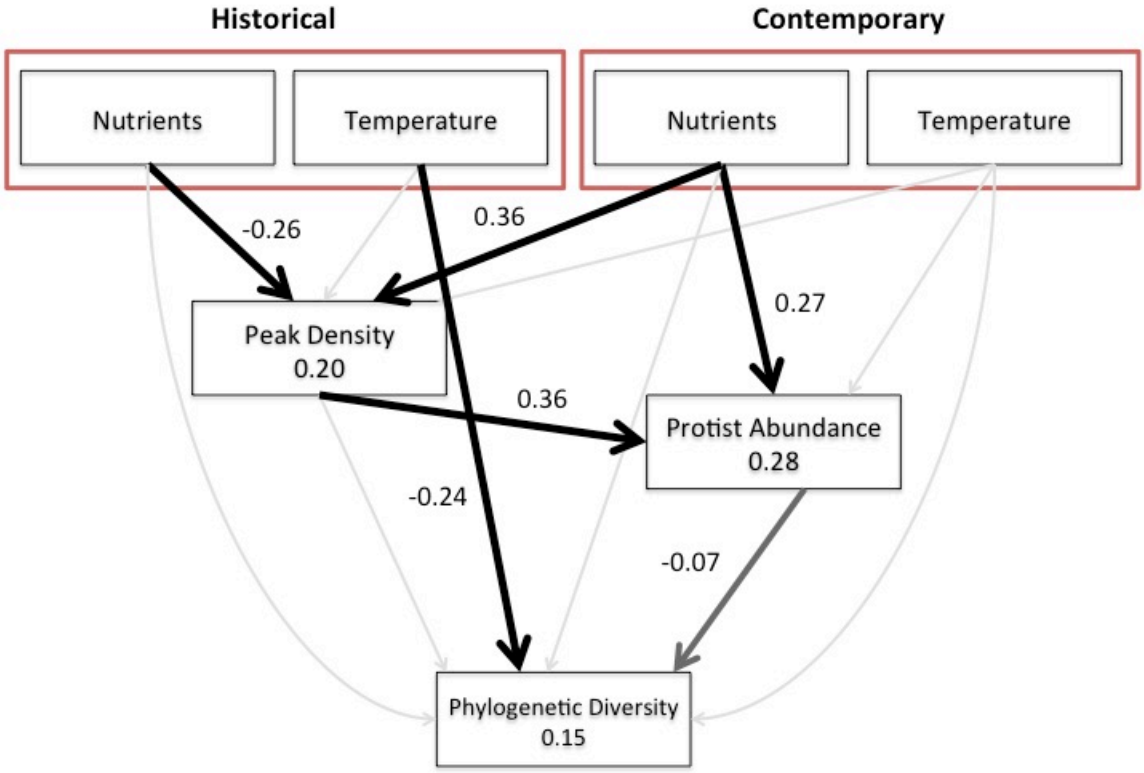

Figure 13 Structural equation model showing the causal effects of treatments and protist peak density and abundance on bacterial alpha diversity (phylogenetic diversity). Values inside of boxes are R^2 values and those beside arrows are standardized coefficients from linear models. Bolded arrows show significant effects while gray arrows are non-significant.

Models that considered bacterial community composition, represented by the first axis scores from the redundancy analysis, showed no significant indirect effects of treatments on the bacterial community mediated by traits or abundance of *Colpidium* (Figs. 14 and 15). Instead, historical nutrients and contemporary temperature had direct effects on community composition, which resulted in an inverse relationship between these treatments and the value of the first axis score. I found no significant effect of abiotic treatments or *Colpidium* traits or abundance on bacterial abundance and very little of the variation in bacterial abundance was explained by these factors (Figs. 16 and 17).

