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Leaf-Associated Bacterial and Fungal Taxa Shifts in Response to Larvae of the Tree Hole Mosquito, *Ochlerotatus triseriatus*

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Abstract

Larvae of the eastern tree hole mosquito, *Ochlerotatus triseriatus* (Say), and related container-breeding species are known to feed upon substrate-associated microorganisms. Although the importance of these microbial resources to larval growth has been established, almost nothing is known about the taxonomic composition and dynamics of these critical microbial food sources. We examined bacterial and fungal community compositional changes on oak leaves tethered in natural tree hole habitats of *O. triseriatus*. We eliminated larvae experimentally in a subset of the tree holes and examined 16S rDNA gene sequences for bacteria and ergosterol concentrations and 18S rRNA gene sequences for fungi collected from leaf material subsamples. Leaf ergosterol content varied significantly with time, but not treatment. Principal component analysis (PCA) was used to compare microbial taxonomic patterns found in leaves incubated with or without larvae present, and we found that larval presence affected both bacterial and fungal groups, either from loosely attached or strongly adherent categories. Bacterial communities generally grouped more tightly when larvae were present, and class level taxa proportions changed when larvae were present, suggesting selection by larval feeding or activities for particular taxa such as members of the Bacteroidetes, Alphaproteobacteria, and Betaproteobacteria classes. Fungal taxa composite scores also separated along PC axes related to the presence of larvae and indicated larval feeding effects on several higher taxonomic groups, including Saccharomycetes, Dothideomycetes, and Chytridiomycota. These results support the hypothesis that larval mosquito feeding and activities altered microbial communities associated with substrate surfaces, potentially leading to decreased food value of the resource and affecting decomposition of particulate matter in the system.

Introduction

Ochlerotatus triseriatus (Say) is a common container-breeding mosquito in eastern North America and the primary vector of La Crosse encephalitis virus. Larvae develop in water-filled tree holes and tires that are normally dominated by heterotrophic microbial activity [23]. The system is driven by both particulate and soluble inputs and by the subsequent microbial processing of plant and animal detritus [27]. Previous studies have addressed the importance of leaf material inputs into tree holes [31] and access to leaf substrate surfaces for browsing larvae [9, 30], but few have examined specific microbial responses to larvae. Most previous sampling and characterization of the biota in tree holes and other phytotelmata has, for practical purposes and because of specific interests in the macroinvertebrate faunal diversity, focused on water column samples [27, 41]. In the only published study thus far to employ molecular techniques to examine tree hole bacterial populations, Bell et al. [3] showed that water column bacterial diversity increased with the size of the habitat; however, that study did not examine invertebrate predation effects.

Of those studies quantifying substrate-associated or biofilm microbial responses to larval mosquito inhabitants of tree holes, only broad categories have been examined such as bacterial abundance, bacterial productivity, or fungal biomass [21-24]. Compared to findings of variable or no measurable larval feeding effects on overall bacterial abundance and productivity in the water column, larvae appear to consistently reduce both numbers and growth rates of bacteria on exposed leaf surfaces [21-24]. However, these studies did not address any possible changes in microbial community composition. Thus far, studies demonstrating mosquito larvae feeding effects on microbial community composition [7, 21, 28, 41] have examined only water column bacteria or protozoan communities. In addition, using measures of ergosterol concentrations in leaf material from larval habitats, previous studies [22, 24] have not consistently demonstrated a larval feeding effect on fungal biomass. Kaufman et al. [23] showed that larvae could reduce leaf ergosterol concentrations per unit surface area, but most likely as a consequence of reducing overall leaf mass. Larval feeding on leaf material is more aptly described as surface browsing rather than shredding of leaf material [35, 42], and we have concluded that much fungal biomass is typically unavailable to larvae because it is associated with the leaf matrix and not necessarily the exposed surfaces [22]. Fungal biomass estimates using ergosterol are dependent, however, on the fungal community present as ergosterol content varies with fungal species [10].

