

Supplementary materials for: Dispersal enhances beta diversity in nectar microbes

Vannette & Fukami

Supplementary Methods 1.

To examine if experimental treatments of bagging or caging influence environmental parameters surrounding flowers, we conducted an experiment at Jasper Ridge Biological Preserve from noon on December 14, 2016 to noon on December 21, 2016. Six of the 45 plants used for the main experiment were chosen, and radiation shields each containing an iButton (temperature loggers; Maxim, San Jose, CA) were set up in the same mesh bags and cages as used in the main experiment near each plant (see the photo below; photo credit: Bill Gomez).

More specifically, at a 90-cm PVC tube post established within 1 m from each plant, a 90 cm-long PVC tube was attached, with three hand-made radiation shields mounted on it. The "exposed" treatment was close to the post, the "bagged" treatment was about 20 cm from the exposed treatment, and the "caged" treatment was on the end of the tube. For the bagged treatment, we opened the seam of two nylon bags used for the bagging treatment in the main experiment, and attached them together with 5 staples to make a larger bag, which was placed over the mounted radiation shield and tied on with string to the pole.

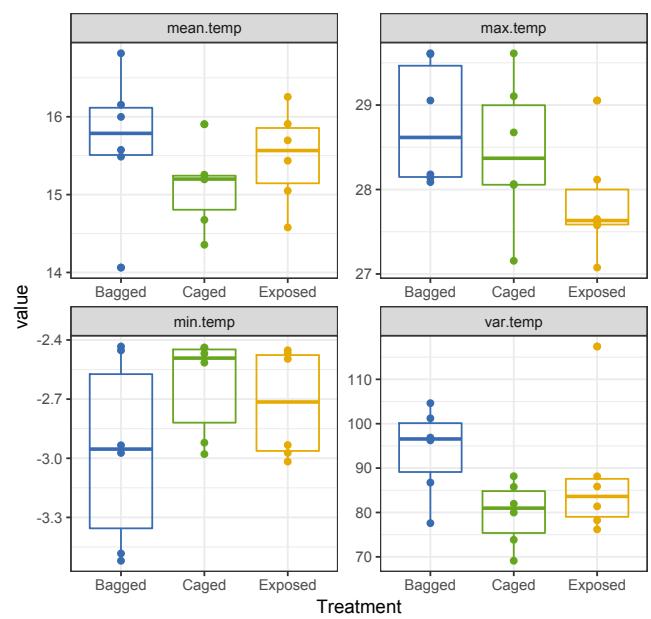
For each iButton, the ambient temperature was recorded every 10 min for seven days in the field where the plants used for the main experiment were located. The seven days they were deployed included sunny, cloudy, and stormy weather. Mean, minimum, maximum, and variance in temperature were calculated for each plant. Temperature was very similar among the treatments (e.g., only about 0.5 C difference in overall mean

temperature), and we found no detectable difference due to treatment imposed (ANOVA $P>0.20$ all comparisons, see figure below).

a)



b)



Supplementary Methods 2.

DNA extraction, PCR, and sequencing

For DNA extraction, 10 µl of each diluted sample was extracted with the DNeasy Blood and Tissue kit (Qiagen, Hilden, Germany) using the nucleated blood protocol. Regions of the ribosomal rRNA gene were amplified using the 16S V4 region for bacteria (515-806) and the ITS1 region for fungi (ITS1f-ITS2) (White *et al.* 1990). Primers contained the Illumina Nextera adapters and linker sequences (Smith & Peay 2014). PCR reactions were carried out in 25 µl reactions including 5 µl of DNA extraction and 0.5 µl of each 10 µM primer in 1x MyTaq HotStart Red Mix (Bioline, Tunton, MA). PCR conditions followed previously published conditions (Smith & Peay 2014). Two replicate PCR reactions were performed for each sample. Amplification was verified by running PCR products on a 1% agarose gel, and replicate reaction products were pooled. Amplicons were cleaned using the Charm Just-A-Plate PCR purification and normalization kits (Charm Biotech, Santa Cruz, CA) and pooled in equimolar concentrations before sequencing. Samples were sequenced together using paired-end, dual indexed (Caporaso *et al.* 2010; Smith & Peay 2014) 2x250 Illumina MiSeq (Illumina, San Diego, CA) at the Stanford Functional Genomics Facility. The amplicon libraries were spiked with 15% PhiX. Negative controls were also amplified using indexed primers and included in the sequencing run. Sequences were obtained from bacterial, but not fungal negative controls.