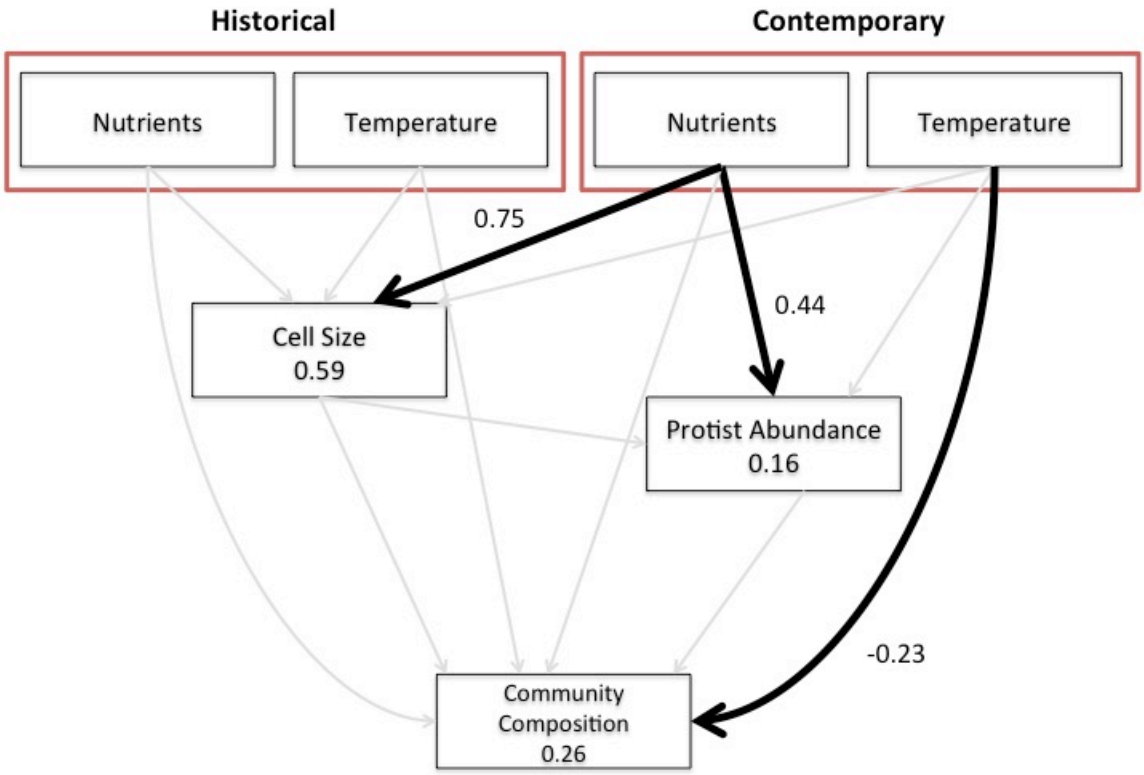

Figure 14 Structural equation model showing the causal effects of treatments and protist cell size and abundance on bacterial community composition (first axis RDA score). Values inside of boxes are R^2 values and those beside arrows are standardized coefficients from linear models. Bolded arrows show significant effects while gray arrows are non-significant.