Our interests in examining the taxonomic composition of major microbial groups on leaf surfaces in tree hole ecosystems are related to our goals of better understanding mosquito production from these and similar habitats. Microbial biomass associated with particulate detritus in tree holes provides a direct source of nutrition to larvae, but perhaps more importantly, microorganisms are key to the transformation of inaccessible nutrients in the particulate material. We have shown, for example, that fungal biomass constitutes a substantial (~10%) portion of the detrital biomass in these habitats and that fungal enzyme activity can be related to mosquito production [23, 24]. Yet, there is little information on what groups comprise this biomass or if only a few species dominate. A notable exception is the survey of fungi associated with leaves in tree holes in Hungary by Gönczöl and Révay

[11]. Similarly, we know that leaf-associated bacteria are harvested by larvae, but no published studies of specific bacterial taxa associated with tree hole detritus are available.

The objectives of this study were to quantitatively describe bacterial and fungal communities associated with decaying leaves in natural tree holes and to relate microbial community composition to larval feeding or other activities. Based on our previous studies, we hypothesized that bacterial communities would show strong responses to larval feeding, but that fungal communities would be largely unaffected. To our knowledge, this is the first replicated study to address the compositional changes of substrate-associated microbial prey items in a larval mosquito habitat.

Materials and Methods

The field study took place in Toumey and Hudson woodlots, near the main campus of Michigan State University, East Lansing, MI. Tree holes in these beech-maple woodlots typically contain dense populations of *O. triseriatus* larvae and have been used previously for study or as sources of material for laboratory microcosms [23, 43]. Senescent oak leaves that had been dried and weighed were tethered with monofilament line tied to the petiole in tree holes in early May. We initially placed two leaves in each of over 23 holes. One randomly chosen leaf was used for subsampling, and the other leaf was used for mass loss estimates. In half of the tree holes, larvae were eliminated with a *Bti* bacterial larvicide formulation (Teknar HP-D, Zoecon Corp., Dallas, TX, see [34]). In tree holes not receiving the larvicide, cadavers of heat-killed *O. triseriatus* larvae from our lab colony were added to account for dead larval biomass in the treated holes. We have not examined the effects of *Bti* on tree hole microbial communities independent of its larvicidal effects; however, the bacterium would be associated mainly with insect cadavers, and we have not recovered sequences or isolates of the organism from water column or leaf material samples in similar previous studies (unpublished data) and in this study. Therefore, we assume here that *Bti* treatment effects on leaf microbial communities were primarily indirect and mediated through larval mortality. Leaves were subsampled periodically before and after treatment using a 1.5-cm diameter cork borer. At each sampling, two leaf discs were placed in 5 ml high performance liquid chromatography (HPLC) grade methanol for later ergosterol analysis, and two discs were placed in 2 ml of filter-sterilized phosphate buffer. All samples were kept on ice during transport. Ergosterol samples were stored in refrigerated, dark conditions, and samples for community analysis were processed immediately for DNA extraction. Ergosterol was extracted and measured as described previously, using HPLC and UV detection and ergosterol standards for quantitation ([22] and references therein).

Two categories of microbial community DNA were extracted: (1) A “loosely attached” fraction obtained by sonicating the leaf material for 12 min in an ice-filled sonicating bath and (2) an “adherent” fraction obtained by pulverizing the previously sonicated leaf discs in liquid nitrogen. Total DNA was subsequently extracted from these individual samples with an Ultra Clean Soil DNA kit (Mo Bio Laboratories, Solana Beach, CA) according to the manufacturer’s instructions. The purified DNA was resuspended in 50 µL of elution buffer and stored at –20°C until polymerase chain reaction (PCR) amplification.

Sequences were prepared from DNA samples extracted 13 days after the leaves were placed in the tree holes (“early decay”) and 13 days after *Bti* treatment (56 days after leaf placement). DNA (~10 ng) was amplified as described below for six individual treehole samples from each treatment. Composite samples were made from six randomly selected tree holes to create the early decay sequences, using ~2 ng from each leaf fraction. Composite samples for each treatment and each leaf fraction were also made from the six individual replicates, using ~2 ng of DNA from each.

