Acquisition and Structuring of Midgut Bacterial Communities in Gypsy Moth (Lepidoptera: Erebidae) Larvae

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ABSTRACT Insects are associated with a diversity of bacteria that colonize their midguts. The extent to which these communities reflect maternal transmission, environmental acquisition, and subsequent structuring by the extreme conditions within the insect gut are poorly understood in many species. We used gypsy moth (*Lymantria dispar* L.) as a model to investigate interactions between egg mass and environmental sources of bacteria on larval midgut communities. Egg masses were collected from several wild and laboratory populations, and the effects of diet, initial egg mass community, and internal host environment were evaluated using 454 16S-rRNA gene pyrosequencing. Wild populations were highly diverse, while laboratory-maintained egg masses were associated with few operational taxonomic units. As larvae developed, their midgut bacterial communities became more similar to each other and the consumed diet despite initial differences in egg mass-associated bacteria. Subsequent experiments revealed that while midgut membership was more similar to bacteria associated with diet than with egg mass-associated bacteria, we were unable to detect distinct, persistent differences attributable to specific host plants. The differences between foliar communities and midgut communities of larvae that ingested them were owing to relative changes in populations of several bacteria phylotypes. We conclude that gypsy moth has a relatively characteristic midgut bacterial community that is reflective of, but ultimately distinct from, its foliar diet. This work demonstrates that environmental acquisition of diverse microbes can lead to similar midgut bacterial assemblages, underscoring the importance of host physiological environment in structuring bacterial communities.

KEY WORDS bacteria, community, midgut, plant–insect interaction, pyrosequencing

Insect guts represent challenging microbial habitats, distinguished by often having extreme environments, relatively stable conditions between intermittent pronounced disturbances, and substantial substrate turnover (Dillon and Dillon 2004, Engel and Moran 2013). Their bacterial communities are taxonomically variable across host species, and can range from having relatively few members (Koch and Schmid-Hempel 2011, Wong et al. 2011, Moran et al. 2012) to being highly diverse (Geib et al. 2009b, Rani et al. 2009, Köhler et al. 2012). The composition of gut microbial communities within individual insect species can also exhibit substantial variation (Broderick et al. 2004, Geib et al. 2009b, Boissière et al. 2012, Tang et al. 2012, Kautz et al. 2013, Wong et al. 2013). It is unclear to what extent this variation is owing to innate differences, environmental inputs, or their interactions. Processes such as maternal acquisition of symbionts, the behavior of the animal including its foraging activities and diet selection, the host's inherent physiology, and environmental exposure can structure an insect's gut bacterial community. These components are not mutually exclusive, but rather interact extensively under natural conditions. The unique behaviors, environmental interactions, feeding ecologies, and gut physiologies, of each insect species, can affect microbial colonization and establishment.

Maternal routes of transmission have typically been the major focus of studies on intracellularly acquired symbionts. Bacteria residing in bacteriocytes of sap-feeding insects are highly conserved, maternally acquired, and physiologically integral symbionts (Baumann 2005). In addition, there are several examples of facultative, transovarially transmitted bacteria that may vary in their tissue associations (Fukatsu et al. 2000, Russell et al. 2013, von Dohlen et al. 2013). Maternal transmission of gut bacteria has been shown to occur via glands (Aksoy et al. 1997), specialized capsule production (Fukatsu and Hosokawa 2002), or deposition on eggs that are subsequently consumed by emerging brood (Lauzon et al. 2009, Bansal et al. 2011, Hosokawa et al. 2012).

Insect behavior can influence bacterial community composition through additional processes, such as di-

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rect transmission between individuals within colonies of social and gregarious species (Minkley et al. 2006, Moran et al. 2012, Woodbury et al. 2013). However, these behaviors are exclusive to insects that have overlapping generations. Relatively plastic behaviors, such as dietary preference, also may cause perturbations to populations and communities of these consortia (Sudakaran et al. 2012). Insect dietary choice has been suggested to be a major factor in shaping midgut bacterial composition in a number of host species (Lundgren and Lehman 2010, Broderick and Lemaitre 2012).

