1   **Linking the development and functioning of a carnivorous pitcher plant's microbial**

2   **digestive community**

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4   *Running title:* Pitcher plant microbial succession and functioning

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26 **ABSTRACT**

27 Ecosystem development theory predicts that successional turnover in community

28 composition can influence ecosystem functioning. However, tests of this theory in natural

29 systems are made difficult by a lack of replicable and tractable model systems. Using the

30 microbial digestive associates of a carnivorous pitcher plant, I tested hypotheses linking host

31 age-driven microbial community development to host functioning. Monitoring the yearlong

32 development of independent microbial digestive communities in two pitcher plant

33 populations revealed a number of trends in community succession matching theoretical

34 predictions. These included mid-successional peaks in bacterial diversity and metabolic

35 substrate use, predictable and parallel successional trajectories among microbial

36 communities, and convergence giving way to divergence in community composition and

37 carbon substrate use. Bacterial composition, biomass, and diversity positively influenced the

38 rate of prey decomposition, which was in turn positively associated with a host leaf's

39 nitrogen uptake efficiency. Overall digestive performance was greatest during late summer.

40 These results highlight links between community succession and ecosystem functioning and

41 extend succession theory to host-associated microbial communities.

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INTRODUCTION

Although the capacity for community composition to mediate ecosystem processes is widely recognized (Hooper *et al.*, 2005), few theoretical (Finn, 1982; DeAngelis, 1992; Loreau, 1998) and empirical studies (Fisher *et al.*, 1982; Schmidt *et al.*, 2007) have investigated community-ecosystem linkages along natural successional gradients. Ecosystem development theory (Odum, 1969) seeks to explain temporal variation in ecosystem properties in terms of community successional turnover. Central to this theory is the prediction that successional turnover can influence elemental cycling rates leading to a coupling of community composition and ecosystem processes through time (Odum, 1969; Huston and Smith, 1987; DeAngelis, 1992; Loreau, 1998). It is worth noting that modern succession theory does not assume directionality toward a stable equilibrium (or 'climax'), but instead recognizes that the temporal trajectories of ecosystems can vary due to the relative influences of general ecological processes (Meiners *et al.*, 2015). Although these predictions have not been immune to critique on both theoretical and empirical grounds, adequately replicated tests in natural communities remain scarce.

The natural microcosms of host-associated microbial communities offer a number of unique advantages for testing ecosystem development hypotheses. First, microbiota can enable identifiable and measureable functions for their hosts (Bäckhed *et al.*, 2005; Lugtenberg and Kamilova, 2009). Next, the habitats being colonized are often nearly identical among closely-related individuals, permitting repeated, independent observations of ecosystem development. Finally, the successional dynamics of host-associated microbiota frequently operate over time scales proportional to the host's lifespan, which can manifest as large shifts in community composition and function over relatively short time periods.

This study uses the microbial digestive communities in developing leaves of the pitcher plant *Darlingtonia californica* (Sarraceniaceae) (Figure 1a) to test the following

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76   hypotheses linking community succession to ecosystem function (Figures 1b-e): First, alpha

77   diversity will either asymptotically increase or be unimodal over the host leaf's lifespan as

78   taxa are recruited from the regional pool and subsequently persist or are excluded by superior

79   competitors (Odum, 1969; Loucks, 1970; Auclair and Goff, 1971; Connell and Slatyer, 1977;

80   Fierer *et al.*, 2010). Consequently, trait diversity (e.g., biochemical pathways, C-substrate

81   use) is also expected to increase as succession proceeds (Odum, 1969). Second, rates of

82   biomass production should decrease over time, as growth-limiting nutrients are lost from the

83   system and/or stored in living biomass — this should manifest as a logistic-like biomass-

84   curve (Odum, 1969; Vitousek and Reiners, 1975; Fierer *et al.*, 2010). Third, beta diversity

85   will increase over time if environmental differences among pitchers cause spatially-variable

86   selection or drift, or decrease over time if different leaves constitute similar selective

87   environments (Christensen and Peet, 1984; Dini-Andreote *et al.*, 2015). Fourth, host

88   ecosystem properties (e.g., nutrient cycling, decomposition) should increase monotonically

89   or be unimodal, concomitant with changes in alpha diversity and biomass, as the

90   accumulation of individuals of different species accelerates the degradation of organic

91   material (Cardinale *et al.*, 2007; Weis *et al.*, 2007; Armitage, 2016). This leads to the

92   prediction that biodiversity and biomass dynamics will set ecosystem processes rates (e.g.,

93   decomposition), which, in turn, will set rates on host functioning (e.g., nutrient uptake rates)

94   (Hooper *et al.*, 2005).

95   To test these hypotheses, I followed cohorts of pitcher leaves over three years and

96   quantified their associated digestive communities through time. In addition, I measured these

97   communities' rates of decomposition, respiration, and their host leaves' nitrogen uptake

98   efficiencies. These data were used to test whether host-associated digestive communities

99   follow general, predictable successional patterns and whether their turnover can influence a

100   host's ability to digest prey and sequester nutrients.

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MATERIALS AND METHODS

103   Complete documentation of the study system, data collection, and statistical analyses are

104   provided in the supplementary materials and methods.

