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Microbes in time: Incorporating bacteria into ecosystem development theory

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Microbes in time: Incorporating bacteria into ecosystem development theory

By

David W. Armitage

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Committee in charge:

Professor Wayne P. Sousa, Chair
Professor Mary E. Power
Professor Mary K. Firestone

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Abstract

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It is widely theorized that population and community processes such as competition, predation, and dispersal influence rates of resource flux within ecosystems. Likewise, the properties of an ecosystem, such as resource availability and space, can feed back onto populations and communities, driving their dynamics and evolutionary trajectories. However, empirical research connecting community and ecosystem-level processes remains a critical missing link between these two disciplines. My dissertation attempts to resolve some of these deficiencies by capitalizing on the tractability and replicability of experimental and natural microbial communities. I use these systems to test a number of theories of community-ecosystem feedbacks.

In chapter 1, I test the theory that a bioregion's time-integrated area and productivity positively drive the extent of diversification in a radiating lineage. This theory of time-integration was developed in response to mismatches in the taxonomic diversity observed in a region (e.g., an island) compared to values predicted from species-area or species-productivity relationships. Time-integration implies that if a region's historical area and productivity were higher than they are today, then its unexpectedly large biodiversity (for its contemporary area and/or productivity) might be explained by historical conditions favoring radiation and a persistence of many or all of these clades as area and/or productivity decreased. To test this theory, I used the bacterium *Pseudomonas fluorescens* SBW25 — a model system for adaptive radiation. I set up independent replicate microcosms that were randomly assigned to different volumes and productivities and transferred every few days so as to experience different environmental histories. By tracking these diversifying communities over time, I demonstrate that time-integrated productivity was the single best predictor of a community's extant diversity whereas "snapshot" measures of contemporary volume and productivity are much less useful predictors. I interpret these results in the context of population growth parameters and extinction rates.

In chapter 2, I present the results of a field study of natural microbial digestive communities occupying leaves of the carnivorous pitcher plant *Darlingtonia californica.* I

combine microscopy, biochemical assays, and community sequencing with respirometry and stable isotope pulse-chase experiments to examine how microbial community succession influences rates of detrital turnover, respiration, and nitrogen cycling in developing micro-ecosystems. I demonstrate that microbial community development and turnover in *D. californica* proceeds in parallel over time with communities becoming more similar to one another. These communities have considerably predictable dynamics such that the bacterial communities from one population can be used to quite accurately predict the ages of pitcher leaves in a different population and year. Furthermore, and in accordance with general successional theory, bacterial communities tended to display unimodal patterns in species diversity over time. This trend appeared driven by differences in the predicted functional properties of bacterial communities. I also encountered unimodal trends in rates of decomposition by the digestive community and nitrogen uptake efficiency by the host leaf. Bacterial diversity and bacterial and midge larvae biomass were positively associated with rates of decomposition, which in turn were positively associated with the efficiency of nitrogen uptake by the host leaf. This study is among the first to demonstrate predictable successional patterns and biodiversity-ecosystem functioning relationships in natural microbial communities.

In chapter 3, I present the results of a laboratory experiment demonstrating a decrease in the strength of biodiversity-ecosystem function (BEF) relationships and competitive interactions during succession in *Darlingtonia californica* leaves. It is often assumed that as ecosystems develop, competition-colonization tradeoffs or niche differences favor the gradual establishment of a biota more successful at competing for resources, leading to increased rates of competitive exclusion and shifting BEF relationships. My approach involved collecting bacterial strains from a cohort of leaves every 11 days over a one-year period and assembling them into communities of varying richness levels such that each community contained either 1, 2, 5, or 10 taxa also isolated from leaves of the same age. By employing an experimental design that allowed for the estimation of individual species' effects as well as their interactions, I show that the relationship between community richness and carbon mineralization rates are most positive during early succession (22-55 days) and gradually decrease over time. Furthermore, diffuse competition was greatest during these same time periods. Together, these results suggest that the effects of species additions or removals on ecosystem processes can vary across time.

Chapter 4 presents an experiment testing a long-held assumption regarding the natural history of *Darlingtonia californica.* Specifically, I test the centuries-old assumption that the unique forked 'fishtail appendage' found on leaves of *D. californica* play an important role in the plant's capture of arthropod prey. In a series of field experiments, I manipulated the presence/absence of the appendage on developing pitcher leaves and compared their prey compositions and biomass. I found that the absence of the fishtail appendage does not significantly impact prey capture success at the level of the individual leaf or within an entire population of leaves. Therefore, contrary to widespread beliefs, the fishtail appendage does not appear to be a critical adaptation enabling carnivory in this species. Instead, I propose three alternative scenarios for the evolutionary maintenance of this structure: 1) as a vestigial structure, 2) as a photosynthetic structure and 3) as a structure serving a potentially mutualistic role with the local insect community.

Dedication

To my mother and father
Mary Elisabeth Snyder, Ph.D.
Richard Hughes Armitage, Ph.D. (1918 – 2007)

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This work could not have been completed without the assistance, input, and patience of numerous colleagues, friends, and family members. First and foremost, I am eternally grateful for the support, mentorship, and friendship provided by my advisor, Wayne Sousa, over the past six years. When Wayne took a chance (that many others avoided) in bringing me to interview for a graduate position, I recall being so immediately struck by the positive, healthy atmosphere of his lab group that I made up my mind to attend within the first hour. In fact, I went so far as to cancel my remaining interviews before I had even been accepted by the department! In my experience, it is rare to find a scientific mentor that practices a healthy work-life balance and is willing to stand up for his or her students with no questions asked. Early on, Wayne keenly suggested that I consider using *Darlingtonia* pitcher plants as a model system in which to conduct my research. I fear this advice may have been the tipping point into a lifelong obsession with the ecology and natural history of carnivorous pitcher plants. It is no surprise that Wayne has mentored so many highly-regarded ecologists over the years, as his advising style, passion for field research, and grasp of the scientific field is certainly worthy of emulation.

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CHAPTER 1 | Experimental evidence for a time-integrated effect of productivity on diversity

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ABSTRACT

The *time-area-productivity* hypothesis is a proposed explanation for global biodiversity gradients. It predicts that a bioregion's modern diversity is the product of its area and productivity, integrated over evolutionary time. I performed the first experimental test of the *time-area-productivity* hypothesis using a model system for adaptive radiation— the bacterium *Pseudomonas fluorescens* SBW25. I initiated hundreds of independent radiations under culture conditions spanning a variety of productivities, spatial extents, and temporal extents. Time-integrated productivity was the single best predictor of extant phenotypic diversity and richness. In contrast, "snapshots" of modern environmental variables at the time of sampling were less useful predictors of diversity patterns. These results were best explained by marked variation in population growth parameters under different productivity treatments and the long periods over which standing diversity could persist in unproductive habitats. These findings provide the first experimental support for time-integrated productivity as a putative driver of regional biodiversity patterns.

INTRODUCTION

The differential distribution of biodiversity across biogeographic regions is simultaneously striking in its apparency and perplexing in its origins (Dobzhansky 1950, Hutchinson 1959, Pianka 1966). A remarkable number of hypotheses have been proposed to explain why such regional differences in diversity exist— many of which place primacy on the strength and fluctuations of physical and biotic processes over evolutionary time (Fischer 1960). In particular, the *time-area-productivity* hypothesis presents a compelling mechanism for explaining global gradients in biodiversity (Mittelbach et al. 2007, Jetz and Fine 2012, Belmaker and Jetz 2015). This hypothesis stresses the simultaneous interacting roles of spatial extent and energy availability integrated over evolutionary time in driving regional diversification. Here, I follow Jetz & Fine's (Jetz and Fine 2012) definition of bioregions as "evolutionary arenas"— geographical regions sharing few species and generally encompassing a single or multiple similar climatic biomes. Time-integration of a bioregion's area and/or productivity allows their historical signatures on diversity dynamics to influence contemporary patterns of species diversity. In other words, time-integration implies that communities equilibrate to a changing environment relatively slowly compared to the timescale over which diversification occurs.

The number of species a bioregion can support is predicted to positively correlate with its spatial extent (Chown and Gaston 2000). This prediction fits with the observation that the larger

tropical bioregions generally contain many more species than smaller, globally-discontinuous temperate regions (Terborgh 1973, Rosenzweig 1995). Originally, the effects of area were attributed to larger bioregions supporting greater population sizes than smaller regions. All else being equal, these larger populations have a greater probability of peripatric speciation (Nuismer et al. 2012) and a decreased probability of extinction due to buffering from catastrophic disturbances (Rosenzweig 1995, Kisel et al. 2011). Larger bioregions are also anticipated to contain more boundaries to gene flow, which should result in the evolution of reproductive isolation (Kisel and Barraclough 2010). These hypotheses have been met with mixed results when observational biodiversity data are fit to models incorporating their bioregional extents (Willig et al. 2003), suggesting that these contemporary "snapshot" measures of area may not be the dominant factors driving global diversity patterns, especially among assemblages containing high levels of endemism.

As with geographic extent, a bioregion's energy supply can set the upper limit on population sizes (Wright 1983, Hurlbert and Jetz 2010). Regions receiving more energy per unit area, such as those in the tropics, can support larger populations of individual species than their temperate counterparts, making them less vulnerable to extinctions and more prone to speciation (Preston 1962, Srivastava and Lawton 1998, Hurlbert 2004). Through its effects on organismal metabolic rates, productivity is predicted to set limits on the total number of diverging populations that an ecosystem can support (Allen et al. 2002). If the assumption of diversity-dependent speciation rates holds true (Rabosky 2013), then this theory provides an avenue for a positive feedback loop between standing and incipient diversity mediated by energy input. Productive environments are also predicted to be more heterogeneous with regard to resource availability and thus may also permit diversification and coexistence via niche partitioning and resource specialization (Abrams 1995). It is widely believed that the fitness advantages of many life history specialists should manifest only above a minimum resource base (Wilbur et al. 1974).

Until now, I have ignored the importance of time as both as a driver of diversity gradients and as a mediator of areal and productivity effects on diversification rates. All else being equal, older bioregions are anticipated to have accumulated more diversity than younger ones (Fischer 1960, Ricklefs and Schluter 1994). This hypothesis is supported by the observation that many extant clades originated in older, tropical bioregions (Mittelbach et al. 2007). Furthermore, because the tempo of diversification occurs on timescales encompassing major climatic and tectonic events, a bioregion's area and productivity are expected to fluctuate to varying degrees which should in turn influence the region's diversification dynamics (Fischer 1960, Terborgh 1973, Ricklefs and Schluter 1994). Opponents of contemporary area-productivity explanations often cite as a counterargument small and/or resource-poor habitat patches containing high biodiversity (McGlone 1996, Fine and Ree 2006). If these patches are relics of once-large and productive habitats, then their contemporary diversities may simply be due to past events promoting diversification and the subsequent long-term maintenance of that diversity in the face of environmental change. By scaling a bioregion's time-integrated area by its average productivity, Fine and Ree (2006) and Jetz and Fine (2012) were able to predict with remarkable accuracy the species richness of their "evolutionary arenas"— a strong argument for the joint roles of historical area, productivity and temporal stability in promoting and maintaining diversity. Crucially, the authors were unable to make similarly accurate predictions using

contemporary "snapshot" measures of area and productivity for trees, endemic vertebrates, and ectotherms – the majority of species included in their analyses.

The environmental stability of a bioregion can theoretically both promote and hinder the generation and maintenance of diversity over evolutionary time. First, greater stability implies more time is available for niche specialization to evolve and selects for continuous, rather than discrete, generations, accelerating rates of recombination (Klopfer 1959, Fischer 1960, Connell and Orias 1964). Likewise, organisms adapted to fluctuating environments are often generalist phenotypes adapted to tolerate ephemeral resources and fluctuating abiotic conditions. Alternatively, environmental fluctuations can lead to negative frequency-dependent population dynamics and create temporal niche opportunities, leading to a greater number of competitors able to coexist (Levins 1979, Petraitis et al. 1989, Chesson and Huntly 1997). These contrasting effects of stability in both promoting and inhibiting coexistence and diversification are not mutually exclusive, and likely depend on spatial and temporal scales.

The bacterium *Pseudomonas fluorescens* SBW25 is a model system for the experimental study of adaptive diversification and coexistence (Rainey and Travisano 1998). Under homogeneous (i.e., shaken) culture conditions, colonies of the strain remain uniform in morphology (called *smooth spreaders*). If cultured under static conditions, however, this ancestral type quickly diversifies into a variety of morphologically distinct niche specialists. These mutants can be categorized into two distinct groups based on colony morphology: the *wrinkly spreaders* and *fuzzy spreaders*, each of which encompasses several unique subtypes including wheel-like, lobate, filamentous, and undulate forms (Fukami et al. 2007). *Wrinkly spreaders* predictably evolve to exploit the air-liquid interface by excreting acetylated cellulose and forming a thick biofilm (Spiers et al. 2003). This niche construction results in a sharp oxygen gradient, paving the way for additional diversification. The genetic basis for these changes is well understood, with the paradox of random beneficial mutations leading to predictable independent radiations explained by large population sizes and rapid growth rates (Spiers et al. 2002). Smaller culture volumes are said to inhibit predictable radiations due to reduced population sizes (Rainey and Travisano 1998). Similarly, the productivity of the growth medium (carbon substrate concentration) also affects the population sizes, growth rates, and relative fitnesses of *P. fluorescens* morphotypes, and therefore can also constrain adaptive radiation (Kassen et al. 2000). Finally, the stability of the culture habitat also influences its diversity dynamics. Intermediate rates of disturbance via imposed population bottlenecks drives diversification rates and resulting community composition in a unimodal pattern, indicating that negative frequency-dependent selection at intermediate productivity and disturbance was equalizing the population sizes (and relative fitnesses) of competing phenotypes (Buckling et al. 2000, Kassen et al. 2004).

Here, I use the *P. fluorescens* SBW25 model system to experimentally test the *time-area-productivity* hypothesis as it relates to the tempo and extent of adaptive diversification. Because the air-water interface habitat is crucial for diversification, I used air-water interface area as a proxy for bioregion extent, though it scales exactly with culture volume in this study. I tested the hypothesis that time-integrated, productivity-scaled area (*TimeAreaProductivity*) is the best predictor of *P. fluorescens* phenotypic diversity owing to the positive effects of area on population size and the positive effects of productivity on both population size and growth rate.

Furthermore, I investigated whether the temporal stability of the culture conditions experienced by each cell line either increased or decreased the extent of *P. fluorescens* diversification. My goal was to compare the utility of these time-integrated variables as predictors of diversity to "snapshots" of the ecosystem taken at the time of sampling. I anticipated time-integrated measures would outperform "snapshot" measures if the time it took diversity to equilibrate to changing abiotic conditions was long relative to the frequency of disturbance. I performed this test in order to experimentally verify the mechanism and relative importance of time-integrated productivity and area as drivers of *P. fluorescens* diversity dynamics.

METHODS

Time-area-productivity experiment

Ancestral *Pseudomonas fluorescens* SBW25 cells were grown in 20 mL KB broth under shaken conditions at 26° C for 2 days (attaining a population density of 3.4 x 10^{10} cells mL^{-1}). Approximately 1 mL of this culture was spun at 10,000 × g for 5 minutes. The supernatant was replaced with sterile phosphate buffer and the pelleted cells re-suspended. The centrifugation and resupply of fresh buffer was repeated three times to wash cells of any residual medium. Cells were then diluted to 10^5 mL^{-1} and starved for 2 hours. I prepared three growth media encompassing a 100-fold difference in nutrient availability— a proxy for productivity. This order of magnitude approximates the variance of primary productivity experienced across all terrestrial biomes (Yuan et al. 2010). Media were prepared by diluting 1x M9-KB broth (NH_4Cl 1 g L^{-1}, Na_2HPO_4 6 g L^{-1}, KH_2PO_4 3 g L^{-1}, NaCl 0.5 g L^{-1}, glycerol 6 g L^{-1}, proteose peptone #3 20 g L^{-1}) 10-fold and 100-fold in M9 salts solution. These three media were aliquoted into flat-bottomed culture vessels spanning two orders of magnitude in volume: 10 mL (6-well plates), 1 mL (48-well plates), and 0.1 mL (96-well plates). This experimental design allowed for growth of bacterial populations in 9 different combinations of habitat volume (0.1, 1, and 10 mL) and resource availability (0.01×, 0.1×, 1×).

Into each habitat I inoculated 50 μL of starved ancestral *P. fluorescens* cells (approximately 1000 isogenic cells). Each of the nine culture conditions were replicated in triplicate and stored at 26° C under static conditions for 24 hours. Aliquots of these cultures were then sampled, briefly vortexed, and stored 20% glycerol at -20° C. From two of the three replicate sets of cultures, I removed, washed, and starved 50 μL, and inoculated them into three different randomly-assigned volume × productivity treatments, for a total of 54 randomized habitats (Fig. S1). These cultures were grown for two days and sampled as previously described. Aliquots were once again taken from 27 of these cultures, washed, and starved before using each one to seed three new randomized habitats for a total of 81 cultures. These were incubated for three days and then sampled and preserved. This schedule forced bacterial populations to remain in or near the exponential phase of growth for the duration of the experiment and did not allow thick biofilms to form and collapse inside of the culture medium, nor allow cultures to deplete the medium and starve. Transfers contained approximately 10^6 cells, which did not represent a significant genetic bottleneck. Dilutions of each 1-, 3-, and 6-day population were made in phosphate buffer and spread on KB agar plates for enumeration. I identified bacterial morphotypes by scoring 100 random colonies per plate. All questionable colonies were re-plated to ensure that they were genetically distinct.

Time-to-equilibrium experiment

I conducted a second experiment to estimate the time scale over which diversity equilibrates to a particular productivity. Washed and starved ancestral bacteria were inoculated into wells containing 1 mL of high, medium, or low-resource broth as previously described. Every three days, a 50 µL aliquot of each culture was used to seed a new well of the same productivity and the rest of the culture was preserved and frozen. After 24 days, 50 µL aliquots from the 8 cultures containing the greatest number of unique morphotypes (all of the high and two of the medium-productivity cultures) were then washed of media, starved, added into 1 mL low-productivity wells, and serially-transferred and preserved every three days for an additional 21 days. Samples from the entire 45-day time series were then plated and scored for morphotype richness and diversity.

Growth curve measurement

I measured growth parameters of *P. fluorescens* SBW25 growing in 100 mL batch cultures in the three productivity treatments. Population growth was approximated as the optical density at 600 nm (OD_{600}) on a Molecular Devices Emax spectrophotometer. I fit Gompertz growth functions to these time series and estimated the 95% confidence intervals for the parameters µ (growth rate), A (carrying capacity), and λ (lag time) using nonlinear least squares. The growth function took the form

$$y = A\exp\left\{-\exp\left[\frac{\mu_m \cdot e}{A}(\lambda - t) + 1\right]\right\} \quad (1)$$

where t is the culture age (hours), e is base of the natural logarithm, and y is the change in OD_{600} values from t_0. Once each culture reached stationary phase, I estimated its population density by plating dilutions of the culture onto solid medium and counting the colonies.

Data collection and statistical analyses

I estimated diversity as previously described (Kassen et al. 2004). Alongside morphotype richness, I estimated the complement (1-λ) of Simpson's index, λ, where $\lambda = \Sigma p_i^2$ and p_i is the frequency of each morphotype in the 100 censused colonies. This metric takes a value between 0 and 1 and represents the likelihood that any two randomly selected colonies belong to different morphotypes. This index was logit-transformed to fulfill the assumptions of linear regression. Time-integrated area (*TimeArea*) and productivity (*TimeProductivity*) were measured by integrating the total areas and productivities experienced by each cell line over their 1-, 3-, or 6-day evolutionary histories. Similarly, *TimeAreaProductivity* is the time-integrated product of culture area and productivity (Jetz & Fine 2012) (Fig. S1). I estimated the stability of *TimeArea*, *TimeProductivity*, and *TimeAreaProductivity* using the coefficient of variation (*CV* = standard deviation/mean). I fit a series of generalized linear (glm) and generalized additive models (gam) to the hypothesized drivers of diversity. I used the slope parameters of glm fits to interpret the magnitude and direction of covariance, while gam fits were used to estimate the shape of the response surface and estimate the proportion of variance explained. Additive models were constrained to a maximum of five basis dimensions to avoid overfitting while permitting quadratic and logistic-like response surfaces. The response variables were richness (modeled as

Poisson-distributed) and logit-transformed diversity (modeled as Gaussian-distributed). Best-fit models were selected using AIC_c and coefficients of determination (R^2). To avoid pseudoreplication, I only analyzed endpoint-communities— those that were not used to seed new microcosms at 1- and 3-day transfers.

To estimate whether equilibrium diversities were reached in the productivity treatments and whether they differed, I fit a series of generalized additive models to the 24-day time series begun with the isogenic ancestral cells. I visually compared the shapes of each productivity treatment's curves and assessed whether the 95% confidence intervals surrounding each estimated mean differed between productivities. I compared these curves to intercept-only null models using likelihood ratio tests and R^2 values. I then performed the same set of analyses on the high-diversity, low-productivity treatments started at day 24. I anticipated that these cultures' diversities would decrease over time, as less fit morphotypes are driven to rarity or extinction and that their diversities would eventually equal those of low-productivity cultures started with ancestral cells. All statistical operations were performed using R (R Development Core Team 2015) and code is available from the author on request.

RESULTS

Time-productivity drives diversity dynamics

A total of 116 independent microcosms were scored for diversity. In total, I identified eight distinct, heritable colony morphotypes, though no single culture contained more than four and all but three were variants of the *wrinkly spreader* phenotype (Fukami et al. 2007) (Fig. S2). In only one case was a morphotype observed to go extinct. I confirmed a relatively weak relationship between *TimeAreaProductivity* and diversity, but not for morphotype richness (Table 1). However, the covariate *TimeProductivity* was the strongest single predictor for both diversity and richness (Fig. 1). Furthermore, linear models incorporating time-integrated productivity explained approximately 33% (diversity) and 26% (richness) more variance than did models using only "snapshot" productivity measured at the time of sampling (Table 1). Neither contemporary "snapshots" of area nor *TimeArea* were found to be associated with diversity or richness. As anticipated, culture age *per se* (*Time*) was also a good predictor of culture diversity. The stability of area over time (CV_{area}) was positively associated with diversity and richness. Inclusion of CV_{area} into the *TimeProductivity* linear and nonlinear models delivered the best predictive accuracies (Fig. 2). For logit-transformed diversity and richness, this increased the percentage variance explained by the best-fit gam models to 72% and 60%, respectively.

Growth rates vary by productivity

By analyzing the strain's growth kinetics under varying nutrient concentrations I determined that differences exist in the strain's growth rates (μ) and carrying capacities (A) among productivity treatments (Table 2). Specifically, diluting the standard KB+M9 medium by 100 has the effect of decreasing the bacterium's growth rate and carrying capacity approximately tenfold (Fig. 3). At steady state, the cultures had estimated population densities of 2.3×10^9, 1.8×10^9, and 2.8×10^8 cells mL^{-1} for the high, medium, and low productivity treatments, respectively.

Historical signatures of productivity are slow to disappear

Diversity and richness in all cultures initially increased over a period of approximately 6-9 days (Fig. 4 & Fig. S3). In high and medium cultures, these values initially increased and then appeared to remain unchanged after 9 days. Diversity in low productivity cultures steadily increased throughout the 24-day period and ended lower than those in high and medium productivities. Average morphotype richnesses for high (5 morphotypes) and medium (4 morphotypes) productivities were both greater than morphotype richness in low-productivity cultures (2 morphotypes) (Fig. 4). For cultures started with ancestral *smooth* cells, *AICc* and R^2 metrics favored time-variant models of diversity over their intercept-only counterparts (Table S1). Morphotype-rich cultures moved into low-productivity media did not decrease in diversity nor richness, and time-dependent models were not improvements over intercept-only null models (Table S1). The expected values from these intercept-only models were within bounds of the endpoint mean diversity and richness values from the high and medium productivity cultures started with the ancestral strain but greater than the mean endpoints of the low-productivity cultures (Fig. 4).

DISCUSSION

Using a model microbial adaptive radiation, I have shown that time-integrated productivity is a primary driver of diversification dynamics in the *P. fluorescens* model system. These data represent the first experimental test of the *time-area-productivity* hypothesis and are consistent with results obtained observational studies (Jetz and Fine 2012, Belmaker and Jetz 2015). Contrary to their findings, however, I failed to identify spatial extent as an important predictor of diversification in this system. A reason for this discrepancy may be that the previous studies were unable to explicitly model *TimeProductivity* separately from *TimeArea* due to a lack of pre-Holocene productivity data. Instead, the authors scaled *TimeArea* by the bioregion's modern productivity. Therefore, this study represents the first to independently test for *TimeProductivity* and *TimeArea* effects. It remains to be determined whether time-integrated productivity is able to explain more variation than time-integrated area in global datasets.

The differences in diversification observed among experimental cultures stemmed from the maximization of population carrying capacities and growth rates during periods of high productivity. Because cultures were maintained at or near exponential-phase growth during the experiment, the positive effects of productivity on growth rates allowed for beneficial mutants to accumulate more rapidly and persist in more productive environments. Contrary to anecdotal evidence (Rainey and Travisano 1998) neither culture volume (i.e., *area*) nor its time-integrated measure was positively associated with the extent of *Pseudomonas* diversification. This occurred despite a two order-of-magnitude difference in population sizes between 10 mL and 0.1 mL cultures. Thus, for *P. fluorescens* SBW25, time-integrated air-water interface area (or culture volume) cannot be considered an important driver of diversification in the presence of other environmental variables affecting population growth.

One explanation for why productivity, rather than area, drove diversification in this experiment is the competitive suppression of *de novo* niche specialists at low nutrient concentrations. Similar to Kassen et al. (2004), even small (0.1 mL), low-productivity cultures at

48 hours contained populations of *P. fluorescens* large enough to produce *wrinkly spreader* mutants. However, the relative fitnesses of these morphotypes rely on a nutrient supply large enough to allow coordinated expression of cellulosic polymer (Spiers et al. 2003, Kassen et al. 2004). Lacking a surplus of growth substrate, wrinkly spreader populations cannot exploit the air-water interface niche and are either driven extinct or maintained at low-frequencies via competition with the ancestral *smooth* morphotype (Kassen et al. 2004). At intermediate and high productivities, all culture sizes contained nutrient concentrations necessary for *wrinkly spreaders* to invade, despite the pleiotropic fitness costs to biofilm production via decreased carbon metabolism (MacLean et al. 2004). In contrast, populations of macro-organisms are often constrained by geographic barriers to sizes much smaller than those observed in this experiment (Preston 1962). Such populations should not diversify, particularly if they are simultaneously being constrained by a low-productivity environment and/or competition (Wright 1983, Rosenzweig and Abramsky 1993). Additionally, larger bioregions are anticipated to contain more barriers to gene flow, which can promote non-adaptive diversification (Terborgh 1973, Gittenberger 1991, Rosenzweig 1995). Such allopatric diversification is not permitted in the relatively homogeneous environment of microbial culture vessels due to their lack of geographic boundaries. However, it is worth noting that no single culture contained all eight morphotypes, and a summation of morphotypes in 10 isolated 1 mL (or < 100 isolated 0.1 mL) wells was unanimously greater than or equal to the diversity of any single 10 mL well, given similar productivities. This finding lends support to the role of geographic barriers in promoting regional diversity through the ecological saturation of convergent morphotypes (Terborgh and Faaborg 1980, Ricklefs 2004). The large effective population sizes (10^6 cells) and overall magnitude and replicability of diversification indicate that divergence via neutral drift is not the cause of this pattern, and it is instead due to selection. In a culture without geographically-isolated populations and limited resources over which to compete, clonal interference could limit the number of coexisting niche specialists (Gerrish and Lenski 1998).

My results are similar to those of Kassen et al. (2000; 2004) in demonstrating inhibition of *P. fluorescens* diversification in low productivity habitats. Unlike these previous studies, however, I did not encounter a negative-quadratic shape to the productivity – diversity relationship. Instead, diversity and richness appeared to asymptote at high time-integrated productivities. This difference may be because Kassen et al.'s 'high productivity' cultures contained nutrient concentrations far greater than the standard 1× King's medium formulation. Furthermore, the extent of *P. fluorescens* diversification tends to follow a sigmoidal trend over the time periods used in this experiment— a pattern observed elsewhere (Fukami et al. 2007). Because two sources of environmental variation are incorporated into the *TimeProductivity* variable, it is probable that the saturating dynamics I observed represent a combination of constraints on diversification imposed by culture age (sigmoidal) and productivity (negative-quadratic). However, equivalent values of *TimeProductivity* can be achieved in a variety of ways. For instance, an intermediate-productivity culture running for three days can equal a high-productivity culture at one day, or a low-productivity culture at six days. Productivity, however, is but one constraint on the diversification dynamics of *P. fluorescens* SBW25. A number of other factors, most notably habitat heterogeneity, temperature, disturbance, and community interactions (e.g., predation and competition) also mediate this model radiation (Rainey and Travisano 1998, Kassen et al. 2004, Fukami et al. 2007, Meyer and Kassen 2007).

I detected an effect of culture area stability on morphotype diversity and richness, but I did not anticipate the association to be positive in direction. Moreover, inclusion of this covariate in my models only resulted in an improvement of 5% variance explained. Models of species accumulation over time often cite the importance of climate-driven habitat stability in driving niche specialization and speciation (Klopfer 1959, Fischer 1960, Connell and Orias 1964). My finding that morphotype richness and diversity *decreased* with environmental stability does not support the prediction that environmental stability *per se* promotes diversification. Rather, this observation suggests that disturbance in terms of periodically imposed population bottlenecks promotes the maintenance of incipient niche specialists. However, these bottlenecks were not especially strong, since approximately 10^6 cells were used to seed new cultures and transfers were made at time intervals over which both community interactions and adaptive diversification can influence diversity dynamics. Buckling et al. (2000) obtained a similar result and concluded that intermediate levels of disturbance acting on diversifying *P. fluorescens* cultures allowed rare genotypes the opportunity to invade an otherwise resistant community. Using the same system, Tan et al. ((Tan et al. 2013) found evidence that temporal variation in niche availability promoted coexistence among morphotypes. However, the positive covariance of CV_{area} with both *Time* and *transfer frequency* hinders the interpretation of this effect in my study. Nonetheless, whichever stability metric was used in my models, the qualitative result was consistent: a negative trend exists between diversity and environmental stability in the model *P. fluorescens* radiation.

Although I only detected a single extinction event during my experiment, it is probable that many undetected *de novo* morphotypes arose in all cultures and were rapidly driven extinct by established competitors. In both experiments, neither diversity nor richness decreased when diverse, high-productivity assemblages were transplanted into low-productivity media. In other words, niche specialist phenotypes unable to establish in low-productivity cultures could persist at their historical relative frequencies despite the reduced habitat quality. Extinction debt is a hypothesis used to explain the maintenance of hyper-diverse assemblages in deteriorating or shrinking habitats (Tilman et al. 1994). In both this experiment and many observational studies present-day area and productivity were poor predictors of regional variance in diversity (Fine and Ree 2006, Jetz and Fine 2012). Rather, *time-integrated* area and productivity were much better explanatory variables. It is noteworthy, however, that Jetz & Fine (Jetz and Fine 2012) found evidence for the primacy of non-time-integrated "snapshot" measures in explaining the richness of non-endemic bird and mammal assemblages. This suggests that time-integrated measures may be less useful when applied to assemblages capable of crossing bioregional boundaries (e.g., via migration). Further work is needed to determine whether biota better modeled with time-integrated variables share certain key traits predisposing them to endemicity.

The primacy of snapshot versus time-integrated variables in explaining regional diversity patterns depends in part on the relative rates at which these systems equilibrate following a disturbance. Historical signatures of area and productivity should manifest if communities return to equilibrium slowly, but should be erased if communities either do not reach a steady state or return to it very quickly. My finding that high-richness cultures placed into low-productivity environments do not lose their derived morphotypes confirms the importance of the cultures' historical conditions in explaining contemporary diversity patterns. The time it took for high, medium, and low-productivity cultures to reach initial equilibria was on the order of one week,

whereas once the communities had developed, they were capable of remaining unchanged for 3 weeks, despite being moved into low-productivity media. While this timeframe is partly a product of the study system's large population sizes, rapid generation times, and relative simplicity, it nonetheless verifies that given certain conditions, time-integrated effects on community structure can persist over evolutionary timescales. Fitness differences that precluded *de novo* morphotypes from establishing in unproductive medium did not appear to affect their long-term persistence if they had originally diversified in a more productive habitat. In other words, the trajectory of diversity in a culture experiencing increasing productivity was not along the same response surface as a culture experiencing a decrease in productivity. Therefore, it is likely that the direction of environmental change can influence the length of time over which time-integrated effects can persist— environments increasing in productivity can be invaded by *de novo* mutants whereas deteriorating environments may prevent such invasions via their suppression by established competitors.

In concert, these results suggest that time-energy effects manifest most strongly during early stages of diversification and persist longest in deteriorating environments. Once niches are sufficiently saturated with *de novo* phenotypes, competitive exclusion of similar low-frequency phenotypes sets the upper limit on richness. In the simple physicochemical habitats used in my experiments, niche saturation occurred relatively quickly and persisted indefinitely. Time-productivity effects may not have been as pronounced had cultures been allowed to remain at equilibria for a longer stretch of time prior to model fitting. However, doing so would have forced the system away from modeling natural diversity dynamics. There is currently limited consensus on whether a strict asymptotic diversification model holds for natural systems, primarily due to the fact that key evolutionary innovations and mass extinctions tend to keep diversification rates from reaching prolonged steady-states (Rabosky 2013). Whether or not the observation of hyper-diverse biotas in sub-optimal habitats or refugia represents extinction debt or evolutionary acclimation requires further investigation, though it is clear from these data that if extinction debt responsible for this observation, it is occurring over a period at least three times as long as the time required for diversity to first appear. Answering this question requires long-term experiments and new methods to identify and characterize novel phenotypes.

In conclusion, high historical energy availability drove the evolution of niche specialists, which were unable to successfully establish in resource-poor environments. Low-productivity cultures inoculated with diverse assemblages from high-productivity habitats, however, did not experience extinctions, implying that a habitat's standing diversity can be decoupled from its contemporary environmental conditions. These results confirm that modern-day "snapshot" ecosystem metrics are at best proxies for explaining regional variation in extant diversity, particularly among endemic species. At worst, these variables can mislead analyses on drivers of biodiversity. Further, these results extend the domain of time-integration hypotheses to bacteria— organisms rarely considered bound by historical biogeographic constraints (Hanson et al. 2012). Going forward, these findings support the need for more historical data on both area and productivity to explain patterns of biodiversity at large spatial and temporal scales.

Table 1. Relative performances of time-integrated, snapshot, and additional covariates in predicting morphotype richness and diversity. Best models ($\Delta AIC_c \leq 2$) are bolded and R^2 represents the shrinkage-adjusted coefficient of determination of observed versus model-predicted values.

Predictor Variables	**Richness**				**Diversity**			
	GLM models		GAM models		GLM models		GAM models	
	ΔAIC_c	R^2	ΔAIC_c	R^2	ΔAIC_c	R^2	ΔAIC_c	R^2
Time	17	0.21	20	0.18	93	0.21	114	0.21
Area	28	0.01	31	0.00	120	0.00	141	0.00
Productivity	13	0.28	15	0.30	69	0.36	89	0.36
AreaProductivity	26	0.05	28	0.08	105	0.12	121	0.17
TimeArea	29	0.00	32	0.00	120	0.00	141	0.00
TimeProductivity	**0**	**0.49**	**0**	**0.56**	5	0.63	6	0.69
TimeAreaProductivity	25	0.06	28	0.06	107	0.11	126	0.06
$C.V._{area}$	22	0.12	25	0.10	108	0.10	129	0.10
$C.V._{productivity}$	28	0.02	28	0.10	116	0.04	123	0.10
$C.V._{area} + C.V._{productivity}$	24	0.12	26	0.16	109	0.11	118	0.16
TimeProductivity + $C.V._{area}$	**0**	**0.54**	**0**	**0.59**	**0**	**0.65**	**0**	**0.72**
TimeAreaProductivity + $C.V._{area}$	21	0.18	24	0.16	95	0.22	115	0.16
Intercept-only null	27	0.00	30	0.00	118	0.00	139	0.00

Table 2. 95% confidence intervals for Gompertz growth model under varying productivities. Parameter μ is growth rate (absorbance units hour^{-1}), A is carrying capacity (absorbance units), and λ is time lag (hours).

	Parameter estimate (95% C.I.)		
Productivity	μ	A	λ
1×	0.037 - 0.055	1.054 - 1.225	16.5 - 19.8
0.1×	0.025 - 0.030	0.830 - 0.927	13.8 - 16.0
0.01×	0.004 - 0.006	0.112 - 0.132	4.5 - 10.3

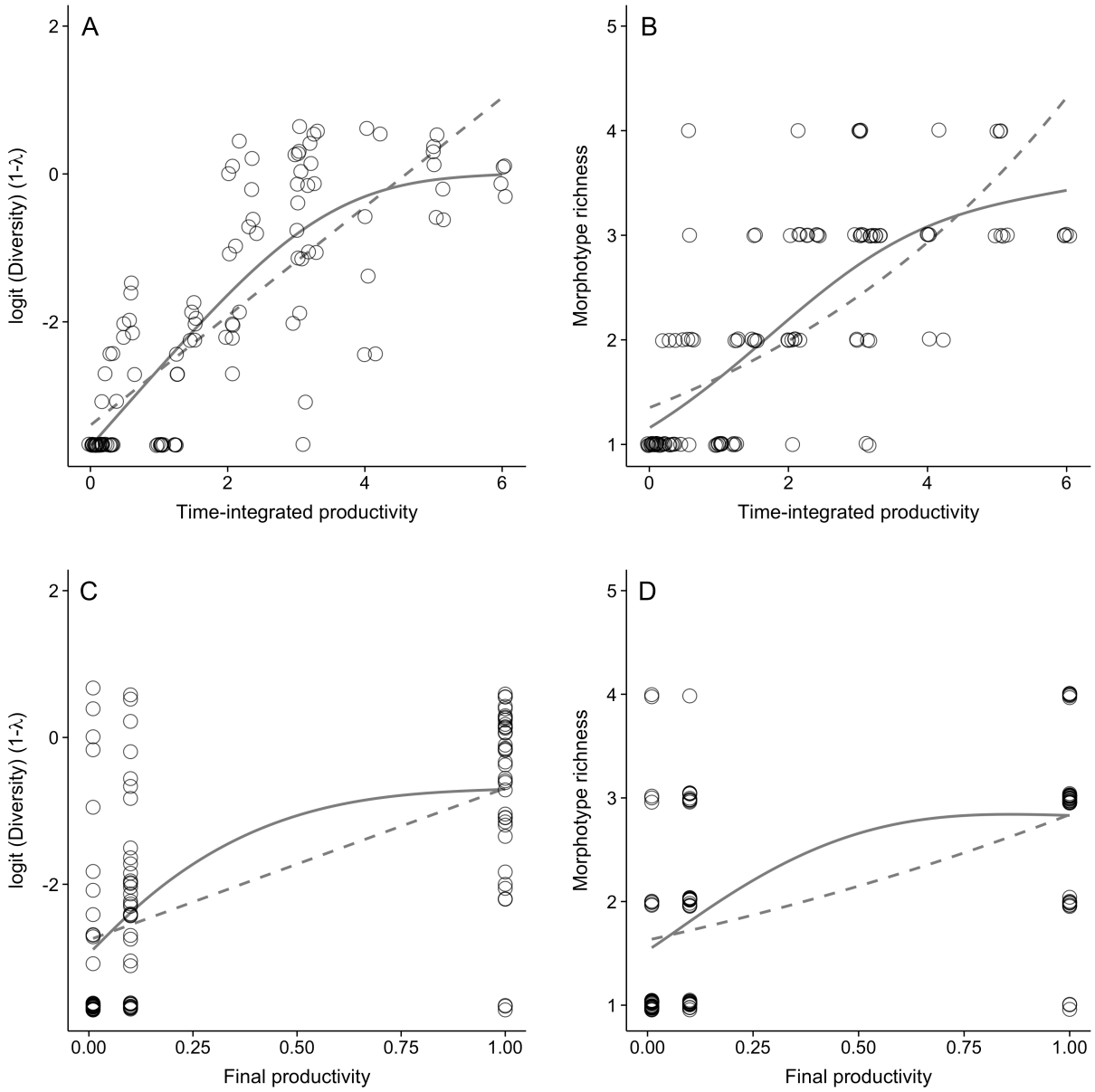

Figure 1. Extent of *P. fluorescens* SBW25 morphotype diversity and richness plotted against time-integrated productivity (A & B) and "snapshot" productivity (C & D). Solid lines denote fitted generalized additive models, dotted lines denote linear models.