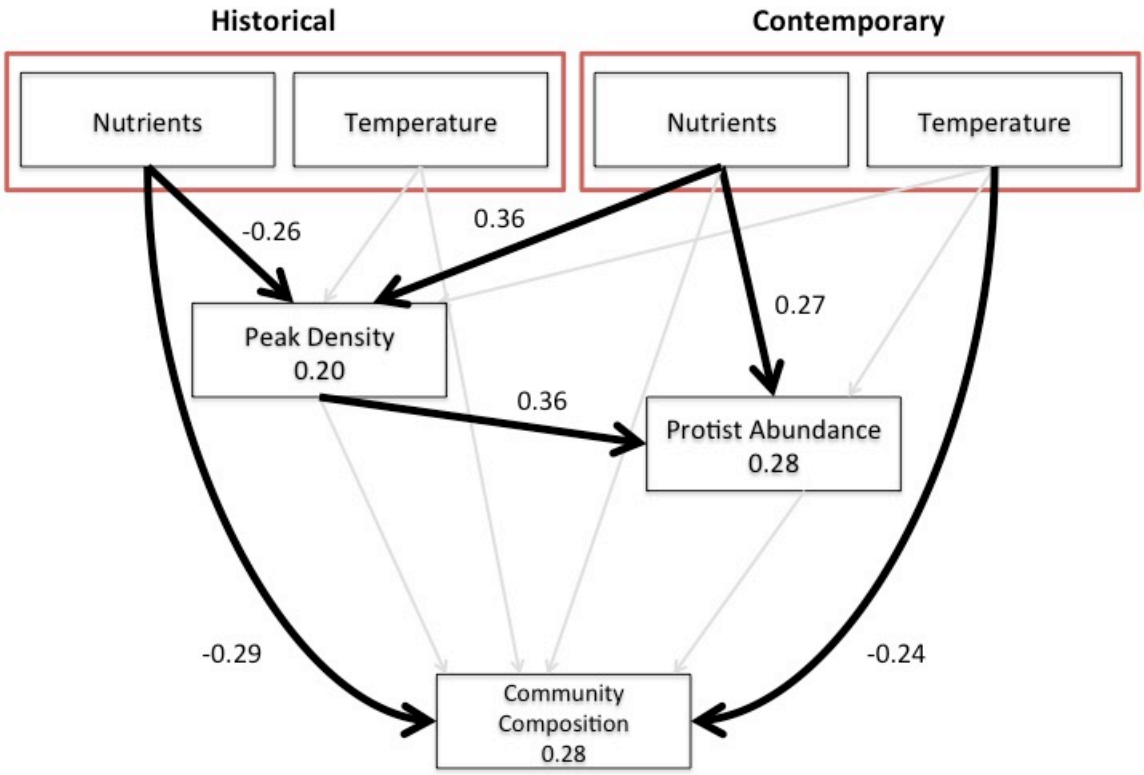

Figure 15 Structural equation model showing the causal effects of treatments and protist peak density and abundance on bacterial community composition (first axis RDA score). Values inside of boxes are R^2 values and those beside arrows are standardized coefficients from linear models. Bolded arrows show significant effects while gray arrows are non-significant.

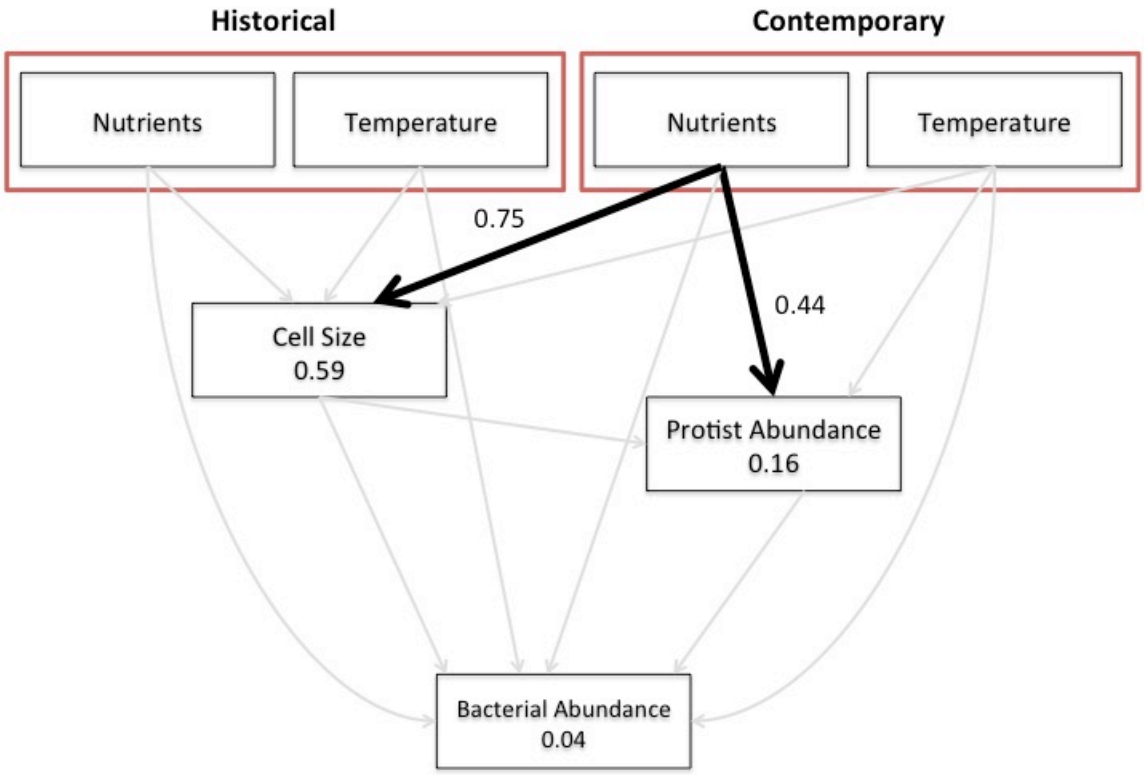

Figure 16 Structural equation model showing the causal effects of treatments and protist cell size and abundance on bacterial abundance (cells/mL). Values inside of boxes are R^2 values and those beside arrows are standardized coefficients from linear models. Bolded arrows show significant effects while gray arrows are non-significant.