105

In situ isotopic labeling of pitcher leaves

107   A stable isotope pulse-chase experiment was used to measure rates of decomposition

108   and nitrogen cycling by the pitchers' aquatic food webs. In early June 2013, I identified and

109   tagged 50 unopened *Darlingtonia* pitcher leaves of equivalent age on different plants

110   growing in a large population in the Plumas National Forest (Plumas Co., CA). Pitcher leaves

111   remain sterile until completing their development and commencing prey capture, and each

112   leaf has a lifespan of approximately 1.5 years. On the day the pitcher leaves first opened in

113   mid-June, I fed gel capsules containing 20 sterile, ^{15}N-enriched fruit flies (*Drosophila*

114   *melanogaster*) to five random leaves, which were then left undisturbed for 11 days. I returned

115   to the site to remove these ^{15}N-labeled pitcher leaves and to feed isotope-labeled flies to 5

116   additional leaves belonging to the same cohort. This process was repeated every 11 days up

117   to day 88 (mid-September), and again on day 365 (June 2014) with 10 leaves. Because the

118   weight of enriched flies (4.25 mg) was much smaller than the average (177 mg) and standard

119   deviations (176 mg) of natural prey masses within a leaf age class, this prey addition was

120   unlikely to significantly overwhelm the natural variation in nutrient levels experienced by

121   pitcher food webs. The 11-day timeframe was chosen based on preliminary data

122   demonstrating peak N incorporation rates by lab-reared plants between 4 and 11 days after

123   prey capture. I repeated this experiment in 2014-2015 in a nearby population of *D.*

124   *californica* and included an additional 166-day sample. The sampled leaves were placed on

125   ice and quickly returned to the lab.

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Quantification of pitcher leaf communities through time

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128   From each freshly-collected leaf I removed 700 microlitres (μL) of fluid for DNA

129   extraction using the PowerSoil microbial DNA isolation kit (MoBio Laboratories, Inc.) and

130   stored the extractions at -80° C. Next, I dissected the pitcher leaves and categorized the state

131   of fruit fly decomposition on an ordinal scale from 0 (no decomposition; flies undamaged) to

132   5 (completely decomposed; head capsules and wings only). I identified and enumerated all

133   protists and living arthropods (primarily *Sarraceniopus* mites and *Metriocnemus* midge

134   larvae) in each leaf's fluid and interior surface under a light microscope and used

135   epifluorescence microscopy to enumerate SYBR-Gold (Thermo Fisher Scientific, Inc.)

136   stained bacterial cells and virus-like-particles bound to 0.02 micrometer (μm) filters. All prey

137   detritus in a leaf was oven-dried at 60° C and weighed.

138

139   *Bacterial community sequencing*

140   Extracted DNA was sent for PCR amplification of the 16S SSU-rRNA genes (primer

141   set 515f/806r) and multiplexed 2×150 bp paired-end sequencing on the Illumina MiSeq at the

142   Argonne National Lab Core Sequencing Facility (Lemont, IL). Sequences were deposited on

143   the MG-RAST public server (http://metagenomics.anl.gov/) server under project ID

144   mgp14344. The QIIME bioinformatics pipeline was used to assemble and cluster reads into

145   97% operational taxonomic units (OTUs) (Caporaso *et al.*, 2010). I calculated each

146   community's alpha diversity (Shannon's H, richness, phylogenetic) and beta diversity

147   (Jensen-Shannon distance and weighted/unweighted UniFrac — a measure of community

148   phylogenetic dissimilarity) using the *vegan* and *PhyloSeq* R packages, and used library size

149   factor (LSF) normalization for all beta diversity metrics (McMurdie and Holmes, 2013; R

150   Development Core Team, 2015; Oksanen *et al.*, 2015; Love *et al.*, 2014). Beta diversities for

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151   each sampling period were estimated using average inter-sample distances, and the results

152   were unchanged when distances-to-centroid were used. I tested whether community

153   composition changed with pitcher age using permutational analysis of variance (Anderson,

154   2001) on samples' Jensen-Shannon distances (JSD) and UniFrac distances and visualized

155   these results using PCoA plots. Results were unchanged when rarefaction was used for

156   normalization.

157   To assess the generality of successional turnover in pitcher communities, I modeled

158   OTU counts using a negative binomial generalized linear model (GLM) (Love *et al.*, 2014).

159   Models were fit using empirical Bayes and OTUs experiencing significant log_2-fold change

160   among time points were identified using Wald *p*-values. I defined the 'successional

161   microbiome' as the subset of OTUs experiencing a statistically significant ($\alpha = 0.01$) $\geq$ 8-fold

162   change in abundance between any two pitcher age classes and used these OTUs to construct

163   an abundance-weighted heat map. The predictive accuracy of this subset of OTUs was

164   assessed by training a random forest machine learning algorithm on OTU counts from the

165   2013 study population and using it to predict the age of samples from the independent 2014

166   study population. Model accuracy was evaluated using the coefficient of determination (R^2)

167   for predicted vs. observed ages along a 1:1 line. The entire bioinformatic/analytical pipeline

168   is illustrated in figure S1.

