RESEARCH ARTICLE

Host species and developmental stage, but not host social structure, affects bacterial community structure in socially polymorphic bees

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Abstract

Social transmission and host developmental stage are thought to profoundly affect the structure of bacterial communities associated with honey bees and bumble bees, but these ideas have not been explored in other bee species. The halictid bees *Megalopta centralis* and *M. genalis* exhibit intrapopulation social polymorphism, which we exploit to test whether bacterial communities differ by host social structure, developmental stage, or host species. We collected social and solitary *Megalopta* nests and sampled bees and nest contents from all stages of host development. To survey these bacterial communities, we used 16S rRNA gene 454 pyrosequencing. We found no effect of social structure, but found differences by host species and developmental stage. *Wolbachia* prevalence differed between the two host species. Bacterial communities associated with different developmental stages appeared to be driven by environmentally acquired bacteria. A *Lactobacillus kunkeei* clade bacterium that is consistently associated with other bee species was dominant in pollen provisions and larval samples, but less abundant in mature larvae and pupae. Foraging adults appeared to often reacquire *L. kunkeei* clade bacteria, likely while foraging at flowers. Environmental transmission appears to be more important than social transmission for *Megalopta* bees at the cusp between social and solitary behavior.

Introduction

Recently documented declines in honey bee (Evans & Schwarz, 2011) and bumble bee (Cameron *et al.*, 2011) populations have motivated studies of the function and community structure of the microbiota associated with these hosts. For example, several studies have shown that the honey bee gut harbors a simple microbiota that is nonetheless quite distinct from other insects (Cox-Foster *et al.*, 2007; Martinson *et al.*, 2011; Moran *et al.*, 2012; Sabree *et al.*, 2012). A metagenomic study of the honey bee gut showed that these microorganisms contain genes that may be involved in defense from pathogens and digestion of pollen (Engel *et al.*, 2012). The bumblebee microbiota is composed of similar taxa to the honeybee microbiota (Koch & Schmid-Hempel, 2011a) and has been shown to protect the bumble bee *Bombus terrestris* from a specialist trypanosome pathogen, *Crithidia bombi* (Koch & Schmid-Hempel, 2011b, 2012).

Both honey bees and bumble bees are social insects, and social transmission is important for the maintenance of their microbiota (Martinson *et al.*, 2012; Koch *et al.*, 2013). Social behaviors such as oral–oral sharing of food (trophallaxis) may allow members of a social insect colony to transmit microorganisms to their colony mates, creating consistent associations. A phylogeny of the lactobacilli associated with a range of social Hymenoptera, however, suggested that honey bees and bumble bees are exceptional in their association with host-specific lactobacilli; solitary and primitively eusocial sweat bees and highly eusocial ants associated with environmentally acquired lactobacilli, whereas honey bees and bumble bees

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associated with phylogenetically derived lactobacilli (McFrederick *et al.*, 2013). Moreover, comparisons of microbial communities in social and solitary hosts are mostly lacking. We previously surveyed microbial communities from two solitary sweat bee nests and two social sweat bee nests, and microbial patterns suggested that sociality may allow for more consistent associations between sweat bees and putatively beneficial lactobacilli (McFrederick *et al.*, 2012). Small sample sizes, however, precluded any definitive conclusions.

Sweat bees in the genus *Megalopta* provide a unique opportunity to compare microbial communities from social and solitary hosts. *Megalopta genalis* and *M. centralis* (formerly *ecuadoria*) are both facultatively social, forming nests with 1–11 females (Wcislo & Gonzalez, 2006). Social and solitary nests are found in the same population in locations such as Barro Colorado Island in Panama (Smith *et al.*, 2003). This social polymorphism allows comparisons of microbial communities in social and solitary nests of the same host species from the same population, thereby controlling for many environmental factors that may influence microbial associations. As facultatively social insects, *M. genalis* and *M. centralis* are at the cusp between solitary and social behavior, and ancient, coevolved relationships between socially transmitted bacteria and *Megalopta* hosts are therefore unlikely. *Megalopta* species, however, exhibit other characteristics of large insect colonies, such as trophallaxis (Wcislo & Gonzalez, 2006), which may allow social transmission within nests (e.g. of crop-inhabiting microorganisms). Trophallaxis may lead to members of a social colony sharing a similar set of microorganisms, and if some of these are beneficial, social behavior may lead to more consistent short-term relationships with beneficial bacteria than solitary behavior. Alternatively, social behavior may increase exposure to diverse bacteria, including pathogens. Consistent with the hypothesis that sociality increases pathogen exposure, the strength of cuticular antimicrobial secretions increases with sociality in bees (Stow *et al.*, 2007), thrips (Turnbull *et al.*, 2011), and wasps (Hoggard *et al.*, 2011).