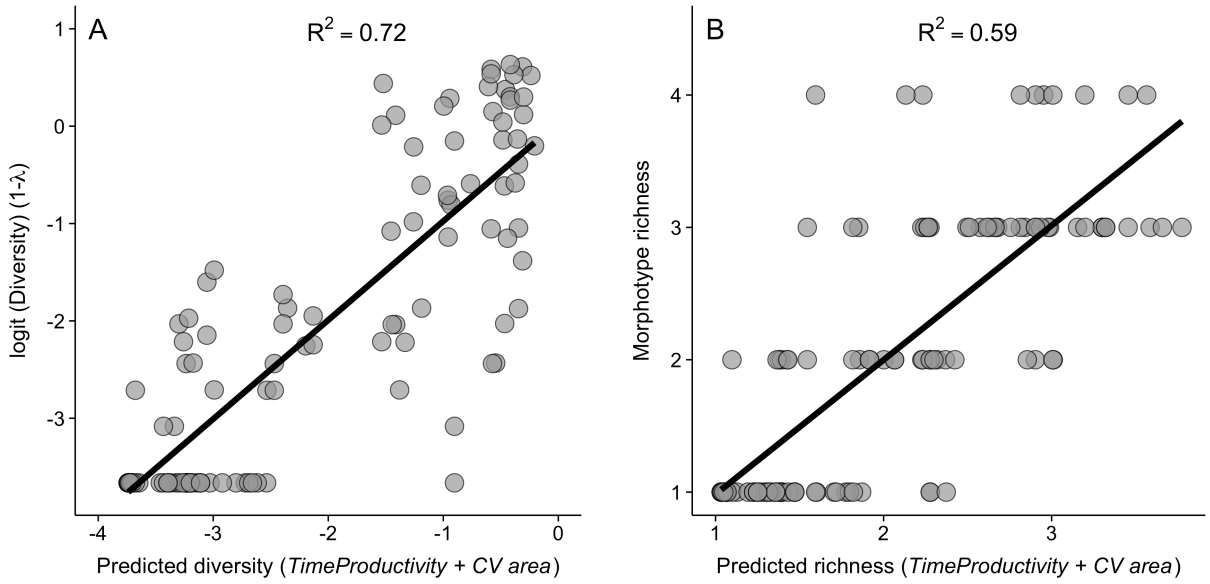

Figure 2. Predicted versus observed values for best fit GAM models for A) logit-transformed morphotype diversity and B) morphotype richness.

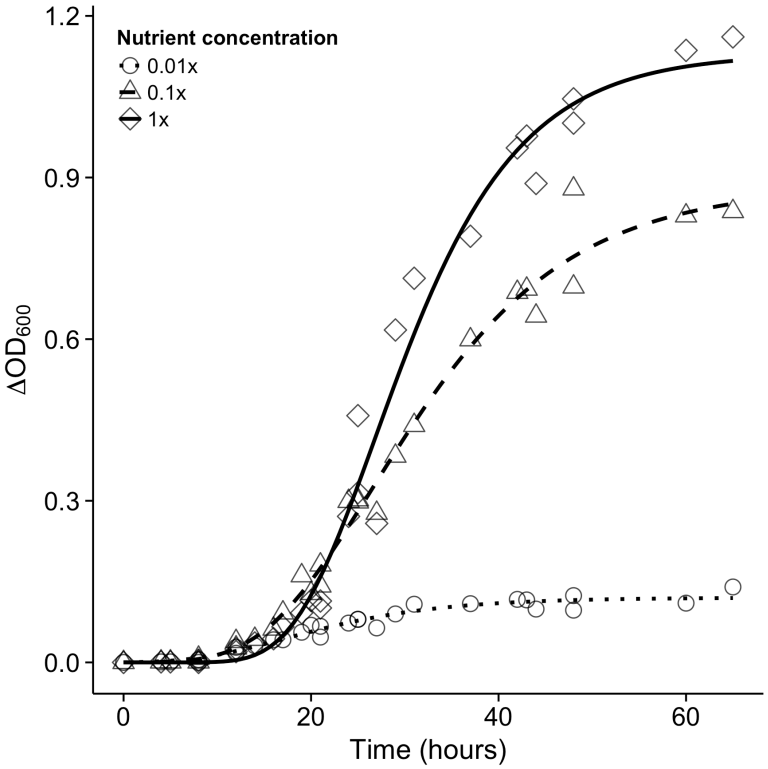

Figure 3. Growth kinetics of *P. fluorescens* SBW25 under the three productivity treatments. Lines denote Gompertz growth curve fits.

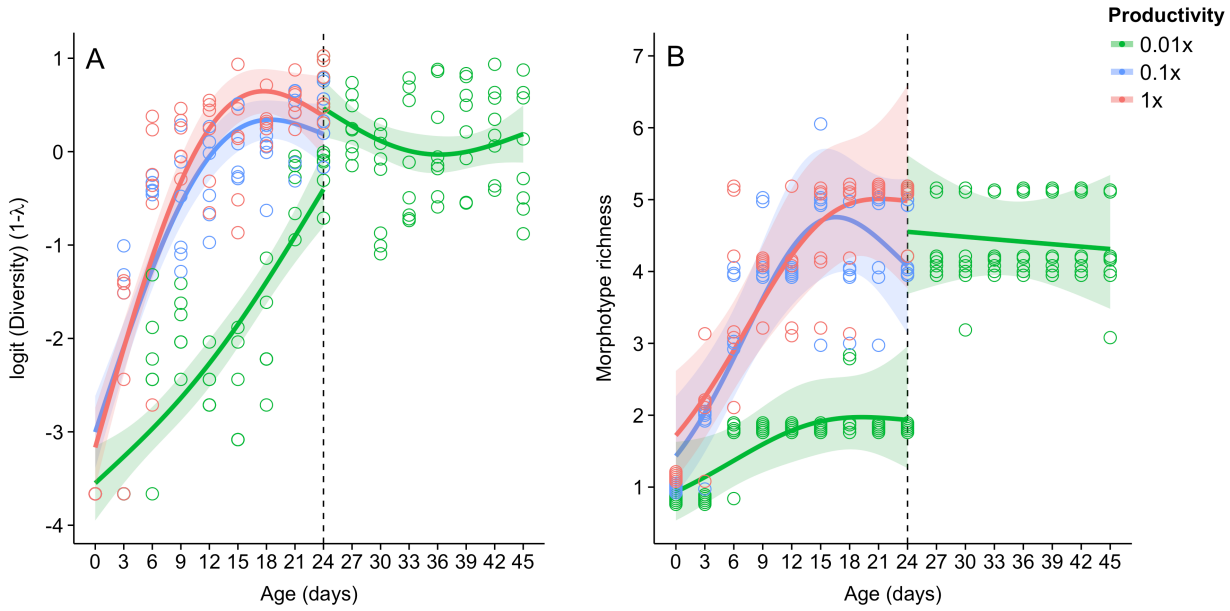

Figure 4. A) Diversity and B) richness dynamics for cultures in high (red), medium (blue), and low (green) productivity media. Dotted line denotes day at which the 8 highest richness cultures were moved into low-productivity environments. Solid lines indicate best-fitting GAM models. Shaded regions denote 95% confidence regions around predicted means.

Table S1. Likelihood ratio test statistics and pseudo-R^2 values for GAM models of diversity and richness in the equilibrium experiment. *Low, medium,* and *high* productivities represent cultures seeded with the ancestral SBW25 strain and grown for 24 days. *Low w/ high richness* category represents the high-richness cultures placed in low-productivity media at day 24.

Productivity	**Predictors**	**Richness**			**logit (Diversity)**		
		χ^2 (d.f.)	*p*-value	R^2	χ^2 (d.f.)	*p*-value	R^2
Low	~ *Age (days)*	-5.3 (-1.5)	< 0.05	0.65	-56.9 (-1.7)	< 0.001	0.70
	~ *1* (intercept only)			0.00			0.00
Medium	~ *Age (days)*	-23.6 (-1.9)	< 0.001	0.72	-67.6 (-2.0)	< 0.001	0.77
	~ *1* (intercept only)			0.00			0.00
High	~ *Age (days)*	-22.8 (-1.8)	< 0.001	0.69	-84.6 (-2.0)	< 0.001	0.80
	~ *1* (intercept only)			0.00			0.00
Low w/ high	~ *Age (days)*	-0.09 (-1.0)	0.77	0.01	-0.52 (-1)	0.21	0.01
richness	~ *1* (intercept only)			0.00			0.00

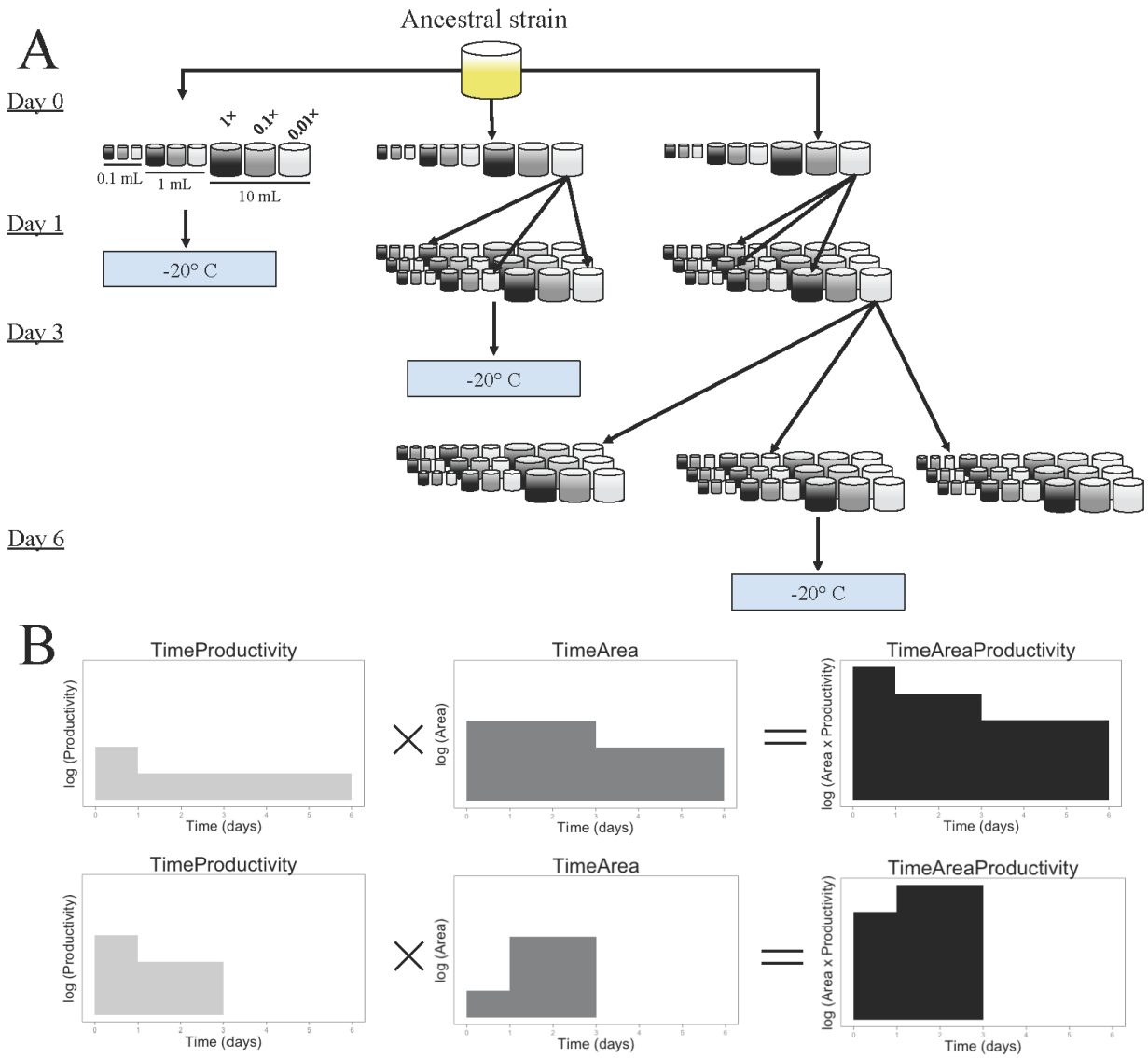

Figure S1. A) Experimental procedure. Ancestral cells underwent periods of growth in area × productivity treatments. After the growth period, they were either sampled and preserved or seeded into new randomized treatments for further growth (denoted by arrows). B) Two examples of how *TimeProductivity, TimeArea*, and *TimeAreaProductivity* are measured and calculated.

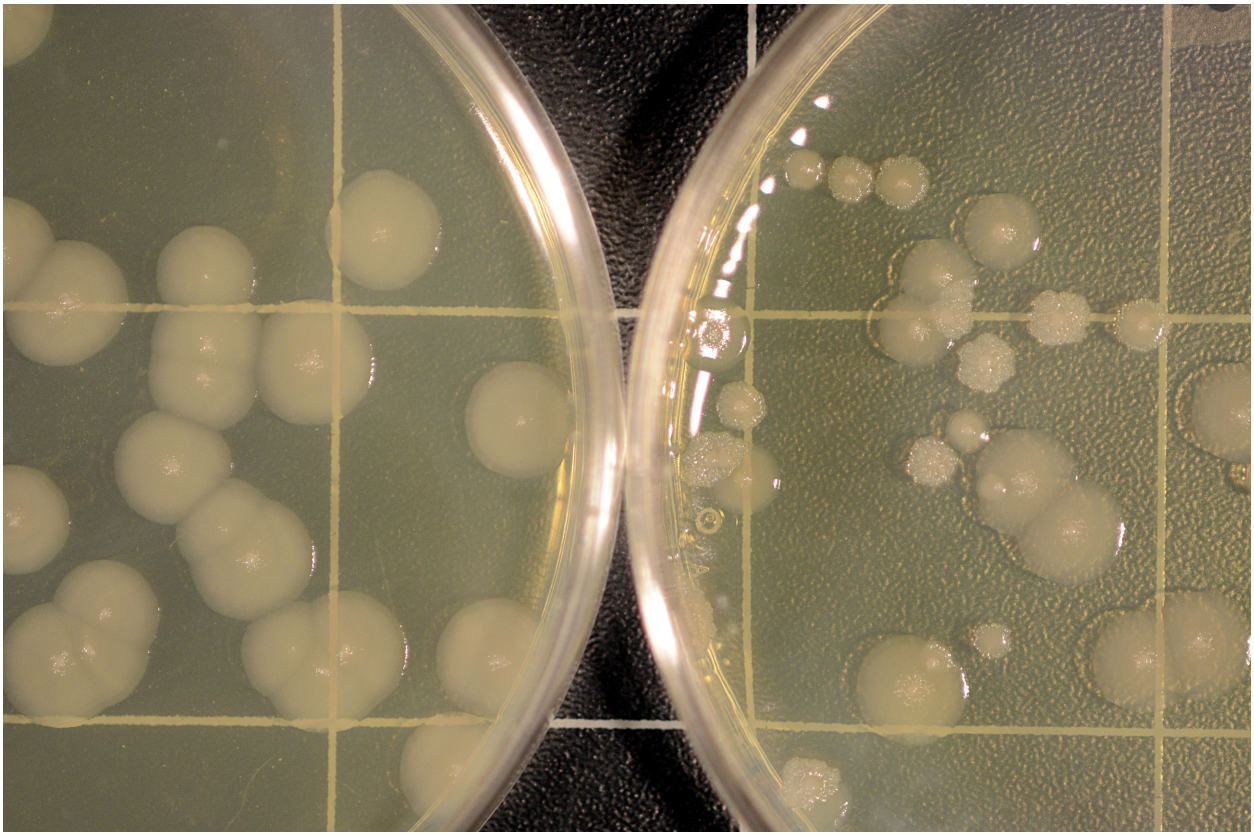

Figure S2. Photograph of ancestral (left) and derived (right) colony morphotypes growing on KB agar. The ancestral plate contains isomorphic colonies while the plate on the right contains three different morphotypes including two *wrinkly spreaders* and the *smooth* ancestral form.

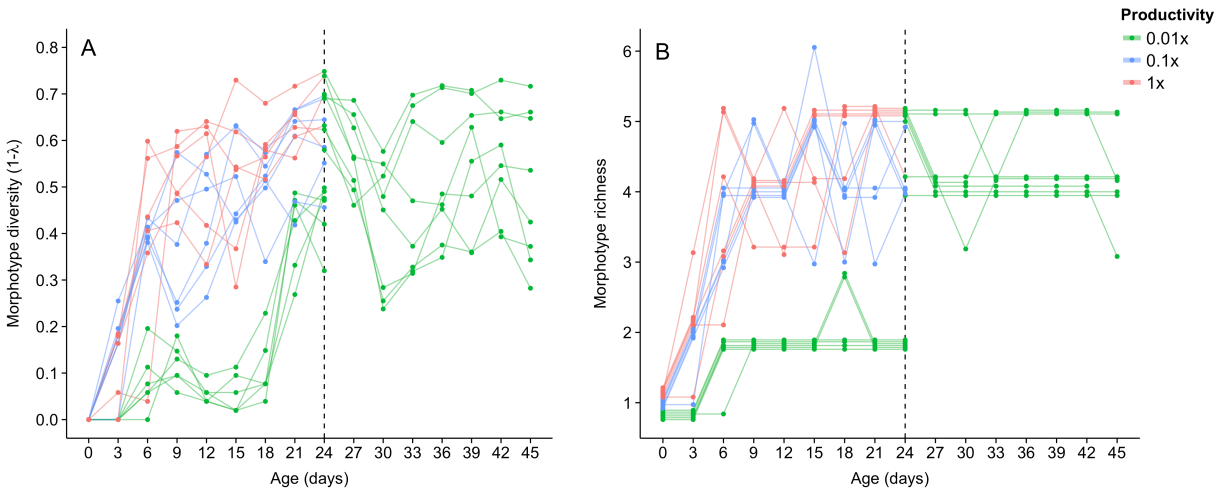

Figure S3. Raw A) diversity and B) richness dynamics over time for cultures in different productivity treatments. Dashed line at t = 24 days denotes time at which 8 highest-diversity treatments were added to low-productivity wells.

LITERATURE CITED

Abrams, P. A. 1995. Monotonic or unimodal diversity-productivity gradients: what does competition theory predict? Ecology 76:2019–2027.

Allen, A. P., J. H. Brown, and J. F. Gillooly. 2002. Global biodiversity, biochemical kinetics, and the energetic-equivalence rule. Science 297:1545–1548.

Belmaker, J., and W. Jetz. 2015. Relative roles of ecological and energetic constraints, diversification rates and region history on global species richness gradients. Ecology Letters 18:563–571.

Buckling, A., R. Kassen, G. Bell, and P. B. Rainey. 2000. Disturbance and diversity in experimental microcosms. Nature 408:961–964.

Chesson, P., and N. Huntly. 1997. The roles of harsh and fluctuating conditions in the dynamics of ecological communities. The American Naturalist 150:519–553.

Chown, S. L., and K. J. Gaston. 2000. Areas, cradles and museums: the latitudinal gradient in species richness. Trends in Ecology & Evolution 15:311–315.

Connell, J. H., and E. Orias. 1964. The ecological regulation of species diversity. The American Naturalist 98:399–414.

Dobzhansky, T. 1950. Evolution in the tropics. American Scientist 38:209–221.

Fine, P. V. A., and R. H. Ree. 2006. Evidence for a time-integrated species-area effect on the latitudinal gradient in tree diversity. The American Naturalist 168:796–804.

Fischer, A. G. 1960. Latitudinal variations in organic diversity. Evolution 14:64–81.

Fukami, T., H. J. E. Beaumont, X.-X. Zhang, and P. B. Rainey. 2007. Immigration history controls diversification in experimental adaptive radiation. Nature 446:436–439.

Gerrish, P. J., and R. E. Lenski. 1998. The fate of competing beneficial mutations in an asexual population. Genetica 102-103:127–144.

Gittenberger, E. 1991. What about non-adaptive radiation? Biological Journal of the Linnean Society 43:263–272.

Hanson, C. A., J. A. Fuhrman, M. C. Horner-Devine, and J. B. H. Martiny. 2012. Beyond biogeographic patterns: processes shaping the microbial landscape. Nature Reviews Microbiology 10:497–506.

Hurlbert, A. H. 2004. Species–energy relationships and habitat complexity in bird communities. Ecology Letters 7:714–720.

Hurlbert, A. H., and W. Jetz. 2010. More than "more individuals": the nonequivalence of area and energy in the scaling of species richness. The American Naturalist 176:E50–E65.

Hutchinson, G. E. 1959. Homage to Santa Rosalia or why are there so many kinds of animals? The American Naturalist 93:145–159.

Jetz, W., and P. V. A. Fine. 2012. Global gradients in vertebrate diversity predicted by historical area-productivity dynamics and contemporary environment. PLoS Biol 10:e1001292.

Juniper, B. B. E., R. J. Robins, and D. M. Joel. 1989. The Carnivorous Plants. Academic Press, London, UK; San Diego, CA.

Kassen, R., A. Buckling, G. Bell, and P. B. Rainey. 2000. Diversity peaks at intermediate productivity in a laboratory microcosm. Nature 406:508–512.

Kassen, R., M. Llewellyn, and P. B. Rainey. 2004. Ecological constraints on diversification in a model adaptive radiation. Nature 431:984–988.

Kisel, Y., and T. G. Barraclough. 2010. Speciation has a spatial scale that depends on levels of gene flow. The American Naturalist 175:316–334.

Kisel, Y., L. McInnes, N. H. Toomey, and C. D. L. Orme. 2011. How diversification rates and diversity limits combine to create large-scale species–area relationships. Philosophical Transactions of the Royal Society of London B: Biological Sciences 366:2514–2525.

Klopfer, P. H. 1959. Environmental determinants of faunal diversity. The American Naturalist 93:337–342.

Levins, R. 1979. Coexistence in a variable environment. The American Naturalist 114:765–783.

Lloyd, F. E. 1942. The Carnivorous Plants. Chronica Botanica. Ronald Press, New York, NY.

MacLean, R. C., G. Bell, and P. B. Rainey. 2004. The evolution of a pleiotropic fitness tradeoff in *Pseudomonas fluorescens*. Proceedings of the National Academy of Sciences of the United States of America 101:8072–8077.

McGlone, M. S. 1996. When history matters: scale, time, climate and tree diversity. Global Ecology and Biogeography Letters 5:309–314.

Meyer, J. R., and R. Kassen. 2007. The effects of competition and predation on diversification in a model adaptive radiation. Nature 446:432–435.

Mittelbach, G. G., D. W. Schemske, H. V. Cornell, A. P. Allen, J. M. Brown, M. B. Bush, S. P. Harrison, A. H. Hurlbert, N. Knowlton, H. A. Lessios, C. M. McCain, A. R. McCune, L. A. McDade, M. A. McPeek, T. J. Near, T. D. Price, R. E. Ricklefs, K. Roy, D. F. Sax, D. Schluter, J. M. Sobel, and M. Turelli. 2007. Evolution and the latitudinal diversity gradient: speciation, extinction and biogeography. Ecology Letters 10:315–331.

Nuismer, S. L., A. MacPherson, and E. B. Rosenblum. 2012. Crossing the threshold: gene flow, dominance and the critical level of standing genetic variation required for adaptation to novel environments. Journal of Evolutionary Biology 25:2665–2671.

Petraitis, P. S., R. E. Latham, and R. A. Niesenbaum. 1989. The maintenance of species diversity by disturbance. The Quarterly Review of Biology 64:393–418.

Pianka, E. R. 1966. Latitudinal gradients in species diversity: a review of concepts. The American Naturalist 100:33–46.

Preston, F. W. 1962. The canonical distribution of commonness and rarity: Part I. Ecology 43:185–215.

Rabosky, D. L. 2013. Diversity-dependence, ecological speciation, and the role of competition in macroevolution. Annual Review of Ecology, Evolution, and Systematics 44:481–502.

Rainey, P. B., and M. Travisano. 1998. Adaptive radiation in a heterogeneous environment.

Nature 394:69–72.

R Development Core Team. 2015. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.

Ricklefs, R. E. 2004. A comprehensive framework for global patterns in biodiversity. Ecology Letters 7:1–15.

Ricklefs, R. E., and D. Schluter, editors. 1994. Species Diversity in Ecological Communities. 1 edition. University Of Chicago Press, Chicago.

Rosenzweig, M. L. 1995. Species Diversity in Space and Time. Cambridge University Press, Cambridge, UK.

Rosenzweig, M. L., and Z. Abramsky. 1993. How are diversity and productivity related? Pages 52–65 *in* R. E. Ricklefs and D. Schluter, editors. Species diversity in ecological communities: historical and geographical perspectives. University of Chicago Press, Chicago.

Spiers, A. J., J. Bohannon, S. M. Gehrig, and P. B. Rainey. 2003. Biofilm formation at the air–liquid interface by the *pseudomonas fluorescens* SBW25 wrinkly spreader requires an acetylated form of cellulose. Molecular Microbiology 50:15–27.

Spiers, A. J., S. G. Kahn, J. Bohannon, M. Travisano, and P. B. Rainey. 2002. Adaptive divergence in experimental populations of *Pseudomonas fluorescens*. I. Genetic and phenotypic bases of wrinkly spreader fitness. Genetics 161:33–46.

Srivastava, D. S., and J. H. Lawton. 1998. Why more productive sites have more species: an experimental test of theory using tree-hole communities. The American Naturalist 152:510–529.

Tan, J., C. K. Kelly, and L. Jiang. 2013. Temporal niche promotes biodiversity during adaptive radiation. Nature Communications 4.

Terborgh, J. 1973. On the notion of favorableness in plant ecology. The American Naturalist 107:481–501.

Terborgh, J. W., and J. Faaborg. 1980. Saturation of bird communities in the West Indies. The American Naturalist 116:178–195.

Tilman, D., R. M. May, C. L. Lehman, and M. A. Nowak. 1994. Habitat destruction and the extinction debt. Nature 371:65–66.

Wilbur, H. M., D. W. Tinkle, and J. P. Collins. 1974. Environmental certainty, trophic level, and resource availability in life history evolution. The American Naturalist 108:805–817.

Willig, M. R., D. M. Kaufman, and R. D. Stevens. 2003. Latitudinal gradients of biodiversity: pattern, process, scale, and synthesis. Annual Review of Ecology, Evolution, and Systematics 34:273–309.

Wright, D. H. 1983. Species-energy theory: an extension of species-area theory. Oikos 41:496–506.

Yuan, W., S. Liu, G. Yu, J.-M. Bonnefond, J. Chen, K. Davis, A. R. Desai, A. H. Goldstein, D. Gianelle, F. Rossi, A. E. Suyker, and S. B. Verma. 2010. Global estimates of

evapotranspiration and gross primary production based on MODIS and global meteorology data. Remote Sensing of Environment 114:1416–1431.

CHAPTER 2 | Linking succession and ecosystem function in a natural host-associated microbiota

ABSTRACT

Ecosystem development theory predicts that the successional turnover of taxa and biomass within communities will drive rates of ecosystem functions such as mineral cycling. Many of this theory's predictions are based on primary-producer controlled communities, and have withstood limited empirical testing. In contrast, donor-controlled communities, particularly host-associated digestive microbiota, have received significant empirical attention, yet few studies attempt to evaluate their findings in the context of ecosystem development theory.
In this study, I employ the natural, replicable digestive communities found in leaves of the carnivorous pitcher plant *Darlingtonia californica* to test hypotheses concerning linkages between community dynamics and ecosystem process rates. I used a combination of community sequencing, fluorescence microscopy, carbon metabolic assays, functional gene prediction, and stable isotope labeling to monitor the development and function of decomposer communities in two cohorts of aging pitcher leaves. I documented a variety of general trends in ecosystem development that matched theoretical predictions, including a hump-shaped trend in bacterial diversity over time, a parallel successional trajectory among independent communities, and a mid-successional peak in carbon metabolic breadth. I found bacterial community composition, biomass, and diversity to influence the rates of prey decomposition, which in turn was correlated to the uptake efficiency of prey-derived nitrogen by the host plant. To my knowledge, these results represent the first attempt to test the predictions of ecosystem development theory using host-associated microbiota.

INTRODUCTION

Although the capacity for community interactions to mediate ecosystem processes is widely recognized (Loreau et al. 2001, Hooper et al. 2005, Cardinale et al. 2006), surprisingly few theoretical (Finn 1982, DeAngelis 1992, Loreau 2010) and empirical studies (Kira and Shidei 1967, Vitousek and Reiners 1975, Fisher et al. 1982, Tang et al. 2014, Lasky et al. 2014) have investigated community-ecosystem linkages along a temporal gradient. This is in spite of the common observation of ecosystems in states of flux due to disturbance, shifting environmental conditions, resource fluctuations, and invasive species (Cowles 1899, Wiens 1977, Connell and Sousa 1983, Fukami and Nakajima 2011). Inspired by the work of Lotka (1925), Eugene Odum (1969) famously posed a number of hypotheses for general patterns of ecosystem development that attempted to explain variation in ecosystem properties during the course of community succession — or the predictable turnover of communities through time following disturbance or colonization of a new habitat. For instance, he reasoned that as diversity and biomass accumulated in ecosystems over time, the ratio of nutrient outputs to inputs will diminish and nutrient cycles become closed (Odum 1969, Vitousek and Reiners 1975). Many of Odum's successional hypotheses, however, have yet to be confronted with replicated data from natural ecosystems (Vitousek and Reiners 1975, Fisher et al. 1982). In this study, I leverage the

replicability and realism of a natural, host-associated microbiota to test Odum's and others' hypotheses for unimodality in biodiversity, biomass production, and ecosystem process rates.

Ecologists have long struggled to formulate a coherent theory of succession, which I define here as the predictable temporal turnover of species in a community. This struggle likely stems from the idiosyncratic nature of community assembly (Walker and Chapin 1987) and has resulted in a scarcity of quantitative, testable predictions. However, a few general predictions can still be made (Miller and terHorst 2012). First, species richness is anticipated to peak mid-succession due to tradeoffs among community members' life history traits (e.g., competition, fecundity, propagule supply rate) (Drury and Nisbet 1973, Connell and Slatyer 1977, Huston and Smith 1987, Tilman 1990). Second, if ecosystem development is strongly deterministic, then replicate communities should develop in parallel over time. Failure to observe these trends among replicate ecosystems could result from a variety of semi-deterministic ecological processes including (but not limited to) initial floristics (Egler 1954), priority effects (Fukami 2004) and neutral dynamics (Hubbell 2001). If these effects dominate community assembly, then there should be few similarities in community composition between replicate ecosystems of similar age. While the hypotheses of parallel development and unimodal diversity dynamics have received substantial theoretical and empirical support, far less is known about 1) successional trends in community members' functional traits (Huston and Smith 1987), and 2) how these changes in community structure affect ecosystem processes such as rates of productivity, decomposition and nutrient cycling (Loreau 1998).

Most studies concerned with community-ecosystem linkages do so from the biodiversity-ecosystem function (BEF) perspective, which seeks to explain the (often positive) covariance between diversity (e.g., taxonomic, phylogenetic, functional) and ecosystem function (e.g., stability, productivity, nutrient cycling) (Loreau et al. 2001). Most BEF studies assemble experimental communities from natural or *ad hoc* species pools and measure their resulting productivities or other functions (Tilman et al. 2001, Jiang 2007, Cardinale 2011). A subset of these studies have tracked the function of experimental communities over multiple time points in order to estimate the effects of community succession on ecosystem functioning (Bell et al. 2005, Weis et al. 2007, Cardinale et al. 2007). Although these studies benefit from tractability and replication, they lack the realism of natural ecosystems by artificially prohibiting immigration of new species into experimental communities. On the other hand, while observational or experimental manipulations of natural ecosystems are desirable from a realism standpoint, these studies often lack substantial replication. Furthermore, the systems in which natural successional dynamics are most often studied tend to experience relatively slow rates of change and so typically employ a chronosequence approach, wherein communities of different ages are measured simultaneously. This 'space-for-time' substitution is valid as long as the ecosystems under investigation share sufficiently similar histories (Pickett 1989). Natural microcosms have been widely-used in ecology to bridge the gap between realism and tractability, and have been successfully used to test successional theory (Kitching 2000, Munguia 2004, Srivastava et al. 2004, Miller and terHorst 2012). In particular, host-associated microbial communities offer a tractable and replicable window into the functional consequences of community assembly and succession.

There are a number of reasons why natural, host-associated microbiota are useful systems in which to study ecosystem development. First, microbiota, beneficial or otherwise, often play a clear role in the wellbeing of the host (Bäckhed et al. 2005, Lugtenberg and Kamilova 2009). In such cases, the ecosystem function being provided can be some aspect of the host's health or fitness. Furthermore, and particularly in the case of obligate endosymbiont communities, the habitats under investigation are often nearly identical among host individuals, which allows for repeated, independent observations of ecosystem development. Finally, the successional dynamics of host-associated microbiota frequently operate over time scales proportional to the host organism's lifespan, which can manifest as large shifts in community composition and ecosystem function over relatively short periods of time. In contrast to other aquatic and terrestrial ecosystems, host-associated communities are typically donor-controlled, meaning they cannot directly influence their resource supply rate. These communities are, however, shaped by the same ecological processes as terrestrial and aquatic systems including colonization, disturbance, and competition (Sousa 1994, Lozupone et al. 2012).

The digestive communities contained within the leaves of carnivorous pitcher plants (families Nepenthaceae, Cephalotaceae, and Sarraceniaceae) are particularly well suited for the study of microbial succession and ecosystem function. These plants have adapted to grow on nutrient-poor substrates by evolving the ability to lure, capture, and digest invertebrate prey using modified, fluid-filled leaves. The captured prey form the base of a simple aquatic food web reliant on proteolytic enzymes produced by the plant and its commensal bacteria, which function to mineralize growth-limiting nutrients (primarily nitrogen and phosphorous) for uptake by the host leaf (Hepburn et al. 1927, Juniper et al. 1989). The pitcher plant *Darlingtonia californica* (Sarraceniaceae) is native to California and Oregon, USA, where it grows in wet meadows on or near serpentine formations. Each plant produces up to 15 pitcher leaves in succession during its 6 month growing season with the first leaf being the year's tallest (up to 0.75 m). Each leaf persists for an average of 1.5 years before senescing. The pitcher leaf completes its vertical growth prior to opening its trapping orifice and its interior chamber is sterile until this point (D. W. Armitage, *unpubl. data*). Compared to other members of Sarraceniaceae, *Darlingtonia* has a unique downward-oriented trapping orifice that minimizes passive dispersal into pitchers and prevents flooding. The pitcher inhabitants either actively colonize newly opened leaves (e.g., the obligate mite *Sarraceniopus darlingtonae* and larvae of the obligate midge *Metriocnemus edwardsi*) or are brought into the leaf by captured prey, which is likely the case for bacteria, fungi, and protists. Most prey capture by *Darlingtonia* typically occurs during a leaf's first two months, after which it ceases nectar production. Like its close relative, *Sarracenia purpurea*, *Darlingtonia* is believed to primarily rely on the actions of its food web for digestion (Hepburn et al. 1927, Lindquist 1975, Naeem 1988, Gallie and Chang 1997). In particular, *S. purpurea* was found to rely almost entirely on the bacteria (as opposed to mites and insect larvae) for nitrogen acquisition (Butler et al. 2008).

To date, there have been few studies of bacterial succession in nature, particularly within host-associated communities. Furthermore, most of these studies lack a clear theoretical context for their results (Scheiner 2013). In this study I use the microbial communities contained in wild *Darlingtonia* pitcher leaves to test a number of hypotheses concerning general patterns of ecosystem development (fig. 1). To accomplish this, I followed a cohort of pitcher leaves one year and measured the diversity, biomass, and functional traits of their associated digestive

communities. In addition, I quantified a number of ecosystem functions such as community respiration, decomposition rates, and host nitrogen uptake efficiency at each time point. Using these data, I test the prediction that community composition and diversity influences ecosystem function and verify this prediction with an independent field experiment. Next, I test for parallel patterns in microbial succession among pitcher leaves by quantifying pitcher leaves' shared 'successional microbiome.' Finally, I use ancestral state reconstruction to estimate the functional genes experiencing significant temporal turnover to estimate succession-driven shifts in traits such as growth rate, motility, and metabolism.

METHODS

Stable isotope labeling of wild pitcher leaves

I conducted the study during June-Oct 2013 and 2014 in two large populations of *Darlingtonia californica* pitcher plants growing near the Butterfly Valley Botanical Area in Plumas National Forest (Plumas Co., CA) (Naeem 1988). These populations were separated by approximately 2 km and contained over 10,000 individual plants. I switched populations between years to mitigate carryover contamination effects from the previous year's isotopic enrichments. Each year, at the beginning of the growing season in early June, I identified and tagged 50 pitcher leaves of equivalent developmental stage on different plants. These leaves represented a single cohort of each plant's first (and largest) pitcher leaves. In order to control for the underground transport of nitrogen between study leaves, I selected leaves from individual clusters of rosettes that were likely derived from different, unconnected genets. Study leaves were never located on neighboring rosette clusters, and were always separated by at least 5 m. I monitored these leaves daily to determine the date at which each leaf's trapping orifice opened and all study leaves began trapping insects on the same day.

To measure rates of decomposition and nitrogen cycling by the pitchers' aquatic food webs, I fed to each pitcher one gelatin capsule filled with 20 sterile, ^{15}N-enriched fruit flies (*Drosophila melanogaster*). I used an existing protocol to create the isotope-labeled flies (Gouw et al. 2011). Briefly, I let adult flies mate and lay eggs in unlabeled yeast (*Saccharomyces cereviseae*) paste. Eggs were harvested by rinsing the paste over 10 μm Nitex mesh and briefly washed in 70% ethanol and sterile deionized water. These eggs were spread onto a sterile Whatman #1 filter and placed into a box containing a sterile cotton sheet soaked in a larval fly diet consisting of 70 mL sterile deionized water, 37.5 μL propionic acid, 240 μL phosphoric acid, 840 μL 10% w/v tegosept, 3 μg ampicillin, 9 g sucrose, and 9 g (dry weight) ^{15}N-labeled yeast. Isotope-labeling of yeast was achieved by culturing yeast in 1.7 g/L YNB without amino acids and ammonium sulfate (Difco) with 20 g/L sucrose and 5 g/L >99 atom% ^{15}N-labeled ammonium sulfate (Cambridge Isotope Laboratories). Yeast cultures were shaken for 24 hours at 30° C. The culture was divided into 250 mL Nalgene tubes and centrifuged at 2,400 × *g* for 20 min at 4° C. The supernatant was removed and the pellets were re-suspended in 10 mL sterile phosphate buffer (PBS) and pooled into sterile 50 mL centrifuge tubes until I obtained the equivalent of 9 g dry weight. Once the eggs were added, the fly box was covered with 10 μm Nitex mesh and placed in a dark growth chamber at 30° C and 95% humidity. Adult flies were collected daily by anesthetization using FlyNap (Carolina Biological Supply, Inc.) and stored at -20° C. Once 2000 flies had been obtained, they were dried for 3 days at 60° C, grouped into lots

of 20 individuals, weighed, autoclaved, and stored in 0.2 mL PCR tubes at -20° C. Four flies were sent to the UC Davis Isotope Facility for gas chromatography - isotope ratio mass spectrometry (GC-IRMS). These flies were found to be enriched to 82 atom% ^{15}N.

