ECOLOGY AND INFLUENCE OF BACTERIA IN GYPSY MOTH – PLANT INTERACTIONS

by

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Thesis Abstract

Microbial symbioses are ubiquitous among insects and plants, but their roles in mediating interactions between them remain poorly understood. The manners in which bacterial symbionts of many insects are acquired, or may be influenced by plant chemical defenses are central to these relationships. Gypsy moth (*Lymantria dispar* L.) is a polyphagous leaf-feeding Lepidoptera. I assessed the contributions of egg-mass and foliar sources of bacteria on larval midgut communities, using culture-independent analyses. Laboratory-reared egg masses had a very low diversity of bacteria, while field-collected populations were highly diverse. Despite differences in egg mass-associated bacteria, larval midguts converged after extended feeding. Larval bacterial communities resembled those of the foliage they consumed, but also differed in the abundances of some members. This indicates various foliar-acquired bacteria respond differently to midgut conditions. To identify roles of midgut bacteria on gypsy moth performance, I used artificial diets containing phenolic glycosides, the principal chemical defense of trembling aspen (*Populus tremuloides*). Larvae were fed bacteria from aspen leaves, field-collected fifth-instar larvae, or mock controls. Larvae inoculated with bacteria from aspen grew larger when phenolic glycosides were included in diet, but not when they were absent. Effects were post-ingestive, as consumption was unaffected. Specific bacteria in larval midguts were differentially affected: *Ralstonia* decreased and *Acinetobacter* increased, when phenolic glycosides were added. Bacterial communities demonstrated high capabilities of degrading phenolic glycosides. Among individual members, *Acinetobacter* most strongly reduced their concentrations. Aspen genotype had little effect on foliar communities, despite widely differing chemical compositions. However, midguts from gypsy moth larvae feeding on trees with different chemical concentrations were strongly influenced by the ratio of phenolic glycosides to condensed tannins in foliage. The limited effects of foliar chemistry on bacterial communities, coupled with the strong effects of these chemicals on bacteria within larval midguts following ingestion, indicate that partitioning of defense chemicals in plant cell vacuoles minimizes effects on phyllosphere residents, but, feeding ruptures these barriers, causing strong interactions between foliar compounds and bacteria within midguts. Overall, these results broaden our understanding of plant-insect-microbe relationships, and identify important interactions among these members.

Thesis Introduction

Microbial symbioses are ubiquitous among arthropods, sometimes being critical to the functioning of the host. These associations can range extensively in their fidelity, acquisition routes, consequentiality, and their functions. For example, mutualistic microbial symbioses can allow insect hosts to access recalcitrant sources of energy (Geib *et al.*, 2008; Reid *et al.*, 2011), produce essential amino acids not present in host diet (Douglas, 1996; Gündüz & Douglas, 2009), supplement sterols (Thompson *et al.*, 2013), defend against natural enemies (Oliver *et al.*, 2003; Scarborough *et al.*, 2005; Dillon *et al.*, 2005), protect mutualistic associates from pathogens (Currie *et al.*, 1999; Scott *et al.*, 2008), modulate host plant defenses (Kaiser *et al.*, 2010; Chung *et al.*, 2013), and ameliorate effects of deleterious allelochemicals (Dowd & Shen, 1990; Kikuchi *et al.*, 2012). It has been suggested that these mutualistic symbioses can expand a host's feeding breadth beyond its own capabilities (Joy, 2013) . In recent years, advent of new sequencing technologies has greatly enhanced explorations into the ecology and evolution of insect – microbe symbioses. In particular, our understanding of mechanisms by which bacteria influence their hosts has been greatly improved. In most insect herbivores, however, the influence of bacteria on the functioning of the host, and how they interact with plant defenses, are poorly understood.

Bacterial symbioses of herbivores associate with various tissues of the insect. They may be restricted to specialized cells in which they conduct distinct mutualistic functions (Baumann, 2005), colonize the entirety of the midgut, or specific locations of the gut tract (Kaltenpoth *et al.*, 2009). Likewise, these relationships may derive from evolutionary feedbacks between a single bacterium with its insect host, or be highly plastic and multipartite, where different bacteria may be functionally substitutable or redundant. The full continuum of these relationships can be observed in insect gut symbioses.

Gut bacteria vary considerably in their acquisition routes and ecologies. Bacteria may be acquired from nestmates (Minkley *et al.*, 2006), maternal deposition (Kaltenpoth *et al.*, 2009; Hosokawa *et al.*, 2012), the environment (Kikuchi *et al.*, 2007; Dematheis *et al.*, 2012), or by some combination of the three. Routes of symbiont transmission may all be critical to the functioning of the host. For example, the relationship between some Heteroptera and environmentally acquired *Bulkholdaria* symbionts appears to be highly conserved across the group (Kikuchi *et al.*, 2011; Garcia *et al.*, 2014). These environmentally based relationships have also been shown to be integral to host (Kikuchi *et al.*, 2012). Some of the most well-described relationships of host - gut bacterial interactions have been documented in Heteroptera and Hymenoptera, but our understanding of these relationships in other insect orders is lacking in comparison.

In Lepidoptera, acquisition routes and ecologies of microbes are not well understood. It has been shown that diet influences composition of the gut consortia (Broderick *et al.*, 2004; Priya *et al.*, 2012). However, whether these changes in microbial membership are due to diet-acquired microbes or effects on maternally sourced microbiota are unclear. The functions of gut bacteria in Lepidoptera remain unclear. Putative nitrogen fixers are present in Lepidoptera (Broderick *et al.*, 2004), but larvae also circumvent nitrogen deprivation through compensatory feeding (Stockhoff, 1993). It is known that these microbes actively colonize and persist in gut tissues (Shao *et al.*, 2014), but unknown whether they are mutualistic, commensals, or latent pathogens.

I investigated the roles of bacteria in interactions between the gypsy moth (*Lymantria dispar* L.) and its host plants. Specifically, I asked: *How do bacteria enable gypsy moth to contend with plant chemical defenses?* Gypsy moth is an ideal system because some information regarding its microbial associates was available (Broderick *et al.*, 2004), but the ecologies of these gut bacteria have not been explored. I focused on trembling aspen (*Populus tremuloides* Michx.) and hybrid poplar (*P. nigra* x *P. nigra*) as models. These were ideal counterparts to the gypsy moth due to the amount of information known about their ecological and biochemical interactions (Lindroth & Hemming, 1990; Hemming & Lindroth, 2000; Lindroth & St. Clair, 2013). There were two major gaps in the literature at the start of this research, which have since received increasing attention. The first was contribution of maternal and environmental sources of bacteria to the guts of Lepidotpera. The second was how do bacteria and plant defense interact, and what are the consequences to either plant or insect host.

My initial hypothesis this was that bacterial associates of gypsy moth enabled this herbivore to better utilize its plant host, and that associates of the plant benefited the plant. As experimental results progressed, the lines demarking these two separate concepts became blurred. It became increasingly clear that compartmentalizing the bacteria of these systems as plant-associated or insect-associated was not useful, and it became more informative that bacteria were its own entity would adapt to their present environmental conditions.

This thesis contains four chapters, which investigates the role of bacteria in gypsy moth – plant interactions. All chapters have been published in, submitted to, or prepared for peer-reviewed publication, and as such some redundancies may be evident.

In Chapter One, I evaluate the contribution of diet- and egg-associated on gypsy moth midgut bacterial communities. I conducted two experiments using laboratory and field-collected egg masses. In the first experiment, I used a single host plant as a diet source, looking at changes in the midgut bacterial community over the development of gypsy moth. Using the information gained from the first experiment, I conducted a second using multiple host plants.

Chapter Two investigates the influence of aspen – associated bacteria on gypsy moth's interaction with the aspen plant defenses, phenolic glycosides. I conducted bioassays, evaluated midgut bacterial community composition, cultured bacteria, and conducted *in vitro* metabolism assays with phenolic glycosides.

Chapter Three evaluates the effects of host plant defense on foliar bacterial communities. I surveyed the bacterial communities of leaves from several aspen genotypes grown in a common garden that vary in their plant defense characteristics.

Chapter 4 investigates the influence of host plant defense phenotype on the bacterial community composition of the gypsy moth midgut. I assessed bacteria associated with foliage of three aspen genotypes, and midguts of insects feeding upon them. Two classes of aspen defense compounds, phenolic glycosides and condensed tannins, were quantified and related to respective bacterial communities.

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Chapter 1

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Acquisition and structuring of midgut bacterial communities in gypsy moth larvae (*Lymantria dispar* L.).

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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 very few OTUs. 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 difference between foliar communities and midgut communities of larvae that ingested them were due 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.

Introduction

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. 2009a, 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. 2009a, 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 due 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). Additionally, 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 direct 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, are 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. While several studies have noted that bacterial communities differ due to diet (Broderick et al. 2004, Geib et al. 2009b, 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, that 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 mid-summer on a variety of surfaces, depositing scales from its abdomen during the process, where they overwinter until spring. Upon 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 due to new community members from diet or changes in bacteria acquired from initial feeding upon 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 masses 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.

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 much many individuals were combined are included in Supplemental Table 1._Egg masses were obtained from two sources: a laboratory-maintained colony (lab) (USDA APHIS) and a field population from the bark of oak trees near Temperance, MI. Dormant two-year-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 two days. 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 approximately 40 leaves. Insects were hatched from non-sterilized egg masses and reared in the same growth chamber at 24°C 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 1st instar (L1) larvae were collected after eclosion. Larvae were reared en masse until 2nd instar, after which they were reared individually in sterile 24-well plates until molting. At 3rd instar (L3), insects were transferred to 12-well plates, and at 4th instar they were transferred to 5.0 cm diameter petri dishes until 5th 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 minutes, 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 approximately 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 500x*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 50mM Tris-HCl (pH 7.0), 1% Triton X-100, and 2mM β-mercaptoethanol. The homogenate was centrifuged at 500 x *g* for 5 min. Supernatant was transferred and centrifuged at 5,500 x *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 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), 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: 95ºC for 2 min, 30 cycles of 95ºC for 20 s, 48ºC for 30 s, 72ºC for 30 s, and a final elongation of 72ºC 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 (MIDs). 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 sec, 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 MIDs and primers, no homopolymer exceeding six nucleotides, and a minimum length of 200 nucleotides. Sequences were aligned to the SILVA SEED database, and 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.

Operational taxonomic unit (OTU) analyses were conducted by clustering at 3% dissimilarity. Since the number of spurious OTUs increases with sampling depth, units were subsampled to least number of sequences (1400). 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 non-metric 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. In order 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 (UPGMA) 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, a total of 162,661 sequences remained. After clustering at 3% dissimilarity, we randomly subsampled to 1,400 sequences per sample.

There were a total of 791 operational taxonomic units (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 standard error) OTUs, and ranged from 30-110. 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 suggested that many OTUs occurred in low abundances. In these wild egg mass samples, 83-89% of OTUs contributed less than 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. Upon 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 due 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 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. UPGMA clustering yielded similar results to ordination-based analyses (Fig. 4A). 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 to the foliage and midgut samples (Fig. 4B). 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 30-56% 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 due 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 was due 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 were 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 (Fig. 1, 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 had extremely low diversity produced 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 (Fig. 1A, Fig. 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 non-hosts 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. Also, 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 bears 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* (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 due 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 pathogens, while wild populations are exposed to potentially transient microbiota and changing weather conditions over a 10-month 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 very similar to each other. In our study, we pooled samples, so variability of community composition among individuals of the same treatments were 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 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 utilized 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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Table 1: Number of observed OTUs at 3%-cutoff and computed diversity metrics. Samples were randomly standardized to contain 1,400 sequences.

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

Figure legends:

Figure 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.'

Figure 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.

Figure 3: UPGMA 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.

Figure 1:

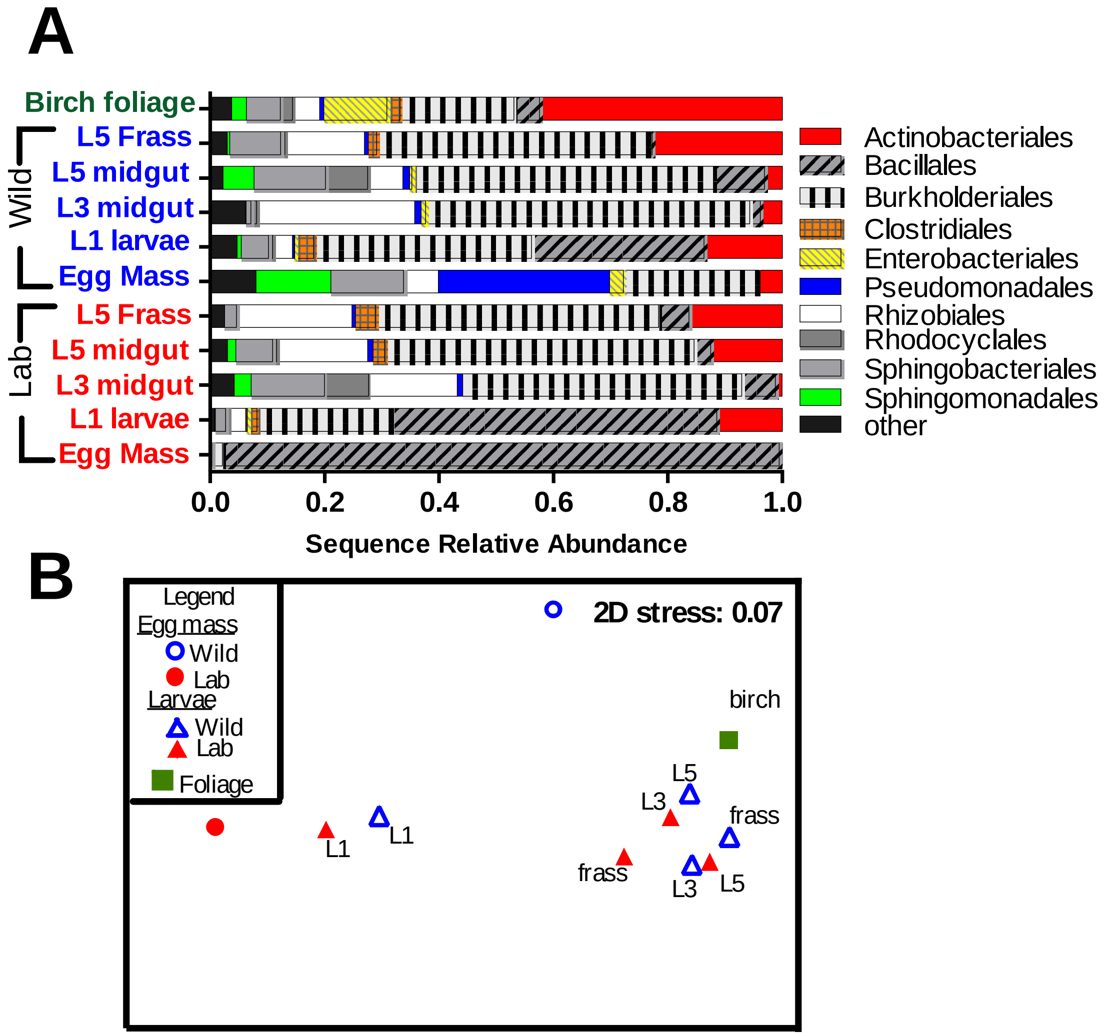

A
Birch foliage
L5 Frass
L5 midgut
L3 midgut
L1 larvae
Egg Mass
Wild
L5 Frass
L5 midgut
L3 midgut
L1 larvae
Egg Mass
Lab
0.0
0.2
0.4
0.6
0.8
1.0
Sequence Relative Abundance
Actinobacteriales
Bacillales
Burkholderiales
Clostridiales
Enterobacteriales
Pseudomonadales
Rhizobiales
Rhodocyclales
Sphingobacteriales
Sphingomonadales
other
B
Legend
Egg mass
Wild
Lab
Larvae
Wild
Lab
Foliage
2D stress: 0.07
birch
L5
L3
frass
L1
L1
frass
L3
L5

Figure 2:

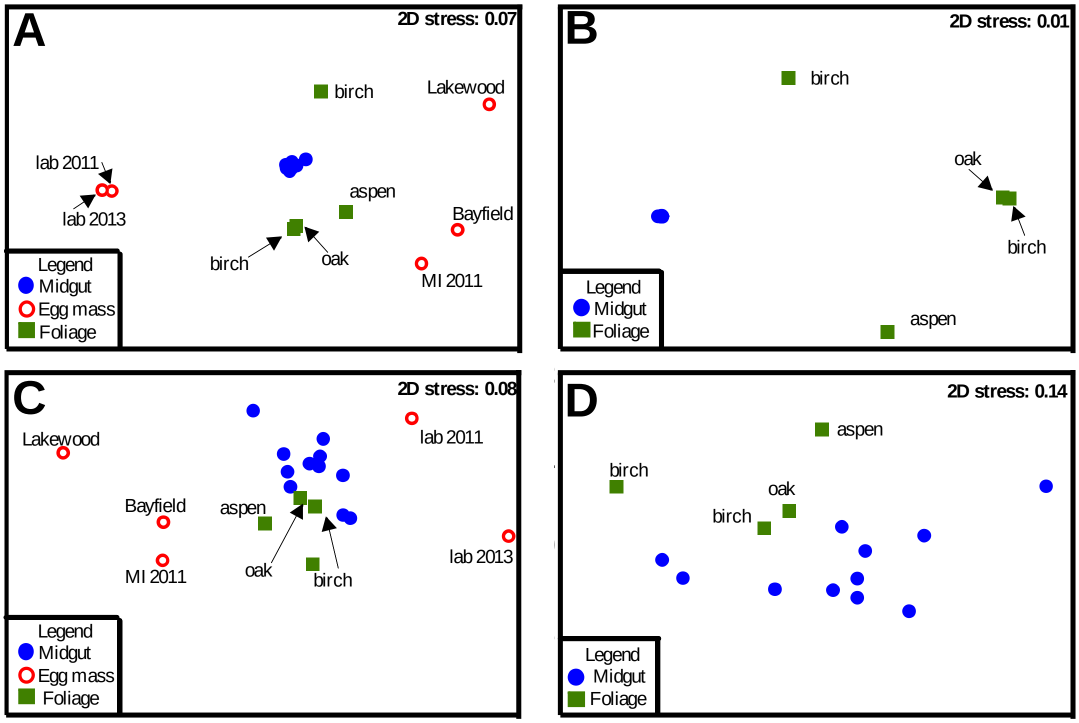
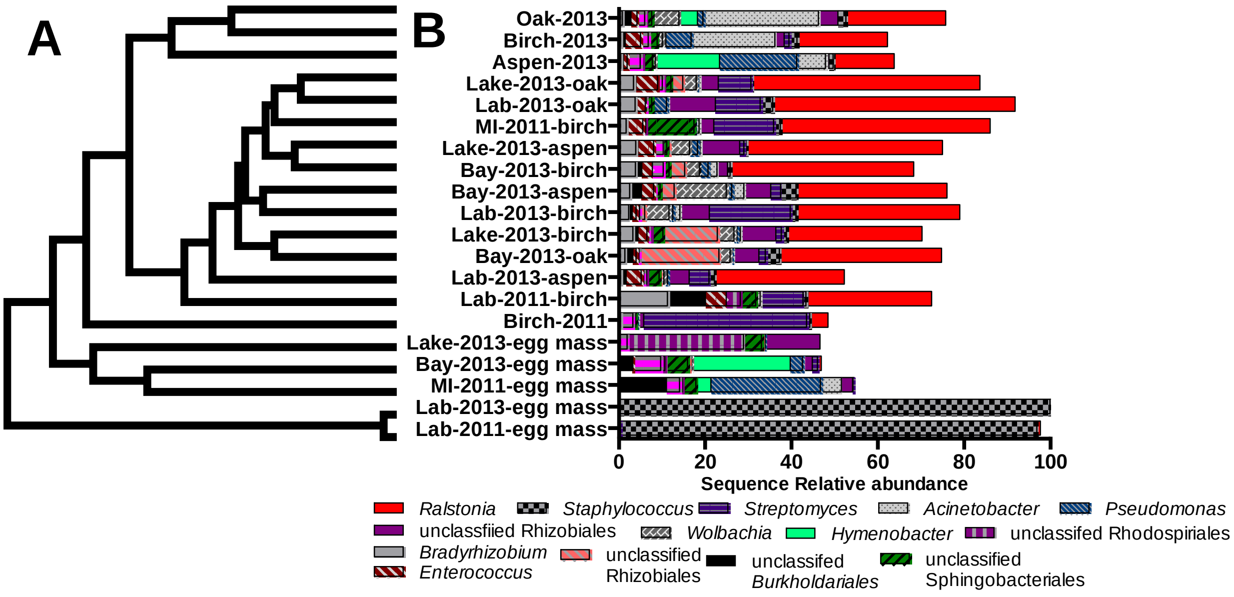

A
2D stress: 0.07
birch
Lakewood
lab 2011
lab 2013
aspen
Bayfield
birch
oak
MI 2011
Legend
Midgut
Egg mass
Foliage
B
2D stress: 0.01
birch
oak
birch
aspen
Legend
Midgut
Foliage
C
2D stress: 0.08
Lakewood
lab 2011
Bayfield
aspen
oak
birch
MI 2011
lab 2013
Legend
Midgut
Egg mass
Foliage
D
2D stress: 0.14
aspen
birch
oak
birch
Legend
Midgut
Foliage

Figure 3:


A
B
Oak-2013
Birch-2013
Aspen-2013
Lake-2013-oak
Lab-2013-oak
MI-2011-birch
Lake-2013-aspen
Bay-2013-birch
Bay-2013-aspen
Lab-2013-birch
Lake-2013-birch
Bay-2013-oak
Lab-2013-aspen
Lab-2011-birch
Birch-2011
Lake-2013-egg mass
Bay-2013-egg mass
MI-2011-egg mass
Lab-2013-egg mass
Lab-2011-egg mass
0
20
40
60
80
100
Sequence Relative abundance
Ralstonia
Staphylococcus
Streptomyces
Acinetobacter
Pseudomonas
unclassfiied Rhizobiales
Wolbachia
Hymenobacter
unclassifed Rhodospiriales
Bradyrhizobium
unclassified Rhizobiales
unclassifed *Burkholdariales*
unclassified Sphingobacteriales
Enterococcus

Supplemental Table 1: Pooling of lifestages in experiments.