Bacterial rRNA Gene Sequences

For bacterial sequences, an approximately 1,300 bp region of a consensus 16S rRNA gene was generated by PCR amplification using the forward primer 63f (5'-CAGGCC TAACAC ATGCAAGTC-3') and reverse primer 1387r (5'-GGGCGGWTGTACAAGGC-3') [32]. The PCR mixture (100 µl) contained 50 µl of FailSafe™ (Epicenter, Madison, WI) PCR PreMix buffer E, 4 µl of each primer, 1 µl of the FailSafe™ PCR enzyme (Taq polymerase), ~10 ng of DNA template, and DNA analysis-grade water to bring the mixture to final volume. PCR cycle parameters were as follows: an initial step at 80°C for 1 min, then 30 cycles of denaturing at 95°C for 1 min, annealing at 60°C for 1 min, extension at 72°C for 1.5 min, followed by a final extension at 72°C for 4 min.

PCR amplicons were each ligated into pGEM-Teasy vectors and transformed into chemically competent *Escherichia coli* JM109 following the manufacturer's protocol (Promega, Madison, WI). *E. coli* JM109 transformants were screened on S-Gal agar plates with ampicillin (100 µg/ml) (Sigma, St. Louis, MO). Randomly picked white colonies per sample were isolated, purified, and each grown overnight at 37°C in 1 ml of Luria–Bertani broth supplemented with ampicillin (100 µg/ml) and submitted to Research Technology Support Facility (RTSF) at Michigan State University for plasmid extraction and sequencing.

Fungal rRNA Gene Sequences

All PCRs for fungal DNA were carried out in 100 µL volumes using the FailSafe™ PCR System as described for bacterial sequence generation. Fungal rRNA genes were amplified with the fungi-specific primers nu-SSU-0817-5' (5'-TTAGCATGGAATAATRAATAGGA-3') and nu-SSU-1536-3' (5'-ATTGCAATGCYCTATCCCCA-3') [4]. Amplification was performed using the following protocol: initial denaturation at 94°C for 2 min, followed by 30 cycles of 94°C for 2 min, 53°C for 30 s, and 72°C for 1 min, with a final extension at 72°C for 7 min. Amplicons were transformed, cloned, and submitted for sequencing as described above.

Data Analysis

Ergosterol concentrations were compared using a multivariate repeated measures analysis of variance (ANOVA). Valid (after chimera checks) bacterial sequences were classified with the Ribosomal Database Project classifier program (rdp.cme.msu.edu) at MSU. Fungal sequences were classified using Basic Local Alignment Search Tool (BLAST - www.ncbi.nlm.nih.gov/BLAST). We used the percentage of sequences falling into class level categories or above as variables for Principal Component Analysis (PCA) of the data.

Scores from components 1–3 and arcsine-square root transformed percentages of taxa categories with high loadings along PC axes were compared by treatment using standard ANOVA procedures. ANOVA results are expressed in the results as $F = F$ statistic, $df =$ degrees of freedom, and $p =$ probability value. We used JMP® Statistical Discovery Software, V5.1 (<http://www.jmpin.com>, SAS Institute, Inc., Cary NC, USA) for all data summaries and statistical tests.

Results

Leaf and Fungal Mass

The mass loss of leaves that were tethered in the tree holes, but not sampled during the experiment, were variable (overall range from 9–49%) and did not differ among *Bti*-treated and control tree holes. The mean mass loss was 26.4% (95% CI = 4.9%) for controls and 23.6% (95% CI = 4.6%) for treated habitats after 56 days exposure.

Ergosterol content of leaf material also was unaffected by treatment, but showed a significant increase with time and no significant interaction of time with treatment (Fig. 1, Table 1).

Bacterial Communities

An average of 76 sequences for individual reps and 81 for composites (over 2,000 total) were used for classification and analysis.