Bioinformatics

Low-quality bases were removed from each sequence using sickle v.1.33, (Joshi & Fass 2011) in paired-end mode with a sliding window quality cutoff. Sequences were merged

using UPARSE (v.8.0, Edgar 2014). Operational taxonomic units (OTUs) were clustered at 97% similarity using UPARSE (v.8.0) and de-novo and reference-based chimaera detection were performed using UNITE UCHIME ITS-trimmed reference dataset (3/11/2015) for fungal sequences and JGI Gold database for bacterial sequences. Taxonomy was assigned using the RDP classifier (Wang *et al.* 2007) trained on either the 16S rRNA training set 14 for bacteria or the Warcup Fungal ITS training set (Deshpande *et al.* 2015) for fungi at bootstrap cutoff of 80%. OTUs that could not be identified to Kingdom or Phylum were discarded, as were bacterial OTUs classified as chloroplast or mitochondria and fungal OTUs classified as *Saccharomyces cerevisiae* due to laboratory contamination with this strain. OTUs with greater than 20 sequences per sample in the negative controls were also removed (Peay *et al.* 2013). Samples were rarefied (Weiss *et al.* 2015) to 500 bacterial and 500 fungal sequences per sample, at which depth the sequencing curves had begun to plateau for most samples (Supplementary Fig. 2). Sequence analyses were performed using the R package phyloseq (McMurdie & Holmes 2013). Rarefied OTU tables were used for all analyses below.

Supplementary Table 1.

Results of ADONIS PERMANOVA performed in vegan, examining the effects of flower dispersal treatment, month, and their interaction on bacterial community composition using Bray-Curtis dissimilarities. Table 1a indicates the results when data from all months are included and 1b when data from late June (when only exposed flowers could be sampled) are excluded. Results are qualitatively similar using other dissimilarity metrics, including Jaccard and Hellinger (Treatment always $P<0.01$).

1a. Data: all nectar samples

Predictor	F	P	partial R ²
Treatment	10.16	0.001	0.093
Month	3.53	0.001	0.03
Treatment * Month	1.77	0.043	0.016

1b. Data: May + June nectar samples

Treatment	7.43	0.001	0.078
Month	3.09	0.012	0.016
Treatment * Month	1.81	0.04	0.019

Supplementary Table 2.

Results of Betadisper analysis performed in vegan, examining the effects of flower dispersal treatment on variance in bacterial community composition within each treatment group. Unless indicated, all analyses indicated that the variance in exposed>caged>bagged. Analyses reported below also include Plant identity as a random effect, although this has minimal effect on the results, and *F* and *P*-values remain relatively unchanged with its addition.

Dataset	Dissimilarity	<i>F</i>	<i>P</i>
All months	Bray-Curtis	23.51	<0.001
All months	Jaccard	5.25	0.0063
All months	Hellinger	24.38	<0.001
May + June	Bray-Curtis	16.69	<0.001
May + June	Jaccard	3.5	0.033
May + June	Hellinger	18.45	<0.001

* exposed
>bagged>caged

Supplementary Table 3.

Results of beta deviation analysis, examining the effects of flower dispersal treatment on distance to the centroid within each treatment group, compared to null model results.

