

Legitimate visitors and nectar robbers of *Aquilegia formosa* have different effects on nectar bacterial communities

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Abstract. Metacommunity structure is strongly influenced by dispersal between habitat patches. Dispersal mode (e.g., active or passively via vector, wind, or water) is recognized to influence metacommunity dynamics, but it is not well understood how within-mode heterogeneity impacts dispersal and community assembly, particularly for microbial communities. Microbes often rely on flower visitors for dispersal among short-lived floral nectar habitats, but it is unclear whether flower visitor guilds (e.g., legitimate visitors vs. larcenists) differentially influence nectar microbial diversity and community structure. We surveyed the community of legitimate nectar foragers and nectar robbers, which damage flowers to obtain floral rewards, of *Aquilegia formosa*. Then, we evaluated how manipulating access by legitimate nectar foragers, primary nectar robbers, and/or secondary nectar robbers influenced the diversity, species composition, and beta diversity of nectar bacteria within individual flowers. A taxonomically diverse insect community visited *A. formosa*, and visitors differentially influenced nectar bacterial community structure at within-flower (local) and among-flower (regional) scales. When legitimate nectar foragers were allowed to access *A. formosa*, we observed an increase in bacterial diversity and changes in bacterial species composition such that common nectar bacteria had higher relative abundances. In contrast, effects of natural and simulated robbing had little effect on bacterial alpha diversity, but simulated robbing decreased the relative abundance of common nectar bacteria, and natural nectar robbing events reduced beta diversity of nectar bacteria. This work highlights the importance of visitor identity on microbial diversity and species composition in flowers, and, more broadly, suggests that vectors can differentially influence metacommunity structure.

Key words: *Aquilegia formosa*; dispersal; floral larceny; flower visitors; legitimate visitor; nectar microbes; nectar robber.

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INTRODUCTION

Dispersal between habitat patches is a major factor driving metacommunity assembly and structure (Leibold et al. 2004). Variation in dispersal rate is typically assumed to arise from neutral processes such as spatial heterogeneity (Jacobson and Peres-Neto 2010) or stochasticity (Lowe and McPeck 2014), but can also arise from variation in organismal traits such as dispersal

mode (e.g., active or passively via wind, water, or vectors; Van De Meutter et al. 2007, Vanschoenwinkel et al. 2008, Ozinga et al. 2009). Many organisms rely on vectors for dispersal to habitat patches, including plants (Nathan 2007), animals (Green and Figuerola 2005), fungi (Malloch and Blackwell 1992), and bacteria (Hellberg and Chu 2016), but the relative influence of different vectors on dispersal and community assembly is not well understood (Vanschoenwinkel et al. 2008).

Vectors may differentially influence assembly due to variation in dispersal rate, the organisms they vector (i.e., portion of the regional species pool), or behavior that modifies habitat characteristics, which can affect the trajectory of community assembly (i.e., niche modification; Fukami 2015).

Phoresis among suitable habitats may represent an important factor shaping the structure of microbial communities, particularly for specialized microbes (Purcell 1982). Although once considered ubiquitous, microbial presence in habitat patches, and especially on ephemeral habitat patches, can be limited by dispersal (Lindström and Langenheder 2012). Variation in dispersal frequency can influence the structure of microbial communities (Vannette and Fukami 2017), and the order of arrival can influence competitive dynamics (Peay et al. 2012). However, for microbial communities, the role of dispersal in driving metacommunity assembly and structure has been difficult to investigate (e.g., Lindström and Langenheder 2012), and there is much to be learned regarding the role of dispersal vectors in structuring microbial communities. Plant-associated microbes mediate many plant traits (Friesen 2013), plant–animal interactions, and the composition of microbial communities can influence the magnitude and direction of these effects (Sugio et al. 2015). Therefore, it is important to understand the factors influencing microbial communities at local (alpha diversity, species composition) and regional scales (beta diversity).

Nectar-inhabiting microbes are an ideal system to study how variation within a dispersal mode can influence microbial community structure. Yeast and bacteria are common inhabitants of floral nectar (Herrera et al. 2009, de Vega et al. 2009, Pozo et al. 2011, Golonka and Vilgalys 2013), and their effects on plant–pollinator interactions can be strong and are dependent on the species of microbes present (Herrera et al. 2013, Vannette et al. 2013, Good et al. 2014, Schaeffer and Irwin 2014). Nectar microbial communities are highly heterogeneous in space and time, varying among nectaries, flowers, plants, plant populations, plant species, and throughout the season (Herrera et al. 2008, 2009, de Vega et al. 2009, Belisle et al. 2012, Golonka and Vilgalys 2013, Jacquemyn et al. 2013, Aizenberg-Gershtein et al. 2017). Whereas heterogeneity in nectar microbial community

composition is well documented, the factors that explain nectar microbial community structure across scales remain elusive (Mittelbach et al. 2015). Dispersal by flower visitors is thought to be a critical factor for nectar microbial community assembly: Excluding visitors eliminates or greatly reduces microbial presence in nectar (Belisle et al. 2012, de Vega and Herrera 2012, Schaeffer et al. 2014). As flowers are short-lived (Willmer 2011) and nectar resources can be depleted rapidly by microbes (Vannette et al. 2013, Good et al. 2014), nectar-inhabiting microbes continually rely on vectors for dispersal to new habitat patches. However, whether flower visitor type is important in influencing nectar microbial community composition is not well understood. Most angiosperms are associated with diverse flower visitor communities (Waser et al. 1996). Because flower visitors vary in many aspects of natural history (e.g., frequency of visitation, floral niche breadth, nectar-foraging behavior, flower handling time, and body size; Willmer 2011), it seems likely that visitors may also vary in their influence on nectar microbial communities.

Here, we explore how two flower visitor guilds, legitimate nectar foragers and nectar robbers, influence nectar microbial communities in western columbine, *Aquilegia formosa*. Legitimate nectar foragers enter flowers as expected given the morphology of the flower (Inouye 1980). In contrast, nectar robbers circumvent specialized floral morphology by chewing through the corolla to access nectar, and secondary robbers utilize already existing robbing wounds to consume nectar (Inouye 1980). We hypothesized that these visitor guilds would differentially influence microbial communities for two reasons. First, legitimate nectar foragers, primary nectar robbers, and secondary nectar robbers of a plant species can be composed of disparate visitor species, which may vector distinct portions of the microbial species pool (although some species can exhibit both legitimate and robbing behaviors depending on an individual's size or foraging bout; Willmer 2011, Richardson and Bronstein 2012). Second, foraging behavior of nectar robbers may influence nectar microbial community assembly if wounding the flower changes the conditions such that nectar becomes more or less habitable to microbes relative to unrobbed flowers (i.e., niche modification).