Based on PCA analysis, both loosely attached and adherent fractions of leaf bacterial communities tended to be more similar and less variable in tree holes with larvae than without (Fig. 2). PC axes 1–3 explained between 77 and 85% of the variance for both fractions. Interestingly, composite samples did not necessarily group with individual replicate samples. PC 1 scores were significantly different between treatments for loosely attached ($F=12.7$, $df=1,10$, $p=0.005$) and for adherent ($F=7.8$, $df=1,10$, $p=0.016$) forms. Highest loadings were for Bacteroidetes, Flavobacteria, and Betaproteobacteria for the loosely attached fraction, and Betaproteobacteria, Alphaproteobacteria, and Bacteroidetes and for the adherent fractions. Differences between treatments within these groups are apparent in the distribution of taxa (Figs. 3 and 4). In the loosely attached fraction, the percentage of sequences for Bacteroidetes ($F=10.7$, $df=1,10$, $p=0.008$) and Betaproteobacteria ($F=16.2$, $df=1,10$, $p=0.002$) were significantly different between treatments. For the adherent fraction, the Alpha- ($F=8.4$, $df=1,10$, $p=0.016$) and Beta-proteobacteria ($F=8.3$, $df=1,10$, $p=0.016$) percentages were significantly different. Major bacterial groups seen in the early decay composite samples were generally present in later samples, and in the loosely attached fraction, the early decay sample was most similar to the later, no-larvae treatment and grouped accordingly with PCA (Figs. 2 and 3).

The most commonly encountered taxa below the class level were those of the Flavobacteriaceae and a family group of uncertain status within the order Burkholderiales (Table 2). Unclassified members of the Betaproteobacteria were the most numerous sequence classifications overall. RDP does not classify sequences below the genus level, and the most commonly identified genus was *Flavobacterium*. Other genera that accounted for

more than 10 total instances of classification were *Phenylobacterium* and *Asticcacaulis* (both Caulobacteriaceae), and *Geothrix* (Acidobacteria). *Phenylobacterium* and *Geothrix* were found only in samples from tree holes treated with *Bti*.

Fungal Communities

An average of 57 sequences for individual reps and 97 for composites (over 1,900 total) were used for classification and analysis.

Fungal communities were variable among tree holes and within treatments (Fig. 5), but individual replicates separated by treatment along PC 1 and 3 for the loosely attached fraction and along PC 3 for the adherent category. PC 1 and PC 3 scores were significantly different between treatments for the loosely attached fraction (PC 1— $F=6.70$, $df=1,10$, $p=0.017$; PC 3— $F=9.26$, $df=1,10$, $p=0.012$). PC 3 scores were significantly different for the adherent ($F=8.13$, $df=1,10$, $p=0.014$) fraction. Letiomycetes, Dothideomycetes, mitosporic Ascomycota, Saccharomycetes, and Chytridiomycota loaded highly along PC 1 and 3 for the loosely attached fraction, while Dothidiomycetes, Sordariomycetes, and Saccharomycetes were most important in construction of PC 3 for adherent samples. The relative abundance of Saccharomycetes, Dothideomycetes, and Chytridiomycota in the loosely attached fraction suggested treatment effects (Figs. 6 and 7), and these were significant when percentages were compared (Saccharomycetes— $F=5.3$, $df=1,10$, $p=0.044$; Dothideomycetes— $F=7.12$, $df=1,10$, $p=0.024$; Chytridiomycota— $F=11.17$, $df=1,10$, $p=0.007$). For the adherent fraction, the Dothideomycetes percentages were significantly different between treatments ($F=11.15$, $df=1,10$, $p=0.008$). All groups present in samples collected after treatment were also present in the early decay composite samples. As with the bacterial sequence classifications, early decay samples for the loosely attached fraction more closely resembled later no-larvae samples and grouped with that treatment in PCA (Fig. 5).

Fungal species that were matched to more than five total sequences across all samples within a fraction are listed in Table 3. All sequence matches listed in Table 2 were at 95% or better matches in the BLAST search results. The most numerous species, and one present in nearly every sample, was *Dimorphospora foliicola*. *D. foliicola* was found 2–3× more often in samples from *Bti*-treated treeholes. Other species that often dominated individual replicate samples were *Monoblepharis* spp. in the Chytridiomycota group and *Cucurbitaria berberidis* from a Dothideomycetes and Chaetothyriomycetes grouping. Species in the Saccharomycetes class, *Williopsis salicorniae*, being the numerical dominant, were found almost exclusively in the loosely attached fraction of samples from treatments with larvae present. Several prominent species in the mitosporic Ascomycota and Leotiomycetes groups were not found in the early decay composite samples.

