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The presence of aggressive ants is associated with fewer insect visits to and altered microbe communities in coffee flowers

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Abstract

The process of dispersal can shape ecological communities, but its influence is thought to be small compared to the effects of environmental variation or direct species interactions, particularly for microbial communities. Ants can influence movement patterns of insects and the microbes they vector, potentially affecting microbial establishment on plants, including in agroecosystems. Here, we examine how the presence of aggressive ants, which can influence floral visitation by bees and other pollinators, shapes the community composition of bacteria and fungi on coffee flowers in farms that differ in shade management intensity. We hypothesized that the presence of aggressive ants should reduce the frequency and diversity of floral visitors. Finally, we predicted that the effects of ants should be stronger in the low-shade farm, which has a less diverse community of floral visitors. We sampled microbial communities from nectar and pistils of coffee flowers near and far from nests of the aggressive ant *Azteca sericeasur* across two farms that vary in shade management and diversity of floral visitors. Bacterial and fungal community composition was characterized using Illumina sequencing of the 16S and ITS regions of the rRNA gene. Consistent with our expectation, *Azteca* presence was associated with a decrease in the number and diversity of visitors, visit duration and number of flowers visited. *Azteca* presence influenced microbial communities, but effects differed between farms. *Azteca* nests were associated with higher bacterial diversity in both farms, but the difference between flowers on trees with and without *Azteca* was greater in the high-shade farm. *Azteca* nests were associated with higher fungal diversity in the high-shade farm, but not the low-shade farm. In addition, the presence of ants was strongly associated with species composition of fungi and bacteria in flowers, but differentiation between ant and no-ant communities was greater in the low-shade farm. Specific operational taxonomic units (OTUs) were differentially associated with the presence of ants. We conclude that indirect interactions that influence dispersal may have large effects on microbial community composition, particularly in ephemeral microbial communities.

Keywords: *Azteca*; Community assembly; *Coffea arabica*; Chiapas; Mexico; Dispersal; Microbial ecology; Microbiome; Nectar chemistry

Introduction

Environmental constraints and direct species interactions are traditionally considered the main determinants of species membership in ecological communities. However, dispersal-driven processes are increasingly recognized for their role in

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community patterns (Ricklefs 1987; Hubbell 2001; Leibold et al. 2004). Theory and a growing body of evidence suggest that dispersal should play an important role in determining community structure and function through its effects on species presence, arrival order, and mass effects (Fukami 2015; Mittelbach & Schemske 2015). In microbial communities, environmental constraints and species interactions (e.g. competition and trophic interactions) are typically thought to structure communities (Lindström & Langenheder 2012; McFall-Ngai et al. 2013), while dispersal has been thought to play a smaller role, because microorganisms often have large effective dispersal distances and are able to survive in a dormant state for long time periods (Becking 1934; Finlay 2002). However, species-area and distance-decay relationships for microorganisms suggest that dispersal limitation may influence community composition (Green & Bohannan 2006; Zhou, Kang, Schadt, & Garten 2008; Vannette, Leopold, & Fukami 2016). In addition, many microorganisms rely on larger animals for transportation (known as phoresis) to specialized habitats. For example, fungal ascospores are carried by bark beetles among tree habitats (Moser, Perry, Bridges, & Yin 1995), and yeasts that grow in ephemeral habitats like floral nectar or rotting fruits often rely on phoresis by pollinators or flies to disperse among these habitats (Starmer, Barker, Phaff, & Fogleman 1986; Starmer & Fogleman 1986; Belisle, Peay, & Fukami 2012). The consequences of variation in dispersal for microbial community composition are not well-understood, particularly when microorganisms rely on phoretic dispersal (but see Ushio et al. 2014; Mittelbach et al. 2015).

Here, we examine the effect of modified dispersal on the diversity and composition of microbial communities in the flowers of *Coffea arabica* (Rubiaceae). We focus on flowers, because these habitats are ephemeral and initially host little to no culturable microorganisms (e.g. Herrera, Canto, Pozo, & Bazaga 2010; Belisle et al. 2012), and as a result, represent the initial stages of community assembly, where effects of dispersal can be disentangled from other processes. We sampled microbial communities on floral stigmas and in nectar of coffee flowers, which are open between 2–5 days (Jiménez-Castano & Castillo-Zapata 1976; Free 1993). We sampled floral nectar and stigmas because they likely differ in suitability for microbial growth and may host different microbial communities (Junker & Keller 2015). Nectar acts as a strong biological filter, and only a phylogenetically restricted subset of microorganisms with particular adaptations has been found to attain high abundance in this environment (e.g. Herrera et al. 2010; Alvarez-Perez, Herrera, & de Vega 2012). Microbial growth can depend on nectar sugar concentration and composition–which determines water activity–and the concentration of secondary compounds (Vannette & Fukami 2016), including caffeine, which is found in coffee nectar. In addition to the utility of microbial communities for testing ecological theory (Srivastava et al. 2004), nectar-inhabiting microbial communities can also differentially influence plant-pollinator interactions (Herrera, Pozo, & Medrano 2013; Vannette, Gauthier, Fukami 2013; Schaeffer & Irwin 2014), depending on their composition. As a result, characterizing taxonomic contribution and species interactions that contribute to variation in composition may be ecologically important in some cases. Here, we characterize microbial composition in coffee nectar and stigmas, its association with sugar composition of nectar and association with the presence of ants, but do not examine functional consequences for plants or pollinators.