Host developmental stage is another important factor that affects bacterial community structure in insects. For example, honey-bee and bumble-bee larvae exhibit different bacterial profiles than adult bees (Mohr & Tebbe, 2006). Although a culture-independent study found that honey-bee larvae appear to harbor few or no gut microorganisms, *Saccharibacter floricola* (referred to as the Alpha 2.2 adult gut phylotype in the honey-bee microbiota literature) was found in those larvae that did harbor gut bacteria (Martinson *et al.*, 2012). A culture-based study found that young larval guts are mostly dominated by Alpha 2.2, while older larvae are dominated by *Fructobacillus fructosus* and *Lactobacillus kunkeei* (Vojvodic *et al.*, 2013). Newly emerged worker honey bees are devoid of gut microorganisms, but acquire the core gut microbiota by the ninth day after emergence (Martinson *et al.*, 2012).

In this study, we investigate whether bacterial community structure differs by host social structure, host developmental stage, or host species. To test the hypothesis that host social behavior affects bacterial community structure, we characterized microbial community diversity from social and solitary nests of *M. genalis* and *M. centralis*. To test the hypothesis that different host stages harbor different bacterial communities, we compared bacterial communities across host developmental stages. To determine whether closely related species host different bacterial communities, we compared communities associated with the two host species.

Materials and methods

Collections

We collected social and solitary nests of *M. genalis* and *M. centralis* on two collection trips to Barro Colorado Island, Panama, lasting from January 13, 2011 to February 5, 2011 and March 9, 2011 to April 12, 2011. *Megalopta genalis* and *M. centralis* construct nests in rotting sticks that have become entangled in vines and lianas in the forest understory (Wcislo *et al.*, 2004), and we searched for nests in enclosed canopy forest where the understory was accessible. *Megalopta genalis* and *M. centralis* are crepuscular and forage for only 60–90 min at dawn and dusk (Wcislo *et al.*, 2004). This crepuscular habit allowed us to collect during the day when all of the members of a nest were present in the nest. Upon discovery of a nest, we plugged the entrance with sterile cotton and brought the nest back to the laboratory on Barro Colorado Island for dissection. We did not individually bag each nest, so it is possible that some cross-contamination of microorganisms between the outsides of the nests occurred. As the nests were plugged upon collection and dissected immediately upon return to the laboratory, samples from inside the nest remained uncontaminated.

In the laboratory, we dissected the nests inside a plastic container turned on its side. We sanitized the container with 10% bleach followed by 100% ethanol. To minimize contamination of the samples with airborne microorganisms, we kept an alcohol flame bulb burning in front of the container. We carefully opened each nest and collected wood from the outside of the nest (haphazardly sampling from the entire stick), from the inner tunnels (also haphazardly sampling the entire nest), and all adults in the nest. *Megalopta centralis* and *M. genalis* form very

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small colonies (range 1–11 workers), with rarely more than four workers, and often just one worker (Wcislo & Gonzalez, 2006). If only one female was present in the tunnels, we considered that nest solitary. If the nest had more than one adult female in the tunnels, we considered that nest social. We flame-sterilized our dissecting tools between each sample. We then carefully opened each brood cell and separately collected the bee (egg, larva, mature larva, pupa, or pharate adult) and cell contents (pollen or frass). We identified mature larvae by the presence of frass in the brood cell, as *Megalopta*, like other halictids, have a discontinuous gut – and therefore do not defecate – until their final instar (Michener, 1974). To investigate cuticular, endosymbiotic, and gut bacteria, we collected the entire insect. To minimize bacterial DNA degradation, we collected all samples into 100% ethanol, immediately froze them at −80 °C, and transported them on ice. As a negative control, we opened tubes in our dissecting container to sample any airborne microorganisms.