On the day the pitcher leaves first opened (6/7/2013 and 6/13/2014), I randomly selected five leaves to receive ^{15}N-enriched fruit flies. I delivered flies into the aquatic digestive zone of the leaves by means of a gel cap, which quickly dissolves in the pitcher fluid. After 11 days, I returned to the site and collected the five complete ^{15}N-labeled pitcher leaves by pinching them off at the rosette. These leaves were carefully placed into vials containing ice-cold sterile spring water and were covered with sterile plastic bags for the 5-hour transport to the lab. Before leaving, 5 more pitchers were randomly fed gel caps containing 20 ^{15}N-labeled flies apiece. This process was repeated every 11 days up to day 88 (mid-September), and again on day 166 (late November). In addition to these leaves, I also fed ^{15}N-labeled flies to 10 pitchers from the previous year's cohort in early June. These leaves represented the first leaves produced by plants in the previous year and were assumed to be approximately 365 days old. These leaves were collected at the same time as the 11-day pitchers.

Pitcher food web quantification

Immediately after returning to the lab, I quantified all components of each pitcher leaf's food web. I began by removing the upper 2/3 of the leaf using a pair of sterilized scissors. The lower portion of the leaf, including the digestive zone and fluid, was placed into a sterile 50 mL falcon tube on ice. I dissected the upper portion of the leaf down its midrib and pinned it on an insect pinning block. I used a dissecting microscope to count and identify all living arthropods on the abaxial surface (Nielsen 1990). Next, I used a pipette to remove all liquid from the lower portion of each pitcher leaf and I recorded the volume of fluid each leaf contained. This fluid was placed in a sterile 15 mL flask on ice. I dissected the bottom portion of the leaf and emptied its contents onto a petri dish filled with 70% ethanol. I counted all mite and midge larvae in the dish and then searched for all fruit fly remains and repeated this process on the sidewalls of the digestive chamber. Then, after thoroughly rinsing each leaf of any residual arthropod material in dH_2O, I dried each dissected leaf in a drying oven for 3 days at 60° C. I did the same for each pitcher's prey items after having removed the midge larvae and any visible fruit flies. I used the condition of the recovered fruit flies to rate the extent of prey decomposition on a 5-point ordinal scale. If all flies were recovered intact with no visible damage (e.g., missing limbs, abdominal segments.), the pitcher was scored a zero. If no flies were recoverable and head-capsules were found, then the pitcher was scored as five. Intermediate values depended on both the number of flies recovered and the extent of their decomposition.

I quantified and identified all microinvertebrates and protists using both Sedgwick Rafter (1 mL) and Palmer (0.1 mL) counting cells. I filled each counting cell with a vortexed aliquot of pitcher fluid containing either Proto-Slo (Carolina Biological, Inc.) or 3% Lugol's iodine solution (Sherr and Sherr 1993). I counted individual organisms in random circular fields until at least 200 had been counted and estimated their abundances using the formula $d = n \div F \cdot A_c \div A_f \div V$, where d is the estimated density of individuals per unit volume, n is the total number of individuals counted across all fields, F is the total number of fields counted,

A_c and A_f are the areas (μm^2) encompassed by the full chamber and the ocular field, respectively, and V is the total dilution-corrected volume (mL) of fluid in the chamber .
If fewer than 200 individuals of a particular taxon could be found in the chamber, I counted the total number of individuals in the chamber. I attempted to identify all micro-invertebrates and protists to genus or species using dichotomous keys (Lee et al. 2000) and previous accounts of pitcher plant-associates (Laird 1969).

Bacterial and viral enumeration

I used epifluorescence microscopy to quantify bacterial and viral abundances in pitcher fluid (Patel et al. 2007). I began by vacuum filtering bulk pitcher fluid through 20 μm Nitex mesh. I created serial dilutions of the filtrate in 0.02 μm-filtered phosphate buffer and added to them sterile sodium pyrophosphate solution to 5 mM. This step causes the elution of particle-bound bacteria and viruses and results in a lower CV between samples (Danovaro et al. 2001). These samples were briefly vortexed and 0.5 mL aliquots of varying dilutions (typically 10^3 for bacteria and 10^4 for viruses) were vacuum-filtered onto 0.02 μm Anodisc 25 filters (Whatman, Inc.). Filters were made in triplicate for each pitcher sample and dilution. After drying, I stained filters with 100 μL 0.5× SYBR-gold nucleic acid stain for 15 minutes in the dark. This stain binds to DNA and produces a green fluorescent signal under blue excitation, and is used to enumerate bacteria, archaea, and virus-like particles (VLPs) (Patel et al. 2007). After a second round of drying, the filters were mounted on glass microscope slides using 0.02 μm-filtered glycerol-based anti-fade solution. To avoid photobleaching of slides during counting, I photographed random fields of each filter at 1000× magnification under blue excitation and counted them later. VLP and bacterial/archaeal abundances were estimated following the protocol of (Patel et al. 2007). All slides were made less than 8 hours after collection from the field and less than 1 hour after pitcher fluid was removed from the plant.

Carbon mineralization assays

I estimated the each pitcher leaf community's potential rate of carbon mineralization using the MicroResp™ respirometry system (Campbell et al. 2003). I added 100 μL of 20 μm-filtered pitcher fluid to 900 μL sterile phosphate buffer in a microcentrifuge tube and centrifuged the mixture at 10,000 × *g* for 5 minutes. I removed the supernatant, added in clean buffer, and resuspended the pellet. This process was repeated twice to wash bacterial communities of any residual medium, and the final bacterial community was starved for 2 hours prior to inoculation into the respirometry chambers. I added 50 μL of starved, dilute pitcher fluid into 750 μL medium comprised of M9 salt solution (NH_4Cl 1 g L^{-1}, Na_2HPO_4 6 g L^{-1}, KH_2PO_4 3 g L^{-1}, NaCl 0.5 g L^{-1}, pH 6.0) and 3 g L^{-1} powder from freeze-dried crickets that had been ground and autoclaved. I inoculated each sample into a sterile 1.2 mL 96-well plate. Onto this plate I clamped a standard 96-well plate containing a redox dye set in purified agar. Carbon dioxide respired from the bacteria in the well passes through a small perforated gasket and into the headspace of the chamber containing the dye. The change in CO_2 concentrations causes the dye to change color. Rates of CO_2-C evolution over time can be estimated using a microplate reader (at 490 nm) and comparing these values to a standard curve. Inoculated MicroResp containers were placed in an incubator for three days at 25° C. Each community's respiration was averaged over six independent replicates. These rates of CO_2 respiration reflect the potential respiration rates of each pitcher's bacterial community in a common habitat, which allowed me to compare

each community's relative performances without the confounding effects of pitcher chemistry and nutrient availability.

I used the Biolog GN2 microplate assay (Biolog Inc., Hayward, CA) to measure the carbon substrate utilization patterns of the microbial communities from an independent collection of 11, 55, and 365 day-old pitchers (10 from each age, 5 per year). Each well of the GN2 microplate contained a different carbon substrate (95 total) and a dye that changes color when reduced by NADP(H). A color change in a well indicated that at least one taxon in the inoculum was capable of growth on the particular C-substrate. Plates were inoculated using the same dilute, filtered, starved communities described above, and incubated for 3 days at 25° C. Each community was run in triplicate, and only wells that showed a color change in all three replicates were scored as positive for metabolism. I used a negative binomial generalized linear model to determine whether the number of metabolized substrates differed among age classes and ran a chi-square likelihood ratio test to compare the fit of the parameterized model to that of an intercept-only null.

Isotope analyses

Once the fluid had been collected and prey removed from each pitcher, I thoroughly washed the leaf tissue in DI water to remove as much remaining arthropod material as possible. Each leaf was then dried for 3 days at 60° C, weighed, and ground using a Wiley Mill. I removed a subsample of this tissue and ground it to a fine powder using a bead beater. These powdered samples were weighed, packaged in tin capsules, and sent to the UC Davis Stable Isotope Facility (Davis, CA) for gas chromatography-isotope ratio mass spectrometry (GC-IRMS). I estimated the nitrogen uptake efficiency (N_{eff}) of each pitcher leaf using the formula

$$N_{eff} = 100 \cdot \frac{[\mathrm{N}_{leaf} \cdot (\mathrm{at\%}^{15}\mathrm{N}_{leaf} - \mathrm{at\%}^{15}\mathrm{N}_{ref})]}{(\mu\mathrm{g}_{fly} \cdot \mathrm{N}_{fly} \cdot \mathrm{at\%}^{15}\mathrm{N}_{fly})} \quad (1)$$

where N_i is the total mass of nitrogen (μg) in leaf or fly tissue, $\mathrm{at\%}^{15}N_i$ are the atom percent measurements of ^{15}N in either leaf tissue, fly tissue, or atmospheric reference samples, and μg_{fly} is the total mass of flies added to the pitcher leaf. This value is an estimate of the total fraction of nitrogen from the added flies that was found in the host pitcher's tissue after 11 days (Butler et al. 2008).

Bacterial community sequencing

I used the MoBio Powersoil DNA Extraction Kit (MoBio Laboratories, Carlsbad, CA) to extract community DNA from 700 μL aliquots of 20 μm pitcher fluid filtrate. After NanoDrop quantification, all samples were stored at -80° C. A control extraction from each Powersoil kit was also performed to check for contamination. Once all samples and blanks were collected, they were sent for sequencing at the Argonne National Laboratory Core Sequencing Facility (Lemont, IL) using Earth Microbiome Project (EMP) protocols (Caporaso et al. 2012). Briefly, this involved PCR amplification of the 16s ribosomal RNA region, including EMP adapters, multiplex barcodes, linker sequences, and primer sequences 515F (5'– AATGATACGGCGACCACCGAGATCTACAC XXXXXXXXXXXX TATGGTAATT GT GTGYCAGCMGCCGCGGTAA – 3') and 806R (5'– CAAGCAGAAGACGGCATACGAGAT

AGTCAGTCAG CC GGACTACNVGGGTWTCTAAT – 3'). The field containing X's represents a unique 12-bp barcode to distinguish pooled samples. Thermocycling followed the EMP protocol: a denaturing step at 94° C for 3 minutes, amplification for 35 cycles at 94° C for 45 seconds, 50° C for 60 seconds, 72° C for 60 seconds, and a final extension at 72° C for 10 minutes (Caporaso et al. 2012). Paired-end 2x150 bp sequencing was carried out on an Illumina MiSeq.

I demultiplexed pooled 16s reads using the QIIME platform (quality parameters $q = 20$ and $p = 0.9$) (Caporaso et al. 2010b) and assembled paired ends using SeqPrep (St. John 2016). Next, I removed chimeric sequences using both reference-based (using the RDP database) and *de novo* UCHIME (Edgar et al. 2011, Cole et al. 2014). I clustered reads into operational taxonomic unites (OTUs) at 97% sequence similarity using an open-reference approach (Rideout et al. 2014). This involved first clustering sequences to representative OTUs in the Greengenes 13.8 database (DeSantis et al. 2006) (Pruesse et al. 2007)using SortMeRNA (Kopylova et al. 2012), and then performing *de novo* clustering on the remaining sequences using SUMACLUST (Mercier et al. 2016). Sequences were then aligned using PyNAST (Caporaso et al. 2010a), assigned to taxonomy using SortMeRNA and the Greengenes database (Bokulich et al. 2015), and used to build a phylogenetic tree with FastTree 2.1 (Price et al. 2010). I removed singletons, OTUs shared with kit blanks, and low-confidence OTUs (those representing less than 0.1% of the sample's total reads) and normalized OTU tables using library size factor (LSF) scaling implemented in the *DESeq2* R package (Love et al. 2014).

Statistical analyses of pitcher communities through time

I combined sample metadata (e.g., pitcher N uptake efficiency, bacterial abundance, pitcher age), phylogenetic data, and OTU tables using the *PhyloSeq* package in R (McMurdie and Holmes 2013, R Development Core Team 2015). Bacterial alpha diversity was measured using Shannon's diversity index (H') and Faith's (Faith 1992) phylogenetic diversity. Temporal differences in bacterial diversity, bacterivore richness, and the abundances of each major food web compartment (detritus, viruses, bacteria, *Polytomella,* mites and midges) were analyzed using ANOVA. I conducted post-hoc analyses using Tukey's range test. When necessary, data were log-transformed for variance stabilization.

I calculated weighted and unweighted UniFrac metrics to characterize the phylogenetic distances between pitcher samples (Lozupone et al. 2011). Using the *vegan* package in R (Oksanen et al. 2015), I clustered these UniFrac distances to produce a hierarchical dendrogram of each pitcher sample and used principal coordinates analysis (PCoA) to visualize the similarities among samples. Similarly, I used PCoA to visualize temporal differences in protist/invertebrate assemblages as well as Biolog plate substrate utilization. I used permutational analysis of variance (PERMANOVA) using the *adonis* function in R (Anderson 2001) to assess whether intra-age dissimilarities were significantly less than inter-age dissimilarities for each data table (bacteria, protists, substrates). To determine whether bacterial communities converged or diverged over time, I compared community dissimilarities among pitcher age classes using ANOVA. I used two different dissimilarity measures: unweighted UniFrac (phylogeny-based) and Jensen-Shannon divergence (JSD; OTU-based).

Defining the successional microbiome

I modeled LSF-normalized OTU counts using a negative binomial generalized linear model (GLM). For an OTU *i* in sample *j*, OTU counts, K_{ij}, were modeled as

$$K_{ij} \sim NB(\text{mean} = \mu_{ij}, \text{dispersion} = \alpha_{ij}) \quad (2)$$

$$\mu_{ij} = s_{ij} q_{ij} \quad (3)$$

$$\log_2 q_{ij} = \beta_i^0 + \beta_i^{\text{A}} x_i^{\text{A}} \quad (4)$$

where x_i^{A} is the pitcher sample's age category, β_i^{A} are parameters describing the log-fold change of an OTU in each age class, and s_{ij} is the OTU and age-specific normalization factor (for further details, see Love et al. 2014). Modeling OTU turnover using their $\log_2$-fold changes in abundance circumvents problems caused by heteroscedastic raw count data. Models were fit using empirical Bayes and OTUs experiencing significant $\log_2$-fold change among time points were identified using multiple testing-corrected Wald *p*-values.

I defined the 'successional microbiome' as the subset of OTUs experiencing a statistically significant ($\alpha = 0.001$) $\geq$ 8-fold change in abundance between any two pitcher age classes. I used these OTUs to build an abundance-weighted heat map and sorted OTUs along the y-axis based on the pitcher age group in which they first appear. I assessed the predictive accuracy of this subset of OTUs using a random forest classifier (Breiman 2001). This classifier builds a "forest" of decision tree models, each based on a random subset of predictor features (here, OTUs) and fits them to a bootstrapped subset of data. The remaining 'out-of-bag' (OOB) samples are used to estimate the classification error of the model. I trained a random forest of 10000 trees using the successional OTU counts from 2013 and used the resulting classifier to predict the age of 2014 samples. The predictive model was fit to minimize the error rate between observed and OOB data. Model accuracy was evaluated using the coefficient of determination (R^2) for predicted vs. observed points along a 1:1 line.

Estimating functional gene turnover during succession

For the subset of closed-reference OTUs detected in my samples, I used the PICRUSt (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States) software (Langille et al. 2013) to predict each OTU's rRNA copy number and functional gene content. The PICRUSt algorithm identifies the closest relatives of each OTU for which annotated genomes are available and performs ancestral state reconstructions to estimate its gene contents. The accuracy of this method depends on the evolutionary distances between an OTU and its nearest reference genome (measured by the 'nearest sequenced taxon index', NSTI), but is generally greater than 75% for samples falling in the range of NSTI values in my samples (0.071 ± 0.01) and can be greater than 90% for certain orthologous gene categories (e.g., amino acid and carbohydrate metabolism) (Langille et al. 2013). I used the Kyoto Encyclopedia of Genes and Genomes (KEGG) database for all gene annotations (Kanehisa et al. 2016). This database classifies individual genes into a hierarchical structure according to the biochemical pathways to

which each gene contributes. To account for variable sequencing depth among samples, PICRUSt predictions were made using an LSF-normalized OTU table.

I estimated the mean weighted rRNA copy number, C_{ij}, for each sample using the method of (Nemergut et al. 2015): $C_{ij} = \sum p_{ij} c_i$, where p_{ij} is the relative abundance of OTU i in sample j and c_i is the PICRUSt-estimated rRNA copy number of OTU i. I used analysis of variance (ANOVA) to determine whether rRNA copy numbers significantly changed over time. Next, I ordinated pitcher samples based on their level 3 KEGG pathway relative abundances using principal components analysis (PCA). In addition, samples were hierarchically clustered based on Euclidean distances among their level 3 KEGG pathways using Ward's method (Ward 1963). To determine genes and pathways that were differentially enriched across time points, I used ANOVA to test for differences in each KEGG pathway's relative abundance across time points. To remove potentially spurious results, I filtered KEGG pathways by p-value ($p \leq 0.01$) and effect size ($\eta^2 \geq 0.26$). All p-values were first corrected for multiple testing using the Benjamini-Hochberg procedure. In addition to KEGG pathways, I evaluated how the abundances of individual KEGG Ortholog genes changed over time using negative binomial generalized linear models, focusing primarily on enzymes involved in the ammonification, nitrate reduction, denitrification, and nitrogen fixation pathways. I tested for a significant effect of age class on gene abundances using a chi-square likelihood ratio test against an intercept-only null model. All tests were run using STAMP 2.0 (Parks and Beiko 2010) and R.

Identifying temporal trends in ecosystem processes

I assessed temporal differences in pitcher ecosystem properties using ANOVA for N uptake efficiency/respiration and a multinomial logit model for the decomposition category (Agresti 2013). The best explanatory models were selected based on (pseudo)-R^2 values (Nagelkerke 1991) and relative values of Akaike's Information Criterion (AIC) (Burnham and Anderson 2003). To investigate the effects of bacterial community composition on ecosystem properties, I ran Mantel tests comparing the pairwise Euclidean distances among samples' ecosystem properties with the unweighted UniFrac distances among the same samples' bacterial communities.

Food web homogenization experiment

Because pitcher leaves of differing ages may physiologically regulate nitrogen uptake, independent of their associated food webs, I conducted a field experiment to separate the effects of the food web and host leaf age on rates of N uptake. During late July 2014 I identified 15 pitcher leaves aged 11 days, 55 days, and > 365 days (5 leaves of each age). I removed and combined the fluid from these leaves and then returned this homogenized mixture back to the host plant. Additionally, I delivered into each leaf a gel capsule containing 20 ^{15}N-enriched fruit flies. I returned after 11 days to harvest and process these pitchers for N-uptake efficiency as described earlier. I used ANOVA to test whether the N-uptake efficiencies of these pitchers with homogenized food webs recapitulated the N-uptake patterns from natural pitcher food webs of equivalent age from the same population.

RESULTS

Pitcher community structure changes over time

Prey biomass in pitcher leaves quickly increased after opening and remained relatively constant until cessation of prey capture during the second growing season (fig. 2a). Bacterial biomass also rapidly accumulated in young pitcher leaves and increased over time during the first growing season to a maximum of 1×10^{11} cells mL^{-1} before significantly declining during the second growing season (fig. 2b). Similarly, virus-like particles, *Sarraceniopus darlingtonae* mites, *Metriocnemus edwardsi* midge larvae, and *Polytomella agilis* flagellates were more abundant in mature pitcher leaves during the first growing seasons (fig. 2c-f). In addition to *P. agilis,* I detected numerous other protists and rotifers, including flagellates *Bodo* sp., *Monas* sp., *Petalomonas* sp., *Rhynchobodo* sp., *Chilomonas* sp., the ciliate *Colpoda* sp., the rotifer *Philodina* sp., and the alga *Chlamydomonas* sp. All of these taxa were observed in 10 or fewer pitcher leaves with no apparent temporal trends in occupancy. Bacterivore richness was not significantly different among time points (fig. 2g). Likewise, there were no apparent temporal trends in beta diversity among time points, and the significant differences in the arthropod/protist community among ages appeared to be driven by the absence of *P. agilis* in the 166-day pitchers (fig. 2h). However, post-hoc analysis revealed 365-day pitchers to be, on average, significantly more dissimilar from one another than young (11 and 22 day) pitchers.

Pitcher bacterial community dynamics are predictable

After quality filtering, my final OTU table consisted of 99 samples with 3642446 total 16s reads representing 762 OTUs. The minimum and maximum number of reads per sample were 21983 and 83157, respectively (mean = 36972), and there were no significant differences in read counts among age classes ($F_{9,89} = 1.3$, $p = 0.26$). Alpha diversity in pitchers significantly differed among age classes. Shannon diversity peaked at the end of the first growing season (fig. 3a) whereas phyogenetic diversity increased over the entire study period (fig. 3b). Communities became more similar over the course of the first growing season, according to the Jensen-Shannon divergence metric and diverged again during the start of the second growing season (fig. 3c). This trend was also observed with phylogenetic (UniFrac) dissimilarity, but only between the 11- and >22-day pitchers (fig. 3d). Furthermore, PERMANOVA analysis of UniFrac distances revealed a significant clustering of pitcher communities by age class (weighted UniFrac: $F_{9,89} = 15.3$, $p = 0.0001$, unweighted UniFrac: $F_{9,89} = 7.9$, $p = 0.0001$) (fig. 4). Of the top 50 most abundant OTUs detected across pitcher samples, most belonged to families Bacteroidetes (fig. 5), Firmicutes (fig. 6), and Proteobacteria (fig. 7). Within each respective family, the genera *Pedobacter, Clostridium*, and *Pseudomonas* were the most common members across all age classes.

Models of read counts using DESeq2 suggested a large number of taxa undergoing significant turnover in pitcher communities. In particular, I identified 74 OTUs that experienced significant ($p < 0.001$) 8-fold turnover among time points (fig. 8). These taxa fell primarily into the phyla Proteobacteria (37 OTUs), Bacteroidetes (16 OTUs) and Firmicutes (14 OTUs). Using these OTUs to train a random forest classifier to predict the pitcher community's age resulted in a high classification accuracy for OOB data (observed vs. predicted $R^2 = 0.80$). Likewise, a

random forest trained on data collected in 2013 was successful at predicting the ages of samples collected from an independent population in 2014 (observed vs. predicted $R^2 = 0.75$) (fig. 9).

Temporal trends in the functional attributes of pitcher microbiota

Based on community metabolic assays, early and late-stage pitcher communities both metabolized significantly fewer carbon substrates than did 55-day communities (fig. 10a). When carbon utilization dissimilarities were plotted in terms of their principal coordinates, the 55-day samples' profiles were less variable than those of 11 and 365-day pitchers (fig. 10b). Furthermore, the 11- and 365-day samples were more similar in terms of their carbon metabolic profiles than they were to the 55-day samples, as evidenced by the overlap in the former two age classes' convex hulls in principal coordinate space.

A principal components plot of samples based on their PICRUSt-predicted level 3 KEGG pathway contents showed pitcher samples to generally separate by age, with the greatest distances between the 11-day and 365-day communities (fig. 11a). Hierarchical clustering of these results supports two distinct clusters: one containing a majority of young communities (11-22 days) and one containing the remainder (fig. 11b). The predicted average number of rRNA gene copies was significantly greater in 11-day pitchers than in any other age class (fig. 12). This trend was also observed in the relative abundances of a number of other predicted KEGG pathways, such as flagellar assembly, motility, chemotaxis, and ABC transporters (fig. 13). Conversely, a variety of predicted metabolic pathways significantly increased over time as pitcher communities aged (fig. 14). In addition, the predicted abundances of a variety of genes involved in protein digestion and nitrogen uptake, nitrogen mineralization, denitrification, and nitrogen fixation all tended to increase in older pitcher communities (figs. 15-18).

Linking community dynamics and ecosystem processes

I detected significant differences in ordinal decomposition categories (fig. 19a) and nitrogen uptake efficiencies (fig. 19b) among pitchers of different ages. Both ecosystem properties tended to increase over the course of the first growing season and subsequently decline in year two. I was unable to detect significant temporal differences in common-garden community respiration rates, although there was still a positive, but non-significant unimodal trend in mean respiration rates over time (fig. 19c). Multinomial logit models predicted bacterial diversity (measured using the Shannon index) and bacterial and midge larvae abundances to positively influence a pitcher's probability of having a higher decomposition score (fig. 20a, table 1). Furthermore a pitcher leaf's nitrogen uptake efficiency was best predicted as a function of decomposition category (fig. 20b, table 1). I detected a weak but significant correlation between pitcher samples' unweighted UniFrac distances and their Euclidean distances in nitrogen uptake efficiency (Mantel $r = 0.10$, $p < 0.05$). I did not detect a correlation between samples' UniFrac distances and differences in respiration rates. In contrast to natural pitcher samples, the nitrogen uptake efficiencies of experimentally-homogenized pitcher food webs did not significantly differ with pitcher leaf age ($F_{2,12} = 0.98$, $p = 0.40$) (fig. 21).

DISCUSSION

Patterns in the development and succession of pitcher plant communities

By tracking the developing 'micro-ecosystems' of *Darlingtonia* pitcher leaf cohorts, I was able to identify a variety of temporal patterns in the structure and function of the leaves' associated communities. Immediately after opening, sterile pitcher leaves are rapidly colonized by bacteria, virus-like-particles (likely bacteriophage viruses), protists, mites and midge larvae, and begin capturing prey. I encountered mid-successional peaks in the abundances of nearly all pitcher associates — a trend also observed in the developing leaves of *Sarracenia purprea* (Miller and terHorst 2012). Unlike other pitcher plants, however, the densities of bacteria and virus-like-particles in *Darlingtonia* were comparable to those found in mammalian and insect digestive tracts (Miller and terHorst 2012, Scott et al. 2013, Takeuchi et al. 2015). These densities (10^{10} – 10^{11} bacterial cells/VLPs per mL) are greater than those reported from nearly every other terrestrial and aquatic habitat (Knowles et al. 2016). As predicted by successional theory (Drury and Nisbet 1973), bacterial diversity also peaked mid-succession, and pitchers' bacterial communities converged over time during the first growing season, but diverged at the start of the second growing season. This temporal convergence has also been observed in the non-bacterial compartments of *Sarracenia purpurea* pitcher leaves (Miller and terHorst 2012) and also on the phyllosphere of crop legumes and *Arabidopsis thaliana* (Maignien et al. 2014, Copeland et al. 2015).

I was able to identify a 'successional microbiome' common to two populations of *Darlingtonia*. This subset of OTUs consisted of bacterial taxa that significantly increased within a particular pitcher age class. A large number of these OTUs were members of clades containing host-associated facultative or obligate anaerobes, such as Enterobacteriaceae, Ruminococcaceae, and Clostridiaceae. It was previously shown that prey capture by *S. purprea* can induce hypoxia in pitcher fluid when bacterial respiration outpaces the rate of oxygen resupply by photosynthesis (Sirota et al. 2013), and in this context the enrichment of anaerobes in *Darlingtonia* leaves is not surprising. Members of the Chitinophagaceae were also greater in older pitchers and may play a role in the degradation of arthropod chitin. The majority of families and genera recovered from both successional and overall bacterial communities mirror those found in the related *Sarracenia purpurea* and *S. alata* (Whitman et al. 2005, Koopman and Carstens 2011, Krieger and Kourtev 2012, Gray et al. 2012). OTUs belonging to genera *Pedobacter* and *Pseudomonas* remained abundant throughout the lifespan of the pitcher cohort. These genera are among the most common members of bulk soil, rhizosphere, and phyllosphere communities (Humphrey et al. 2014) and have been previously recovered from both *Sarracenia* pitchers as well as in pitchers of the distantly-related *Nepenthes* (Nepenthaceae) (Gray et al. 2012, Takeuchi et al. 2015). Finally, many of the bacterial families that were enriched during later time points in pitcher communities (e.g., Rhizobiaceae, Sphingomonadaceae, and Comamonadaceae) also did so in the *Arabidopsis* phyllosphere (Maignien et al. 2014), implying that there may be some subset of the phyllosphere successional microbiome that is shared more broadly across plant taxa.

Temporal trends in the functional attributes of pitcher communities

The mid-successional increase in the amount of carbon substrates utilized by each community mirrors the unimodal trend in taxonomic diversity. This trend in biochemical diversity was predicted by Odum (Odum 1969) in response to Cooke's (Cooke 1967) observation of a mid-succession peak in algal carotenoid diversity. In addition, samples from early and late-stage leaves were also more dissimilar both in terms of their diversities, as well as their carbon substrate profiles, suggesting that divergence in microbial community composition may drive divergence in carbon substrate metabolism. However, the diversity and metabolic data were collected from different pitcher samples, prohibiting a direct test of their correlation.

Many PICRUSt-predicted functional genes and pathways also significantly changed over the course of succession. Transport pathways and motility-related pathways such as chemotaxis and flagellar assembly experienced some of the most significant declines in abundance as pitcher leaves developed. In addition, the average predicted rRNA copy number (per taxon) was significantly higher in early-stage pitchers— a successional pattern that has been observed in a variety of other habitats (Nemergut et al. 2015). All of these genomic properties have previously been shown to predict a taxon's rate of response to unpredictable nutrient conditions (Klappenbach et al. 2000, Livermore et al. 2014, Yawata et al. 2014) and thus contribute to a its fitness when introduced into novel environments. This is precisely what occurs as sterile pitchers are colonized by bacteria from insect guts and other habitats. This observation lends support to and extends to bacteria the life-history tradeoff model of succession wherein ruderal, fast-responding taxa are eventually excluded via competition or direct antagonism (Odum 1969, Connell and Slatyer 1977, Huston and Smith 1987). And while species interactions could not be directly measured in this study, a previous experiment using lab-reared bacterial strains isolated from the same *Darlingtonia* leaves over time found that competition among the isolates was lowest in 11-day pitchers and strongest during mid-successional stages (D. W. Armitage, *in review*).

In contrast to motility and ribosomal genes, predicted metabolic gene abundances significantly increased in pitchers' microbial communities over time. As prey accumulate in pitcher leaves, bacterial taxa possessing genes for lipid, carbohydrate, nucleotide, and amino acid metabolism increase in relative abundance. As has been observed in studies of corpse decomposition (Metcalf et al. 2016), pathways contributing to the deamination of amino acids and production of the volatiles cadaverine and putrescine were enriched during mid-succession. These foul-smelling compounds may serve to lure insects to pitcher leaves which then deposit eggs or become prey (Nielsen 1990, Aitkenhead-Peterson et al. 2015). PICRUSt-predicted genes encoding for nearly every step of the nitrogen cycle, including N fixation (presumably via enrichment of Rhizobiaceae or Bradyrhizobiaceae) were increased in abundance during succession. In particular, many genes involved in nitrogen mineralization were predicted to be greatest during mid-succession — a period that also corresponded to pitchers' greatest rates of N uptake efficiency. Denitrification genes were also significantly enriched as succession proceeded, indicating that certain taxa may effectively deprive late-stage pitchers of N, although the magnitude of this effect and its negative impact on the host plant's overall N budget may not be significant. However, it is important to keep in mind that the metagenomic data I present here are predictions based on ancestral state reconstructions from the subset of OTUs contained in the

Greengenes database. A more rigorous test of temporal trends in functional gene abundances could instead utilize metatranscriptomics to measure gene expression dynamics.

Temporal trends in ecosystem properties

Two of the three ecosystem properties I measured (N uptake efficiency and decomposition extent) were significantly associated with the age of pitcher leaves. The extent of fruit flies' decomposition over 11 days was greatest in mid-aged pitchers and a large portion of its variance was explained by bacterial diversity, bacterial abundance, and midge larval abundance. The positive effects of bacterial and *Metriocnemus* midge larval abundances on decomposition rate is not surprising, given that these groups rely almost exclusively on insect corpses to support their growth, while *Sarraceniopus* mites and protists (the two other primary food web components) feed primarily on bacterial cells and biofilms (Fashing 2005). Naeem (Naeem 1988) experimentally manipulated the numbers of mites and midges in *Darlingtonia* pitchers and found that the rate of prey biomass decrease was positively associated with the ratio of midge to mite abundances. A similar study of *Sarracenia purpurea* determined that the biomass of midge larvae, but not of mites nor protists positively influenced rates of prey decomposition (Baiser et al. 2011).

I encountered a positive effect of bacterial diversity on the extent of fruit fly decomposition — an observation is in line with the predicted positive influence of microbial diversity on ecosystem process rates (Loreau 2001, Ekschmitt et al. 2001). To my knowledge, this is the first biodiversity-ecosystem function relationship detected in a natural host-associated decomposer community. Loreau (Loreau 2001) reasoned that microbial diversity would contribute to enhanced decomposition only if the number of organic compounds able to be metabolized by the community increased with diversity. This theoretical prediction is supported by my observation that both bacterial diversity and community metabolic breadth were greatest during mid-succession (ca. 55 days). To date, the few studies to investigate microbial diversity and decomposition rates *in situ* have arrived at conflicting results (Hättenschwiler et al. 2011), but the pattern is generally supported among the few experimental tests of the hypothesized relationship (Bell et al. 2005), including in an experiment utilizing bacterial isolates from the same *Darlingtonia* population studied here (D.W. Armitage, *chapter 3*).

The fraction of fly-derived nitrogen detected in pitcher tissues after 11 days peaked in 77-day old leaves and declined during the second year. This trend contrasts with the majority of ecosystem development theory, which instead predicts that rates of N loss from the food web (e.g., due to leaching) will be lowest mid-succession, when rates of biomass productivity are maximized (Odum 1969, Vitousek and Reiners 1975, Finn 1982, DeAngelis 1992, Loreau 2010). However, these theories focus on primary-producer-controlled (so-called "green") food webs in which the N pool is growth-limiting and immobilized in long-lived organic tissue. On the other hand, donor-controlled (i.e., "brown") food webs may not experience as severe N limitation during periods of rapid detrital accumulation. This may be particularly true in *Darlingtonia* and other host-associated digestive communities for two reasons. First, bacterial biomass turnover is rapid, leading to an increase in the release of N from constant mortality, independent of the resource supply rate. For instance, the lysis of bacterial cells by viruses, which are abundant in pitcher fluid, is predicted to be a major source of bioavailable N in aquatic habitats (Fuhrman

1999). Second, the rapid accumulation of detritus with a low C:N ratio may buffer the food web from nitrogen losses to the host plant. Because bacteria are predicted to outcompete plants for bioavailable N (Kaye and Hart 1997), the relatively large fraction of prey-derived N found in pitcher foliar tissue indicates that in this system, N is not particularly growth-limiting for bacteria. However, it is difficult to translate the results of plant-soil microbe competition studies to aquatic communities.

Successional change in N uptake efficiency, particularly between a leaf's first and second years, was positively associated with rates of fly decomposition as well as weakly associated with the phylogenetic composition of the bacterial community. In addition, the most parsimonious model for N uptake efficiency included both bacterial diversity and abundance as covariates. Furthermore, I failed to detect the mid-succession peak in N uptake efficiency among pitcher leaves containing experimentally homogenized bacterial communities. In concert, these results point to the importance of the composition of the pitcher microbial community in mediating the transfer of N from prey to host — a function critical to the fitness of a host plant adapted to life in nitrogen-poor soils. Prey-derived nitrogen uptake efficiencies by *Sarracenia* (40 – 60 %) and *Darlingtonia* (57 – 90%) have previously been estimated using stable isotopes and my measured values fall within this range (27 – 96%, median = 60%) (Schulze et al. 1997, Butler et al. 2008). In the *Sarracenia* study, the authors were unable to detect a difference in the N uptake efficiencies between pitcher leaves containing either food webs seeded with the complete food web and pitcher leaves containing bacteria only (Butler et al. 2008). They conclude, as I do here, that bacteria are the primary facilitators of N transfer to the host plant.

Conclusions

By combining a ^{15}N stable isotope pulse-chase experiment with microbial community sequencing, metabolic fingerprinting, and functional gene predictions, I have demonstrated a number of general trends in a host-associated digestive community undergoing primary succession. In particular, my data support and extend hypotheses of semi-deterministic successional trajectories and a mid-successional peak in diversity to host-associated bacterial communities. In *Darlingtonia californica*, pitcher leaves' microbial communities are characterized by a predictive 'successional microbiome' shared among populations. Bacterial taxa detected in early-successional leaves were predicted to possess traits conferring fitness advantages in unpredictable habitats, including high counts of genes coding for ribosomal RNA, motility, chemotaxis, and membrane transport. In contrast, high carbon substrate diversities and abundances of metabolic genes characterized mid- and late-successional communities. I detected a positive influence of bacterial diversity and biomass on rates of prey decomposition, which in turn was predicted to influence the efficiency of prey-derived nitrogen uptake from the host plant. Future work should build on these findings by investigating the consequences of nutrient limitation on host-microbe interactions and its effect on successional patterns. In concert, these results represent a step towards integrating host-associated microbiota and phyllosphere communities into classical theories of succession and ecosystem development. Looking ahead, these types of replicated natural systems appear promising for studies attempting to bridge population, community, and ecosystem scales.

Table 1. Model selection results of multinomial logit and linear regression models for decomposition category and nitrogen uptake efficiency, respectively. Bolded values indicate the best-performing models based on AIC and R^2 values. AIC values falling within 9 units of one another were considered equally parsimonious.