Sample:	Pooled?	Number Pooled
egg mass	no	n/a
L1 larvae	yes	10
L3 larvae	yes	10
L5 larvae	yes	10
L5 frass	yes	10
foliage	yes	portions of 20-30 leaves from 2-3 trees of same species

Chapter 2

Published as:

Plant-associated bacteria degrade defense chemicals and reduce their adverse effects on an insect defoliator.

in Oecologia

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Abstract:

Phytophagous insects must contend with numerous secondary defense compounds that can adversely affect their growth and development. The gypsy moth (*Lymantria dispar*) is a polyphagous herbivore that encounters an extensive host and chemical range. We used this folivore and a primary component of aspen chemical defenses, phenolic glycosides, to investigate if bacteria detoxify phytochemicals and benefit larvae. We conducted insect bioassays using bacteria enriched from environmental samples, analyses of microbial community in midguts of bioassay larvae, and *in vitro* phenolic glycoside metabolism assays. Inoculation with bacteria enhanced larval growth in the presence, but not absence, of phenolic glycosides in artificial diet. This effect of bacteria on growth was observed only in larvae administered bacteria from aspen foliage. The resulting midgut community composition varied among the bacterial treatments. When phenolic glycosides were included in diet, the composition of midguts in larvae fed aspen bacteria was significantly altered. Phenolic glycosides increased population responses by bacteria that we show metabolize these compounds in liquid growth cultures. Several aspects of these results suggest that vectoring or pairwise symbiosis models are inadequate for understanding microbial mediation of plant-herbivore interactions in some systems. First, bacteria that most benefitted larvae were initially foliar residents, suggesting that toxin-degrading adaptions of phyllosphere inhabitants indirectly benefit herbivores upon ingestion. Second, assays with single bacteria did not confer the benefits to larvae obtained with consortia, suggesting multi- and inter-microbial interactions are also involved. Our results show that bacteria mediate insect interactions with plant defenses, but are community specific and highly complex.

Introduction:

Insect herbivores must contend with a wide variety of plant-derived toxins, which have broad ranges of activities, are highly variable in space and time, and exhibit a high diversity of molecular structures and physical properties. Further, these barriers to utilizing plants may intensify upon initial feeding, when induced responses can greatly increase concentrations of constitutive compounds, alter their relative abundances, and biosynthesize new compounds not previously present (Kessler and Baldwin 2002). Insects can partially contend with toxins through behavioral, physiological, and biochemical mechanisms, including detoxification enzymes such as esterases, glutathione-s-transferases, and cytochrome P450 monoxidases (Li *et al.* 2007). However, detoxification systems can be overcome by high toxin concentrations, and they incur physiological costs (Carriere *et al.* 1994).

Our understanding of how microbial associates can mediate plant-insect interactions is rapidly improving. Plant-associated microbes may directly (Clay 1988) and indirectly (Pineda *et al.* 2010) influence herbivores. Insect herbivores are associated with microbes that can supply essential nutrients (Douglas 1996), contribute to expansion of diet breadth (Chu *et al.* 2013), modulate plant responses to attack (Kaiser *et al.* 2010; Chung *et al.* 2013), and defend against natural enemies (Kellner 2002; Oliver *et al.* 2003). Insect-associated microorganisms may also suppress opportunistic pathogens (Cardoza et al. 2006; Koch and Schmid-Hempel 2012) and produce volatiles that are exploited as cues by predators (Boone *et al.* 2008). These relationships can range from being closed, obligate, and pairwise to open and extensively multipartite.

Bacteria-insect associations are considered to be ubiquitous, yet in most cases our understanding of how they function or are maintained is poorly understood. While environmental sources of microorganisms have been used extensively in chemical remediation efforts, it has now been shown that insect-associated microbes can metabolize insecticides (Kikuchi *et al.* 2012), heavy metals (Senderovich and Halpern 2013), and plant defense chemicals (De Fine Licht et al. 2013; Boone et al. 2013; Hammerbacher et al. 2013). The source of bacteria can vary extensively from being environmentally acquired (Kikuchi *et al.* 2012) and relatively indiscriminate from host plant communities (Adams *et al.* 2013), to being predominantly egg-associated (Senderovich and Halpern 2013). However, the extent to which microbial metabolism of plant defenses influences insect performance, or the composition of associated microbes and their feedbacks, is unknown.

Gypsy moth (*Lymantria dispar*) is an insect folivore distinguished by its broad host range and intermittent landscape scale population outbreaks that cause considerable tree mortality and alter forest composition. This insect must contend with both low nutrient content of foliage and a large variety of host-plant chemicals that vary extensively within and among hosts. Gypsy moth larvae use several mechanisms to metabolize or inactivate secondary products, such as its highly alkaline midgut (Govenor *et al.* 1997) and detoxification enzymes (Lindroth and Hemming 1990; Janković-Hladni *et al*. 1997). These larvae also contain a midgut bacterial community that is influenced by ingested, foliar bacteria (Broderick et al. 2004). Specifically, most bacterial members of gypsy moth midgut communities are obtained primarily from foliage in their diet, but the structure of this community is subsequently determined largely by the host insect's internal physiology (Mason & Raffa *unpublished data*). The roles of these bacteria in mediating gypsy moth–plant interactions are unknown.

Quaking aspen (*Populus tremuloides*) is among gypsy moth's preferred hosts and is defended from these herbivores through production of several secondary chemicals, of which phenolic glycosides are critically important. Increasing concentrations of phenolic glycosides decrease gypsy moth growth and development (Hemming and Lindroth 1995). Concentrations of these compounds vary (2% to 25 % dry weight) with tree genotype, phenology, demography, and environmental conditions (Hemming and Lindroth 1995; Donaldson *et al*. 2006). While the exact mechanisms of phenolic glycoside detoxification are still unclear (Boeckler *et al.* 2011), there is substantial evidence that metabolism by esterases is involved (Lindroth 1989; Hemming and Lindroth 2000), as larval growth and survival increases with the level of enzyme activity (Lindroth and Weisbrod 1991). However, high phenolic glycoside concentrations overwhelm these enzyme systems resulting in reduced growth accompanied by increased mortality and pathogen susceptibility (Hwang *et al.* 1995). Since esterases are prevalent amongst bacteria, we postulated that gypsy moth might benefit from microorganisms that mediate interactions with phenolic glycosides.

We evaluated whether bacteria associated with the gypsy moth-aspen system facilitate the ability of larvae to contend with host plant defense chemicals under controlled, laboratory conditions using a four-pronged approach: 1.) Does bacterial inoculation of laboratory populations of gypsy moth improve larval performance in artificial diet, and in a manner that relates to concentrations of phenolic glycosides? 2.) Do phenolic glycosides incorporated into artificial diets in concentrations mimicking foliar quantities affect larval midgut bacterial communities? 3.) Can pure culture isolates of bacteria reduce concentrations of phenolic glycosides incorporated into bacterial growth medium? 4.) Can individual bacterial isolates provide the same results as complete communities when larvae are fed artificial diet amended with phenolic glycosides?

Methods:

Question 1: Do bacteria influence gypsy moth performance, and if so, are improvements related to overcoming negative effects of phenolic glycosides?

Experimental overview: We conducted bioassays that included three bacterial treatments and three phenolic glycoside treatments for a total of nine unique treatment combinations. Larvae were randomly selected from non-sterilized egg masses, reared on artificial diet, and randomly administered one phenolic glycoside by bacteria treatment. Individual larva served as our unit of replication and had a minimum of 11 larvae per treatment combination.

Rearing conditions: Gypsy moth egg masses were obtained from a USDA APHIS rearing facility (Otis Air National Guard Base, MA). Larvae were maintained on artificial diet (MP Biomedical, Solon, OH) in a growth chamber at 25°C with 50-70% relative humidity using previously described methods (Broderick *et al.* 2004). Larvae were reared en mass in 25-cm plastic petri dishes until 3rd instar (L3), at which time they received bacterial inoculations.

Bacterial preparation and larval inoculations: Previous work demonstrates that the consortia within gypsy moth are both acquired from foliage and structured by the insects' internal environment (Broderick *et al.* 2004, Mason & Raffa *unpublished data*), so we used both leaves and midguts as sources of bacteria. We enriched two environmental sources of bacteria: aspen leaves from trees maintained for several years in cold frames, and midguts from a wild population in Wisconsin, where gypsy moths are currently abundant in oak-dominated stands. Samples were homogenized by pulverizing with steel beads. 100 µL of homogenate was inoculated into 5 mL of dilute tryptic soy broth (5 g/L) amended with 100 mg cyclohexamide to retard fungal growth. Samples were incubated with shaking at 25°C for 18h, concentrated by centrifugation, and washed with phosphate buffered saline (PBS). Gypsy moths were inoculated by feeding with 10^6 cells as quantified by a hemocytometer. Newly molted larvae were provided a standard diet disk (1mm X 3 mm diameter) with either of the bacterial enrichments or PBS control in 24-well culture plates. Only larvae that consumed the entire diet disk were used in the bioassay. Insects were then starved for 24h.

Amendments of diet with phenolic glycosides: Crude extracts of phenolic glycosides were obtained from quaking aspen foliage as described in Lindroth et al. (1986). Extracts had a purity of approximately 90%, and contained predominately tremulacin and salicortin. The artificial diet was amended with 3 or 6% (w/v) crude extract phenolic glycosides dissolved in water, or with an equal volume of water added. The L3 larvae were weighed after starvation and maintained on a randomly assigned diet treatment in individual 30 mL plastic cups until molting into the 4th stadium (L4). All insects molted by the ninth day of the stadium (see results). L4 larvae were anesthetized, and midguts were aseptically dissected and frozen at -80°C. Larval carcass and the remaining diet were dried to a constant mass to quantify growth and consumption.

Statistical analyses: Larval growth and consumption were analyzed using two-way cross-factorial analysis of covariance (ANCOVA) in R v. 3.0.1 (R Core Development Team 2012). Phenolic glycoside concentration and bacterial source were fixed effects, and larval initial dry weight was used as a covariate. Initial dry weights of diet and larvae were determined from a ratio-based relationship with fresh weight calculated from ten separate individuals at the start of the feeding trial ($R^2 > 0.91$). Pairwise comparisons were conducted using the R package ‘agricolae’ using an LSD test.

Question 2: Is the bacterial community composition of gypsy moth midguts influenced by phenolic glycosides?

Experimental overview: We conducted culture-independent analysis of bacterial community composition using 454 16S-rRNA gene pyrosequencing. Three larvae from each treatment combination from Question 1 were randomly selected for the analysis.

DNA extraction and 16S-pyrosequencing analyses: We conducted 16S-rRNA gene pyrosequencing to assess how larval midgut bacterial communities respond to phenolic glycoside treatments. Three samples per bacterial-phenolic glycoside treatment were randomly selected for a total of 27 samples. DNA was extracted from midguts using a CTAB protocol with an addition of a phenol-chloroform cleanup step. Two-step 16S PCR amplification was conducted using the protocols and primers 799F-mod6 and 1392R described in Hanshew *et al.* (2013). Pyrosequencing was conducted on a 454 GS Junior (Roche, Indianapolis, IN).

Data processing and analysis: All 454 pyrosequencing data were processed in mothur v. 1.29.2 (Schloss *et al.* 2009). Data were denoised via PyroNoise and sequences were processed so as to allow for no differences in multiplex identifiers and primers, a maximum homopolymer exceeding six nucleotides and a minimum length of 200 nt. Sequences were aligned using the SILVA SEED database, and chimeras were detected using UCHIME and removed. Sequences were classified with an 80% confidence threshold and those classified as chloroplast, eukaryotic, or unknown at the Kingdom taxonomic level were removed from further analyses. Operational taxonomic unit (OTU) analyses were conducted by clustering at 3% dissimilarity. All samples were randomly subsampled to the unit with the fewest number of OTUs. Bray-Curtis dissimilarities were computed, and non-metric multidimensional scaling (MDS) was conducted in Primer-E (v. 6.0). Statistical analyses of diversity metrics and individual OTUs were conducted in R. Diversity metrics were analyzed using an ANOVA with phenolic glycosides treated as a factor, while individual OTUs were analyzed using phenolic glycosides as a continuous variable. Pairwise comparisons were conducted using a LSD test with the R package 'agricolae.'

Sequences from this experiment were deposited in NCBI Sequence Read Archive under the project accession SRP036073.

Question 3 Do bacteria associated with gypsy moth reduce concentrations of phenolic glycosides in vitro*?*

Experimental overview: Pure culture isolates of *Acinetobacter* and *Ralstonia* (see results) were obtained from gypsy moth guts. Phenolic glycosides were inoculated in liquid medium after 24h of bacterial cell growth and analyzed after 24h post-inoculation. There were three replicates of each control and bacterial treatment.

Isolation and culturing of isolates: We conducted a targeted culturing approach to obtain representative bacterial cultures of the two OTUs having the largest shifts in abundance in our previous experiments. Bacteria were isolated from midguts of gypsy moth larvae feeding on aspen foliage, using casamino acid-peptone-glucose (CPG) medium (Denny and Hayward 2001) and yeast-glucose medium (Kikuchi *et al.* 2011). Briefly, the larval midgut was sterilely removed from larvae as previously described, homogenized in PBS, and applied to the solid media with standard plate-spreading technique. Cultures were streaked for isolation, and subcultured at least twice, or until there was a single morphology. Cultures were then maintained on CPG medium. Between the two media, approximately twenty bacterial cultures with unique morphologies were isolated. Bacteria were identified by amplification and sequencing of the16S-rRNA gene and confirmed using NCBI blastN. We used representatives of the genera of *Ralstonia* and

Acinetobacter in subsequent experiments as they exhibited the most dramatic population shifts in our 16S-rRNA pyrotag experiments (see results). Sequences of the isolates used in these experiments were deposited to the NCBI Genbank sequence database under the ascension numbers KJ364529 and KJ364530.

***In vitro* metabolism assay:** Experiments evaluating bacterial metabolism of phenolic glycosides were conducted with an effort to minimize background levels of degradation. Specifically, we minimized the duration in which phenolic glycosides were in water and avoided heat sterilization. Treatments were conducted in triplicate and included untreated controls at 0 and 24h post- phenolic glycoside inoculation, *Ralstonia sp.* A3-1, *Acinetobacter sp.* R7-1, and heat-killed *Acinetobacter* cells. In 10 mL glass culture tubes, 10^6 colony forming units (CFUs) were added to 350 μL of liquid CPG medium (pH 7.2) for a total volume of 400 μL. After 18h, phenolic glycosides were added to control and bacteria-inoculated tubes. The crude extract described earlier was suspended in water and sterilized using a 1.0 μm PES syringe filter. Phenolic glycosides concentrations were approximately 1.5% w/v after addition. Cultures were incubated at 25°C with shaking (180 RPM) for 24h. After 24h, samples were diluted and centrifuged at 16,000 x *g* for 5 min. and supernatants were collected. Deuterated salicylic acid was used as an internal standard and was added to the sample supernatant for a final concentration of 0.1 mg/mL. Samples were passed through a 0.45 μm PTFE filter prior to liquid chromatography analysis.

Analysis of phenolic glycosides: Samples were analyzed by ultra-high performance liquid chromatography with negative electrospray ionization single quadrupole mass spectrometry using a Waters integrated Acquity I-Class UPLC (Milford, MA). Samples were injected (2 μL) onto a Waters Acquity CSH C-18 (2.1 x 100 mm, 1.7 μm) column and separated at 40°C on a gradient of water (solvent A) and acetonitrile (solvent B), both acidified with 0.1% formic acid, at a flow rate of 0.5 mL/min. Gradient conditions were: 1-15% B over 0-4 min, 15-25% B (4-12 min), 25% B (12-16 min), 25-31% B (16-20 min), 31-62% B (20-23 min), 62-99% B (23-24 min), 99% B (24-25 min). The mass spectrometer was operated under negative ionization mode (200 – 700 m/z) with a cone potential of 30 V. Commercially available salicin and laboratory-purified compounds of tremulacin, tremuloidin, and salicortin were suspended in methanol and used as analytical standards. Content of phenolic glycosides in samples were determined from calculated standard curves fit by quadratic models ($R^2 > 0.98$).

We assessed further putative breakdown products (described in Boeckler *et al.* 2011) on a subset of our samples. Samples were run on UPLC-MS as previously described under negative ionization mode (100 – 700 m/z). Products were identified and quantified by using masses that were deprotonated with a formate adduct (Keefover-Ring, *personal communication*). Among the products we looked for were the HCH moiety (200.03 m/z), 6-HCH (157.05 m/z), and benzoic acid (167.12 m/z).

Statistical analyses: Contents of individual phenolic glycosides were analyzed using an ANOVA with bacterial treatment treated as a fixed effect. Pairwise comparisons were conducted using the package ‘agricolae’ in R with a Bonferroni *P*-value adjustment.

Question 4: Are single species of phenolic glycoside-degrading bacteria sufficient to protect gypsy moth larvae?

Bioassay and analysis: We conducted a larval bioassay under identical conditions and experimental design as in Question 1. Newly molted L3 larvae were either inoculated with PBS or pure cultures consisting of 10^6 CFUs of *Ralstonia* or *Acinetobacter*. Larvae were fed commercial diet alone or amended with 4% w/v crude extract phenolic glycosides and reared to

L4. Midguts were removed and larval dry weights were assessed. Data were analyzed as previously described using an ANCOVA with initial weight being a covariate and pairwise comparisons were completed using an LSD test.

Results:

Bacteria improve gypsy moth performance in the presence, but not absence, of phenolic glycosides.

Phenolic glycosides ($F_{2,123} = 118.44$, $P < 0.001$), bacterial inoculation ($F_{2,123} = 17.672$, $P < 0.001$), and their interaction ($F_{4,123} = 3.541$, $P = 0.009$) influenced gypsy moth larval growth. Larvae grew well regardless of bacterial treatment when administered diets that did not contain phenolic glycosides (Fig. 1A). Phenolic glycosides at 3% and 6% w/v reduced growth relative to the control by 46% and 65%, respectively. Bacterial communities enriched from aspen leaf sources enhanced gypsy moth growth under phenolic glycoside treatments ($P < 0.05$). Larvae inoculated with bacteria from aspen foliage were 77% larger than the non-inoculated larvae at the moderate phenolic glycoside concentration, and 64% larger at the high concentration. These effects on growth were not attributable to initial larval mass ($F_{1,123} = 0.245$, $P = 0.6215$).

Larval consumption of diet (Fig. 1B) was decreased by phenolic glycoside concentration ($F_{2,123} = 92.143$, $P < 0.001$). Neither bacterial inoculation ($F_{2,123} = 1.421$, $P = 0.2455$), nor its interaction with phenolic glycosides ($F_{4,123} = 2.043$, $P = 0.0924$) affected feeding. Stadium duration ranged from 6-9 days (Supplemental Fig. 1). Development time increased under phenolic glycoside inclusion ($F_{2,123} = 6.559$, $P = 0.002$), but was unaffected by inclusion of bacteria ($F_{2,123} = 0.389$, $P = 0.679$), and the bacterial interaction with phenolic glycosides ($F_{2,123} = 0.786$, $P = 0.537$).

Ingestion of phenolic glycosides alters the composition of midgut bacterial communities.

After removal of low-quality and contaminating sequences, we had 97,073 high-quality sequences that we rarified to 1600 sequences per sample. We detected 278 total OTUs at 97% similarity with sample richness ranging from 2-64. Larvae inoculated with the environmental samples of bacterial had an increased number of OTUs ($F_{2,18} = 38.741$, $P < 0.001$), Chao1 richness estimates ($F_{2,18} = 25.8$, $P < 0.001$), Simpson diversity, ($F_{2,18} = 10.226$, $P = 0.001$) and Shannon diversity ($F_{2,18} = 163.57$, $P < 0.001$) compared to the control (Table 1). Phenolic glycosides had marginally significant effects on number of OTUs ($F_{2,18} = 3.527$, $P = 0.051$) and Chao1 estimates Chao1 estimates ($F_{2,18} = 3.152$, $P = 0.067$), a significant effect on Shannon diversity ($F_{2,18} = 14.697$, $P < 0.001$), and had no effect on Simpson diversity ($F_{2,18} = 3.067$, $P = 0.714$). An interaction between phenolic glycosides and bacteria was only present in Shannon diversity estimates ($F_{2,18} = 10.081$, $P < 0.001$) Chao1 and Good's coverage estimates indicated that we approached sampling saturation for our samples.

At the completion of our bioassay, control larvae contained very few taxa (Fig. 2A), as opposed to the larvae receiving inoculations of environmental bacterial enrichments. Control larvae were dominated (97%) by one OTU in the order Lactobacilliales. Larvae receiving inoculations from the gypsy moth midgut bacteria had similar community compositions to what we have previously found to naturally inhabit gypsy moth (Mason & Raffa, *unpublished data*). In both the controls and the midgut inoculations, there were only minor order-level differences attributed to phenolic content (Fig. 2A), and there was no difference in clustering patterns in our ordination analysis (Fig. 2C).

In contrast to the other samples, the midgut communities of larvae inoculated with bacteria from aspen foliage underwent changes in response to the presence of phenolic glycosides. Amending larval diet with phenolic glycosides resulted in a major decrease in the abundance of *Ralstonia* ($F_{1,7} = 19.39$, $P = 0.003$) (80% to 4%), and corresponding increases in several other bacterial orders (Fig. 2A). The most dramatic response to phenolic glycosides was in *Acinetobacter*, which rose from < 0.1% to 31% ($F_{1,7} = 7.13$, $P = 0.032$) (Fig. 2B). On the whole, ordination analyses indicated the midgut community structure of the aspen bacterial inoculations were more variable than the other samples in response to phenolic glycosides (Fig. 2C).

Bacteria reduce concentrations of phenolic glycosides in vitro; Variation in between-species capabilities is consistent with population shifts following larval ingestion of phenolic glycosides.

In the previous experiments, we found that inoculation with bacteria from aspen reduced the negative effects of phenolic glycosides on gypsy moth growth, and that the ingestion of phenolic glycoside shifted the abundances of *Ralstonia* and *Acinetobacter*. Therefore, we focused on these two genera in our metabolism experiments. We obtained pure-culture representatives from gypsy moth midguts fed aspen leaves, and evaluated their abilities to reduce phenolic glycoside concentrations *in vitro*.