Insect host physiology can also be an important driver of microbial interactions (Dillon and Dillon 2004). Insect digestive systems often contain multiple compartments, and variable conditions among and within hosts can yield different distributions of bacteria (Kaltenpoth et al. 2009). Insect midguts usually constitute extreme environments, ranging from very acidic (pH 2.8) to highly basic (pH $>$ 11; Clark 1999) and having low oxygen tensions (0–6.0 mm HG; Johnson and Barbehenn 2000). Furthermore, pathogenic and commensal bacteria can trigger host immunological responses against many microbes (Buchon et al. 2013). In order for bacteria to establish and survive in animal guts, they must have the phenotypic plasticity to contend with a diversity of hosts (Rawls et al. 2006), ingested metabolites (Kohl and Dearing 2012), and microbial competitors (Dillon et al. 2005, Robinson et al. 2010, Bando et al. 2013).

The impact of the external environment on transmission, and its interaction with the host internal environment, is critical, yet understudied aspects of insect gut communities. Some insect species acquire gut-colonizing *Burkholderia* spp. from soil, which have widespread association across many insect host species (Kikuchi et al. 2007, 2011a). In addition, dietary sources of microbiota may represent a source of potentially helpful, harmful, or seemingly inert bacteria. Although several studies have noted that bacterial communities differ because of diet (Broderick et al. 2004, Geib et al. 2009a, Pinto-Tomás et al. 2011, Anderson et al. 2012, Colman et al. 2012, Jones et al. 2013), few have surveyed the diet they consumed (Su et al. 2010, Brucker and Bordenstein 2012, Dematheis et al. 2012, Priya et al. 2012). Recently, it has been noted that in at least one system, *Drosophila*, frequent replenishment of gut microbiota by bacteria colonizing food is essential for continued maintenance of its communities (Blum et al. 2013).

Gypsy moth (*Lymantria dispar* L.) is a leaf-feeding insect previously shown to contain distinct and diverse microbiota, which are potentially influenced by a variety of exogenous and endogenous components that introduce variation in community structure (Broderick et al. 2004). The larvae are highly polyphagous, capable of consuming several hundred species of plants (Liebhold et al. 1995). The larval midguts are well-buffered (Appel and Maines 1995), highly alkaline (Schultz and Lechowicz 1986), and have low oxygen tensions (Johnson and Barbehenn 2000). Female adults oviposit in a single egg mass in midsummer on a variety of surfaces, depositing scales from its abdomen during the process, where they overwinter until spring. On eclosion, neonate larvae consume a portion of their eggshell, then disperse by wind and feed solitarily (Leonard 1970). A previous census of community membership suggests gypsy moth midgut consortia are influenced by both population and diet sources (Broderick et al. 2004). However, whether these differences are owing to new community members from diet or changes in bacteria acquired from initial feeding on eggs immediately after eclosion is unknown.

The goal of this study was to determine the predominate forces influencing midgut bacterial community composition in gypsy moth. We assessed the influence of dietary and egg mass-associated bacterial components in acquisition and composition of midgut bacteria using high-throughput 16S-rRNA gene amplicon sequencing. Diet represents a potential environmental source of gut bacteria, while egg masses may include maternal and environmental sources. We used egg mass sources of laboratory and field origin with the intention to provide very different starting community compositions. Egg mass sources included three wild populations and a historically lab-reared population. Eclosed larvae were provided three different host plant species as diet sources. Changes in community composition were assessed throughout insect development using two insect populations feeding on a single foliar diet source. We conducted a second experiment using three insect populations and three plant dietary sources on a single developmental stage to confirm our conclusions under different diet regimes.

Materials and Methods

Insect and Plant Rearing, Maintenance, and Sample Collection. *Experiment 1: How Does Gypsy Moth Bacterial Communities Differ Through Development on the Same Host Plant?* As some samples were pooled in this study, a detailed description of which samples were pooled and how many individuals were combined is included (Supp Table 1 [online only]). Egg masses were obtained from two sources: a laboratory-maintained colony (lab; U.S. Department of Agriculture Animal and Plant Health Inspection Service [USDA APHIS]) and a field population from the bark of oak trees near Temperance, MI. Dormant 2-yr-old white birch (*Betula papyrifera* Marshall) trees were obtained from the Wisconsin Department of Natural Resources (Wilson Nursery, Boscobel, WI) and planted in 20-cm-diameter pots with Metro Mix soil media, and fertilized with 10-14-10 Osmocote slow release fertilizer. Trees were grown in cold frames outside of the University of Wisconsin-Madison Walnut Street Greenhouses, and watered by flood irrigation every 2 d. Foliage was collected daily with sterilized forceps into sterile plastic collection bags and transported to the laboratory. Leaves were cut into 2–3 cm squares with flame-sterilized instruments.