Decomposition Category			**Nitrogen Uptake Efficiency**		
Predictor Variables	ΔAIC	*pseudo-*R^2	Predictor Variables	ΔAIC	R^2
~ *Community age (A)*	32	0.71	~ *Community age (A)*	22	0.24
~ *Bacterial abundance (B)*	23	0.37	~ *Bacterial abundance (B)*	27	0.04
~ *Bacterial diversity (D)*	26	0.34	~ *Bacterial diversity (D)*	36	0.05
~ *Bacterivore richness (R)*	61	0.05	~ *Bacterivore richness (R)*	42	0.00
~ *Log midge abundance (M)*	45	0.20	~ *Log midge abundance (M)*	41	0.01
~ *Log mite abundance (N)*	60	0.05	~ *Log mite abundance (N)*	41	0.00
~ *B + D*	**0**	0.56	~ *Decomposition category (C)*	9	0.16
~ *B + D + M*	**3**	0.59	~ *A + C*	**5**	**0.26**
~ *A + B + D + M*	24	**0.82**	~ *A + B + D + C*	**0**	0.23
~ 1 (intercept-only null)	55	0.00	~ 1 (intercept-only null)	40	0.00

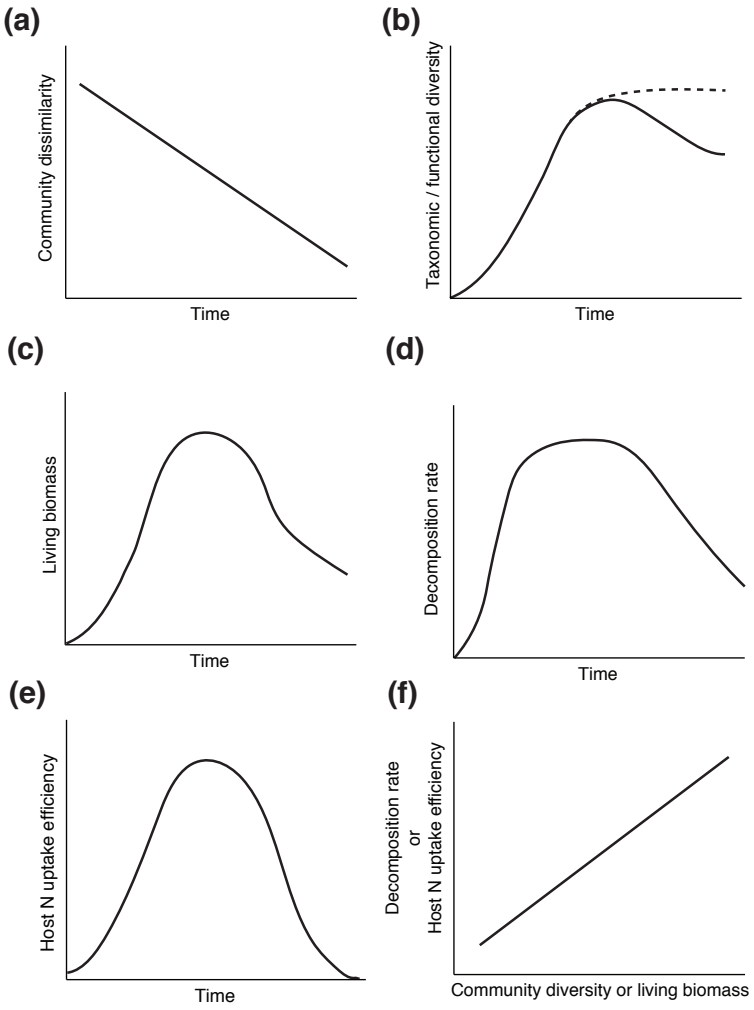

Figure 1. Trends in community and ecosystem properties anticipated during succession within *Darlingtonia* pitcher leaves (Cooke 1967, Odum 1969, Drury and Nisbet 1973, Kinzig et al. 2001, Loreau 2001).

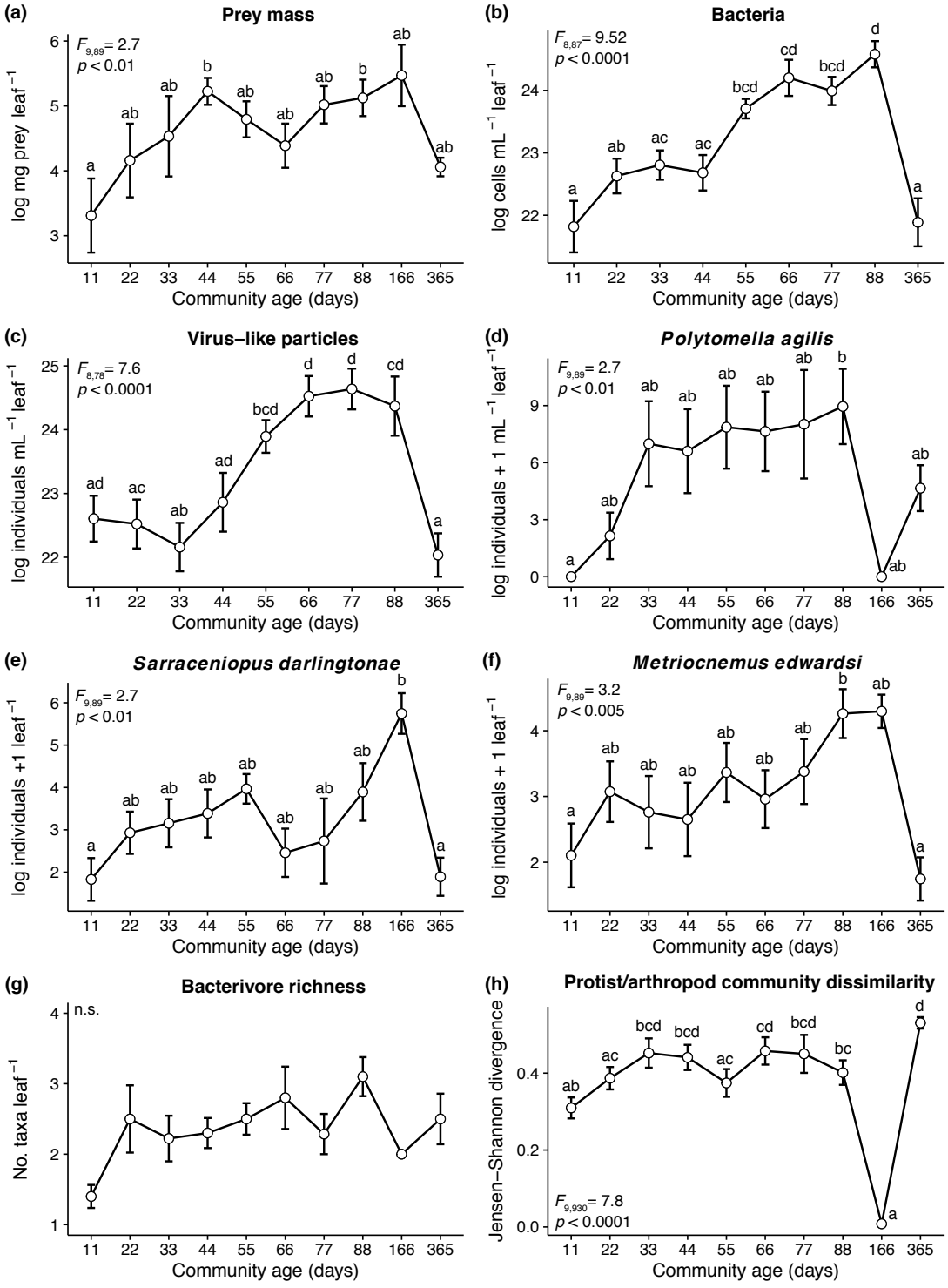

Figure 2. (a) – (f) Temporal trends in the abundances of pitcher community members. (g) Bacterivore richness did not significantly change among pitcher leaves of different ages. (h) Within-age Jensen-Shannon divergence in the pitcher protist/arthropod community. In each graph, shared letters above groups indicate no significant Tukey pairwise differences ($p > 0.05$).

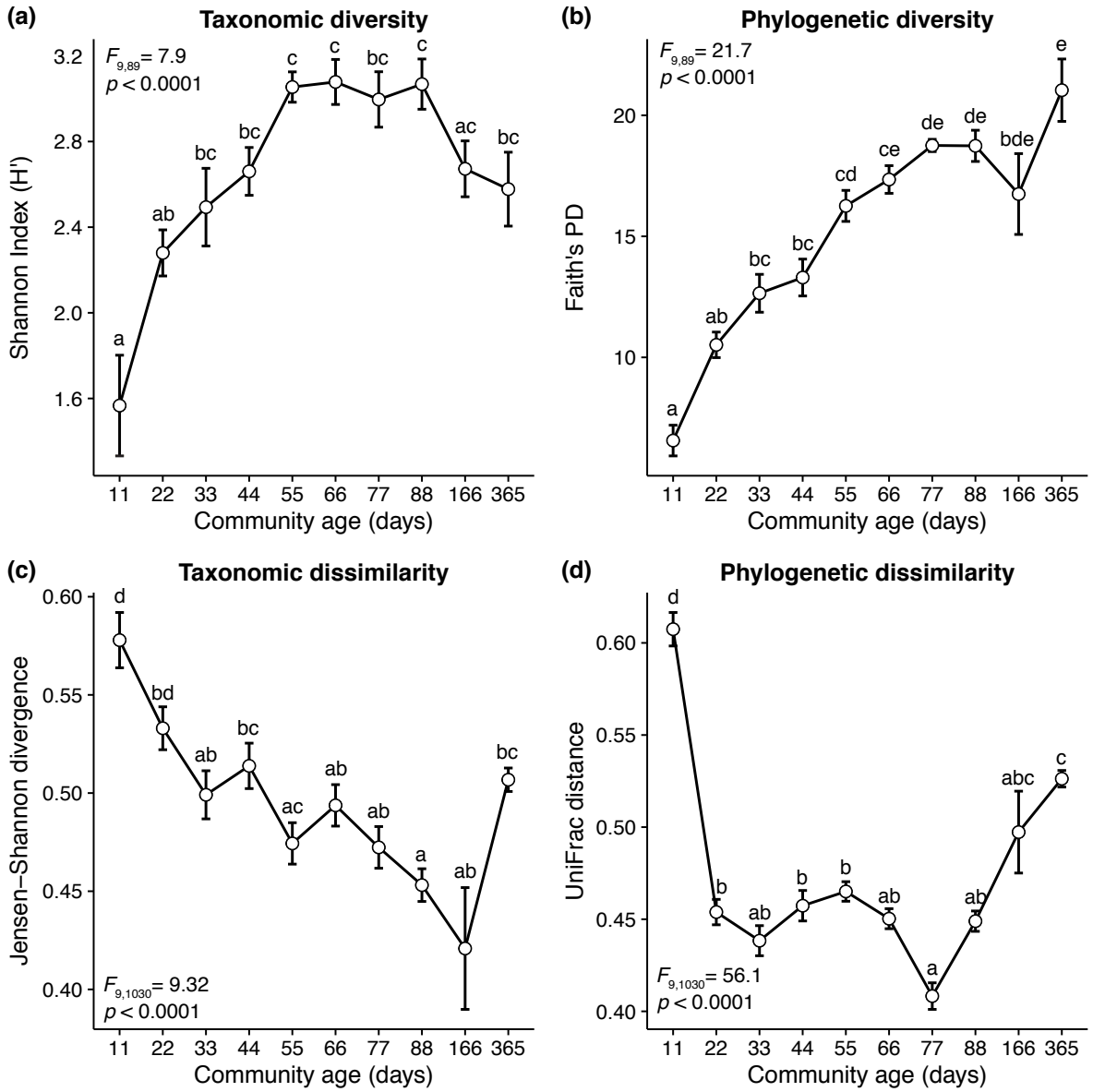

Figure 3. Temporal trends in the (a) OTU taxonomic and (b) phylogenetic diversities of pitcher communities. After opening, these pitchers tend to become more similar to one another over time in both (c) 97% OTU and (d) phylogenetic composition. In each graph, shared letters above groups indicate no significant pairwise differences ($p > 0.05$).

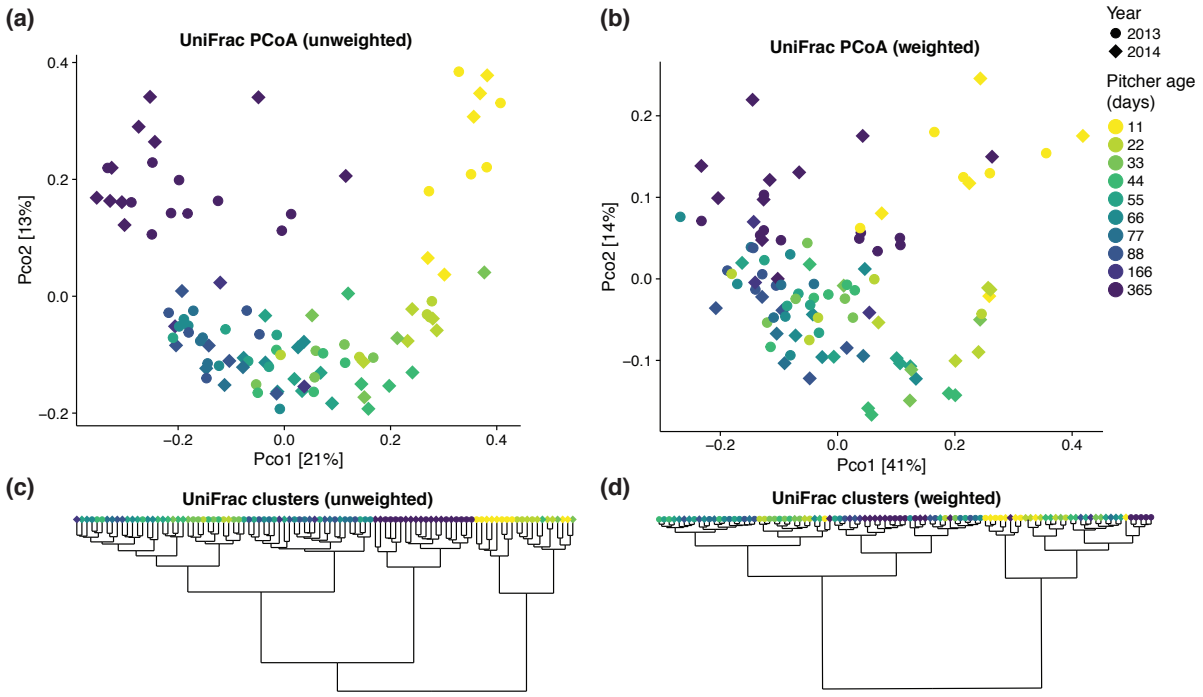

Figure 4. Principal coordinate (PCoA) plots for (a) unweighted and (b) weighted UniFrac distances between samples. The percentages of variance explained by the principal coordinates are displayed on each axis. Plots (c) and (d) display the results of hierarchical clustering of these UniFrac distances.

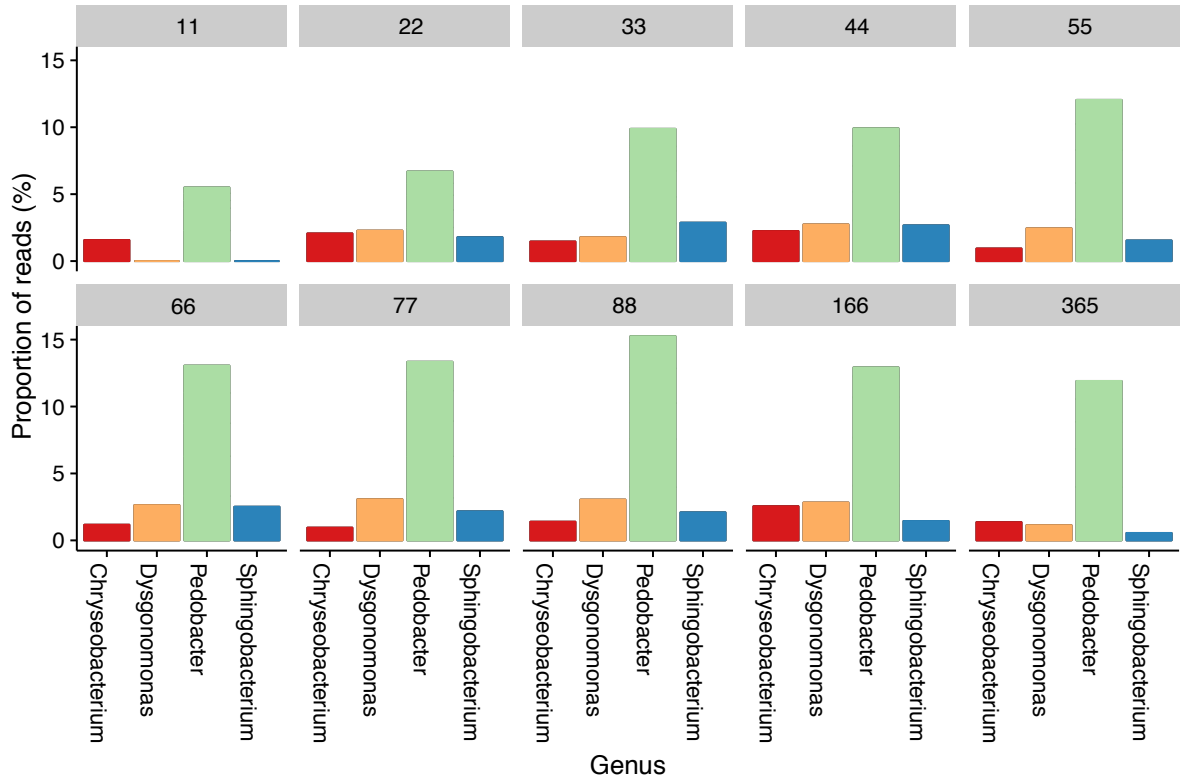

Figure 5. Trends in the top 50 most abundant OTUs belonging to phylum Bacteroidetes. Values denote the relative proportions of each genus compared to the rest of the top 50 most abundant OTUs.

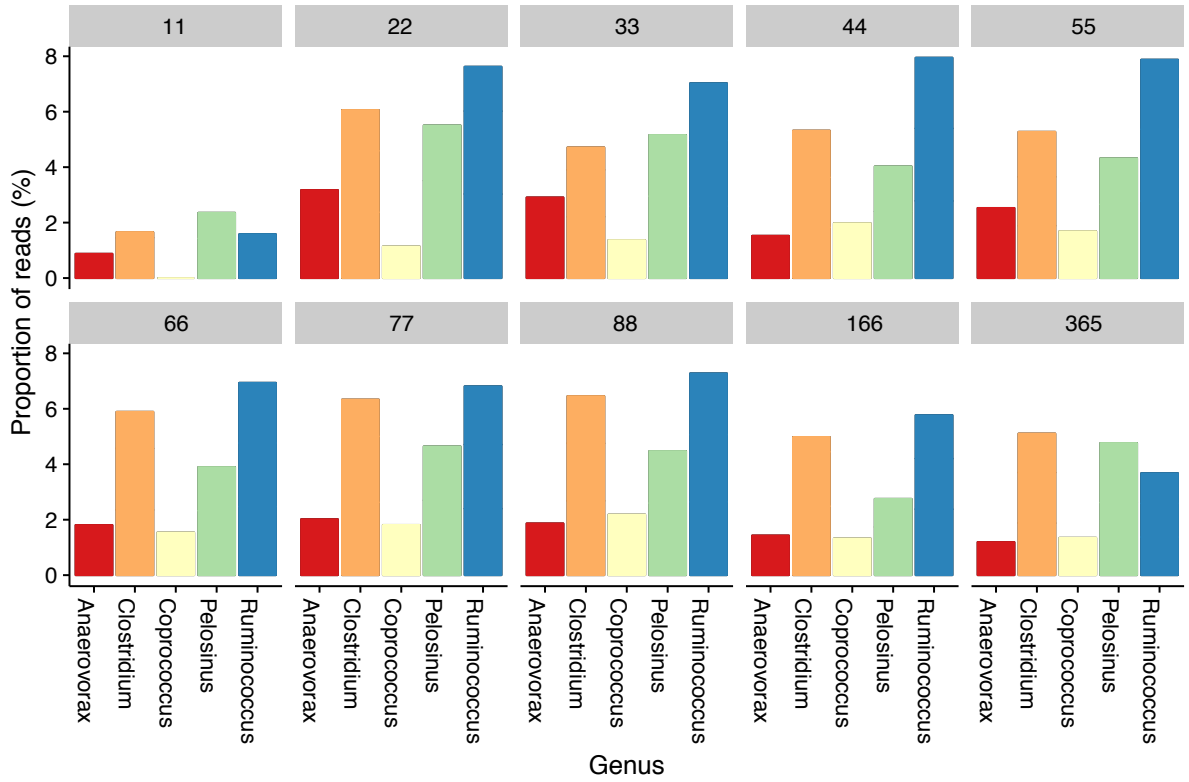

Figure 6. Trends in the top 50 most abundant OTUs belonging to phylum Firmicutes. Values denote the relative proportions of each genus compared to the rest of the top 50 most abundant OTUs.

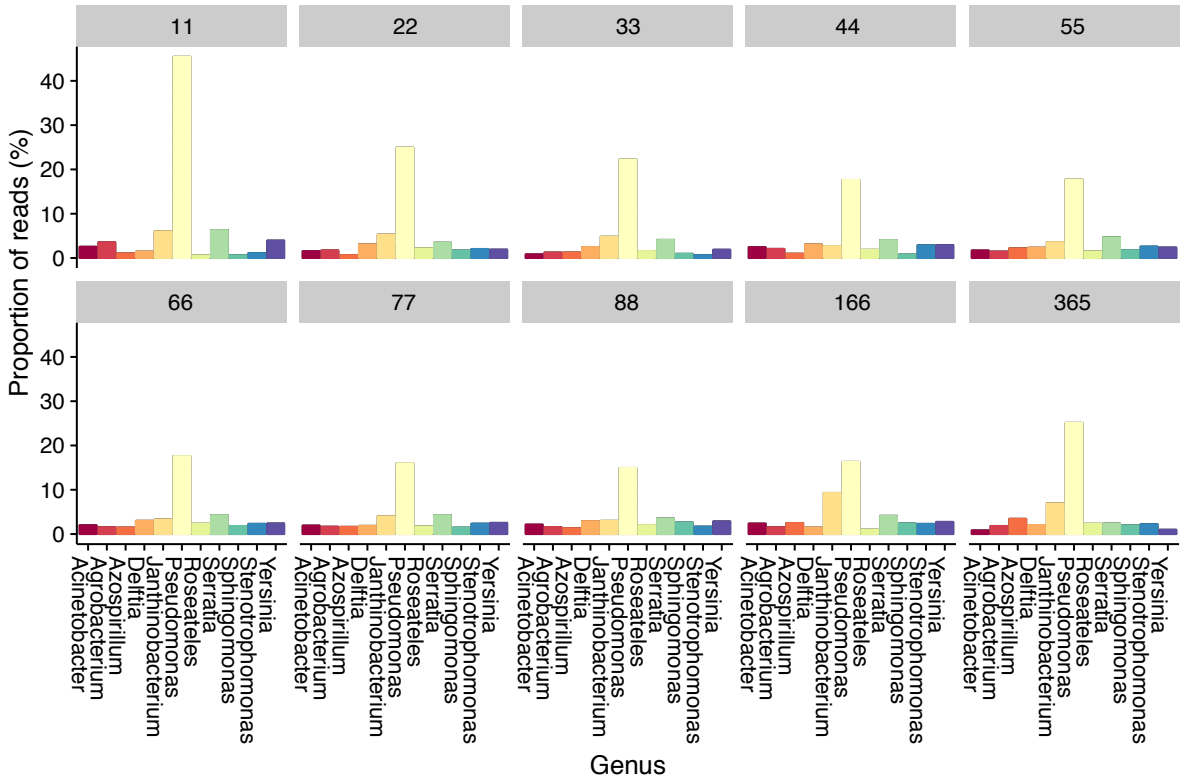

Figure 7. Trends in the top 50 most abundant OTUs belonging to phylum Proteobacteria. Values denote the relative proportions of each genus compared to the rest of the top 50 most abundant OTUs.

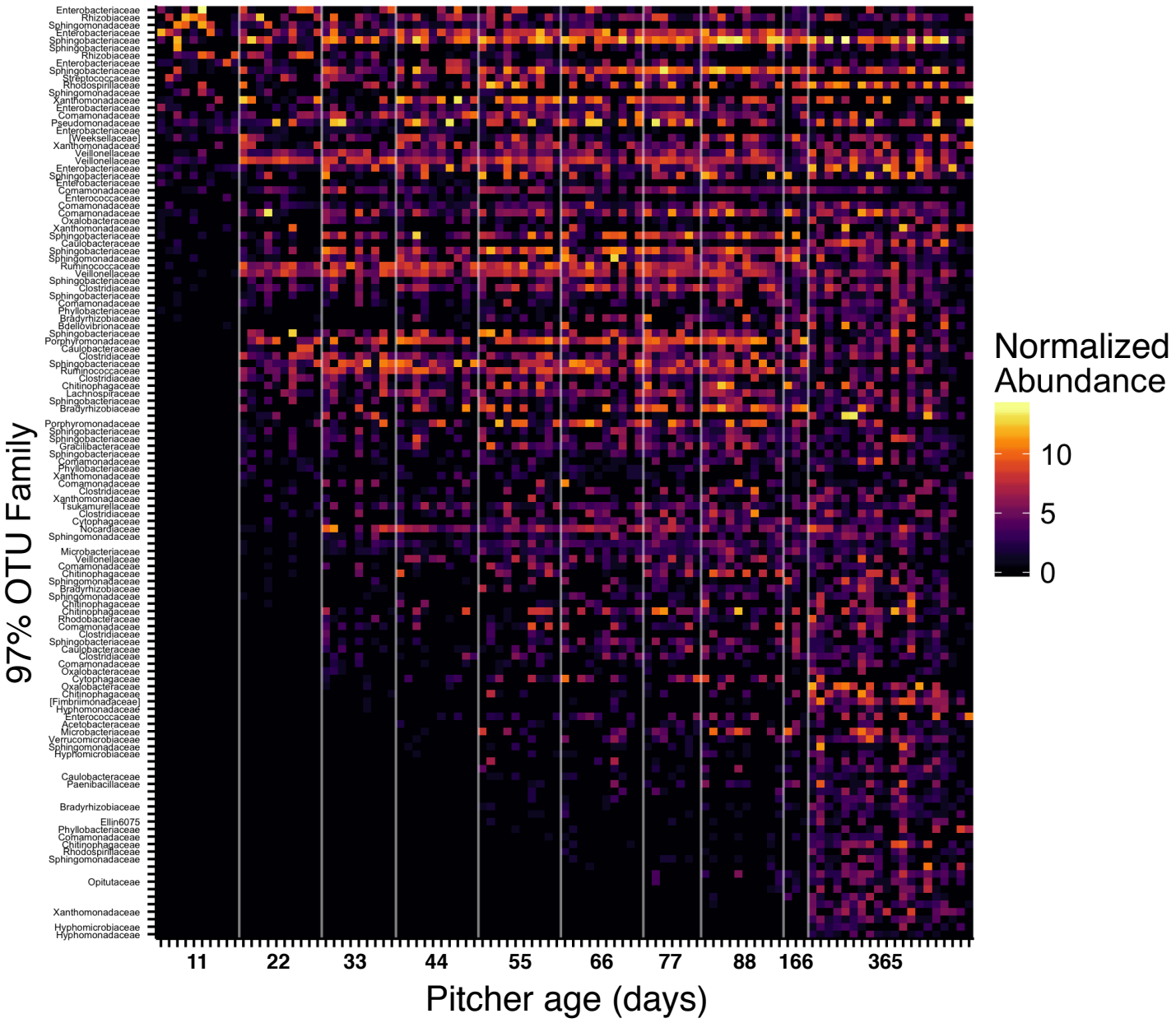

Figure 8. Abundance-weighted heat map of OTUs that experienced significant ($p < 0.001$) 8-fold or greater turnover between time points. OTUs are labeled by family and ordered based on the community age in which they were first detected. No label indicates that the OTU could not be classified to the family level using the Greengenes reference database.

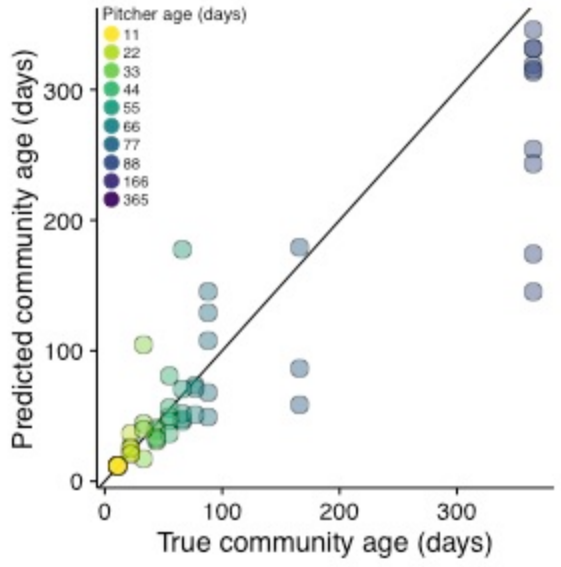

Figure 9. Observed versus predicted ages of 2014 pitchers classified using a random forest classifier trained on 2013 data ($R^2 = 0.75$). The solid line represents perfect 1:1 predictive accuracy.

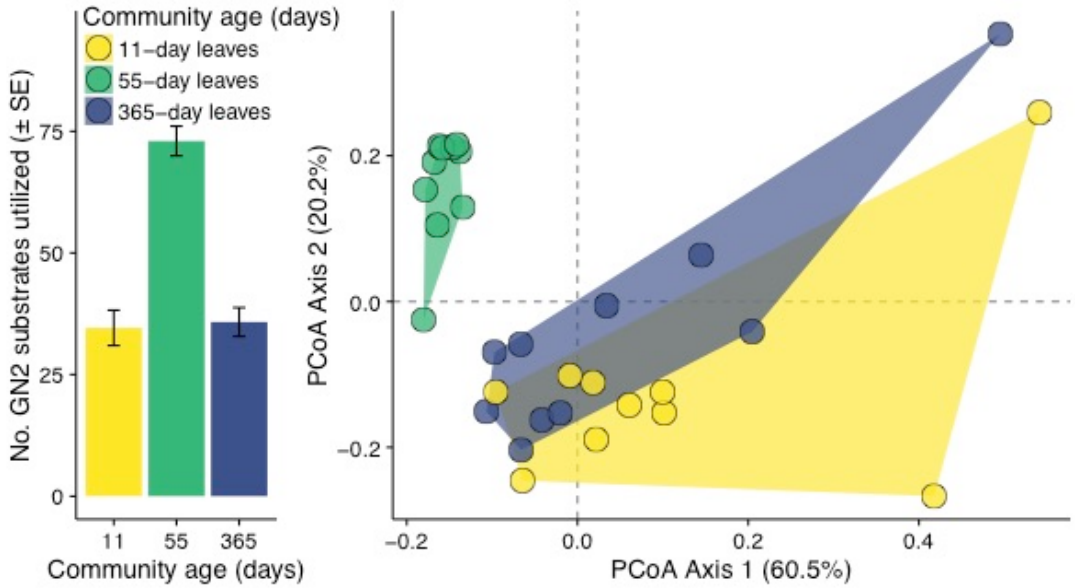

Figure 10. (a) Mid-stage pitcher communities are able to metabolize a greater number of Biolog GN2 substrates than are early and late-stage communities. (b) PCoA plot of GN2 metabolic profiles show a separation between 55-day pitchers and 11/365 day pitchers. Distances are based on Jaccard similarities between GN2 microplates.

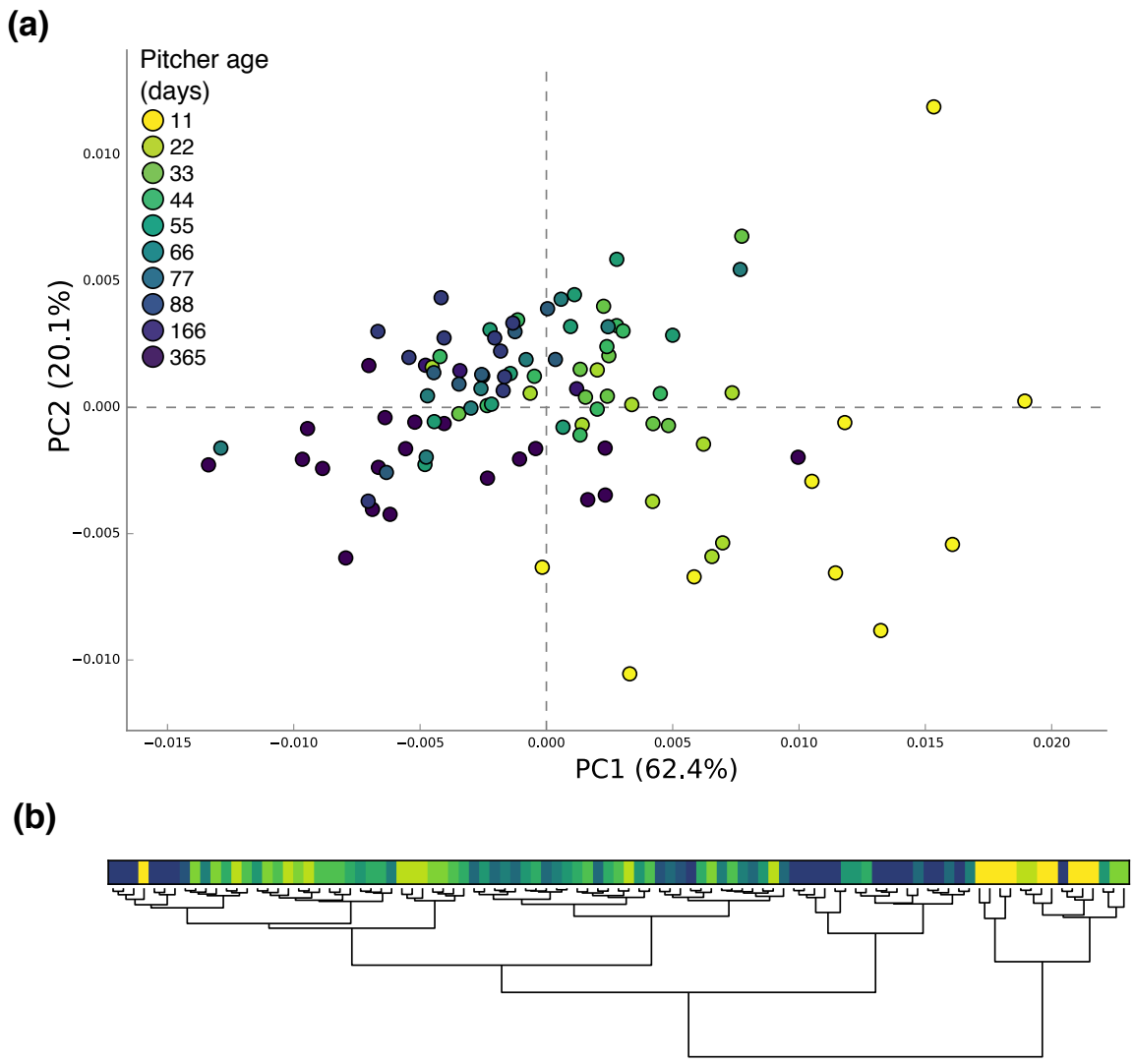

Figure 11. (a) Pitcher samples' PICRUSt-predicted metagenome contents generally separate by age along their first two principal component axes. (b) Hierarchical clustering results for these samples supports two major clades: one comprised of early (22-33 day) samples and the other comprised of middle- and late-stage samples.

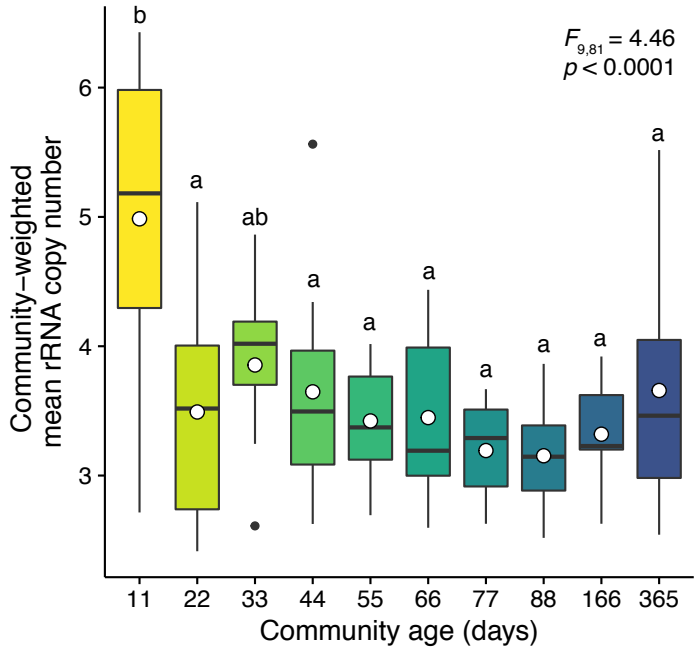

Figure 12. PICRUSt-predicted average rRNA copy numbers for OTUs in a sample. White points denote mean for each age class. Early-stage (11 day) pitcher communities contained OTUs with more rRNA copies than were predicted for later pitcher communities. Shared letters above groups indicate no significant post-hoc pairwise differences ($p > 0.05$). Boxplots are colored by age for consistency among figures (key in fig. 4).

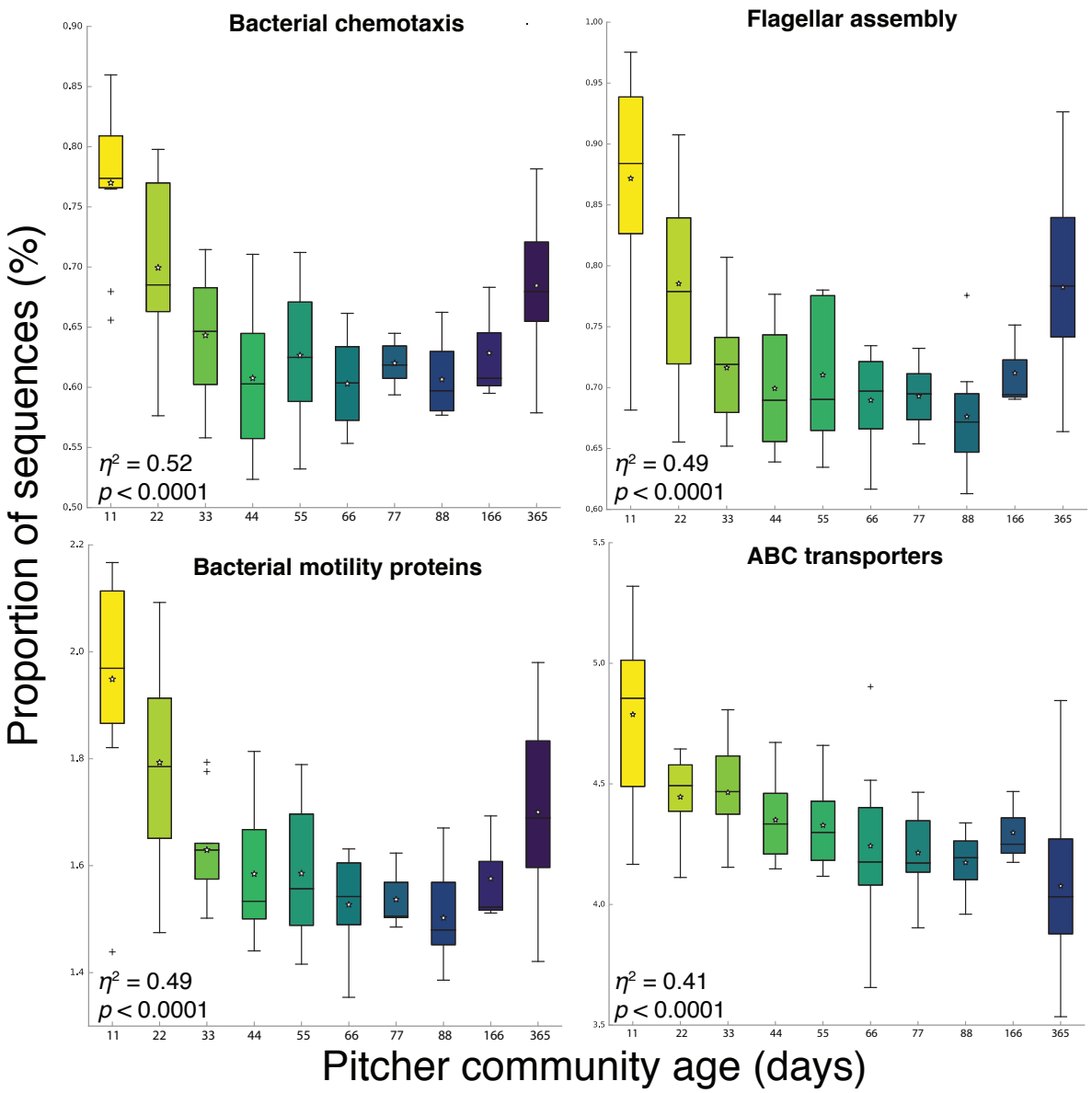

Figure 13. Patterns in the within-age class relative abundances of PICRUSt-predicted KEGG pathways. White points denote mean values for each age class. Pathways shown here are more abundant within young pitcher samples. P-values show the significant of age effects and η^2 values are a measure of age effect size. Boxplots are colored by age for consistency among figures (key in fig. 4).