Salicortin and tremulacin comprised the majority of the phenolic glycosides used in our extracts. We observed large differences in phenolic glycoside contents with *Acinetobacter* compared to the controls. *Acinetobacter sp.* R7-1 elicited significant reductions of salicortin (70%) and tremulacin (88%). It also caused an 8.7 fold increase in salicin content, although the absolute change was relatively minor compared to the reduction of the other compounds. There were no effects of bacteria on the content of tremuloidin in the samples ($F_{4,10} = 2.843$, $P = 0.082$) (Fig. 3D). *Ralstonia sp.* A3-1 and heat-killed *Acinetobacter sp.* R7-1 cells did not affect the contents of salicortin, tremuloidin, or salicin compared to the control at inoculation, and the reductions seen in tremulacin (32% and 26%, respectively) were not significantly different from the control after 24h. The controls at inoculation and 24h post-inoculation had similar levels of salicortin, slightly reduced (11%) concentrations of tremulacin, and there were no differences in amounts of immediate degradation products of tremuloidin or salicin.

The decrease in phenolic glycoside content was not accompanied by corresponding increases in degradation products, such as benzoyl or HCH-derived groups. Therefore, it is likely that the *Acinetobacter* cultures are further degrading the compounds beyond simple enzymatic cleavage of phenolic glycosides' glycosidic and ester linkage.

Addition of individual isolates cannot emulate full-community benefits to gypsy moth

We inoculated gypsy moth larvae with the pure cultures of bacteria we used in the *in vitro* metabolism assay. As before, inclusion of phenolic glycosides reduced larval growth (Supplemental Fig. 2; $F_{1,43} = 26.496$, $P < 0.001$). However, inoculation of single cultures of bacteria ($F_{2,43} = 0.372$, $P = 0.692$), and their interaction with phenolic glycosides ($F_{2,43} = 0.120$, $P = 0.888$) exerted no effects on the growth of the gypsy moth larvae.

Discussion

Our results demonstrate that bacteria associated with gypsy moth can reduce concentrations of phenolic glycosides that inhibit gypsy moth growth (Fig. 3). This supports research in other systems that have shown microorganisms metabolize plant terpene (Boone *et al.* 2013) and stilbene defenses (Hammerbacher *et al.* 2013). Our results further demonstrate that these bacteria assist larvae in contending with plant chemical defenses, as the bacterial inoculations improved insect performance (Fig. 1). In recent studies, investigators have shown that bacteria can suppress the induction of plants defenses to the insect's benefit (Chung *et al.* 2013), reduce concentrations of insecticidal defense chemicals *in vitro* (Boone *et al.* 2013), and expand an insect's host breadth through increased production of cysteine proteases (Chu *et al.* 2013). Our study presents the first example of the consumption and establishment of specific midgut bacteria consortia enhancing insect larval growth in response to plant-derived chemical defenses.

The influence of bacterial inoculation on gypsy moth performance was dependent upon source. Larvae fed bacteria from aspen foliage were better able to contend with the aspen-derived defenses than the other treatments. This was unexpected, as an initially more intuitive prediction might be that plant-associated microbes benefit plants and insect-associated microbes benefit insects. However, "insect-associated bacteria" may be a relatively uninformative and overly strict categorical designation in gypsy moth, as its gut bacteria are largely acquired from foliage rather than vertically transmitted through eggs (Mason, *unpublished data*). This parallels similar results with bark beetles in which bacteria degrade terpenes toxic to the beetle and its fungal symbionts, utilize terpenes as a carbon source, and are enriched with genes encoding terpene-degrading enzymes, yet these associates occur equally in the plant host and beetles (Adams et al. 2011; Adams et al. 2013; Boone et al. 2013). It appears, then, that benefits to some herbivores may be a byproduct of the bacterial community's need to contend with plant toxins in their environment, and even more so when these toxins increase upon herbivore attack. In aspen, increased contact between foliar bacteria and phenolic glycosides occurs by rupturing of vacuoles. For some systems then, simple models of vectoring or paired symbiotic relationships may be less useful than more open models, in which the bacteria's ability to respond to the environmental conditions of both plant and insect hosts confer collateral benefits to the herbivore. There are likely limits to these effects, as bacterial degradation may be overcome by high phytochemical concentrations (Boone et al. 2013).

The complexity of bacterial-plant-insect interactions is further illustrated by the specificity of responses in the midgut communities to the plant toxins. When administered phenolic glycosides, the larvae fed bacteria from aspen experienced increased abundances of *Acinetobacter* and decreases in *Ralstonia* in their midguts (Fig. 2). These population shifts are consistent with the metabolism data (Fig. 3) showing that *Acinetobacter*, but not *Ralstonia*, reduces phenolic glycoside content. In contrast, the larvae not fed bacteria, or fed bacteria consortia enriched from gypsy moth midguts, did not change. The differences associated with inoculation source may arise from several potential mechanisms. The *Acinetobacter* cells that seeded the midgut communities in larvae fed aspen bacteria may have been better adapted to or primed for contending with phenolic glycosides, as related to the other bacteria. In contrast, environmental sources of bacteria other than aspen did not have members that increased following administration of phenolic glycosides. Naturally occurring populations of bacteria may have different enzymatic capacities that can mediate ecological interactions. For example, bacteria-salicin interactions can mediate effects of amoeboid predators (Sonowal et al. 2013). In a taxonomically distant and structurally different mammalian gut system, diet history can affect differential microbial responses to plant toxins (Kohl and Dearing 2012), similar to what we observed in gypsy moth midguts. While the response of *Acinetobacter* was the most dramatic, several other OTUs also shifted in abundance to some degree in response to the phenolic glycoside treatments (Fig 2A). This suggests that a multitude of interactions are occurring.

These results support other studies showing that pure-cultures of fungi and bacteria do not elicit the same effects alone as when they interact with other community members (Brenner *et al.* 2008; Aylward *et al.* 2013). Specifically, our results suggest that interactions among bacterial community members, and interactions between bacteria and the host insect, contribute to herbivore detoxification systems. Pure cultures of phenolic glycoside-degrading *Acinetobacter*, did not enhance larval growth in the same manner as complete communities. Several mechanisms could explain how additional bacteria in the consortia may directly or indirectly enhance gypsy moth growth in response to phenolic glycosides (Field *et al.* 1995). First, additional bacteria may enable *Acinetobacter* to tolerate and function under the extreme conditions of the midgut. Second, other bacteria may also contribute to the metabolism of phenolic glycosides, such as by enzymatic degradation or contributing molecular oxygen (Ettwig *et al.* 2010; Imlay 2011) to cytochrome P450-like enzymes (Feyereisen 1999). Lastly, other bacteria in the system may provide some unknown function, possibly unrelated to phenolic glycoside detoxification. Regardless of mechanism, these findings further underscore the value of working with bacterial consortia, and incorporating ecological context, as opposed to individual isolates.

Our findings contribute to a growing body of evidence that microorganisms mediate numerous facets of plant-herbivore interactions. These interactions are likely widespread, and future studies should explore comparisons among generalist and specialist insects, and across a range of host plant chemistries. Our study also showed that these interactions are highly complex, as the source of the bacteria determined the extent of the interaction. Hence, current models of symbiotic mutualisms or vectoring may be too simplified to describe many of these associations. Further testing of this model is necessary.

The results presented here illustrate a direct benefit of bacterial detoxification to an herbivore under controlled conditions, and future work can improve our understanding of these processes in nature. In the gypsy moth, future studies should evaluate the specific bacteria-bacteria or bacteria-host interactions that mediate the effects of phenolic glycosides on larval growth. These studies should include both culture based methods and natural settings to emulate the environmental conditions encountered by microbiota and their host. Specifically, development of *in vitro* growth media systems is needed to more closely emulate the pH and redox conditions of lepidopteran midguts, which may directly (Ruuhola et al. 2003) or indirectly influence degradation. Furthermore, evaluating these interactions using host plants, including their multiple sources of variability, and with multiple feeding bouts, is needed. Our results indicate that aspen provides a useful model to conduct such work. Evaluating how interactions among members of the midgut community contribute to plant defense degradation, and how these microbes interface with the herbivore's detoxification system would further our knowledge of functioning under varying environmental conditions.

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Table 1: Average OTU diversity and estimates or richness and coverage. Different letters represent significant differences at *P* < 0.05.

Sample [a]	Observed OTUs	Good's Coverage	Chao1	1/Simpson	Shannon
<u>Control</u>					
0%	7.33 d	0.997	10.06 e	1.01 c	0.04 c
3%	5.67 d	0.998	8.33 e	1.02 c	0.05 c
6%	15.67 dc	0.998	18.33 de	1.12 c	0.32 c
<u>Aspen inoculated</u>					
0%	26.67 bc	0.996	33.50 cd	1.51 c	0.96 b
3%	39.33 ab	0.995	53.50 ab	7.32 a	2.62 a
6%	29.33 b	0.996	42.17 bc	7.18 ab	2.382 a
<u>Midgut inoculated</u>					
0%	32.67 b	0.998	40.67 bc	4.62 abc	2.22 a
3%	50.00 a	0.995	61.25 a	6.45 ab	2.50 a
6%	36.67 b	0.998	37.17 bcd	3.44 bc	2.16 a

a. Samples include the bacterial inoculation and the concentration of ingested phenolic glycosides (0, 3, 6%).

Figure legends:

Figure 1: Effects of inoculation with bacteria and amending artificial diet and phenolic glycosides on gypsy moth larval growth (A) and consumption (B). Bars represent means + 1 standard error. Columns with different letters are significantly different (ANCOVA followed by LSD test: $P < 0.05$).

Figure 2: Midgut communities following the administration of bacterial and phenolic glycoside treatments in Fig. 1, as assessed by 16S-rRNA pyrosequencing. A: Average abundance (n = 3) of Silva-derived orders (calculated with an 80% cutoff threshold; ‘other’ includes those amounting to < 1.0% of the total abundance. B: OTUs that exhibited the greatest degree of change in abundance due to phenolic glycosides in the aspen inoculation. C: MDS plot constructed with Bray-Curtis dissimilarities calculated from OTUs designated at 97% similarity. Symbols coloring represent different phenolic glycoside concentrations: control inoculation - blue; midgut inoculation - black; aspen inoculation - red.

Figure 3: Effects of bacterial source on phenolic glycoside contents in samples. Phenolic glycosides are salicortin (A), tremulacin (B), salicin (C), and tremuloidin (D). Bars represent means + 1 standard error. Bars with different letters are significantly different (ANOVA followed by Bonferroni correction: $P < 0.05$).

Supplemental Figure 1: Influence of bacterial consortia and phenolic glycosides on the duration of the 3rd larval instar. Bars represent means + 1 standard error. Columns with different letters are significantly different (ANCOVA followed by LSD test: $P < 0.05$).

Supplemental Figure 2: Influence of individual bacteria and phenolic glycosides on the L3 growth. Bars represent means + 1 standard error. Columns with different letters are significantly different (ANCOVA followed by LSD test: $P < 0.05$).

Figure 1:

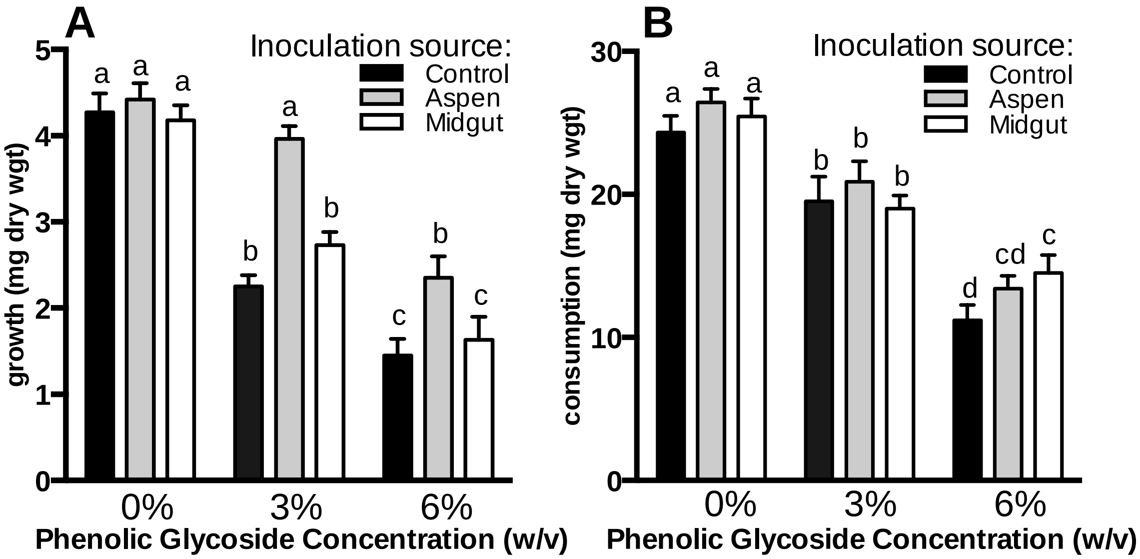

A
Inoculation source:
Control
Aspen
Midgut
growth (mg dry wgt)
5
4
3
2
1
0
a
a
a
b
a
b
c
b
c
0%
3%
6%
Phenolic Glycoside Concentration (w/v)
B
Inoculation source:
Control
Aspen
Midgut
consumption (mg dry wgt)
30
20
10
0
a
a
a
b
b
b
d
cd
c
0%
3%
6%
Phenolic Glycoside Concentration (w/v)

Figure 2:

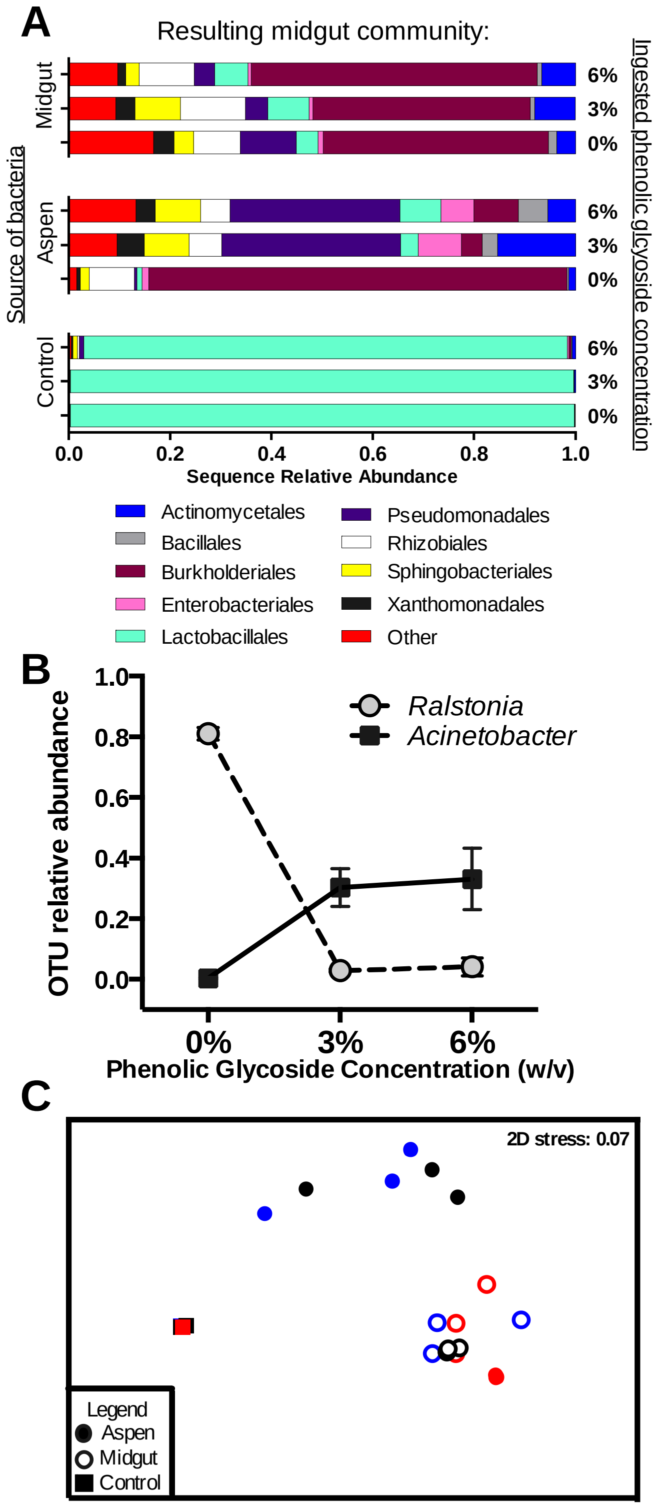
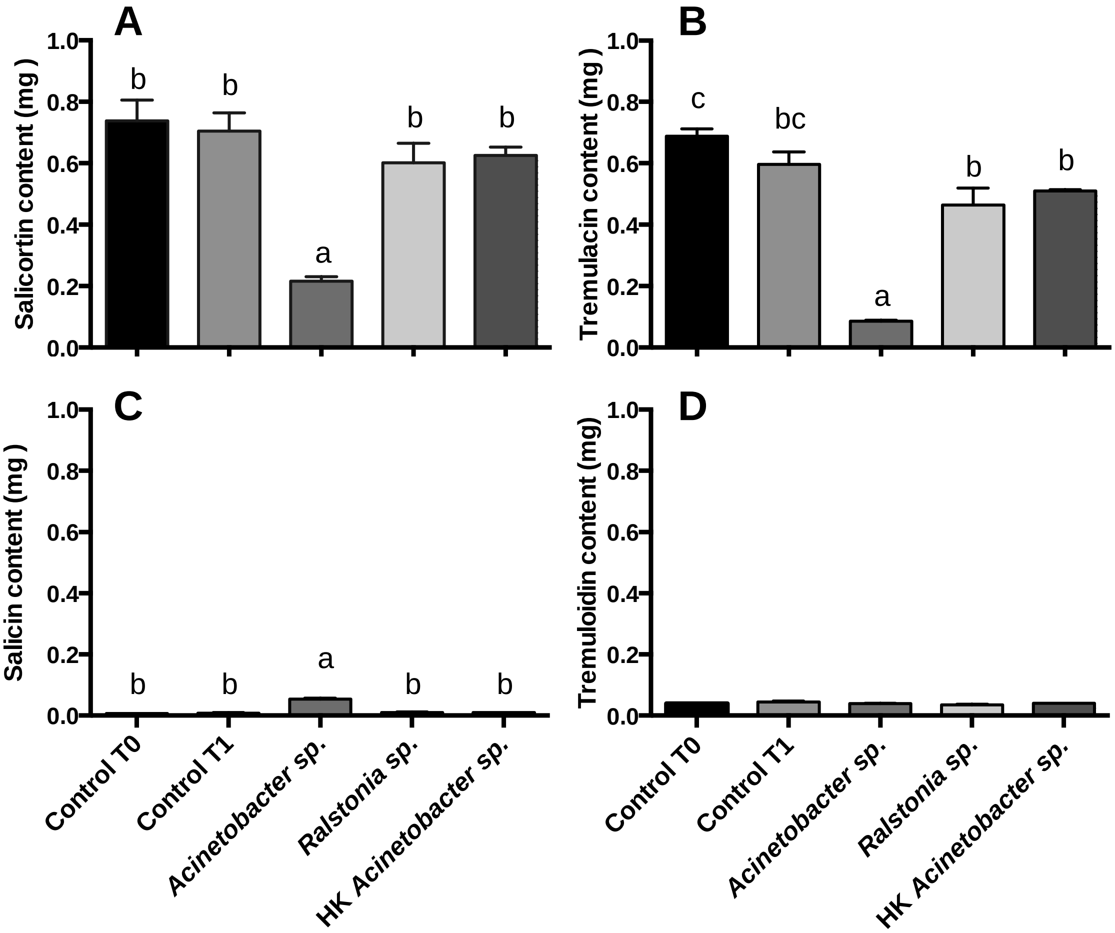
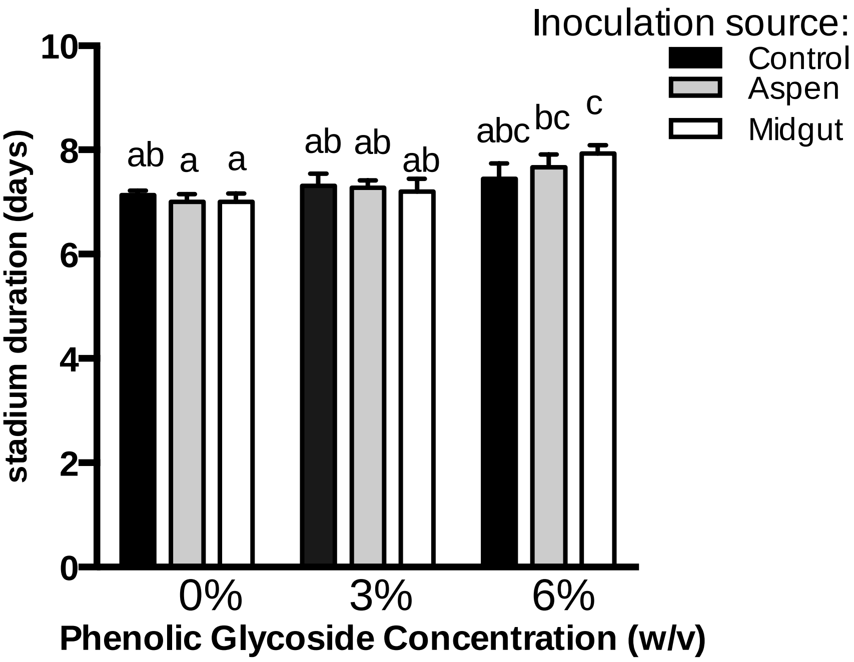

A
Resulting midgut community:
Source of bacteria
Midgut
Aspen
Control
Ingested phenolic glcyoside concentration
6%
3%
0%
0.0
0.2
0.4
0.6
0.8
1.0
Sequence Relative Abundance
Actinomycetales
Bacillales
Burkholderiales
Enterobacteriales
Lactobacillales
Pseudomonadales
Rhizobiales
Sphingobacteriales
Xanthomonadales
Other
B
OTU relative abundance
1.0
0.8
0.6
0.4
0.2
0.0
Ralstonia
Acinetobacter
0%
3%
6%
Phenolic Glycoside Concentration (w/v)
C
2D stress: 0.07
Legend
Aspen
Midgut
Control

Figure 3:


A
Salicortin content (mg)
1.0
0.8
0.6
0.4
0.2
0.0
b
b
a
b
b
B
Tremulacin content (mg)
1.0
0.8
0.6
0.4
0.2
0.0
c
bc
a
b
b
C
Salicin content (mg)
1.0
0.8
0.6
0.4
0.2
0.0
b
b
a
b
b
D
Tremuloidin content (mg)
1.0
0.8
0.6
0.4
0.2
0.0
Control T0
Control T1
Acinetobacter sp.
Ralstonia sp.
HK Acinetobacter sp.
Control T0
Control T1
Acinetobacter sp.
Ralstonia sp.
HK Acinetobacter sp.