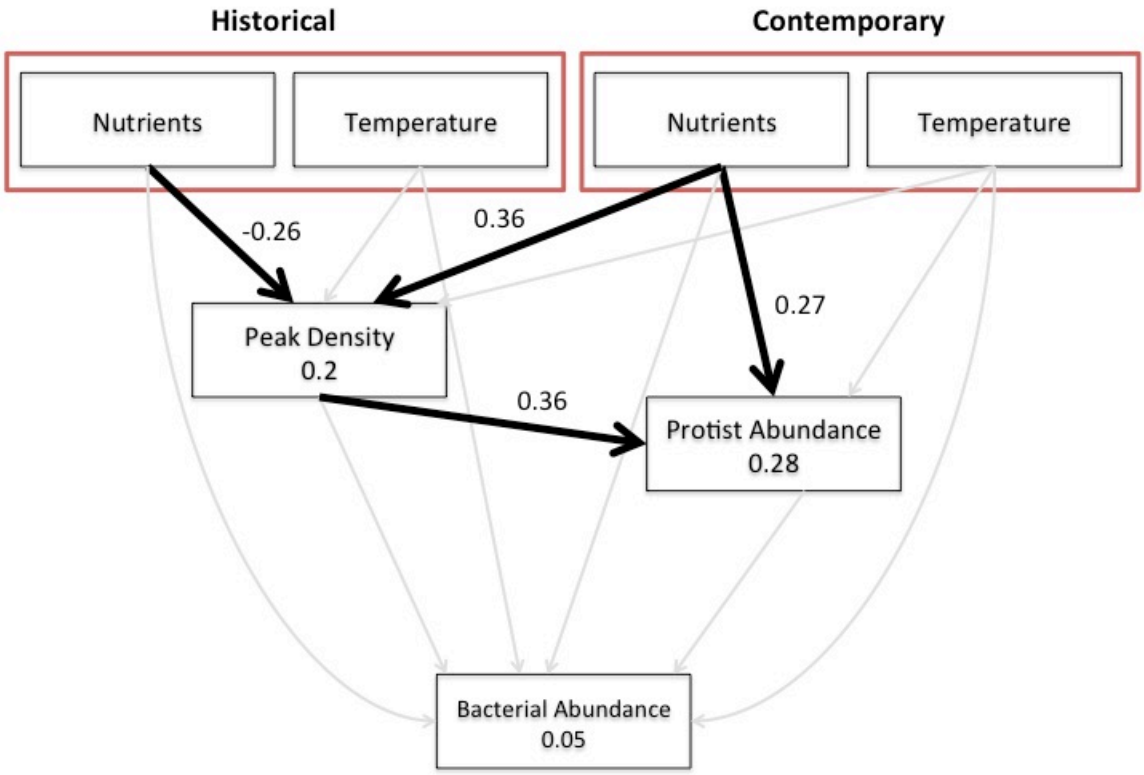

Figure 17 Structural equation model showing the causal effects of treatments and protist peak density and abundance on bacterial abundance (cells/mL). Values inside of boxes are R^2 values and those beside arrows are standardized coefficients from linear models. Bolded arrows show significant effects while gray arrows are non-significant.

	Per-Capita Growth Rates (Day 31) (Gamma)		Cell Size (Gamma)		Peak Density (Gaussian)		Abundance (Day 31) (Gaussian)	
	F	P	F	P	F	P	F	P
Hist Nutr	54.410	**< 0.001**	3.390	0.070	2.880	0.095	33.492	**< 0.001**
Hist Temp	0.008	0.930	0.052	0.820	0.225	0.637	0.871	0.354
Contemp Nutr	10.471	**0.003**	114.556	**< 0.001**	7.784	**0.007**	23.771	**< 0.001**
Contemp Temp	0.564	0.457	0.209	0.650	0.436	0.511	0.871	0.354
Peak Density	-	-	1.784	0.187	-	-	7.972	**0.006**
Cell Size	-	-	-	-	1.605	0.210	0.306	0.582
Hist Temp * Hist Nutr	4.879	**0.033**	0.127	0.723	0.710	0.403	0.306	0.582
Contemp Temp * Contemp Nutr	0.000	0.985	6.951	**0.011**	0.020	0.888	0.423	0.518
Hist Temp * Contemp Temp	1.244	0.272	1.920	0.171	0.832	0.365	0.040	0.842
Hist Temp * Contemp Nutr	2.110	0.154	0.000	0.994	0.044	0.834	5.832	**0.019**
Hist Nutr * Contemp Temp	0.008	0.930	0.910	0.344	1.399	0.241	0.154	0.696
Hist Nutr * Contemp Nutr	0.374	0.544	5.589	**0.021**	5.225	**0.026**	0.003	0.954
Hist Temp * Hist Nutr * Contemp Temp	2.782	0.103	0.114	0.737	1.020	0.317	1.224	0.273
Hist Temp * Hist Nutr * Contemp Nutr	7.855	**0.008**	0.078	0.781	1.966	0.166	15.641	**< 0.001**
Hist Temp * Contemp Temp * Contemp Nutr	3.946	0.054	0.386	0.537	0.317	0.576	2.746	0.103
Hist Nutr * Contemp Temp * Contemp Nutr	0.946	0.337	3.252	0.076	0.007	0.934	4.751	**0.033**
Parent Sample (Hist Temp * Hist Nutr)	15.022	**< 0.001**	-	-	-	-	-	-
Hist Temp * Hist Nutr * Contemp Temp* Contemp Nutr	1.197	0.280	0.422	0.5183	0.013	0.910	0.098	0.755

Table 3 Summary table showing F-ratios and p-values for all factors in the final models used to analyze *Colpidium* response variables. The distribution used to model each response variable is listed below the variable in parentheses.