454 pyrosequencing

DNA extraction, PCR, and 454 FLX titanium pyrosequencing were performed at Molecular Research LP using previously published protocols (Dowd *et al.*, 2008; Sen *et al.*, 2009; Ishak *et al.*, 2011; Engel *et al.*, 2013). We used the 28F (5′-GAGTTTGATCNTGGCTCAG-3′) and 519R (5′-GTNTTACNGCGGCKGCTG-3′) primer pair, which amplifies the V1, V2, and V3 regions of the 16S rRNA gene. We combined our newly collected data with publicly available data from a previous study of two *M. genalis* nests which used the same primers and sequencing protocols as the current study (McFrederick *et al.*, 2012). The new data are publicly available on NCBI's sequence read archive (SRA accession number SRP027357). Altogether, we obtained samples from 12 nests of *M. genalis* and six nests of *M. centralis* (Table 1).

Bioinformatics

For sequence quality control and analysis, we followed the 'BEEBOOK' standard methods for analyzing the honey-bee gut microbiota (Engel *et al.*, 2013). We first denoised the 454 data using AMPLICONNOISE (Quince *et al.*, 2011). We then imported the denoised data into QIIME (Caporaso *et al.*, 2010) for further quality control and analyses. We removed barcodes and forward and reverse primer sequences and discarded any sequences < 200 bases in length or with homopolymer runs > 6 bases, with mismatches to the primers or barcodes, or with ambiguous bases. To cluster sequences sharing 97% or greater sequence identity into operational taxonomic units (OTUs), we used USEARCH (Edgar, 2010) as implemented in QIIME. For chimera detection, we used UCHIME (Edgar *et al.*, 2011) within the QIIME pipeline. To assign OTUs to taxonomy, we used the Ribosomal Database Project's Naïve Bayesian Classifier and the greengenes database (McDonald *et al.*, 2012). We removed singleton OTUs (i.e. single sequences that did not cluster into OTUs with other sequences). As honey bee-associated microorganisms have been shown to be poorly classified using standard 16S rRNA gene databases (Newton & Roeselers, 2012; Sabree *et al.*, 2012), we ran additional BLAST searches against NCBI's GenBank for the 70 most abundant OTUs.

Table 1. Number of microbial communities analyzed, listed by species and social status of the nest

Sample size (# of nests)	Social	Solitary
M. genalis	21 (4)	28 (8)
M. centralis	19 (3)	36 (3)

Number of unique nests from which the individual samples were taken is in quotation marks.

To explore beta diversity, we used QIIME to calculate UNIFRAC distances (Hamady & Lozupone, 2009). We first aligned our sequence data against the greengenes database. Next, we removed empty columns resulting from the alignment and masked variable regions so that they were excluded from the phylogenetic analysis. To inspect and trim the resulting alignment, we used the program Mesquite (Maddison & Maddison, 2011) and trimmed the alignment to 332 bases. To build a phylogenetic tree, we used the QIIME implementation of FASTTREE (Price *et al.*, 2009). We then ran UNIFRAC on two separate datasets, which we subsampled to standardize the number of sequences per sample: (1) insect samples only (subsampled to 1074 sequences); (2) all samples (subsampled to 1032 sequences per sample). We then calculated UNIFRAC distances and performed nonmetric multidimensional scaling (NMDS) ordination of the UNIFRAC distance matrixes according to standard methods (Hout *et al.*, 2013).

To determine which OTUs were differentially abundant between the two host species, we used the program METASTATS, which uses nonparametric *t*-tests for abundant taxa and Fisher's exact test for low-abundance taxa and corrects for multiple comparisons (White *et al.*, 2009). We also used METASTATS to detect differential abundance between OTUs associated with the pollen and larvae samples as compared to the rest of the samples. To determine which OTUs associated with the axes in our NMDS analyses, we used the cor.test command in R (R Core Development Team, 2013) to conduct Pearson's correlations between the NMDS axes and (1) the most abundant OTU (a *Lactobacillus* OTU); (2) all *Lactobacillus* OTUs; and (3) all *Wolbachia* OTUs.

To explore alpha diversity, we plotted the ten most abundant phylotypes by sample using the heatmap.2

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function in the gplots package of the R program. To explore clustering patterns based on the abundances of the 10 most abundant phylotypes, we used hierarchical clustering to create a dendrogram by which the columns (= sample) of the heatmap were ordered. We calculated the probability of an interspecific encounter (PIE), which is the probability that two randomly sampled individuals (i.e. sequences) from the community are different species (Hurlbert, 1971). To test for significant differences in OTU richness or PIE between social and solitary nests of the two different species, we conducted two-way ANOVAS using the aov function of the program R (R Core Development Team, 2013). We plotted the residuals and log-transformed both OTU richness and PIE so that the residuals met the assumption of normality (Quinn & Keough, 2002).