Supplemental Figure 1:



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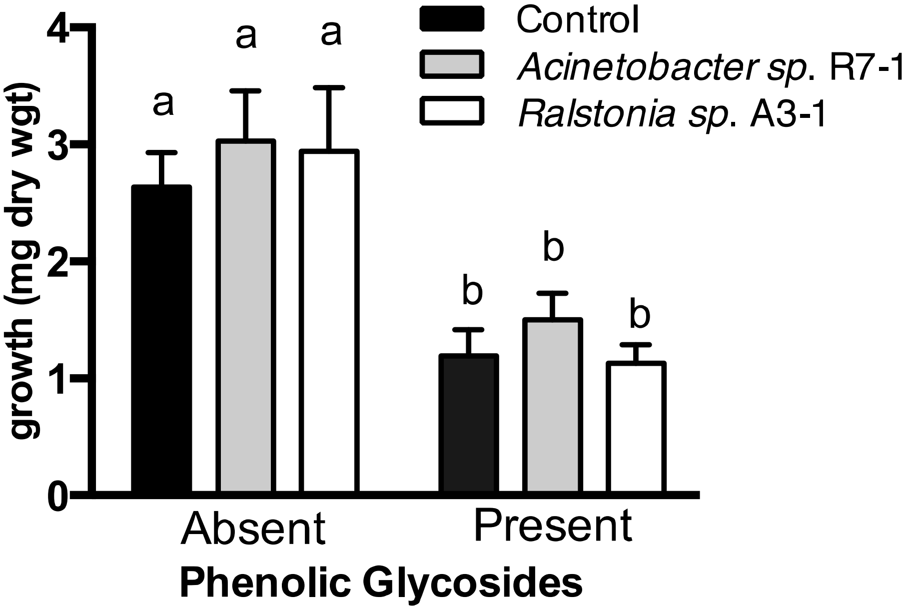

Chapter 3

Submitted for publication as:

Foliar bacterial community of trembling aspen in a common garden.

Charles J. Mason, Jesse A. Pfammatter, Liza M. Holeski and Kenneth F. Raffa

Abstract:

Microbial associations with plants are widely distributed, and are structured by a number of biotic and physical factors. Among biotic factors, the host plant genotype may be integral to these plant-microbe interactions. Trees in the genus *Populus* have become models for studies in scaling effects of host plant genetics and in plant – microbe interactions. Using 454 pyrosequencing of the 16S-rRNA gene, we assessed the foliar bacterial community of seven genotypes of mature trembling aspen trees (*Populus tremuloides* Michx.) grown in a common garden. Trees were selected based on prior analyses showing clonal variation in their concentration of chemicals conferring resistance against insect herbivores. At broad taxonomic designations, the bacterial community of trembling aspen was similar across all plant genotypes. At finer taxonomic scale, the foliage of these trees varied in their community composition, but there was no distinct pattern to colonization or abundance related to plant genotype. The most abundant operational taxonomic units (OTUs) were classified as *Ralstonia, Bradyrhizobium, Pseduomonas,* and *Brucella.* These OTUs varied across the common garden, but there was no significant effect of host plant genotype, or spatial position on the abundance of these members. Our results suggest that aspen genotype is less important in the structuring of foliar bacterial communities than other factors, such as environmental conditions, interactions among microbes, and plant intracellular structures that separate secondary compounds from residents of this phyllosphere.

Introduction:

Plant - microbe symbioses are ubiquitous in natural ecosystems and occur among all plant organs (Compant et al., 2011; Schulz and Boyle, 2005). Both fungal and bacteria associates can be located on the plant surface (Mercier and Lindow, 2000; Osono, 2008), or in vascular tissue (Hallmann et al., 1997). Often these symbiotic relationships do not have any apparent effects on the host (Faeth and Fagan, 2002), but other relationships range from pathogenic to mutualistic (Saikkonen et al., 1998). In plant foliage, benefits may arise from these associations through production of plant growth promoters (Altomare et al., 1999; Compant et al., 2010), resistance to abiotic stressors (Ruiz-Lozano et al., 1995; Sziderics et al., 2007), production of insecticidal defenses (Schulz et al., 2002), and inducible defense priming against both necrotrophic and biotrophic organisms (Balint-Kurti et al., 2010; Conrath et al., 2006). Different symbiotic microbial species and strains can elicit unique plant responses to various environmental interactions (Klironomos, 2003).

Numerous biotic and abiotic factors influence the microbial colonization of host plants. Geographic location (Gottel et al., 2011; Knief et al., 2010; Redford et al., 2010), nutrient characteristics and availability (Mercier and Lindow, 2000), access to water (Beattie and Lindow, 1994), temperature (Brandl and Mandrell, 2002), UV light (Kadivar and Stapleton, 2003), and temporal variation (Redford and Fierer, 2009) are among the principal abiotic factors shown to influence microbe associations with plants. Features of both the host and microbe, and their interactions, can affect the degree of the association. Genetic variation of the microbiota (Achouak et al., 2004; Weyens et al., 2011), responses of microbiota to plants secretions (De-la-Peña et al., 2012), as well as plant species and genotype can be key components in structuring fungal (Bailey et al., 2005; Bever et al., 1996; Pan et al., 2008) and bacterial communities

(Bulgarelli et al., 2012; Redford et al., 2010). Plant phenotypic traits that can affect microbial associations include leaf characteristics (Hunter et al., 2010; Reisberg et al., 2013) and chemical composition of foliage (Ruppel et al., 2008; Yadav et al., 2005).

Trees of the genus *Populus* have emerged as models for studying effects of host genetics on environmental processes (e.g., DeWoody et al., 2013; Lindroth et al., 2007; Whitham et al., 2006) and of plant – microbe interactions (van der Lelie et al., 2009). *Populus* include some of the most widely distributed and ecologically and economically important tree species in the Northern Hemisphere (Stettler et al., 1996). Plant genotype has been shown to mediate *Populus* interactions with fungal colonizers of foliage (Bailey et al., 2005), endophytic bacteria (Ulrich et al., 2008), lichins (Lamit et al., 2011) and belowground interactions with both bacteria and mycorrhizal fungi inhabiting the rhizosphere (Schweitzer et al., 2008; Shakya et al., 2013). Plant secondary chemistry can be an important factor in fungal endophyte associations (Bailey et al., 2005). *Populus* produce two major groups of defense chemicals, condensed tannins and phenolic glycosides (Lindroth and Hwang, 1996; Lindroth and St. Clair, 2013), which vary considerably among genotypes (Holeski et al., 2012; Hwang and Lindroth, 1997; Osier and Lindroth, 2001). These compounds have differential effects on biotic threats, whereby phenolic glycosides exert stronger adverse effects on herbivores, particularly Lepidoptera (Hwang and Lindroth, 1997), and condensed tannins have been correlated with inhibition of fungi (Holeski et al., 2009).

In a recent study, bacteria of trembling aspen (*Populus tremuloides* Michx.) were shown to mediate interactions between the lepidopteran folivore gypsy moth (*Lymantria dispar* L.) and the *Populus* defense chemicals phenolic glycosides (Mason et al. in press). In this study, we aimed to assess whether aspen genotypes known to vary in foliar defense chemistry influence bacterial community composition. We hypothesized that different plant genotypes growing in the same environment would harbor different bacterial communities. We used a common garden approach to test this hypothesis using seven aspen genotypes. We conducted bacterial 454 pyrosequencing using the 16S-rRNA gene of DNA extracted from the foliage tissue sampled from these trees, including both external and internal residents in our communities.

Methods:

Site description and sample collection: Aspen leaves were sampled from genotypes grown in a 10-year old common garden at the University of Wisconsin-Madison Arlington Agricultural Research Station. Details of the plot layout and establishment are in Holeski et al. (2009). Briefly, genotypes were arranged in a randomized complete block design with individual trees spaced 3 m apart (Fig. 1). The common garden contained twelve aspen genotypes, and we selected a subset of seven that are known to provide a range of plant chemistries based on previously published work (Holeski et al., 2009; Hwang and Lindroth, 1997).

Fully expanded leaves were collected in early June 2012. A single ramet from the lowest branch (3 – 4 m) was removed from the north side of trees with pole pruners. Seven undamaged leaves showing no symptoms of herbivory or diseases were removed. Leaves were detached at the petiole and placed into one sterile plastic bag for each tree. Leaves were transported to the laboratory on ice, flash frozen, and stored at -80ºC until DNA extraction.

DNA extraction: Samples were processed in three sets in random order. Leaves were homogenized in liquid nitrogen in a mortar and pestle. 250-400 mg of ground leaf material was added to sterile 2 mL screw-cap microcentrifuge tubes, after which 500 µL preheated (65ºC) CTAB buffer preheated was added. Samples were incubated at 65ºC for 1 hour, and homogenate was vortexed every 15 min. After incubation, an equal volume of phenol: chloroform: isoamyl alcohol (24:24:1) was added, samples were briefly vortexed, and centrifuged at 4ºC. Supernatant was collected, transferred to a fresh tube, and an equal volume of chloroform: isoamyl alcohol (24:1) was added. Samples were centrifuged, supernatant was collected, and an equal volume of cold 100% isopropanol was added and incubated overnight at -20ºC to precipitate DNA. DNA was pelleted and rinsed twice with 70% ethanol before eluting in sterile TE (10 mM Tris-HCl, 1.0 mM EDTA; pH 8.0).

16S-rRNA gene amplification: PCR and sequencing conditions followed protocols detailed in Hanshew et al. (2013). Briefly, the V6V8 region of the 16S-rRNA gene was amplified using the primers 799F-mod6 and 1392R. Triplicate PCRs were conducted using 25 ng template DNA, 0.25 μL Herculase II DNA polymerase (Agilent, Santa Clara, CA), 1.0 nM dNTPs, 0.5 μL DMSO, 5 μL buffer, 300 nM forward and reverse primers, and water totaling a final volume of 25 μL. Reaction conditions were: 95ºC for 2 min, 30 cycles of 95ºC for 20 s, 48ºC for 30 s, 72ºC for 30 s, and a final elongation of 72ºC for 3 min. PCR products were pooled and gel-extracted with a Zymoclean Gel DNA Recovery Kit (Zymo Research, Irvine, CA). Second PCR was completed using 2 μL of the recovered PCR product. Primers in the second PCR contained the A- and B- adaptors required for 454 sequencing along with 5 bp multiplex identifiers (MIDs). 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 completed on a GS-Junior (Roche, Indianapolis, IN) using modifications described in Hanshew et al. (2013).

454 Data processing: The 454 16S-rRNA amplicon data were processed in mothur v.1.32.0 following recommendations outlined in the Schloss standard operating procedures (Schloss et al., 2011, 2009). Data were denoised via PyroNoise. Sequences were processed to allow for no differences in MIDs and primers, no homopolymer exceeding six nucleotides, and a minimum length of 200 nucleotides. Sequences were aligned to the SILVA SEED database, and a preclustering step was conducted to reduce sequencing errors. Chimeras were detected using UCHIME and discarded (Edgar et al., 2011). Sequences were classified using a mothur formatted Ribosomal Database Project training set (v. 9.0) at an 80% confidence threshold (Wang et al., 2007). Sequences classified as chloroplast, eukaryotic, or unknown at the Kingdom taxonomic level were removed from further analyses. Operational taxonomic unit (OTU) analyses were conducted by clustering at 97% similarity. Units were randomly subsampled to 2500. Diversity metrics of OTUs were computed at this time using the standardized data.

Statistical analyses: Dissimilarity matrices were computed in mothur using the Bray-Curtis dissimilarity index and the Jaccard similarity coefficient. Bray-Curtis dissimilarities incorporated abundance, and Jaccard distances assessed using members presence or absence. The pair-wise distances were used to conduct non-metric multidimensional scaling (MDS) in PRIMER-E v. 6.0. A permutation-based MANOVA was conducted between samples in PRIMER using Bray-Curtis and Jaccard dissimilarities with plant host genotype as a fixed effect. The PERMANOVA was conducted using 9,999 permutations with Type I sums of squares.

Diversity metrics, individual OTUs, and spatial analysis of individual OTUs were analyzed in the R v. 3.0.1 programing environment. Trends of the most abundant individual OTUs were analyzed with an ANOVA. OTU abundances were analyzed with an ANOVA using with genotype as a fixed effects. OTUs that did not meet the assumption of normality were $\log(x + 1)$ transformed prior to analysis. Spatial patterns for the four most abundant OTUs within the garden were assessed with visual mean abundance (Fig. 4) and semi-variogram plots (supplemental Fig. 2). Visual abundance plots for each OTU were created by colorimetrically overlaying mean aggregated OTU abundance for each tree as it positioned in our samples. Semi-variograms for the four most abundant OTUs were calculated (including all genotypes) at a max distance of half the garden (15m) and using the geoR package v. 1.7-4.

Results:

After curation and quality control, 16S-rRNA gene pyrosequencing yielded 159,017 total sequences. The number of sequences per sample ranged from 990 to 7669 prior to subsampling. Two samples were discarded at the subsampling procedure. One had a low number of sequences compared to the other samples. The other was dominated by one OTU that was classified as a putative phytoplasma and was considered an outlier to the remainder of the samples.

The number of OTUs in individual samples ranged from relatively low (15) to comparatively diverse (86), with an average of 34.6 (std. error = 2.68). Estimates of sampling depth indicated the number of sequences obtained for the study was sufficient (Supplemental Fig. 1). OTUs richness was not influenced by genotype of the host tree ($F_{6,33} = 1.461$ $P = 0.222$). Moreover, aspen genotype did not have an effect on Shannon ($F_{6,33} = 1.10$ $P = 0.383$) or Simpson ($F_{6,33} = 1.277$ $P = 0.295$) estimates of diversity.

Taxonomic designations of sequences at order-level classification did not reveal distinct differences of foliar bacterial composition among genotypes (Fig. 2). Ten orders comprised the majority of abundance, with unclassified bacteria comprising approximately 8% of the total. Burkholdariales was the most prevalent order across all samples. Some trends appeared to be present, so multivariate analyses on the OTUs were conducted. Nonmetric multidimensional ordination (MDS) plots were generated using the distance matrices constructed from OTUs using Bray-Curtis dissimilarities and Jaccard index dissimilarities. MDS plots indicated no clear grouping of samples by genotype (Fig. 3). A PERMANOVA indicated that genotype did not influence community membership (Jaccard dissimilarities; Pseudo-$F_{6,33} = 0.979$ $P = 0.609$) or structure (Bray-Curtis dissimilarities; Pseudo-$F_{6,33} = 0.854$ $P = 0.860$). However, some clustering appeared to occur among some samples of WAU2 and SAU3 genotypes (Fig. 3B).

Although there was no clear difference in bacterial membership on the community as a whole, we assessed whether genotype effects were present among individual members. We analyzed the influence of genotype on the four most abundant individual OTUs, which comprised between 20 and 90% of the relative abundance with an average of 50% across all samples. At the genus level, these OTUs were classified as *Ralstonia*, *Brucella*, *Pseudomonas*, and *Bradyrhizobium* (Fig. 4 A-D). None of these OTUs were affected by host plant genotype ($P > 0.30$), with the exception of *Pseudomonas* where the effect was marginal ($\log\{y+1\}$ transformed; $F_{6,33} = 1.969$ $P = 0.099$).

We evaluated the influence of sample position on the abundance of these OTUs to evaluate potential spatial effects (Supplemental Fig. 2). *Ralstonia* OTUs varied in abundance across the common garden, but there was no statistically significant effect (Fig. 4E). Similar patterns were present with the *Brucella* and *Bradyrhizobium* OTUs (Fig. 4F, 4H). The *Pseudomonas* OTU had higher abundances in the 2nd and 3rd rows that were sampled than elsewhere in the plot (Fig. 4G). In these rows, abundances were highest when associated with two trees of the aspen genotype SAU3. Elsewhere in the plot, the SAU3 genotype did not have high abundances.

Discussion:

We evaluated the influence of host plant genotype on foliar bacterial community composition in a common garden of trembling aspen. Genotype did not exert strong effects on either the entire community or individual OTUs (Fig. 3; Fig. 4). Although the abundances of

OTUs varied throughout the common garden, a spatial pattern of the OTUs was not detected in our survey (Fig. 4; Supplemental Fig. 2). These results suggest that if trembling aspen genotype influences the commensal bacteria associated with foliage, it is minor compared to other environmental and biological factors. The bacterial consortium of trembling aspen is quite consistent at both order and OTU levels, and some features of the aspen leaf environment may influence the composition of these communities.

Contrary to our hypothesis, host plant genotypes of varying secondary chemical composition did not influence the bacterial consortia associated with foliar tissues. The possibility that a spatial influence at the plot scale obscured potential genotypic differences was not supported. A likely explanation involves a spatial influence at the micro-scale, specifically that compartmentalization of the major phytochemicals of aspen within plant cells limits contact with phyllosphere residents. Defense chemicals, like phenolic glycosides and condensed tannins, are compartmentalized in the vacuoles of foliar cells (Lees et al., 1993; Wink, 1993). This compartmentalization may serve as a barrier to the phyllosphere bacteria, as they would predominantly reside in the external portions of cells on the foliar surface or in the vascular tissues. This contrasts with some other systems, in which the plants exude their metabolites through glands onto the foliar surface, coming into direct contact with the microorganisms (Yadav et al., 2005). Likewise, exudates from root tissues of some plants can structure rhizosphere bacterial communities, but, in *Populus*, root communities are only weakly explained by host plant genotype (Shakya et al., 2013). The genotypes we selected did not have foliar exudates. Thus, other members of *Populus* and Salicaceae exude foliar and extrafloral secretions might yield different results (Greenaway et al., 1992; Thadeo et al., 2008). Overall, it seems likely that species – level features of trembling aspen may be more important in affecting bacterial community composition than intra - specific nuances of foliar chemistry and physical characteristics. These data support previous work that found trees within a species had very similar bacterial communities at the plot level (Redford et al., 2010).

The most abundant bacterial OTUs found within the common garden are known to be plant and environmentally associated microbes. These and other members of the same bacterial phyla and families been frequently found associated with plants (Vorholt, 2012), and we have previously found them associated with trembling aspen foliage (Mason and Raffa in press). Almost all of the abundant OTUs were distributed throughout the common garden plot (Fig 4E-H), suggesting sources that seed the community, whether they reside internally or externally, are accessible to the foliar tissue. *Ralstonia* and *Pseudomonas* are commonly considered as potential plant pathogens, but also can colonize the phyllosphere as commensals or mutualists (Hol et al., 2013). The roles of these foliage-colonizing bacteria in aspen biology are unknown.

The *Pseudomonas* OTU had a higher abundance associated with the leaves of two trees, both from the SAU3 genotype, compared with other genotypes in the common garden (Fig. 4G). Several possible mechanisms could contribute to these high incidences. Although we sampled visibly undamaged leaves, those associated with these trees may have had ruptured cells. This could result in chemical changes to the phyllosphere causing the newly available nutrients as well as the defense metabolites. Alternatively, the high abundance may be indicative of the initiation of a pathogenic infection. We only sampled one time point, and these bacterial communities can vary temporally (Redford and Fierer, 2009). However, it is unclear from these data if the associated *Pseudomonas* are indeed plant pathogens.

These results demonstrate that host plant genotype has an overall minor effect on bacterial community composition of aspen foliage. Rather, interactions among microbiota and the abiotic environment may more strongly affect community composition, with limited effects of defense chemicals imposed by intracellular compartmentalization. Future studies should investigate how foliar morphological characteristics and interactions among microbes affect bacterial community composition. The dynamics of these consortia should also be assessed through time. The influence of herbivory on releasing defense chemicals from vacuoles, and thus potentially influencing microbial associates, should also be investigated. Given the ability of bacterial associates to strongly mediate interactions between plants and herbivores (Mason et al. *in press*), understanding how variation in host plant characteristics can influence bacterial communities is particularly important to understanding plant - herbivore - microbe relationships, and their implications to both evolutionary theory and resource management.

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Figure 1: Plot layout of the aspen genotypes selected for this study. Trees were planted 3 m apart in both X- and Y- coordinates. Trees were selected from every other row, with exception of one from row two (DAN1), because of tree mortality from the first row. Locations of the two trees excluded from the statistical analyses are not present in the figure. Samples are arranged in this and in subsequent figures by increasing phenolic glycoside concentration as evaluated by Holeski et al. (2009).

Figure 2: Mean relative abundance of sequences at the order taxonomic classification. Trees are arranged in increasing phenolic glycoside content based on measurements conducted by Holeski et al. (2009).

Figure 3: Nonmetric multidimensional scaling plots of the Jaccard (A) and Bray-Curtis (B) dissimilarity matrices made from bacterial OTUs. Jaccard assesses shared OTU membership without taking relative abundance into account, and Bray-Curtis assesses membership with the abundance of the OTUs.

Figure 4: Average number of sequences of the four most abundant OTUs (A-D) and the abundance of those OTUs throughout the common garden (E-H). Taxonomic designation of the OTUs was based on RDP classifier. Bars are +1 SE. Genotype did not have a significant effect on the number of sequences of all the OTUs ($P > 0.05$). OTU3 (C) was $\log(x + 1)$ transformed to help meet normality assumptions. The difference in abundance of OTU3 was influenced by high abundances of two particular plants (G). The two samples removed from the analysis were not included in the spatial plots.