	Bacterial Abundance (Day 31) (Gamma)		Community Composition (N/A)	
	F	P	F	P
Hist Nutr	1.779	0.184	12.459	**0.001**
Hist Temp	0.181	0.671	12.425	**0.001**
Contemp Nutr	0.203	0.653	148.875	**0.001**
Contemp Temp	0.181	0.671	1.294	0.251
Colpidium	6.184	**0.014**	83.869	**0.001**
Hist Temp * Hist Nutr	0.151	0.698	5.864	**0.002**
Contemp Temp * Contemp Nutr	4.841	**0.030**	2.098	0.085
Hist Temp * Contemp Temp	0.318	0.574	1.489	0.189
Hist Temp * Contemp Nutr	0.024	0.877	4.132	**0.009**
Hist Nutr * Contemp Temp	0.404	0.526	0.64	0.613
Hist Nutr * Contemp Nutr	0.022	0.884	4.218	**0.007**
***Colpidium* * Hist Temp**	0.027	0.870	12.337	**0.001**
***Colpidium* * Hist Nutr**	2.257	0.136	13.528	**0.001**
***Colpidium* * Contemp Temp**	0.235	0.629	1.393	0.229
***Colpidium* * Contemp Nutr**	0.015	0.904	25.915	**0.001**
Hist Temp * Hist Nutr * Contemp Temp	0.000	0.987	0.367	0.849
Hist Temp * Hist Nutr * Contemp Nutr	0.01	0.922	3.617	**0.014**
Hist Temp * Contemp Temp * Contemp Nutr	0.055	0.815	0.923	0.403
Hist Nutr * Contemp Temp * Contemp Nutr	3.117	0.080	0.134	0.980
***Colpidium* * Hist Temp * Hist Nutr**	0.995	0.321	11.419	**0.001**
***Colpidium* * Contemp Temp * Contemp Nutr**	3.271	0.073	0.279	0.920
***Colpidium* * Contemp Nutr * Hist Nutr**	0.019	0.890	3.153	**0.022**
***Colpidium* * Contemp Nutr * Hist Temp**	0.401	0.528	1.502	0.207
***Colpidium* * Contemp Temp * Hist Nutr**	0.034	0.855	2.087	0.087
***Colpidium* * Contemp Temp * Hist Temp**	0.016	0.900	0.222	0.963
Parent Sample (Hist Temp * Hist Nutr)	-	-	3.431	**0.001**
Hist Temp * Hist Nutr * Contemp Temp * Contemp Nutr	1.153	0.285	0.814	0.467
***Colpidium* * Hist Temp * Hist Nutr * Contemp Temp**	0.335	0.564	0.542	0.687
***Colpidium* * Hist Temp* Hist Nutr * Contemp Nutr**	0.065	0.799	2.374	0.072
***Colpidium* * Hist Temp * Contemp Nutr * Contemp Temp**	0.097	0.756	0.298	0.902
***Colpidium* * Hist Nutr* Contemp Nutr* Contemp Temp**	1.711	0.193	0.42	0.798
***Colpidium* * Hist Temp * Hist Nutr * Contemp Temp * Contemp Nutr**	0.509	0.477	0.875	0.441

Table 4 Summary table showing F-ratios and p-values for all factors in the final models used to analyze bacterial community response variables. The distribution used to model each response variable is listed below the variable in parentheses.

Discussion

This project examined multiple aspects of the eco-evolutionary effects of environmental change by testing four main hypotheses. First, I tested the hypothesis that the historical and contemporary temperature and nutrient environments experienced by *Colpidium* will affect two ecologically relevant traits, peak density and cell size. I found that both traits were affected by the historical nutrient treatment, but the strength of this effect depended on the contemporary environment. Second, I tested whether the effects of historical temperature and nutrients were additive and found that they are, suggesting that their combined effects can be predicted based on their individual effects. Third, I investigated whether protists and bacteria differed in their response to environmental change. Although *Colpidium* were affected by both the contemporary and historical environments, bacterial abundance was only affected by the contemporary environment. Finally, I tested the hypothesis that the environment would have an effect on species interactions. I found that historical and contemporary treatments altered bacterial diversity indirectly through effects on *Colpidium* traits and abundance.