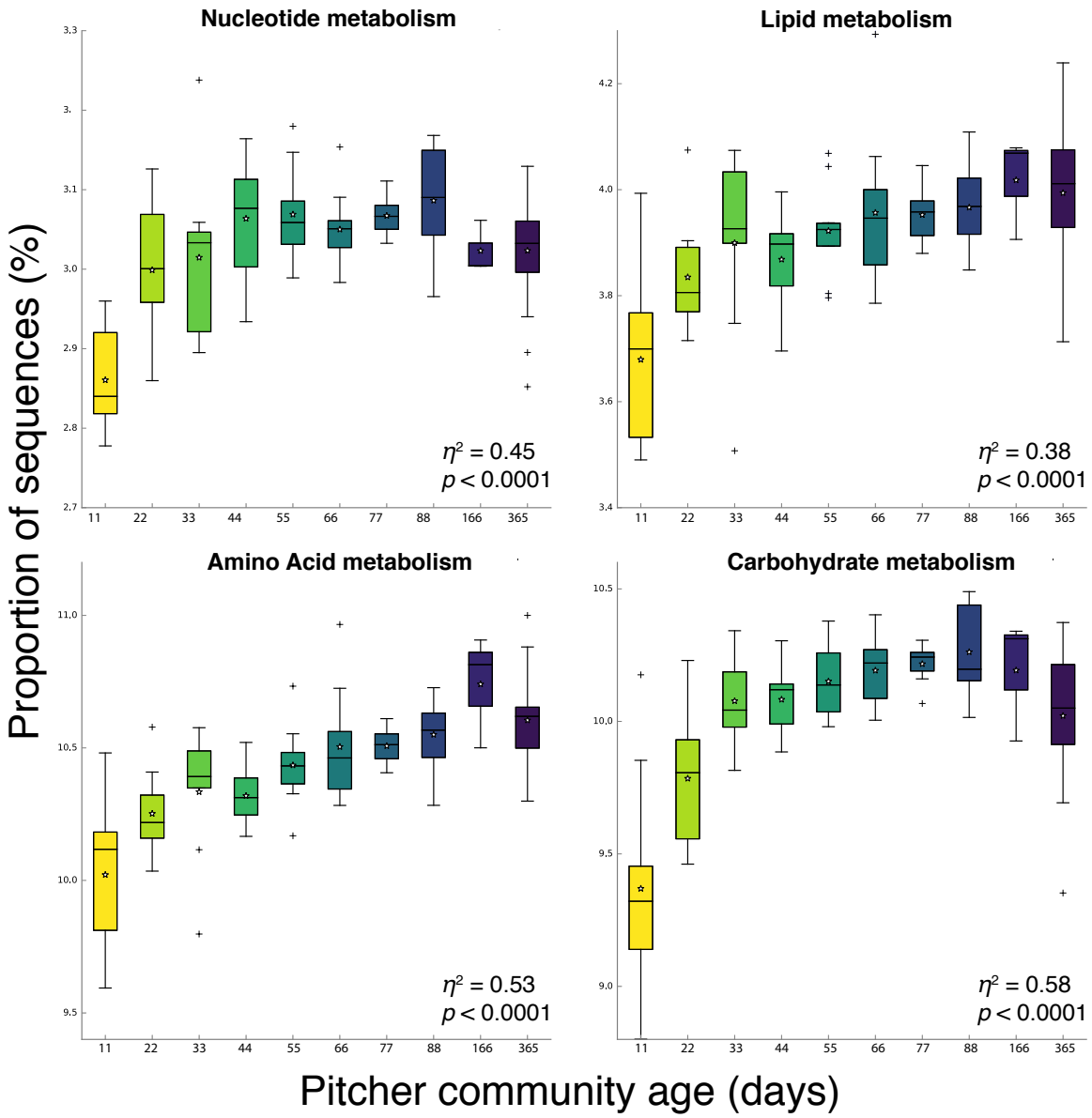

Figure 14. PICRUSt-predicted KEGG pathways for major metabolic pathways. Pathways shown here are more abundant within older pitcher samples. White points denote mean values for each age class. P-values show the significant of age effects and η^2 values are a measure of age effect size. Boxplots are colored by age for consistency among figures (key in fig. 4).

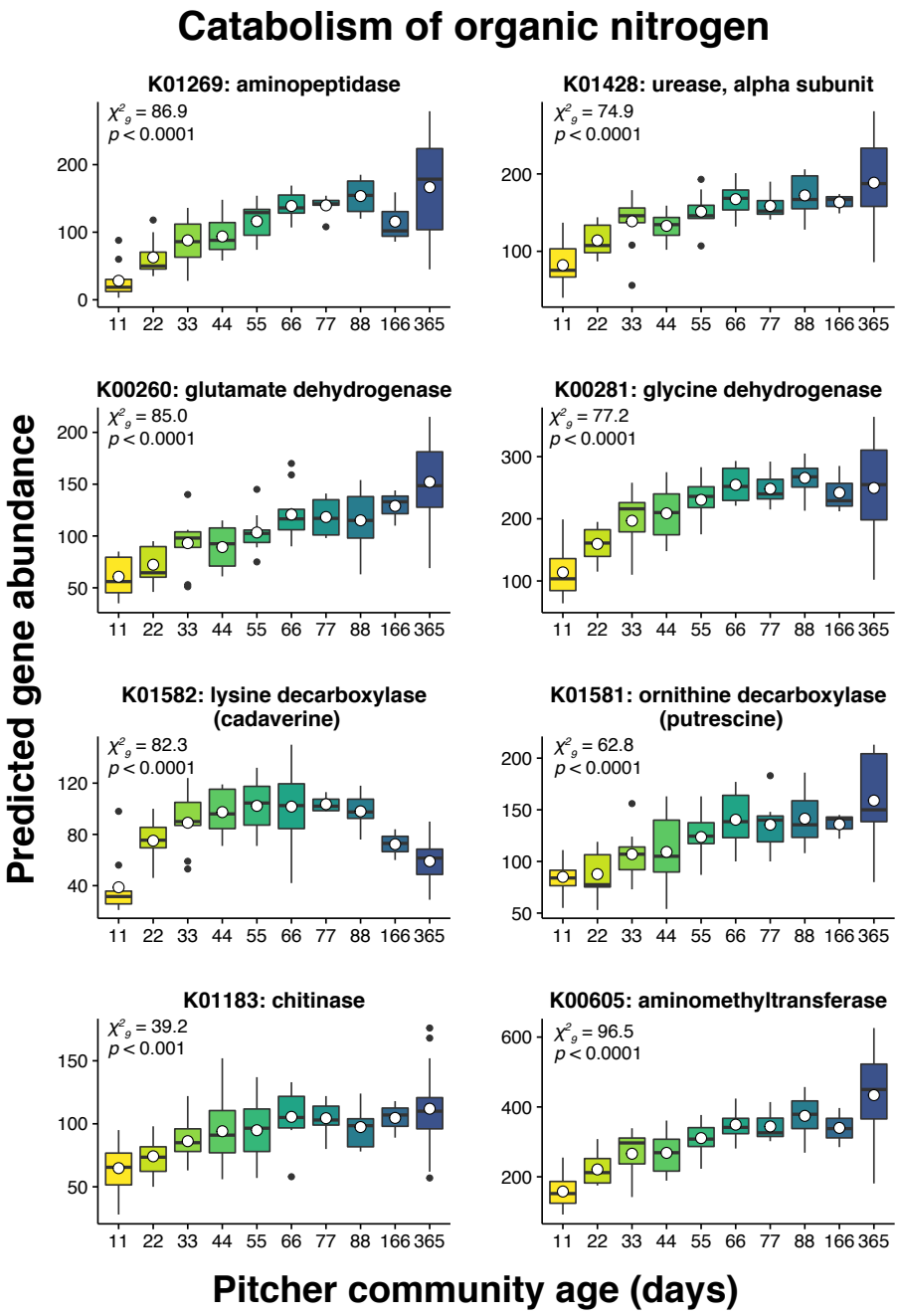

Figure 15. Temporal trends in the abundances of PICRUSt-predicted KEGG ortholog genes involved in the catabolism and uptake of organic nitrogen and its conversion to ammonium. White points denote mean values for each age class. χ^2 is the likelihood ratio test statistic for the effect of pitcher age on gene abundance. Boxplots are colored by age for consistency among figures (key in fig. 4).

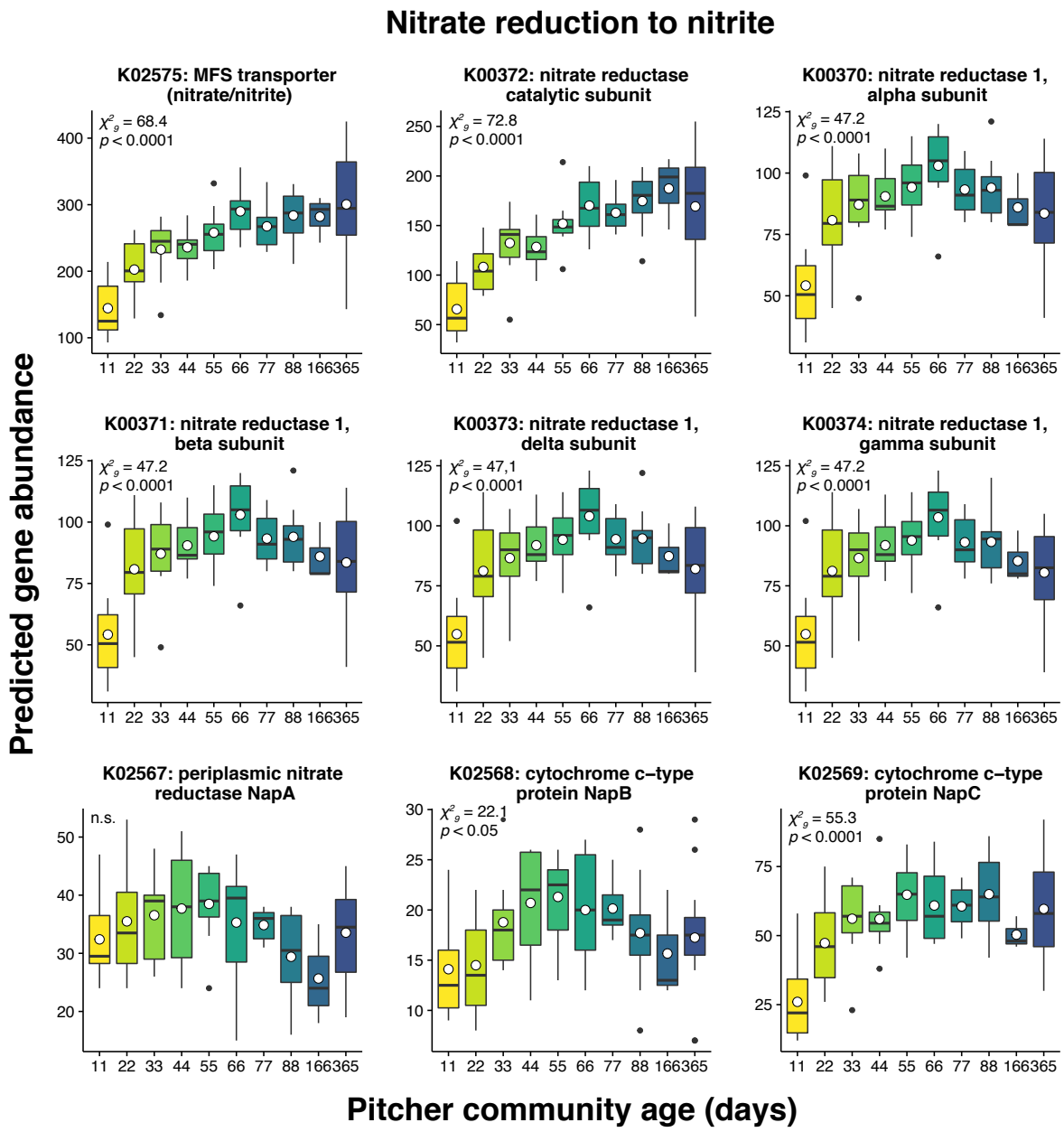

Figure 16. Temporal trends in the abundances of PICRUSt-predicted KEGG ortholog genes involved in the reduction of nitrate to nitrite. White points denote mean values for each age class. χ^2 is the likelihood ratio test statistic for the effect of pitcher age on gene abundance. Boxplots are colored by age for consistency among figures (key in fig. 4).

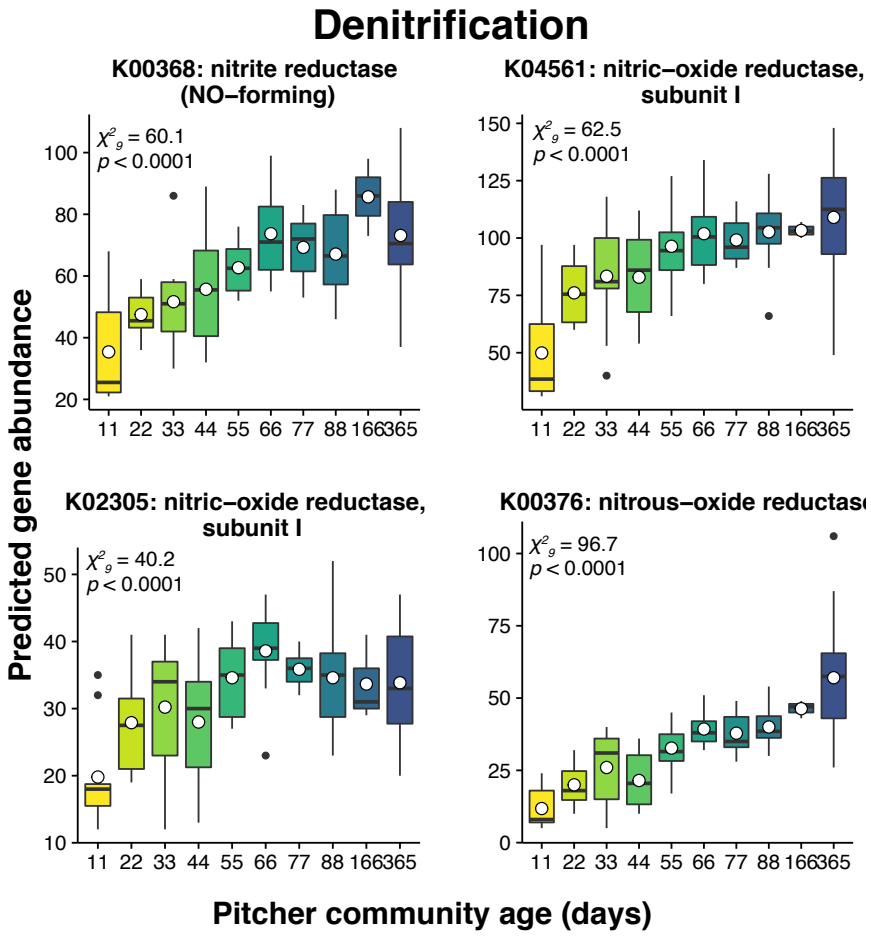

Figure 17. Temporal trends in the abundances of PICRUSt-predicted KEGG ortholog genes involved in the conversion of nitrite to gaseous N_2. White points denote mean values for each age class. χ^2 is the likelihood ratio test statistic for the effect of pitcher age on gene abundance. Boxplots are colored by age for consistency among figures (key in fig. 4).

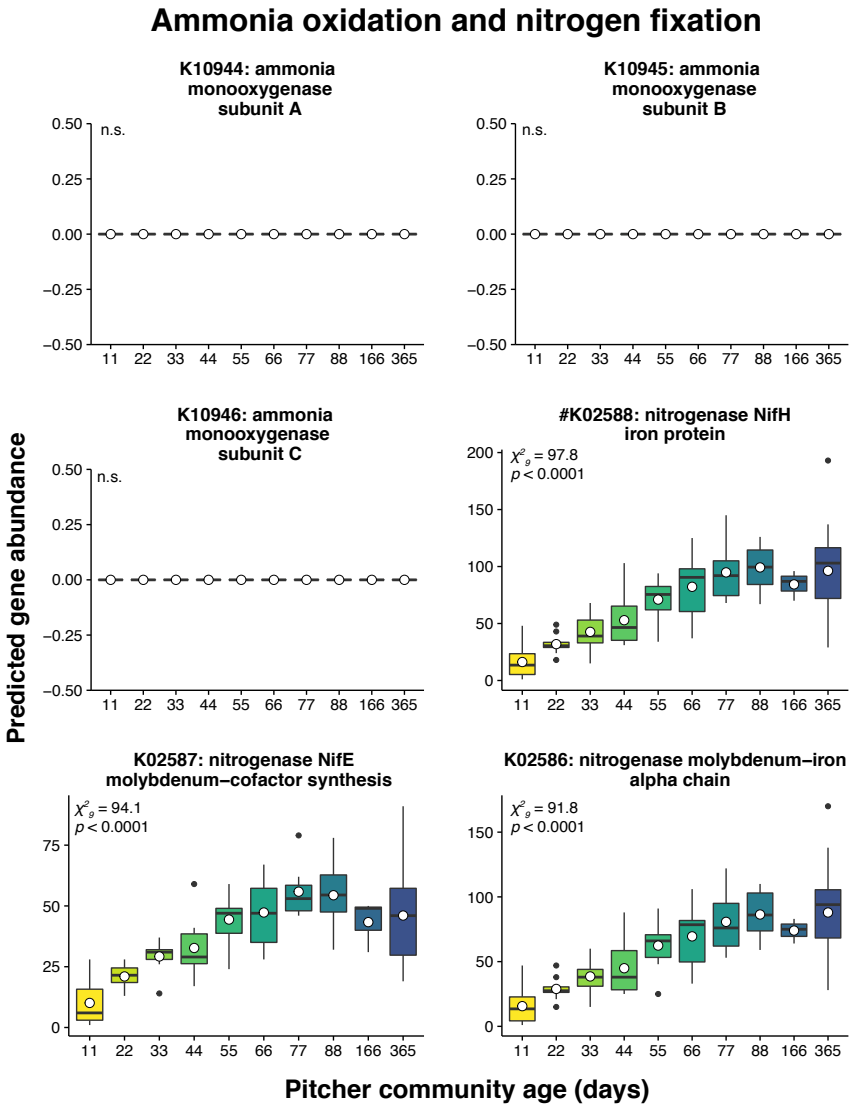

Figure 18. Temporal trends in the abundances of PICRUSt-predicted KEGG ortholog genes involved in ammonia oxidation and nitrogen fixation. White points denote mean values for each age class. χ^2 is the likelihood ratio test statistic for the effect of pitcher age on gene abundance. Boxplots are colored by age for consistency among figures (key in fig. 4).

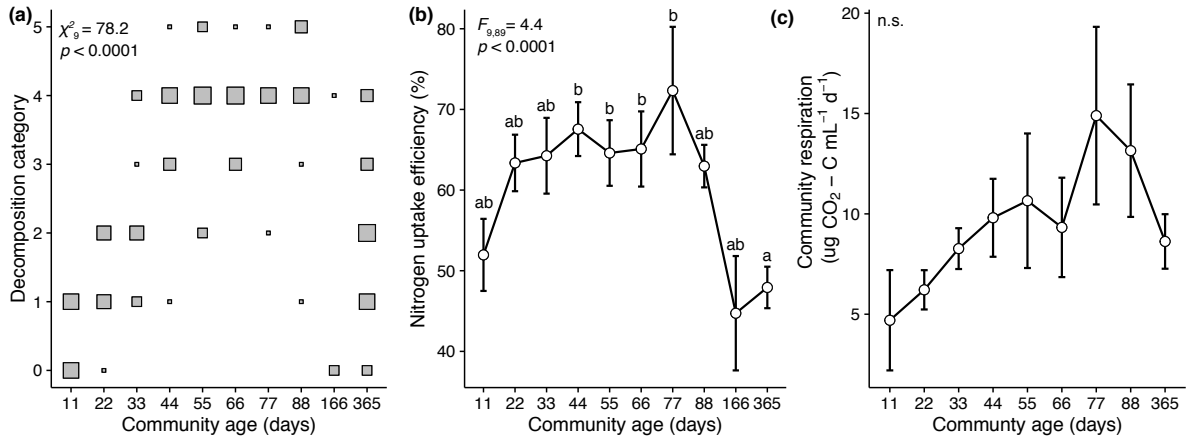

Figure 19. (a) The frequencies of decomposition classes for pitchers of different ages. Square size is proportional to relative frequency of a particular decomposition category for that age class. χ^2 is the likelihood ratio test statistic for the effect of pitcher age on the fit of a multinomial logit distribution to predict decomposition categories. (b) Pitcher nitrogen uptake efficiency changes as a function of time, and is significantly lower in late-stage pitcher leaves. (c) The potential respiration rates of pitcher communities do not significantly change over time. ANOVA *F* statistics for the effects of age are presented.

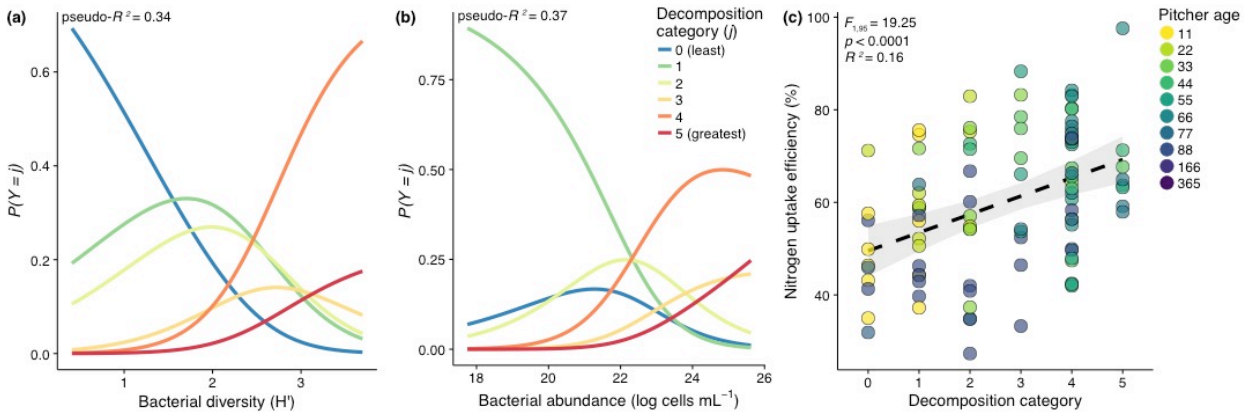

Figure 20. The probability of a pitcher having a higher decomposition score increases with both (a) bacterial diversity and (b) bacterial biomass. Curves represent multinomial logit model fits for each decomposition category. Pseudo-R^2 values are deviance-based approximations of model fit. (c) The extent of prey decomposition is positively associated with the percentage of prey-derived nitrogen found in the host leaf's foliar tissue.

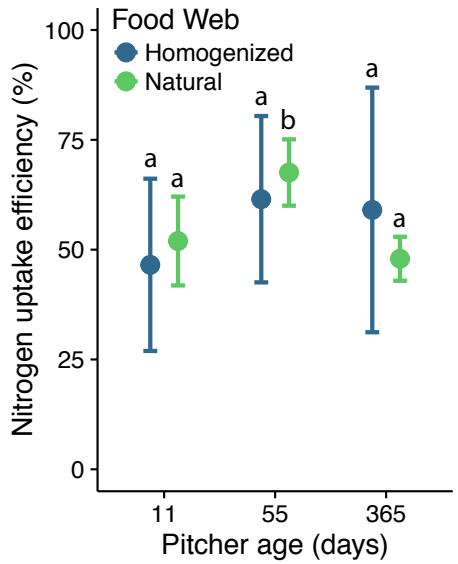

Figure 21. Homogenizing the food webs of 11, 55, and 365-day pitchers and placing them back into the plants removes the significant differences observed in natural pitcher communities of the same ages. Letters above the groups represent the within-treatment contrasts. Different letters within each group represent statistically different ($p < 0.05$) mean values between age classes.

LITERATURE CITED

Agresti, A. 2013. Categorical Data Analysis. Third edition. John Wiley & Sons, Inc., Hoboken, NJ.

Aitkenhead-Peterson, J. A., M. B. Alexander, J. A. Bytheway, D. O. Carter, and D. J. Wescott. 2015. Applications of soil chemistry in forensic entomology. Pages 283–294 *in* J. K. Tomberlin and M. E. Benbow, editors. Forensic Entomology: International Dimensions and Frontiers. CRC Press, Boca Raton, FL.

Anderson, M. J. 2001. A new method for non-parametric multivariate analysis of variance. Austral Ecology 26:32–46.

Bäckhed, F., R. E. Ley, J. L. Sonnenburg, D. A. Peterson, and J. I. Gordon. 2005. Host-bacterial mutualism in the human intestine. Science 307:1915–1920.

Baiser, B., R. S. Ardeshiri, and A. M. Ellison. 2011. Species richness and trophic diversity increase decomposition in a co-evolved food web. PLOS ONE 6:e20672.

Bell, T., J. A. Newman, B. W. Silverman, S. L. Turner, and A. K. Lilley. 2005. The contribution of species richness and composition to bacterial services. Nature 436:1157–1160.

Bokulich, N., J. R. Rideout, E. Kopylova, E. Bolyen, J. Patnode, Z. Ellett, D. McDonald, B. Wolfe, C. Maurice, R. Dutton, P. J. Turnbaugh, R. Knight, and J. G. Caporaso. 2015. A standardized, extensible framework for optimizing classification improves marker-gene taxonomic assignments. PeerJ PrePrints 3:e1502.

Breiman, L. 2001. Random forests. Machine Learning 45:5–32.

Burnham, K. P., and D. R. Anderson. 2003. Model Selection and Multimodel Inference: A Practical Information-Theoretic Approach. Springer Science & Business Media.

Butler, J. L., N. J. Gotelli, and A. M. Ellison. 2008. Linking the brown and green: nutrient transformation and fate in the *Sarracenia* microecosystem. Ecology 89:898–904.

Campbell, C. D., S. J. Chapman, C. M. Cameron, M. S. Davidson, and J. M. Potts. 2003. A rapid microtiter plate method to measure carbon dioxide evolved from carbon substrate amendments so as to determine the physiological profiles of soil microbial communities by using whole soil. Applied and Environmental Microbiology 69:3593–3599.

Caporaso, J. G., K. Bittinger, F. D. Bushman, T. Z. DeSantis, G. L. Andersen, and R. Knight. 2010a. PyNAST: a flexible tool for aligning sequences to a template alignment. Bioinformatics 26:266–267.

Caporaso, J. G., J. Kuczynski, J. Stombaugh, K. Bittinger, F. D. Bushman, E. K. Costello, N. Fierer, A. G. Peña, J. K. Goodrich, J. I. Gordon, G. A. Huttley, S. T. Kelley, D. Knights, J. E. Koenig, R. E. Ley, C. A. Lozupone, D. McDonald, B. D. Muegge, M. Pirrung, J. Reeder, J. R. Sevinsky, P. J. Turnbaugh, W. A. Walters, J. Widmann, T. Yatsunenko, J. Zaneveld, and R. Knight. 2010b. QIIME allows analysis of high-throughput community sequencing data. Nature Methods 7:335–336.

Caporaso, J. G., C. L. Lauber, W. A. Walters, D. Berg-Lyons, J. Huntley, N. Fierer, S. M. Owens, J. Betley, L. Fraser, M. Bauer, N. Gormley, J. A. Gilbert, G. Smith, and R.

Knight. 2012. Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. The ISME Journal 6:1621–1624.

Cardinale, B. J. 2011. Biodiversity improves water quality through niche partitioning. Nature 472:86–89.

Cardinale, B. J., D. S. Srivastava, J. Emmett Duffy, J. P. Wright, A. L. Downing, M. Sankaran, and C. Jouseau. 2006. Effects of biodiversity on the functioning of trophic groups and ecosystems. Nature 443:989–992.

Cardinale, B. J., J. P. Wright, M. W. Cadotte, I. T. Carroll, A. Hector, D. S. Srivastava, M. Loreau, and J. J. Weis. 2007. Impacts of plant diversity on biomass production increase through time because of species complementarity. Proceedings of the National Academy of Sciences 104:18123–18128.

Cole, J. R., Q. Wang, J. A. Fish, B. Chai, D. M. McGarrell, Y. Sun, C. T. Brown, A. Porras-Alfaro, C. R. Kuske, and J. M. Tiedje. 2014. Ribosomal Database Project: data and tools for high throughput rRNA analysis. Nucleic Acids Research 42:D633–D642.

Connell, J. H., and R. O. Slatyer. 1977. Mechanisms of succession in natural communities and their role in community stability and organization. The American Naturalist 111:1119–1144.

Connell, J. H., and W. P. Sousa. 1983. On the evidence needed to judge ecological stability or persistence. The American Naturalist 121:789–824.

Cooke, G. D. 1967. The pattern of autotrophic succession in laboratory microcosms. BioScience 17:717–721.

Copeland, J. K., L. Yuan, M. Layeghifard, P. W. Wang, and D. S. Guttman. 2015. Seasonal community succession of the phyllosphere microbiome. Molecular Plant-Microbe Interactions 28:274–285.

Cowles, H. C. 1899. The ecological relations of the vegetation on the sand dunes of Lake Michigan. Part I.-geographical relations of the dune floras. Botanical Gazette 27:95–117.

Danovaro, R., A. Dell'Anno, A. Trucco, M. Serresi, and S. Vanucci. 2001. Determination of virus abundance in marine sediments. Applied and Environmental Microbiology 67:1384–1387.

DeAngelis, D. L. 1992. Dynamics of Nutrient Cycling and Food Webs. Chapman & Hall, London ; New York.

DeSantis, T. Z., P. Hugenholtz, N. Larsen, M. Rojas, E. L. Brodie, K. Keller, T. Huber, D. Dalevi, P. Hu, and G. L. Andersen. 2006. Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. Applied and Environmental Microbiology 72:5069–5072.

Drury, W., and I. Nisbet. 1973. Succession. J. Arnold Arbor. 54:331–368.

Edgar, R. C., B. J. Haas, J. C. Clemente, C. Quince, and R. Knight. 2011. UCHIME improves sensitivity and speed of chimera detection. Bioinformatics 27:2194–2200.

Egler, F. E. 1954. Vegetation science concepts I. Initial floristic composition, a factor in old-field vegetation development with 2 figs. Vegetatio 4:412–417.

Ekschmitt, K., A. Klein, B. Pieper, and V. Wolters. 2001. Biodiversity and functioning of ecological communities — why is diversity important in some cases and unimportant in others? Journal of Plant Nutrition and Soil Science 164:239–246.

Faith, D. P. 1992. Conservation evaluation and phylogenetic diversity. Biological Conservation 61:1–10.

Fashing, N. J. 2005. Biology of Sarraceniopus darlingtoniae (Histiostomatidae: Astigmata), an obligatory inhabitant of the fluid-filled pitchers of *Darlingtonia californica* (Sarraceniaceae). Phytophaga 14:299–305.

Finn, J. T. 1982. Ecosystem succession, nutrient cycling and output-input ratios. Journal of Theoretical Biology 99:479–489.

Fisher, S. G., L. J. Gray, N. B. Grimm, and D. E. Busch. 1982. Temporal succession in a desert stream ecosystem following flash flooding. Ecological Monographs 52:93–110.

Fuhrman, J. A. 1999. Marine viruses and their biogeochemical and ecological effects. Nature 399:541–548.

Fukami, T. 2004. Assembly history interacts with ecosystem size to influence species diversity. Ecology 85:3234–3242.

Fukami, T., and M. Nakajima. 2011. Community assembly: alternative stable states or alternative transient states? Ecology Letters 14:973–984.

Gallie, D. R., and S. C. Chang. 1997. Signal transduction in the carnivorous plant *Sarracenia purpurea*. Regulation of secretory hydrolase expression during development and in response to resources. Plant Physiology 115:1461–1471.

Gouw, J., B. J. Tops, and J. Krijgsveld. 2011. Metabolic labeling of model organisms using heavy nitrogen (^{15}N). Pages 29–42 *in* K. Gevaert and J. Vandekerckhove, editors. Gel-Free Proteomics. Humana Press.

Gray, S. M., D. M. Akob, S. J. Green, and J. E. Kostka. 2012. The bacterial composition within the Sarracenia purpurea model system: local scale differences and the relationship with the other members of the food web. PLoS ONE 7:e50969.

Hättenschwiler, S., N. Fromin, and S. Barantal. 2011. Functional diversity of terrestrial microbial decomposers and their substrates. Comptes Rendus Biologies 334:393–402.

Hepburn, J. S., F. M. Jones, and E. Q. John. 1927. The biochemistry of the American pitcher plants: biochemical studies of the North American Sarraceniaceae. Transactions of the Wagner Free Institute of Science of Philadelphia 1927:1–95.

Hooper, D. U., F. S. Chapin, J. J. Ewel, A. Hector, P. Inchausti, S. Lavorel, J. H. Lawton, D. M. Lodge, M. Loreau, S. Naeem, B. Schmid, H. Setälä, A. J. Symstad, J. Vandermeer, and D. A. Wardle. 2005. Effects of biodiversity on ecosystem functioning: a consensus of current knowledge. Ecological Monographs 75:3–35.

Hubbell, S. P. 2001. The Unified Neutral Theory of Biodiversity and Biogeography (MPB-32). Princeton University Press.

Humphrey, P. T., T. T. Nguyen, M. M. Villalobos, and N. K. Whiteman. 2014. Diversity and abundance of phyllosphere bacteria are linked to insect herbivory. Molecular Ecology

23:1497–1515.

Huston, M., and T. Smith. 1987. Plant succession: life history and competition. The American Naturalist 130:168–198.

Jiang, L. 2007. Negative selection effects suppress relationships between bacterial diversity and ecosystem functioning. Ecology 88:1075–1085.

Juniper, B. B. E., R. J. Robins, and D. M. Joel. 1989. The Carnivorous Plants. Academic Press, London, UK; San Diego, CA.

Kanehisa, M., Y. Sato, M. Kawashima, M. Furumichi, and M. Tanabe. 2016. KEGG as a reference resource for gene and protein annotation. Nucleic Acids Research 44:D457–D462.

Kaye, J. P., and S. C. Hart. 1997. Competition for nitrogen between plants and soil microorganisms. Trends in Ecology & Evolution 12:139–143.

Kinzig, A. P., S. W. Pacala, and D. Tilman. 2001. The Functional Consequences of Biodiversity: Empirical Progress and Theoretical Extensions. Princeton University Press.

Kira, T., and T. Shidei. 1967. Primary production and turnover of organic matter in different forest ecosystems of the western Pacific. Japanese Journal of Ecology 17:70–87.

Kitching, R. L. 2000. Food Webs and Container Habitats: The Natural History and Ecology of Phytotelmata. Cambridge University Press.

Klappenbach, J. A., J. M. Dunbar, and T. M. Schmidt. 2000. rRNA operon copy number reflects ecological strategies of bacteria. Applied and Environmental Microbiology 66:1328–1333.

Knowles, B., C. B. Silveira, B. A. Bailey, K. Barott, V. A. Cantu, A. G. Cobián-Güemes, F. H. Coutinho, E. A. Dinsdale, B. Felts, K. A. Furby, E. E. George, K. T. Green, G. B. Gregoracci, A. F. Haas, J. M. Haggerty, E. R. Hester, N. Hisakawa, L. W. Kelly, Y. W. Lim, M. Little, A. Luque, T. McDole-Somera, K. McNair, L. S. de Oliveira, S. D. Quistad, N. L. Robinett, E. Sala, P. Salamon, S. E. Sanchez, S. Sandin, G. G. Z. Silva, J. Smith, C. Sullivan, C. Thompson, M. J. A. Vermeij, M. Youle, C. Young, B. Zgliczynski, R. Brainard, R. A. Edwards, J. Nulton, F. Thompson, and F. Rohwer. 2016. Lytic to temperate switching of viral communities. Nature 531:466–470.

Koopman, M. M., and B. C. Carstens. 2011. The microbial phyllogeography of the carnivorous plant *Sarracenia alata*. Microbial Ecology 61:750–758.

Kopylova, E., L. Noé, and H. Touzet. 2012. SortMeRNA: fast and accurate filtering of ribosomal RNAs in metatranscriptomic data. Bioinformatics (Oxford, England) 28:3211–3217.

Krieger, J. R., and P. S. Kourtev. 2012. Bacterial diversity in three distinct sub-habitats within the pitchers of the northern pitcher plant, *Sarracenia purpurea*. FEMS Microbiology Ecology 79:555–567.

Laird, D. D. 1969. Pitcher plant, *Sarracenia purpurea* L., as an ecosystem. Master's Thesis, University of British Columbia, Vancouver.

Langille, M. G. I., J. Zaneveld, J. G. Caporaso, D. McDonald, D. Knights, J. A. Reyes, J. C. Clemente, D. E. Burkepile, R. L. Vega Thurber, R. Knight, R. G. Beiko, and C.

Huttenhower. 2013. Predictive functional profiling of microbial communities using 16S rRNA marker gene sequences. Nature Biotechnology 31:814–821.

Lasky, J. R., M. Uriarte, V. K. Boukili, D. L. Erickson, W. John Kress, and R. L. Chazdon. 2014. The relationship between tree biodiversity and biomass dynamics changes with tropical forest succession. Ecology Letters 17:1158–1167.

Lee, J. J., G. F. Leedale, and P. C. Bradbury, editors. 2000. An illustrated guide to the protozoa. Second edition. Society of Protozoologists, Lawrence, KS.

Lindquist, J. A. 1975. Bacteriological and ecological observations on the northern pitcher plant, *Sarracenia purpurea* L. Ph.D dissertation, University of Wisconsin, Madison, WI.

Livermore, J. A., S. J. Emrich, J. Tan, and S. E. Jones. 2014. Freshwater bacterial lifestyles inferred from comparative genomics. Environmental Microbiology 16:746–758.

Loreau, M. 1998. Ecosystem development explained by competition within and between material cycles. Proceedings of the Royal Society B: Biological Sciences 265:33–38.

Loreau, M. 2001. Microbial diversity, producer–decomposer interactions and ecosystem processes: a theoretical model. Proceedings of the Royal Society of London B: Biological Sciences 268:303–309.

Loreau, M. 2010. From Populations to Ecosystems: Theoretical Foundations for a New Ecological Synthesis (MPB-46): Theoretical Foundations for a New Ecological Synthesis (MPB-46). Princeton University Press, Princeton, NJ.

Loreau, M., S. Naeem, P. Inchausti, J. Bengtsson, J. P. Grime, A. Hector, D. U. Hooper, M. A. Huston, D. Raffaelli, B. Schmid, D. Tilman, and D. A. Wardle. 2001. Biodiversity and ecosystem functioning: current knowledge and future challenges. Science 294:804–808.

Lotka, A. J. 1925. Elements of Physical Biology. Williams and Wilkins, Baltimore.