Within coffee agroecosystems, aggressive arboreal ants are dominant ecological players with the potential to influence microbial dispersal both directly and indirectly. Ants can facilitate the dispersal of specific microbes (de Vega & Herrera 2013), including entomopathogenic fungi and fungi that parasitize rust in coffee systems (Philpott 2010; Vandermeer, Perfecto, & Philpott 2010; Jackson, Zemenick, & Huerta 2012) and yeast to floral nectar (de Vega & Herrera 2013). Aggressive ants also influence the abundance, diversity and composition of other insects that may vector microorganisms. Specifically, aggressive ants (e.g., Argentine ants, red imported fire ants, and pavement ants) are renowned for negatively affecting pollination services in many plant species, both by chasing pollinators (bees) away from plants, stealing nectar without pollinating (Inouye 1980), causing mechanical damage to floral tissue, or leaving deterrent scent marks on flowers (Lach 2007; LeVan, Hung, McCann, Ludka, & Holway 2014; Sidhu & Rankin 2016). In coffee agroecosystems, the dominant arboreal ant *Azteca sericeasur* tends scale insects on coffee plants, defends scales and coffee plants from both herbivores and predators, and is aggressive towards a number of other ant species (Vandermeer et al. 2010). Because this ant is generally aggressive towards all insects it encounters, and because other aggressive ant species negatively affect pollinators, we hypothesize that *A. sericeasur* alters pollinator visits to coffee flowers, ultimately influencing diversity and structure of the microbial communities in coffee flowers.

Coffee is grown under varying management intensity, and cultivation conditions may also influence microbial dispersal. At one extreme, coffee is cultivated in the understory of a diverse and dense canopy of shade trees and at the other extreme, coffee is produced under intensive management with minimal shade tree cover (Perfecto, Rice, Greenberg, & Van der Voort 1996; Moguel & Toledo 1999). Shade coffee farms with high vegetation complexity support a higher abundance, richness, and a distinct community composition of many insects, including pollinators and other floral visitors, than do farms with less complex vegetation or without shade (Klein, Steffan-Dewenter, Buchori, & Tscharntke 2002; Armbrecht, Rivera, & Perfecto 2005; Philpott et al. 2008a; Jha & Vandermeer 2010). Changes in the insect species present, and their interactions with one another may also influence the floral microbial community.

In this study, we tested the hypothesis that the presence of aggressive ants influences microbial communities in flowers. We hypothesized that ant presence will (1) reduce floral

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visitation, thereby resulting in (2) decreased microbial diversity if flower microbial communities are dispersal-limited, (3) differences in microbial species composition in flowers on plants with and without ants, and (4) more homogenous microbial communities in ant-associated flowers compared to flowers far from ant colonies. To examine this hypothesis, we surveyed flowers at multiple sites with and without ants, in two coffee farms that differed in agricultural management practices. We examined floral visitation rates and visitor diversity, characterized microbial community composition, and assessed nectar chemistry across these sites, in flowers of *C. arabica*.

Materials and methods

Study system and sampling

Our sampling efforts focused on two farms within the Soconusco region of Chiapas, Mexico that vary in shade management intensity. The high-shade farm, Finca Irlanda (15°, 11′N, 92°, 20′W), is classified as commercial polyculture and the low-shade farm, Finca Hamburgo (15°, 10′N, 92°, 19′W), is classified as shaded monoculture (Moguel & Toledo 1999). Both farms are very large (>280 ha), are located between 900–1150 m. a.s.l., and receive >4000 mm rain per year. The two farms differ in number of floral visits and in visitor communities with the high-shade farm hosting twice as many species of floral visitors (15 vs. 8) and receiving significantly more visits by Africanized honeybees, native social bees, and native solitary bees (Jha & Vandermeer 2009). At the time of floral visitor observations (see below), there was just one managed honey bee apiary located between the two farms (Jha & Vandermeer 2009).

The aggressive ant *A. sericeasur* (hereafter, *Azteca*) is the dominant ant in this ecosystem and is characterized by a patchy distribution (presence/absence) in both farms (Perfecto & Vandermeer 2008; Vandermeer et al. 2010). *Azteca* has been observed visiting flowers and contacting nectar, but its contribution to pollination success is unclear, and likely relatively rare in most locations (Klein, Steffan-Dewenter, & Tscharntke 2003; Philpott, Uno, & Maldonado 2006). However, where it occurs, this species, like other aggressive ants, may deter insect visitation to flowers or influence the frequency of floral visitation (e.g. Altshuler 1999; Philpott et al. 2006).

We conducted behavioral observations to examine how the presence of *Azteca* ants influences visit duration and the diversity of flying insects visiting coffee flowers. We conducted 30 10-min observations on two paired coffee plants with and without *Azteca* between 8:45 AM and 12:15 PM during the peak coffee flowering in February 2008. All observations were conducted in the high-shade site. We haphazardly selected pairs of coffee plants – one within 2 m of a shade tree containing an *Azteca* nest, and the other >5 m away from the *Azteca* nest. Prior to conducting pollinator observations, we counted the number of *Azteca* ants passing a fixed location on the coffee plant. Then, at the same time (with two observers), we observed visits to flowers of the two coffee plants. For 10 min, we observed two adjacent, haphazardly selected branches on each coffee plant, approximately 1 m above ground, noting the number of visits, the duration of visits, the number of flowers on those two branches visited by each visitor, and the identity of floral visitors to species, where possible, or to genus or morphospecies (see Supplementry Appendix A: Table 1). We also counted the total number of open flowers on the two coffee branches.

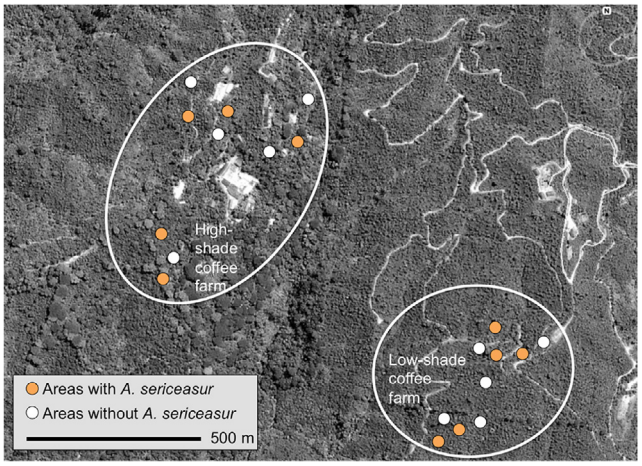

Fig. 1. Map of coffee farm study sites in Chiapas, Mexico. We worked in one high-shade and one low-shade coffee farm, and in those farms selected sites with and without nests of the aggressive ant *Azteca sericeasur*. We collected nectar from plants within 2 m of nests (with ants) or at least 15 m from nests (without ants). Coffee nectar and pistils were collected from four plants per site during peak flowering. IKONOS satellite image was purchased by SMP from DigitalGlobe.