169

170   *Estimating microbial community traits*

171   The Biolog GN2 microplate assay (Biolog Inc., Hayward, CA) was used to measure

172   the carbon substrate use patterns of the microbial communities from an independent

173   collection of 11, 55, and 365 day-old pitchers (10 from each age in 2014). These time-points

174   were chosen to represent early, middle, and late-stage communities. Plates were inoculated in

175   triplicate using the same dilute, filtered, starved communities described above, and incubated

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176 for 3 days at 25° C. I regressed substrate counts against leaf age using a negative binomial

177 GLM to determine whether the number of metabolized substrates differed among leaf

178 community age. To visualize differences in substrate profiles between age classes, I plotted

179 samples onto principal coordinate (PCoA) axes based on their Jaccard distances.

180   I used ancestral genome reconstruction implemented by the PICRUSt software

181 (Langille *et al.*, 2013) to predict the rRNA copy number and functional gene contents for the

182 subset of OTUs in my samples present in the greengenes database (nearest sequenced taxon

183 index = 0.071 ± 0.01 SEM). I estimated the mean weighted rRNA copy number of each

184 pitcher sample (Nemergut *et al.*, 2015) and then evaluated their temporal turnover using

185 ANOVA. Pitcher samples were then ordinated based on their predicted level 3 KEGG

186 pathway relative abundances (Kanehisa *et al.*, 2016) using principal components analysis

187 (PCA) and then hierarchically clustered. I filtered KEGG pathways using ANOVA *p*-values

188 ($p \leq 0.01$) and effect sizes ($\eta^2 \geq 0.26$) in order to identify genes and pathways (focusing

189 primarily on enzymes involved in protein degradation and nitrogen transformation) that were

190 predicted to be differentially enriched across time points. The predictive nature of these data

191 precluded statistical hypothesis testing, and are treated as speculative hypotheses.

192

193 **Quantification of pitcher ecosystem properties through time**

194   Empty pitcher leaves were thoroughly rinsed, dried at 60° C, homogenized in a bead-

195 beater, weighed, and analyzed for ^{15}N using an isotope ratio mass spectrometer at the UC

196 Davis Stable Isotope Facility (Davis, CA). I used the fly and leaf ^{15}N measurements to

197 estimate the total amount of fly-derived ^{15}N found in a leaf's tissue after 11 days, which is

198 interpreted to be the host leaf's nitrogen uptake efficiency.

199   To estimate each pitcher microbial community's potential C-respiration rate, I

200 inoculated starved, washed pellets of pitcher bacteria into deep-well plates containing 800 μL

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201   sterile medium comprised of M9 salt solution and ground cricket powder. I used the

202   MicroResp^{TM} respirometry system to measure the rates of CO_2-C respired from cultures over

203   three days at 25° C. These rates of CO_2 respiration reflect the potential respiration rates of

204   each pitcher's bacterial community in a common environment.

205   I assessed temporal variation in pitcher ecosystem properties using ANOVA for N

206   uptake efficiency/carbon respiration and a multinomial logit model for the fly decomposition

207   category (Agresti, 2013). Covariates in these models included bacterial biomass and

208   diversity, midge larvae abundance, leaf dry weight, and leaf age. Best-fit models were

209   identified pluralistically using a combination of R^2 and small-sample adjusted Akaike

210   Information Criterion (AIC$_c$) statistics (Burnham and Anderson, 2003). To investigate

211   whether bacterial community composition influenced host functioning, I ran a Mantel test to

212   assess whether pairwise Euclidean distances among samples' N uptake efficiencies covaried

213   with their pairwise JSD or UniFrac dissimilarity metrics.

214

215   **Verifying the effects of community structure on host function**

216   Pitcher leaves of differing ages might physiologically regulate nitrogen uptake independent

217   of their associated food webs, which can obscure food web effects. To account for this, I ran

218   a field experiment to separate the effects of the food web and host leaf age on rates of N

219   uptake. During late July 2014 I identified 15 pitcher leaves aged 11 days, 55 days, and > 365

220   days (5 leaves of each age), intended to represent young, middle-aged, and senescing pitchers

221   based on developmental trends observed the previous year. The fluid from these leaves was

222   removed and mixed in equal parts to form a homogenate. 5 mL aliquots of these

223   homogenized communities were then returned to the host plants. Additionally, 20 ^{15}N-

224   enriched fruit flies were delivered into each leaf. I returned after 11 days to harvest and

225   process these pitchers for N-uptake efficiency as previously described. I used ANOVA to test

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226   whether the N-uptake efficiencies of these pitchers with homogenized food webs

227   recapitulated the N-uptake patterns from natural pitcher food webs of equivalent age from the

228   same population.

229

230   **RESULTS**

231   **Temporal changes in the *Darlingtonia* food web**

232   The dynamics of dead and living biomass were qualitatively similar to the predictions in

233   figure 1a. Pitcher leaves' prey biomass varied widely among leaves of the same age, and

234   mean prey masses quickly increased after opening and remained relatively stable throughout

235   the plant's lifespan (Figure 2a). Bacterial biomass also rapidly accumulated in young pitcher

236   leaves and increased over time during the first growing season to a maximum of 1×10^{11} cells

237   mL^{-1} before declining during the second growing season (Figure 2a). Virus-like particles,

238   *Sarraceniopus darlingtonae* mites, and *Polytomella agilis* flagellates also increased in

239   abundance during the first growing season (Figs. 2a, S1). In addition to *P. agilis,* I detected

240   numerous other eukaryotes, including *Bodo*, *Monas*, *Petalomonas*, *Rhynchobodo*,

241   *Chilomonas*, *Colpoda*, *Philodina*, and *Chlamydomonas*, but these taxa were observed in 10

242   or fewer pitcher leaves with no apparent temporal trends in occupancy or richness (Figure

243   S2). Likewise, I did not detect a temporal trend in bacterivore beta diversity among time

244   points until they diverged in year 2 (Figure S2).