Approximately 25–40% of each leaf was pooled, flash-frozen, and stored at −80°C until DNA extraction, and the remainder was provided to the larvae. Foliage material was collected off of two trees, and the molecular analysis included some portion from ≈40 leaves. Insects were hatched from nonsterilized egg masses and reared in the same growth chamber at 24°C and 50–70% relative humidity across all experiments. Larvae from a single egg mass from each source were reared in sterile 10-cm-diameter plastic petri dishes. Samples of each egg mass (unpooled) and 10 first-instar (L1) larvae were collected after eclosion. Larvae were reared en masse until second instar, after which they were reared individually in sterile 24-well plates until molting. At third instar (L3), insects were transferred to 12-well plates, and at fourth instar, they were transferred to 5.0-cm-diameter petri dishes until fifth instar (L5). Upon molting into L3 and L5, 10 larvae from each source were starved for 18 h, anesthetized by placing in −20°C for 10 min, surface sterilized with 95% ethanol, and their midguts were dissected. The 10 larval midguts of each lifestage were pooled and stored at −80°C. The frass deposited by L5 larvae during the starvation period was collected and stored for future analysis.

Experiment 2: How Does Gypsy Moth Bacterial Communities Differ on Different Host Plants? The second experiment was performed with insects obtained from the same lab-reared colony, and two field-collected populations separated by 255 km near Bayfield, WI, and Lakewood, WI. Field populations were collected from the bark of hardwood trees. Leaves of white birch, quaking aspen (*Populus tremuloides* Michx.), and white oak (*Quercus alba* L.) were collected from mature trees on the UW-Madison campus. As described in the first experiment, leaves were collected in sterile plastic bags, transported to the laboratory, fed to the larvae, and a portion was pooled, frozen, and stored at −80°C. Foliage material was collected was collected off of two trees and included some portion from ≈20 leaves. This second experiment was conducted under identical conditions, in the same growth chamber as the first experiment, but was terminated after larvae had molted into L3 and midguts were dissected.

DNA Extraction. DNA was extracted from gypsy moth tissues using the Epicenter Master Pure Complete DNA and RNA purification Kit (Illumina, Madison, WI), according to the manufacturer's protocols with minor modifications. Tissues were homogenized in 2.0-ml screw-cap vials with one 3-mm-diameter bead in 500 μl T&C buffer. Samples were centrifuged at 500 × *g* for 3 min, supernatant was collected, and the remaining manufacturer's directions were followed. If contamination from reagents or kits was present, it was below detectable levels based on our methods.

DNA was extracted from plant foliage using the protocol of Ikeda et al. (2009) with minor modifications. Leaves collected throughout the experiment were homogenized in liquid nitrogen and suspended in 50 mM Tris-HCl (pH 7.0), 1% Triton X-100, and 2 mM β-mercaptoethanol. The homogenate was centrifuged at 500 × *g* for 5 min. Supernatant was transferred and centrifuged at 5,500 × *g* for 20 min. The resulting pellet was used for DNA extraction described above sans modifications.

16S-rRNA Gene Amplification and 454 Pyrosequencing. We included appropriate negative controls at all steps in our PCRs reactions. Controls were completed using identical protocols as stated below, but with the inclusion of the TE buffer (10 nM Tris-HCl, 1.0 nM EDTA. pH 8.0) used to elute DNA. If contamination from reagents or kits was present, it was below detectable levels based on our methods.

Two-step PCR procedures were performed to amplify the V6V8 region of the 16S rRNA gene using the primers 799F-mod6 and 1392R, as described in Hanshew et al. (2013). PCR reactions of the pooled samples were done in triplicate containing 50 ng template DNA, 0.5 μl Herculase II DNA polymerase (Agilent, Santa Clara, CA), 1.0 nM dNTPs, 1.0 μl DMSO, 10 μl buffer, 300 nM forward and reverse primers, and water, totaling a final volume of 50 μl. Reaction conditions were—95°C for 2 min, 30 cycles of 95°C for 20 s, 48°C for 30 s, 72°C for 30 s, and a final elongation of 72°C for 3 min. The triplicate reactions of each sample were combined and gel extracted using a Zymoclean Gel DNA Recovery Kit (Zymo Research, Irvine, CA). Secondary PCR was completed using 2 μl of the recovered PCR product. Primers in the second PCR contained the A- and B- adaptors along with 5 bp multiplex identifiers. PCR conditions were identical except only 10 cycles were performed. The ≈700 bp product were purified by gel extraction and quantified by an Invitrogen Qubit Fluorometer (Life Sciences, Grand Island, NY). Equimolar concentrations of samples were diluted and pooled for 454 pyrosequencing.