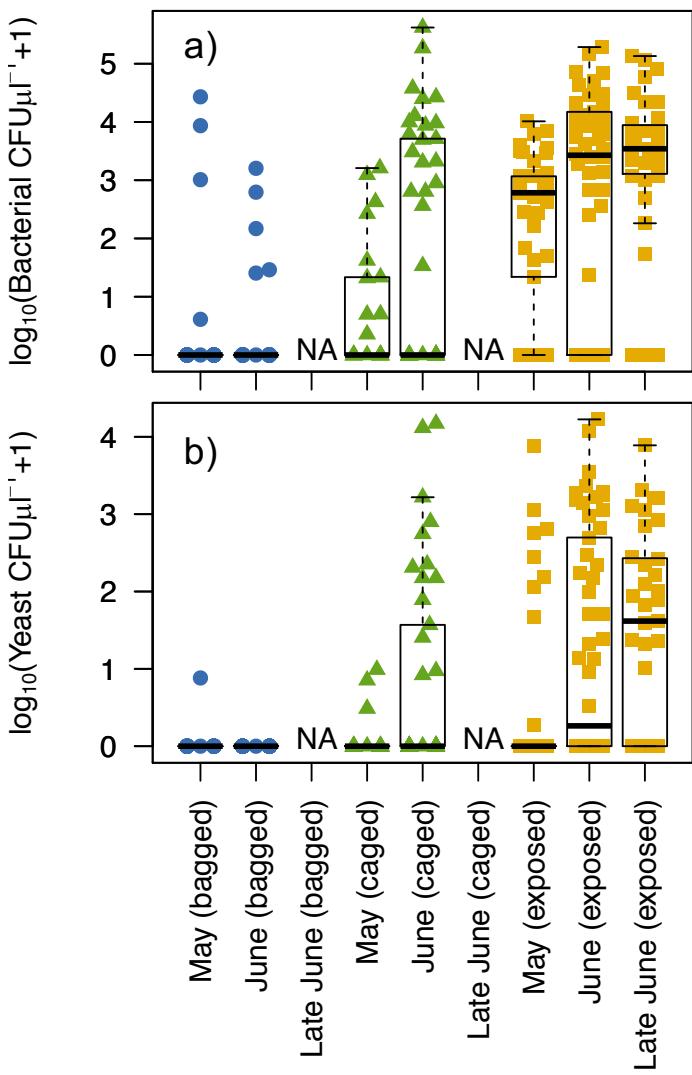
Analyses reported below also include Plant identity as a random effect in mixed models. Month could not be included in the mixed model with the full dataset because this resulted in an unbalanced design.

Data used	Model type	Predictors	F-value	P-value	Adj R² (full model)*
Full dataset	linear				
		Treatment	9.16	0.0002	
		Full model		0.0002	0.078
Full dataset	linear mixed model				
		Treatment	9.16	0.0002	
May + June	linear				
		Month	7.1	0.008	
		Treatment	10.43	<0.0001	
		Month * Treatment	4.52	0.01	
		Full model		0.0002	0.1
May + June	linear				
		Treatment	7.95	0.0005	
		Full model		0.0005	0.075
May + June	linear mixed model				
		Month	0.42	0.51	
		Treatment	8.17	0.0005	
		Month * Treatment	4.52	0.013	

*adj. R2 only calculated for linear models

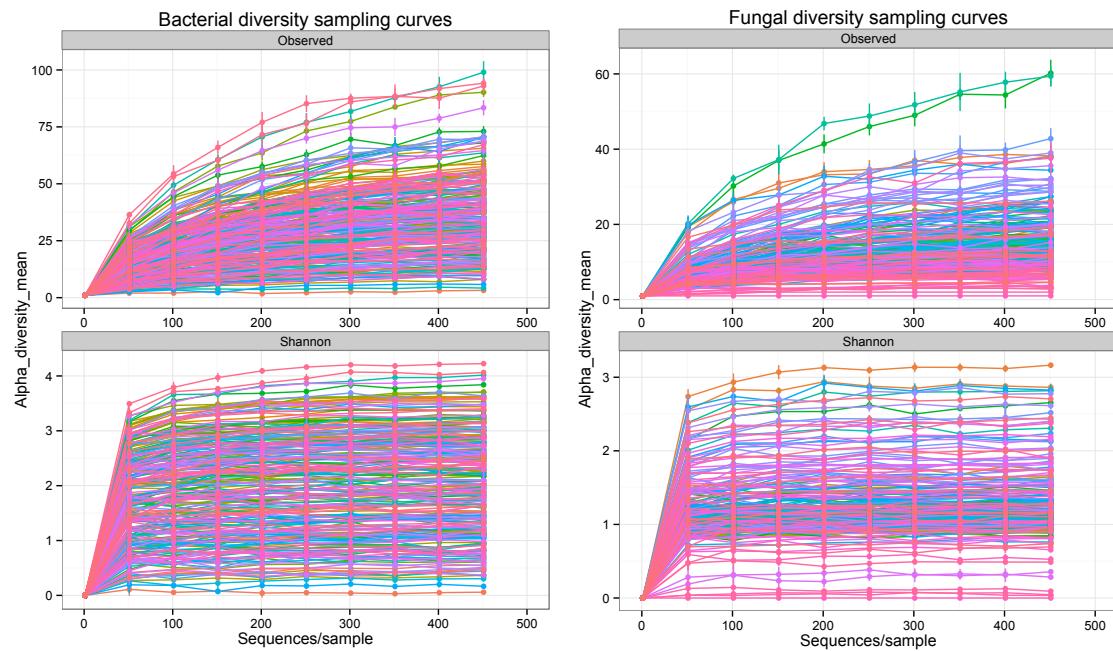
Supplementary Figure 1.