Fig. 1:

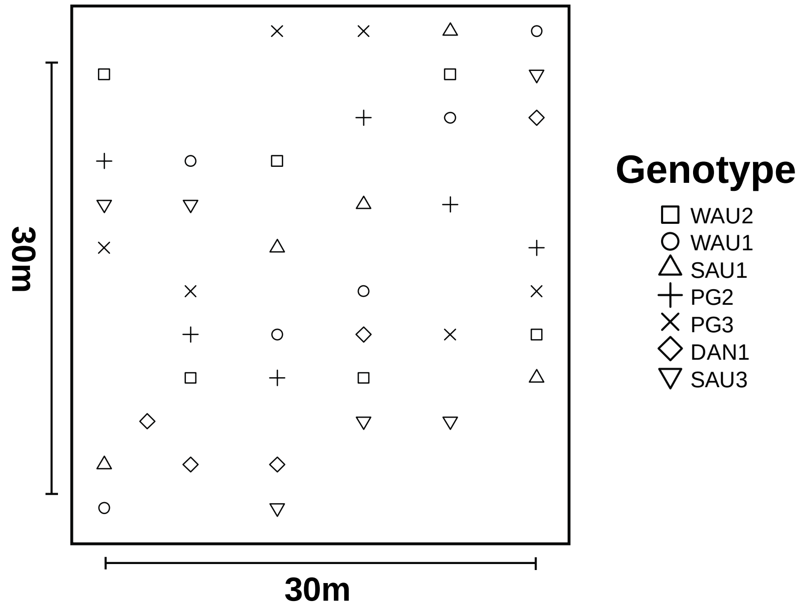


Fig. 2:

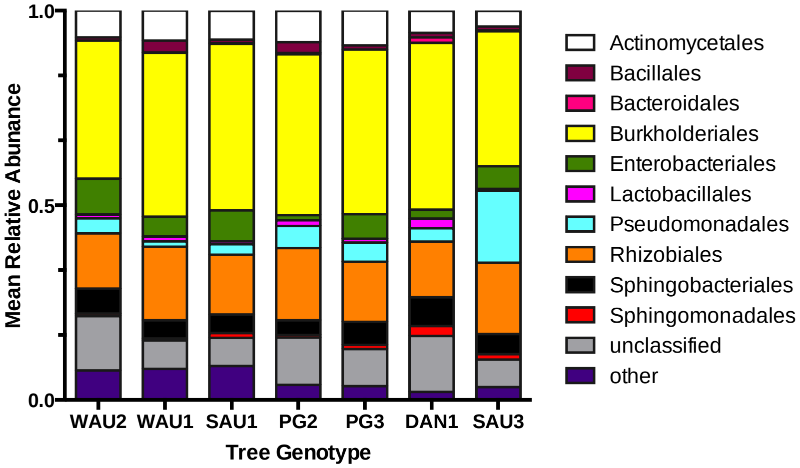

1.0
0.5
0.0
Mean Relative Abunance
WAU2
WAU1
SAU1
PG2
PG3
DAN1
SAU3
Tree Genotype
Actinomycetales
Bacillales
Bacteroidales
Burkholderiales
Enterobacteriales
Lactobacillales
Pseudomonadales
Rhizobiales
Sphingobacteriales
Sphingomonadales
unclassified
other

Fig. 3:

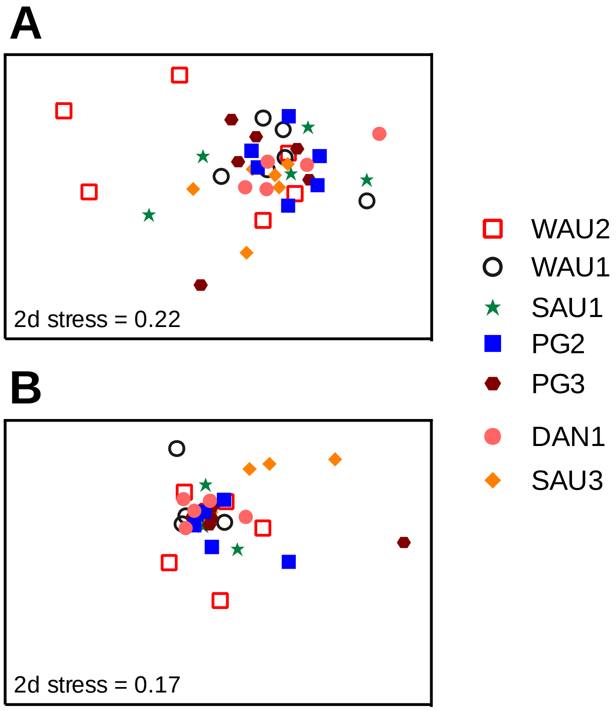
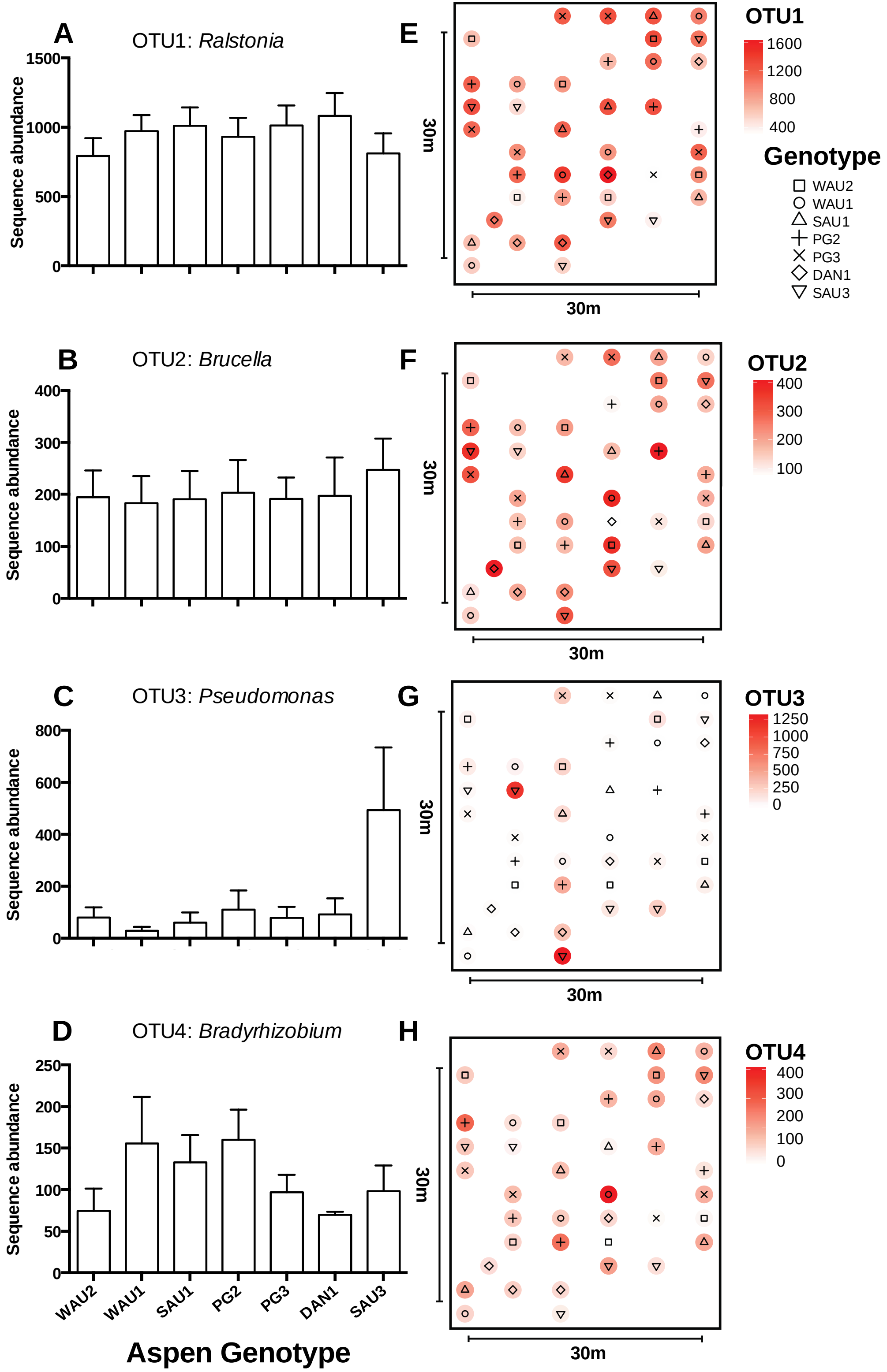

A
2d stress = 0.22
B
2d stress = 0.17
WAU2
WAU1
SAU1
PG2
PG3
DAN1
SAU3

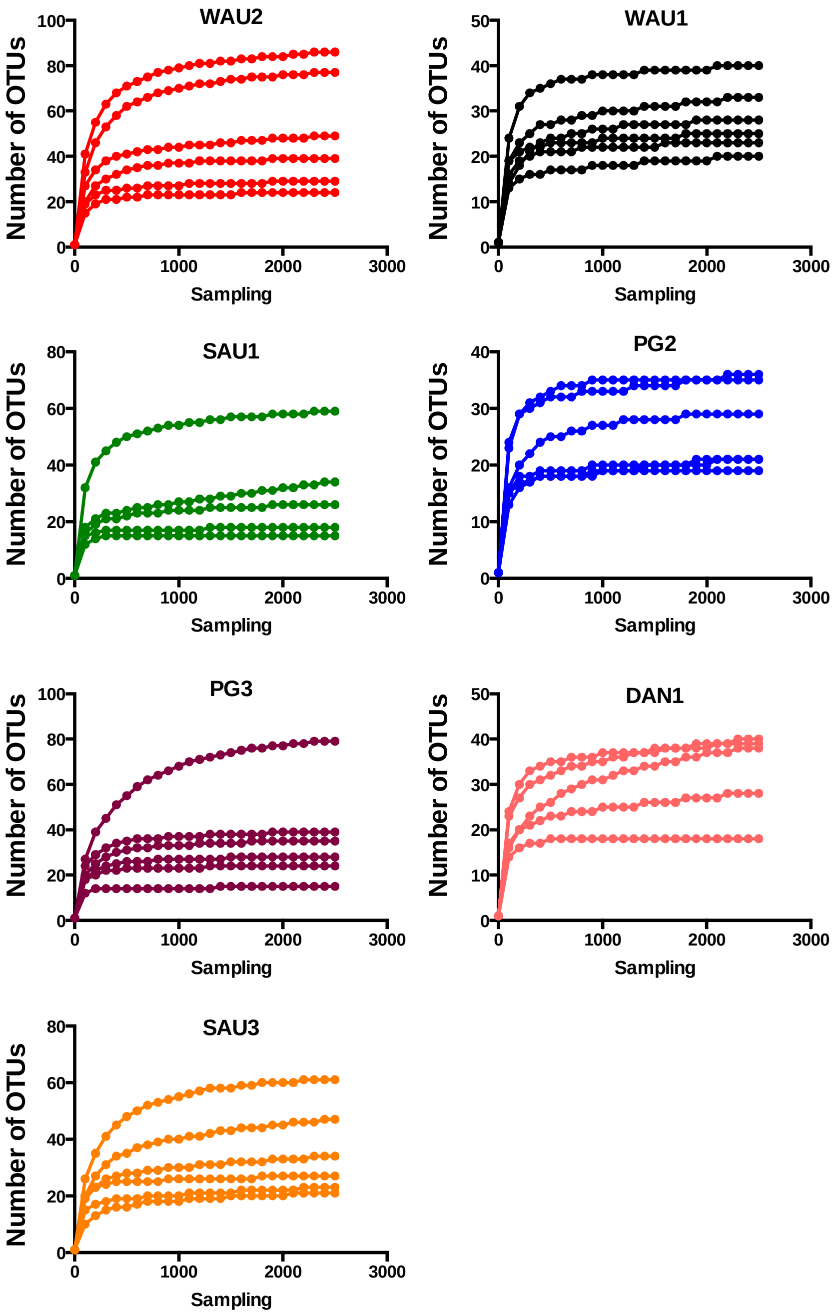
Fig. 4:


A
OTU1: *Ralstonia*
Sequence abundance
1500
1000
500
0
E
OTU1
1600
1200
800
400
Genotype
□ WAU2
○ WAU1
△ SAU1
+ PG2
× PG3
◇ DAN1
▽ SAU3
30m
30m
B
OTU2: *Brucella*
Sequence abundance
400
300
200
100
0
F
OTU2
400
300
200
100
30m
30m
C
OTU3: *Pseudomonas*
Sequence abundance
800
600
400
200
0
G
OTU3
1250
1000
750
500
250
0
30m
30m
D
OTU4: *Bradyrhizobium*
Sequence abundance
250
200
150
100
50
0
WAU2
WAU1
SAU1
PG2
PG3
DAN1
SAU3
Aspen Genotype
H
OTU4
400
300
200
100
0
30m
30m

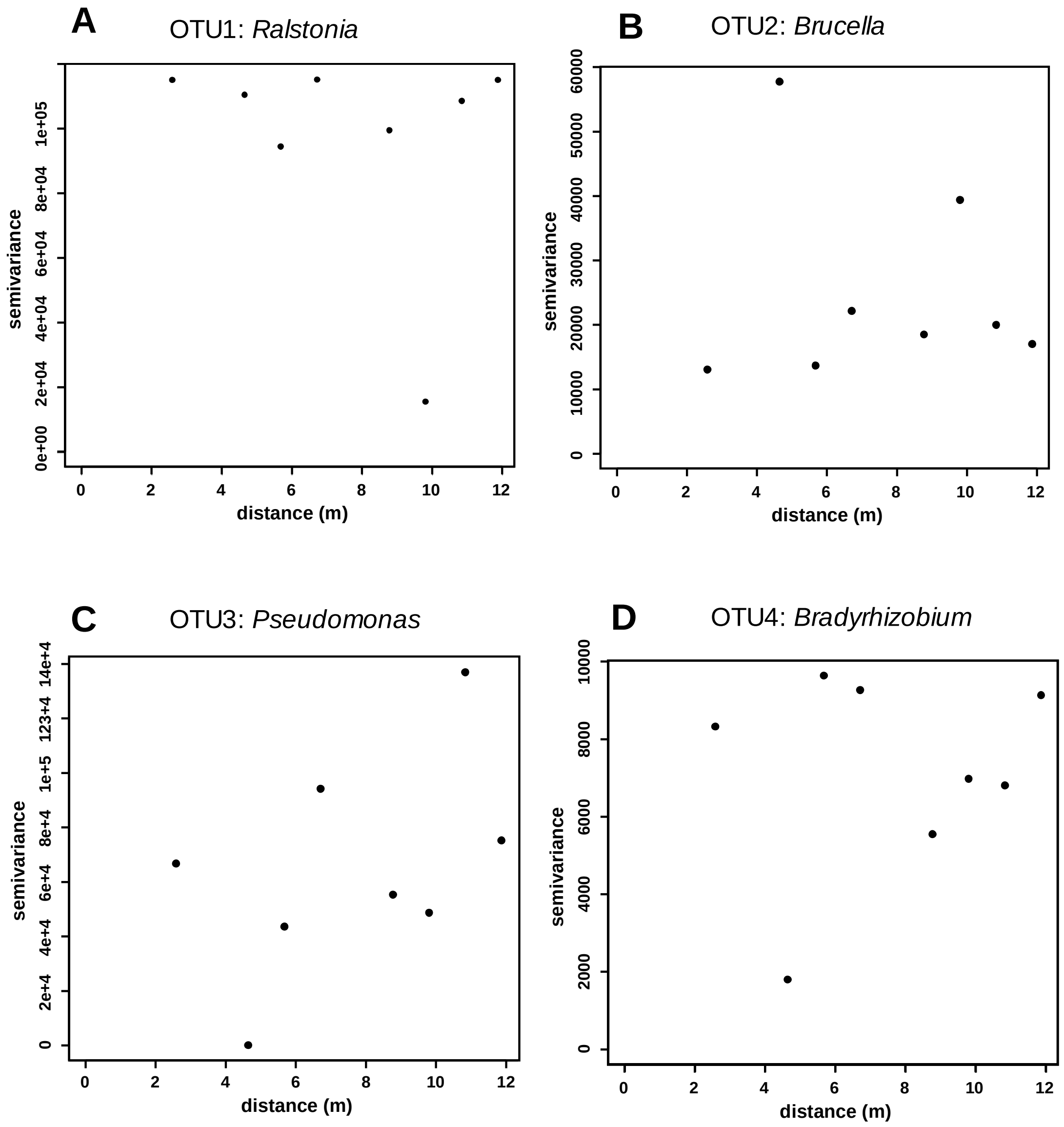
Supplemental Figure 1: Rarefaction curves of observed OTUs foliar bacterial communities of the genotypes after subsampling in mothur. Level of diversity differed among and between genotypes. All rarefaction curves reached an asymptote, indicating the level of sampling was sufficient.

Supplemental Figure 2: Semivariograms of the four most abundant OTUs (A-D). Semivariance was estimated form mean aggregated OTU abundance at a max distance of half the size of the garden (15m). Low semivariance at shorter distances relative to high semivariance at larger distances (logarithmic fit to the data points) would be indicative of spatial correlation. Spatial correlation is not apparent for any OTU.

Supplemental Fig. 1:



Supplemental Fig. 2:



Chapter 4

Submitted for publication as:

Aspen defense chemicals influence the midgut bacterial community of gypsy moth.

Charles J. Mason, Kennedy Rubert-Nason, Richard L. Lindroth, and Kenneth F. Raffa

Abstract:

Microbial symbionts are becoming increasingly recognized as mediators of many aspects of plant – herbivore interactions. However, the influence of plant chemical defenses on gut associates of insect herbivores is less well understood. We used gypsy moth (*Lymantria dispar* L.), and trembling aspen (*Populus tremuloides* Michx.) genotypes that vary in chemical defenses, to assess the influence of foliar chemistry on bacterial communities of larval midguts. We evaluated the bacterial community composition of foliage, and of midguts of larvae feeding on those leaves, using next-generation high-throughput sequencing. Plant defense chemicals did not influence the composition of foliar communities. In contrast, both phenolic glycosides and condensed tannins affected the bacterial consortia of gypsy moth midguts. The two most abundant operational taxonomic units were classified as *Ralstonia* and *Acinetobacter*. The relative abundance of *Ralstonia* was higher in midguts than foliage when phenolic glycoside concentrations were low, but lower in midguts when phenolic glycosides were high. In contrast, the relative abundance of *Ralstonia* was lower in midguts than foliage when condensed tannin concentrations were low, but higher in midguts when condensed tannins were high. *Acinetobacter* showed a different relationship with host chemistry, being relatively more abundant in midguts than in foliage when condensed tannin concentrations were low, but lower in midguts when condensed tannins were high. *Acinetobacter* tended to be more abundant in midguts of insects feeding on genotypes with high phenolic glycoside concentrations. These results show that plant defense chemicals influence herbivore midgut communities, which may in turn influence host utilization.

Introduction:

Microbial-arthropod symbioses are ubiquitous interactions that are often critical to insect functioning, performance, and survival. Symbionts of insect herbivores have been shown to mediate interactions with natural enemies (Oliver et al. 2003, Scarborough et al. 2005, Łukasik et al. 2013), enable access to recalcitrant sources of carbon (Geib et al. 2008), contribute to nitrogen provisioning (Gündüz and Douglas 2009, Morales-Jiménez et al. 2013, Ayayee et al. 2014), circumvent plant defenses (North et al. 1997, Chung et al. 2013), and augment detoxification of plant-derived toxins (Dowd and Shen 1990, Kikuchi et al. 2012, Boone et al. 2013, Hammerbacher et al. 2013). Mechanisms of acquisition vary extensively. For example, midgut bacteria can be acquired from both maternal (Kaltenpoth et al. 2009, Hosokawa et al. 2012) and environmental sources (Kikuchi et al. 2007). In many social insects, eukaryotic and bacterial members of the midgut are transmitted among nest mates through stomodeal and proctodeal trophallaxis (Noda et al. 2007). In leaf-feeding Lepidoptera, midgut bacteria appear to be seeded primarily through dietary sources, but are subsequently influenced by larval host plants within midgut environmental conditions (Broderick et al. 2004, Priya et al. 2012, Mason and Raffa, 2014).

Plant defense chemicals pose significant barriers to the abilities of insect herbivores to utilize plant substrates (Mithöfer and Boland 2012). In addition to being deleterious to the insect host, these defenses may also affect microbial associates. Plant defense chemicals have been shown to influence vectored (Klepzig et al. 1996) and plant pathogenic fungi (Holeski et al. 2009). Additionally, differences in the ability of bacteria associated with various bark beetles to tolerate terpenoid defenses may relate to differences in the beetles' life history strategies (Adams et al. 2011, Boone et al. 2013). Both bacterial and fungal symbionts of insects possess enzymes, or genes encoding for enzymes, that are typically associated with toxin detoxification (DiGuistini et al. 2011, Adams et al. 2013). The toxin-metabolizing capacities of these symbionts vary in presence and level of activity (Hammerbacher et al. 2013, Sonowal et al. 2013).

The European gypsy moth (*Lymantria dispar* L.) is an important defoliator of deciduous trees in North America. The gypsy moth is highly polyphagous (Liebhold et al. 1995), and thus encounters a diversity of plant chemistries. The gypsy moth larval midgut poses an extreme environment to microorganisms, being both highly alkaline (Appel and Maines 1995) and relatively hypoxic (Johnson and Barbehenn 2000). Bacteria residing in the midgut tissues are mostly obtained from consumption of the host plant (Broderick et al. 2004), but their communities are subsequently structured by the environmental conditions of the midgut (Mason and Raffa 2014).

Trembling aspen (*Populus tremuloides* Michx.) foliage contains a suite of chemical defenses that can deter feeding by herbivores, greatly reducing their growth and development (Boeckler et al. 2011, Lindroth and St. Clair 2013). The most prominent chemical defenses produced by aspen consist of phenolic glycosides and condensed tannins (Lindroth and Hwang 1996). Phenolic glycosides are aspen’s principal chemical defense against folivorous lepidopterans, and vary considerably among genotypes (Hemming and Lindroth 1995, Hwang and Lindroth 1997, Osier and Lindroth 2001). In *Populus*, condensed tannins have been shown to be negatively correlated with the colonization of foliar fungal communities (Bailey et al. 2005) and pathogens (Holeski et al. 2009), but tend to have limited effects on the performance of Lepidoptera (Osier et al. 2000, Barbehenn et al. 2009, Barbehenn and Constabel 2011). Like many plant secondary metabolites, condensed tannins and phenolic glycosides are thought to be stored in cell vacuoles in aspen (Lees et al. 1993, Wink 1993, Payyavula et al. 2009). Upon herbivore feeding, this storage structure ruptures, causing toxin release and deleterious effects to the herbivore. Despite these defenses, gypsy moth may exploit aspen by selecting less well-defended trees and detoxifying or tolerating allelochemicals.