454 pyrosequencing was conducted on a GS Junior with Titanium Chemistry (Roche, Indianapolis, IN). Modifications to emPCR to accommodate longer amplicons included increasing Amp mix to 297 μl, Amp Primer to 104 μl, and decreasing water to 359 μl. Thermocycler conditions were modified to—94°C for 4 min, 50 cycles of 94°C for 30 s, 60°C for 10 min, and storage at 10°C. No other modifications to manufacturer's protocols were made.

Data Processing and Analysis. The 454 16S-rRNA amplicon data were processed in mothur v.1.29.2 following recommendations outlined in the Schloss standard operating procedures (Schloss et al. 2009, 2011). Data were denoised via PyroNoise. Sequences were processed as to allow for no differences in multiplex identifiers and primers, no homopolymer exceeding six nucleotides, and a minimum length of 200 nucleotides. Sequences were aligned to the SILVA SEED database, and we performed a preclustering step to reduce sequencing errors. Chimeras were detected using UCHIME and discarded (Edgar et al. 2011). Sequences were classified using the SILVA SEED database at an 80% confidence threshold and those classified as chloroplast, eukaryotic, or unknown at the Kingdom taxonomic level were removed from further analyses.

Table 1. Number of observed OTUs at 3% cutoff and computed diversity metrics

Sample	Observed OTUs	Good's coverage	Chao1	1/Simpson	Shannon
APHIS 2011 eggs					
Egg mass	18	0.995	22.67	1.077	0.235
L1 whole larvae	54	0.991	69.00	2.795	1.842
L3 midgut	34	0.999	34.00	7.512	2.561
L5 midgut	39	0.998	40.00	3.758	2.239
L5 frass	37	0.994	55.00	4.293	2.243
Michigan 2011 eggs					
Egg mass	185	0.945	304.63	10.054	2.563
L1 whole larvae	104	0.976	141.05	6.610	3.419
L3 midgut	34	0.994	41.20	3.348	2.741
L5 midgut	49	0.995	54.60	6.315	1.879
L5 frass	48	0.992	61.00	4.109	2.501
APHIS 2013 eggs					
Egg mass	2	0.999	2.00	1.001	0.005
L3 midgut-Birch	43	0.997	48.00	5.293	2.414
L3 midgut-Oak	32	0.996	33.75	2.803	1.674
L3 midgut-Aspen	110	0.990	116.00	9.763	3.453
Bayfield 2013 eggs					
Egg mass	206	0.941	354.83	21.762	4.071
L3 midgut-Birch	72	0.998	72.30	5.607	2.893
L3 midgut-Oak	40	0.997	45.00	5.580	2.460
L3 midgut-Aspen	49	0.997	50.67	6.538	2.674
Lakewood 2013 eggs					
Egg mass	205	0.932	383.36	13.655	3.639
L3 midgut-Birch	63	0.995	66.50	7.649	2.899
L3 midgut-Oak	58	0.994	67.00	3.451	2.242
L3 midgut-Aspen	43	0.9985	44.00	4.456	2.428
Foliage diet					
Birch foliage 2011	73	0.991	81.75	5.948	2.649
Birch foliage 2013	43	0.996	50.50	9.264	2.806
Oak foliage 2013	67	0.989	86.00	5.746	2.563
Aspen foliage 2013	70	0.996	72.63	10.628	3.056

Samples were randomly standardized to contain 1,400 sequences.

Operational taxonomic unit (OTU) analyses were conducted by clustering at 3% dissimilarity. Because the number of spurious OTUs increases with sampling depth, units were subsampled to least number of sequences (1,400). The Shannon and Simpson diversity indices, Chao1 richness estimates (Chao 1984), and Good's coverage (Good 1953) were calculated using the OTUs. The Yue–Clayton index (Yue and Clayton 2005) and the Jaccard similarity coefficient were used to assess community structure and membership, respectively. The pair-wise distances were used to conduct nonmetric multidimensional scaling (MDS) in PRIMER-E (v.6.0). We conducted three separate MDS ordination analyses using different insect life stages and diet from samples in the first experiment (2011), egg masses, L3, and diet samples from the second experiment (2013), and a subset containing L3 and diet samples. A permutation-based MANOVA was conducted between third-instar gut samples in PRIMER using Yue–Clayton and Jaccard dissimilarities. To determine specific bacterial genera that may contribute to the communities, genera phylotypes were designated, samples were subsampled, and the resulting designations were used to compute Bray–Curtis dissimilarities to conduct unweighted pair-group method using arithmetic mean (unweighted pair-group method with arithmetic average) clustering in mothur.