To sample nectar and microbes, we chose five sites near *Azteca* nests (within 2 m) and five sites far from *Azteca* nests (at least 15 m away) within each farm (Fig. 1). In each site, we selected four coffee plants all within 6 m of each other. All plants in sites with *Azteca* nests were within 2 m of a shade tree with an ant nest. *Azteca* presence on a plant was verified by shaking plants and searching for ant foragers. On each plant, we haphazardly sampled nectar from four open flowers and collected pistils from four different flowers. All nectar samples were collected with 10 μl microcapillary tubes. We collected pistils by holding the tip over a micro-centrifuge tube and clipping the base with scissors. All samples were diluted with 30 μl of sterile water and frozen within 3 h following analysis. Open flowers were chosen haphazardly within each plot for sampling. All samples were collected between 5 March and 16 April 2013, with low-shade (Hamburgo) sites sampled on one date, and high-shade (Irlanda) sites sampled on 3 separate dates, depending on when flowering occurred at each site.

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Sample processing, DNA extraction and sequencing

Samples were transported on ice to Stanford University, where nectar samples were split into three aliquots: one for DNA extraction, one for sugar analyses, and a final for analysis of caffeine. For both nectar and pistil samples, 15 μl of the aqueous sample was extracted using the Qiagen Dneasy Blood and Tissue kit using the nucleated blood protocol (Qiagen, Valencia, CA, USA). Regions of the ribosomal rRNA gene were amplified using the 16S V4 region for bacteria (515-806) and ITS1 region for fungi (ITS1f-ITS2) primers (Caporaso et al. 2012; Smith & Peay 2014). Primers contained the Illumina Nextera adapters and linker sequences (Smith & Peay 2014). PCR reactions were carried out in 25 μl reactions including 5 μl of DNA extraction and 0.5 μl of each 10 μM primer in 1x MyTaq HotStart Red Mix (Bioline, Tunton, MA). PCR conditions followed previously published conditions (Smith & Peay 2014). Two replicate PCR reactions were performed for each sample, amplification was verified by running PCR products on a 1% agarose gel, and replicate reaction products were pooled. Amplicons were cleaned using Charm Just-A-Plate PCR purification and normalization kits (Charm Biotech, Santa Cruz, CA), and pooled in equimolar concentrations before sequencing. Samples were sequenced together using paired-end, dual indexed 2×250 Illumina MiSeq (Caporaso et al. 2010; Smith & Peay 2014) at the Stanford Functional Genomics Facility. The amplicon libraries were spiked with 15% PhiX. Negative extraction controls were also amplified using indexed primers and included in the sequencing run.

Bioinformatics

Low-quality bases were removed from each sequence using sickle v.1.33 (Joshi & Fass 2011) in paired end mode with a sliding window quality cutoff. Sequences were merged using UPARSE v.8.0, (Edgar & Flyvbjerg 2015). Operational Taxonomic Units (OTUs) were clustered at 97% using UPARSE (v.8.0) and de-novo and reference-based chimaera detections were performed using UNITE UCHIME ITS-trimmed reference dataset (3/11/2015) for fungal sequences, and JGI Gold database for bacterial sequences. Taxonomy was assigned using the RDP classifier (Wang, Garrity, Tiedje, & Cole 2007) trained on either the 16S rRNA training set 14 for bacteria, or the Warcup Fungal ITS training set (Deshpande et al. 2015) for fungi at bootstrap cutoff of 80%. OTUs that could not be identified to Kingdom or Phylum were discarded, as were bacterial OTUs classified as chloroplast or mitochondria. Dynamic filtering was used to control for possible cross-sample contamination (Peay, Baraloto, & Fine 2013). OTUs with 5 or more sequences in any of the negative control samples were also removed. This resulted in samples sequenced at a mean depth of 4526 sequences/sample for bacteria and 3331 seq/sample for fungal sequences. Because sequencing depth varied widely among samples, samples were rarefied (Weiss et al. 2015) to 200 bacterial and 400 fungal sequences per sample, at which depth sequencing curves had plateaued (see Supplementry Appendix A: Fig. 1) and we were able to retain the majority of samples. Rarefaction and cleaning were performed using the R package phyloseq (McMurdie & Holmes 2013). Rarefied OTU tables were used for all analyses below.

Nectar chemical analyses

We also quantified the sugar concentration and composition, and caffeine concentration within each nectar sample. Briefly, diluted nectar was filtered through a 0.22 μm pore size centrifugal filter and diluted 200-fold for sugar analysis or 4-fold for caffeine analysis. Sugar separations were achieved using a water:acetonitrile gradient (Vannette et al. 2013) on a Luna amide column (50×2 mm, 3 μm, Phenomenex) and saccharides detected and quantified using an evaporative light scattering detector (Waters, Milford MA, USA). Caffeine was detected and quantified using UV at 280 nm following separation on a BEH C18 column (Acquity, 50×2.1 mm, 1.7 μm), (Vannette & Fukami 2016). A series of external standards was used to quantify caffeine, sucrose, glucose, and fructose in original nectar samples. Pistil samples were not analyzed for sugar or caffeine concentrations because they do not secrete nectar.

Statistical analysis

We used paired *t*-tests to assess the effects of *Azteca* ant presence on floral visitor diversity, visit duration, and floral visitation. We conducted a separate analysis for *Apis*-specific variables because *Apis* represented a large fraction of visits on both plants (91.4% of visits with *Azteca* 88% of visits without *Azteca*).