The historical nutrient environment affected both cell size and peak density. *Colpidium* from a high historical nutrient environment had larger cells if they were grown in a low contemporary nutrient environment (Fig. 5B). In high nutrient environments, *Colpidium* populations become more dense and experience more intense intraspecific competition, which could be the selective agent driving the increase in cell size in the historical high nutrient environment. Previous work found that larger protists are better interspecific competitors (Kneitel 2002) so it is possible that this trait is also beneficial in intraspecific competitive interactions. In the high contemporary nutrient environment,

Colpidium from the low historical nutrient environment had significantly higher peak density than the populations from the high historical nutrient environment (Fig. 6), perhaps because those from the low historical nutrient group adapted to use resources more efficiently. These effects of historical nutrient increase on traits persisted even after 9-18 protist generations in their contemporary nutrient environment, indicating evolutionary effects of the historical nutrient environment on *Colpidium* traits.

Interestingly, these evolutionary effects of historical nutrients on *Colpidium* traits were dependent on their contemporary environment. Although protists grown in a low contemporary nutrient environment differed in their cell size depending on their evolutionary history, those grown in high contemporary nutrient environments did not (Fig. 5B). In the contemporary high nutrient treatment, it is possible that maternal effects caused *Colpidium* to grow larger regardless of their historical nutrient treatment. Maternal effects can alter the biological resources allocated to progeny. This usually occurs through differences in initial offspring resources (i.e. yolk sac size, endosperm allocation, etc.) and/or initial offspring size (Mousseau and Fox 1998). Unlike the evolutionary effects imposed by historical nutrients, maternal effects would not persist for more than a few generations once the environmental factor causing them (in this case, high contemporary nutrients) was removed. Similarly, the peak densities of *Colpidium* from low historical nutrient environments and high historical nutrients environments was different in the high contemporary nutrient treatment but not in the low contemporary nutrient treatment (Fig. 6), where nutrients were presumably limiting to populations regardless of their historical nutrient environment. This suggests that the critical assumption of eco-evolutionary feedbacks tested here – that evolutionary effects depend

upon the ecological context in which they are expressed – is a reasonable one, although more studies in other systems are needed in order to fully confirm the validity of this assumption.

Under global change conditions, species are not only expected to respond ecologically (i.e. abundance, range shifts, phenology, etc.) (Walther *et al*. 2002) but also evolutionarily (i.e. trait evolution) (Strauss *et al.* 2008). The traits that I measured evolved in response to their historical environment but these historical effects also depend on their contemporary environment. This evolution was mainly due to clonal selection since the primary mode of reproduction for *Colpidium* is asexual. However, sexual recombination does occur rarely in this species and cannot be discounted as potentially contributing to genetic variation in the microcosm populations (Dunthorn, Foissner and Katz 2008). Rapid evolution has been proposed as one mechanism that would allow species to persist in the face of global change (Kinnison and Hairston 2007). However, the dependence of these evolutionary effects on the contemporary environment adds an additional layer of complexity in predicting how species will respond to environmental factors. It is also clear that not all species can evolve rapidly and even those that do may not respond evolutionarily to all environmental stressors. For example, neither of the traits I measured was affected by historical temperature treatments. If accurate predictions are to be made about how species will respond evolutionarily to environmental change, a number of factors must be considered including the sensitivity of traits in question, the stressor they are responding to, and the environmental context in which traits are being expressed.

Although global change literature predicts non-additive effects of multiple stressors (Folt *et al*. 1999), I found that the combined effects of historical temperature and historical nutrient treatments on *Colpidium* traits did not differ significantly from the additive value that would be expected based on their individual effects. There are at least two possibilities that would explain this result. First, global change literature predicts non-additive effects on ecological response variables so it is possible that evolutionary responses differ from ecological responses in this regard. However, non-additive effects of multiple agents of selection do occur and can alter the trajectory of evolutionary responses (terHorst *et al*. in press). Another possibility is that there was little or no evolutionary response to one or both historical treatments. This is likely since the effects of historical temperature treatments were not significant for either trait. If this is the case, the combined effects would appear to be additive but in reality would be pairwise with the treatment that did have a significant effect on traits – historical nutrients.