Deposition of Sequences. All sequences obtained in this study were deposited in the NCBI Sequence Read Archive under the accession number SRP030624.

Results

Composition and Diversity of Bacterial Communities Associated With Gypsy Moth. The 454 pyrosequencing of the 16S rRNA gene amplicons from the plant and gypsy moth samples yielded 169,144 sequences after removal of low-quality reads. Following removal of unclassified and contaminating plastid and chimeric sequences, 162,661 sequences remained. After clustering at 3% dissimilarity, we randomly subsampled to 1,400 sequences per sample.

There were in total 791 OTUs across all samples at the 3% cutoff after processing and subsampling. The 10 most abundant OTUs comprised 62% of the total abundance, and the 50 most abundant comprised 88%. Observed OTUs ranged from 2 to 206 per sample (Table 1). Wild egg mass samples were the most diverse, lab-reared egg masses were the least diverse, and the other larval samples and diet had intermediate levels of diversity. Gypsy moth midguts harbored an average of 51.8 (±6.4 SE) OTUs, and ranged from 30 to 110. Chao1 and Good's coverage estimates indicated that we had reached sampling saturation in all samples with exception of the wild egg masses. The high inverse Simpson diversity estimates in these samples sug-

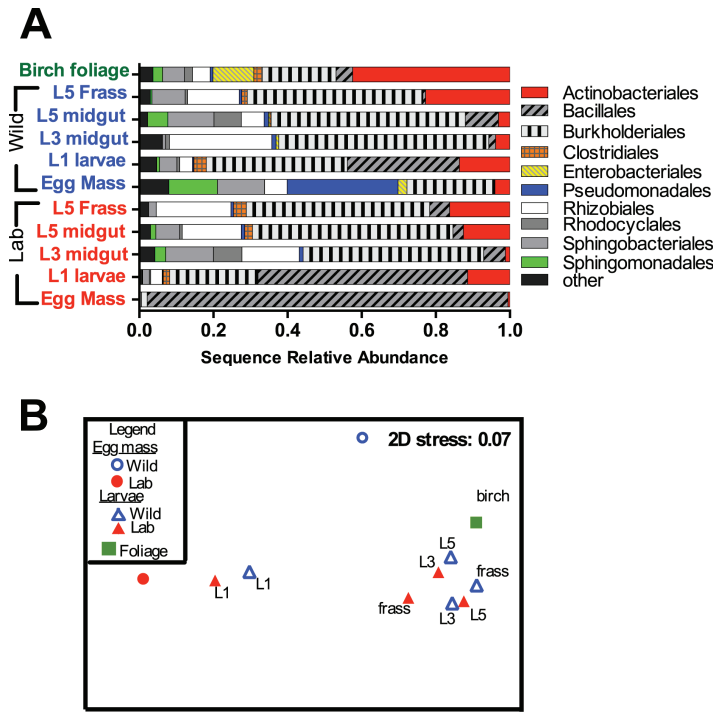

Fig. 1. Order-level designations (A) and OTU-based Yue–Clayton dissimilarity 2D-MDS plot (B) of gypsy moth developmental stages (egg mass, L1–L5, frass) and the birch foliage diet. Egg masses are from a single source, while all other samples were pooled from 10 individually reared samples. Only samples from the first experiment (2011) were included in this analysis. Determination of orders was conducted with an 80% minimum confidence threshold using the SILVA SEED database. Any orders comprising <1.0% of total abundance were included in the group "other." (Online figure in color.)

gested that many OTUs occurred in low abundances. In these wild egg mass samples, 83–89% of OTUs contributed <0.01% of the total abundance.

In the first experiment, 23 orders of bacteria were associated with at least one developmental stage or foliar diet of gypsy moth (Fig. 1A). Ten orders constituted the majority (97%) of these sequences. Lab egg masses were predominately associated with Bacillales (97.3%). The Michigan wild egg mass source had a different starting bacterial community composition than that of the lab population egg masses. Bacillales were not detected in the egg mass, with predominant bacterial belonging to the orders Sphingomonadales (13.1%), Sphingobacteriales (12.6%), Pseudomonadales (29.9%), and Burkholderiales (23.7%). Larval instars followed similar trends regardless of egg mass source. On feeding, larvae had similar bacterial composition and were dominated by Burkholderiales (≈50%), which was found in L3 midguts, L5 midguts, and L5 frass. The bacterial composition of the birch foliage diet included many members found in the insect tissues, but in different abundances. Actinobacteriales comprised 42% of the total abundance and Burkholderiales comprised 19.9%, while Enterobacteriales comprised 11% and Rhizobiales 4.8%.