Total density of colony-forming units on a) R2A plates supplemented with cycloheximide or b) yeast media agar plates supplemented with chloramphenicol. Each point represents CFU density in a single flower of *Mimulus aurantiacus* with dispersal treatments (bagged, caged, or exposed). No data are available for bagged or caged flowers in late June (indicated as NA). In the boxplots, hinges represent first and third quartiles, error bars indicate 95% confidence intervals, and center bar represents the median.



Supplementary Figure 2.

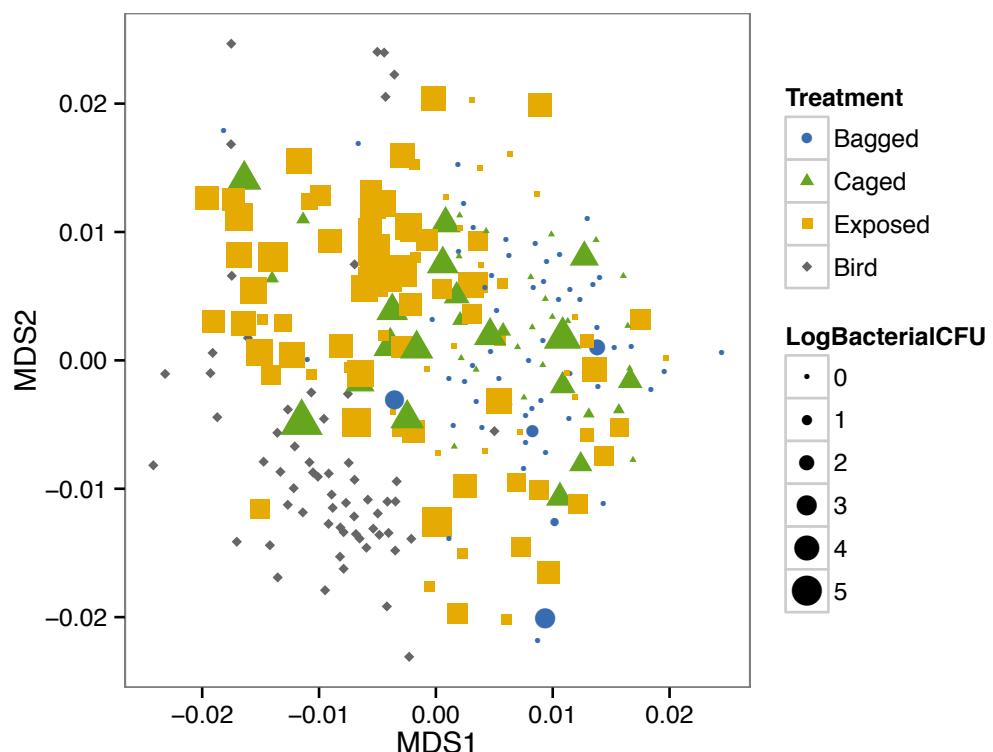
Sampling curves for bacterial and fungal diversity. Each line represents an individual nectar sample, and error bars around each point indicate the standard deviation among values of 5 resampling draws for observed diversity (richness) or Shannon diversity.