To explore whether access by different flower visitor guilds differentially influences nectar bacterial communities of *A. formosa*, we (1) surveyed the community of legitimate nectar foragers and nectar robbers, and (2) evaluated how manipulating access by legitimate nectar foragers, primary nectar robbers, and/or secondary nectar robbers influenced nectar bacterial communities within flowers (alpha diversity, species composition) and among flowers (beta diversity). We hypothesized that (1) flowers accessible by both legitimate nectar foragers and robbers would have a higher alpha diversity of bacteria than flowers for which we blocked visitor access, and (2) flowers accessible by legitimate nectar foragers would have higher relative abundances of common nectar bacteria.

MATERIALS AND METHODS

Study system

Western columbine, *A. formosa* (Ranunculaceae), is an herbaceous perennial that produces relatively few red and yellow nodding flowers with long nectar spurs (Chase and Raven 1975). The species is protandrous, and stamens tend to mature halfway through a flower's life, typically ranging from 5 to 10 d (Chase and Raven 1975). *Aquilegia formosa* is considered to be pollinated solely by hummingbirds and long-tongued bees (Fulton and Hodges 1999). Hodges et al. (2004) noted that unlike nectar-foraging hummingbirds and hawkmoths that visit flowers at all stages, bees may tend to focus on male-phase flowers since they visit *A. formosa* primarily to collect pollen. Although former studies have focused on hummingbird and bee visitors, our preliminary observations revealed a diverse community of flower visitors, including primary and secondary nectar robbers that were comprised of bees, wasps, and flies. The experiment was conducted in a ~2460 m² high elevation wet meadow at the University of California's Sagehen Creek Field Station (2400 m, 39°25'11.52" N, 120°18'27.18" W). The meadow is dominated by herbaceous perennial flowering plants, is surrounded by a sub-alpine old-growth pine–fir forest, and contained over two hundred *A. formosa* individuals.

Flower visitation

In 2015, flowers were manipulated using six experimental treatments (described below) and

observed to estimate visitation rates of legitimate nectar feeders, nectar robbers, and pollen foragers. Haphazardly selected experimental flowers were observed from a distance of at least 5 m for 133 15-min time periods (33.25 h total). Short focal length binoculars were used to aid detection of small flower visitors. For all visiting events observed, we noted the type of visitation attempt (i.e., legitimate nectar feeding or nectar robbing) and whether the attempts were successful (i.e., if the visitor accessed nectar), and when possible, a crude taxonomic description of the visitor (e.g., bumble bee, other bee, fly).

In 2016, patches of non-experimental flowers were observed for sixteen 30-min time periods (8 h total) to estimate visitation rates of different flower visitor guilds. Any visitors observed during these time windows were collected for identification purposes. Visitors were also collected opportunistically throughout the experiment. All visitors collected were identified using the keys in Thorp et al. (1983), Michener et al. (1994), and with the help of taxonomic experts (see *Acknowledgments*).

Experimental treatments

Within the study site, focal flower buds were haphazardly chosen from different stems (presumed to be different individuals), labeled with green tape on the pedicel, and enclosed within green mesh bags. Experimental buds were checked daily to assess their development and health, and to remove arthropods as necessary (e.g., aphids). At anthesis, the bag was removed and a randomly selected treatment was applied (N = 20/treatment). Three types of experimental manipulations were used (Fig. 1). Legitimate nectar foragers were excluded by plugging the entrance to each nectar spur with cotton (spurs plugged). Nectar robbers were excluded by capping each nectar spur with a small plastic tube (spurs capped). Simulated robbing was achieved by piercing the apex of each nectar spur with an ethanol and flame-sterilized dissecting needle to create a wound approximately the same size and location as a natural robbing wound (spurs pierced).

Using these methods, six treatments were established (Table 1): legitimate forager exclusion (spurs plugged), nectar robber exclusion (spurs capped), all visitor exclusion (spurs plugged and capped), accessible by all visitors

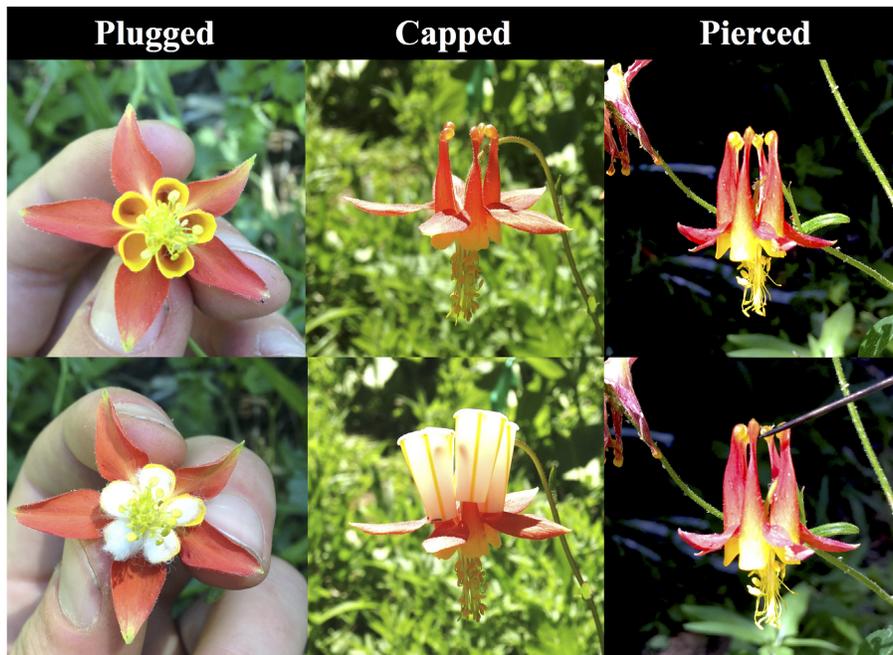


Fig. 1. Experimental manipulations to the corolla of *Aquilegia formosa* flowers utilized in the study: plugging, capping, and piercing each nectar spur of a flower.