We conducted finer OTU taxonomic designations, constructed dissimilarity matrices, and conducted MDS ordination analyses to determine if the order-level differences were present. Supporting the observations in order-level differences, the different insect life stages had formed different groups (Fig. 1B). Both egg masses were outside of one major group, which was composed of the midguts and frass samples. The L1 larvae both were outside of the midgut and frass group but distanced from the corresponding egg mass. The birch diet was the closest sample to the midguts and frass samples, but was outside of the grouping.

Despite Initial Differences Arising From Different Diets and Egg Sources, Midgut Communities Within Gypsy Moth Larvae Become Highly Similar Through Larval Development. We conducted a second experiment to partition the extent to which the community structures we observed in our first experiment were owing to the source of egg masses or larval diet. Along with the laboratory-reared egg masses, we included two additional egg masses from two field populations, three foliage diets, and the midguts of the insects of these nine population-by-diet sources. Similar to the ordination analysis for the first experiment, egg masses were positioned away from foliage, from midgut samples, and from each other (Fig. 2A). The lab and field-collected egg mass sources were different from the other samples and each other. Midguts ordinated tightly together, with the foliage samples distinct from each other and of this group. To obtain better resolution, we removed egg masses from the analysis and conducted another MDS that focused on diet and midguts (Fig. 2B). No discernible clustering of larval

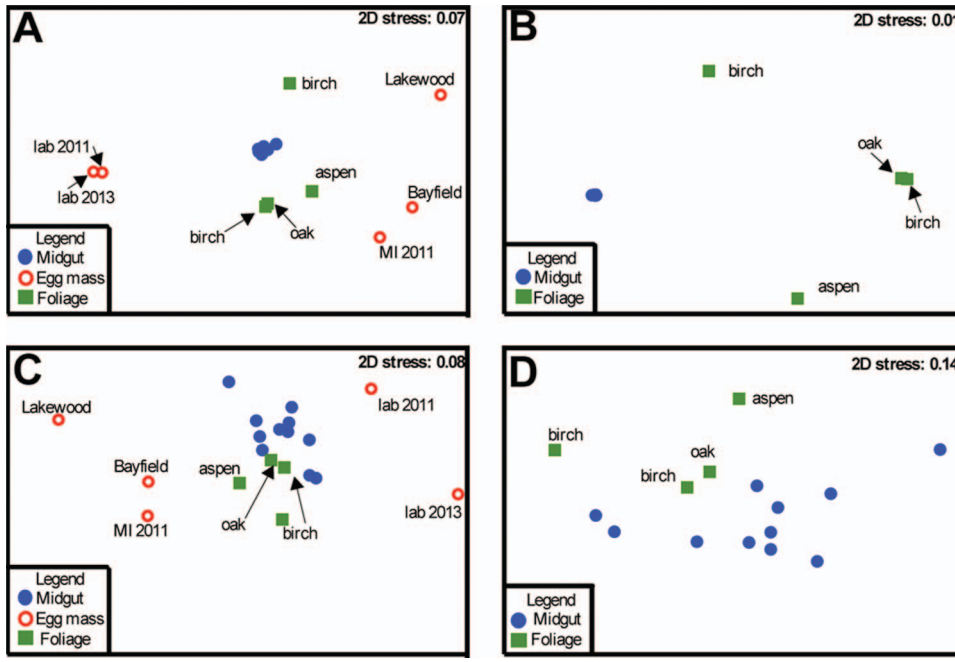

Fig. 2. 2D-MDS plots including (A and C) egg masses (open circles), all pooled foliage samples (oak, birch 2011, birch 2013, and aspen; squares), and L3 midgut samples (closed circles) and (B and D) only foliage and L3 midguts. Ordination plots in panels A and B were performed with Yue–Clayton-based dissimilarities and panels C and D with Jaccard-based dissimilarities. Data from both experiments (2011 and 2013) were included for a total of 11 midgut samples. (Online figure in color.)

midguts by either population source or diet was observed. Community membership as determined by Jaccard pairwise distances followed similar trends. Midgut bacterial communities were more similar to foliar diet communities than to egg masses (Fig. 2C), but did not form distinct groupings (Fig. 2D).