Discussion

The use of rRNA gene sequence data to describe and identify microbial groups independent of culturing is now commonplace. The technique's utility in quantifying relative abundances of microbial populations, however, remains somewhat questionable as PCR bias and variable rRNA gene copy number make extrapolation from sequence abundance data to population abundance difficult [2, 5, 8]. Our comparison of relative abundance of sequences

is valid within the context of the study because we used the same techniques on all samples and were primarily interested in how taxa changed with treatment, not as an estimate of absolute population sizes. The results of the sequence analyses collectively suggest that larval grazing/presence can be an important force in shaping the communities of microorganisms associated with large particulate matter in tree hole systems and that larvae are ultimately changing their microbial resource base and potentially altering detritus decomposition dynamics.

Bacterial Communities

Major bacterial taxa found in this study appear to be typical of freshwater habitats that are often dominated by Betaproteobacteria when sequence data are used [2, 26]. Overall, relatively few sequences were classified only to the level of Eubacteria, suggesting that although there may be unique species present, the bacterial flora of treeholes is not markedly different from other freshwater habitats. The taxa represented (Table 2) also likely reflect inputs from surrounding soils, as the tree holes studied were located at the base of the tree, forming in cavities between buttressing roots.

The main effect of larvae on leaf bacteria was to depress members of the Alphaproteobacteria and Bacteroidetes classes and to enhance the relative abundance of Betaproteobacteria. Within the Alphaproteobacteria, the Bradyrhizobiaceae and Caulobacteriaceae groups appeared to benefit most from larval removal. The latter family is noted for its stalked forms and may simply be susceptible to direct larval grazing. The Bacteroidetes phylum (includes the *Cytophaga/Flavobacterium/Bacteroides*—CFB) includes important polymer degrading bacteria and would be expected to be prominent on leaf material [26]. We have observed in previous studies (unpublished data, submitted manuscript) that *Flavobacterium* spp. are reduced in the presence of larvae, particularly in water column samples, and have targeted this group for additional genetic study [6]. A somewhat surprising result here is that larvae appeared to have had little impact on the Flavobacteriaceae, and instead, reduced other Bacteroidetes members, the Porphyromonadaceae (Table 2) that are typically anaerobic, heretofore found mainly in gut systems and the human oral cavity (e.g., [16, 39]). The fact that anaerobic microhabitats might arise on submerged leaf material is not surprising, and our measurements of dissolved oxygen that indicate saturation levels of below 10% are common in tree holes. Our observation that larvae affected Flavobacteria in the water column (unpublished data, submitted manuscript) and did not have the same degree of influence on leaf surface Flavobacteria is consistent with the idea that bacteria attached to particles are more resistant to some kinds of predation [20]. It is noteworthy that the CFB group, in general, seems susceptible to metazoan predation [15, 26], however, previous studies have generally investigated only the responses of bacterioplankton to zooplankton grazing [29, 37].

The Betaproteobacteria are an extremely diverse group metabolically, often involved in transformations of nitrogen compounds. As the effect here was observed on both the loosely attached and the adherent community, it may be that larvae affected this group of bacteria indirectly (e.g., ammonia excretion). It may also be that members of this group tend to be more resistant to digestion, and thus, proliferate at the expense of other groups that are

susceptible [19, 25]. However, larval feeding on leaf material would dislodge and displace even digestion resistant forms, implying a rapid recolonization and proliferation of those bacteria. It is likely that the increased dominance of Betaproteobacteria members in the presence of larvae also reflected a stronger ability to adhere to the substrate.

It is well-established that larval mosquitoes feed extensively on bacterial communities [35], but there is little information about how those communities might be subsequently altered. Kaufman et al. [21] showed that *O. triseriatus* larvae reduced some Gammaproteobacteria in the water column and concurrently enhanced the numbers of facultative anaerobes. Most research documenting changes in bacterial community composition related to invertebrate feeding has been done in zooplankton/bacterioplankton systems and has focused on the cascade of effects involving the protozoan trophic level [37]. Metazoan feeding effects on bacteria associated with surfaces is not well-studied, and we do not yet know if protozoans are intermediaries in any larval feeding effect.