245

246   **Composition and convergence of pitcher bacterial communities**

247   After quality filtering of 16S amplicon sequences, the final OTU table consisted of 3 642 446

248   total reads representing 762 97% OTUs. The minimum and maximum number of reads per

249   sample (n = 99) were 21 983 and 83 157, respectively (mean = 36 972), and read counts did

250   not differ among age classes ($F_{9,89} = 1.3$, $p = 0.26$). Of the top 50 most abundant OTUs

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251   detected across pitcher samples, the majority belonged to families Bacteroidetes (Figure S3),

252   Firmicutes (Figure S4), and Proteobacteria (Figure S5). As hypothesized in figure 1a,

253   bacterial alpha diversities (Shannon's H') peaked at the end of the first growing season and

254   experienced a slight decrease after day 88 (Figure 2b), whereas phylogenetic diversity

255   increased over the entire study period (Figure S2). Taxonomic richness was highly correlated

256   with phylogenetic diversity (Pearson's $r = 0.96$) — increasing over time with the greatest

257   variation among the 365-day samples. In contrast with the prediction in figure 1c, however,

258   community composition tended to converge (i.e., beta diversity decreased) during the course

259   of the first growing season, and diverge again during the start of the second growing season,

260   according to both taxonomic (Figure 2c) and phylogenetic (Figure S2) dissimilarity metrics.

261   Furthermore, permutational ANOVA on Jensen-Shannon and UniFrac distances revealed a

262   structuring of pitcher bacterial communities by age class (table S1) and parallel successional

263   trajectories between years (Figs. 3a, S6).

264   A subset of OTUs experienced particularly strong temporal turnover (Figure 4).

265   These taxa fell primarily into the phyla Proteobacteria (37 OTUs), Bacteroidetes (16 OTUs)

266   and Firmicutes (14 OTUs). Using these OTUs to train a random forest classifier to predict the

267   pitcher community's age resulted in a high classification accuracy for withheld data

268   (observed vs. predicted $R^2 = 0.80$). Likewise, a random forest trained on 2013 data was

269   successful at predicting the ages of samples collected from the independent 2014 population

270   ($R^2 = 0.75$) (Figure S8), implying that observed community trajectories are parallel and

271   generalizable between individuals and populations.

272

273   **Temporal trends in the functional attributes of pitcher microbiota**

274   Assays of pitcher communities' carbon substrate use patterns mirrored trends in taxonomic

275   and phylogenetic alpha and beta-diversities — namely, early and late-stage pitcher

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276   communities both metabolized significantly fewer carbon substrates than did 55-day

277   communities (Figure 5a). Furthermore, 11-day and 365-day pitchers' substrate profiles were

278   much more variable than and clustered apart from the 55-day samples. (Figs. 3b, 5b).

279   A PCA plot of samples' reconstructed metagenomes predicted pitcher samples to

280   separate by age, with the greatest distances between the 11-day and 365-day communities

281   (Figure S8). The average number of rRNA gene copies per taxon was predicted to be greater

282   in 11-day pitchers than in any other age class (Figure S9). This trend was also observed in the

283   relative abundances of a number of other predicted KEGG pathways, such as flagellar

284   assembly, motility, chemotaxis, and ABC transporters (Figure S10). Conversely, a variety of

285   metabolic pathways were predicted to increase over time (Figure S11). Likewise, the

286   abundances of genes involved in nitrogen cycling (deamination, nitrogen mineralization,

287   denitrification, and nitrogen fixation) were also predicted to increase over a pitcher leaf's

288   lifespan (Figs. S12-S15).

289

290   **Linking community dynamics and ecosystem properties**

291   Prey decomposition was unimodal over leaves' lifespans, peaking at 44-88 days (Figure 6a).

292   This increased decomposition, however, did not herald similar temporal differences in

293   common-garden community respiration rates, although there was still a positive, non-

294   significant unimodal trend in mean respiration rates over time (Figure S2). Multinomial logit

295   models predicted bacterial diversity, bacterial abundance, and midge abundance to positively

296   influence a pitcher's probability of having a higher decomposition score (Figures 6b and 6c,

297   Table 1). Leaf nitrogen uptake efficiency also increased during the first growing season and

298   subsequently declined at the start of year 2 (Figure 6d), and was found to be positively

299   associated with decomposition extent and leaf dry mass (Figure 6e, Table 1). Additionally,

300   there was a weak but significant positive correlation between pitcher samples'

301 JSD/unweighted UniFrac distances and their Euclidean distances in nitrogen uptake

302 efficiencies (JSD Mantel $r = 0.08$, $p < 0.05$; UniFrac Mantel $r = 0.10$, $p < 0.05$). Finally, in

303 contrast to natural pitcher samples collected in 2014, the nitrogen uptake efficiencies of

304 experimentally-homogenized pitcher food webs did not differ between leaf age classes ($F_{2,12}$

305 $= 0.98$, $p = 0.40$) (Figure 7).