Colonization of gypsy moth midgut tissues by bacteria originating from aspen foliage augments larval detoxification of phenolic glycosides, in experimentally amended artificial diets (Mason et al. 2014). In that study, some bacteria were shown to be more sensitive to phenolic glycosides than others. Those that exhibited increased relative abundances in host midguts in response to phenolic glycosides also had greater *in vitro* phenolic glycoside degrading capacities. However, whether differences in aspen foliar chemistry influence bacterial community composition in the midgut is unknown. Here, we investigated the interaction among foliar bacterial communities, trembling aspen genotypes, and the midgut community of gypsy moth. We conducted bacterial 16S-rRNA gene pyrosequencing on paired host plant and gypsy moth larval midgut samples. Phenolic glycoside and condensed tannin concentrations of these trees were quantified to assess if variation in tree chemical defenses may explain bacterial community composition of foliage and the larval midgut.

Materials & Methods:

Insect rearing and plant propagation: Gypsy moth egg masses were obtained from a laboratory culture maintained by a USDA APHIS rearing facility (Otis, MA, USA). Larvae were reared on commercial wheat germ based artificial diet (MP Biomedical, Santa Ana, CA, USA) in sterile 9.5 cm diameter plastic petri dishes until molting into the third stadium (L3).

Aspen genotypes were maintained in a common garden at the University of Wisconsin-Madison were cloned by Knight Hollow Nursery, Inc. (Middleton, WI, USA) using tissue culture propagation following the method of Sellmer et al. (1989). Three aspen genotypes were selected to provide a range of phenolic glycoside and condensed tannin concentrations, based on previously published analyses (Hwang and Lindroth 1997, Holeski et al. 2009). We transplanted individual microcuttings into Deepots pots (Stueue & Sons, Tangent, OR, USA) containing a 1:1 mix of MetroMix 366PSC (Sun-Gro, Agawam, MA, USA) and torpedo sand. We maintained the trees outdoors in cold-frames during summer (2011), overwintered them in a greenhouse (2011 – 2012), and returned them to the cold-frames the following spring (May, 2012) until conducting our experiment (June, 2013). We applied water-soluble fertilizer (20-10-20 N-P-K) to the trees once per week between July and mid-September, 2011.

Experimental feeding and tissue collection: Three replicate trees of each genotype were randomly selected for analysis. After molting into the third stadium, individual gypsy moth larvae were paced into sterile 5.5-cm diameter plastic petri dishes and provided with two leaves from a single replicate tree.. Leaf position was standardized from the first fully expanding leaf among all genotypes and replicates. Forceps were surface disinfected by rinsing in 2% bleach followed by 70% ethanol. Leaves at LPI 3 and 4 were collected at the petiole and transported to the laboratory in sterile plastic bags. Larvae were allowed to feed for 72h. We anesthetized larvae at -20ºC for 15 minutes, surface-sterilized, and removed their midguts by dissection. Tissues were stored at -80ºC until DNA extraction.

Leaf samples were collected for DNA extraction and phytochemical analysis at the same time as tissue collection of insects. For microbial community analyses, five leaves from each tree were removed, placed into a sterile plastic bag, frozen, and stored at -80ºC until processing. Tissues for phenolic glycoside analysis were collected in paper envelopes, flash frozen, and stored at -20ºC until freeze drying.

Analysis of foliar defense chemistry: Aspen foliage was analyzed for phenolic glycoside and condensed tannin concentrations to verify our assumption that the genotypes differed in these constituents. Foliage was lyophilized and homogenized with 30 3 mm steel beads in 20 mL plastic scintillation vials.

Phenolic glycosides were extracted from 20-30 mg of foliage in 1.5 mL of ice-cold methanol with sonication. Extracts were diluted and analyzed by ultra-high performance liquid chromatography (UHPLC) with negative electrospray ionization single quadrupole mass spectrometry using a Waters integrated Acquity I-Class UPLC (Milford, MA) according to a modified method of Abreu et al. (2011). Briefly, samples (2 µL) were injected onto a Waters Acquity CSH C-18 column (2.1 x 100 mm, 1.7 µm) and separated at 40°C on a gradient of water and acetonitrile, both acidified with 0.1% formic acid, at a flow rate of 0.5 mL/min. The mass spectrometer was operated in negative ionization mode, with selective ion recording of the salicinoid-formate adducts. Operating and data acquisition conditions for the mass spectrometer were as follows: cone potential, 30 V; capillary potential, 2500 V; extractor potential, 3 V; RF lens potential, 0.1 V; source temperature, 120 °C; desolvation temperature, 250 °C; desolvation gas flow, 500 L/h; cone gas flow, 10 L/h; infusion rate, 5 □L/min; dwell time, 0.025 s. Calibrations were based on internal standardization by salicylic acid-d$_6$ (Sigma-Aldrich, St. Louis, MO, USA), using four-point (15 – 1500 mg/L), salicin, salicortin, tremulacin, quadratic models. The salicin calibration standard was purchased from Sigma-Aldrich (St. Louis, MO, USA) and the tremulacin and salicortin calibration standards were extracted from aspen foliage by liquid-liquid extraction (Lindroth et al. 1986) and purified by normal phase liquid chromatography (Still et al. 1978).

Condensed tannins were extracted from 20-30 mg of ground foliage in cold 70% acetone containing 10 mM ascorbic acid as an antioxidant with sonication. Extracts were quantified spectrophotometrically using acidified butanol according to Porter et al. (1986). Condensed tannin standards were purified from aspen according to Hagerman and Butler (1980).

DNA extraction: Foliage was homogenized in liquid nitrogen in a mortar and pestle. Ground leaf material was added to sterile 2 mL screw-cap microcentrifuge tubes. 500 μL of preheated (65ºC) cetrimonium bromide (CTAB) buffer was added to the ground tissue. Samples were incubated at 65ºC for 1 hour, mixing homogenates every 15 min. After incubation, an equal volume of phenol:chloroform:isoamyl alcohol (24:24:1) was added, samples were briefly vortexed, and centrifuged at 13,200 × *g* for 10 min at 4ºC. Supernatant was collected, transferred to a fresh tube, and an equal volume of chloroform: isoamyl alcohol (24:1) was added. Samples were centrifuged, supernatant was collected, and DNA was precipitated by adding an equal volume of cold 100% isopropanol and overnight incubation at -20ºC. Samples were centrifuged and DNA pellets were rinsed twice with 70% ethanol before eluting in sterile TE (10 mM Tris-HCl, pH 8.0; 1.0 mM EDTA, pH 8.0).

Insect midgut samples were homogenized in 500 μL hot CTAB with a 4 mm steel bead by bead beading in 2 mL plastic screw cap tubes. All subsequent steps were identical to the DNA extraction used for the plant tissue.

16S-rRNA gene amplification and sequencing: PCR reactions were performed as described in Hanshew et al. (2013). The V6V8 region of the 16S rRNA gene was amplified using the primers 799F-mod6 and 1392R. PCR reactions were done in triplicate containing 25 ng template DNA, 0.25 μL Herculase II DNA polymerase (Agilent Technologies, Santa Clara, CA, USA), 1.0 nM dNTPs, 0.5 μL DMSO, 5 μL buffer, 300 nM forward and reverse primers, and water totaling a final volume of 25 μL. Reaction conditions were: 95ºC for 2 min, 30 cycles of 95ºC for 20 s, 48ºC for 30 s, 72ºC for 30 s, and a final elongation of 72ºC for 3 min. Triplicates were pooled for each sample, and lanes were extracted from the gel using a Zymoclean Gel DNA Recovery Kit (Zymo Research, Irvine, CA, USA). The second PCR was completed using 2 μL of the recovered PCR product. Primers in the second PCR contained the 454 A- and B- adaptors along with 5 bp multiplex identifiers (MIDs). PCR conditions were identical except only 10 cycles were performed. The ~700 bp product was purified by gel extraction and quantified by an Invitrogen Qubit Fluorometer (Life Sciences, Grand Island, NY, USA). Pyrosequencing was completed on a Roche 454 GS-Junior (Roche Diagnostics, IN, USA) using modifications described in Hanshew et al. (2013).

454 Data processing: The 454 16S-rRNA amplicon data were processed in mothur v.1.32.0 following the Schloss standard operating procedures (Schloss et al. 2009, 2011). Data were denoised via PyroNoise. Sequences were processed so as to allow for no differences in MIDs 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 a mothur formatted Ribosomal Database Project training set (v. 9.0) at an 80% confidence threshold (Wang et al. 2007). Sequences classified as chloroplast, eukaryotic, or unknown at the Kingdom taxonomic level were removed from further analyses. Operational taxonomic unit (OTU) analyses were conducted by clustering sequences at a 97% similarity cutoff. Units were standardized by randomly subsampling to 2500.

Statistical analyses: Concentrations of aspen secondary metabolites were calculated as dry weight percentages. Chemical concentrations were analyzed in the R v. 3.0.1 programing environment (R Core Team 2013) using an ANOVA. Samples were assessed for normality and pairwise comparisons were conducted using an LSD test with the package 'agricolae' v. 1.1-4.

In mothur, OTUs were used to calculate Shannon and Simpson diversity metrics, and to compute dissimilarity matrices with the Bray-Curtis dissimilarity index and the Jaccard similarity coefficient. Bray-Curtis dissimilarities incorporate abundance, while Jaccard dissimilarities use presence and absence of OTUs. The pair-wise distances were used to conduct a principal coordinates analysis (PCO) in PRIMER-E v. 6.0. We conducted linear regression analysis of the axis scores computed from the PCO analysis using phenolic glycosides and condensed tannins as predictive variables. Linear regression was conducted on the PCO axis scores from the first axis of the PCO conducted on the Bray-Curtis dissimilarity matrix. This analysis was not conducted on subsequent axes, or from the PCO produced from the Jaccard matrix, because the axes explained a relatively small percentage of the variation (see Results).

We found that two OTUs comprised 25% - 98% (average = 48%) of the relative abundance across the samples, so we assessed the effects of genotype and ingestion on them individually. Numbers of OUT sequences were log(y+1) transformed to fit normality assumptions, and analyzed in R with the statistical package 'nlme' v. 3.1-109. OTUs were analyzed with an ANOVA using genotype and herbivory as fixed effects and plant as a random effect. Differences in OTU relative abundances between the paired foliar and midgut met normality assumptions, and samples were analyzed using an ANOVA with genotype as a fixed effect followed with pairwise comparisons conducted with the package 'agricolae.' We then assessed how the phenolic glycosides and condensed tannin concentrations influenced the differences in relative abundance between foliage and midgut samples of the most abundant OTUs using linear regression.

Results:

Foliar concentrations of phenolic glycosides (Fig. 1A) and condensed tannins (Fig. 1B) differed among plant genotypes. SAU3 had significantly higher (45%) concentrations of phenolic glycosides than WAU2, but not PG2 ($F_{2,6} = 5.583$, $P = 0.043$). Salicortin, tremulacin, and salicin were the most prevalent phenolic glycosides. Tremulacin and salicortin comprised 98% of the total dry weight percentage in approximately a 6:5 ratio. PG2 had the highest concentration of condensed tannins; it was significantly higher (43%) than SAU2, but not WAU2 ($F_{2,6} = 10.057$, $P = 0.012$). Concentrations of phenolic glycosides did not correlate with condensed tannin concentration among clones ($P = 0.755$; $R^2 = 0.015$).

454 pyrosequencing yielded 68,857 high-quality sequences prior to subsampling. After processing and subsampling, OTUs ranged from 18-109 among both midgut and foliage samples. Samples from foliage had an average of 50% more OTUs than samples from midguts ($F_{1,6} = 7.145$, $P = 0.037$), but neither genotype nor its interaction with whether the community was foliar or midgut influenced the number of OTUs. There was no effect ($P > 0.05$) of genotype, foliar or midgut tissues, or their interaction, on Shannon and Simpson diversity indices of the bacterial communities.

Two-dimensional ordination analyses were conducted with Bray-Curtis and Jaccard dissimilarity matrices computed from OTUs. Overall, plots generated from principal coordinates analysis (PCO) did not appear to form distinct groups (Fig. 2). The Bray-Curtis PCO did not produce tight clustering of groups in two-dimensions (Fig. 2A). Samples from the midguts formed loose groupings by genotype, but there was substantial overlap from communities of foliage samples. Foliar and midgut samples from several genotypes were oriented in close proximities, indicating similar bacterial community composition. In contrast, midgut samples appeared to form a gradient along BC-PCO1, which described 40.8% of the variation between the communities. Larvae feeding on SAU3 tended to have a higher value along BC-PCO1 than either WAU2 or PG2. Foliar samples did not have such a pattern. The PCO constructed using Jaccard dissimilarities yielded separation between sample types. Midgut samples tended to cluster on the right side of the graph, while foliar samples were to the left (Fig. 2B). However, axes in Jaccard PCO explained very little (J-PCO1: 11.7%; J-PCO2: 9.7%) of the total variation in the communities.

The midgut samples appeared to arrange along the x-axis by genotype (Fig 2A; BC-PCO1). Therefore, we assessed whether foliar defense chemicals explained the orientation of leaf and midgut bacterial communities along this coordinate. We conducted linear regression using the phenolic glycoside and condensed tannin concentrations as explanatory variables for the axis scores of BC-PCO1 (Fig. 3). Total phenolic glycoside (Fig. 3A), tremulacin (Fig. 3B), salicortin (Fig. 3C), and condensed tannin concentration (Fig. 3B) did not describe the position of the foliar samples along the BC-PCO1. In contrast to the foliar samples, tremulacin (Fig. 3F), salicortin (Fig. 3G), and total phenolic glycosides described the variation observed in the bacterial communities in midgut samples along this coordinate (Fig. 3E). Midguts from insects that fed on trees having higher phenolic glycoside concentrations had larger axis scores. Condensed tannins also helped explain some of this variation in the axis scores of the midgut samples, but to a lesser extent (Fig. 3H). The relationship between the axis score and foliar condensed tannin concentration was the opposite of that for phenolic glycosides; higher condensed tannins correlated with a smaller axis score.

We assessed the effects of genotype and ingestion on the relative abundances of the two most prevalent OTUs, *Ralstonia* and *Acinetobacter* (Fig. 4). *Ralstonia* abundances were not influenced by genotype ($F_{2,6} = 1.703$, $P = 0.260$) or whether they were on the foliar or in the midgut environment ($F_{1,6} = 0.734$, $P = 0.424$). However, interactions between foliar and midgut properties influenced OTU abundances ($F_{2,6} = 5.294$, $P = 0.047$). *Ralstonia* abundances were larger in the midguts of insects feeding on the WAU2 and PG2 genotypes than on the corresponding foliage (Fig. 4B), but were smaller in the gut than foliage when feeding upon the SAU3 genotype ($F_{2,6} = 11.722$, $P = 0.008$). In contrast, total *Acinetobacter* abundances (Fig. 4A) were not influenced by genotype ($F_{2,6} = 0.901$, $P = 0.452$), environment ($F_{1,6} = 0.295$, $P = 0.606$), or by their interaction ($F_{2,6} = 2.476$, $P = 0.164$). The difference in the relative abundance of *Acinetobacter* between foliage and the larval midgut varied among plant genotypes (Fig. 4B). *Acinetobacter* had lower relative abundances in the midgut compared to the foliage of WAU2 and PG2, but had increased abundances in the midguts of insects feeding on SAU3 compared to the corresponding foliage ($F_{2,6} = 4.832$, $P = 0.056$).

We analyzed the difference between *Ralstonia* and *Acinetobacter* relative abundance using total phenolic glycoside and condensed tannin concentrations as separate continuous variables. Phenolic glycoside concentrations described the differences in *Ralstonia* abundances between foliar and midgut samples (Fig. 5A), but not for *Acinetobacter* ($R^2 = 0.154$; $P = 0.297$). *Ralstonia* had lower abundances in the midguts than the foliage when insects fed on plants with higher phenolic glycoside contents. While not statistically significant, higher abundances *Acinetobacter* in the midgut tended to occur when insects consumed trees with high phenolic glycoside concentrations (Fig. 5B). Condensed tannin concentration described these relationships better than phenolic glycosides. With high foliar tannins, the abundance of *Ralstonia* in the midgut increased compared to corresponding plant foliage (Fig. 5C), while the abundances of *Acinetobacter* in the midgut decreased (Fig. 5D).

Discussion:

We assessed the influence of aspen genotypes that vary in defense chemistries on bacterial communities of leaves and in midguts of gypsy moth larvae . Gypsy moth larvae exhibited a different composition of bacteria in their midgut than of the leaves they were consuming. Phenolic glycoside and condensed tannin concentrations did not influence foliar bacterial communities. However, both groups of compounds influenced the bacterial composition in larval midguts. This suggests that the partitioning in cellular vacuoles limits effects of metabolites on bacteria of foliage, but upon insect feeding and rupturing of the plant cells, these compounds affect both the herbivore and the community structure of insect midgut bacteria. We propose a general model of these processes in Figure 6. In this model, different foliar chemical concentrations yield different bacterial communities when secondary metabolites are released. Similar responses by herbivore-associated bacterial communities to plant chemical defenses have been documented in mammal (Kohl and Dearing 2012) and arthropod guts (Mason et al. 2014), and in sub-epidermal plant tissues excavated by bark beetles (Adams et al. 2013) and leaf miners (Humphrey et al. 2014).

Several behavioral and physiological factors could potentially influence the composition of larval midgut bacterial communities. Gypsy moth larvae acquire the majority of their community from foliage they consume. Potential variation in feeding sites within or among plants, along with the accompanying microbial associates, could affect these relationships. For example, in our study we found that the bacterial community of both midgut and foliar samples varied considerably within a clone (Fig. 2). Therefore, the ingestion of bacteria that either benefit or impair larval success (Mason et al. 2014) may vary in time and space. However, despite the initial variation in the communities that seed the gypsy moth midgut, the resulting midgut consortia become more similar under similar chemical conditions (Fig. 3). As gypsy moth acquires bacteria, these symbionts face a radically different environment from that of the foliar surface. Fluids are much more abundant in the gut than on the foliage, the pH is much higher (Appel and Maines 1995), and oxygen tensions are much lower (Johnson and Barbehenn 2000). Under these new and extreme environmental conditions, different bacteria may be better suited to colonize the midgut, while others are more restricted by this barrier (Mason and Raffa, 2014). Within this context, host plant defense chemistries introduce an additional component to the structuring of insect midgut bacterial communities (Fig. 3).

The effects of foliar chemical defenses on midgut bacterial communities could arise from two nonexclusive processes: directly on the microbial community members, or indirectly by affecting the host midgut and physiological processes. Many plant chemicals are toxic to both insect and bacteria. Bacteria show differential tolerances to plant chemical defenses, and also vary in their toxin metabolic capacities (Anderson et al. 1993, Adams et al. 2011, Boone et al. 2013, Miller et al. 2014). The two most abundant OTUs detected in this study, *Acinetobacter* and *Ralstonia*, have previously been shown to change in relative abundance due to phenolic glycosides in artificial diets, and to vary in their *in vitro* metabolic capacities (Mason et al. 2014). Indirect effects on community composition may be mediated through changes in the gut. Phenolic glycosides are more damaging than condensed tannins to the midguts of lepidopterans. Deterioration of the midgut lumen induced by phenolic glycosides could potentially alter sites at which bacteria colonize. Although the exact colonization sites of microbiota within the gypsy moth midgut are unknown, bacteria in other Lepidoptera have been shown localize in the mucosal layer (Shao et al. 2014).

The relative extents to which phenolic glycosides, condensed tannins, and their interactions affect gypsy moth midgut bacterial composition remain unclear. In *Populus,* condensed tannins have been shown to reduce litter decomposition rates (Driebe and Whitham 2000), and colonization by commensal (Bailey et al. 2005) and phyotpathogenic fungi (Holeski et al. 2009). However, effects of condensed tannins on bacteria are less understood. Likewise, considerably more research has been conducted on how phenolic glycosides affect herbivores than microbial associates. Because concentrations of phenolic glycosides and condensed tannins were not correlated within aspen clones, it is probable both chemical groups exert some effects. Specifically, these compounds may affect the bacteria differently, whereby some species have low tolerance for one class of compounds while others can tolerate compounds more effectively. However, which of these processes predominate, and how they interact, is not known. Our data suggest that plant phytochemistry mediates the competition among bacteria within the midgut, producing communities with distinct structures despite initial variation in acquisition.

The results presented here show that bacterial community composition in the gypsy moth midgut is influenced by aspen foliar defense chemistry. The midgut bacterial sequences that increased in association with genotypes under high phenolic glycoside concentrations have been shown to metabolize these compounds (Mason et al. 2014). However, we do not know how these changes would affect herbivore fitness when feeding on these various genotypes. Future research is needed to determine how relationships between plant defense chemistry and midgut communities affect herbivore performance, the relative roles of different chemical components, and what properties of gut physiology contribute to gut community structure across a broad range of insect herbivores.

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Fig. 1: Mean phenolic glycoside and condensed tannin concentrations of three aspen genotypes. Different letters represent statistically significant differences at $P < 0.05$. Bars represent +1 standard error.

Fig. 2: Principal coordinates analysis plots constructed using Bray-Curtis (A) and Jaccard (B) dissimilarity matrices of 97% similarity bacterial OTUs. Bacterial communities originate from either foliage (open shapes) or gypsy moth midgut tissues (filled shapes).

Fig 3: Relationship of foliar total phenolic glycoside (A and E), the phenolic glycosides tremulacin (B and F) and salicortin (C and G), and condensed tannin concentration (D and H) with the axis scores of the first principal coordinate from Fig. 2A (x-axis; BC-PCO1). The principal coordinate analysis was generated from Bray-Curtis dissimilarities. Different shapes represent different aspen genotypes being WAU2 (Δ), PG2 [O], and SAU3 [□]. The top row (open shapes) includes samples from foliage (A-D) and the bottom row (closed shapes) contains samples from midguts of gypsy moth larvae feeding on foliage (E-H). BC-PCO1 axis scores of the foliage samples were not influenced by foliar chemical composition.