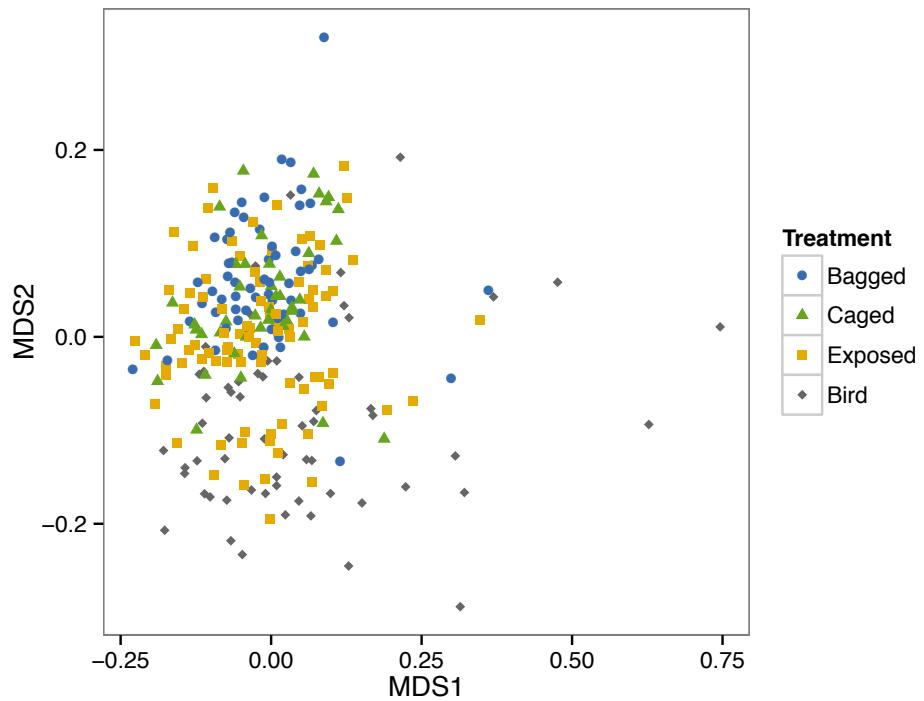
Supplementary Figure 3.

Non-metric multidimensional scaling ordination of bacterial communities found in with *Mimulus aurantiacus* floral nectar or on *Calypte anna* hummingbird bills. Ordinations are based on a) Bray-Curtis and scaled by Bacterial CFU density in nectar (\log_{10} CFU μl^{-1} +1). Bird samples are not scaled by bacterial densities. In b), ordination is based on Unifrac dissimilarity matrices. In c) NMDS ordination is based on Jaccard dissimilarity. In d) PCoA ordination is based on Bray-Curtis dissimilarities. Bacterial taxa found only on bird bills (and not in nectar) were excluded from the analyses. Flowers were subjected to three dispersal treatments: exposed to all pollinators, caged to exclude large-bodied pollinators, or bagged to exclude floral visitors.

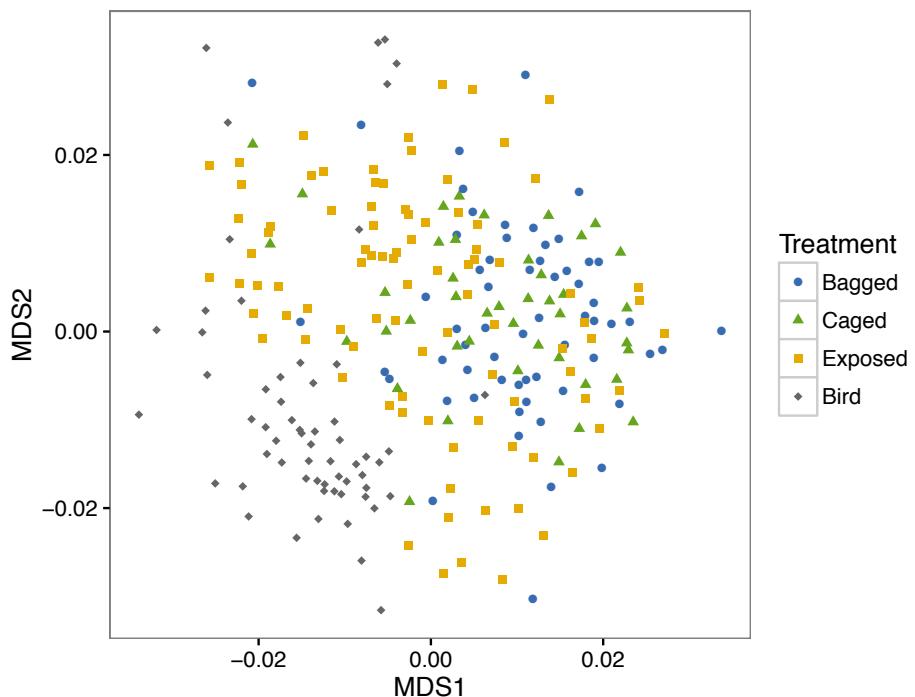
a)