We used ANOVA to assess the interactive effects of floral tissue, ant presence, and farm identity on microbial diversity using OTU richness and Shannon diversity index, calculated using the natural logarithm in the R package vegan (Oksanen et al. 2012), and the interaction between ant presence and farm identity. Estimates of bacterial and fungal diversity were considered separately. Significance was assessed using *F*-tests using the drop1 function (type III sums of squares) in R. Sampling curves to estimate total microbial diversity at each farm, in the presence and absence of ants, were created using unconditional estimates (Colwell et al. 2012), implemented in vegan (Oksanen et al. 2012).

To visually assess the similarity of microbial communities inhabiting individual flower samples, we used NMDS to ordinate communities based on Bray–Curtis dissimilarities. We used a permutational ANOVA to assess statistically the variation in community composition explained by floral tissue, ant presence and farm identity. We examined if samples from farms or from sites where ants were present or absent were

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Table 1. The number and frequency of floral visitors observed visiting coffee branches on plants with and without the aggressive ant *Azteca sericeasur*[a] in a high-shade coffee farm in Chiapas, Mexico. See visitors list in Supplementry Appendix A Table 1. Na indicates that no statistical test was performed (for cumulative species numbers only).

Variable	Coffee plants with *Azteca*	Coffee plants without *Azteca*	*t*	*df*	*P*
No. *Azteca* ants	19.63 ± 2.34	0 ± 0	8.398	29	<0.001
No. flowers	46.47 ± 3.87	43.80 ± 2.84	0.596	29	0.556
No. species of visitors (cumulative)	2	8	Na	Na	Na
No. species of visitors	1.03 ± 0.33	1.37 ± 0.12	−2.567	29	0.016
No. visits (cumulative)	35	76	Na	Na	Na
No. visits	1.17 ± 0.31	2.53 ± 0.33	−3.139	29	0.004
No. *Apis* visits	1.07 ± 0.29	1.97 ± 0.26	−2.619	29	0.014
No. flowers visited per visitor	1.77 ± 0.48	3.48 ± 0.37	−2.877	29	0.007
Duration of visits (sec) per visitor	14.16 ± 5.13	23.54 ± 2.19	−2.387	13	0.033
Duration *Apis* visits (sec)	14.97 ± 5.44	21.48 ± 2.21	−1.757	12	0.104

[a] All variables (except for those denoted as cumulative) are mean values across 30 10-min observations of floral visitors to two branches per coffee plant. Plants with and without *Azteca* were separated by ∼5 m and were observed simultaneously. Values refer to mean ± SE. Statistical results are for paired t-tests.

more variable (differential among-sample variance) using betadisper in vegan (Oksanen et al. 2012). We also examined which fungal or bacterial OTUs were differentially abundant between ant and no-ant flowers in a consistent fashion across farms. We compared observed OTU abundance to repeated permutations of the data using the SAMseq function in the package samr (Li & Tibshirani 2013). Low-abundance OTUs (<10 observations, or in fewer than 3 samples) were removed before analysis to avoid spurious results. We report OTUs that were differentially abundant at q = 0, where q-values are false-discovery-rate adjusted, and q = 0 represents the most conservative significance level. The FunGuild database was used to assign guild and functional information to differentially abundant OTUs (Nguyen et al. 2016).

We also examined if ant presence, farm identity, microbial composition (PCoA axes from Bray–Curtis ordination), or microbial diversity was associated with nectar characteristics, including nectar volume, sugar concentration, the ratio of monosaccharides: total sugars in nectar, or the concentration of caffeine in nectar. Multiple regression followed by stepwise model simplification by AIC was used to examine associations between predictors and each nectar characteristic.

For ANOVA, PERMANOVA, and other permutational analyses, individual flower samples were used as the unit of replication. All statistical analyses were performed in the R environment (R Core Development Team 2012).

Results

The presence of the ant *A. sericeasur* was associated with fewer total visits to coffee flowers, and a smaller number of floral visitor species (Table 1). There was no difference in floral abundance on coffee plant branches with and without *Azteca* ants, but the number of visits to flowers, number of visits by honeybees (*Apis mellifera*), number of flowers visited, number of floral visitor species, and duration of visits were all lower on plants with *Azteca* (Table 1).

We found a high diversity of microbial taxa in coffee flowers, with an estimated 1387 bacterial and 2151 fungal OTUs detected overall (based on 16S and ITS region, respectively). Bacterial sequence pools were dominated by OTUs assigned to the Phyla Proteobacteria and Actinobacteria, while Bacteriodetes and Firmicutes were also common (Fig. 2A). In fungal sequence pools, yeasts in the Classes Saccharomycetes and Tremellomycetes, and members of the Eurotiomycetes and Sordariomycetes were also common (Fig. 2B). Microbial diversity in individual flowers was, in most cases, greater on plants that were near *Azteca* nests (Fig. 3, Supplementry Appendix A: Table 2), but the strength and direction of this effect depended on the farm identity and microbial kingdom considered (bacteria vs. fungi). In bacteria, species richness and diversity were higher in the low-shade farm (Fig. 3A and C), and the difference in bacterial diversity associated with the presence of an *Azteca* nest was more pronounced in the high-shade farm. Fungal richness and abundance-weighted diversity (H') were greater in the presence of an *Azteca* nest in the high-shade farm (similar to bacterial communities), but this pattern was reversed in the low-shade farm (Fig. 3B and D). Nectar and pistil microbial communities did not differ significantly in OTU richness for fungal or bacterial communities (see Supplementry Appendix A: Table 2), but bacterial abundance-weighted diversity (H') was slightly higher in nectar compared to pistil samples ($F_{2,155} = 5.6$, $P = 0.02$). Within an individual flower, bacterial richness was generally greater in the low-shade farm, whereas no systematic difference was observed in fungal diversity between farms. However, total microbial richness (cumulative number of OTUs across all samples) was between 30–50% lower in the high-shade environment on plants far from ant nests (see Supplementry Appendix A, Fig. 2) compared to all other sample types, and this pattern held for both bacterial and fungal communities.