Love, M. I., W. Huber, and S. Anders. 2014. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biology 15:1–21.

Lozupone, C. A., J. I. Stombaugh, J. I. Gordon, J. K. Jansson, and R. Knight. 2012. Diversity, stability and resilience of the human gut microbiota. Nature 489:220–230.

Lozupone, C., M. E. Lladser, D. Knights, J. Stombaugh, and R. Knight. 2011. UniFrac: an effective distance metric for microbial community comparison. The ISME journal 5:169–172.

Lugtenberg, B., and F. Kamilova. 2009. Plant-growth-promoting rhizobacteria. Annual Review of Microbiology 63:541–556.

Maignien, L., E. A. DeForce, M. E. Chafee, A. M. Eren, and S. L. Simmons. 2014. Ecological succession and stochastic variation in the assembly of *Arabidopsis thaliana* phyllosphere communities. mBio 5:e00682–13.

McMurdie, P. J., and S. Holmes. 2013. phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. PLOS ONE 8:e61217.

Mercier, C., F. Boyer, A. Bonin, and E. Coissac. 2016. SUMATRA and SUMACLUST: fast and exact comparison and clustering of sequences. https://git.metabarcoding.org/obitools/sumatra/wikis/home.

Metcalf, J. L., Z. Z. Xu, S. Weiss, S. Lax, W. V. Treuren, E. R. Hyde, S. J. Song, A. Amir, P. Larsen, N. Sangwan, D. Haarmann, G. C. Humphrey, G. Ackermann, L. R. Thompson, C. Lauber, A. Bibat, C. Nicholas, M. J. Gebert, J. F. Petrosino, S. C. Reed, J. A. Gilbert, A. M. Lynne, S. R. Bucheli, D. O. Carter, and R. Knight. 2016. Microbial community assembly and metabolic function during mammalian corpse decomposition. Science 351:158–162.

Miller, T. E., and C. P. terHorst. 2012. Testing successional hypotheses of stability, heterogeneity, and diversity in pitcher-plant inquiline communities. Oecologia 170:243–251.

Munguia, P. 2004. Successional patterns on pen shell communities at local and regional scales. Journal of Animal Ecology 73:64–74.

Naeem, S. 1988. Resource heterogeneity fosters coexistence of a mite and a midge in pitcher plants. Ecological Monographs 58:215–227.

Nagelkerke, N. J. D. 1991. A note on a general definition of the coefficient of determination. Biometrika 78:691–692.

Nemergut, D. R., J. E. Knelman, S. Ferrenberg, T. Bilinski, B. Melbourne, L. Jiang, C. Violle, J. L. Darcy, T. Prest, S. K. Schmidt, and A. R. Townsend. 2015. Decreases in average bacterial community rRNA operon copy number during succession. The ISME Journal.

Nielsen, D. W. 1990. Arthropod communities associated with *Darlingtonia californica*. Annals of the Entomological Society of America 83:189–200.

Odum, E. P. 1969. The strategy of ecosystem development. Science 164:262–270.

Oksanen, J., F. G. Blanchet, R. Kindt, P. Legendre, P. R. Minchin, R. B. O'Hara, G. L. Simpson, P. Solymos, M. H. H. Stevens, and H. Wagner. 2015. vegan: Community Ecology Package.

Parks, D. H., and R. G. Beiko. 2010. Identifying biologically relevant differences between metagenomic communities. Bioinformatics 26:715–721.

Patel, A., R. T. Noble, J. A. Steele, M. S. Schwalbach, I. Hewson, and J. A. Fuhrman. 2007. Virus and prokaryote enumeration from planktonic aquatic environments by epifluorescence microscopy with SYBR Green I. Nature Protocols 2:269–276.

Pickett, S. T. A. 1989. Space-for-time substitution as an alternative to long term studies. Pages 110–135 *in* G. E. Likens, editor. Long-Term Studies In Ecology: Approaches and Alternatives. Springer-Verlag, New York, NY.

Price, M. N., P. S. Dehal, and A. P. Arkin. 2010. FastTree 2 – approximately maximum-likelihood trees for large alignments. PLOS ONE 5:e9490.

Pruesse, E., C. Quast, K. Knittel, B. M. Fuchs, W. Ludwig, J. Peplies, and F. O. Glöckner. 2007. SILVA: a comprehensive online resource for quality checked and aligned ribosomal RNA sequence data compatible with ARB. Nucleic Acids Research 35:7188–7196.

R Development Core Team. 2015. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.

Rideout, J. R., Y. He, J. A. Navas-Molina, W. A. Walters, L. K. Ursell, S. M. Gibbons, J. Chase, D. McDonald, A. Gonzalez, A. Robbins-Pianka, J. C. Clemente, J. A. Gilbert, S. M. Huse, H.-W. Zhou, R. Knight, and J. G. Caporaso. 2014. Subsampled open-reference clustering creates consistent, comprehensive OTU definitions and scales to billions of sequences. PeerJ 2:e545.

Scheiner, S. M. 2013. The ecological literature, an idea-free distribution. Ecology Letters 16:1421–1423.

Schulze, W., E. D. Schulze, J. S. Pate, and A. N. Gillison. 1997. The nitrogen supply from soils and insects during growth of the pitcher plants *Nepenthes mirabilis*, *Cephalotus follicularis* and *Darlingtonia californica*. Oecologia 112:464–471.

Scott, K. P., S. W. Gratz, P. O. Sheridan, H. J. Flint, and S. H. Duncan. 2013. The influence of diet on the gut microbiota. Pharmacological Research 69:52–60.

Sherr, E. B., and B. F. Sherr. 1993. Preservation and storage of samples for enumeration of heterotrophic protists. Pages 207–212 *in* P. F. Kemp, B. F. Sherr, E. B. Sherr, and J. J. Cole, editors. Handbook of methods in aquatic microbial ecology. Lewis Publishers, London, UK.

Sirota, J., B. Baiser, N. J. Gotelli, and A. M. Ellison. 2013. Organic-matter loading determines regime shifts and alternative states in an aquatic ecosystem. Proceedings of the National Academy of Sciences of the United States of America 110:7742–7747.

Sousa, W. P. 1994. Patterns and processes in communities of helminth parasites. Trends in Ecology & Evolution 9:52–57.

Srivastava, D. S., J. Kolasa, J. Bengtsson, A. Gonzalez, S. P. Lawler, T. E. Miller, P. Munguia, T. Romanuk, D. C. Schneider, and M. K. Trzcinski. 2004. Are natural microcosms useful model systems for ecology? Trends in Ecology & Evolution 19:379–384.

St. John, J. (2016). SeqPrep. https://github.com/jstjohn/SeqPrep.

Takeuchi, Y., S. Chaffron, M. M. Salcher, R. Shimizu-Inatsugi, M. J. Kobayashi, B. Diway, C. von Mering, J. Pernthaler, and K. K. Shimizu. 2015. Bacterial diversity and composition in the fluid of pitcher plants of the genus *Nepenthes*. Systematic and Applied Microbiology 38:330–339.

Tang, J., S. Luyssaert, A. D. Richardson, W. Kutsch, and I. A. Janssens. 2014. Steeper declines in forest photosynthesis than respiration explain age-driven decreases in forest growth. Proceedings of the National Academy of Sciences 111:8856–8860.

Tilman, D. 1990. Constraints and tradeoffs: toward a predictive theory of competition and succession. Oikos 58:3–15.

Tilman, D., P. B. Reich, J. Knops, D. Wedin, T. Mielke, and C. Lehman. 2001. Diversity and productivity in a long-term grassland experiment. Science 294:843–845.

Vitousek, P. M., and W. A. Reiners. 1975. Ecosystem succession and nutrient retention: a hypothesis. Bioscience 25:376–381.

Walker, L. R., and F. S. Chapin. 1987. Interactions among processes controlling successional change. Oikos 50:131–135.

Ward, J. H. 1963. Hierarchical grouping to optimize an objective function. Journal of the American Statistical Association 58:236–244.

Weis, J. J., B. J. Cardinale, K. J. Forshay, and A. R. Ives. 2007. Effects of species diversity on community biomass production change over the course of succession. Ecology 88:929–939.

Whitman, R. L., S. E. Byers, D. A. Shively, D. M. Ferguson, and M. Byappanahalli. 2005. Occurrence and growth characteristics of Escherichia coli and enterococci within the accumulated fluid of the northern pitcher plant (*Sarracenia purpurea* L.). Canadian Journal of Microbiology 51:1027–1037.

Wiens, J. A. 1977. on competition and variable environments: Populations may experience "ecological crunches" in variable climates, nullifying the assumptions of competition theory and limiting the usefulness of short-term studies of population patterns. American Scientist 65:590–597.

Yawata, Y., O. X. Cordero, F. Menolascina, J.-H. Hehemann, M. F. Polz, and R. Stocker. 2014. Competition–dispersal tradeoff ecologically differentiates recently speciated marine bacterioplankton populations. Proceedings of the National Academy of Sciences 111:5622–5627.

CHAPTER 3 | Time-variant species pools shape competitive dynamics and diversity-ecosystem function relationships

ABSTRACT

Biodiversity-ecosystem function (BEF) experiments often employ common garden designs, drawing samples from a local biota. However, the communities from which taxa are sampled may not be at equilibrium. I assembled pools of aquatic bacterial strains isolated at different time points from leaves on the pitcher plant *Darlingtonia californica* to evaluate the role of a dynamic, host-associated microbiota on the BEF relationship. I constructed experimental communities using bacteria from each time point and measured their respiration rates. Communities assembled from mid-successional species pools showed the strongest positive relationships between community richness and respiration rates, driven primarily by linear additivity among isolates. Diffuse competition was common among all communities but greatest within mid-successional isolates. These results demonstrate the dependence of the BEF relationship on the temporal dynamics of the local species pool, implying that ecosystems may respond differently to the addition or removal of taxa at different points in time during succession.

INTRODUCTION

The rates at which ecosystems cycle nutrients are predicted to be set in large part by the actions of their constituent organisms (Odum 1969, DeAngelis 1992, Loreau 2010). Over the past two decades, this conceptual unification of community and ecosystem scales has been empirically evaluated using the biodiversity - ecosystem function (BEF) framework (Loreau et al. 2001, Hooper et al. 2005, Cardinale et al. 2006). This research commonly reports a positive covariance between species richness and community biomass production and is hypothesized to be jointly driven by community members' differential contributions to ecosystem properties (selection effects) and their degree of niche overlap (complementarity effects) (Loreau and Hector 2001). Communities, however, are dynamic and can experience both gradual successional turnover and rapid state transitions (e.g., Odum 1969, Connell and Slatyer 1977a, Connell and Sousa 1983, Scheffer 2009) . Thus, a comprehensive theory linking biodiversity to ecosystem function must explicitly account for the effects of community turnover through time (Kinzig and Pacala 2001, Mouquet et al. 2002).

The majority of BEF experiments track the productivity of monocultures and polycultures assembled from taxa randomly drawn from a natural biota or from *ad hoc* combinations of experimentally tractable organisms such as algae or protists. In these experimental communities, the magnitude and drivers of the BEF relationship are often found to change over time (Tilman et al. 2001, Fox 2004, Ruijven and Berendse 2005, Spehn et al. 2005, Bell et al. 2005, Weis et al. 2007, Cardinale et al. 2007, Gravel et al. 2011, Doherty et al. 2011, Reich et al. 2012, Lasky et al. 2014). While these experiments have contributed fundamental insights into the temporal dynamics of BEF relationships, they do not account for a dynamic

species pool. In other words, the groups of species used to seed these communities represent either a *snapshot* of a natural community at a particular point in time (fig. 1A) or a collection of species that may be differentially distributed across time such that two species added into a community do not necessarily co-occur under natural settings (fig. 1B). Communities assembled from a dynamic species pool, however, may show different BEF relationships over time due to the shifting identities and interactions of the constituent taxa (fig. 1C).

Whereas most biodiversity-ecosystem function experiments have been conducted using plant and algal communities, the framework has also been successfully extended to other taxonomic groups (van der Heijden et al. 1998, Lefcheck and Duffy 2015). In particular, bacterial communities have been the subject of numerous BEF studies, owning to their importance in regulating ecosystem processes (Bell et al. 2005, Jiang 2007, Salles et al. 2009, Langenheder et al. 2010, Gravel et al. 2011, Venail and Vives 2013). Because natural bacterial communities often exhibit marked turnover through time (Redford and Fierer 2009, Koenig et al. 2011) they provide an opportunity to investigate the strength and drivers of the BEF relationship over a temporal gradient.

Carnivorous pitcher plants in the family Sarraceniaceae are a group for which bacterial communities provide a particularly critical function. These plants have evolved to capture arthropod prey by means of a conical leaf in which trapped insects are drowned by fluid secreted from the host (Lloyd 1942, Juniper et al. 1989). Digestion is facilitated both by enzymes produced by the plant (Hepburn et al. 1927, Gallie and Chang 1997) and by the bacteria residing in the fluid (Hepburn et al. 1927, Lindquist 1975, Butler et al. 2008). These bacterial communities are dynamic and change predictably over time such that they are more similar between two leaves at the same time point than within a single leaf at two different points in time (Koopman et al. 2010).

The pitcher plant *Darlingtonia californica* (Torr.) is hypothesized to rely heavily on bacteria for prey digestion (Hepburn et al. 1927). The pitcher leaves of this species are produced at regular intervals throughout the June-October growing season and are sterile prior to opening (Armitage, unpublished data). Once the leaves fully develop, they quickly begin trapping insects, and bacterial biomass skyrockets to over 10^9 cells mL^{-1} (Armitage, unpublished data). After approximately two months, a leaf slowly ceases to capture prey yet remains photosynthetically active for a second growing season. Bacterial diversity in *Darlingtonia* pitchers also changes over time, as has been documented by both culture-independent molecular approaches as well as among bacterial cultures isolated from different aged leaves (Armitage, unpublished data). These temporal isolates provide a unique opportunity to experimentally test the relationship between biodiversity and ecosystem functioning along a natural microbial successional gradient.

My goals for this study were twofold. First, I investigated whether the contribution of bacterial richness to rates of carbon mineralization changed over time along a natural successional gradient in *Darlingtonia* leaves. I anticipated results mirroring those of (Lasky et al. 2014) and (Weis et al. 2007), wherein the positive effect of species richness on ecosystem function decreased during succession. Second, I used these data to estimate the relative influences of individual species and their interspecific interactions (such as competition) on the BEF relationship (Bell et al. 2009). The strength of interspecific competition among bacterial

strains growing in a polyculture can be approximated as the difference between the community's predicted respiration in the absence of any interference (i.e., the sum of the strains' monoculture respiration rates) and the community's realized respiration rate, given the mono- and polycultures have equal total starting densities (Foster and Bell 2012, Keddy 2012). If strains in a polyculture do not inhibit one another through resource competition or direct antagonism, then the community's rate of carbon respiration will not significantly differ from the additive monoculture expectation (Foster and Bell 2012). This measure of competitive inhibition is anticipated to increase over time if, for instance, a competition-colonization tradeoff results in the dominance of early pitcher leaves by less-competitive, ruderal taxa which are later excluded by superior competitors (Connell and Slatyer 1977b). Alternatively, the bacterial taxa dominating late-stage pitchers may be specialists on recalcitrant carbon resources and therefore may not contribute significantly to ecosystem function, compared to early, fast-growing colonists. In this case, I anticipated a negative trend in competitive inhibition over time. In order to experimentally test these hypotheses, I assembled synthetic microbial communities using pools of bacterial strains isolated from a cohort of pitcher leaves at regular intervals and measured their rates of carbon mineralization.

METHODS

Sample collection & strain isolation

I collected samples during 2014 in a single large patch of *Darlingtonia californica* pitcher plants growing in the Butterfly Valley Botanical Area, Plumas National Forest (Plumas Co, CA). *Darlingtonia* plants begin producing leaves in mid-June, which remain closed and sterile until they reach their maximum height. Upon prey capture, leaves secrete a fluid that facilitates bacterial growth (volume = 7 ± 0.94 mL; pH = 6.18 ± 0.17). At the start of the growing season in 2014, I identified and tagged 5 emerging pitcher leaves of equivalent developmental stage on individual plants spaced at least 50 m apart. The exact date of each pitcher's opening was recorded, allowing me to return to tagged leaves throughout their lifespan and know the precise age of their associated communities. I visited this cohort of leaves every 11 days to collect samples of pitcher fluid. To remove pitcher fluid without damaging the leaf, I used an insect aspirator connected to a flexible tube to gently agitate the pitcher contents and then siphon 0.5 mL pitcher fluid into a sterile centrifuge tube. I performed this on all 5 pitchers, making sure to sterilize the tubing and rubber stopper with 70% ethanol and a 4% bleach-tween solution between samples. When pitcher leaves did not contain enough fluid to be aspirated I would add 2 mL of sterile phosphate buffer (pH 6) to the leaf, wait 5 minutes, gently agitate the fluid, and then collect a 0.5 mL sample. The centrifuge tubes were placed on ice and immediately returned to the lab. I replaced the fluid collected from each pitcher with an equal volume of sterile phosphate buffer to minimize damage to the leaf tissue and its associated aquatic food web.

I spread dilutions of each leaf's contents onto R2A agar plates (pH 6) and incubated them at 25° C. After 48 hours, I isolated all visually distinct colony phenotypes onto their own plates based on color and colony morphology. I verified the genetic basis for these isolates' colony morphologies by serially re-plating them three times and observing no phenotypic changes. I selected bacterial strains to be used in the experiment based on their ability to grow on R2A agar and their distinct colony morphologies/pigmentations, cell morphologies (viewed under 1000×

phase contrast microscopy), and behavior in liquid R2A culture (e.g., biofilm-forming, relative growth rates). Although 16s ribosomal RNA identification of colony isolates would verify the unique identities of each strain, such information would not have provided much additional insight. Even if ribosomal RNA sequences suggested two isolates belonged to the same operational taxonomic unit (defined as 97% similarity in 16s rRNA), there is no guarantee that these isolates share similar ecological niches, particularly given their divergence in phenotypic characters (e.g., Shapiro et al. 2012).

From this remaining pool of bacterial isolates, I selected the 10 most abundant strains to represent the pool of isolates for a given pitcher age. I repeated this process using the same five pitcher leaves every 11 days until day 88, and then returned to the population in early 2015 to resample the leaves. Pooling pitchers of a given age was necessary, as there were never 10 distinct cultivable phenotypes fitting my selection criteria within a single leaf, although I attempted to minimize this bias by preferentially selecting strains originating from the same pitcher leaves. Despite this caveat, strain overlap among pitchers appeared high based on colony morphology, and both plating and rRNA amplicon surveys of pitchers revealed their communities to strongly cluster by age (Armitage, *chapter 2*). This trend was also observed in a study on the related species *Sarracenia alata* (Koopman et al. 2010). In a study from the same population of *Darlingtonia*, 147 bacterial isolates were isolated from 44-day and 365-day pitchers using the same methods as this study. These strains were assigned a taxonomy based on their 16s ribosomal rRNA genes. The genera of nearly every isolate matched with the 25 most abundant taxa recovered from culture-independent 16s amplicon sequencing of similar-aged pitchers (Armitage, *chapter 2*). To estimate a leaf's rate of prey capture, I collected prey material from 10 pitcher leaves of identical age and divided its dry mass by the number of days it had been open. Supplemental figure S1 provides a graphical walkthrough of the experimental procedure.

Microcosm experiment

I combined the 10 strains isolated from each time point into 1-, 2-, 5-, and 10-strain communities using the random partitions design introduced by Bell *et al*. (Bell et al. 2009). My experiment consisted of 4 partitions (P), each containing 4 species richness treatments (R) and $10/R$ randomized communities within each $P \times R$ treatment (supplemental fig. S2). Every experimental community was replicated 3 times. This design ensures that all species are equally represented within and among richness levels. It also permits the statistical separation of species effects and richness effects on ecosystem processes without the need for measuring the contribution of an individual species to the properties of the polyculture. This enables the user to estimate species' contributions to emergent ecosystem properties (e.g. carbon mineralization rates). Furthermore, it relaxes the requirement for a full-factorial experimental design, which becomes intractable as the number of species increases. In total, I assembled 216 communities per time point, resulting in a total of 1944 cultures spanning 9 source community ages and 4 levels of richness.

The bacterial strains used to seed the experimental communities were grown in shaken R2A broth to mid-log phase and diluted using PBS to an OD_{600} of 0.25. Aliquots of these isolates were then added into microcentrifuge tubes and centrifuged at $10{,}000 \times G$ for 5 minutes at 4° C.

Next, I removed the supernatant broth from the tubes and washed the pellet of R2A medium by adding 1 mL sterile PBS, re-suspending the pellet, and centrifuging it again. This step was repeated twice and the pellets were left to starve for 2 hours at room temperature to consume any residual medium. These pellets were then agitated and seeded into 1.2 mL 96-well plates containing 700 µL of sterile growth medium. This medium contained an M9 salt solution (NH_4Cl 1 g L^{-1}, Na_2HPO_4 6 g L^{-1}, KH_2PO_4 3 g L^{-1}, NaCl 0.5 g L^{-1}, pH 6.0) into which I added 3 g L^{-1} of powder from freeze-dried crickets that had been ground and autoclaved. Each strain was then introduced into its community at the volume required to keep the total number of cells across richness treatments equal (100 µL total inoculum). Once assembled, plates were sealed using a sterile, perforated rubber gasket. Over this gasket I placed an inverted 96-well plate containing cresol red dye and $NaHCO_3$ set in 1% purified agar. CO_2 respired by the microbial communities passes through the perforated gasket where it reacts with the agar following the equation $CO_2 + H_2O + HCO_3^- \rightarrow 2\ CO_3^- + 3\ H^+$. This redox reaction induces a colorimetric change in the dye that can be read on a 96-well spectrophotometer (Campbell et al. 2003).

All experimental communities from a source pitcher age were run simultaneously on at 25° C for three days. I estimated percentage of CO_2-C in each agar well by measuring its absorbance at 570 nm and comparing these values to a calibration curve. Next, I estimated the rate of carbon respired by each community using the ideal gas formula

$$\mu g\ CO_2 - C\ mL^{-1}\ d^{-1} = \left\{ \frac{\frac{\%CO_2}{100} \cdot H \cdot \frac{44}{22.4} \cdot \frac{12}{44} \cdot \frac{273}{273+T}}{V} \right\} \cdot \frac{1}{t} \quad (1)$$

where H is the headspace volume of the culture well (400 µL), T is the temperature (25° C), V is the volume of medium (800 µL), and t is the incubation time (3 days). After three days, I removed the colonies and plated the 10-species communities onto agar in order to assess whether extinctions had taken place. This procedure was repeated every 11 days using different pools of isolates collected from the same pitchers.

Statistical analyses

To assess how drivers of the BEF relationship differed among time points, I fit a linear model to community respiration rates (Bell et al. 2009). This model took the form

$$y = \beta_0 + \beta_{LR} x_{LR} + \beta_{NLR} x_{NLR} + \left(\sum_i^S \beta_i\, x_i \right) + \beta_Q x_Q + \beta_M x_M + \varepsilon \quad (2)$$

where y is a community or ecosystem process (e.g., respiration rate), β_{LR} is the effect of species richness measured on a continuous scale (linear richness, x_{LR}), β_{NLR} is the effect of species richness measured on a categorical scale (non-linear richness, x_{NLR}), β_i is the impact of an individual species presence in the productivity of its community, β_Q is the effect of the particular species pool used in each $P \times R$ treatment, and β_M is the effect of a particular species composition within each species pool, β_0 is the intercept, and ε is the error term.

By estimating the linear richness term prior to the nonlinear richness and species' impact terms, the latter two terms become orthogonal. The species impact (β_i) terms sum to zero and reflect the relative influence an individual strain exerts on the community's respiration. The nonlinear richness term (β_{NLR}) can be interpreted as the magnitude of deviations from linear species richness effects. Nonzero values of β_{NLR} reflect the influence of facilitative and competitive interactions on ecosystem processes when all pairwise interactions among species cannot be statistically evaluated. I used least squares to estimate the model coefficients and an F-test to determine the statistical significance of each variable. Because species impact models consisted of 10 parameters, the overall significance of the parameterized β_{NLR} and β_i models were assessed using a likelihood ratio test against intercept-only null models. Model terms were entered in the order in which they appear in equation 2: nonlinear richness (β_{NLR}) and species impacts (β_i) were estimated from the residuals of the model containing the linear richness (β_{LR}) term.

I estimated the effects of the source pitchers' ages and prey capture rates, as well as experimental communities' richness on rates of CO_2 respiration using linear regression. Source community age and prey capture rate were perfectly collinear and so their effects could not be simultaneously estimated. To aid in the interpretation of interactions, predictors were centered to their mean values prior to model fitting. I assessed the pairwise differences among community ages using Tukey's range test ($\alpha = 0.05$).

I estimated the extent to which strains inhibit one another's potential CO_2 production in polyculture by calculating the difference between a community's predicted and observed respiration rates. The predicted values were calculated by summing all community members' average monoculture respiration rates. The difference between a polyculture's predicted and observed respiration values will equal zero if there are no inhibitory effects between members of the community (i.e., all taxa in a polyculture perform as well they do in monoculture) (Foster and Bell 2012, Keddy 2012, Fiegna et al. 2015). Alternatively, direct antagonism (e.g., antibiotic production) or resource competition is anticipated to result in respiration rates less than the additive prediction (Foster and Bell 2012). Similarly, facilitative interactions such as cross feeding are expected to increase polyculture respiration compared to predicted rates (Foster and Bell 2012). I used ANCOVA to test the null hypothesis that the mean differences between predicted and observed respiration rates were equal among community ages, controlling for richness effects. Pairwise differences between centered predictor variables were assessed using Tukey's range test. All models were fit using R v3.1 (R Development Core Team 2015).

Community metabolic fingerprinting

I measured the carbon metabolic profiles of each 10-species community using the GN2 microplate assay (Biolog, Inc.). I inoculated each dilute, starved, 10-strain mixture onto the 96-well GN2 plate, which consisted of 95 unique carbon compounds and a blank. The inoculum contained dye that turns violet when reduced by NADPH. The optical absorbance at 570 nm of these wells is proportional to the productivity of the community on the particular substrate. The pattern of metabolite use provides a unique metabolic "fingerprint" that can serve as a basis for the comparison of different communities. I performed this assay on each 10-species community

in triplicate. After three days of incubation at 25° C I visually scored each well. If a violet color had developed in the same well across the three replicates, I scored that substrate as an electron donor for at least one strain in the community. With these data, I constructed a logical matrix from which I calculated the pairwise Jaccard distances between each community's carbon metabolic profiles. Next, I ordinated these distances using Principal Coordinates Analysis (PCoA) and visually assessed the results on a plot of the first two principal coordinates.

Pairwise antagonism assay

I performed spot assays to determine whether a particular bacterial strain directly inhibits the growth of a co-occurring strain. I created lawns of focal strains by spreading log-phase broth cultures onto two plates containing R2A agar and letting them dry for three hours. Onto these lawns I spotted 2 µL log-phase broth culture of each co-occurring isolate using a flame sterilized 48-pin replicator. Each spot was replicated four times on the same plate, resulting in 8 cross-inoculations per strain pair (excluding sterile blanks). After 24 hours at 25° C, I searched for zones of clearing surrounding a colony using a dissecting microscope. I considered the spotted strain to be inhibitory to the focal strain if unambiguous zones of clearing surrounded at least 6 replicate spots.

RESULTS

After 3 days, I found an average of 6.9 (se = 0.18, range = 5-9) bacterial strains remaining in each 10-species community, and there were no significant differences in the proportions of surviving strains among source community ages ($F_{8,27} = 2.3$, $p = 0.06$). Thus, although the strains' relative abundances changed throughout the incubation period, no single, dominant strain was able to exclude the majority of others. I detected significant differences between the mean respiration rates of bacterial communities isolated from pitcher leaves of different ages (table 1, fig. 2). Post-hoc analysis revealed respiration rates to be greatest among bacterial communities isolated from pitcher leaves between 22 and 55 days old (fig. 2). This pattern was consistent under all four richness treatments, although there was a general tendency for variance in respiration rates among treatments to increase when more strains were present (fig. 2). Bacterial richness had a significantly positive effect on overall respiration rates, independent of age ($\beta_R = 0.05 \pm 0.007$; table 1), although there was a significant interaction between richness and source community age (table 1). Likewise, the mean prey capture rate of pitchers within an age cohort was significantly positively associated with overall rates of carbon respiration ($\beta_{capture} = 0.06 \pm 0.012$; table 1).

The effect of linear richness (β_{LR}) on respiration rates was significantly positive for all source community ages except those from days 88 and 365 (fig. 3). This positive effect of richness on respiration was greatest for isolates from pitcher leaves between 22 and 66 days old, and tended to increase from days 11 to 22 and then slowly decrease towards zero throughout the rest of the pitchers' lifespan (fig. 4A). For each bacterial isolate pool, I detected both significant nonlinear richness effects (β_{NLR}) and individual species (β_i) effects (table S1), and the relative influence of nonlinear richness effects was greater than overall species effects for the majority of time points (fig. 4B).

The average differences between expected and observed respiration rates initially increased between samples collected from 11-day and 22-day pitchers, and then declined with source community age (fig. 5). The magnitude of this inhibitory effect increased with species richness (fig. 5, table 1). I only detected 12 antagonistic interactions between eight pairs of strains (out of 405 total). These interactions occurred only in 11- and 44-day source pools. Furthermore, there was no detectable temporal trend among source pool ages in either the total number of carbon substrates utilized (fig. S3) or in their multivariate Jaccard similarities (fig. S3).

DISCUSSION

Dynamic species pools impact BEF relationships

I encountered a unimodal association between the age of the bacterial source community and rates of carbon mineralization, independent of taxonomic richness. This implies that when placed into identical environments, bacterial strains isolated from leaves of intermediate ages (22 to 55 days old) were better able to mineralize carbon in the growth medium. This result could not be explained by differences in the taxon pools' carbon metabolic profiles. Rather, the increase in strains' average respiration rates during this period coincided with the greatest rates of prey capture by the pitcher leaf. It is possible that the relatively low respiration rates of late-stage source communities reflect an adaptive strategy for living in nutrient-replete environment. This is supported by the observation of lower average ribosomal RNA copy numbers — a trait correlated with growth rate— as succession proceeds (Nemergut et al. 2015, Armitage *chapter 2*) . However, information on all strains' relative performances across different nutrient concentrations would be required to experimentally verify this hypothesis.

The effects of a community's richness on respiration rates were generally positive, but varied over time such that the slope estimates were unimodal, peaking in pitcher leaves of intermediate age. These positive BEF relationships appeared to be driven by linear, additive contributions of taxa, as evidenced by the positive linear richness terms and weak species impact terms. This observation implies that, on average, community members had similar relative respiration rates and moderate levels of niche overlap. This interpretation is supported by the rarity of both competitive exclusions and direct antagonistic interactions between taxa in all species pools. However, competitive inhibition of a community's potential maximum respiration rate was common in all 5- and 10- species polycultures and peaked in communities assembled from intermediate-aged pitcher leaves — an observation supported by significant nonlinear richness terms in many of the communities. This general pattern of diffuse competition in polycultures may not be typical of bacteria within pitcher plants due to my isolation procedure (Foster and Bell 2012). By using a single medium to isolate bacteria, it is likely that the strains I sampled were more phenotypically similar to one another than to a random sample of all bacteria in a pitcher leaf. Thus, the strains used in this study should be considered members sampled from a guild of aerobic, heterotrophic bacteria and are expected to compete with one another for resources and express similar rates of carbon respiration. A useful follow-up to this experiment would investigate the effects of increasing the phenotypic diversity of the species pool by adding strains obtained using a broader range of media.

Competition among isolates is predicted to decrease in bacterial communities over time due to divergent evolution (Lawrence et al. 2012). The relatively low levels of competitive inhibition among strains from late-stage pitcher leaves may represent indirect evidence of divergence. This scenario is plausible, given the rapid generation times and population sizes of the isolates. A recent study by Fiegna et al. (Fiegna et al. 2015) showed that the experimental evolution of bacterial isolates over 5 weeks can result in the relaxation of competition. Although such an effect is possible in natural systems, its demonstration would require tracking individual bacterial lineages over time and regularly assaying their competitive interactions. Miller and Kneitel attempted this by measuring the degree of competitive inhibition of four bacterial colony morphotypes isolated from the same pitcher leaves 7 days and 42 days after opening (Ellison et al. 2003). The authors found that the competitive abilities (relative to a common bacterial competitor) of two of the four strains decreased with pitcher age while two did not appear to change (Ellison et al. 2003). These results match my observation of increased competitive inhibition of potential respiration on a similar timescale (11-day and 44-day leaves). Besides relaxing competition, the evolution of specialization via divergent selection has been observed to increase net BEF effects in experimental populations of plants (Zuppinger-Dingley et al. 2014), fishes (Harmon et al. 2009), and bacteria (Gravel et al. 2011). Although population divergence was not tracked in my experiment, my results do not support the role of increased specialization increasing net BEF effects.

To date, few studies have directly estimated the impacts of natural successional dynamics in the context of biodiversity and ecosystem functioning (Wacker et al. 2009, Petermann et al. 2010, Lasky et al. 2014). Using 15 years of observational data from regenerating tropical forest plots, the Lasky *et al.* documented a decreasing effect of species richness on rates of aboveground biomass production in mid- and late-successional tropical forest plots (Lasky et al. 2014). These results matched both theoretical predictions (Kinzig and Pacala 2001) and experimental studies in which diversity effects were tracked over time within individual microcosms without immigration (Bell et al. 2005, Weis et al. 2007). In contrast, Wacker *et al.* (2009) found increasing diversity effects on ecosystem function during succession in temperate herbaceous plant communities. As in my study, however, they encountered the relatively low net diversity effects at early-successional time points (Wacker et al. 2009). My results conform to those of other BEF time-series experiments, despite marked differences in design. In concert, our findings challenge the common observation that the effects of richness on productivity become more positive over time (Cardinale et al. 2007), though further investigation is necessary to uncover the mechanisms leading to these contrasting outcomes.

Potential drivers of BEF relationships

All previous experimental studies measuring the BEF relationship over time do so using communities with finite resources and no immigration. Consequently, the closed nature of these systems may have influenced the resulting community dynamics and ecosystem processes. My study, however, measured individual "snapshots" of communities assembled from a temporal gradient of natural, open source pools. Furthermore, my microcosms were assembled with equal starting concentrations of bacterial strains and resources, which may have prevented communities from becoming resource limited prior to measuring their respirations. Despite these differences, however, decreases in BEF relationships of both static species pools over time and

dynamic species pools at a single time point suggest that similar ecological processes may influence these patterns.

Shifts towards non-significant or negative BEF relationships can be caused by functional redundancy, wherein the majority of taxa within a community are functionally equivalent and the loss of any member will not result in a decrease in ecosystem functioning (Allison and Martiny 2008, Reich et al. 2012). In my communities, low variation in respiration among monocultures from 11-day and >66-day source pools supports increased functional redundancy in these communities. This is because functional redundancy requires redundant species to contribute equally to ecosystem function. A community comprised of functionally redundant taxa should also show a marked difference between predicted and observed respiration rates, since redundant taxa will compete for shared carbon resources. In my experiment, however, communities assembled from 11-day and >66-day leaves had relatively low levels of competitive inhibition. Furthermore, a fair assessment of functional redundancy would require information on the phenotypic overlap of individual strains (Allison and Martiny 2008).

A second mechanism for generating non-positive BEF relationships is the negative selection effect (Loreau and Hector 2001, Jiang 2007, Jiang et al. 2008). This phenomenon occurs when the competitively dominant taxa in a community are those that contribute least to the measured ecosystem function. Like the positive selection effect in BEF literature (Loreau and Hector 2001), the negative selection effect implies that competitive interactions will drive communities towards monodominance. Two lines of evidence from my experiments suggest that the negative selection effect does not occur in late-stage source communities. First, I did not detect any trends towards increasing rates of competitive exclusions in late-stage source communities. Second, these communities had some of the lowest nonlinear richness (species interaction) effects signifying a low contribution of species interactions to respiration rates.

Conclusions

In leaves of the pitcher plant *Darlingtonia californica,* bacterial carbon mineralization is a process critical for the plant's acquisition of prey-derived nutrients. In this and many other host-associated systems, the diversity of the microbiota is predicted to influence rates of ecosystem flux. Using bacterial strains isolated from pitcher leaves at regular intervals over a one-year period, I tested whether the successional dynamics of pitcher leaves' natural bacterial source communities would affect the relationship between an experimental community's taxonomic richness and respiration rate. I determined the magnitude of this relationship to be unimodal with source community age. This positive richness effect on respiration was driven both by linear, additive species impacts and diffuse competition among strains in polyculture. This study represents an initial attempt to integrate biodiversity-ecosystem function effects over successional time and concludes that the functional consequences of diversity loss on a host or ecosystem can be time-dependent. Future studies on biodiversity-ecosystem function relationships are encouraged to adopt a dynamic species pool framework to increase the generalizability of their results.

Table 1. ANCOVA results for total respiration and respiration differences. Respiration rates were log-transformed to satisfy homoscedasticity. Richness and prey capture rates were treated as a continuous variables and age as a categorical variable with contrasts summing to zero. Marginal (type 3) sums-of-squares (SS) are presented.

Response	**Covariate**	**DF**	**SS**	***F***	***p***	$\mathbf{R^2}$
Log respiration rate	Intercept	1	17.35	317.35	< 0.001	0.17
	Source community age	8	12.89	12.89	< 0.001	
	Species richness	1	47.03	47.03	< 0.001	
	Interaction term	8	14.52	3.311	< 0.001	
	Residuals	1926	1056			
Response	**Covariate**	**DF**	**SS**	***F***	***p***	$\mathbf{R^2}$
Log respiration rate	Intercept	1	6.85	11.28	< 0.001	0.07
	Average leaf capture rate	1	16.58	27.32	< 0.001	
	Species richness	1	0.28	0.46	0.5	
	Interaction term	1	4.44	7.32	< 0.01	
	Residuals	1940	1177.32			
Response	**Covariate**	**DF**	**SS**	***F***	***p***	$\mathbf{R^2}$
Expected - observed respiration	Intercept	1	689	154	< 0.001	0.89
	Source community age	8	116	3.3	< 0.01	
	Species richness	1	7480	1675.0	< 0.001	
	Interaction term	8	1133	31.7	< 0.001	
	Residuals	270	1206			

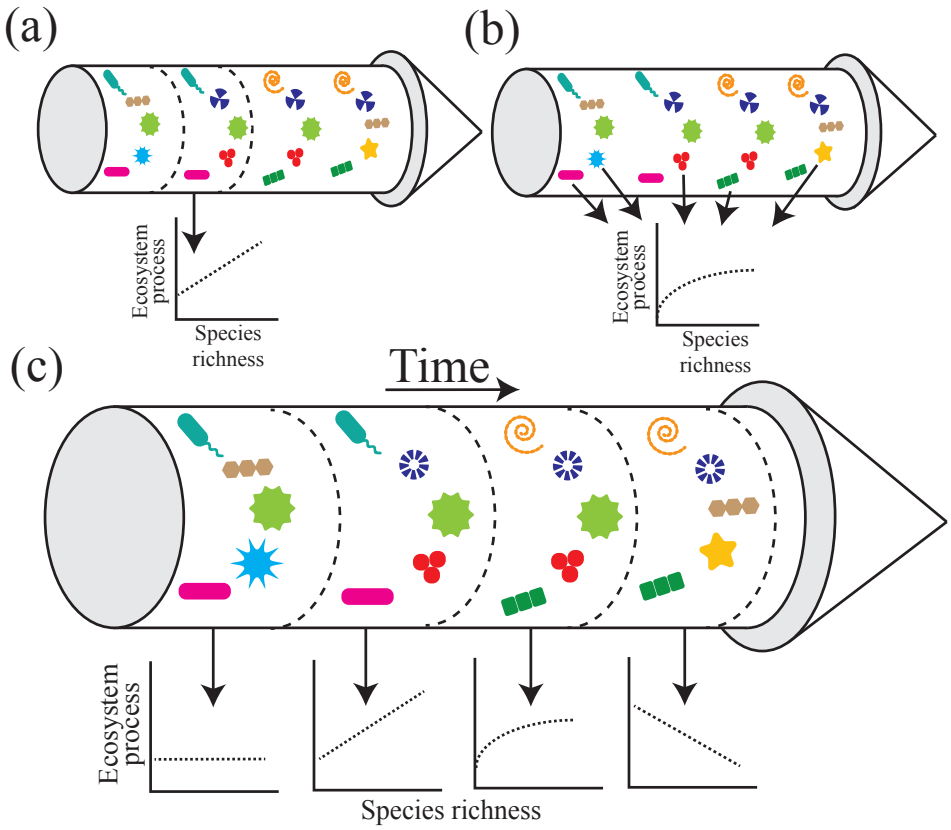

Figure 1. Conceptual diagram of species pool selection in biodiversity-ecosystem function experiments. Species pools are typically either chosen by sampling a community at a single point in time (a) or from species, which may not co-occur at a particular time point (b). Far fewer studies have taken the approach of measuring BEF relationships over a temporally dynamic species pool (c).