306

DISCUSSION

307

308 As predicted, community diversity and biomass were positively associated with rates of prey

309 decomposition, and the extent of decomposition was positively associated with the fraction

310 of prey-derived nitrogen removed from the food web by the host leaf. In concert, these

311 results imply that the services these digestive communities provide their hosts are time-

312 dependent — highlighting important, general linkages between the temporal dynamics of

313 communities and rates of ecosystem or host function.

314

Temporal patterns in community composition

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316 The logistic-like accumulation of both bacterial diversity and biomass in developing pitcher

317 leaves aligns with both predictions from succession models (Figure 1b) and time series of

318 animal gut communities (e.g., Koenig *et al.*, 2011; Jemielita *et al.*, 2014). However, it is

319 important to note that both bacterial and midge abundances decreased over the winter —

320 likely in response to the cessation of prey capture. A unimodal or monotonic increase in

321 diversity over time is anticipated for open systems experiencing high rates of immigration

322 and low rates of extinction, which is the likely state of pitcher leaves during their first

323 growing season. Once leaves cease to produce prey attractants, prey capture becomes more

324 stochastic (Wolfe, 1981). Because of this, bacterial communities may experience extinctions

325 under diminishing resource levels or continue to accumulate diversity if prey capture

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326   continues to occur. This may explain the increased variation in diversity among year-old

327   leaves.

328   Because pitcher plant leaves are similar in habitat structure and resource composition

329   at a particular point in time, it is not surprising that bacterial communities converged in

330   composition over the first growing season. This convergence can be attributed to common

331   selection pressures acting on a shared pool of immigrants, which would serve to homogenize

332   communities (see Vellend, 2016). This convergence is supported by the converging carbon

333   substrate and OTU profiles of pitcher communities from two different populations.

334   Successional convergence has also been documented in non-bacterial communities from the

335   pitcher plant *Sarracenia purpurea* (Miller and terHorst, 2012), other phyllosphere bacterial

336   communities (Copeland *et al.*, 2015), the human gut (Palmer *et al.*, 2007), and more

337   generally, across a variety of terrestrial (e.g., Christensen and Peet, 1984) and aquatic

338   ecosystems (e.g., Moorhead *et al.*, 1998).

339   Contrasting with this pattern, year-old leaves contained higher microbial beta

340   diversities than those observed in preceding time points, implying communities diverged

341   over the winter. This is likely the consequence of stochastic prey capture amplifying

342   differences in leaves' ratios of labile to recalcitrant metabolic substrates. If this ratio

343   constitutes a reasonably strong selection gradient, then this heterogeneity should drive

344   divergence among communities (Eisenhauer *et al.*, 2013; Dini-Andreote *et al.*, 2015).

345   Alternatively, stochastic drift can drive community divergence when the number of

346   individuals is small (Orrock and Watling, 2010; Vellend, 2016). In *Darlingtonia* leaves,

347   however, drift is likely minimal, since bacterial population sizes are probably too large to be

348   influenced by demographic stochasticity.

349   Temporal variation in propagule supply can also lead to community divergence

350   (Evans *et al.*, 2017). This might occur when a fraction of ageing leaves, whose communities

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351   had previously been homogenized by a sustained input from a common microbial pool,

352   suddenly experience a more stochastic supply of immigrants. If the species comprising the

353   common immigrant pool also vary over time, then discontinuous, stochastic prey input could

354   drive divergence in communities in the absence of drift and selection effects. A 55-year study

355   of old-field communities observed similar patterns of convergence giving way to divergence

356   driven by dispersal limitation (Meiners *et al.*, 2015). Nonlinear temporal trends in beta

357   diversity have also been identified in host-associated and groundwater microbial

358   communities (e.g., Marino *et al.*, 2014; Zhou *et al.*, 2014), though the processes governing

359   these patterns remain vague. Pitcher microbial communities offer a tractable system in which

360   to experimentally assess the relative influences of deterministic vs. stochastic dispersal on

361   beta diversity.

362

363   **Temporal trends in communities' functional attributes**

364   Leaf communities' carbon metabolic profiles had temporal patterns similar to OTU beta

365   diversity, implicating a link between community composition and metabolic functioning.

366   However, microbial community sequences were not generated from the leaves used for

367   Biolog assays, prohibiting a direct test of this hypothesis. Many of the genes predicted to be

368   enriched in young pitchers (ribosomal RNA copy number, chemotaxis/motility genes) have

369   been linked to a taxon's responsiveness to unpredictable nutrient conditions (Klappenbach *et*

370   *al.*, 2000; Livermore *et al.*, 2014; Nemergut *et al.*, 2015). These predictions are in accordance

371   with successional tolerance and inhibition models, wherein ruderal, fast-responders are

372   eventually joined or outcompeted by more growth-efficient forms (Connell and Slatyer,

373   1977; Huston and Smith, 1987; Tilman, 1990).

374   Metabolic pathways contributing to amino acid demamination and N mineralization

375   were predicted to be enriched during mid-succession — a pattern also detected during

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376   microbial succession on decomposing corpses (Metcalf *et al.*, 2016). Similar successional

377   increases in metabolic genes have been documented in host-associated (Koenig *et al.*, 2011)

378   and aquatic (Teeling *et al.*, 2012) bacterial communities. In concert with the community

379   metabolic assays, these findings demonstrate, in principle, how bacterial communities'

380   taxonomic and functional profiles can undergo predictable changes over a host's lifespan in

381   accordance with predictions derived from succession models. The next step is to relate these

382   community changes to the services they provide the host organism.