Results

Beta diversity

Using only the insect samples, NMDS ordination of UNIFRAC distance matrixes indicated that bacterial communities did not cluster by host social structure, but instead clustered by host species and developmental stage of the host (Fig. 1). We found similar patterns in our analysis of all samples (see Supporting Information, Data S1 and Fig. S1). Clustering by host species was strong whether the UNIFRAC matrix was unweighted (Fig. 1a) or weighted by bacterial relative abundance (Fig. 1b). In contrast, clustering by host developmental stage was present but not as strong in the unweighted (Fig. 1a) as in the weighted (Fig. 1b) analysis. Adonis analysis (permutational MANOVA) supported these findings: (1) host social structure was not significant (unweighted UNIFRAC matrix: $R^2 = 0.02$, $P = 0.54$; weighted UNIFRAC matrix: $R^2 = 0.01$, $P = 0.64$); while (2) host species (unweighted UNIFRAC matrix: $R^2 = 0.05$, $P < 0.001$; weighted UNIFRAC matrix: $R^2 = 0.38$, $P < 0.001$); and (3) host developmental stage (unweighted UNIFRAC matrix: $R^2 = 0.10$, $P = 0.003$; weighted UNIFRAC matrix: $R^2 = 0.21$, $P = 0.002$) were significant. The sample type clusters, however, exhibited unequal dispersion, which can cause false-positive results for permutational MANOVA (Anderson, 2001). Although the significance level was likely inflated by unequal dispersion among sample types, the agreement between