Table 1. Experimental manipulations applied to each of the six treatments, and how treatments influenced the ability of different groups of flower visitors to access the nectar.

| Treatment | Access to visitors | | |
|--------------------------|----------------------------|-------------------|---------------------|
| | Legitimate nectar foragers | 1° nectar robbers | 2° nectar robbers |
| Spurs plugged | No | Yes | If naturally robbed |
| Spurs capped | Yes | No | No |
| Spurs plugged and capped | No | No | No |
| Un-manipulated | Yes | Yes | If naturally robbed |
| Spurs pierced | Yes | Yes | Yes |
| Spurs pierced and capped | Yes | No | No |

(un-manipulated), accessible by secondary robbers (spurs pierced), and a final treatment that served to isolate the effects of the robbing wound while excluding robber visitation (spurs pierced and capped). Flowers were checked daily for signs of senescence and to ensure the integrity of treatments. Once flowers began to senesce, as indicated by drooping or discoloration of sepals and elongation of the stigma, they were collected. Experimental flowers were exposed to visitors in the field for 3.52 ± 1.09 d (mean \pm standard deviation). Excised flowers were placed in a cooler, and nectar was collected from all

viable spurs with 5- or 10- μ L microcapillary tubes and frozen in 30 μ L PCR water within 6 h of collection.

Assessment of nectar bacterial communities

DNA from floral nectar was extracted from each sample using the Qiagen DNeasy 96 Blood and Tissue Kit following the manufacturer's protocols. Extracted DNA was sent to the Microbiome Resource Center (Halifax, Nova Scotia, Canada) for amplicon library preparation and MiSeq Illumina sequencing using ITS2 and 16S V4-V5 primers for fungi and bacteria, respectively.

Amplification of chloroplast DNA in 16S reactions was reduced via the addition of pPNA PCR blockers (GGCTCAACCCTGGACAG; PNA Bio, Newbury Park, California, USA).

Sequence data were cleaned and grouped into operational taxonomic units (OTUs) using the UPARSE OTU pipeline. First, low-quality trailing bases were removed with sickle (Joshi and Fass 2011). Next, read pairs were merged with usearch version 5.1 (Edgar 2010), but due to the low merging success rate (32.6%), only forward reads were used in subsequent steps and analyses (McFrederick et al. 2017). Singletons were removed and sequences clustered into OTUs with a 97% similarity cutoff with usearch. Chimeras were removed using de novo detection in usearch and reference-based removal with the GOLD database (<http://drive5.com/uchime/gold.fa>). Operational taxonomic units were then classified taxonomically with the Ribosomal Database Project (RDP) Naive Bayesian rRNA Classifier version 2.11 (Wang et al. 2007). Bacterial taxonomical hierarchy was classified by referencing the RDP 16S rRNA training set 16, and fungi with the Warcup Fungal ITS trainset 2 (Deshpande et al. 2016). Operational taxonomic unit and taxonomy tables were assembled and analyzed in R (R Core Team 2016) using the phyloseq package (McMurdie and Holmes 2013).

PCR using fungal (ITS2) primers was largely unsuccessful (Appendix S2: Fig. S1, Table S1), so only bacterial sequence data from 16S amplicons were utilized for analyses. From these amplicon pools, 179 cyanobacteria/chloroplast OTUs were removed. Negative controls for DNA extraction and PCRs were blank. In total, 2927 bacterial OTUs were detected. Sampling curves revealed that a depth of 1000 reads was sufficient in capturing OTU richness and Shannon diversity of samples (Appendix S2: Fig. S2). Therefore, only samples with 1000 reads or more were included in diversity and community composition analyses. Samples were rarefied to an even depth (1000 reads) to remove bias of unequal sampling depth. After rarefaction, 71 out of 82 samples with sequence data were retained and 174 OTUs were represented in the dataset. Below, we report results from the experiment performed in 2016 (see Appendix S1 for field experiment performed in 2015, where smaller sample size precluded most formal analyses).

Statistical analysis

To assess whether the probability of successful bacterial amplification in each sample varied among manipulations or with nectar volume, we employed binomial models in R (R Core Team 2016). We interpreted no/low amplification as an indicator of bacterial abundance that was below the detectability threshold of the methods used. Utilizing only those samples with detectable bacteria, we performed the following comparisons.

Effect of access by legitimate nectar foragers on nectar bacteria.—To assess the effects of access by legitimate nectar foragers on nectar bacterial communities, we compared all flowers with legitimate nectar feeders excluded (spurs plugged and spurs plugged and capped; N = 14) to flowers that allowed visitation by legitimate nectar feeders (spurs capped, spurs pierced, spurs pierced and capped, and un-manipulated; N = 35). We first compared OTU richness and Shannon diversity between groups using ANOVA for parametric data or the Kruskal-Wallis (KW) test for non-parametric data as determined by the Shapiro-Wilk test of normality ($\alpha = 0.05$). Operational taxonomic unit richness was compared with KW test and Shannon diversity with ANOVA (no covariates were included in the models). Multivariate community dispersion (beta diversity) and species composition based on Bray-Curtis distances were compared using the functions `betadisper` and `adonis`, respectively, in the R package `vegan` (Oksanen et al. 2017). Finally, we identified bacterial OTUs that were differentially abundant between flowers with and without plugged spurs using the R package `DESeq2` (Love et al. 2014).

Effect of simulated robbing wounds on nectar bacteria.—To evaluate whether the wound created by nectar robbing influences the diversity or species composition of nectar bacteria, flowers inaccessible by robbers (spurs capped; N = 14) were compared to flowers that were subject to artificial robbing and immediately capped to block access by secondary robbers (spurs pierced and capped; N = 7). We first compared OTU richness and Shannon diversity between groups using ANOVA for parametric data or the KW test for non-parametric data as determined by the Shapiro-Wilk test of normality at $\alpha = 0.05$ level. Both OTU richness and Shannon diversity were

compared across groups with ANOVA (no covariates were included in the models). Multivariate community dispersion (beta diversity) and species composition (based on Bray-Curtis distances) were compared using the functions `betadisper` and `adonis`, respectively, as above. Finally, we identified bacterial OTUs that were differentially abundant between flowers with and without simulated robbing wounds using the package `DESeq2` (as above).