383

384   **Linking community properties to host functioning**

385   In agreement with succession hypotheses (Figure 1d), detrital processing rates by the pitcher

386   leaf communities varied over time, and were positively associated with detritivore

387   abundances (bacteria, midge larvae) and bacterial diversity. Loreau (2001) reasoned that

388   microbial diversity would enhance decomposition only if the number of organic compounds

389   able to be metabolized by the community increased with alpha diversity. This prediction is

390   supported by observations of peak bacterial diversity coinciding with peak carbon metabolic

391   diversity during mid-succession (ca. 55 days). To date, the few studies to investigate

392   microbial diversity and decomposition rates *in situ* have arrived at conflicting results

393   (Hättenschwiler *et al.*, 2011) but a positive relationship is common in the few experimental

394   tests using bacteria (Nielsen *et al.*, 2011), including in a lab experiment using bacterial

395   isolates from the same *Darlingtonia* population studied here (Armitage, 2016). More

396   generally, microbial community composition is anticipated to set ecosystem process rates

397   (Figure 1e). — especially when the effects of environmental variation are minimal (Graham

398   *et al.*, 2016).

399   From a host plant's perspective, decomposition by its commensal biota should set

400   limits on its rate of N sequestration. In *Darlingtonia*, the state of fly digestion explained a

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401   some of the variance in N uptake efficiency, though there was still a large amount of

402   unexplained variance to account for. More convincingly, a follow-up experiment failed to

403   detect the same mid-succession peak in N uptake efficiencies among pitcher leaves

404   containing experimentally homogenized bacterial communities. The related pitcher plant

405   *Sarracenia purpurea* also relies heavily on its bacterial community for nitrogen processing

406   (Butler *et al.*, 2008). Furthermore, microbial community composition is an important

407   determinant of nitrogen mineralization rates in soil (Balser and Firestone, 2005; Strickland *et*

408   *al.*, 2009), and changes in N mineralization can track microbial community change over time,

409   independent of environmental variation (Balser and Firestone, 2005). In concert, these results

410   highlight the potential for the pitcher microbial communities to mediate N transfer from prey

411   to host — a function critical to the fitness of a host plant adapted to life in nitrogen-poor

412   soils.

413   Contrary to predictions from succession models (Vitousek and Reiners, 1975; Finn,

414   1982; Loreau, 1998), maximal rates of N loss from the *Darlingtonia* food web occurred

415   during periods of high (rather than low) standing biomass. This mismatch may be explained

416   by differences between donor-controlled food webs, which receive pulses of bioavailable N

417   at regular intervals, and primary producer-controlled food webs, in which the N pool is

418   slowly renewed *in situ* and quickly immobilized (Fierer *et al.*, 2010). As a consequence,

419   donor-controlled food webs may not experience strong competitive pressure to sequester

420   growth-limiting nutrients. This may be particularly true in *Darlingtonia* and other digestive

421   communities for two reasons. First, rapid bacterial turnover (e.g., via viral lysis & protozoan

422   grazing) serves to increase the concentration of bioavailable N. Second, pitcher leaves'

423   continuous accumulation of low C:N detritus (relative to plant-based food webs) may buffer

424   the food web from a loss of nitrogen to the host plant.

425

17

426 **Succession or seasonality?**

427 Because study leaves belonged to the same cohort, their temporal dynamics may reflect the

428 effects of seasonal forcing rather than succession. Although winter temperatures drive the

429 plants into a state of dormancy, their leaves persist, and I have observed active populations of

430 mites, midges, and bacteria in pitcher leaves underneath snow cover, suggesting that the food

431 web still functions during the winter months. Furthermore, these brief cold periods are

432 unlikely to have caused strong population bottlenecks or extinctions, given the large bacterial

433 biomasses observed across pitcher leaves. Seasonal forcing should cause community

434 composition to be cyclical over an annual cycle, yet communities collected from 11 and 365-

435 day samples on the same day were strongly dissimilar, implying that under nearly identical

436 external environmental conditions, communities show measurable age-related differences —

437 an observation in line with previous studies (Thompson *et al.*, 1993; Redford and Fierer,

438 2009; Williams *et al.*, 2013; Metcalf *et al.*, 2016).

439

440 **Cross-system considerations**

441 It is now recognized that community and ecosystem dynamics are shaped by unique

442 combinations of disturbances, competition, and dispersal (Meiners *et al.*, 2015). And

443 although succession is most frequently defined in terms of species turnover, it is reasonable

444 to redefine it as the change in average trait values or gene frequencies within a community.

445 Such change could influence the functioning of the host plant if, for instance, selection

446 favored a more efficient processing or storage of nitrogen by commensal organisms. The

447 potential for rapid evolutionary change to influence ecosystem properties has been

448 documented (Harmon *et al.*, 2009), yet theory integrating ecosystem development and

449 evolution is scarce (Loreau, 1998). In doing so, care must be taken to avoid ascribing

450 adaptive properties to ecosystems (i.e., treating ecosystems as 'super-organisms') (Odum,

18

451   1969). However, because many host-associated systems serve functions critical to their

452   hosts' fitnesses, they may be expected to more closely align with Odum's controversial

453   predictions for increasing stability and productivity. Tests of these predictions (e.g., Beaver,

454   1985; Neutel *et al.*, 2007) using existing quantitative frameworks (Finn, 1982; DeAngelis,

455   1992; Loreau, 1998) would be difficult but valuable contributions toward a unified theory of

456   communities and ecosystems.