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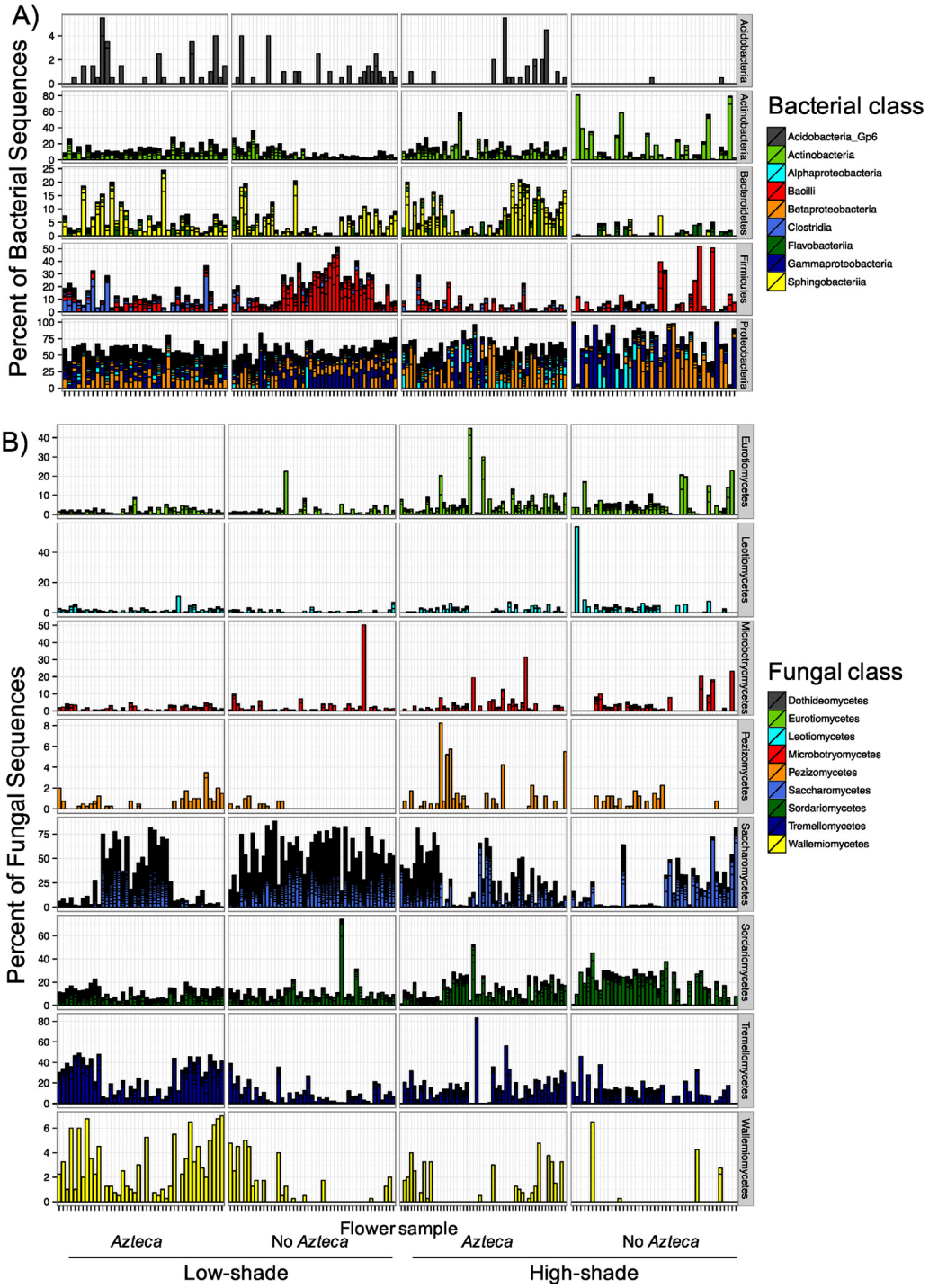

Fig. 2. Relative abundance of bacterial OTUS, by Phylum (A) and fungal OTUs, by Class (B), with each OTU colored by Class. Bars represent percent of OTUs in sequence pools from single coffee flowers (both nectar and pistil samples are shown) in a low-shade and a high-shade coffee farm in Chiapas, Mexico. Flowers were collected near to the nest of the aggressive ant *Azteca* or far from an *Azteca* nest. Percent for each OTU was calculated for Bacterial and Fungal pools separately.

The composition of microbial communities differed among floral structures, and with ant presence and farm identity. Nectar and pistil microbial communities differed somewhat in microbial community composition (Table 2, Fig. 4). However, ant presence, farm identity, and the interaction between the two explained a greater proportion of variation in microbial species composition overall (∼12–15%; Table 2). Bacterial and fungal communities were strongly differentiated by the presence of *Azteca* nests in the low-shade farm, but the difference in community composition