Previous work found that, in plant-based food webs, trophic functional groups differ in their response to environmental perturbation (Petchy *et al*. 1999). In particular, Petchy and colleagues (1999) found that bacterivores are particularly resistant to warming and become dominant in systems under warming conditions. However, no attention was paid to differences between the responses of bacterivores and bacteria. I did find evidence of difference in response to environmental conditions between *Colpidium* and the bacterial community. In particular, only the contemporary environment affected bacterial abundance (Figs. 9A and 9B) but both the historical and contemporary environment affected *Colpidium* abundance (Figs. 8A and 8B). Since bacterial abundance is more affected by contemporary environment than by historical environment, they may

be more sensitive to changes in the contemporary environment. Protist abundance depends on both contemporary and historical factors so the effects of a rapidly changing contemporary environment may be buffered by historical effects. This is consistent with the findings of Petchy and colleagues (1999) who note that bacterivores (like *Colpidium*) are less sensitive to warming than other trophic functional groups. These differences are likely due to differences in generation time between trophic levels. Bacteria typically reproduce far more quickly than *Colpidium* so what may be considered "contemporary" time for protists may be "historical" time for bacteria. In addition, bacteria are known for being plastic in their physiology, particularly their metabolism, so they respond to changes in environment more quickly than eukaryotes typically do (Justice *et al.* 2008).

Historical and contemporary environments altered the interaction between *Colpidium* and the bacterial community. In a low contemporary nutrient environment, the composition of the bacterial community was significantly different depending on whether *Colpidium* was present or absent (Fig. 10A). However, in the high contemporary nutrient environment, there was no significant difference between bacterial communities grown with or without *Colpidium* (Fig. 10A). This result suggests that nutrient enrichment can mitigate the effects of *Colpidium* on the bacterial community, perhaps because additional resource availability allows bacterial species that would otherwise be vulnerable to extinction via predation to maintain their abundance in spite of predation pressures. In other words, some species are superior competitors making them better able to access resources but, as an evolutionary trade-off, are more susceptible to predation. These taxa will receive a fitness benefit from increased nutrients in the high nutrient environment. Other taxa are better at avoiding predators but are poor competitors and their fitness

suffers in a high nutrient environment due to more intense levels of competition. This "equalizing mechanism" thereby minimizes fitness differences between species (Chesson 2000) and results in similar bacterial community compositions with and without protists if high levels of nutrients are available.

Structural equation models also show that bacterial phylogenetic diversity is negatively affected by treatments directly and indirectly, through changes in *Colpidium* abundance and traits (Figs. 12 and 13). Specifically, contemporary and historical nutrients have a positive effect on *Colpidium* abundance, peak density, and cell size. Peak density also has a positive effect on *Colpidium* abundance because populations that can use resources more efficiently can maintain higher abundances. *Colpidium* abundance ultimately has a negative effect on bacterial phylogenetic diversity, meaning that when *Colpidium* are more abundant, the bacterial community is less diverse. This result could also explain why the presence of *Colpidium* increases bacterial abundance (Fig. 9B). In a bacterial community with several hundred species, there is likely to be functionally redundant species that compete for the same ecological niche (Allison and Martiny 2008). Through grazing, *Colpidium* reduces the diversity of a community, through size specificity or preference (Fenchel 1980, Dopheide *et al.* 2011), and potentially decreases the number of species competing for the same resources. The remaining species can then reach higher abundances because they are not experiencing the negative ecological effects of competition on population growth rates and carrying capacity. Another possible mechanism for this result is that *Colpidium* may have allowed more bacteria to gain access to nutrient in my experiment by mixing any nutrients that fell to the bottom of tubes back into solution. *Colpidium* have been shown to be useful in hydrocarbon

bioremediation because the mechanical action of their cilia help emulsify oil (Rogerson and Berger 1983). Kaunzinger and Morin (1998) also observed a small but significant increase in bacterial abundance when *Colpidium* were present in laboratory microcosms, which they attributed to ratio-dependent predator dynamics or evolutionary response of bacteria to *Colpidium*.