457

458   **Conclusions**

459   By combining a ^{15}N stable isotope pulse-chase experiment with observations of

460   community dynamics, I have confirmed a number of successional hypotheses in natural,

461   host-associated microbial digestive communities. In particular, my data support and extend

462   the hypotheses of parallel community trajectories and mid-successional peaks in functional

463   and taxonomic diversity to host-associated bacterial communities. In concert, these results

464   represent a step towards integrating host-associated microbial communities into classical

465   conceptual models of ecosystem development and demonstrate a coupling of community

466   dynamics and host functioning. Looking ahead, more theoretical and experimental work is

467   needed before we can identify definitive links between community dynamics and host

468   functioning, and I believe that the continued experimental use of replicated, natural host-

469   associated communities offers a productive path forward.

470

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632
633 **FIGURE LEGENDS**
634

635 **Figure 1.** Predictions for successional patterns in *Darlingtonia* leaves. **(a)** Conceptual model

636 for the interactions between host leaf (shaded oval) and its digestive food web (boxes).

637 Dashed grey arrows denote ecological processes hypothesized to influence food web

638 dynamics and host functioning. **(b)** α-diversity and living biomass are predicted to increase in

639 pitcher leaves after opening, and eventually either saturate or decrease, consistent with

640 observations made across a variety of ecosystems (Odum, 1969; Loucks, 1970; Auclair and

641 Goff, 1971; Vitousek and Reiners, 1975; Connell and Slatyer, 1977; Peet and Christensen,

642 1988; Alday *et al.*, 2011). **(c)** Compositional differences among leaf communities (β-

643 diversity) may either decrease or increase depending on whether selection is homogenous or

644 variable among leaves (Christensen and Peet, 1984; Dini-Andreote *et al.*, 2015; Meiners *et*

645 *al.*, 2015). **(d)** Ecosystem or host function is anticipated to be unimodal or saturating over a

646 successional gradient (van Ruijven and Berendse, 2005; Cardinale *et al.*, 2007; Weis *et al.*,

647 2007; Armitage, 2016), — a pattern predicted to be influenced by **(e)** the positive effects of

24

648 α-diversity and living biomass on ecosystem function (Hooper *et al.*, 2005; Bell *et al.*, 2005).

649 Dashed lines denote alternative hypotheses.

650

651 **Figure 2.** Trends in community composition during succession. **(a)** Insect prey biomass

652 rapidly increased in leaves after opening and remained relatively steady throughout the

653 remainder of the leaf's lifespan, while bacterial and midge larval abundances steadily

654 increased throughout leaves' first growing season, and then sharply declined after the first

655 year. **(b)** Bacterial alpha diversities increased and then leveled off in middle-aged pitcher

656 communities, dropping slightly during year 2. **(c)** Conversely, leaf bacterial beta diversities

657 decreased during the first growing season and increased at the beginning of year 2. In each

658 graph, shared letters above groups indicate no significant pairwise differences ($p > 0.05$).

659 Points denote mean values $\pm$ SEM.

660

661 **Figure 3.** Principal coordinate (PCoA) plots for **(a)** Jensen-Shannon distances between

662 samples, demonstrating convergence and approximately-parallel successional trajectories in

663 between-population community structures over time, and **(b)** Jaccard distances between

664 Biolog ^{TM} plates for communities of different ages, demonstrating the convergence of

665 metabolic profiles in mid-successional pitcher leaves and overlapping metabolic profiles for

666 young and senescing leaves. The percentages of variance explained by the principal

667 coordinates are displayed on each axis. Points denote yearly centroid values $\pm$ SEM.

668

669 **Figure 4**. Abundance-weighted heat map of 97% OTUs that experienced significant ($p <$

670 0.01) 8-fold or greater turnover between time points for the **(a)** 2013 Blackhawk Creek and

671 **(b)** 2014 Butterfly Valley study populations. Tick marks on X-axis denote individual pitcher

672 samples. OTUs are labeled by family and ordered based on the community age in which they

673 were first detected regardless of year. Random forest models trained on OTU abundances

674 from 2013 were able to predict the ages of 2014 samples with 75% accuracy (Figure S7).

675

676 **Figure 5. (a)** Mid-successional pitcher communities were capable of metabolizing

677 significantly more Biolog GN2 plate C-substrates than were early- and late-stage

678 communities. **(b)** Mid-successional pitcher communities were much more similar to one

679 another in terms of their carbon metabolic profiles than were early- and late-stage pitchers.