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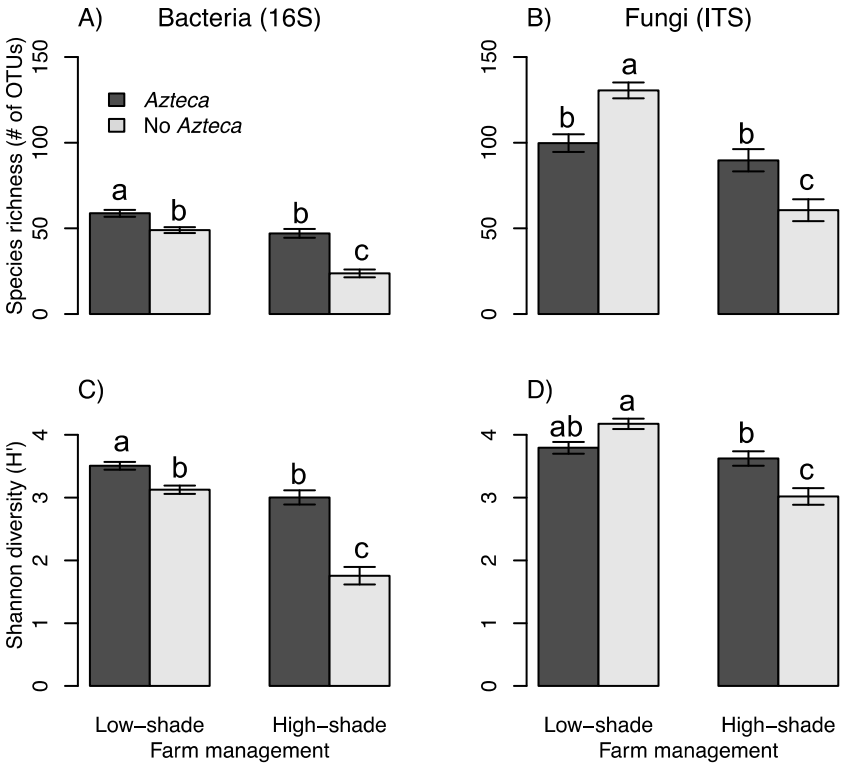

Fig. 3. The species richness (A,B) and Shannon diversity (C,D) ±SEM of bacterial (A,C) and fungal (B,D) communities collected from coffee flower nectar and pistil samples from a high-shade and a low-shade coffee farm in Chiapas, Mexico. Presence of an aggressive ant (*Azteca sericeasur*) and farm identity were both associated with bacterial and fungal diversity. Letters indicate results of a Tukey HSD test ($P < 0.05$). ANOVA tables are presented in Supplementry Appendix A: Table 2.

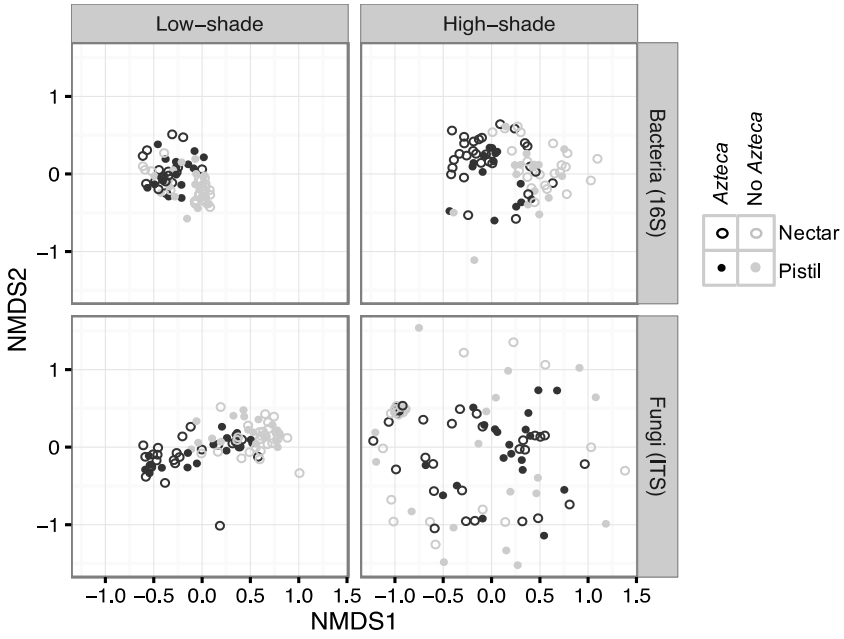

Fig. 4. NMDS ordination of bacterial and fungal communities collected from coffee flowers in a low-shade and a high-shade coffee farm in Chiapas, Mexico. Point character indicates the floral tissue sampled, including nectar or pistil. Separate ordinations, based on Bray–Curtis dissimilarities, were performed for Bacteria and Fungi. Point color indicates the presence (dark gray) or absence (light gray) of a nest of the aggressive ant *Azteca sericeasur* (see methods for full details).

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Table 2. PerMANOVA table summarizing the effects of proximity to a nest of the aggressive ant *Azteca sericeasur*, farm identity, and floral tissue (nectar vs. pistil), and two-way interactions among predictors on the species composition of fungi (ITS region) and bacteria (16S). F-values, followed by significance level (*P*-values), and R^2 values were calculated using the adonis function in the vegan package (Oksanen et al. 2012).

Taxa	Source	Ant	Farm	Tissue	Ant × farm
Fungi	*F*	6.02***	12.89***	1.5*	5.91***
	R^2	0.03	0.06	0.01	0.03
Bacteria	*F*	7.70***	13.50***	3.3***	6.90***
	R^2	0.04	0.07	0.02	0.04

*** $P < 0.001$.
* $P < 0.05$.

was less pronounced in the high-shade farm, particularly for fungal communities (Fig. 4). In addition, both bacterial and fungal communities were more variable in the high-shade farm (bacteria $P < 0.001$, fungi $P < 0.001$). Further, fungal communities (but not bacterial communities) were more similar in the presence of ants (betadisper: bacteria $P = 0.81$, fungi $P < 0.001$).

Differences in community composition were not sensitive to the dissimilarity method used, as analyses performed using Jaccard and weighted Unifrac distances (for bacteria only) were qualitatively similar to those reported in Table 2 (results not shown).