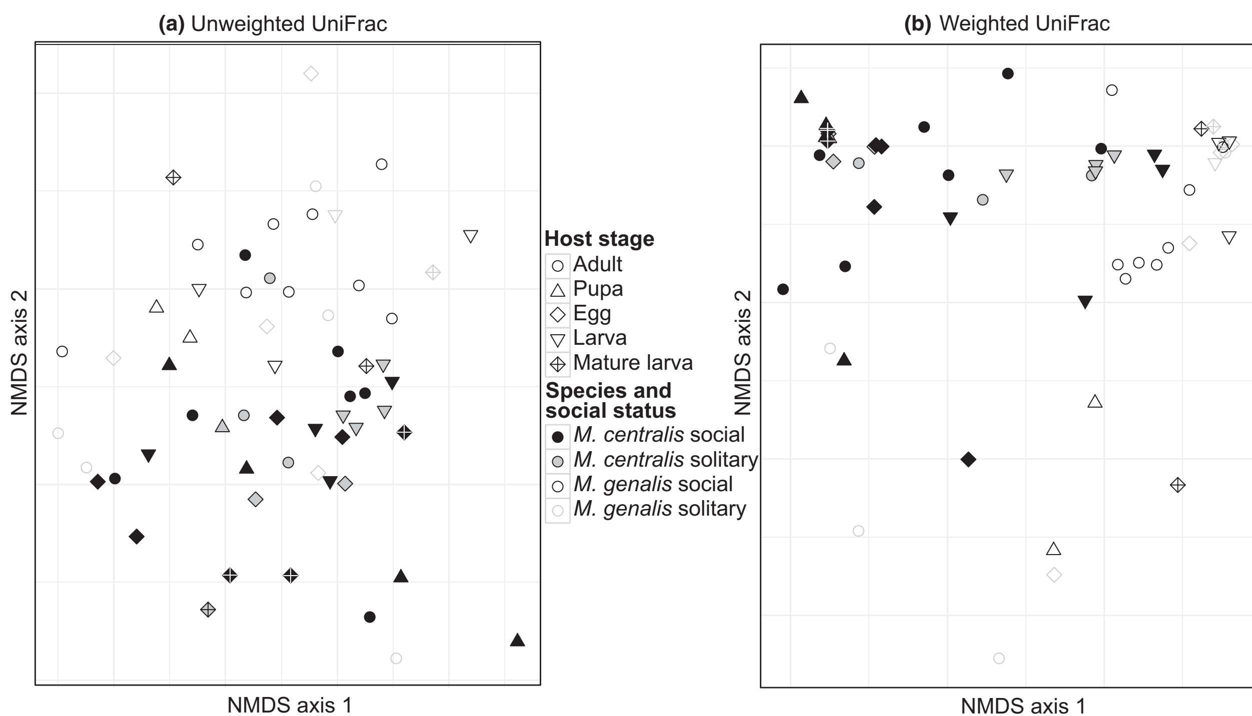

Fig. 1. Ordination of UNIFRAC distance matrixes of bacterial microbiota of *Megalopta* bees using NMDS: (a) unweighted (b) weighted by OTU abundance. Two-dimensional solutions, stress = 0.19 (unweighted) and 0.09 (weighted). Samples are labeled by host species and social structure of the nest from which the sample originated (shading and open vs. closed shapes) and developmental stage of the insect (shape), as indicated in the figure legend. Microbiota cluster by host species in both (a) and (b). The weighted ordination exhibits greater clustering by host developmental stage, with a cluster of *Megalopta centralis* samples dominated by *Wolbachia* (upper left), a cluster of *M. genalis* adult, larva, and egg samples dominated by *Lactobacillus kunkeei* (upper right), and a loose cluster of *M. centralis* larvae and adult samples dominated by both *Wolbachia* and *L. kunkeei* (upper middle).

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visual inspection of the clusters and the Adonis analysis suggests that the pattern is real. For example, in the weighted analysis, bacterial communities from several *M. centralis* mature larvae, adults, pupae, and eggs formed a cluster (Fig. 1b, top-left). While there was some dispersion of bacterial communities associated with *M. centralis* larvae, most clustered closely to several *M. centralis* adult-associated communities (Fig. 1b top-middle). Several *M. genalis* larvae and adults and three *M. genalis* eggs formed a tight cluster, with two mature *M. genalis* larvae falling close by (Fig. 1b, top-right). Although five adult *M. genalis* social samples clustered in the weighted UNIFRAC analysis, in both weighted and unweighted analyses, adult-associated communities were extremely dispersed. Pearson's correlations between abundant OTUs and the weighted NMDS axes showed that *Wolbachia* strongly negatively correlated with NMDS axis 1 ($r = -0.75$, $P < 0.001$), while *L. kunkeei* strongly positively correlated with NMDS axis 1 ($r = 0.74$, $P < 0.001$).

These results were verified by hierarchical clustering based on the top-ten phylotypes found in each community, where the deepest divergence was between species, while within these clusters, there was further clustering based on sample type (Fig 2). A large cluster of bacterial communities from pollen provisions, adults, and larvae of both species were dominated by *L. kunkeei*, whereas mature larvae and pupae-associated communities exhibited low relative abundance of *L. kunkeei*. The deepest split in the hierarchical clustering analysis occurred between *M. centralis* samples dominated by *Wolbachia* and those samples not dominated by *Wolbachia*, which were mainly *M. genalis* samples but also included some (mostly noninsect) *M. centralis* samples. That *Wolbachia* drives the difference in communities from the two host species was confirmed by a METASTATS analysis, which showed that of the 250 OTUs that exhibited significant differential abundance between the two host species, *Wolbachia* exhibited the greatest difference in mean abundance ($P = 0.0009$).

Alpha diversity

Rarefaction plots indicated that our sampling depth accurately characterized the bacterial diversity of the majority of these samples (Fig. S2). After quality control, our sequencing depth ranged from 1032 to 11 979 (average 4094) sequences per sample. The number of OTUs found in each sample varied greatly, with the greatest