Fungal Communities

As we've reported previously [22, 24], larval feeding effects on overall fungal biomass as estimated by ergosterol concentrations were not readily apparent. In contrast to previous studies, we observed here that the dominant fungal groups were similar, and therefore, ergosterol comparisons are a valid surrogate for biomass comparisons [10]. Although overall biomass did not change with treatment, fungal groups did respond to larvae. Invertebrate feeding on fungi associated with detritus is a common feature of decomposer food webs, and most information has come from studies of leaf decay in freshwater streams (reviewed in [12]). Although aquatic invertebrates are known to prefer some fungal species over others and may alter fungal communities accordingly [12, 38], there is no evidence that mosquito larvae actively select particular groups. Any selective feeding is more likely a consequence of having a growth form or spores that would expose tissues to larvae browsing on leaf surfaces.

The fungal taxa reported here represent the first such investigation of tree hole fungi species for North America. In a study in Hungary, Gönczöl and Révay [11] cataloged approximately 40 different species based on induced sporulation from detritus collected from tree holes. Perhaps partly because of methodological differences, our list of species overlapped very little with that from Hungarian tree holes. Only *Dimorphospora foliicola* was common to both, although the *Tricladium* genus was also shared. *D. foliicola* appears to be a ubiquitous and perhaps opportunistic colonizer, as it has also been found on wood and leaf material in the U.S. and European streams [13, 14]. *D. foliicola* was found to be dominant on decaying maple leaves in a stream section enriched with inorganic nutrients [14], so its dominance in the tree holes we studied is consistent with the nutrient enrichment we have found in these systems [42].

From a broader taxonomic perspective, larval presence enhanced members of the Dothideomycete and Saccharomycete group, while depressing the Chytridiomycota. Larvae might have been predicted to reduce groups like chytrids and yeasts, as the members are not known for their ability to degrade senescent plant material polymers [44]. Neither group is characterized by invasive hyphae that would penetrate the leaf matrix and presumably make

them less accessible to larvae except when sporulating. It is therefore surprising to see that yeasts increased with larval presence. However, yeasts in biofilms may respond positively to predation if only the yeasts' exopolymer is harvested [18]. The decline in chytrids with larval presence is also counter-intuitive, as many are parasites or commensals of invertebrates [44]. However, the Monoblepharidales are considered general saprophytes and produce mycelia [1] that would be available for larval grazing. The enhancement of Dothidiomycetes by larval presence may, as we suggested for the Betaproteobacteria above, be related to direct grazing resistance or reduced competition because of larval harvest of competing groups. Although larvae did not affect the Leotiomyces group as a whole, its dominant member, *Dimorphospora foliicola*, was less abundant in treatments with larvae. An uninvestigated aspect of this study is the likelihood that larvae were consuming spores of the fungal groups present and not necessarily vegetative biomass. It is unclear if 18S sequence analyses used here preferentially assess sporulating taxa, but interpretation of differences in relative abundances in the fungal groups may be more complex than those of bacteria.

The finding that *O. triseriatus* larvae affected bacterial and fungal communities from both the loosely attached and adherent fractions was not expected. In general, larval feeding effects on both bacterial and fungal communities in the loosely attached fraction were more pronounced, but the effect on the adherent fraction was still evident in PCA of major groups. Our previous observations of larval feeding effects on bacteria associated with leaves [22], and from behavioral observations of *O. triseriatus* feeding behavior [42], would have suggested that the loosely attached community would be most dramatically altered. We contend that larval feeding likely affected the loosely attached fraction directly (ingestion, dislodgment), and impacts on the remaining, more adherent fraction were indirect. However, Tall et al. [40] showed that different stream macroinvertebrate groups ingested loosely attached and adherent diatom community components and that this was partially related to size (age) of the invertebrate. *O. triseriatus* larvae in our tree holes were a mixture of instars (II-IV), and this size range may have contributed to more complete utilization of the leaf microbial components.