Effect of natural robbing on nectar bacteria.—We evaluated the influence of natural nectar robbing on nectar bacterial diversity and species composition by comparing all flowers with exposed spurs (spurs plugged or un-manipulated) that were naturally robbed ($N = 4$) to those that were not robbed ($N = 15$). We first compared OTU richness and Shannon diversity between groups using ANOVA for parametric data or the KW test for non-parametric data as determined by the Shapiro-Wilk test of normality at $\alpha = 0.05$ level. Operational taxonomic unit richness was compared with the KW test, and Shannon diversity across groups was compared with ANOVA (no covariates were included in the models). Multivariate community dispersion (beta diversity) and species composition based on Bray-Curtis distances were compared using the functions `betadisper` and `adonis`, respectively, as above. Finally, we identified bacterial OTUs that were differentially abundant between flowers with and without natural robbing wounds with the package `DESeq2` (as above).

Effect of access by secondary robbers on nectar bacteria.—To determine the effect of accessibility by secondary nectar robbers on nectar bacterial diversity and species composition, we compared flowers with artificial wounds accessible by secondary robbers (spurs pierced; $N = 4$) to flowers with exposed spurs that were not naturally robbed and therefore inaccessible by secondary robbers (un-manipulated; $N = 15$). We first compared OTU richness and Shannon diversity between groups using ANOVA for parametric data or the KW test for non-parametric data as determined by the Shapiro-Wilk test of normality at $\alpha = 0.05$ level. Operational taxonomic unit richness was compared across groups with the KW test, while Shannon diversity was compared across groups using ANOVA (no covariates were included in the models). Multivariate

community dispersion (beta diversity) and species composition based on Bray-Curtis distances were compared using the functions `betadisper` and `adonis`, respectively, as above. Finally, we identified bacterial OTUs that were differentially abundant between flowers with and without simulated robbing wounds accessible to secondary nectar robbers with the package `DESeq2` (as above).

RESULTS

Flower visitation

During 33.5 h of timed observations of experimental flowers in 2015, we observed 31 arthropods attempt to forage on floral resources (mean = 0.96 visits per hour). The majority of visitors foraged for pollen (18), six visitors were potential legitimate nectar foragers (four inserted their head or whole body into a nectar spur, while the other two were blocked by cotton), and the other seven visitors landed on capped spurs and were considered to be potential nectar robbers.

There was no difference in visitation rate to flowers with vs. without spurs plugged (KW $\chi^2_1 = 3.22$, $P = 0.07$), with vs. without spurs capped (KW $\chi^2_1 = 3.21$, $P = 0.07$), or with vs. without simulated robbing (KW $\chi^2_1 = 2.12$, $P = 0.15$), indicating that experimental manipulations did not render flowers unattractive to visitors. Therefore, any between-treatment differences in microbial communities are unlikely to be driven by visitation frequency. Given the observed rates of visitation and floral longevity, and assuming daylight-active visitors (14 h), we estimate that on average, each experimental flower could have experienced 45.6 visit attempts, including 19.1 from potential nectar foragers.

During eight hours of timed observations of non-experimental flower patches in 2016, 14 pollen foragers, four nectar robbers, and two legitimate nectar feeders were collected. Overall, including timed observations and opportunistic collections in 2015 and 2016, we observed 18 species foraging for floral resources (Table 2). Legitimate nectar foragers include the bees *Bombus flavifrons*, *Bombus mixtus*, *Hylaeus mesillae*, and *Osmia* sp. and the ant *Leptothorax calderoni*. Species observed robbing nectar included the bees *Bombus bifarius* and *B. mixtus* and the fly *Platycheirus* sp. II. Although

Table 2. *Aquilegia formosa* flower visitors observed during timed observations and opportunistic collections at the study site.

| Visitor order and family | Species | Pollen forager? | Nectar forager? | |
|--------------------------|---------------------------------------|-----------------|-----------------|--------|
| | | | Legitimate | Robber |
| Hymenoptera | | | | |
| Andrenidae | <i>Andrena nigrocaerulea</i> | Yes | No | No |
| Apidae | <i>Bombus bifarius</i> | Yes | No | Yes |
| | <i>Bombus flavifrons</i> | Yes | Yes | No |
| | <i>B. mixtus</i> | Yes | Yes | Yes |
| | <i>Bombus vosnesenskii</i> | Yes | No | No |
| Colletidae | <i>Hylaeus mesillae</i> | No | Yes | No |
| Halictidae | <i>Lasioglossum dialyctus</i> sp. D | Yes | No | No |
| | <i>Lasioglossum sensu strictu</i> sp. | Yes | No | No |
| Megachilidae | <i>Osmia</i> sp. | No | Yes | No |
| Formicidae | <i>Leptothorax calderoni</i> | Yes | Yes | No |
| Diptera | | | | |
| Anthomyiidae | Unknown sp. | Yes | No | No |
| Syrphidae | <i>Chrysotoxum</i> sp. | Yes | No | No |
| | <i>Lapposyrphus laponicus</i> | Yes | No | No |
| | <i>Meligramma trianguliferum</i> | Yes | No | No |
| | <i>Platycheirus albimanus</i> | Yes | No | No |
| | <i>Platycheirus</i> sp. I | Yes | No | No |
| | <i>Platycheirus</i> sp. II | No | No | Yes |
| Coleoptera | | | | |
| Coccinellidae | <i>Hippodamia cf. quinquesignata</i> | Yes | No | No |

hummingbirds (*Selasphorus* spp.) were occasionally observed visiting *A. formosa* at our study site as legitimate nectar foragers, they did not visit *A. formosa* during timed observations in 2015 or 2016.

Effects of treatment on nectar volume and bacterial amplification

Experimental flowers contained 2.24 ± 3.25 μL (mean \pm SD) nectar per spur (range: 0–15.52 μL). Flowers that were damaged or eaten by deer (N = 28) were discarded, leaving a final sample size of 126 flowers to submit for sequencing. Plugging spurs significantly increased nectar volume (Fig. 2a; KW $\chi^2 = 23.5$, df = 1, $P < 0.001$), capping spurs to exclude robbers had no influence on nectar volume (Fig. 2b; KW $\chi^2 = 0.58$, df = 1, $P = 0.45$), and piercing spurs significantly reduced nectar volume (Fig. 2c; KW $\chi^2 = 13.4$, df = 1, $P < 0.001$), which may have been due to the difficulty of collecting nectar from spurs with artificial robbing wounds. After taking into account the positive effect of nectar volume, we found that the probability of bacterial amplification was significantly reduced by plugging spurs, but was not influenced by spur capping or piercing (Table 3).