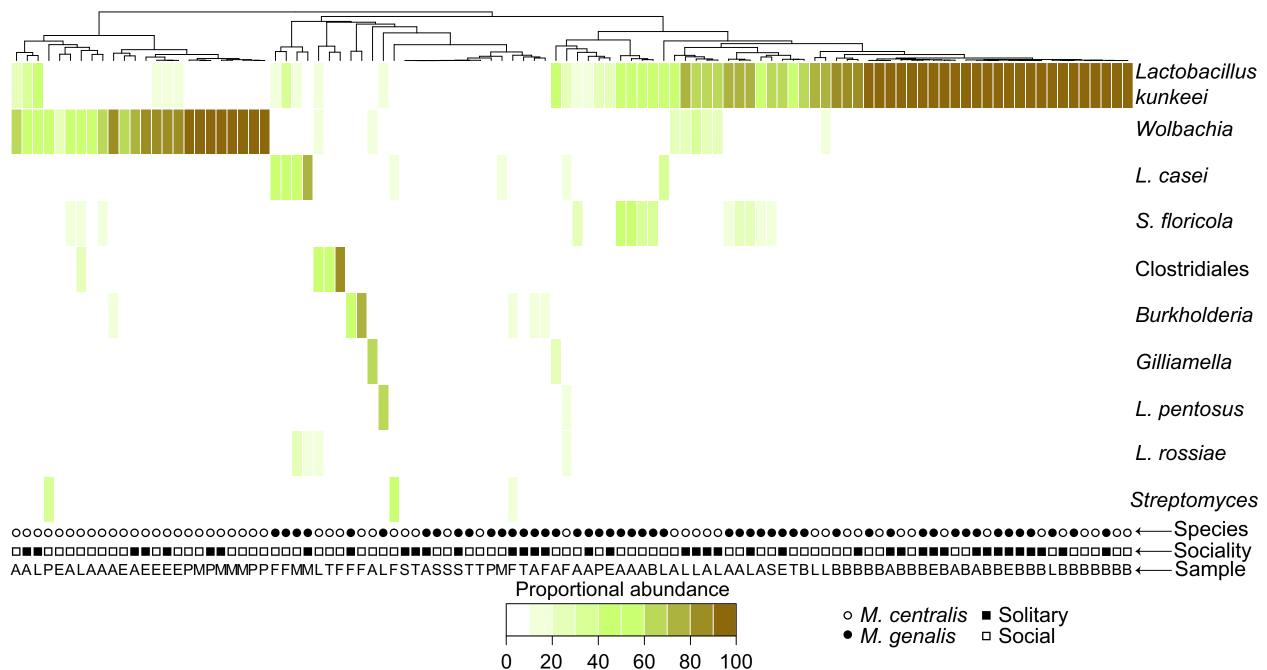

Fig. 2. Heatmap of the ten most abundant bacterial phylotypes (row) by sample (column). Proportional abundance of the phylotype in each sample is presented by shade/color as indicated by the scale bar at the bottom, with darker shading indicating greater proportional abundance. The samples (columns) are ordered by hierarchical clustering based on community dissimilarity as indicated by the dendrogram at the top. The samples are coded by species (open circles = *Megalopta centralis* and closed circles = *M. genalis*), sociality (open squares = solitary and closed squares = social), and sample type (a = adult, b = pollen provision, e = egg, f = frass, l = larva, m = mature larva, p = pupa, s = substrate surrounding nest, and t = tunnel of nest).

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richness found in samples taken from the tunnels of the nest or the substrate surrounding the nest (Fig. 3). Within sample type, however, there was also variation in OTU richness. For example, bacterial communities associated with adult bees varied from nine, the lowest OTU richness of any sample, to 292 OTUs. The PIE among members of these communities also varied, but was highest in substrate and tunnel bacterial communities and lowest in pollen provision bacterial communities (Fig. 3). A two-way ANOVA revealed that OTU richness was not significantly different in social or solitary samples ($F_{1,100}$ = 0.73, P = 0.39), by host species ($F_{1,100}$ = 0.26, P = 0.62), or by host species and social structure interaction ($F_{1,100}$ = 0.03, P = 0.86). We found the same result for PIE, which did not differ in social or solitary samples ($F_{1,100}$ = 0.55, P = 0.462), by host species ($F_{1,100}$ = 1.95, P = 0.16), or by host species and social structure interaction ($F_{1,100}$ = 0.06, P = 0.80).

The bacterial communities associated with *Megalopta* species and their nests included both novel bacteria and bacteria that have been previously found in association with wild bees (Fig. 2). Several of the most abundant bacteria, namely an undescribed relative of *L. kunkeei*