Bacterial communities without *Colpidium* grown in low nutrient environments had a higher abundance of taxa known for hydrolyzing chitin (Fig. 11). Chitin is the primary molecule that composes the exoskeletons of insects, which are the prey of *S. purpurea*. High abundance of chitinolytic taxa is beneficial to *S. purpurea* because it would allow for more efficient decomposition of insect prey and faster release of nitrogen and phosphorus to the plant itself. The fact that these beneficial bacteria were more abundant when *Colpidium* were absent suggest that *Colpidium* may have a negative effect on the mutualistic relationship between *S. purpurea* and its bacterial inquilines. *Colpidium* may even have a parasitic relationship with *S. purpurea*. This provides empirical support for theoretical models of the interactions between *S. purpurea* and its inquiline community (Mouquet *et al.* 2008). Through modeling stoichiometric constraints on prey decomposition and nutrient cycling, Mouquet and colleagues (2008) found that the bacteria-plant interaction is mutualistic while bacterivore-plant interactions are parasitic. Taxa, such as *Burkholderia, Pedobacter, Azospirillum,* and others that were found in high abundance even in samples were *Colpidium* were present (Fig. 11), may be more resistant to the effects of predation by bacterivores, allowing for the bacterial inquiline community to maintain its function when bacterivores are in high abundance. In order to achieve a more mechanistic understanding of these interactions, future research

should focus on the functional metagenome and metatranscriptome of bacterial inquiline communities of *S. purpurea* with and without *Colpidium*, as well as other ciliates that feed on the bacterial community, to determine if these ciliates reduce the ability of the bacterial community to decompose insect prey.

I found that the peak density in the initial *Colpidium* stock was far greater than peak density in any of the treatments by the end of the experiment (Fig. 6). In natural pitcher plants, bacteria must break down detritus sequentially, beginning with carbon sources that are immediately available on the exterior of insect prey (mainly chitin). Other forms of carbon are made available once the exterior of the insect is decomposed (e.g. amino acids such as glycine) or chitin is hydrolyzed (e.g. N – acetylglucosamine). Bacteria that require those carbon sources for their metabolism remain metabolically inactive and in low abundance until those metabolites become available. As a result, *Colpidium* from natural environments, where resources are more difficult to access and bacterial are less abundant, adapted to be more efficient at using these minimal resources. In experimental microcosms, a variety of carbons sources were made available all at once. So, bacteria using a range of metabolisms were able to be abundant simultaneous and, potentially, overall bacterial abundance was greater. This led *Colpidium* populations in experimental microcosms, where resources are more readily accessible, to become less efficient than those from the original stock and, instead, allocated energy toward more advantageous traits. Unfortunately, I did not count bacterial abundance in naturally occurring pitchers so I cannot quantitatively confirm this hypothesis.

The fact that my experimental microcosms showed greater species richness and phylogenetic diversity than my field samples (Table 1) was a surprising result because it

is well-known that only a small fraction of bacteria can be cultured in a laboratory (Staley and Konopka, 1985) so one would expect field samples to be more diverse. It is possible that the experimental microcosms were contaminated, with live bacterial cells or free bacterial DNA, potentially through the nutrient mixture or the autoclaved water. This is unlikely to affect comparisons between experimental treatments because all tubes could have been equally contaminated in this way. In addition, potential contamination does not change my ecological interpretations because the most abundant taxa in my samples are typically associated with aquatic freshwater environments, such as *Telmatospirillum* (Sizova *et al.* 2007) and *Aquitalea* (Lau *et al.* 2006), and plants, such as *Azospirillum* (Tarrand *et al.* 1978) and *Luteibacter* (Johansen *et al.* 2006), which is what one would expect in the *S. purpurea* inquiline community.

Although the structural equation models presented here find no direct effects of treatments on bacterial community diversity, ordination through redundancy analysis shows that treatments did have an effect on bacterial community composition when *Colpidium* was not present. This discrepancy could simply be due to the samples that were used for each of these analyses. While the PERMANOVA and redundancy analysis took advantage of all 160 data points, the structural equation model was reserved to only the 80 samples that had both bacteria and *Colpidium*. Structural equation modeling is informed by theoretical causal relationships and, because of this, I did not include samples where protist abundance was directly manipulated (the bacteria only treatments) in these models. If I had been able to include all samples in my structural equation models, I predict that I would have found more evidence for direct effects of treatments on bacterial alpha diversity. With that in mind, the structural equation models presented

here do show strong evidence for indirect effects of treatments that are mediated through protist populations and are, thus, still useful in informing the questions posed for my research.

Microcosm experiments have proven to be useful in exploring ecological and evolutionary processes and provide insight into natural communities that would otherwise be difficult or impossible to attain (Srivastava *et al*. 2004). Current ecological hypotheses that attempt to explain ecosystem response to global change often fail to account for the effects of rapid evolution, which can have an impact on the application of these hypotheses. Although it was not within the scope of my thesis, I believe the data collected through this project could be extrapolated to other ecosystems to predict how populations will respond to global change factors in light of evolution, trophic level and non-additive effects. The future success of management and conservation of natural systems rests upon the best possible understanding of not only the ecological implications but also, and perhaps even more importantly, the evolutionary consequences of environmental change.

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