Although the probability of bacterial amplification was influenced by nectar volume and spur plugging, nectar volume was not significantly related to OTU richness ($R^2 = 0.02$, $P = 0.17$). Therefore, any differences in OTU richness between treatments presented below should not be driven by confounding effects of nectar volume. In contrast, flowers with greater nectar volume tended to have greater bacterial Shannon diversity ($R^2 = 0.11$, $P = 0.01$). Thus, differences in OTU Shannon diversity may be weakly influenced by nectar volume.

Treatments did not influence longevity of experimental flowers or the abundance of aphids on experimental flowers (Appendix S2: Fig. S3).

Effect of treatments on nectar bacterial communities

When all treatments were included in a single analysis, we found that nectar bacterial species composition differed among treatments (Appendix S2: Fig. S4; $F_{5,43} = 2.21$, $R^2 = 0.21$, $P = 0.001$). There was no difference in multivariate dispersion (beta diversity) between treatments ($F_{5,43} = 1.27$, $P = 0.32$).

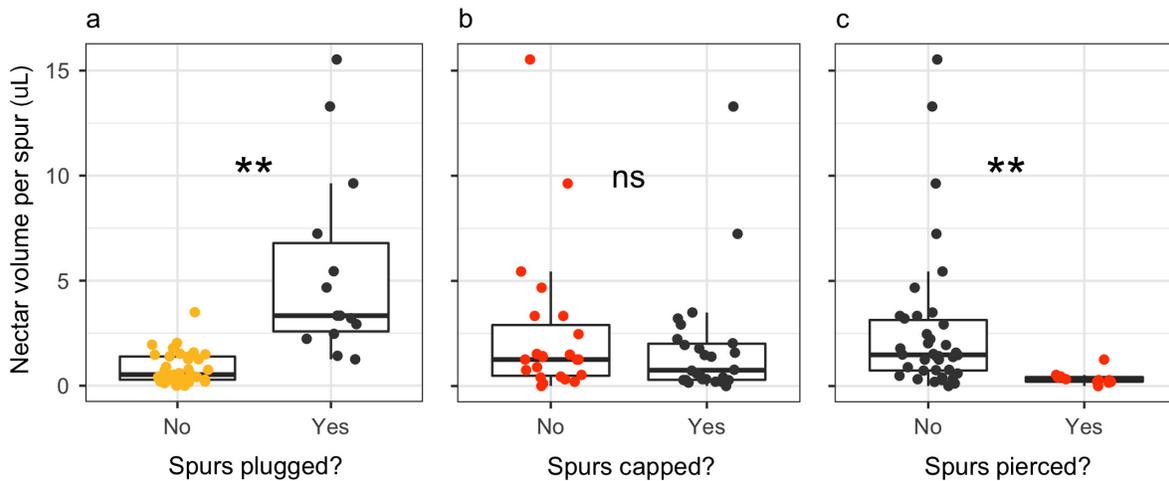


Fig. 2. The effect of experimental manipulations on nectar volume. (a) Plugged spurs had a significantly higher nectar volume than un-plugged spurs. (b) Capping spurs had no significant effect on nectar volume. (c) Pierced spurs had a significantly lower nectar volume than unpierced spurs. Significant differences at the $\alpha = 0.001$ value are demarcated with “**,” and non-significant values with “ns.”

Table 3. Spur plugging and nectar volume influenced the probability of bacterial amplification.

| Spur condition | Bacteria amplified? | | Binomial model results | |
|----------------|---------------------|-----|------------------------|-------------------------|
| | No | Yes | Effect of manipulation | Effect of nectar volume |
| Spurs plugged? | | | | |
| Yes | 26 | 14 | $z = -2.6$ | $z = 2.80$ |
| No | 49 | 35 | $P = 0.01$ | $P < 0.01$ |
| Spurs capped? | | | | |
| Yes | 39 | 26 | $z = 0.20$ | $z = 2.10$ |
| No | 36 | 23 | $P = 0.83$ | $P = 0.03$ |
| Spurs pierced? | | | | |
| Yes | 28 | 11 | $z = -1.00$ | $z = 1.70$ |
| No | 47 | 38 | $P = 0.31$ | $P = 0.08$ |

Effect of access by legitimate nectar foragers on nectar bacteria.—Access by legitimate visitors significantly increased OTU richness (Fig. 3a; KW $\chi^2_1 = 5.58$, $P = 0.018$) and Shannon diversity (Fig. 3b; $F_{1,47} = 8.66$, $P = 0.005$). Bacterial species composition also differed with access by legitimate visitors (Fig. 3c; $F_{1,47} = 4.99$, $R^2 = 0.10$, $P = 0.001$). However, access by legitimate visitors did not affect beta diversity ($F_{1,47} = 1.64$, $P = 0.204$). Three OTUs had a significantly higher relative abundance in flowers with plugged spurs, all of which were un-classified OTUs (OTUs 6, 17, and 2202; Fig. 4a, Appendix S2: Table S2). Six OTUs had significantly higher relative abundance in flowers with

un-plugged spurs that were accessible by legitimate nectar foragers: *Enterococcus* (OTU 278), *Lactococcus* (OTU 19), an Enterobacteriaceae (OTU 76), an Enterococcaceae (OTU 3), and two OTUs identified with confidence to order, a Bacillales (OTU 426) and a Lactobacillales (OTU 16). *Leuconostoc* (OTU 37) had a marginally higher relative abundance in flowers with un-plugged spurs. Many of these important OTUs belong to genera that have been isolated from floral nectar, including *Enterococcus* (Álvarez-Pérez and Herrera 2013), *Lactococcus* (Lenaerts et al. 2017), and *Leuconostoc* (Álvarez-Pérez and Herrera 2013, Samuni-Blank et al. 2014). Others belong to families that have been