Fig. 4: Abundances of the two most prevalent OTUs across all samples. Number of sequences of the two OTUs classified as *Ralstonia* and *Acinetobacter* from each sample (A) and the differences in relative abundance between the paired midgut and foliage samples (B). Bars represent 1 standard error.

Fig. 5: Difference in relative abundance between foliar and midgut populations of *Ralstonia* and *Acinetobacter* OTUs in relation to dry weight concentrations of phenolic glycosides (A and B) and condensed tannins (C and D). The dashed line at zero indicates there was no difference between the abundance in the larval midgut and foliage. Any number above zero indicates the OTU had a higher relative abundance in the midgut than the foliage.

Fig. 6: Conceptual diagram of interactions among phyllosphere bacteria, foliar chemistry, and herbivores. Bacteria (*Ra* = *Ralstonia*; *Ac* = *Acinetobacter*; X, Y, Z = other species) on aspen foliage experience little contact with phenolic glycosides (PGs) or condensed tannins (CTs). Gypsy moth larvae acquired their midgut bacteria by consuming foliage. Herbivory ruptures plant cells, releasing chemical defenses. Foliar chemicals interact with foliar bacteria, jointly structuring the midgut community. When insects feed on foliage with a ratio of higher phenolic glycosides to condensed tannin, *Acinetobacter* exceed *Ralstonia*. The opposite chemical environment favors a community in which *Ralstonia* yield *Acinetobacter*. The extent to which these changes are mediated through direct contact with the phytochemical, or indirectly through the host, is unclear.

Fig. 1:

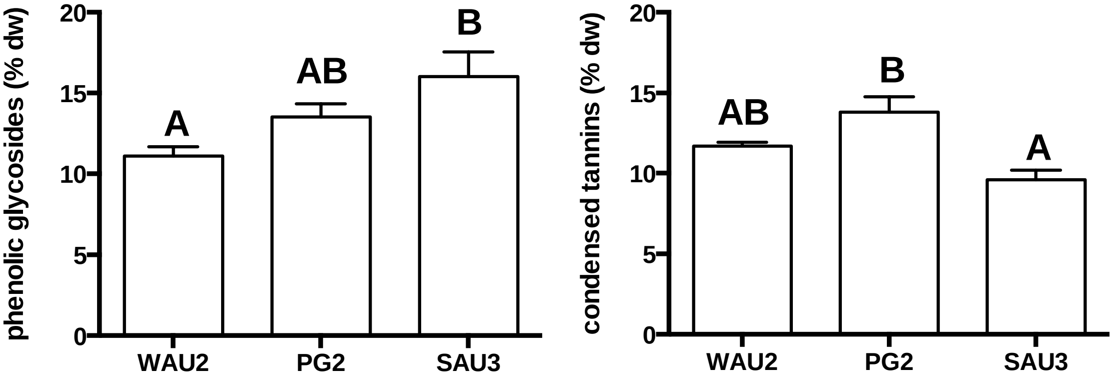

phenolic glycosides (% dw)
20
15
10
5
0
A
AB
B
WAU2
PG2
SAU3
condensed tannins (% dw)
20
15
10
5
0
AB
B
A
WAU2
PG2
SAU3

Fig. 2:

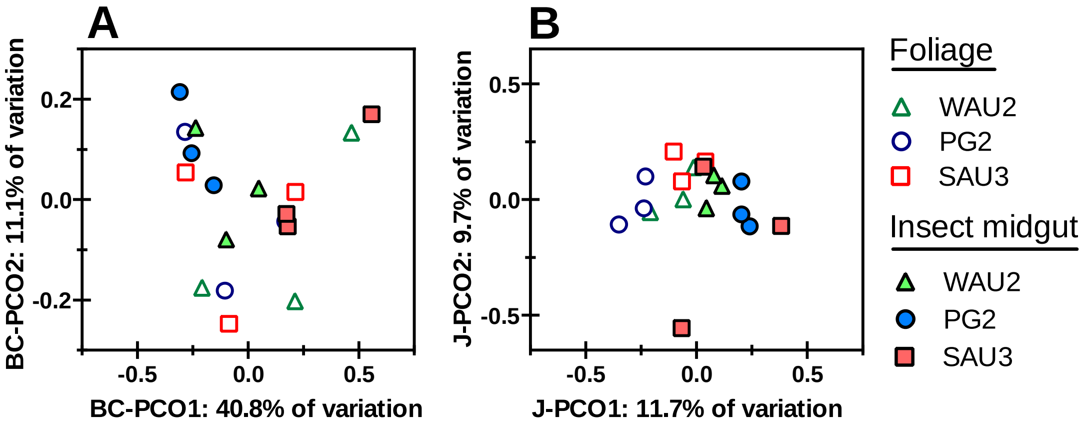

A
B
BC-PCO2: 11.1% of variation
0.2
0.0
-0.2
-0.5
0.0
0.5
BC-PCO1: 40.8% of variation
J-PCO2: 9.7% of variation
0.5
0.0
-0.5
-0.5
0.0
0.5
J-PCO1: 11.7% of variation
Foliage
WAU2
PG2
SAU3
Insect midgut
WAU2
PG2
SAU3

Fig 3:

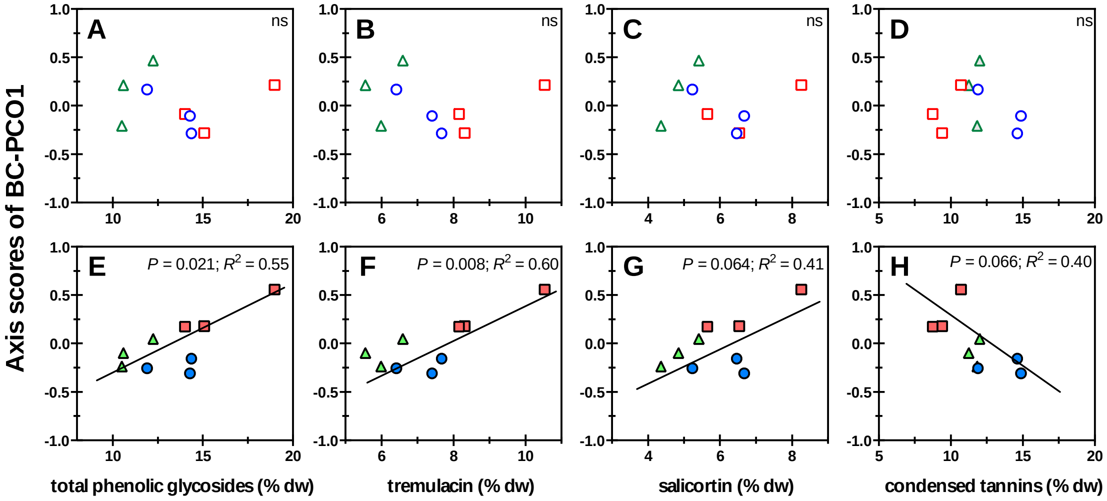

Axis scores of BC-PCO1
A
B
C
D
ns
E
P = 0.021; R2 = 0.55
F
P = 0.008; R2 = 0.60
G
P = 0.064; R2 = 0.41
H
P = 0.066; R2 = 0.40
1.0
0.5
0.0
-0.5
-1.0
total phenolic glycosides (% dw)
tremulacin (% dw)
salicortin (% dw)
condensed tannins (% dw)

Fig. 4:

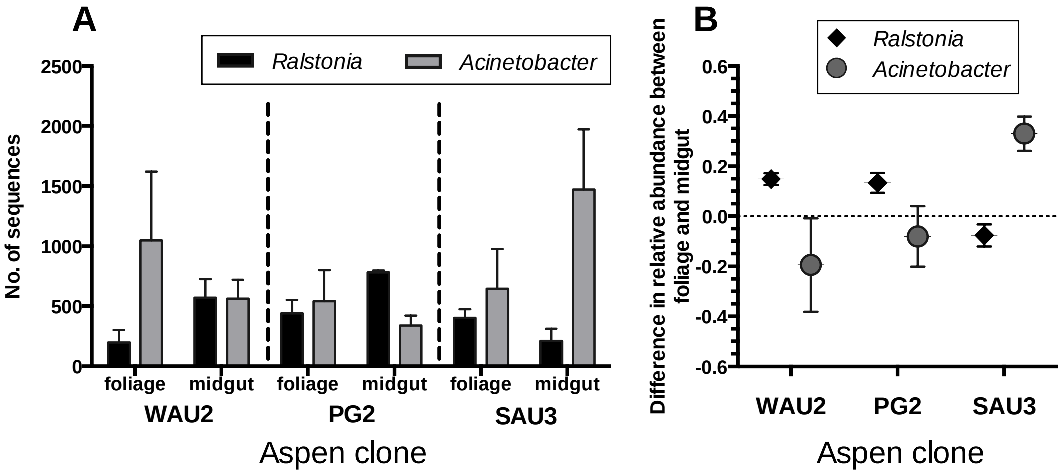

A
Ralstonia
Acinetobacter
No. of sequences
2500
2000
1500
1000
500
0
foliage
midgut
foliage
midgut
foliage
midgut
WAU2
PG2
SAU3
Aspen clone
B
Ralstonia
Acinetobacter
Difference in relative abundance between foliage and midgut
0.6
0.4
0.2
0.0
-0.2
-0.4
-0.6
WAU2
PG2
SAU3
Aspen clone

Fig. 5:

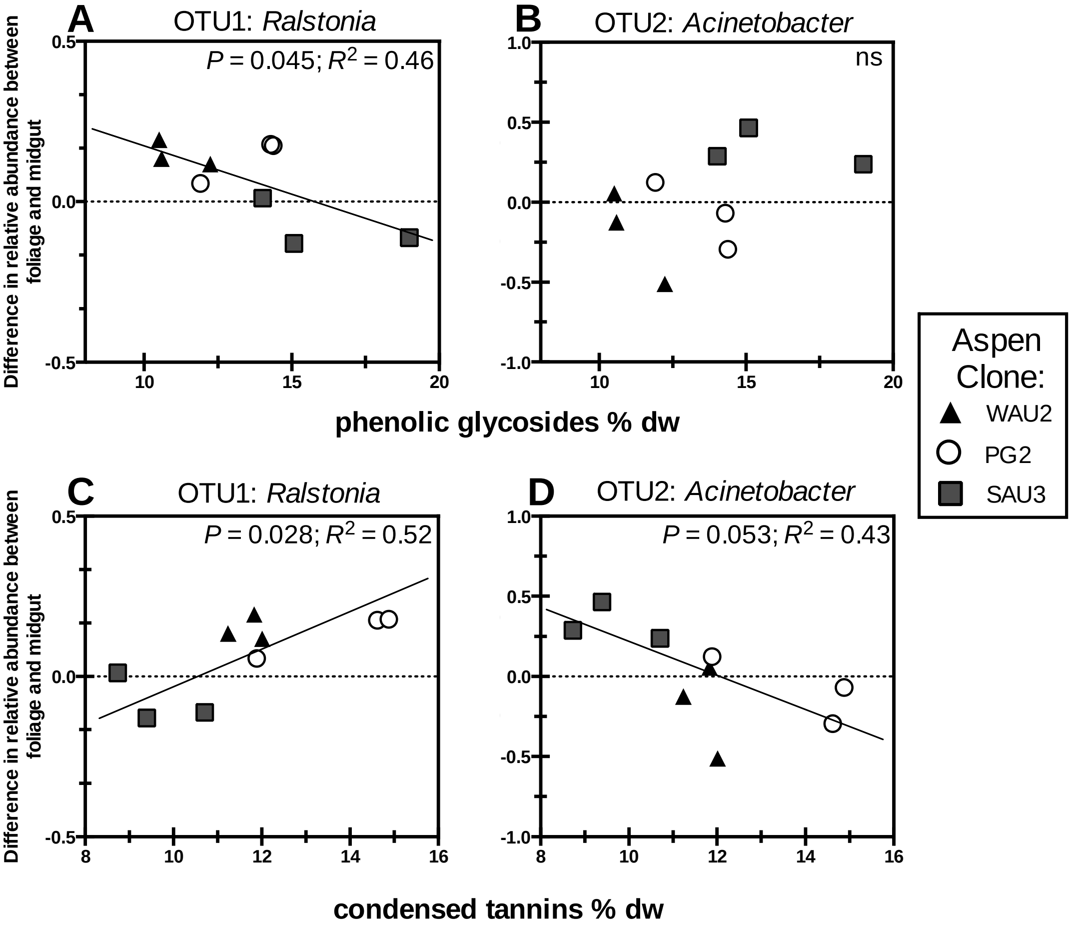
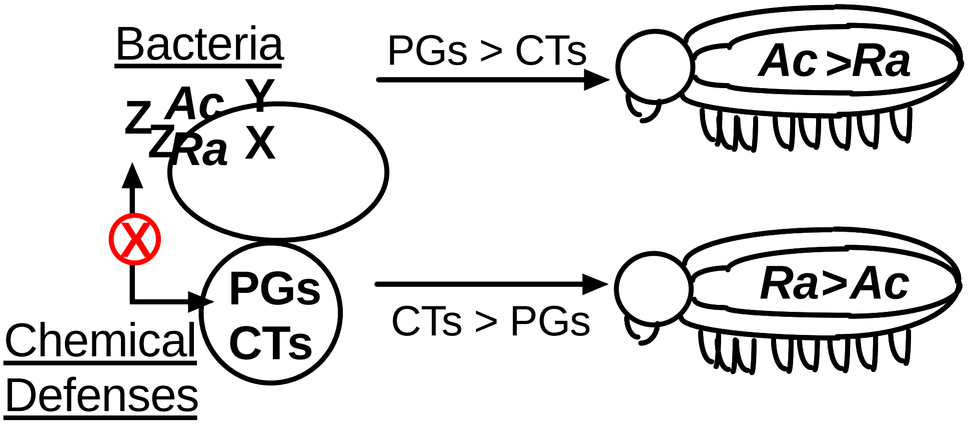

A
OTU1: Ralstonia
P = 0.045; R2 = 0.46
B
OTU2: Acinetobacter
ns
C
OTU1: Ralstonia
P = 0.028; R2 = 0.52
D
OTU2: Acinetobacter
P = 0.053; R2 = 0.43
Difference in relative abundance between foliage and midgut
0.5
0.0
-0.5
1.0
-1.0
10
15
20
8
12
14
16
phenolic glycosides % dw
condensed tannins % dw
Aspen Clone:
WAU2
PG2
SAU3

Fig. 6:


Bacteria
Z Ac Y
Z Ra X
PGs > CTs
Ac >Ra
PGs
CTs
Chemical
Defenses
CTs > PGs
Ra>Ac

Thesis Conclusions

1. **Bacterial communities of the gypsy moth midgut are obtained primarily from the foliage it consumes, but are subsequently structured by the midgut environment.** Gypsy moth larvae had a similar community composition to that of its foliage. However, the communities were more distinct from foliage in later instars. Some bacteria species proliferated in the midgut, while others became substantially reduced in abundance compared to the diet. This indicates that certain bacteria are better adapted than others to the extreme conditions of the gypsy moth midgut.
2. **Egg mass - associated bacteria have an overall minor contribution to the bacterial community of the larval midgut.** Different sources of egg masses had varying community composition, where field-collected egg masses were highly diverse and those from a rearing facility were dominated by one taxa. Despite there large differences in starting bacterial communities, the resulting midguts were very similar. In most cases, these differences were attributed to bacteria that are absent from the egg mass community.
3. **After extended feeding, gypsy moth midgut bacteria are relatively stable throughout development.** Only minor differences were observed between the midgut bacterial community composition of third and fifth instar larvae. Moreover, frass was also similar to midguts. This indicates that these communities do not undergo drastic changes in structure in response to additional immigration. The similarities of the frass bacterial

communities to the midgut suggest that hindgut conditions do not substantially alter the bacteria.

4. **Despite feeding on different host trees, larvae develop similar midgut bacterial communities.** Gypsy moth larvae feeding on different host plant species ultimately attained a similar community composition. This similarity arises despite differences in bacterial communities among different host plants. This further supports the view that features of the midgut ultimately result in stable midgut bacterial communities.
5. **Bacteria can improve gypsy moth larval growth, but only in the presence of the aspen defense compounds, phenolic glycosides.** Gypsy moth larvae that were inoculated with bacteria enriched from aspen leaves had increased growth when phenolic glycosides were included in artificial diet. This was not accompanied by increases in consumption. The growth response was not observed when phenolic glycosides were excluded from diet. This was not due to the addition of bacteria to the insect, as enrichments from insect midguts were not different from the water controls.
6. **Bacteria respond differently to phenolic glycosides in the larval midgut and *in vitro*.** Insects inoculated with aspen bacteria had different communities when phenolic glycosides were included in the diet. These changes were attributed to two OTUs, *Ralstonia* and *Acinetobacter*. In the larval midguts, *Ralstonia* decreased in relative abundance with phenolic glycoside inclusion in the diet. Conversely, *Acinetobacter* increased in relative abundance under the same conditions. These bacteria corresponded to the phenolic glycoside degradation capacities of different bacteria. When phenolic glycosides were added to growth medium, *Ralstonia* did not degrade phenolic glycosides to an appreciable level. However, *Acinetobacter* significantly reduced phenolic

glycosides. Degradation appears to be fairly complete, as there was little accumulation of immediate breakdown products of phenolic glycosides.

7. **Plant genotype and phenotypic have small effects on the foliar bacteria community of trembling aspen.** Foliar bacterial communities were not influenced by host plant genotype or phenotypic differences. Rather, it appears that the community composition of established stands is influenced by other factors. Foliar bacterial communities of trees grown in the common garden had very little variation, but trees grown in the cold frame. The differences were not explained by host plant genotype, or phenotypic characteristics.
8. **Concentrations of foliar defense chemistry influence the bacteria community in larval midguts.** Although aspen genotype and phenotype did not influence foliar bacterial communities, they did influence the bacterial composition of the gypsy moth midgut. This is likely due to the partitioning of defense compounds within the aspen, where these compounds are stored in vacuoles. When leaves are ruptured upon feeding by herbivores, defense chemicals and foliar communities come into contact. The extent to which interactions between foliar chemistry and bacteria are mediated by direct influence on the microbes, indirectly through the host, or a combination, is unknown.
9. **Changes in bacterial abundance in the gypsy moth midgut relate to different chemical classes.** Different chemical classes elicited different bacterial abundances in the gut. Concentrations of phenolic glycosides and condensed tannins did not covary, but both partially explained variation in midgut bacterial communities of gypsy moth. When trees had higher concentrations of condensed tannins than phenolic glycosides, *Ralstonia* had higher relative abundances. In contrast, when there were higher phenolic glycoside than condensed tannin concentrations, *Acinetobacter* had higher relative abundances.

Appendix 1

Bacteria in gypsy moth oral secretions do not inhibit poplar inducible defenses.

Summary:

In this experiment, I tested the influence of bacteria in gypsy moth larval oral secretions on hybrid poplar inducible defenses. I used antibiotics to prevent colonization of bacteria, and used wounding with oral secretion application to induce defenses. Hybrid poplars were propagated from green wood cuttings, maintained under greenhouse conditions, and used larval bioassays to assess induction. While there was an induced response to the wounding and oral secretion application, I did not observe an effect of bacterial removal on the induced defenses in poplar. This suggests that, at least in this system, gypsy moth-associated bacteria are not involved in the manipulating host plant inducible defenses.

Introduction:

Plants are capable of eliciting defensive responses to insect herbivores and microbial pathogens. The signaling pathways that initiate changes in plant defense interact extensively (Felton & Korth, 2000; Thaler *et al.*, 2002). Herbivores contain multiple properties that plants may serve as cues for, and also cause more mechanical damage to plant tissue than microbial pathogens (Felton & Tumlinson, 2008). Microbes are capable of overcoming host defense by minimizing signals until a threshold is reached. However, some groups of microbes have features that allow plants to key into defense, elicit signaling cascades, and prevent colonization even if they are not pathogenic (Garcia-Brugger *et al.*, 2006). Herbivores are frequently associated with microbes. These associates can be intracellular and obligate, or colonize different body components and be significantly more facultative. These microbial components may affect plant defensive signaling. Indeed, symbiotic fungi have been shown to elicit defensive signaling against bark beetles in conifers (Paine *et al.*, 1997). Bacteria have received recent attention in how they may affect plant responses against herbivores. For example, the bacterium, *Wolbachia,* in leaf miners can inhibit plant senescence (Kaiser *et al.*, 2010) and several bacteria in Colorado potato beetle oral secretions can inhibit tomato inducible defenses, effectively masking the effects of herbivory (Chung *et al.*, 2013).

In this experiment, I investigated whether the removal of bacteria in gypsy moth affected the influence of oral secretions on induced responses of hybrid poplar to herbivory. I used a hybrid poplar clone previously identified to be highly inducible (Havill & Rafffa, 1999), and conducted insect bioassays on the leaves of the induced trees.

Materials and Methods:

Plant and insect culturing:

Hybrid poplar clone NC5271 (*P. nigra* x *P. nigra*) was vegetatively propagated from greenhouse-maintained tissue. Shoots were collected from actively growing branches. Tissue was cut into several 5 cm segments allowing for approximately 0.5 cm above the bud and petiole. Leaves were left intact to the segments. Cuttings were dipped into distilled water, and then into Hormex #8 Rooting Powder (Chatsworth, CA). Cuttings were planted into Metro Mix soil media in a flat containing poriferations. Propagules were maintained under a 15 s misting regimen occurring every 10 minutes at a 16:8 light dark photoperiod until roots were established. Plants were potted into 3:1 Metro Mix - peat moss mixture in Classic 300 pots. Trees were fertilized with 15-15-15 Osmocote slow-release fertilizer. Trees were maintained in a greenhouse (16:8 photoperiod, 24°C) for 4-6 weeks prior to the experiment.

Two groups of gypsy moth larvae were reared for this experiment. Both groups were obtained from a USDA lab-maintained colony (Buzzards Bay, MA), and reared on artificial diet in a growth chamber (16:8 photoperiod, 24C, 50% humidity). The first group was reared to induce plant tissues. Larvae were either reared with, or without an antibiotic cocktail included in the artificial diet. The antibiotics included 500 mg / L of rifampin, gentamycin, penicillin, and streptomycin. Insects were reared until 5th instar. Oral secretions were collected from larvae using a capillary pipet. Oral secretions were stored at 4°C for, at most, 48 h. The second group was reared as bioassay organisms. Upon molting into the 3rd instar, larvae were isolated and starved for 24 h prior to bioassay.