680

681 **Figure 6.** Trends in ecosystem properties during succession. **(a)** The frequencies of

682 decomposition classes for pitchers of different ages. Square size is proportional to relative

683 frequency of a particular decomposition category for that age class. χ^2 is the likelihood ratio

684 test statistic for the effect of pitcher age on the fit of a multinomial logit distribution to

685 predict decomposition categories. **(b & c)** The probabilities of observing high decomposition

686 rates increases with both bacterial diversity and bacterial biomass. Curves represent fitted

687 proabilities of multinomial logit models, and individual curves can be interpreted as logistic

688 regression fits for each decomposition category. **(d)** Pitcher leaves' nitrogen uptake

689 efficiencies change over time, and are significantly lower in late-stage pitcher leaves. Points

690 denote mean values ± SEM. **(e)** The extent of prey decomposition is positively associated

691 with the percentage of prey-derived nitrogen found in the host leaf's foliar tissue. The dashed

692 line denotes the best-fit linear model ± 95% CI.

693

694 **Figure 7.** Homogenizing the food webs of 11, 55, and 365 day pitchers and placing them

695 back into the plants removes the significant differences observed in natural pitcher

696 communities of the same ages. Letters above the groups represent the within-treatment

697 contrasts. Points denote mean values ± SEM (n = 5).

26

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_c and R^2 statistics. AIC_c values falling within 9 units of the top model were considered equally parsimonious.

Decomposition Category			Nitrogen Uptake Efficiency		
Predictor Variables	ΔAIC_c	$pseudo\text{-}R^2$	Predictor Variables	ΔAIC_c	R^2
~ Community age [1] (A)	32	0.71	~ Community age [1] (A)	418	0.17
~ Bacterial abundance (B)	23	0.37	~ Bacterial abundance (B)	420	0.04
~ Bacterial diversity (D)	26	0.34	~ Bacterial diversity (D)	430	0.05
~ Bacterivore richness (R)	61	0.05	~ Log midge abundance (M)	436	0.00
~ Log midge abundance (M)	45	0.20	~ Log mite abundance (N)	436	0.01
~ Log mite abundance (N)	60	0.05	~ Leaf dry mass (P)	410	0.23
~ B + D	**0**	0.56	~ Decomposition category (C)	14	0.16
~ B + D + M	**3**	0.59	~ A + C + P	**4**	**0.37**
~ A + B + D + M	24	**0.82**	~ A + B + D + P + C	**0**	0.34
~ 1 (intercept-only null)	55	0.00	~ 1 (intercept-only null)	434	0.00

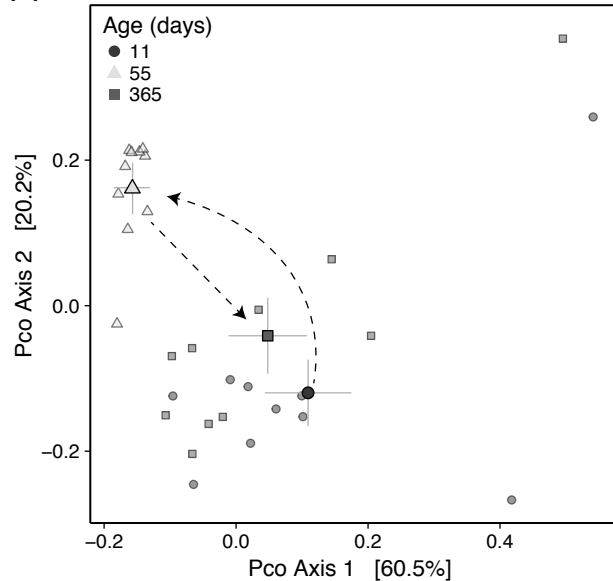698   [1] Age covariate was modeled as quadratic

(a) Host leaf

External environment

Digestive food web

Host environment

Drift

Species interactions

Sp.1   Sp.2
Sp.3
Trait A
Trait B
Trait C

Sp.3   Sp.4
Sp.5
Trait C
Trait D
Trait E

Sp.5   Sp.6   Sp.7
Trait C   Trait E
Trait F
Trait G

Time

Prey capture & immigration

Decomposition & Mineralization

Host nutrient uptake

Host nutrient budget
N P P K N
P N K P N

(b) α - diversity or living biomass vs Time since establishment

(c) β - diversity vs Time since establishment

(d) Ecosystem or host function vs Time since establishment

(e) Ecosystem or host function vs α - diversity or living biomass

Figure 1

Figure 2

(a) Bacterial community composition

Age (days)
□ 11
□ 22
□ 33
□ 44
■ 55
■ 66
■ 77
■ 88
■ 166
■ 365

Year
● 2013
▲ 2014

Pco Axis 1 [19%]
Pco Axis 2 [15.6%]

(b) Community carbon metabolic profile

Age (days)
● 11
▲ 55
■ 365

Pco Axis 1 [60.5%]
Pco Axis 2 [20.2%]

Figure 3

(a) 2013-2014 (b) 2014-2015

Families
S Sphingomonadaceae
E Enterobacteriaceae
V Veillonellaceae
X Xanthomonadaceae
R Ruminococcaceae
Cl Clostridiaceae
B Bradyrhizobiaceae
C Chitinophagaceae
   Other / Unclassified

Normalized
abundance
0   5   10

Pitcher age (days)

Figure 4

(a) Metabolic *α*-diversity

$\chi^2_2 = 32.7$
$p < 0.0001$

No. GN2 substrates utilized (± SE)

Community age (days)

(b) Metabolic *β*-diversity

$F_{2,132} = 93.1$
$p < 0.0001$

Jaccard distance (± SE)

Community age (days)

Figure 5

Figure 6

Figure 7