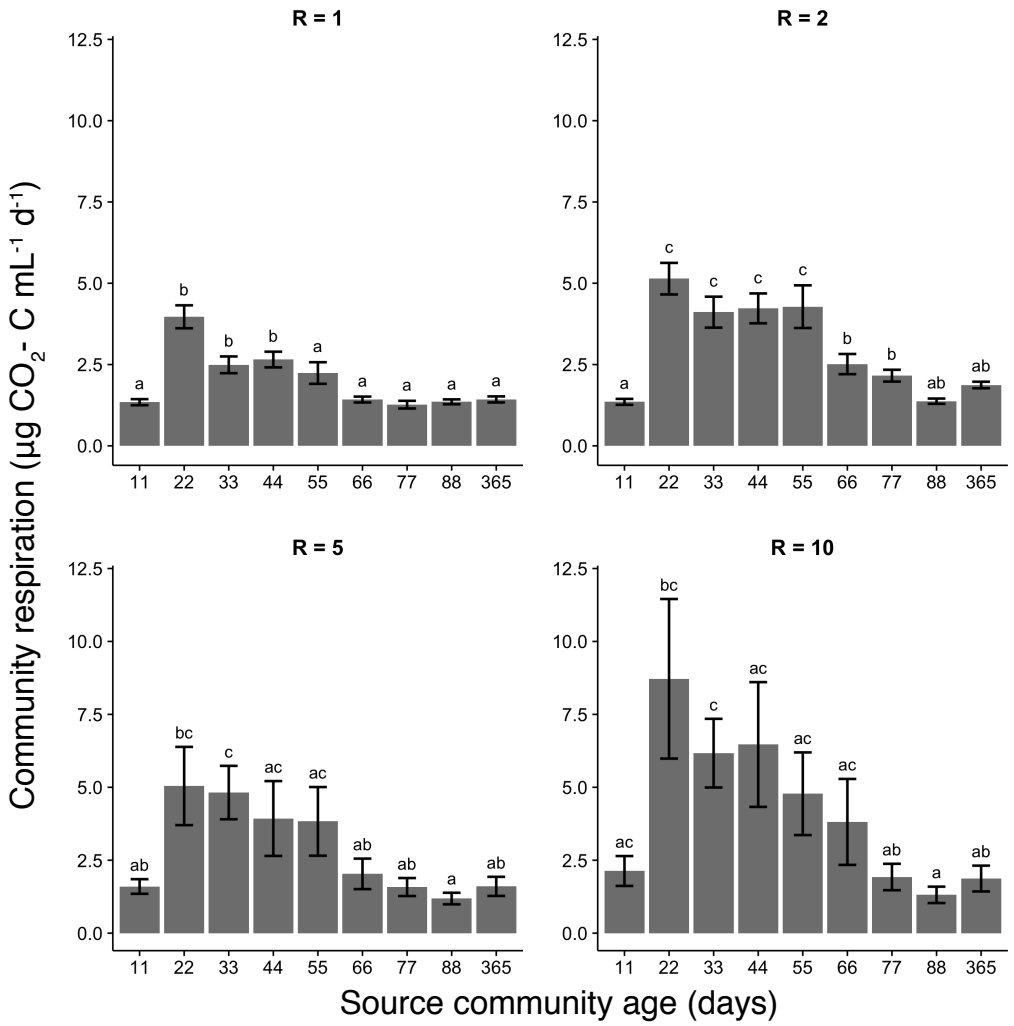

Figure 2. Mean rates of carbon mineralization for synthetic bacterial communities were unimodal over time, independent of richness (*R*). Different letters within each richness level indicate significant differences between ages according to Tukey's range test ($p < 0.05$). Bars denote standard errors.

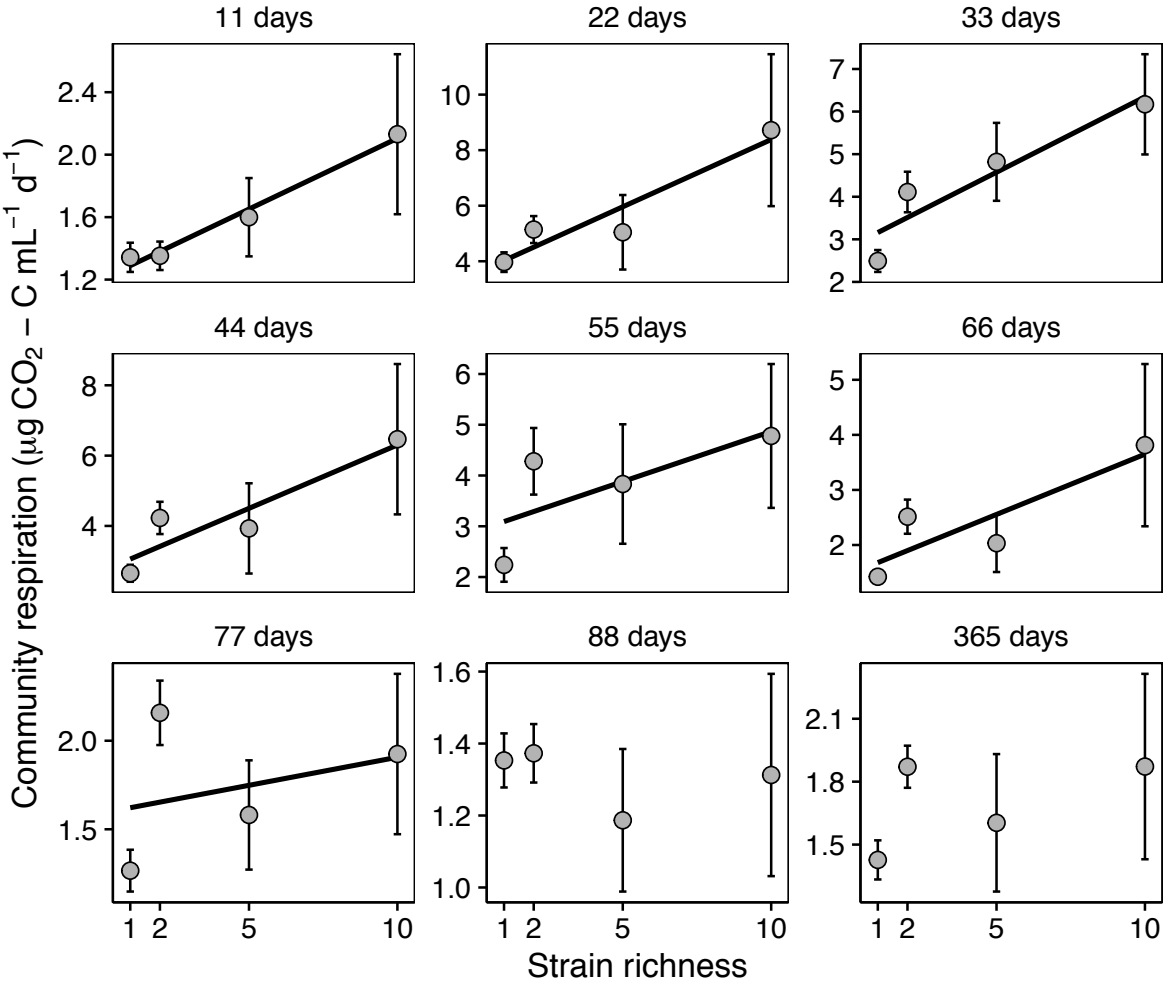

Figure 3. Relationships between strain richness and community respiration for synthetic bacterial communities assembled from pitchers of different ages. Black lines denote significant linear richness fits for individual communities within each age group ($p < 0.05$). Mean values for the response variables are presented for clarity. Bars denote standard error measurements.

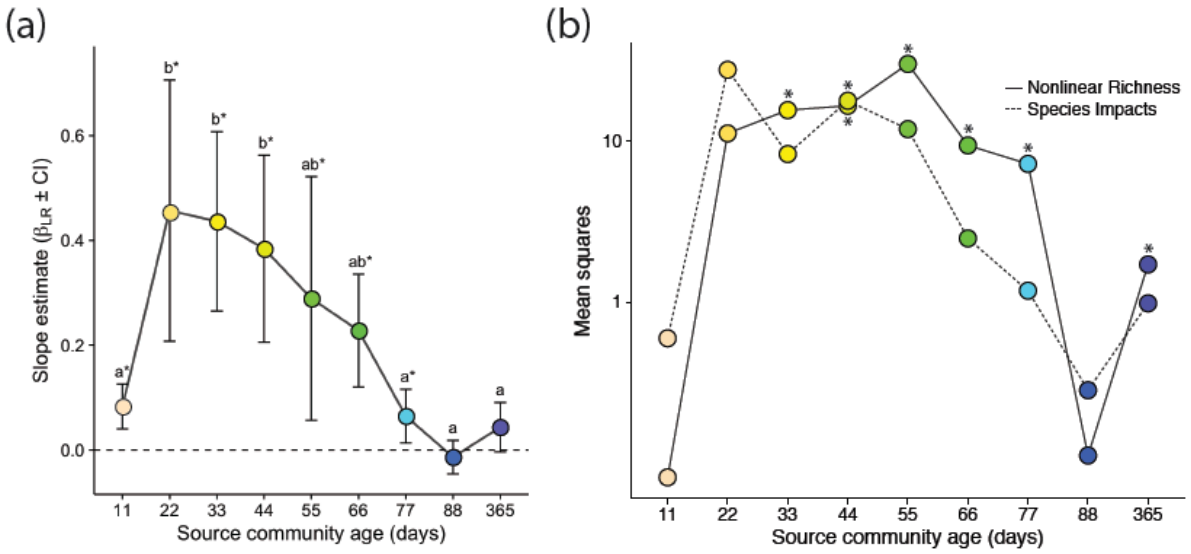

Figure 4. (a) Linear richness (β_{LR}) regression coefficients as a function of source community age. Bars denote 95% confidence intervals and shared letters between ages signify an overlap between the two estimates. Asterisks (*) denote coefficients found to be significantly greater than zero (F-test, $p < 0.05$). (b) Mean square estimates for the combined species impact (β_i) and nonlinear richness (β_{NLR}) parameters. These values represent the relative contributions of species-specific effects and species interactions, respectively, on rates of carbon mineralization once the effects of linear richness have been accounted for. Asterisks (*) within points indicate that the coefficient's inclusion into the model provides a statistically improved fit over a null intercept-only model (χ^2 likelihood ratio test, $p < 0.05$).

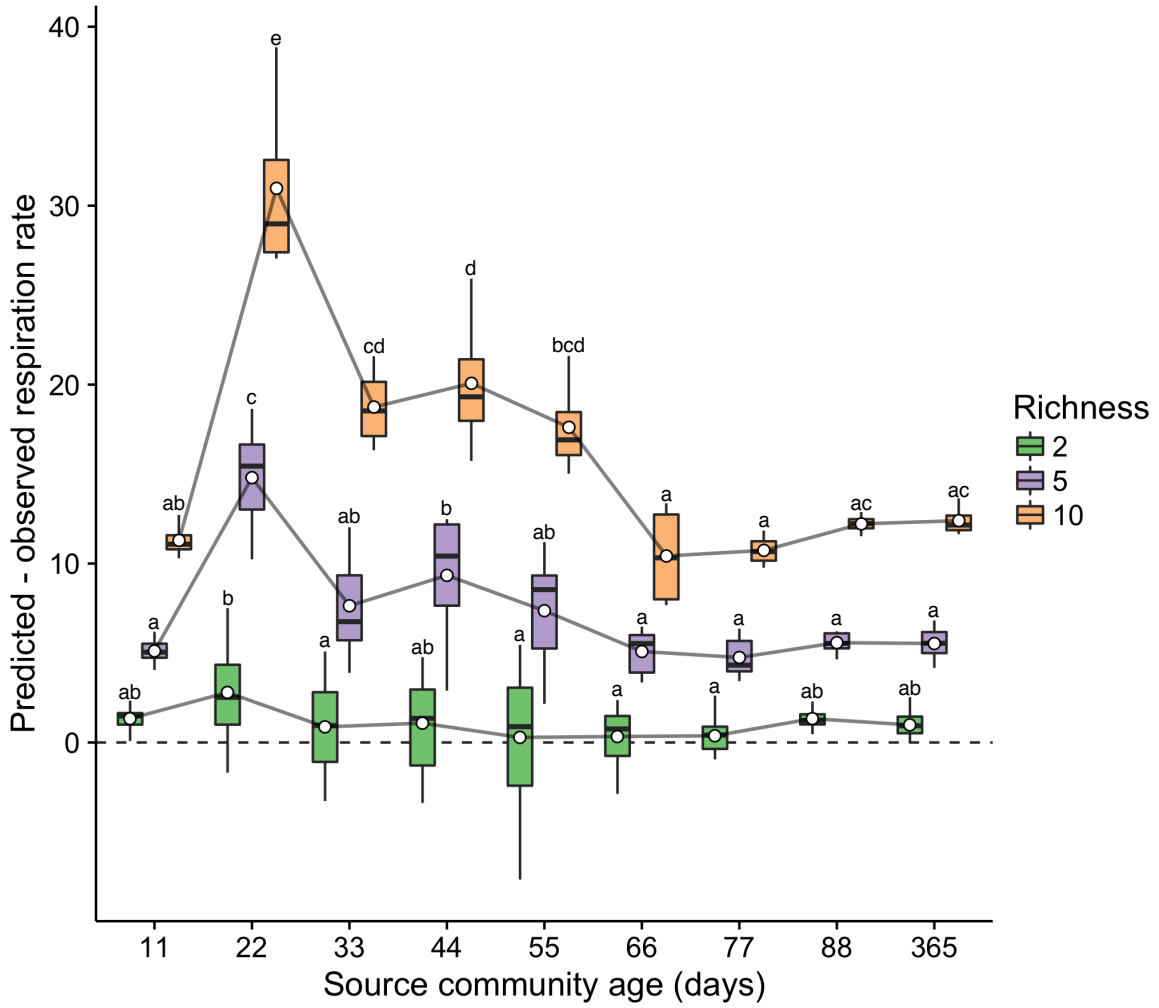

Figure 5. Relative suppression of bacterial respiration in polycultures. Values of zero indicate that the sum of community members' respirations in their respective monocultures equaled the community's performance in polyculture. Values greater than zero indicate a greater suppression of potential respiration and may signify interspecific competitive or antagonistic interactions. Boxplots denote quartiles of raw data and white points are mean values. Letters shared by points within a richness group indicate that their means do not significantly differ from one another (Tukey's range test, $p < 0.05$).

Table S1. Ordinary least squares estimates for parameters in equation 2 (see main text). Rows labeled "ns" denote the effect was not statistically different from zero.

Age	Parameter		Mean	SE	*t*	*p*
11 days	Intercept	β_0	1.23	0.07	18.00	< 0.001
	Linear richness	β_{LR}	0.08	0.02	3.80	< 0.001
	Species effect	β_i =				
		β_1	-0.13	0.13	-0.96	ns
		β_2	0.12	0.13	0.91	ns
		β_3	0.05	0.13	0.36	ns
		β_4	0.02	0.13	0.18	ns
		β_5	0.18	0.13	1.40	ns
		β_6	0.07	0.13	0.54	ns
		β_7	0.17	0.13	1.33	ns
		β_8	-0.09	0.13	-0.73	ns
		β_9	-0.11	0.13	-0.83	ns
		β_{10}	-0.28	0.13	-2.14	< 0.05
	Nonlinear richness	β_{NLR} =				
		$R = 1$	0.03	0.06	0.41	ns
		$R = 2$	-0.05	0.09	-0.52	ns
		$R = 5$	-0.05	0.14	-0.34	ns
		$R = 10$	0.07	0.20	0.34	ns

Age	Parameter		Mean	SE	*t*	*p*
22 days	Intercept	β_0	3.66	0.40	9.16	< 0.001
	Linear richness	β_{LR}	0.46	0.13	3.61	< 0.001
	Species effect	β_i =				
		β_1	-0.02	0.77	-0.03	ns
		β_2	1.12	0.74	1.52	ns
		β_3	-1.13	0.74	-1.53	ns
		β_4	0.34	0.74	0.46	ns
		β_5	1.33	0.76	1.75	ns
		β_6	-0.81	0.75	-1.08	ns
		β_7	1.19	0.75	1.59	ns
		β_8	0.04	0.75	0.05	ns
		β_9	-0.98	0.75	-1.31	ns
		β_{10}	-1.07	0.77	-1.39	ns
	Nonlinear richness	β_{NLR} =				
		$R = 1$	-0.15	0.37	-0.41	ns
		$R = 2$	0.56	0.52	1.09	ns
		$R = 5$	-0.90	0.82	-1.10	ns
		$R = 10$	0.49	1.16	0.42	ns

Table S1, continued

Age	Parameter		Mean	SE	*t*	*p*
33 days	Intercept	β_0	2.44	0.27	8.88	< 0.001
	Linear richness	β_{LR}	0.44	0.09	5.03	< 0.001
	Species effect	β_i =				
		β_1	-0.13	0.53	-0.25	ns
		β_2	-0.10	0.51	-0.20	ns
		β_3	-0.23	0.51	-0.45	ns
		β_4	0.14	0.52	0.28	ns
		β_5	0.20	0.53	0.37	ns
		β_6	-0.11	0.52	-0.21	ns
		β_7	1.28	0.52	2.46	< 0.05
		β_8	-0.57	0.52	-1.09	ns
		β_9	0.27	0.52	0.51	ns
		β_{10}	-0.75	0.53	-1.40	ns
	Nonlinear richness	β_{NLR} =				
		R = 1	-0.38	0.25	-1.51	ns
		R = 2	0.80	0.36	2.26	< 0.05
		R = 5	0.20	0.56	0.36	ns
		R = 10	-0.63	0.80	-0.79	ns

Age	Parameter		Mean	SE	*t*	*p*
44 days	Intercept	β_0	2.59	0.29	9.06	< 0.001
	Linear richness	β_{LR}	0.38	0.09	4.25	< 0.001
	Species effect	β_i =				
		β_1	0.66	0.54	1.21	ns
		β_2	0.48	0.52	0.92	ns
		β_3	-0.86	0.52	-1.65	ns
		β_4	-0.62	0.52	-1.19	ns
		β_5	0.90	0.54	1.67	ns
		β_6	-0.27	0.53	-0.51	ns
		β_7	1.22	0.53	2.31	< 0.05
		β_8	-0.60	0.53	-1.14	ns
		β_9	-0.16	0.53	-0.30	ns
		β_{10}	-0.74	0.54	-1.37	ns
	Nonlinear richness	β_{NLR} =				
		R = 1	-0.32	0.26	-1.25	ns
		R = 2	0.87	0.36	2.40	<0.05
		R = 5	-0.58	0.57	-1.02	ns
		R = 10	0.03	0.81	0.04	ns

Table S1, continued

Age	Parameter		Mean	SE	*t*	*p*
55 days	Intercept	β_0	2.48	0.29	9.06	< 0.001
	Linear richness	β_{LR}	0.29	0.09	4.25	< 0.05
	Species effect	β_i =				
		β_1	0.18	0.74	0.25	ns
		β_2	-1.00	0.72	-1.38	ns
		β_3	-0.47	0.71	-0.67	ns
		β_4	0.68	0.70	0.96	ns
		β_5	0.19	0.72	0.26	ns
		β_6	0.89	0.71	1.26	ns
		β_7	0.71	0.71	1.00	ns
		β_8	-0.71	0.71	-1.00	ns
		β_9	0.33	0.69	0.47	ns
		β_{10}	-0.83	0.77	-1.08	ns
	Nonlinear richness	β_{NLR} =				
		$R = 1$	-0.53	0.34	-1.54	ns
		$R = 2$	1.17	0.49	2.40	< 0.05
		$R = 5$	-0.08	0.77	-0.10	ns
		$R = 10$	-0.56	1.09	-0.52	ns

Age	Parameter		Mean	SE	*t*	*p*
66 days	Intercept	β_0	1.42	0.17	8.23	< 0.001
	Linear richness	β_{LR}	0.23	0.06	4.17	< 0.001
	Species effect	β_i =				
		β_1	-0.03	0.34	-0.08	ns
		β_2	-0.05	0.33	-0.15	ns
		β_3	-0.06	0.33	-0.18	ns
		β_4	-0.40	0.33	-1.22	ns
		β_5	0.34	0.34	1.01	ns
		β_6	0.65	0.33	1.95	ns
		β_7	0.19	0.33	0.59	ns
		β_8	-0.12	0.33	-0.36	ns
		β_9	-0.13	0.33	-0.38	ns
		β_{10}	-0.40	0.34	-1.18	ns
	Nonlinear richness	β_{NLR} =				
		$R = 1$	-0.23	0.16	-1.42	ns
		$R = 2$	0.64	0.22	2.86	<0.01
		$R = 5$	-0.53	0.35	-1.49	ns
		$R = 10$	0.11	0.50	0.23	ns

Table S1, continued

Age	Parameter		Mean	SE	*t*	*p*
77 days	Intercept	β_0	0.14	0.08	17.49	< 0.001
	Linear richness	β_{LR}	0.07	0.03	2.48	< 0.05
	Species effect	β_i =				
		β_1	-0.12	0.16	-0.76	ns
		β_2	-0.01	0.15	-0.06	ns
		β_3	-0.01	0.15	-0.06	ns
		β_4	-0.20	0.15	-1.29	ns
		β_5	0.36	0.16	2.27	< 0.5
		β_6	0.28	0.15	1.83	ns
		β_7	0.14	0.15	0.88	ns
		β_8	-0.06	0.15	-0.41	ns
		β_9	0.01	0.15	0.09	ns
		β_{10}	-0.39	0.16	-2.46	< 0.5
	Nonlinear richness	β_{NLR} =				
		$R = 1$	-0.24	0.07	-3.52	<0.001
		$R = 2$	0.59	0.10	6.08	<0.001
		$R = 5$	-0.18	0.15	-1.21	ns
		$R = 10$	-0.16	0.22	-0.75	ns

Age	Parameter		Mean	SE	*t*	*p*
88 days	Intercept	β_0	0.14	0.05	26.57	< 0.001
	Linear richness	β_{LR}	-0.01	0.02	-0.85	ns
	Species effect	β_i =				
		β_1	-0.04	0.10	-0.41	ns
		β_2	0.06	0.10	0.58	ns
		β_3	0.13	0.10	1.30	ns
		β_4	-0.13	0.10	-1.32	ns
		β_5	0.16	0.10	1.66	ns
		β_6	0.08	0.10	0.78	ns
		β_7	-0.07	0.10	-0.72	ns
		β_8	0.03	0.10	0.33	ns
		β_9	-0.02	0.10	-0.21	ns
		β_{10}	-0.20	0.10	-1.94	ns
	Nonlinear richness	β_{NLR} =				
		$R = 1$	0.00	0.05	-0.04	ns
		$R = 2$	0.03	0.07	0.47	ns
		$R = 5$	-0.11	0.11	-1.05	ns
		$R = 10$	0.08	0.15	0.54	ns

Table S1, continued

Age	Parameter		Mean	SE	*t*	*p*
365 days	Intercept	β_0	1.50	0.08	19.75	< 0.001
	Linear richness	β_{LR}	0.04	0.02	1.81	ns
	Species effect	β_i =				
		β_1	-0.11	0.15	-0.73	ns
		β_2	0.30	0.14	2.16	ns
		β_3	0.11	0.14	0.75	ns
		β_4	-0.18	0.14	-1.26	ns
		β_5	0.30	0.14	2.05	ns
		β_6	-0.06	0.14	-0.42	ns
		β_7	0.09	0.14	0.66	ns
		β_8	-0.02	0.14	-0.16	ns
		β_9	-0.22	0.14	-1.58	ns
		β_{10}	-0.21	0.15	-1.43	ns
	Nonlinear richness	β_{NLR} =				
		$R = 1$	-0.11	0.07	-1.69	ns
		$R = 2$	0.29	0.10	2.97	< 0.01
		$R = 5$	-0.11	0.15	-0.73	ns
		$R = 10$	-0.06	0.22	-0.28	ns

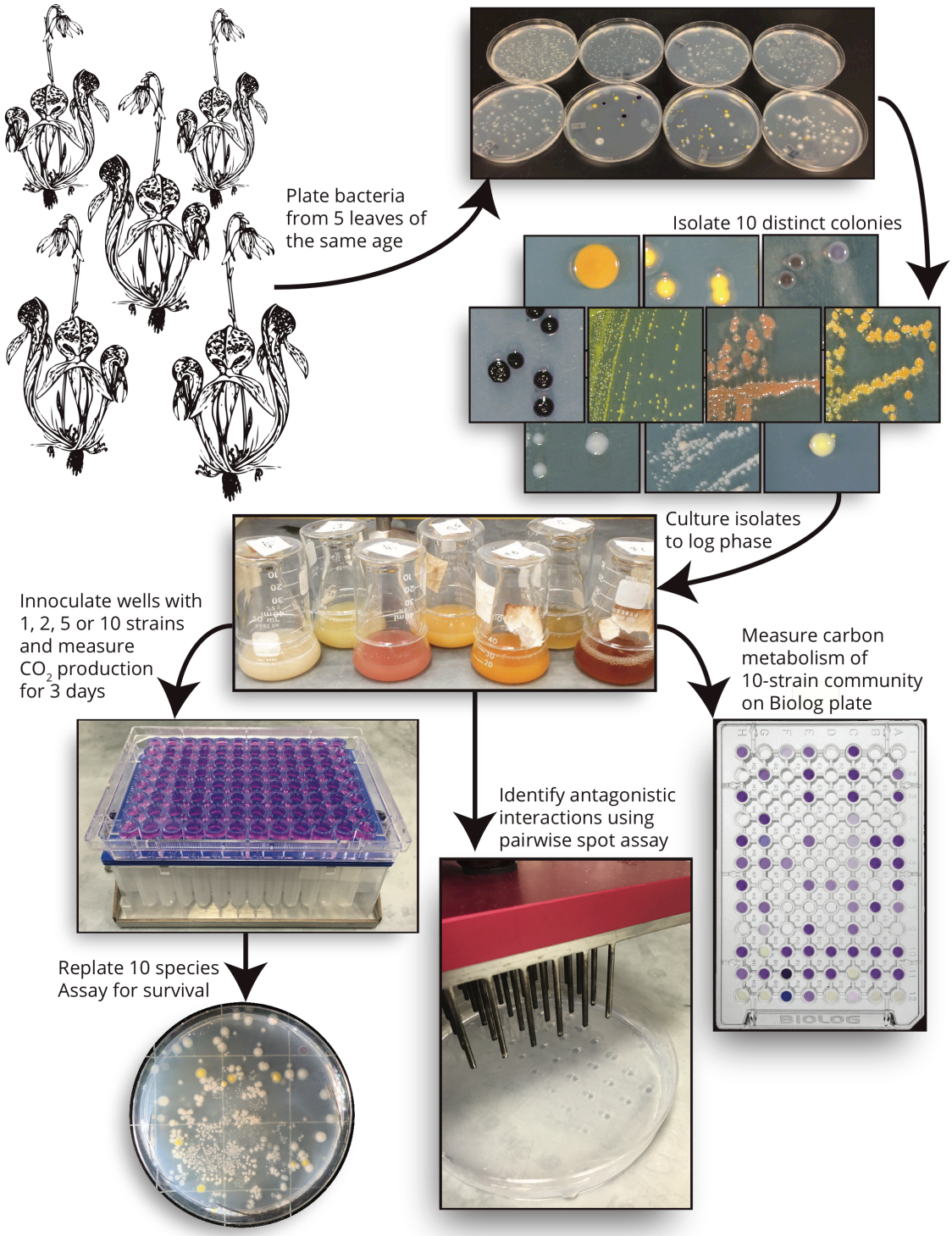

Figure S1. Graphical flowchart of the experimental procedure. This process was repeated every 11 days until day 88 using the same pitcher leaves. Five additional year-old leaves were also sampled.

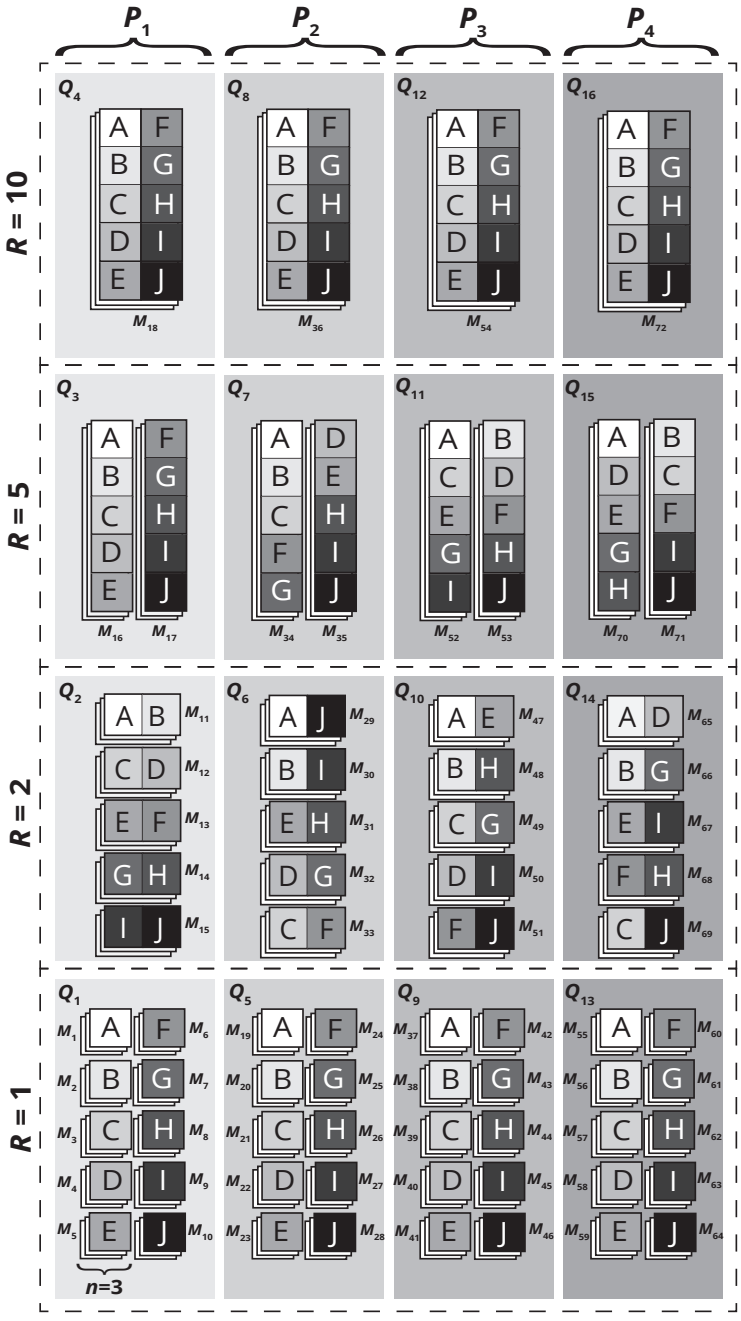

Figure S2. Diversity-ecosystem function experimental design, modified from (Bell *et al.* 2009). Each lettered box represents one of 10 individual bacterial strains isolated from a source pitcher leaves of a particular age. Each partition *P* represents a unique assignment of species to communities, *M*, where a species is chosen without replacement. These communities span 4 levels of species richness and are replicated three times per species pool *Q*. This design was used for each set of bacterial isolates from each source community age.

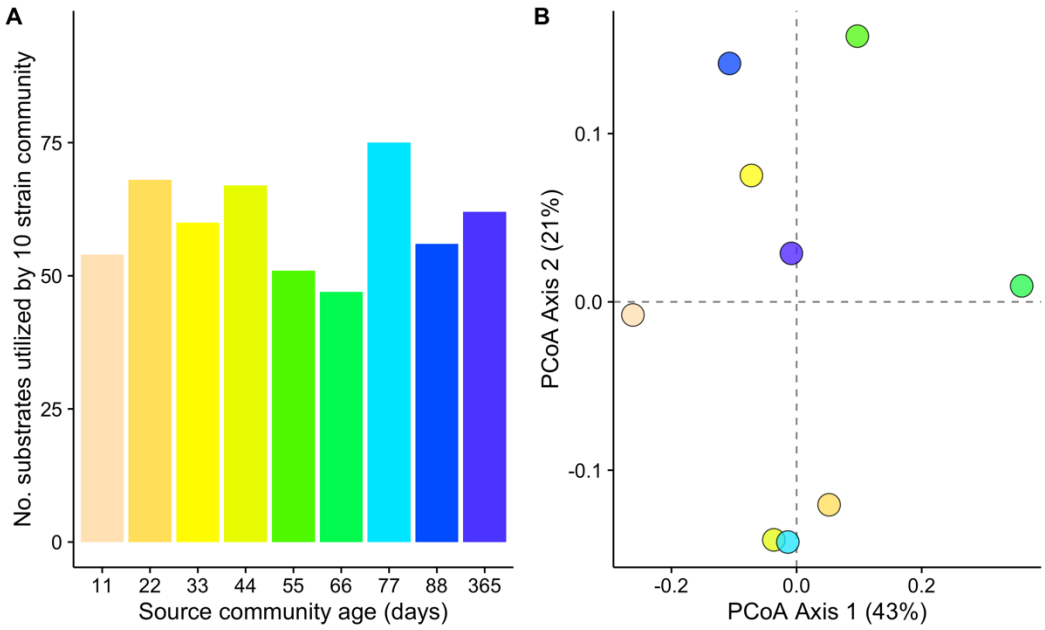

Figure S3. (a) The number of carbon substrates metabolized by each 10-species community on the Biolog GN2 microplate. (b) Jaccard distances between each community's carbon utilization profile plotted on first two principal coordinates. Colors represent source community ages as in (a).

LITERATURE CITED

Allison, S. D., and J. B. H. Martiny. 2008. Resistance, resilience, and redundancy in microbial communities. Proceedings of the National Academy of Sciences of the United States of America 105:11512–11519.

Bell, T., A. K. Lilley, A. Hector, B. Schmid, L. King, and J. A. Newman. 2009. A Linear Model Method for Biodiversity–Ecosystem Functioning Experiments. The American Naturalist 174:836–849.

Bell, T., J. A. Newman, B. W. Silverman, S. L. Turner, and A. K. Lilley. 2005. The contribution of species richness and composition to bacterial services. Nature 436:1157–1160.

Butler, J. L., N. J. Gotelli, and A. M. Ellison. 2008. Linking the brown and green: nutrient transformation and fate in the Sarracenia microecosystem. Ecology 89:898–904.

Campbell, C. D., S. J. Chapman, C. M. Cameron, M. S. Davidson, and J. M. Potts. 2003. A rapid microtiter plate method to measure carbon dioxide evolved from carbon substrate amendments so as to determine the physiological profiles of soil microbial communities by using whole soil. Applied and Environmental Microbiology 69:3593–3599.

Cardinale, B. J., D. S. Srivastava, J. Emmett Duffy, J. P. Wright, A. L. Downing, M. Sankaran, and C. Jouseau. 2006. Effects of biodiversity on the functioning of trophic groups and ecosystems. Nature 443:989–992.

Cardinale, B. J., J. P. Wright, M. W. Cadotte, I. T. Carroll, A. Hector, D. S. Srivastava, M. Loreau, and J. J. Weis. 2007. Impacts of plant diversity on biomass production increase through time because of species complementarity. Proceedings of the National Academy of Sciences 104:18123–18128.

Connell, J. H., and R. O. Slatyer. 1977a. Mechanisms of succession in natural communities and their role in community stability and organization. The American Naturalist 111:1119–1144.

Connell, J. H., and W. P. Sousa. 1983. On the evidence needed to judge ecological stability or persistence. The American Naturalist 121:789–824.

DeAngelis, D. L. 1992. Dynamics of Nutrient Cycling and Food Webs. Chapman & Hall, London ; New York.

Doherty, J. M., J. C. Callaway, and J. B. Zedler. 2011. Diversity–function relationships changed in a long-term restoration experiment. Ecological Applications 21:2143–2155.

Ellison, A. M., N. J. Gotelli, J. S. Brewer, D. L. Cochran-Stafira, J. M. Kneitel, T. E. Miller, A. C. Worley, and R. Zamora. 2003. The evolutionary ecology of carnivorous plants. Advances in Ecological Research 33:1–74.

Fiegna, F., A. Moreno-Letelier, T. Bell, and T. G. Barraclough. 2015. Evolution of species interactions determines microbial community productivity in new environments. The ISME Journal 9:1235–1245.

Foster, K. R., and T. Bell. 2012. Competition, not cooperation, dominates interactions among culturable microbial species. Current Biology 22:1845–1850.

Fox, J. W. 2004. Effects of algal and herbivore diversity on the partitioning of biomass within and among trophic levels. Ecology 85:549–559.

Gallie, D. R., and S. C. Chang. 1997. Signal transduction in the carnivorous plant Sarracenia purpurea. Regulation of secretory hydrolase expression during development and in response to resources. Plant Physiology 115:1461–1471.

Gravel, D., T. Bell, C. Barbera, T. Bouvier, T. Pommier, P. Venail, and N. Mouquet. 2011. Experimental niche evolution alters the strength of the diversity-productivity relationship. Nature 469:89–92.

Harmon, L. J., B. Matthews, S. Des Roches, J. M. Chase, J. B. Shurin, and D. Schluter. 2009. Evolutionary diversification in stickleback affects ecosystem functioning. Nature 458:1167–1170.

van der Heijden, M. G. A., J. N. Klironomos, M. Ursic, P. Moutoglis, R. Streitwolf-Engel, T. Boller, A. Wiemken, and I. R. Sanders. 1998. Mycorrhizal fungal diversity determines plant biodiversity, ecosystem variability and productivity. Nature 396:69–72.

Hepburn, J. S., F. M. Jones, and E. Q. John. 1927. The biochemistry of the American pitcher plants: biochemical studies of the North American Sarraceniaceae. Transactions of the Wagner Free Institute of Science of Philadelphia 1927:1–95.