We identified several taxa that were differentially abundant ($q = 0$; see Supplementry Appendix A: Tables 3 and 4) between flowers in the presence and absence of *Azteca* ant nests, potentially driving the differences in richness and species composition observed. The relative abundance of some microbial taxa was associated with the presence of ants. Bacterial genera, including *Phyllobacterium* (Rhizobiales), *Uruburuella* (Neisseriales), *Roseomonas* (Rhodospirillales), and *Sedimenibacterium* (Sphingobacteriales) were more abundant in the presence of ants, whereas *Pantoea* (Enterobacteriales), *Pluralibacter* (Enterobacteriales), *Lactobacillus* (Lactobacillales), *Acidovorax* (Burkholderiales), *Dietzia* (Actinomycetales) and *Corynebacterium* (Actinomycetales) were more abundant, on average, in the absence of ants (see Supplementry Appendix A, Table 3). Across both farms, the Basidiomycetous yeasts *Cryptococcus*, *Sporobolomyces*, and *Fibulobasidium*, as well as putative saprotrophs (e.g. *Ganoderma*, *Stereum*, and *Penicillium*) were more abundant in the presence of ants (see Supplementry Appendix A, Table 4). Ascomycetes in the Class Dothideomyces were among those common in the absence of ants, as well as the Eurotiomycete in the Genus *Exophiala*. In addition, we detected fungal taxa of interest, including *Lecanicillium* (entomopathogenic and mycoparasitic fungus that can negatively influence coffee rust), *Cephalosporium* (white halo fungus), and *Beauveria* (an entomopathogenic fungus), although mostly at low abundance so we could not determine differential abundance among sampling locations or if they were associated with ants.

Nectar characteristics did not differ significantly between farms (Fig. 5). Plants near ant nests had significantly higher sugar concentrations in floral nectar (Fig. 5B, $P = 0.017$), but ant presence was not associated with other nectar parameters, including sugar composition (proportion of sugars monosaccharides) or caffeine concentration in nectar ($P > 0.1$). Bacterial community composition was associated with the concentration of caffeine in nectar (PCoA axis 1, $P = 0.02$, $R^2 = 0.07$). However, there was no significant association between nectar sugar composition and any predictors included in the model ($P > 0.1$).

Discussion

Our results suggest that the presence of the aggressive ant *A. sericeasur* decreases visitation by pollinators and other floral visitors to coffee flowers, and is associated with distinct microbial composition within flowers. Interestingly, the difference in microbial diversity and species composition ascribed to ant presence depended on the farm identity. Ants were associated with higher nectar sugar concentration, but this did not vary between farms. Strong association between ant presence and microbial species composition suggests that differential dispersal or species interactions contribute to the observed microbial community patterns, although variation in nectar chemistry, including sugar and caffeine concentration in nectar, may contribute to differences in microbial species composition.

Several mechanisms may explain the observed differences in microbial communities in areas with and without *Azteca* ants. As predicted, ants were associated with differential species abundance and composition of insect visitors to flowers, resulting in fewer visits, fewer species of visitors, and shorter visit duration (Table 1). However, in contrast to our expectations, flowers near *Azteca* nests tended to have similar or more diverse microbial communities than flowers without *Azteca*. This suggests that either microbial dispersal is not limited by the frequency and abundance of floral visitation in this system, or is a direct effect of ants. Because ants decrease visit duration, ants might actually promote higher floral visitor movement (Altshuler 1999; Philpott et al. 2006), and facilitate microbial dispersal among flowers. Conversely, ants might influence microbial dispersal or survival directly, either by introducing particular microbial taxa (de Vega & Herrera 2013) or influencing microbial survival in nectar. For example, we observed ant visitation to flowers and nectar consumption through robbed flowers, suggesting that direct dispersal may occur. On the other hand, antimicrobial secretions from ant metapleural or other glands, or hygienic ant behaviors could instead negatively affect microbial growth on plants where ants are present (Beattie, Turnbull, Hough, & Knox 1986; Veal, Trimble, & Beattie 1992). Finally, it is a possibility that ants may choose to forage on flowers that host

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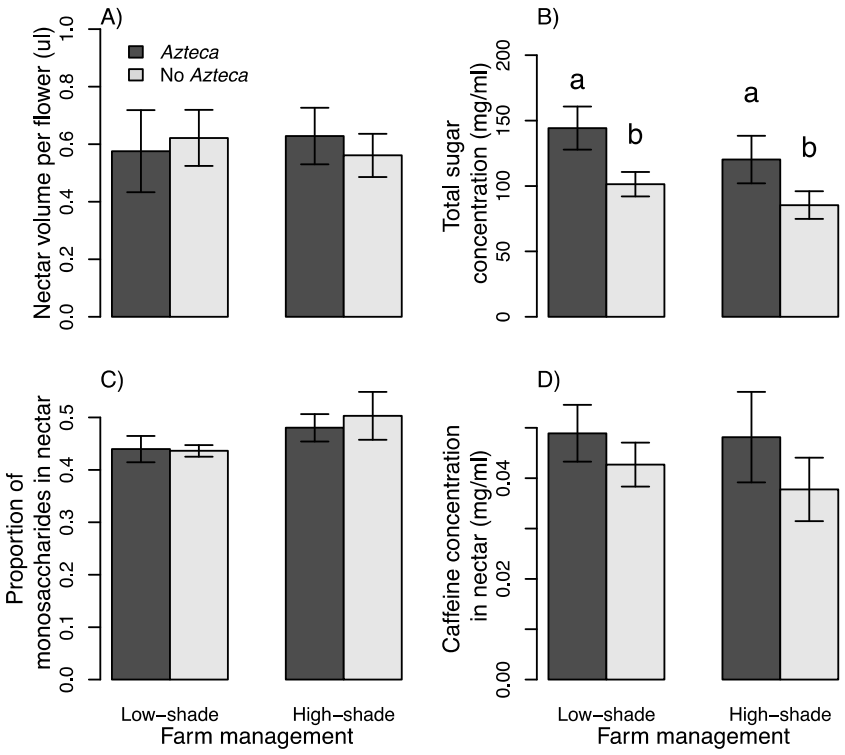

Fig. 5. Characteristics of floral nectar collected from coffee plants with (*Azteca*) and without (No *Azteca*) the aggressive ant species *Azteca sericeasur* in one high-shade and one low-shade coffee farm in Chiapas, Mexico. Nectar parameters included (A) nectar volume, (B) total sugar concentration, (C) the proportion of monosaccharides in nectar and (D) caffeine concentration. Bars represent means ± SEM. Letters indicate results of a Tukey HSD test indicating a difference in the mean between plants with and without *Azteca* ($P < 0.05$).

a particular microbial community. Further work is necessary to distinguish among these hypotheses.