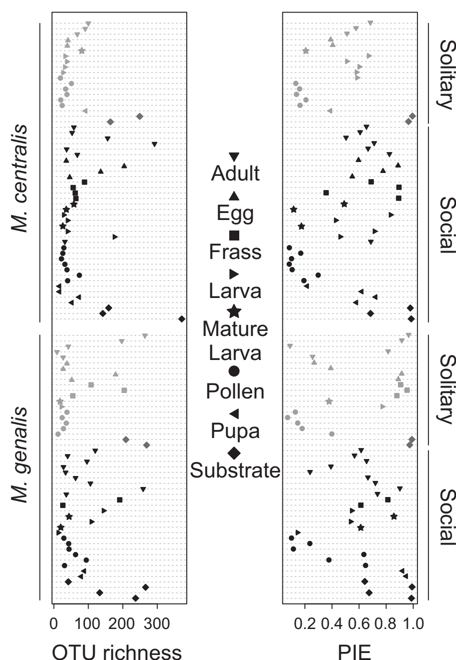

Fig. 3. Alpha diversity organized by host species (labeled on the left of the figure), social status of the nest from which the sample originated (labeled on the right of the figure), developmental stage for bee samples, or sample type for nonbee samples (labeled by shape as indicated in the figure legend). The chart on the left is the observed number of OTUs in each sample, while the chart on the right is the probability of an interspecific encounter (PIE), a measure of community evenness.

(*Bacilli*), *S. floricola* (*Alphaproteobacteria*), and *F. tropaeoli* (*Bacilli*), are known to occur on flowers or belong to groups of bacteria frequently associated with flowers (Endo *et al.* 2009, 2011,; Jojima *et al.*, 2004). We previously reported the presence of *L. kunkeei* relatives and *S. floricola* associated with *M. genalis* (McFrederick *et al.*, 2012). Another OTU shared 95% sequence identity to *Gillamella apicola* (*Gammaproteobacteria*), a bacterium specific to honey bees and bumble bees (Kwong & Moran, 2013), and 97% sequence identity to a close relative of *G. apicola* isolated from a bumble bee (GenBank accession number HM215025). Other abundant bacteria have no known close relatives, such as the undescribed *Clostridiales* (*Clostridia*) and a bacterium that shared 87% sequence identity to an uncultured *Nitrospira* (*Nitrospira*) species (GQ183206). The undescribed *Clostridiales* shared 94% sequence identity to an unknown bacterium isolated from *Apis dorsata* (Ahn *et al.*, 2012) and 93% sequence identity to an unknown bacterium that we recently found associated with the alfalfa leafcutter bee *Megachile rotundata* (McFrederick *et al.*, 2014). Although uncommon, we found the honey-bee pathogen *Melissococcus plutonius* (*Bacilli*) associated with a larva, two pupae, and a frass sample. In addition, we found OTUs that were assigned to the genus *Paenibacillus*, which includes the causative agent of American foulbrood, and an OTU that was assigned to the genus *Spiroplasma* (*Mollicutes*), which also contains honey-bee pathogens (Evans & Schwarz, 2011), but additionally contains mutualists of mycophagous fruit flies (Jaenike *et al.*, 2010).

Discussion

Bacterial communities associated with wild bees in the genus *Megalopta* did not differ by host social structure, but instead differed by host species and developmental stage of the host. A similar set of environmentally acquired (e.g. *L. kunkeei*) and maternally inherited (i.e. *Wolbachia*) microorganisms dominated *Megalopta*-associated bacterial communities, regardless of host social structure. For example, an OTU closely related to *L. kunkeei* dominated many samples from both social and solitary nests, indicating that environmental transmission may be more important for the persistence of this association than social transmission, or that environmental transmission masks any effect of social transmission. *Megalopta centralis* and *M. genalis* have pollen diets dominated by just a few plant species (Smith *et al.*, 2012), meaning that social and solitary foragers visit the same floral resources and therefore are likely exposed to similar environmental microorganisms. Social structure therefore does not affect bacterial communities associated with the socially polymorphic *M. centralis* or *M. genalis*.

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Studies of honey bees and bumble bees suggest that social transmission is important for the maintenance of their host-specific, distinctive microbiota. Martinson *et al.* (2012) found that honey-bee larvae and newly emerged workers lacked the distinct honey-bee microbiota, but that these bacteria were present after contact with hive materials and oral–oral sharing of food (trophallaxis). Koch & Schmid-Hempel (2011b) and Koch *et al.* (2013) also found evidence for social transmission of the bumble bee microbiota. Newly emerged bumble bees that were fed sterile sugar water lacked bumble bee-specific gut bacteria, while newly emerged bumble bees that were exposed to their nestmates' feces acquired these bacteria (Koch & Schmid-Hempel, 2011b). Additionally, Koch *et al.* (2013) found that queen bumble bees that were allowed social interaction tested positive for *Gilliamella* and *Snodgrasella* more consistently than queens that were kept in sterile conditions. Social transmission therefore seems to be important for the maintenance of the honey-bee and the bumble-bee microbiota.