Hooper, D. U., F. S. Chapin, J. J. Ewel, A. Hector, P. Inchausti, S. Lavorel, J. H. Lawton, D. M. Lodge, M. Loreau, S. Naeem, B. Schmid, H. Setälä, A. J. Symstad, J. Vandermeer, and D. A. Wardle. 2005. Effects of biodiversity on ecosystem functioning: a consensus of current knowledge. Ecological Monographs 75:3–35.

Jiang, L. 2007. Negative selection effects suppress relationships between bacterial diversity and ecosystem functioning. Ecology 88:1075–1085.

Jiang, L., Z. Pu, and D. R. Nemergut. 2008. On the importance of the negative selection effect for the relationship between biodiversity and ecosystem functioning. Oikos 117:488–493.

Juniper, B. B. E., R. J. Robins, and D. M. Joel. 1989. The Carnivorous Plants. Academic Press, London, UK; San Diego, CA.

Keddy, P. A. 2012. Competition. Springer Science & Business Media.

Kinzig, A. P., and S. W. Pacala. 2001. Successional biodiversity and ecosystem functioning. Pages 175–212 *in* A. P. Kinzig, S. W. Pacala, and G. D. Tilman, editors. The functional consequences of biodiversity: empirical progress and theoretical extensions. Princeton University Press, Princeton, NJ.

Koenig, J. E., A. Spor, N. Scalfone, A. D. Fricker, J. Stombaugh, R. Knight, L. T. Angenent, and R. E. Ley. 2011. Succession of microbial consortia in the developing infant gut microbiome. Proceedings of the National Academy of Sciences 108:4578–4585.

Koopman, M. M., D. M. Fuselier, S. Hird, and B. C. Carstens. 2010. The carnivorous pale pitcher plant harbors diverse, distinct, and time-dependent bacterial communities. Applied and Environmental Microbiology 76:1851–1860.

Langenheder, S., M. T. Bulling, M. Sloan, and Prosser. 2010. Bacterial biodiversity-ecosystem functioning relations are modified by environmental complexity. PLOS ONE 5:e10834.

Lasky, J. R., M. Uriarte, V. K. Boukili, D. L. Erickson, W. John Kress, and R. L. Chazdon. 2014. The relationship between tree biodiversity and biomass dynamics changes with tropical forest succession. Ecology Letters 17:1158–1167.

Lawrence, D., F. Fiegna, V. Behrends, J. G. Bundy, A. B. Phillimore, T. Bell, and T. G. Barraclough. 2012. Species interactions alter evolutionary responses to a novel environment. PLoS Biol 10:e1001330.

Lefcheck, J. S., and J. E. Duffy. 2015. Multitrophic functional diversity predicts ecosystem functioning in experimental assemblages of estuarine consumers. Ecology 96:2973–2983.

Lindquist, J. A. 1975. Bacteriological and ecological observations on the northern pitcher plant, *Sarracenia purpurea* L. Ph.D dissertation, University of Wisconsin, Madison, WI.

Lloyd, F. E. 1942. The Carnivorous Plants. Chronica Botanica. Ronald Press, New York, NY.

Loreau, M. 2010. From Populations to Ecosystems: Theoretical Foundations for a New Ecological Synthesis (MPB-46): Theoretical Foundations for a New Ecological Synthesis (MPB-46). Princeton University Press, Princeton, NJ.

Loreau, M., and A. Hector. 2001. Partitioning selection and complementarity in biodiversity experiments. Nature 412:72–76.

Loreau, M., S. Naeem, P. Inchausti, J. Bengtsson, J. P. Grime, A. Hector, D. U. Hooper, M. A. Huston, D. Raffaelli, B. Schmid, D. Tilman, and D. A. Wardle. 2001. Biodiversity and ecosystem functioning: current knowledge and future challenges. Science 294:804–808.

Mouquet, N., J. L. Moore, and M. Loreau. 2002. Plant species richness and community productivity: why the mechanism that promotes coexistence matters. Ecology Letters 5:56–65.

Nemergut, D. R., J. E. Knelman, S. Ferrenberg, T. Bilinski, B. Melbourne, L. Jiang, C. Violle, J. L. Darcy, T. Prest, S. K. Schmidt, and A. R. Townsend. 2015. Decreases in average bacterial community rRNA operon copy number during succession. The ISME Journal.

Odum, E. P. 1969. The strategy of ecosystem development. Science 164:262–270.

Petermann, J. S., A. J. F. Fergus, C. Roscher, L. A. Turnbull, A. Weigelt, and B. Schmid. 2010. Biology, chance, or history? The predictable reassembly of temperate grassland communities. Ecology 91:408–421.

R Development Core Team. 2015. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.

Redford, A. J., and N. Fierer. 2009. Bacterial succession on the leaf surface: a novel system for studying successional dynamics. Microbial Ecology 58:189–198.

Reich, P. B., D. Tilman, F. Isbell, K. Mueller, S. E. Hobbie, D. F. B. Flynn, and N. Eisenhauer. 2012. Impacts of biodiversity loss escalate through time as redundancy fades. Science 336:589–592.

Ruijven, J. van, and F. Berendse. 2005. Diversity–productivity relationships: Initial effects, long-term patterns, and underlying mechanisms. Proceedings of the National Academy of Sciences of the United States of America 102:695–700.

Salles, J. F., F. Poly, B. Schmid, and X. Le Roux. 2009. Community niche predicts the

functioning of denitrifying bacterial assemblages. Ecology 90:3324–3332.

Scheffer, M. 2009. Critical Transitions in Nature and Society. Princeton University Press, Princeton, NJ.

Shapiro, B. J., J. Friedman, O. X. Cordero, S. P. Preheim, S. C. Timberlake, G. Szabó, M. F. Polz, and E. J. Alm. 2012. Population genomics of early events in the ecological differentiation of bacteria. Science 336:48–51.

Spehn, E. M., A. Hector, J. Joshi, M. Scherer-Lorenzen, B. Schmid, E. Bazeley-White, C. Beierkuhnlein, M. C. Caldeira, M. Diemer, P. G. Dimitrakopoulos, J. A. Finn, H. Freitas, P. S. Giller, J. Good, R. Harris, P. Högberg, K. Huss-Danell, A. Jumpponen, J. Koricheva, P. W. Leadley, M. Loreau, A. Minns, C. P. H. Mulder, G. O'Donovan, S. J. Otway, C. Palmborg, J. S. Pereira, A. B. Pfisterer, A. Prinz, D. J. Read, E.-D. Schulze, A.-S. D. Siamantziouras, A. C. Terry, A. Y. Troumbis, F. I. Woodward, S. Yachi, and J. H. Lawton. 2005. Ecosystem effects of biodiversity manipulations in European grasslands. Ecological Monographs 75:37–63.

Tilman, D., P. B. Reich, J. Knops, D. Wedin, T. Mielke, and C. Lehman. 2001. Diversity and productivity in a long-term grassland experiment. Science 294:843–845.

Venail, P. A., and M. J. Vives. 2013. Phylogenetic distance and species richness interactively affect the productivity of bacterial communities. Ecology 94:2529–2536.

Wacker, L., O. Baudois, S. Eichenberger-Glinz, and B. Schmid. 2009. Diversity effects in early- and mid-successional species pools along a nitrogen gradient. Ecology 90:637–648.

Weis, J. J., B. J. Cardinale, K. J. Forshay, and A. R. Ives. 2007. Effects of species diversity on community biomass production change over the course of succession. Ecology 88:929–939.

Zuppinger-Dingley, D., B. Schmid, J. S. Petermann, V. Yadav, G. B. De Deyn, and D. F. B. Flynn. 2014. Selection for niche differentiation in plant communities increases biodiversity effects. Nature 515:108–111.

CHAPTER 4 | The cobra's tongue: Rethinking the function of the 'fishtail appendage' on the pitcher plant *Darlingtonia californica*

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ABSTRACT

Carnivorous pitcher plants employ a variety of putative adaptations for prey attraction and capture. One example is the peculiar forked "fishtail appendage"— a foliar structure widely presumed to function as a prey attractant on adult leaves of *Darlingtonia californica* (Sarraceniaceae). This study tests the prediction that the presence of the appendage facilitates prey capture and can be considered an example of an adaptation to the carnivorous syndrome. In a field experiment following a cohort of *Darlingtonia* leaves over their growing season, before the pitcher traps opened, the fishtail appendages from half of the leaves were removed. Additionally, all appendages were removed from every plant at two small, isolated populations. After 54 and 104 d, prey items were collected to determine whether differences in prey composition and biomass existed between experimental and un-manipulated control leaves. Removal of the fishtail appendage did not reduce pitcher leaves' prey biomass nor alter their prey composition at either the level of individual leaves or entire populations. Fishtail appendages on plants growing in shaded habitats contained significantly greater chlorophyll concentrations than those on plants growing in full sun. These results call into question the longstanding assumption that the fishtail appendage on *Darlingtonia* is an adaptation critical for the attraction and capture of prey. I suggest alternative evolutionary explanations for the role of the fishtail structure and re-propose a hypothesis on the mutualistic nature of pitcher plant–arthropod trophic interactions.

INTRODUCTION

Carnivorous pitcher plants (families Sarraceniaceae, Nepenthaceae, and Cephalotaceae) share a number of convergent traits enabling the attraction and capture of animal prey. These include modified fluid-filled pitcher leaves, low-friction cuticles, false exits, trichomes, and extrafloral nectaries (Lloyd 1942, Juniper et al. 1989). Many other pitcher plant traits, however, have yet to be rigorously verified as critical adaptations to the carnivorous syndrome, despite over a century of literature assigning them such a functional role. In actuality, many unique features of pitcher plants, when closely scrutinized, suggest alternative adaptive scenarios (e.g., Joel, 1988). For instance, certain morphological characters of *Nepenthes* (Nepenthaceae) pitcher organs have been shown to serve mutualistic, rather than predatory, roles with arthropods and mammals (Clarke et al., 2010; Grafe et al., 2011; Bazile et al., 2012). Likewise, many leaf traits hypothesized to facilitate prey capture in *Sarracenia* (Sarraceniaceae) serve either an alternative or no detectable function upon empirical evaluation (Cresswell, 1993; Green and Horner, 2007; Bennett and Ellison, 2009; Schaefer and Ruxton, 2014).

Darlingtonia californica Torr. (Sarraceniaceae), the sole member of its genus, is the only pitcher plant native to the western United States. It is associated with habitats containing a constant source of cold, flowing water on or downstream of serpentine formations. A recent phylogenetic analysis suggests *Darlingtonia* diverged from the clade giving rise to *Sarracenia* and *Heliamphora* sometime during the Oligocene (25–44 million years ago) (Ellison et al., 2012). However, due to a paucity of fossil evidence, the phenotype of the common ancestor to these clades is unknown (Wong et al., 2015). While the pitcher leaves of *Darlingtonia* clearly share morphological features with other members of Sarraceniaceae, they are arguably the most complex within the family (James, 1885; Lloyd, 1942; Franck, 1975) (Fig. 1). Foliar traits unique to *Darlingtonia* include the presence of transparent light-transmitting fenestrations, a downward-oriented trap entrance, and the twisting of developing leaves around their vertical axes such that consecutive leaves on a rosette end up facing different directions. Perhaps the most striking feature of the *Darlingtonia* leaf is its "fishtail appendage"— a unique forked structure hanging off the apex of the leaf's pitcher entrance. This feature has inspired the taxon's common name: the cobra plant or cobra lily. At its base, the appendage merges with the nectar roll, which encircles the margin of the pitcher entrance. The nectar roll continues along the margins of the fishtail appendage until terminating distally. The fishtail appendage's nectar glands, red venation, and short hairs oriented toward the pitcher entrance have led researchers to treat the structure as a prey attractant (Austin, 1875–1877; Lloyd, 1942; Juniper et al., 1989; Schnell, 2002; Ellison and Farnsworth, 2005), but this function has not yet been experimentally verified.

The leaves of *Darlingtonia* vary in size both within and among individual plants. Pitcher leaves can be classified into two morphological types: juvenile and adult. Juvenile leaves are small and tubular (less than 5 cm in length), and found on plants less than 2 years of age. These lack many of the specialized morphological features of adult leaves (e.g., the bifurcated fishtail appendage) and may function to trap small arthropods (Franck, 1976). The more complex adult leaves produced by mature plants vary considerably in size (5 cm to >1 m tall), but are otherwise morphologically identical. Leaf height, mass, and chlorophyll content tend to increase with shade (D. W. Armitage, unpublished data). During the growing season (early June–October), an individual rhizome continuously produces leaves in a rosette, with each successive leaf decreasing in size. The pitcher entrances remain closed until the leaf reaches its maximum height, then the fishtail appendage rapidly develops and begins producing nectar. Concurrently, the semitransparent distal portion of the leaf swells and forces open the trapping orifice. Small adult pitcher leaves are often recumbent, and their fishtail appendage may function as a ramp for small terrestrial arthropods to access the nectar roll (Lloyd, 1942).

If the presence of the fishtail appendage enhances the luring and trapping prey items, then its removal from an adult pitcher should result in a decrease of successful capture events compared with a pitcher on which it is present. However, this effect may only manifest in small adult pitchers lying prone along the ground. Alternatively, since individual *Darlingtonia* ramets can produce upward of 10 pitcher leaves per growing season, there may be a ramet or genet-level benefit of the appendage that does not manifest at the level of the individual pitcher leaf. For instance, expression of the appendage by an isolated stand of leaves may serve as a long-distance attractant for prey insects, which otherwise may not congregate in the vicinity of the plant. If the absence of these appendages does not negatively impact a plant's accumulation of prey, then

they should not be considered a critical adaptation for prey capture. I tested this hypothesiss using a manipulative field experiment in which I removed the fishtail appendages from individual large (erect) and small (prone) *Darlingtonia* leaves before their maturation. I tracked and compared the taxonomic composition and accumulated biomass of captured prey between manipulated and control leaves over a 3-mo growing season. Additionally, I removed all appendages from two small, isolated patches of *Darlingtonia* to test whether the presence of the appendage at the population level influenced rates of prey capture.

METHODS

The study was done in Plumas National Forest (Plumas County, California, USA), where numerous, patchy populations of *Darlingtonia* occur in spring-fed seeps ranging from between <10 to thousands of individual ramets, each possessing upward of 10 pitcher leaves (exact coordinates are available on request). During late May 2014, I tagged 60 individual developing, unopened pitcher leaves belonging to two size classes: large (n = 40, height > 50 cm, erect) and small (n = 20, height < 15 cm, growing recumbently). These leaves were located in a small (<1000 total leaves), isolated (>1 km from closest known patch) population of *Darlingtonia* growing in a 30 m^2 spring-fed seep in the Butterfly Valley Botanical Area, Plumas National Forest. One single leaf was selected per rosette to minimize bias caused by a plant's genotype and to decrease impacts on the organism. These focal leaves were spaced a minimum of 50 cm apart. I removed the developing fishtail appendage using a pair of fingernail clippers from a random sample of half of the leaves. Because insects may also be attracted to the scent of a pitcher leaf's putrefying detritus, I removed fishtail appendages before pitchers opened and commenced trapping.

After 52 d, I harvested 10 large leaves from experimental and control treatments. Prey accumulation in all small leaves was at the limit of detection, and so none were removed. All other large and small leaves were harvested after 104 d. I removed the prey contents of each leaf and stored them in vials of 70% ethanol. Most arthropod prey are rapidly dismembered and digested by the pitchers' associated aquatic food web, leaving only the exoskeleton behind. This digestion prevents the unambiguous identification prey items, although the remaining material (primarily head capsules and wings) permitted identification of individuals to the level of order, and many to suborder or family. This material was then dried at 60°C for 48 h and weighed. I tested for differences in prey biomass between leaves with and without their fishtail appendages using an analysis of variance (ANOVA) with pitcher size and sampling date as covariates. Additionally, I tested whether the age and presence of the appendage on large pitchers had an effect on the taxonomic composition of prey insects using the permutational analysis of variance algorithm adonis implemented in the vegan R package (Anderson, 2001; Oksanen et al., 2015). I performed this analysis twice using raw compositional data primarily at the suborder and family levels and the same data grouped at the order level.

I conducted a second experiment to test the hypothesis that fishtail appendages play a role in prey attraction or capture at the level of individual plants or small founding populations. Early in the 2015 growing season, I located two small, isolated patches of *Darlingtonia* comprising between 100 and 250 pitchers and located at least 500 m downstream from the nearest neighboring populations. Because *Darlingtonia* populations tend to expand downstream along

spring-fed seeps, these small founding populations probably represented a single genet dispersed as a seed from larger populations growing upstream. From these populations, I removed fishtail appendage from all leaves. I marked 20 unopened leaves of equivalent developmental stage, and returned to the site approximately every 20–30 d to remove the appendages from developing leaves. After 104 d, I removed the 20 marked pitchers from each plot and took them to the laboratory for prey biomass measurement. I compared the per-leaf prey biomasses from these "population-level" removal treatments to those of the 2014 "leaf-level" removals and control treatments using ANOVA. The 2014 data were necessarily used as controls because no other unmanipulated and similarly isolated plots of *Darlingtonia* could be located in the region. However, there were no interannual differences in prey capture rates among unmanipulated leaves sampled from larger nearby populations (D. W. Armitage, unpublished data). To increase statistical power, I pooled data from the two experimental populations because there were no significant differences between the populations' means and variances.

Because *Darlingtonia* can grow in shaded habitats, its fishtail structure may also serve a photosynthetic role. To investigate this possibility, I removed an additional 20 fishtail appendages from 10 leaves growing in full sunlight and 10 plants growing in heavy shade for chlorophyll analysis. These leaves were taken from a medium-sized population growing upstream of the 2015 experimental plots. I estimated the total chlorophyll [Chl*a* + Chl*b*] concentration of each appendage using an Apogee CCM-200 chlorophyll content meter (Apogee Instruments, Logan, Utah, USA). This device outputs a chlorophyll content index (CCI) that I converted into total chlorophyll content (mg/cm^2) using a standard calibration equation (R^2 = 0.96) (Richardson et al., 2002). Each fishtail appendage was measured in triplicate (at its midpoint and along both wings), and I compared the averaged estimates using a Student's *t* test. I performed all data analysis in R (v. 3.1.1) (R Core Team, 2015). When necessary, data were log-transformed to meet the assumptions of homogeneity and normality of residual variances.

RESULTS

Prey biomass increased with leaf age, but removal of appendages did not impact prey biomass accumulation (Table 1, Fig. 2A). Likewise, I did not detect an effect of population-level appendage removals on prey biomass ($F_{2,56}$ = 2.1, P = 0.133) (Fig. 2B). Despite marked interleaf variability in prey composition, I found no evidence for discrimination in prey taxa between *Darlingtonia* leaves with and without fishtail appendages, but prey composition differed significantly between 52-d- and 104-d-old leaves (Table 1; Appendix S1, see Supplemental Data with the online version of this article). The majority of prey in all large pitchers belonged to the orders Hymenoptera (11.4 ± 2.0/leaf; primarily families Vespidae and Ichneumonidae), Coleoptera (9.9 ± 2.3/leaf; primarily family Cerambycidae), and Diptera (3.9 ± 0.9/leaf). However, individual large-bodied lepidopterans and orthopterans occasionally greatly contributed to prey biomass. Small pitchers contained far fewer prey than large pitchers, and the prey assemblage contained only small dipterans and coleopterans <3 mm in body length (Appendix S1). Chlorophyll content was relatively low in fishtail appendages growing in both full sun and shade, but appendages from shaded plants contained significantly higher chlorophyll concentrations than plants in full sun (t = 4.6, df = 16.9, P < 0.0005) (Fig. 3).

DISCUSSION

Contrary to expectations, removal of the fishtail appendage from leaves of *Darlingtonia* did not hinder the plant's ability to lure and capture prey items, nor did it change the taxonomic composition of the captured prey. This outcome was observed in both large and small adult pitchers and was not influenced by the leaf's age. Furthermore, the complete removal of fishtail appendages from entire isolated patches of *Darlingtonia* did not negatively impact individual leaves' abilities to trap prey compared to both untreated populations and experimental leaves surrounded by neighbors possessing the appendage. In concert, these findings suggest that the presence of fishtail appendages within relatively isolated, small populations of *Darlingtonia* may not function to attract prey into the patch nor into a leaf's trapping orifice. These results call into question the assumption that all nectary-bearing structures on pitcher plant leaves are critical adaptations to the carnivorous syndrome.

The fishtail appendage of *Darlingtonia* may instead represent a vestigial or exapted structure. Members of the sister genus *Sarracenia* possess an offshoot of the nectar roll that forms an operculum "hood", replete with extrafloral nectaries along its outer rim, red venation along the medial axis, and hairs on its underside. Similar, convergent structures can also be found on the majority of *Nepenthes* (Nepenthaceae) and *Cephalotus* (Cephalotaceae) species. In these genera, the operculum shows remarkable interspecific variation but is generally believed to function both in prey capture and the prevention of flooding and dilution of the pitcher chamber from rainwater (Juniper et al., 1989). Within Sarraceniaceae, *Sarracenia purpurea* and members of the neotropical genus *Heliamphora* lack an operculum, although derivatives of this structure manifest in *S. purpurea* as a vertical extension of the nectar roll possessing all of its characteristic traits and in *Heliamphora* as a reduced appendage possessing enlarged nectar glands and no hairs (Adams and Smith, 1977). In the latter, flooding is mitigated via a small slit in the leaf's keel serving as a drain (Lloyd, 1942). In *Darlingtonia* and the morphologically similar *Sarracenia psittacina*, the flooding problem is ameliorated by the species' downward-facing pitcher openings. In these taxa, an inflated, semitransparent, hairless "hood" wraps over the entire upper portion of the pitcher leaf, and the operculum covering is either lost completely (as in *S. psittacina*) or remains as the fishtail appendage on *Darlingtonia* (Lloyd, 1942).

The fishtail appendage of *Darlingtonia* and the opercula of *Sarracenia* and *Heliamphora* share many developmental and morphological features. The pitcher leaves of Sarraceniaceae undergo nearly identical patterns of early histogenesis wherein the apical tissue of the leaf primordium gives rise to the operculum in *Sarracenia* or the hood and fishtail appendage in *Darlingtonia* (Lloyd, 1942; Franck, 1975; Fukushima et al., 2015). Arber (1941) observed that the leaves of Sarraceniaceae share a similar pattern of venation wherein the majority of veins converge at the midrib's apex at exactly the point where the opercula and fishtail appendage originate. The author hypothesized that this clustering of veins results in a disproportionate supply of nutrients and localized hypertrophy, which, in turn, initiates the development of the pitcher's operculum or fishtail appendage (Arber, 1941). On adult leaves of *Darlingtonia* and *Sarracenia*, nectar glands ring the margins of their appendages, and the positioning and location of hairs on the undersides of both structures is identical, although the hairs are reduced in *Darlingtonia* (Adams and Smith, 1977). In *Sarracenia*, these hairs are hypothesized to inhibit the foothold of insects, which then fall directly into the pitcher trap (Lloyd, 1942). In *Darlingtonia*,

however, these hairs would cause potential prey to fall from the leaf. These observations are consistent with the fishtail appendage of *Darlingtonia* being homologous with the operculum of *Sarracenia* and the nectar spoon of *Heliamphora.*

Given the morphological plasticity of the fishtail and opercular appendages in Sarraceniaceae, these structures may serve roles contingent upon their local environments. It was hypothesized that plants' investments in traits that facilitate carnivory come with a cost to photosynthetic efficiency such that they can only thrive in habitats where they will not experience strong competition with noncarnivorous plants for light, water, or nutrients (Givnish et al., 1984). Thus, plants should reduce their investment in carnivorous organs in low-light environments and instead reallocate growth to photosynthetic tissues (Zamora et al., 1998; Thorén et al., 2003). Using the size of the fishtail appendage as a proxy for a plant's investment in prey capture, Ellison and Farnsworth (2005) concluded that, contrary to their expectations, *Darlingtonia* plants were unable to regulate the size of the fishtail appendage under changing photosynthetic conditions. The authors also detected remarkably low overall photosynthetic rates for all plants measured (Ellison and Farnsworth, 2005). Unlike other members of Sarraceniaceae, however, *Darlingtonia* can be found growing in shaded habitats (D. W. Armitage, personal observations), where the plants' fishtail appendages contain greater chlorophyll concentrations than plants growing in full sun. This finding suggests that the fishtail appendage might serve a photosynthetic role in light-limited habitats. Therefore, the structure should not be assumed to be independent of a plants' photosynthetic abilities nor a proxy for investment in carnivory.

The fishtail appendage of *Darlingtonia* may instead be involved in supporting local insect populations—a phenomenon that has also been observed in the genus *Nepenthes* (Bazile et al., 2012). Rebecca Austin (1875–1877), the first botanist to study the natural history of *Darlingtonia* in the wild, noted that flies would regularly alight on the fishtail appendage and feed on its nectar, while only a very small minority were drawn into the leaf and trapped. The low capture probability of *Darlingtonia* was later estimated to be approximately 1.7% of vespulid wasps that had already landed on a pitcher leaf (Dixon et al., 2005). The probability of successful prey capture (compared with the overall number of visits) in other pitcher plant species is similarly low, and experimental and observational studies have failed to support the hypothesized roles of numerous other foliar traits in prey capture (Cresswell, 1993; Green and Horner, 2007; Bennett and Ellison, 2009; Schaefer and Ruxton, 2014). These findings, along with the common observation of insects feeding on pitcher leaves and avoiding capture, beg an alternative explanation for the fishtail appendage's role. Joel (1988) hypothesized that the relationship between pitcher plants and insects represents a mutualistic, rather than a predator–prey interaction. The author reasoned that because pitcher plants are commonly found in hydric, sunny habitats, their liberal nectar offerings do not negatively impact a plant's water and carbon budgets. Pitcher plants in the family Sarraceniaceae are often the dominant nectar-bearing members of their local communities and supply nectar to insects continuously throughout the growing season. Nectivorous insects quickly learn to exploit this resource, and the behavior is maintained as long as insect mortality via entrapment is not common enough to be selected against. With its bountiful nectar glands, the fishtail appendage on *Darlingtonia* may facilitate insects' feeding on the plant's nectar while remaining incidental to prey capture. Additional sources of "unsafe" nectar along the margins of the pitcher entrance may tempt the occasional insect into entering the trap while the "safe" nectar presented on the fishtail appendage may

promote and maintain visitation by nectivorous insects. The plant would then benefit from the small proportion of insects lured into the pitcher trap. However, my data do not support this hypothesis, since the per capita prey biomass in isolated, appendage-free populations was not significantly lower than in control populations, although a nonsignificant negative trend was observed. An alternative approach toward testing this hypothesis would involve quantifying the relative contributions of pitcher nectar to the diets of the local insect community. Such a study could be carried out using stable isotope ratios of nitrogen, which are anticipated to differ between the nectars of carnivorous and non-carnivorous plants, and likewise between insects selectively feeding on either nectar source (e.g., Zanden and Rasmussen, 2001).

In conclusion, by experimentally removing the unique fishtail appendages on leaves of *Darlingtonia californica,* I demonstrated that the absence of the structure does not hinder a leaf's ability to capture prey. This finding contrasts with over a century of descriptive literature categorizing the structure as an adaptation to the luring and trapping of prey. There are at least three potential alternative explanations for this structure's evolutionary persistence: (1) as a vestigial homologous structure under little to no selection; (2) as a facultatively photosynthetic structure facilitating carbon acquisition in shaded habitats; and (3) as a source of "safe" nectar encouraging visitation by nectivorous insects and supporting their local populations while entrapping a small proportion of visitors. These alternative roles need not be mutually exclusive and likely depend on environmental factors. Adaptive explanations for the unique morphological and physiological traits possessed by carnivorous plants pervade the literature. However, barring experimental verification, these adaptive hypotheses remain speculative. Going forward, researchers are encouraged to take a more pluralistic approach in their treatments of carnivorous plants by considering alternative evolutionary explanations for the unique foliar features of these plants.

Table 1. ANOVA and PERMANOVA models and test statistics for the effects of appendage removal, pitcher leaf age (days), and pitcher leaf size (mg) on prey biomass (mg) and prey composition in *Darlingtonia californica* leaves. No significant interactions were found among any covariates.

Response variable	Covariate	df	SS	*F*	*P*
Prey biomass	Fishtail appendage	1	5.71	1.952	0.168
	Pitcher age	1	142.3	48.679	**<0.0001**
	Pitcher size	1	5.8	1.984	0.165
	Residuals	56	163.7		
Prey composition (orders)	Fishtail appendage	1	0.230	1.131	0.324
	Pitcher age	1	2.489	12.236	**<0.0001**
	Residuals	36	7.324		
Prey composition (raw)	Fishtail appendage	1	0.497	1.558	0.101
	Pitcher age	1	2.033	6.377	**<0.0001**
	Residuals	36	11.479		

Figure 1. Extent Mature leaf of *Darlingtonia californica* with fully formed fishtail appendage.

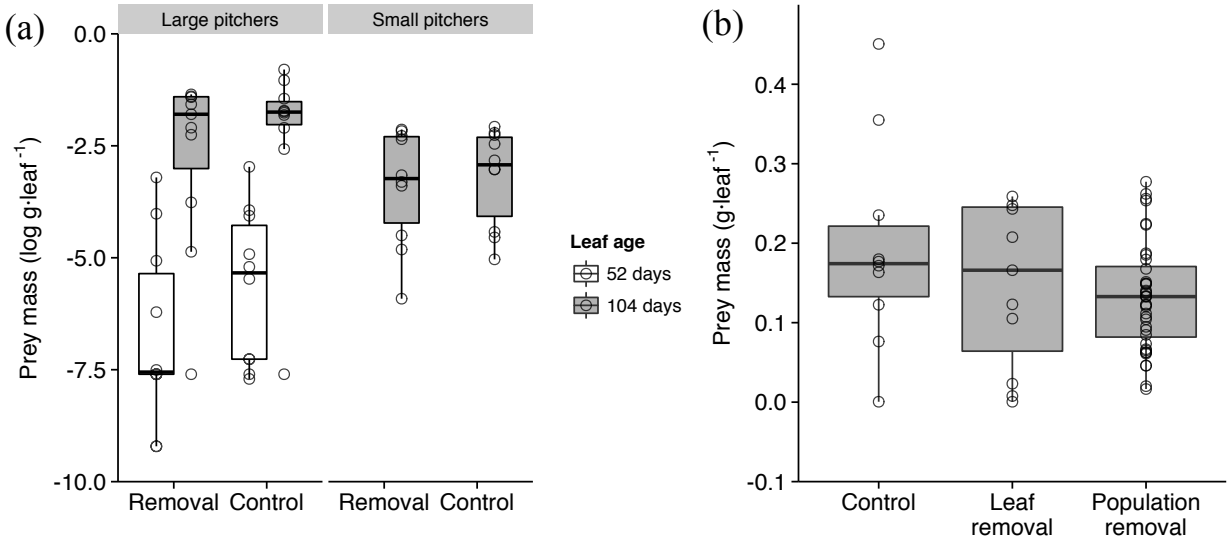

Figure 2. (a) Prey biomass as a function of fishtail appendage presence (*x*-axes), pitcher size (facets), and pitcher age (shading); (b) prey biomass as a function of appendage removal treatment. Boxes denote 25th through 75th percentiles; bolded lines denote median values.

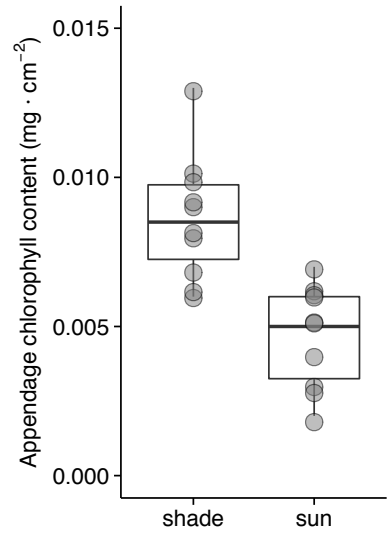

Figure 3. Total chlorophyll content of fishtail appendages from sun- and shade-growing *Darlingtonia* populations. Boxes denote 25th through 75th percentiles; bolded lines denote median values.

Table S1. Counts and identities of all insect corpses recovered from *Darlingtonia* pitchers

Tongue presence	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	1
Leaf age	52	52	52	52	52	52	52	52	52	52	52	52	52	52	52	52	52	52	52	52	104	104	104	104	104	104	104	104	104	104	104	104	104	104	104	104	104	104	104
Blattodea sp.																									1														
Buprestidae																																1	1						
Cantharidae																											1						1						
Carabidae																												1											
Cerambycidae																					1	9	9	1	2	2		14	3	5			6	15	10	42	13	4	5	5
Coccinellidae																							1											1		1				
Coleoptera sp.														1					2			4	1						3			11	2			2	1		2
Elateridae	3											1										1																	
Meloidae																									8														
Chironomidae											5																			5									
Diptera sp.								1			1		2	2	1	4			2	3	7	9	2			3	2	4	1		7	1	11	5	4	4	3	3	1	2
Muscidae														1															1										
Sarcophagidae																						1																	
Tephritidae																														1									
Tipulidae																						1	3					1											
Collembola sp.																																					1		
Cicadellidae																					1												4						
Apoidea											1										2	1			23	2		3			9			10			1	7	1	
Braconidae																														1									
Chrysididae			1																																				
Formicidae				1	2		2						4		1	1				1		2			1															
Halicdidae		1	1		1																																		
Hymenoptera sp.							2					1								3							2	1		2			1	5	1	1	3	2		1
Ichneumonidae	9								1		1					1		1	8		2	6	11				2	3		4	3		5	4	10	7	6	3		5
Sphecidae																						1							1										
Vespidae		1			1		2		1	1			2				1				10		9		7	8							1	4	2		1		9	
Vespoidea																									3	12													9	
Lepidoptera sp.		1		3				1									3					2	1		1									3	5			1	2
Mantidae										1																													
Mecoptera sp.																									2	1													1	
Chrysopidae																						1											2						
Hemerobiidae																																	1						
Zygoptera	3																		1		1	1	1					1		3				1		1			
Acrididae																																			1	1			
Total count	15	3	2	4	4	0	6	2	2	2	8	2	8	3	3	6	4	1	13	7	24	39	38	1	48	28	7	27	7	18	25	1	39	53	31	62	29	21	27	17

LITERATURE CITED

Adams, R. M., II, and G. W. Smith. 1977. An S.E.M. survey of the five carnivorous pitcher plant genera. American Journal of Botany 64:265–272.

Anderson, M. J. 2001. A new method for non-parametric multivariate analysis of variance. Austral Ecology 26:32–46.

ARBER, A. 1941. On the morphology of the pitcher-leaves in *Heliamphora, Sarracenia, Darlingtonia, Cephalotus*, and *Nepenthes*. Annals of Botany 5:563–578.

Austin, R. M. 1875, 1877. Selected letters to W.M. Canby. Delaware Historical Society.

Bazile, V., J. A. Moran, G. Le Moguédec, D. J. Marshall, and L. Gaume. 2012. A carnivorous plant fed by its ant symbiont: a unique multi-faceted nutritional mutualism. PLoS ONE 7:e36179.

Bennett, K. F., and A. M. Ellison. 2009. Nectar, not colour, may lure insects to their death. Biology Letters 5:469–472.

Clarke, C., J. A. Moran, and L. Chin. 2010. Mutualism between tree shrews and pitcher plants. Plant Signaling & Behavior 5:1187–1189.

Cresswell, J. E. 1993. The morphological correlates of prey capture and resource parasitism in pitchers of the carnivorous plant *Sarracenia purpurea*. American Midland Naturalist 129:35–41.

Dixon, P. M., A. M. Ellison, and N. J. Gotelli. 2005. Improving the precision of estimates of the frequency of rare events. Ecology 86:1114–1123.

Ellison, A. M., E. D. Butler, E. J. Hicks, R. F. C. Naczi, P. J. Calie, C. D. Bell, and C. C. Davis. 2012. phylogeny and biogeography of the carnivorous plant family Sarraceniaceae. PLoS ONE 7.

Ellison, A. M., and E. J. Farnsworth. 2005. The cost of carnivory for *Darlingtonia californica* (Sarraceniaceae): evidence from relationships among leaf traits. American Journal of Botany 92:1085–1093.

Franck, D. H. 1975. early histogenesis of the adult leaves of *Darlingtonia Californica* (Sarraceniaceae) and its bearing on the nature of epiascidiate foliar appendages. American Journal of Botany 62:116–132.

Franck, D. H. 1976. Comparative morphology and early leaf histogenesis of adult and juvenile leaves of *Darlingtonia californica* and their bearing on the concept of heterophylly. Botanical Gazette 137:20–34.

Fukushima, K., H. Fujita, T. Yamaguchi, M. Kawaguchi, H. Tsukaya, and M. Hasebe. 2015. Oriented cell division shapes carnivorous pitcher leaves of *Sarracenia purpurea*. Nature Communications 6:6450.

Givnish, T. J., E. L. Burkhardt, R. E. Happel, and J. D. Weintraub. 1984. Carnivory in the bromeliad *Brocchinia reducta*, with a cost/benefit model for the general restriction of carnivorous plants to sunny, moist, nutrient-poor habitats. The American Naturalist 124:479–497.

Grafe, T. U., C. R. Schöner, G. Kerth, A. Junaidi, and M. G. Schöner. 2011. A novel resource–service mutualism between bats and pitcher plants. Biology Letters 7:436–439.

Green, M. L., and J. D. Horner. 2007. The relationship between prey capture and characteristics of the carnivorous pitcher plant, *Sarracenia Alata* Wood. The American Midland Naturalist 158:424–431.

James, J. F. 1885. How the pitcher plant got its leaves. The American Naturalist 19:567–578.

Joel, D. M. 1988. Mimicry and mutualism in carnivorous pitcher plants (Sarraceniaceae, Nepenthaceae, Cephalotaceae, Bromeliaceae). Biological Journal of the Linnean Society 35:185–197.

Juniper, B. B. E., R. J. Robins, and D. M. Joel. 1989. The Carnivorous Plants. Academic Press, London, UK; San Diego, CA.

Lloyd, F. E. 1942. The Carnivorous Plants. Chronica Botanica. Ronald Press, New York, NY.

Oksanen, J., F. G. Blanchet, R. Kindt, P. Legendre, P. R. Minchin, R. B. O'Hara, G. L. Simpson, P. Solymos, M. H. H. Stevens, and H. Wagner. 2015. vegan: Community Ecology Package.

R Development Core Team. 2015. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.

Richardson, A. D., S. P. Duigan, and G. P. Berlyn. 2002. An evaluation of noninvasive methods to estimate foliar chlorophyll content. New Phytologist 153:185–194.

Schaefer, H. M., and G. D. Ruxton. 2014. Fenestration: a window of opportunity for carnivorous plants. Biology Letters 10:20140134.

Schnell, D. E. 2002. Carnivorous plants of the United States and Canada. Timber Press, Portland:468 pp.

Thorén, L. M., J. Tuomi, T. Kämäräinen, and K. Laine. 2003. Resource availability affects investment in carnivory in *Drosera rotundifolia*. New Phytologist 159:507–511.

Wong, W. O., D. L. Dilcher, C. C. Labandeira, G. Sun, and A. Fleischmann. 2015. Early Cretaceous Archaeamphora is not a carnivorous angiosperm. Frontiers in Plant Science 6.

Zamora, R., J. M. Gómez, and J. A. Hódar. 1998. Fitness responses of a carnivorous plant in contrasting ecological scenarios. Ecology 79:1630–1644.

Zanden, M. J. V., and J. B. Rasmussen. 2001. Variation in δ15N and δ13C trophic fractionation: Implications for aquatic food web studies. Limnology and Oceanography 46:2061–2066.