We conducted phylotype-level analyses to evaluate putative bacterial genera that may influence the differences between the samples. Unweighted pair-group method with arithmetic average clustering yielded similar results to ordination-based analyses (Fig. 3A). Midgut bacterial communities were found to be more similar to each other than to the bacterial composition of the ingested diet or to their corresponding egg source. Several OTUs contributed to these clusters; *Ralstonia* was particularly high in abundance in the larval midguts compared with the foliage and midgut samples (Fig. 3B). The foliage had low (4.0%) to moderate (25%) abundances of *Ralstonia* sequences, in contrast to the higher levels of the larval midguts that ranged from 30 to 56% abundance. The abundances of *Ralstonia* in larval midguts did not correspond to either their abundances in the foliage the larvae consumed or their egg masses (0–1.3%) from which they originated. The differences between the foliage and midgut communities were also owing to other OTUs. For example, *Acinetobacter* were detected in birch and oak foliage in 2013 in high abundances (21–26%), but were uncommon in larval samples (0–2.7%). Similar trends existed with birch in 2011 with *Streptomyces* and in aspen with *Pseudomonas*. The major differences among larval gut samples were owing to lower phylotype abundances, but these differences did not appear attributable to either diet or population source. Additional abundant bacteria were unclassified at the genus level. One phylotype associated with both diet and midgut sources was classified as *Wolbachia* with the SILVA SEED. This is more likely an artifact of the length of the sequence and classifier, rather than of contamination from other sources outside of our study.

Discussion

This study identified factors that contribute to the establishment and structuring of bacterial communities in the midguts of gypsy moth larvae. These communities appeared to arise from multiple sources, including egg masses and diet. We found that communities associated with egg masses differ extensively, and range from very high (field collected) to low diversity (Figs. 1 and 2). However, these differences in bacterial communities of egg masses did not translate into which bacteria ultimately inhabited the corresponding larval midgut tissues. Instead, midgut bacterial communities more closely resembled those communities associated with the host plant diet, suggesting that seeding by phyllosphere inhabiting bacteria was a more influential factor in initial acquisition of bacteria, than the initial transmission from egg masses (Fig. 2). For example, the laboratory egg masses that had extremely low diversity produced

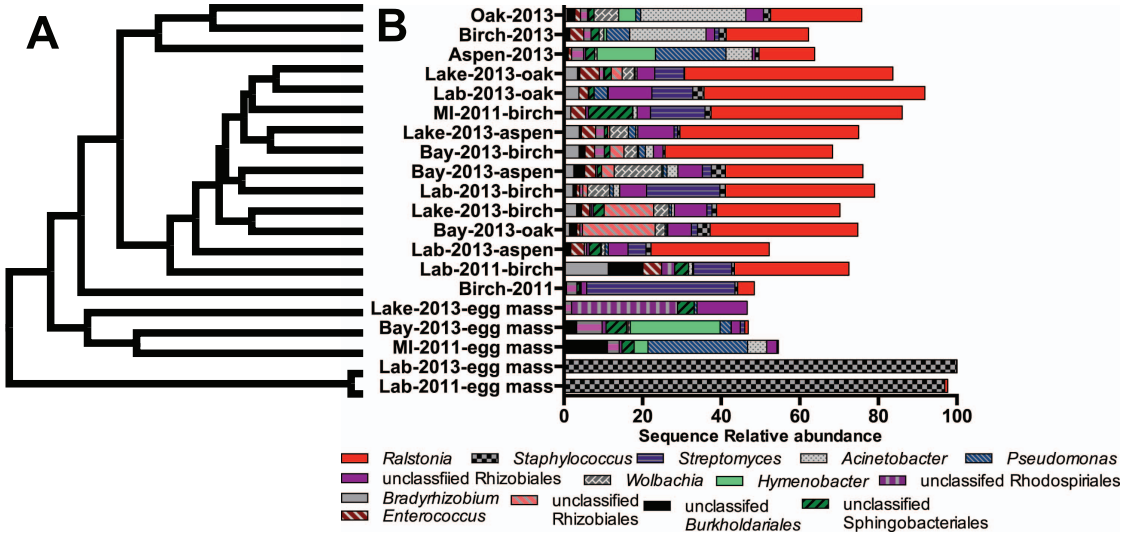

Fig. 3. Unweighted pair-group method with arithmetic average dendrogram of phylotypes using Bray–Curtis dissimilarity (A) and relative abundances of the 14 most abundant 3% cutoff phylotypes across all samples (B). Phylotypes unable to be classified by genus using the SILVA SEED database were designated unclassified with their corresponding order-level classification. (Online figure in color.)

larvae that had midguts with much higher diversity after feeding on foliage, supporting the role of dietary seeding in acquisition of midgut bacteria. Despite differences associated with the plant species consumed, larval midgut communities ultimately attained a similar consortium. The physiological and biochemical conditions within the host insect midgut appear to play an important role in ultimately structuring these communities.