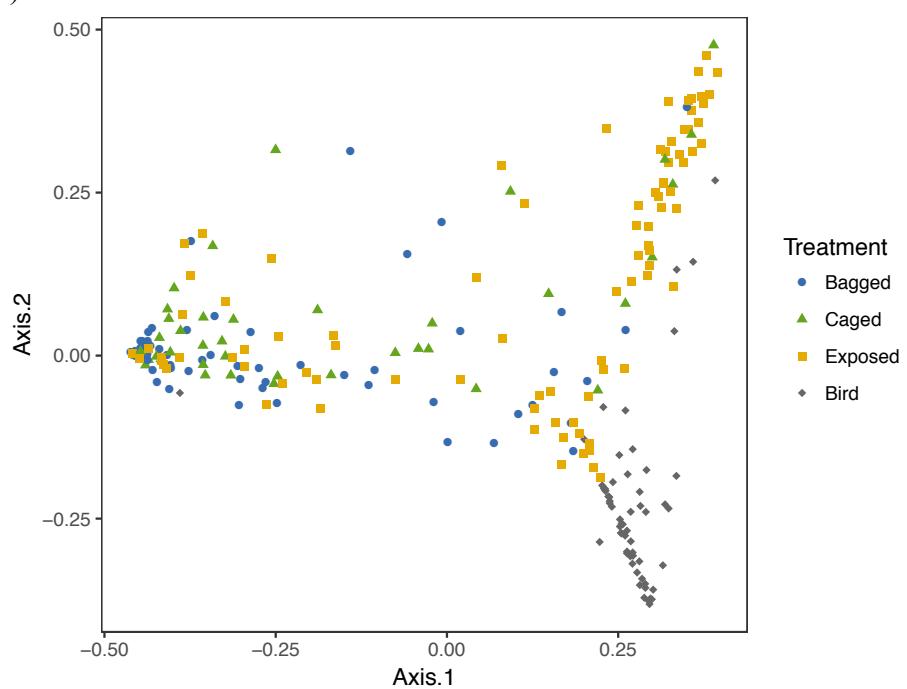
b)



c)