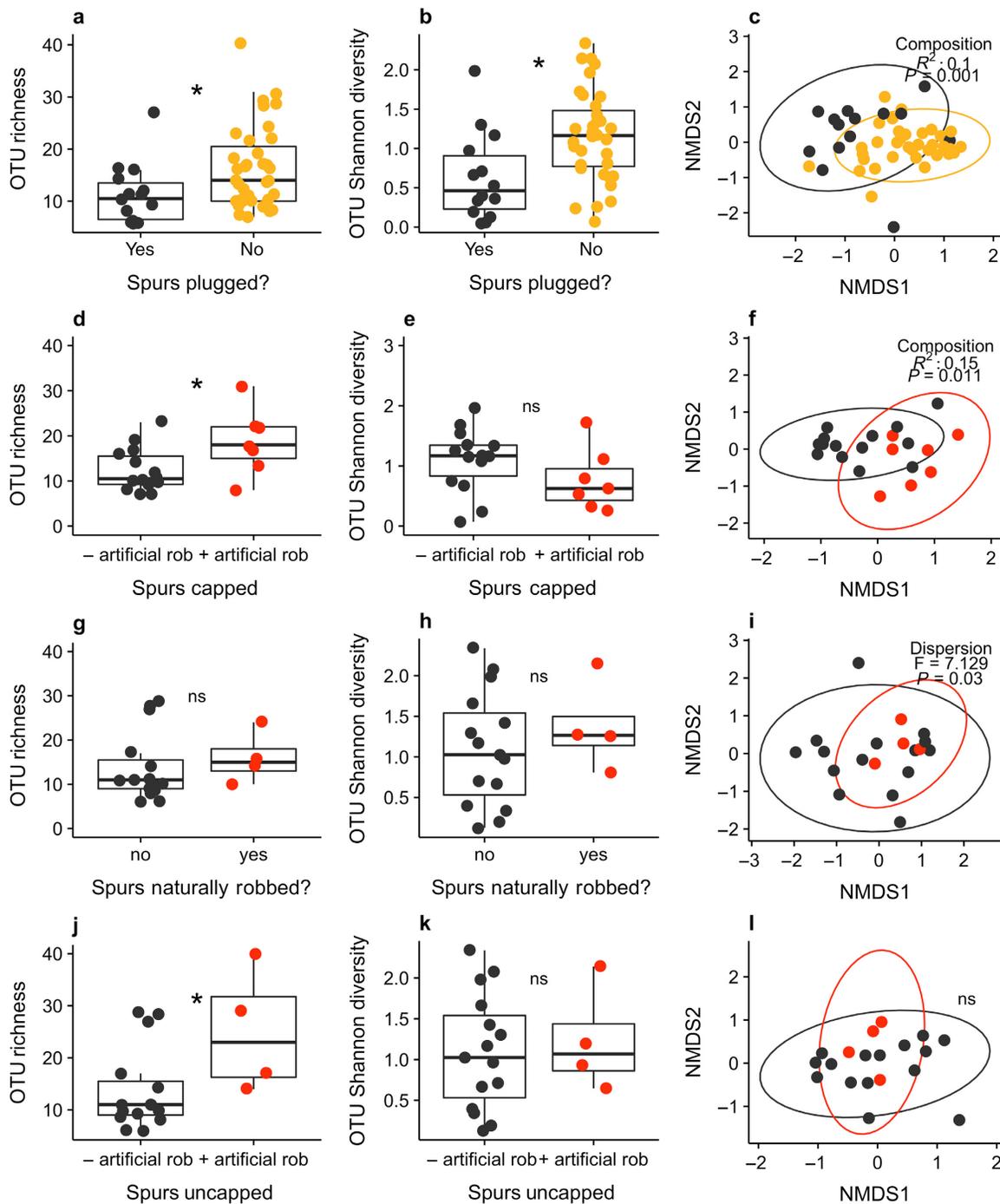


Fig. 3. The influence of access by legitimate nectar foragers (a–c) and nectar robbing (d–l) on bacterial operational taxonomic unit (OTU) richness (left column), Shannon diversity (middle column), and community structure (right column). Yellow points show effects of legitimate nectar foragers, and orange-red points show effects of nectar robbing relative to controls. Significant differences at the $\alpha = 0.05$ value are demarcated with “*,” and non-significant values with “ns.” All NMDS values were plotted with Bray-Curtis distances with two dimensions and stress equal to 0.21 (c), 0.16 (f), 0.14 (i), and 0.17 (l).

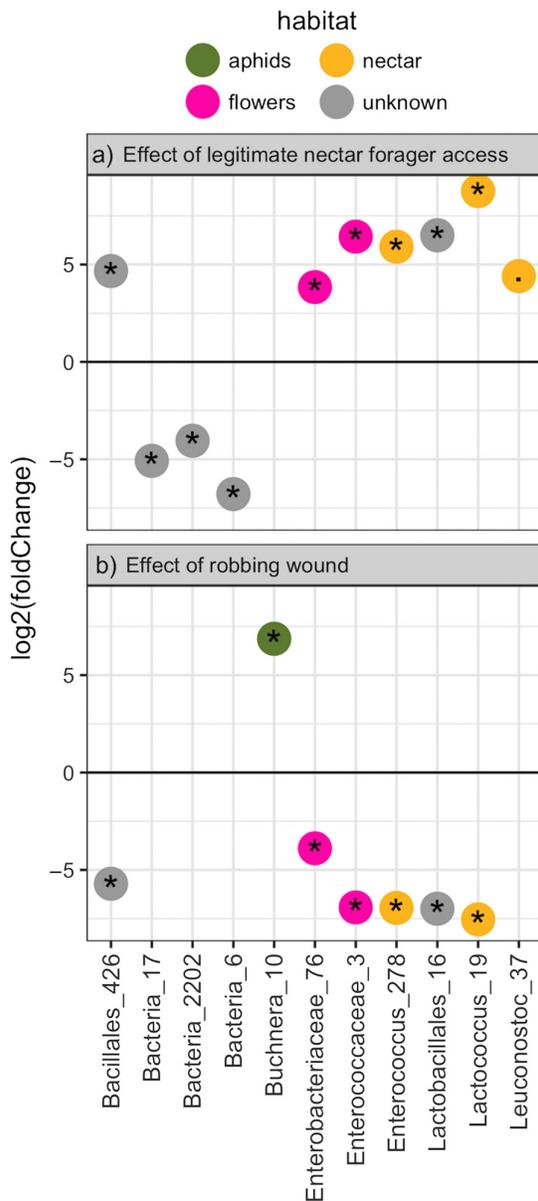


Fig. 4. Differentially abundant bacterial operational taxonomic units (OTUs) from the nectar of *Aquilegia formosa* experimentally manipulated to allow different floral visitation. (a) Effect of legitimate nectar forager access. Positive values denote higher relative abundances in flowers with un-plugged spurs that were accessible by legitimate nectar foragers. (b) Effect of simulated robber wound. Negative values denote lower relative abundances in flowers with pierced and capped spurs. Significant differences are shown with "*" for alpha = 0.05 level and "." for alpha = 0.1 level. See Appendix S2: Table S3 for detailed taxonomic assignments of OTUs.

isolated from floral tissue, including Enterobacteriaceae (Jacquemyn et al. 2013, Shade et al. 2013, Junker and Keller 2015, McFrederick et al. 2017) and Enterococcaceae (Álvarez-Pérez and Herrera 2013). Also, many of these taxa have been recently isolated from hummingbird bills (C. Lee et al., unpublished manuscript).