The fact that larval effects on surface-associated microbial communities were demonstrated is also surprising because in contrast to previous studies done in microcosms (e.g., [22, 24]), elimination of larvae in the experimental tree holes did not eliminate other prominent leaf browsers. Larvae of scirtid beetles (Coleoptera: Scirtidae) were present (but not quantified) in the tree holes and were presumably active in both treated and untreated replicates. These beetle larvae, in contrast to mosquito larvae, are considered scrapers and shredders of leaf material [36]. Interactions of scirtids with mosquito larvae are thought to result in facilitation of mosquito growth [36]. It may be that beetles and mosquitoes affect different microbial groups and that this demonstration of a larval mosquito effect reflects changes in the microbial community over a background of changes induced by scirtid larvae. We are currently investigating mosquito-scirtid interactions as they relate to leaf microbial community structure. We also did not account for potentially complex interactions of larval grazing and protozoans in the system when investigating microbial responses. As in planktonic systems, protozoan predation on microbial biofilm components can affect

community composition [17, 33], and larval effects seen here could be mediated through removal of protists from the leaf surface.

The study demonstrates that mosquito larvae alter substrate-associated microbial communities in natural habitats. Although mechanisms remain to be demonstrated, the effects are most likely both direct and indirect, involving grazing, removal of competitors, and changes in nutrient moieties or concentration. The consequences of altering the microbial community likely are lower availability and quality of microbial food resources for larvae. Other ramifications of larvae-induced changes might be a change in function that impacts, positively or negatively, detritus decomposition dynamics. It has been shown in some studies that larval grazing enhances leaf decomposition rates [9, 23], and this may be occurring through stimulation of fungal or bacterial groups rather than direct abrasion by larval feeding.

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Figure 1.
Ergosterol concentrations in tree hole leaf material during the experiment

Figure 2.

Principal component analysis of bacterial 16S rRNA gene sequence classifications at the class level (or above) for loosely attached and adherent fractions. PC 1–3 explained 85 and 77% of the overall variance for loosely attached and adherent fractions, respectively

Figure 3. Percentage of bacterial 16S rRNA gene sequences in class level (or above) taxonomic categories from loosely attached leaf fraction for composite samples and individual replicates (mean $\pm$ SE, $n=6$)

Figure 4. Percentage of bacterial 16S rRNA gene sequences in class level (or above) taxonomic categories from adherent leaf fraction for composite samples and individual replicates (mean $\pm$ SE, $n=6$)

Figure 5. Principal component analysis of fungal 18S rRNA gene sequence classifications at the class level for loosely attached and adherent fractions. PC 1–3 explained 79 and 83% of the variance for loosely attached and adherent fractions, respectively

Figure 6. Percentage of fungal 18S rRNA gene sequences in class level (or above) taxonomic categories from the loosely attached leaf fraction for composite samples and individual replicates (mean $\pm$ SE, $n=6$)

Figure 7. Percentage of fungal 18S rRNA gene sequences in class level (or above) taxonomic categories from the adherent leaf fraction for composite samples and individual replicates (mean±SE, $n=6$)

Table 1

Repeated measures analysis (MANOVA) results for leaf ergosterol values showing F statistics (F), degrees of freedom (df), and probability values (p) for main effects and their interaction (Time $\times$ Treatment)

Effects	F	df	p
Larval presence	0.2436	1, 16	0.6283
Time	3.6627	3, 14	0.0388
Time $\times$ treatment	0.6687	3, 14	0.5851