Plant induction and bioassay:

Trees were randomly selected and assigned a treatment. Leaf numbers were assigned on trees starting with the first fully expanded leaf. The first leaf was designated a leaf position index (LPI) of one. Induction treatments were done to LPI 6, and LPI 4 was used as the bioassay leaf. Tree defenses were induced by wounding by a hemostat and application of 10 μL of larval oral secretions. Oral secretions were applied evenly over the wound site with an autoclaved paintbrush. Control treatment was conducted by applying 10 μL of sterile water over the induction leaf.

24 h post-induction LPI 4 leaves were removed at the petiole, placed into plastic bags, and transported to the lab on ice. Leaves were inserted into a water pik, and placed into a 100 mm – diameter arena. Bioassay larvae were weighed and randomly assigned a treatment. Larvae were allowed to feed on material for two days after the start of the bioassay. Upon completion, insects were weighed again, and relative growth rate for the larvae was calculated. Data were analyzed using an analysis of variance with a Tukey’s HSD post-hoc correction.

Results:

Gypsy moth larvae that fed on leaves from trees receiving wounding and oral secretion application had significant reductions in their growth (F = 4.344, *P* = 0.021). Wounding and oral secretions reduced larval growth rates by 83% (Fig. 1). However, larval oral secretions from those fed antibiotics did not influence host plant induction. Antibiotic inclusion in the artificial diet limited the number of culturable bacteria present in gypsy moth oral secretions. No bacteria were cultured in the antibiotic treatments, while a full complement was present when antibiotics were excluded.

Discussion:

Recent studies have indicated that symbiotic bacteria are able to mediate host plant responses to herbivory to benefit insect feeding on that material (Kaiser *et al.*, 2010; Chung *et al.*, 2013). The results of my study suggest that this phenomenon does not appear to occur gypsy moth – poplar interactions. This was not a factor of bacteria being absent from both control and antibiotic treatments, as bacteria were isolated from insects that were not fed antibiotics. Possible include bacterial species associated with these components, and the amount of bacteria present in the secretions. Different bacteria have different features that could be integral for modulating host plant responses. For example, flagellated bacteria can induce these components readily. In the Colorado potato beetle system, the flagella of the bacteria in the oral secretions were partially responsible for the inhibition of induction (Chung *et al.*, 2013). Bacterial titers may also play an important role in affect host plant induction (Casteel *et al.*, 2012), and the amount of bacteria in gypsy moth oral secretions may not be high enough to inhibit responses.

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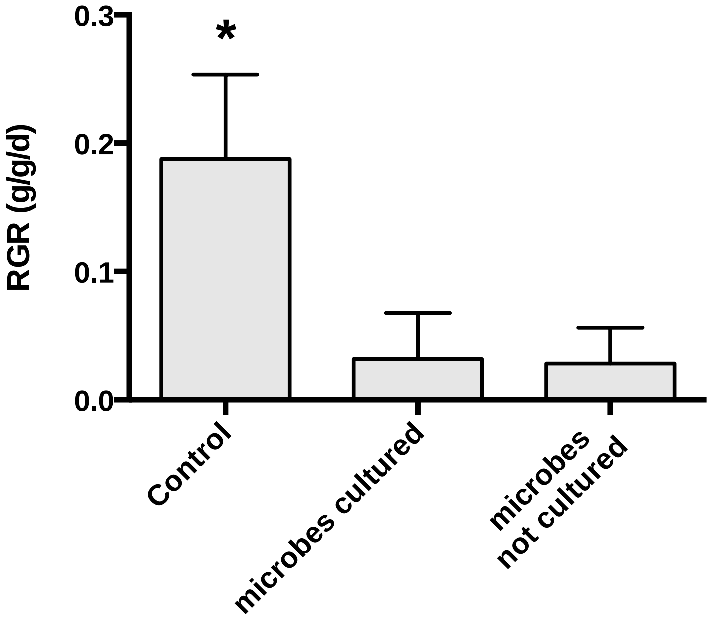
Figure 1: Relative growth rates of 3rd instar gypsy moth larvae feeding on induced or non-induced leaves of hybrid poplar. Induction was done with oral secretions from larvae fed artificial diets with antibiotics present (bacteira not cultured) or absent (bacteria cultured). Asterisk indicates significant difference at $P < 0.05$.



Appendix 2:

Larval feeding does not alter the foliar community of hybrid poplar.

Summary:

In these experiments, I investigated the effect of gypsy moth herbivory on bacterial communities of hybrid poplar foliage using 454 pyrosequencing. I found that while there were differences between communities of the larval midgut and foliage, there were only minor differences in bacterial communities among foliar samples. These results suggest that foliar bacterial communities are resistant to disturbance associated with gypsy moth herbivory, but do undergo disturbances once consumed and encountering the midgut.

Introduction:

Microbial communities are susceptible to both biotic and abiotic disturbances which can have drastic effects on community composition and functioning (Shade *et al.*, 2012). Among biotic agents, there are multiple mechanisms by which they can alter the community or habitat. These can include the facilitation of introduction of new members, changing chemical natures of the environment, physically changing aspects of the environment, or by some combination. These changes range from minor to drastic. Insects are among organisms that may drastically alter microbial communities.

In the field of plant – herbivore interactions, it has become increasingly recognized that microbiota may affect the relationship, but also that the converse is true. Recent examples of insects disturbing plant microbial communities include pollinators (Aleklett *et al.*, 2014), leaf miners (Humphrey *et al.*, 2014), bark beetles (Adams *et al.*, 2013), and folivores (Müller *et al.*, 2003). In addition to directly disturbing the communities, systemic inducible defenses can also affect plant – microbial relationships. For example, induction by phytohormone application can influence the structure of these communities (Kniskern *et al.*, 2007).

In this study, I investigated the effects of gypsy moth (*Lymantria dispar* L.) herbivory on the foliar bacterial communities of a hybrid poplar previously shown to be systemically inducible (Havill & Rafffa, 1999). I assessed the bacterial composition of leaves receiving, and not receiving herbivory and compared them to corresponding larval midguts. I hypothesized that local and systemically induced leaves would have different bacterial communities than that of the control trees.

Methods:

Plant and insect culturing: Hardwood cuttings of hybrid poplar clone NC5271 (*P. nigra* x *P. nigra*) were obtained from dormant trees maintained in a common garden. In early Summer 2012, cuttings were soaked cuttings in distilled water for 48 h at 25ºC under a 16:8 light – dark photoperiod to break dormancy. Trees were potted in 7.5 c diameter cone pots in MetroMix soil media, which were positioned to allow the bottom portion of the cone was submerged in a flat of water. After observing visible rooting, trees were transplanted to 3.8L plastic pots in MetroMix, fertilized with 14-14-14 Osmocote slow-release fertilizer, and transported outdoors to cold frames. Plants were flood irrigated daily for two months before our experiment.

Gypsy moth egg masses were obtained from a USDA APHIS laboratory culture (Otis, MA). Insects were reared on wheat germ artificial diet in a growth chamber (25ºC, 50-70% RH). Larvae were maintained until 4th instar, at which point they were used in our experiment.

Experimental design and tissue collection: A graphical depiction of the experiment and the bacterial communities that we evaluated is observed in Fig. 1. At the time of the experimental treatments, plants were transported into a greenhouse having the aforementioned conditions. All trees had five undamaged leaves collected prior to the experiment. Trees were randomly selected and assigned treatments. Single 4th instar gypsy moth larvae were caged onto entire treatment trees using mesh cloth. Cages were sterilized in 10% bleach, rinsed in autoclaved distilled water, and air-dried. Both herbivory and no herbivory treatments were caged. Insects were allowed to feed for 72 h. Samples of leaves that were partially eaten were collected, along with samples of uneaten leaves from the same tree. All leaf samples were collected in a sterile plastic bag and stored at -80ºC until processing. At the same time, leaves were sampled from control trees. Midguts from the larvae feeding on the trees were ascetically removed and stored until DNA extraction.

DNA extraction: Plant material was homogenized in liquid nitrogen in a mortar and pestle. 250-400 mg of ground leaf material was added to sterile 2 mL screw-cap microcentrifuge tubes after which 500 μL preheated (65ºC) CTAB buffer preheated was added. Samples were incubated at 65ºC for 1 hour, and homogenate was vortexed every 15 min. After incubation, an equal volume of phenol: chloroform: isoamyl alcohol (24:24:1) was added, samples were briefly vortexed, and centrifuged at 4ºC. Supernatant was collected, transferred to a fresh tube, and an equal volume of chloroform: isoamyl alcohol (24:1) was added. Samples were centrifuged, supernatant was collected, and an equal volume of cold 100% isopropanol was added and incubated overnight at -20ºC to precipitate DNA. DNA was pelleted and rinsed twice with 70% ethanol before eluting in sterile TE (10 mM Tris-HCl, pH 8.0; 1.0 mM EDTA, pH 8.0).

Larval midgut tissue was homogenized in 2 mL vials in 500 μL CTAB using a 4 mm diameter steel bead. All subsequent steps were identical to the procedures done for the plant tissue.

16S-rRNA gene amplification: PCR and sequencing conditions followed protocols described in Hanshew et al. (2013). The V6V8 region of the 16S-rRNA gene was amplified 799F-mod6 and 1392R. Triplicate PCRs were conducted using 25 ng template DNA, 0.25 μL

Herculase II DNA polymerase (Agilent), 1.0 nM dNTPs, 0.5 µL DMSO, 5 µL buffer, 300 nM forward and reverse primers, and water totaling a final volume of 25 µL. Reaction conditions were: 95ºC for 2 min, 30 cycles of 95ºC for 20 s, 48ºC for 30 s, 72ºC for 30 s, and a final elongation of 72ºC for 3 min. PCR products were pooled and gel extracted with a Zymoclean Gel DNA Recovery Kit (Zymo Research, Irvine, CA). Second PCR was completed using 2 µL of the recovered PCR product. Primers in the second PCR contained the A- and B- adaptors required for 454 sequencing along with 5 bp multiplex identifiers (MIDs). PCR conditions were identical except only 10 cycles were performed. The ~700 bp product were purified by gel extraction and quantified. Equimolar concentrations of samples were diluted and pooled for 454 pyrosequencing. 454 pyrosequencing was completed on a GS-Junior using modifications described in Hanshew et al. (2013).

454 Data processing: The 454 16s-rRNA amplicon data were processed in mothur v.1.32.0 following recommendations outlined in the Schloss standard operating procedures (Schloss *et al.*, 2009, 2011) with an effort to minimize sequencing errors. Data were denoised via PyroNoise. Sequences were processed as to allow for no differences in MIDs and primers, no homopolymer exceeding six nucleotides, and a minimum length of 200 nucleotides. Sequences were aligned to the SILVA SEED database, and a preclustering step was conducted to reduce sequencing errors. Chimeras were detected using UCHIME and discarded (Edgar *et al.*, 2011). Sequences were classified using a mothur formatted Ribosomal Database Project training set (v. 9.0) at an 80% confidence threshold (Wang *et al.*, 2007). Sequences classified as chloroplast, eukaryotic, or unknown at the Kingdom taxonomic level were removed from further analyses. Samples were found to have a sequence determined to be bacteria, but otherwise was unclassified which was removed from the analysis. Operational taxonomic unit (OTU) analyses were conducted by clustering at 97% similarity.

Statistical analyses: In mothur, dissimilarity matrices were computed using Bray-Curtis dissimilarities. The pair-wise distances were used to conduct non-metric multidimensional scaling (MDS) in PRIMER-E v. 6.0. A permutation-based MANOVA was conducted between samples in PRIMER using Bray-Curtis with herbivory treatment as a fixed effect. The PERMANOVA was conducted using 9,999 permutations with Type I sums of squares using an unrestricted permutation. Numbers of sequences of the three most abundant individual OTUs were analyzed using an ANOVA in R v. 3.0.1 with post hoc comparisons conducted with a Tukey HSD test in the 'agricolae' package. Larval midguts and leaves receiving herbivory were analyzed using a two-sample t-test.

Results:

Sequence curation and general trends

454 pyrosequencing yielded xxx sequences across the 56 samples in the study. During the processing of sequences, a portion of the abundance were classified as Bacteria, but unclassified at finer taxonomic levels. NCBI BLASTn indicated that closest matches of these sequences were plastid sequences. Therefore, I removed these sequences from our analysis, and subsampled to 675 sequences for each sample. Rarefaction estimates indicated that adequate sampling at this level was achieved. A number of samples had low numbers of sequences after quality curation, so they were removed from subsequent analyses. One additional sample found to be very different from all others, was considered an outlier, and was removed from subsequent analyses.

The number of OTUs in the samples ranged from 10 to 34. Overall, the five most abundant OTUs comprised approximately 80% of the total abundance. In comparing the poplar foliage samples, there was no difference between samples on the number of observed OTUs ($F_{3,31} = 2.56$; $P = 0.072$), Simpson diversity indices ($F_{3,31} = 1.78$; $P = 0.172$), but there were differences in Shannon diversity ($F_{3,31} = 2.87$; $P = 0.052$) where the control had higher estimates.

Herbivory does not alter leaf foliar bacterial communities.

In the first set of analyses, I only evaluated the bacterial communities associated with the poplar foliage. An MDS plot of the data did not reveal any clear clustering of the leaf bacterial community samples by treatment (Fig. 2A). With the exception of three samples, there was very little separation between samples. These observations were supported with a PERMANOVA of the Bray-Curtis dissimilarity matrix with these treatments.

While abundances of the most prevalent individual OTUs varied among treatments (Fig 3), a pattern related to presence or absence of herbivory was not present. There were no differences between *Ralstonia* and *Bradyrhizobium* among herbivory treatments. *Acinetobacter* had significantly higher abundances ($F_{3,31} = 4.018$; $P = 0.016$) associated with uneaten leaves from trees receiving herbivory than either controls or partially consumed leaves.

Bacterial communities in gypsy moth midguts differ from the leaves receiving herbivory.

In contrast to the poplar foliage, the gypsy moth larval midguts had very different bacterial communities. MDS ordination analysis showed that with midguts included, the foliar samples were clustered together (Fig 2B). In contrast, the larval midguts were positioned along the perimeter of the cluster. Additionally, the midgut samples had a degree of variation that was not observed in the foliar samples.

Since the larval midguts were intimately associated with the poplar foliage that they consumed, I compared the most abundant OTUs with the foliage that received herbivory. Larval midgut samples had lower abundances of *Ralstonia* than the foliage (Fig 4A; $t = 7.316$, $P <$

0.001), and tended to have higher abundances of *Acinetobacter* (Fig 4C), where the effect was marginally significant (t = 2.032, *P* = 0.08). In contrast, there was no significant difference in the abundances of *Bradyrhizobium* among the foliage and larval midguts (Fig 4B).

Discussion:

Contrary to my hypothesis, there were few discernible differences among foliar bacterial communities. I did not observe significantly different communities, regardless of localized herbivory or systemic induction. This contrasts with other studies finding effects of leaf feeding on bacterial communities (Müller *et al.*, 2003; Humphrey *et al.*, 2014) However, differences of bacterial communities among foliage and gypsy moth larval midguts were observed, where different bacteria were either fewer or greater in abundance than the foliage. This supports some of my other findings in the system (Mason and Raffa 2014, Mason et al 2014, chapter 4), in which the midgut conditions exert effects that cause shifts in bacterial populations.

There are a few possibilities for the minor effects of gypsy moth feeding on foliar bacterial communities. One may be the nature of the feeding. In contrast to leaf miners, gypsy moth consumes large portions of the leaf. Therefore, chemical changes in response to herbivory may only be present along the feeding edge. Also, gypsy moth larvae may not harbor bacteria on its exterior that could adequately colonize foliage. Finally, there may be features of hybrid poplar that enable bacterial communities resistant to this type of disturbance. In one of my previous chapters, I found that aspen genotype had minor effects on bacterial communities, and similar processes may be present here.

Ultimately, these results provide further guidance for future studies in this system. The limited effects of herbivory on foliar bacteria, but apparently stronger effects of the midgut once those microbes are consumed, has been observed frequently. Identifying the factors in which these microbes respond, and their subsequent effects on the larval host, are areas of research that should be pursued.

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Figure 1: Diagram of experimental design.

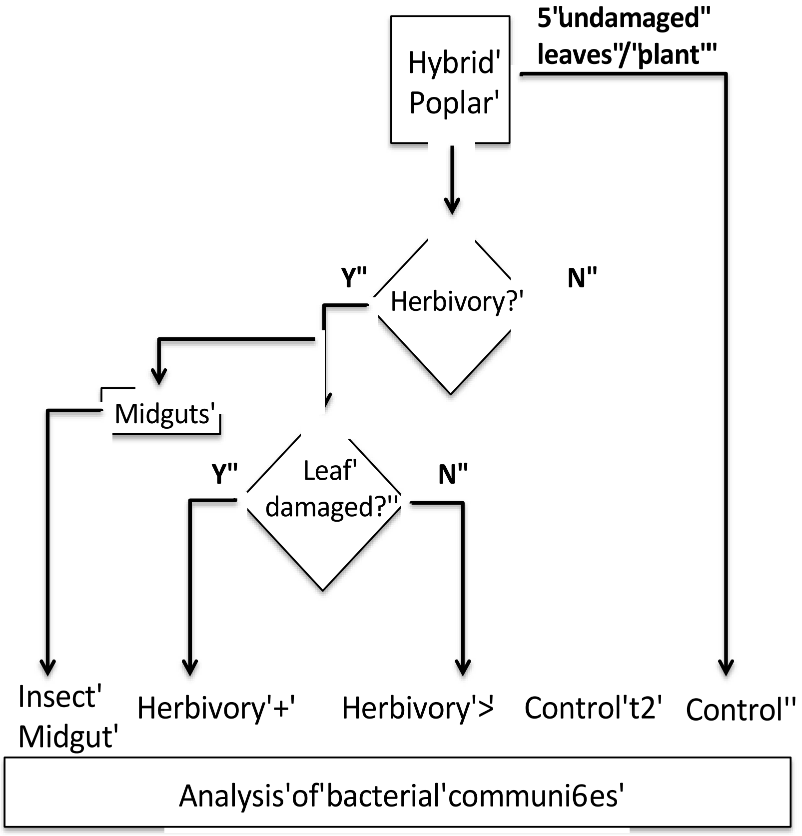

Figure 2: MDS plots of bacterial communities of poplar foliage excluding (A) or including (B) the larval midgut samples. MDS plots were created using OTUs at a 97% similarity cutoff with Bray-Curtis dissimilarites.

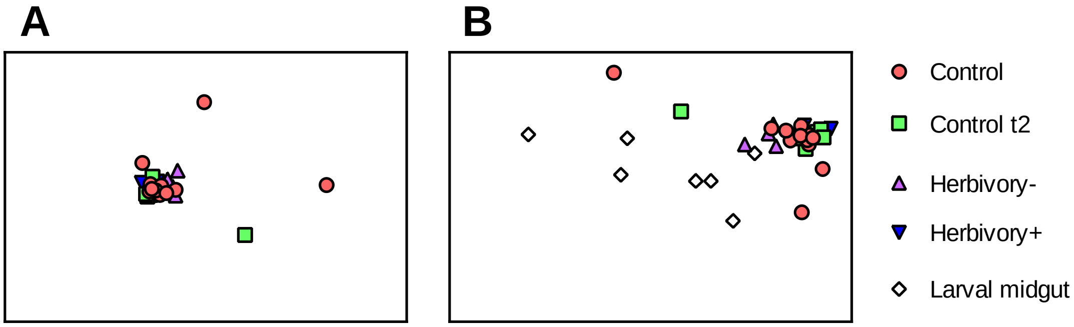

Figure 3: Number of sequences of the three most abundant OTUs across all samples. Bars represent +1 standard error. Asterisks indicate a significant difference at $P < 0.05$.

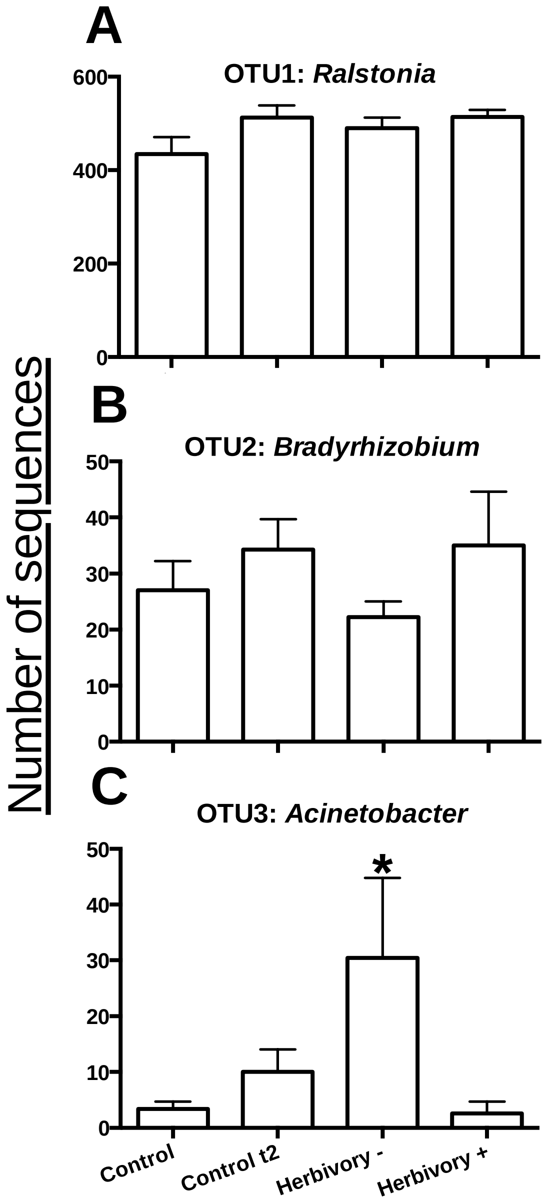

Figure 4: Number of sequences of the three most abundant OTUs across all samples. Bars represent +1 standard error. Asterisks indicate a significant difference at $P < 0.05$.