Effect of simulated robbing wounds on nectar bacteria.—Piercing spurs significantly increased OTU richness (Fig. 3d; $F_{1,19} = 5.74$, $P = 0.03$), but did not significantly influence Shannon diversity (Fig. 3e; $F_{1,19} = 1.94$, $P = 0.18$) or community dispersion ($F_{1,19} = 2.50$, $P = 0.14$). However, piercing spurs did have a significant effect on bacterial species composition (Fig. 3f; $F_{1,19} = 3.39$, $R^2 = 0.15$; $P = 0.01$). In flowers with pierced spurs, *Buchnera* (OTU 10), an obligate aphid endosymbiont (Baumann et al. 1995), had a significantly higher relative abundance, and six OTUs had a significantly lower relative abundance: *Enterococcus* (OTU 278), *Lactococcus* (OTU 19), an Enterobacteriaceae (OTU 76), an Enterococcaceae (OTU 3), a Bacillales (OTU 426), and a Lactobacillales (OTU 16; Fig. 4b; Appendix S2: Table S3).

Effect of natural robbing on nectar bacteria.—A pre-experimental survey of 140 haphazardly censused flowers revealed a natural robbing rate of 22.7% (108/684 spurs). During the experiment, four out of 19 (21.1%) flowers with exposed, un-plugged spurs were naturally robbed (each with one spur robbed). We found that natural robbing had no effect on OTU richness (Fig. 3g; KW $\chi^2_1 = 0.91$, $P = 0.34$) or Shannon diversity (Fig. 3h; $F_{1,17} = 0.28$, $P = 0.60$). However, bacterial communities in naturally robbed flowers had lower beta diversity (Fig. 3i; $F_{1,17} = 7.13$, $P = 0.03$) compared to flowers with exposed spurs that were not naturally or artificially robbed. Natural robbing had no significant effect on community composition ($F_{1,17} = 1.14$, $R^2 = 0.06$, $P = 0.32$). There were no significant differences in OTU abundances in robbed flowers relative to un-robbed flowers.

Effect of access by secondary robbers on nectar bacteria.—Accessibility by secondary nectar robbers significantly increased OTU richness (Fig. 3j; KW $\chi^2_1 = 4.66$, $P = 0.03$), but had no significant effect on Shannon diversity (Fig. 3k; $F_{1,17} = 0.13$, $P = 0.73$), community dispersion ($F_{1,17} = 0.18$, $P = 0.67$), or community composition (Fig. 3l;

$F_{1,17} = 0.83$, $R^2 = 0.05$, $P = 0.60$). There were no significant differences in OTU abundances in flowers that were accessible by secondary nectar robbers relative to flowers that were not accessible by secondary nectar robbers.

DISCUSSION

By assessing the influence of legitimate nectar foragers and nectar robbers, we show that the guild of flower visitor can differentially influence nectar bacterial community composition both within and among flowers. Allowing access by legitimate nectar foragers to *A. formosa* decreased nectar volume, increased bacterial diversity, and altered bacterial species composition. Common nectar bacteria were more abundant in flowers that were accessible to legitimate nectar foragers. Although piercing spurs to simulate the isolated effects of robbing wounds also increased bacterial OTU richness, this manipulation altered species composition of nectar bacteria in a way that reduced the relative abundance of common nectar bacteria. In cases of natural nectar robbing events, the beta diversity of nectar bacteria was reduced. Overall, we show that similar to heterogeneity across dispersal modes, heterogeneity within a dispersal mode (i.e., different vector guilds) influences community structure in different ways, and may be an important factor to consider in studies of dispersal, community assembly, or metacommunity dynamics.

In *A. formosa*, as in other systems, nectar microbial communities are diverse and heterogeneous, and dependent on floral visitation. Notably, many of the bacterial genera associated with differences in floral visitation (Fig. 4) have been previously isolated from floral nectar or floral tissue, including *Enterococcus* (OTU 278; Álvarez-Pérez and Herrera 2013), *Lactococcus* (OTU 19; Lenaerts et al. 2017), and *Leuconostoc* (OTU 37; Álvarez-Pérez and Herrera 2013, Samuni-Blank et al. 2014). Other OTUs were assigned to families that have been isolated from nectar and floral tissue, including an un-classified Enterococcaceae (OTU 3; Álvarez-Pérez and Herrera 2013) and an un-classified Enterobacteriaceae (OTU 76), a family of bacteria that has previously been isolated from nectar (Jacquemyn et al. 2013), bees and pollen (McFrederick et al. 2017),

stamina and styles (Junker and Keller 2015), and apple flowers (Shade et al. 2013). In contrast to bee-pollinated *A. vulgaris* and *A. pyrenaica cazorlensis*, in which most nectar samples contained yeasts (Herrera et al. 2008), we had very limited success in amplifying fungal sequences. This may have been due to low abundance of fungi in the system (Appendix S2: Table S4), differential methods employed in our study vs. Herrera et al. (2008; PCR vs. microscopy), differences in nectar sugar composition (Mittelbach et al. 2015), infrequent visitation by hummingbirds at the study site (Belisle et al. 2012, Vannette and Fukami 2017), bacterial dominance (Tucker and Fukami 2014), or other mechanisms.