Table 2

Bacterial family level sequence abundance from leaf material

Taxonomic group	Loosely attached fraction			Adherent fraction		
	Larvae	No larvae	Early decay	Larvae	No larvae	Early decay
Unclassified Eubacteria	3	15	1	1	12	1
Proteobacteria	18	7	1	18	4	1
<u>Alphaproteobacteria</u>	37	17		19	19	6
Sphingomonadales		1			4	
Sphingomonadaceae	18	8		8	11	5
Rhizobiales	2	3	1	4	24	1
Bradyrhizobiaceae	1	30		10	40	
Rhodobacterales		2	1		5	
Rhodobacteraceae	3	8		2	14	
Caulobacterales		2			2	
Caulobacteraceae	22	42		16	42	
<u>Betaproteobacteria</u>	134	23	1	144	40	16
Burkholderiales	23	42	2	30	24	3
Burkholderiaceae		4			5	
Comamonadaceae	29	1		21	12	5
Oxalobacteraceae	7			13	2	
Incertae sedis	90	6		85	37	1
Rhodocyclales		11	11		6	
Rhodocyclaceae	25	8		26	25	10
<u>Gammaproteobacteria</u>	4	46		4	30	
Methylococcaceae	2			1	3	
Enterobacteriaceae	1	2				
Pseudomonadales		1	18			
Pseudomonadaceae	6	1		13	2	
Bacteroidetes	11	21	5	29	59	10
<u>Bacteroidetes</u>		34				
Bacteroidales	6	51	37	8	21	2
Porphyromonadaceae	4	52		4	26	
<u>Flavobacteria</u>						
Flavobacteriales		3	2		13	
Flavobacteriaceae	122	91		55	46	22
<u>Firmicutes</u>		1			1	
Clostridiales		11	1		19	
Acidobacteriales		6	1		6	
Acidobacteriaceae	2	9		1	12	1
Actinobacteria						
<u>Actinobacteria</u>					3	
Actinomycetales		3		5		

Major groupings from Figs. 3 and 4 are underlined. Deltaproteobacteria are not included because only five sequences total were found. Larvae and No larvae columns are sums of composite and individual samples.

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Table 3

Common fungal species sequence abundance from leaf material

Species or species group	Loosely attached fraction			Adherent fraction		
	Larvae	No larvae	Early decay	Larvae	No larvae	Early decay
<u>Ascomycota incertae sedis</u>						
<i>Oidiodendron tenuissimum</i>	19	6		23	3	2
<u>Chytridiomycota</u>						
<i>Monoblepharella</i> sp.		21	1	37	23	
<i>Monoblepharis</i> sp.	2	26	3	38	33	
<i>Oedogoniomyces</i> sp.				27		
<u>Dothideomycetes</u>						
<i>Alternaria alternata</i>	7	1				
<i>Dothideomycete</i> spp.	15	1	4	10		2
<i>Leptosphaeria bicolor</i>				5	2	1
<i>Leptosphaeria maculans</i>				6		3
<i>Paraphaeosphaeria</i> sp.	26	3	1			
<i>Pleosporales</i> sp.	32	1		7		
<i>Setomelanomma holmii</i>	5			10		1
<i>Shiraia bambusicola</i>	6	7		1	4	3
<u>Dothideomycetes et</u>						
<i>Chaetothyriomycetes incertae sedis</i>	0					
<i>Aureobasidium pullulans</i>	6		1			
<i>Cucurbitaria berberidis</i>	91	28	6	6	10	4
<i>Didymocrea sadasivani</i>	7			1		
<i>Lojkania enalia</i>	1	6		1		
<u>Leotiomycetes</u>						
<i>Chloroscypha enterochroma</i>	7	5	6	10	21	
<i>Dimorphospora foliicola</i>	43	113	25	77	221	43
<i>Hymenoscyphus ericae</i>				21	2	4
<i>Lambertella tubulosa</i>	7			35	12	
<i>Pezicula carpinea</i>				11		
<i>Potebniamyces pyri</i>	9			2		
<u>Mitosporic Ascomycota</u>						
<i>Phoma</i> sp.	4	21			9	3
<i>Scleroconidioma sphagnicola</i>	1	1	6			
<i>Scytalidium hyalinum</i>	8	38	32		8	
<i>Tricladium patulum</i>	3	3		8	7	
<i>Tricladium splendens</i>	3	5		2	4	
<i>Tumularia aquatica</i>	9	9		16	23	
<u>Saccharomycetes</u>						
<i>Williopsis salicorniae</i>	83	3			6	
<u>Sordariomycetes</u>						

Species or species group	<u>Loosely attached fraction</u>			<u>Adherent fraction</u>		
	Larvae	No larvae	Early decay	Larvae	No larvae	Early decay
<i>Sordariomycete</i> sp		1	5			
<i>Trichoderma viride</i>	13	1	1			
<u>Homobasidiomycetes</u>						
<i>Armillaria cepistipes</i>	6			2	4	

Only those with more than five (total across fraction and treatment) sequences are listed. Underlined taxa are major groupings from Figs. 6 and 7.