In concordance with our hypotheses, floral microbial communities with *Azteca* nests present were generally distinct from communities with no *Azteca* (Fig. 4). Further, fungal communities were more similar in the presence of Azteca nests, suggesting homogenization through increased visitation frequency by other organisms or direct effects of ants on microbial communities. Ants were associated with a higher sugar concentration in floral nectar, which could influence microbial establishment, relative growth in nectar, or the production of pathogenic or stress-tolerant spores (e.g. Rangel et al. 2015). In addition, competitive interactions among microbial taxa in flowers (e.g. Peay et al. 2013; Vannette & Fukami 2014) and resulting changes in community diversity may contribute to the observed community patterns. As a result, the specific mechanisms by which ants influence microbial communities remain unclear and addressing this question will require additional data on ant behavior, dispersal frequency, abundance, and the resulting abundance of microorganisms.

Difference in ant effects between farms

We documented pronounced variation in microbial species composition between farms, and in the effects of ants on microbial communities. High-shade and low-shade coffee farms frequently differ in terms of arthropod, bird, and plant diversity (Klein et al. 2002; Philpott et al. 2008b; Jha & Vandermeer 2010; Jha et al. 2014), with greater plant and arthropod pollinator diversity in high-shade farms (Jha & Vandermeer 2010). The large difference in species composition between farms could be attributed to differences in species composition of floral visitors (Jha & Vandermeer 2010), or their abundance. In contrast to our expectations, we found that microbial communities were not more diverse either at the local or cumulative scale in the high-shade farm, and were often actually less diverse overall in the absence of ant nests. However, microbial communities within flowers at the high-shade farm were more variable, with greater dissimilarity in the species composition among individual flowers. This supports the hypothesis that a greater diversity of floral visitors results in more variable microbial communities deposited on flowers in the high-shade farm. In addition, the association between *Azteca* presence and microbial species composition was less pronounced in the high-shade farm. However, we only examined one high-shade and one low-shade farm, with sites separated by ∼500 m. Although it is clear from other studies that the abundance and species richness of floral visitors differ between these sites, some of the colonies may forage in both high and low shade sites studied. In addition, the differences observed between farms could be a result of management, or of other spurious differences between the two farms, and the results should

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be treated with caution. But the differences described here warrant further exploration. In addition, we only assessed the effects of *Azteca* on floral visitation in the high-shade farm, but it is unlikely that ant behavior varies significantly among farms given other documented impacts of *Azteca* ants in the two farms (Moorhead, Philpott, & Bichier 2010; Murnen, Gonthier, & Philpott 2013). These results imply that aggressive ants are associated with pronounced differences in microbial species composition, and that the biotic context mediates the strength and direction of this effect on microbial communities.

Implications for coffee

In other species of plants, microbial composition can influence nectar characteristics, and can have subsequent effects on floral visitors, and pollination services (Herrera et al. 2013; Vannette et al. 2013; Good, Gauthier, Vannette, & Fukami 2014). Although we did not examine microbial effects on pollination in the current study, previous work in the system suggests a link between ant presence, floral visitation and fruit set. Philpott et al. (2006) showed that coffee plants exposed to both ants and floral visitors experienced higher fruit weights, but only in the high-shade farms. Although experimental work is required, differences in fruit set or fruit weight may be linked to microbial patterns documented here. For example, microbial differences could indicate visitation by more effective pollinators, or microorganisms could be directly involved in promoting fruit development. For example, in other systems, microbial growth in flowers can influence pathogen infection of fruit (Ippolito & Nigro 2000). There have also been reports that the presence of fungi from the genus *Epicoccum*, which were common far from ant nests in our study, is linked with decreased severity of the coffee berry disease (Colletotrichum), perhaps through antagonistic interactions (e.g. Gichuru 2005).

In addition, microbial composition of coffee berries has been linked to variation in flavor development, particularly when cherries are processed using wet and semi-dry methods (Gilberto, Soccol, Brar, Neto, & Soccol 2015). Moreover, in coffee, one of the fungal genera (*Sporobolomyces* sp.) associated with ant presence has been associated with the suppression of toxigenic *Aspergillus* in coffee (Melo Pereira et al. 2016). Further experimental work will be necessary to examine if microbial community composition in flowers is linked to microbial composition in fruits, or can influence fruit set, development, flavor, and infestation of fruits by pathogens or toxigenic microorganisms.

Conclusions

In conclusion, our results suggest that the presence of aggressive ants can influence visitation to flowers and alter microbial diversity and species composition within flowers. Although manipulative experiments are required to establish the mechanism, these results suggest that aggressive ants can modify the behavior of microbial dispersal agents, with consequences for microbial community patterns. These results demonstrate that trait-mediated indirect interactions (Werner & Peacor 2003; Schmitz, Krivan, & Ovadia 2004) may have consequences for the initial stages of community assembly. Initial colonist identity can modify subsequent community structure through priority effects, including environmental modification, niche pre-emption, or other mechanisms (Vannette & Fukami 2014; Fukami 2015). Because many microorganisms, particularly specialized microbes, rely on phoresis for dispersal (Herrera et al. 2010; Belisle et al. 2012), trait-mediated effects on foraging and behavioral patterns of phoretic agents may be an understudied but potentially important factor influencing community assembly patterns.

Sequence data

Sequence data have been deposited at NCBI SRA under BioProject ID # PRJNA377879.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.baae.2017.02.002.

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