Bacterial amplification was highly variable, and many samples did not yield detectable bacterial sequences (Table 3). This may be due to variability in microbial abundance within individual flowers (e.g., Herrera et al. 2009, Rering et al. 2017). Similar to various studies where bagged flowers eliminated or greatly reduced microbial presence in nectar (Belisle et al. 2012, de Vega and Herrera 2012, Schaeffer et al. 2014), failure to amplify bacteria from *A. formosa* flowers was related to vector accessibility, but only when manipulating access by legitimate nectar foragers (Table 3). The bacteria detected in flowers made inaccessible by legitimate nectar foragers, and robbers could be explained by the presence of alternate vectors such as thrips or aphids, both of which were commonly observed on *A. formosa* flowers at our site. Flower visitors that are small or presumed to be inefficient pollinators have rarely been explored as important nectar microbial vectors; however, one study showed that mirids can function as efficient vectors (Samuni-Blank et al. 2014). Nectar bacteria could also be dispersed via other (non-phoretic) modes such as rain or wind, as is the case for other plant-associated bacteria (Lindemann and Upper 1985). As there were no rain events during the course of our 2016 experiment, we can rule out rain as a key vector of bacteria to *A. formosa* nectar. All flowers would have been exposed to wind dispersal after the flowers opened and before treatments were applied. However, given the morphology of *A. formosa* nectaries (nectaries are located at the end of long and narrow nectar spurs, whose opening points downward), wind is likely not as important as it might be in

flowers with exposed nectaries. We hypothesize that low amplification success was likely due to low bacterial abundance within individual flowers and not lack of effective dispersal agents. Pooling nectar or flowers prior to extracting bacterial DNA can circumvent this issue (e.g., Shade et al. 2013), but at the cost of assessing variation among individual flowers. Future work could pool flowers or utilize emerging methods that perform well even with low microbial abundances. Although there was low amplification success and therefore a low number of samples to compare nectar bacterial community structure, we were still able to uncover effects of access by different vectors on bacterial community structure in terms of diversity, composition, and beta diversity utilizing flowers with detectable bacteria.

The degree to which variation in nectar microbial diversity and community structure is driven by variation in flower visitor composition is poorly understood. Variation in nectar microbial species composition has previously been attributed to dispersal frequency (Vannette and Fukami 2017) and competitive exclusion via strong priority effects (Peay et al. 2012). Here, we found that access by legitimate nectar foragers decreased nectar volume (Fig. 2a), increased the probability of bacterial amplification (Table 3), and significantly influenced nectar bacterial diversity and species composition, whereas access by primary and secondary nectar robbers had only weak effects (Fig. 3). For flowers with detectable bacteria, those with accessible (un-plugged) spurs had a significantly higher alpha diversity of nectar bacteria than flowers with plugged spurs (Fig. 3a, b). Flowers accessible by legitimate nectar foragers also had higher relative abundances of common nectar bacteria than those with spurs plugged, including *Enterococcus*, *Lactococcus*, *Leuconostoc*, and the un-classified members of the families Enterobacteriaceae and Enterococcaceae (Fig. 4a, Appendix S2: Table S2). These findings are in accord with studies attributing nectar microbial dispersal to primary pollinators of plants that are legitimate nectar foragers (de Vega and Herrera 2012, Herrera et al. 2013, Vannette et al. 2013, Schaeffer et al. 2014) and suggest that microbial dispersal is frequent enough to compensate for potential

consumption of nectar and nectar bacteria by legitimate nectar foragers (Hausmann et al. 2017).

It is also possible that plugging spurs influenced bacterial diversity and species composition via pathways other than blocking access by legitimate nectar foragers. Cotton plugs may have precluded other dispersal modes such as rain or wind, although, as discussed above, these pathways are not likely to be as important as animal vectors in our system. Cotton plugs also may have altered the floral microenvironment. However, as with previous studies that have excluded visitors with mesh bags, it is unknown how our manipulations influenced the floral microenvironment, and we cannot rule out the possibility that cotton altered nectar to make it less habitable to nectar bacteria. Future work should assess the influence of manipulations on the floral microenvironment and their consequences for nectar microbial growth and community structure.

In contrast to access by legitimate nectar foragers, nectar robbing wounds seem to alter species composition of nectar bacteria via lower relative abundances of common nectar bacteria. Flowers with simulated robbing wounds had significantly lower relative abundances of *Enterococcus*, *Lactococcus*, a member of Enterobacteriaceae, and a member of Enterococcaceae (Fig. 4b, Appendix S2: Table S3). Since artificial robbing wounds reduced the relative abundance of common nectar bacteria, it seems that corolla damage from nectar robbers may influence bacterial species composition. We hypothesize that by chewing a hole in the corolla, robbers may reduce the habitability of nectar to common nectar bacteria. Reduced habitability could be due to exposure to plant defenses (e.g., secondary metabolites, which in some cases can reduce microbial growth in nectar; Vannette and Fukami 2016) or higher rates of nectar evaporation following a robbing event (increasing the already high osmotic stress of the nectar environment; Lachance 2006). Therefore, nectar robbing may potentially serve as a form of niche modification, a phenomenon in which the activity of organisms alters their environment in a way that creates new niches for other organisms (Shaani et al. 2018). In addition, nectar robbers may alter species composition by providing a pathway for floral surface microbes to colonize

nectar. For example, flowers with pierced spurs were associated with higher relative abundances of *Buchnera* (OTU 10), an obligate aphid endosymbiont (Baumann et al. 1995). It was common for aphids to congregate on the exterior of flowers regardless of treatment (Appendix S2: Fig. S3b), so the higher relative abundance of *Buchnera* in these flowers was likely due to propagules leaking into nectar from the exterior surface of the spur. It is possible that other floral surface bacteria could colonize nectar after entering through robbing wounds. Providing entrance to bacteria on the flower surface could serve as an explanation for the weak effects of robbing on OTU alpha diversity (Fig 3).

Whereas access by legitimate nectar foragers did not influence beta diversity, flowers visited by nectar robbers had a significantly lower beta diversity than un-robbed flowers (Fig. 3i). This reduction in the heterogeneity of bacterial species composition could potentially be due to niche modification by robbing wounds only allowing a subset of bacteria from the species pool to persist. Alternatively, the observed reduction in beta diversity with nectar robbing could be due to reduced visitation rates by legitimate visitors to these flowers (Willmer 2011). We do not have a reliable estimate of visitation rate to naturally robbed flowers, but did not find an effect of simulated robbing on flower visitation during timed observations in 2015. However, as there were only four natural robbing events, more work is necessary to disentangle the importance and pathways by which nectar robbers influence species composition and turnover across habitat patches in nectar bacterial metacommunities.

Overall, we found that access to flowers by legitimate nectar foragers and nectar robbers significantly affected bacterial community structure, but in different ways. Legitimate nectar feeders significantly increased bacterial alpha diversity, whereas nectar robbing was associated with a significant reduction in bacterial beta diversity. Visitor-influenced differences in the size of the regional species pool or niche modification may explain these patterns. Although the mechanisms remain to be determined, this work suggests that heterogeneity within a vector mode can influence community structure in vectored organisms.

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