The convergence of dramatically different initial communities, arising from different egg mass and foliar communities, into a very similar midgut consortia was attributed to population shifts of specific bacterial taxa (Figs. 1A and 3B). This suggests that certain taxa are likely better suited for colonizing and proliferating in gypsy moth midgut tissues than others. That is, gypsy moth guts act as an environmental filter that alters the abundances of ingested microbiota. Two potential environmental feedbacks contributing to this filtering may relate to pH and oxygen tensions. Shifts in bacterial communities of nonhosts have been observed along pH gradients and oxygen availability (Lauber et al. 2009, Shrestha et al. 2009), which are extremely high and limited, respectively, in gypsy moth larval midguts. It is currently unknown if there are conserved functions of gut microbiota in gypsy moth, but some members may be involved in nutrient provisioning (Belda et al. 2011, Engel and Moran 2013).

Previous studies on gypsy moth (Broderick et al. 2004) and other Lepidoptera (Xiang et al. 2006, Pinto-Tomás et al. 2011, Priya et al. 2012) have indicated diet is a key factor influencing larval midgut community composition. Our results support these conclusions in general, but suggest subsequent host-induced structuring may be greater than previously thought. This may arise in part from differences in experimental design, methodologies, and analyses. Previous work on gypsy moth evaluated bacterial membership and maintained plants in the greenhouse (Broderick et al. 2004), whereas we used outdoor plants exposed to natural conditions and evaluated community structure. Furthermore, Broderick et al. (2004) used plants with a broader range of host-plant chemistries, so there may be limits to the community similarities that we observed. The relatively minor differences we observed in gypsy moth midgut communities over multiple years bear some resemblance to *Burkholderia* symbionts in various stinkbug species, in which environmental sources of bacteria are consistently obtained every generation from diet (Kikuchi et al. 2007, 2011b). Gypsy moth guts do not contain the same extent of morphological compartmentalization as these Heteroptera, so physiological features of the entire system may primarily structure the overall community, perhaps with variation in these features providing more specialized sites for bacterial colonization and proliferation.

Several other recent studies have reported differences in microbiota between laboratory and field populations, specifically in *Drosophila melanogaster* Meigan (Wong et al. 2013) and the lepidopteran *Heliconius erato* (L.) (Hammer et al. 2014). In contrast to the work with *Heliconius*, we observed small differences in larval instars attributed to the historical artifact of rearing conditions. This is possibly owing to our study focusing on the midgut tissues, as opposed to whole insects. Differences in the diversities of bacterial communities in laboratory versus wild egg masses likely correspond to different degrees of environmental exposure. Laboratory colonies of the gypsy moth are often reared under axenic conditions to minimize patho-

gens, while wild populations are exposed to potentially transient microbiota and changing weather conditions over a 10-mo period. Overall, our results support the need to incorporate ecological context, i.e., bacteria consumed through diet, into laboratory-based experiments.

Despite large differences in starting egg mass and diet-associated phyllosphere communities, the resulting communities of the gypsy moth midgut were similar to each other. In our study, we pooled samples, so variability of community composition among individuals of the same treatments was not evaluated. Investigating this potential variability, especially at the time of initial acquisition, is needed in future work. Likewise, little is known about the functions of midgut bacteria in this host. Additional studies are needed assess the potential advantageous or deleterious (Broderick et al. 2006) effects of various midgut-residing bacteria in gypsy moth. Similarly, it would be important to know if the midgut community retains this stability when gypsy moth feeds on relatively resistant host plants. Our study used highly palatable plant hosts to ensure gypsy moth consumption and development, and perhaps less preferred species within its broad host range would alter community composition. This insect could serve as a model for comparative studies of bacterial associations among foliar feeding insects that have different midgut physiological constraints. The high polyphagy of the gypsy moth makes it a uniquely well-suited system for consistent comparisons with other folivorous insect species.

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