Our work differs from the work with honey bees and bumble bees in that we compared microbial communities from solitary nests and nests just across the threshold of social behavior. We found that social interactions such as trophallaxis did not influence bacterial community structure nor create more similar microbial communities associated with members of social nests compared to solitary nests. *Megalopta* species do not need social transmission to associate with the putatively beneficial *L. kunkeei*; instead, *Megalopta* appear to acquire *L. kunkeei* from the environment each generation, paralleling the acquisition of *Burkholderia* symbionts by the stink bug *Riptortus clavatus* (Kikuchi *et al.*, 2007).

In contrast to social structure, host developmental stage influenced the structure of *Megalopta*-associated bacterial communities. Pollen provisions were dominated by *L. kunkeei* bacteria, which are likely obtained by bees from flowers (McFrederick *et al.*, 2012). This fructophilic bacterium appears to thrive on the pollen and nectar provisions that the adult bees obtain from flowers. Several eggs seemed to have become covered with *L. kunkeei* from the pollen provisions on which they were laid. The bacterial communities from these egg samples therefore clustered with other *L. kunkeei* dominated bacterial communities. *Lactobacillus kunkeei* dominated many of the communities associated with young larvae, suggesting that *L. kunkeei* also thrives in the larval gut. The majority of mature larvae, however, lacked *L. kunkeei*, indicating that *L. kunkeei* was lost when the mature larvae voided their guts. Several mature larvae, however, still harbored communities dominated by *L. kunkeei*, possibly because they had not yet completely voided their guts. Pupae also have empty guts, and *L. kunkeei* was completely lacking from five of six pupae-associated communities. Many of the adult-associated communities were once again dominated by the *L. kunkeei* relative. Environmental transmission, likely from flowers, therefore appears to be the main mechanism by which adults reacquire the bacterium that dominated their guts when they were larvae. Whether flower- and bee-associated bacteria such as *L. kunkeei*, *S. floricola*, and *F. tropaeoli* are predominately bee-associated, pollen provision-associated, flower-associated, or simply thrive in all three niches due to the presence of similar resources requires further study.

The significant difference between *M. centralis* and *M. genalis*-associated bacterial communities was driven in large part by differential presence and relative abundance of *Wolbachia* in the two species. As we previously reported (McFrederick *et al.*, 2012), *Wolbachia* was present in *M. genalis*, but occurred in only a few samples at relative abundances < 1.6%. In contrast, *Wolbachia* was present in all but one of the *M. centralis* insect samples, often at very high relative abundance. Functional assays are needed to determine whether *Megalopta*-associated *Wolbachia* are sex ratio distorters (Werren *et al.*, 2008), nutritional mutualists (Hosokawa *et al.*, 2010), or defensive mutualists (Hedges, 2008; Teixeira *et al.*, 2008; Unckless & Jaenike, 2011). In particular, it would be interesting to determine whether *Wolbachia* infection has different fitness consequences for *M. centralis* and *M. genalis*, which would help explain the differential prevalence in these two species.

There is still much future work to be done with the bacterial microbiota of wild bees. Although the *L. kunkeei* relative exhibits several general properties that mean it is likely to be beneficial to its hosts (McFrederick *et al.*, 2013), its actual function in wild bees remains unknown. The highly derived *Clostridium* relative that is related to but distinct from a *Clostridium* relative associated with *Megachile rotundata* (McFrederick *et al.*, 2014) deserves further study to determine whether it is host-specific and what effect it may have on host fitness. We also detected the honey-bee pathogen *M. plutonius* and relatives of the honey bee pathogen *Paenibacillus larvae*. Whether these bacteria were spread to *Megalopta* from feral honey-bee colonies that occur in Panama or whether they are endemic to *Megalopta* and honey bees remains an open question. The honey-bee and bumble-bee microbiota have already provided fascinating insights into insect–microorganism interactions. The microbiota of wild bees promises to hold insights that are just as fascinating.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Data S1. Methods and results.

Fig. S1. UNIFRAC distance matrix (weighted by OTU abundance) ordination using nonmetric multidimensional scaling (NMDS).

Fig. S2. Rarefaction curves of the number of observed OTUs from individual samples coded by host species and social status of the host's nest, as indicated in the figure legend.

Fig. S3. UNIFRAC distance matrix (weighted by OTU abundance) ordination using principal coordinates analysis (PCoA).

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