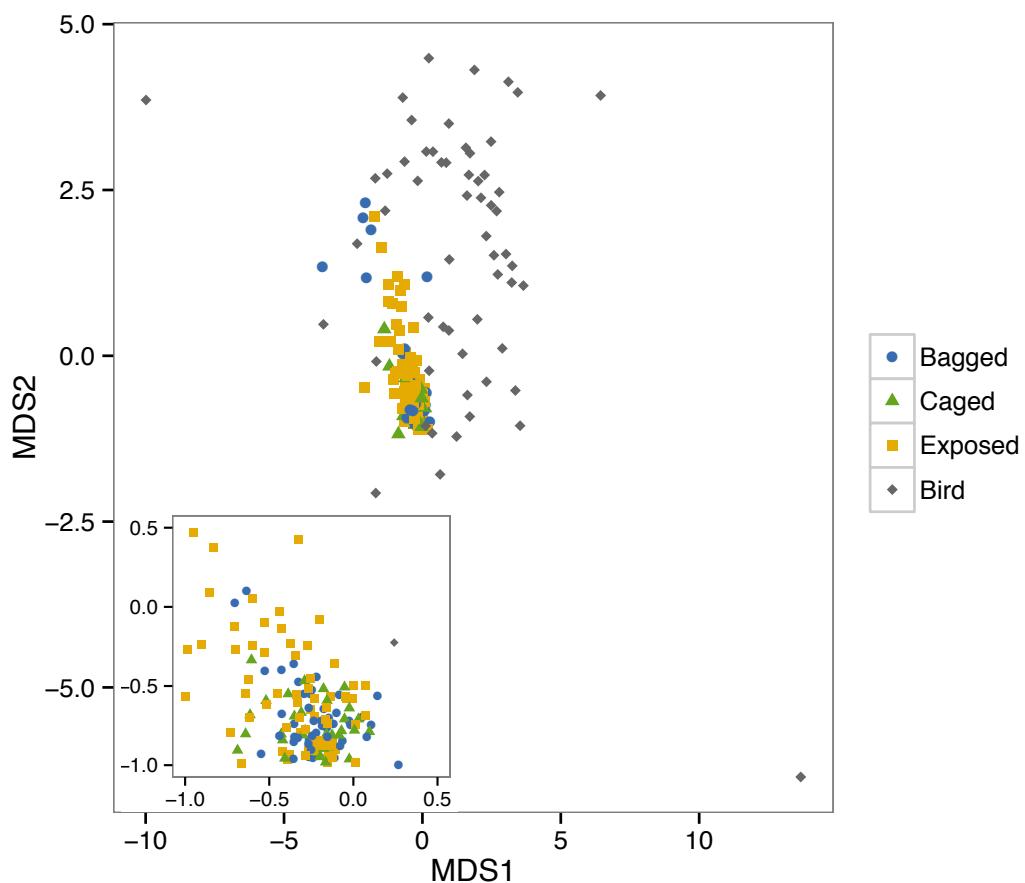


d)



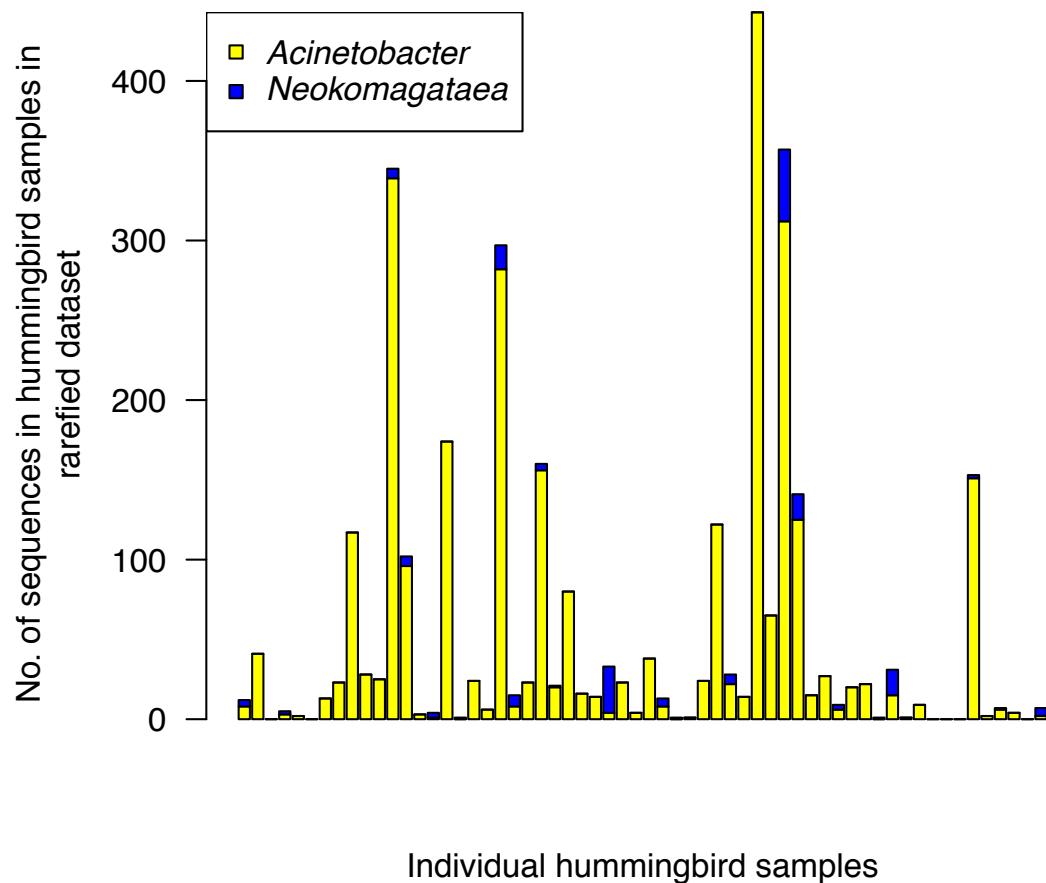
Supplementary Figure 4.

Non-metric multidimensional scaling ordination of fungal communities found in with *Mimulus aurantiacus* floral nectar or on *Calypte anna* hummingbird bills. The NMDS was conducted using Bray-Curtis dissimilarities calculated from rarefied OTU tables. Samples from all months are included in the NMDS. Inset shows a zoomed-in version of the same NMDS to better distinguish interior points.



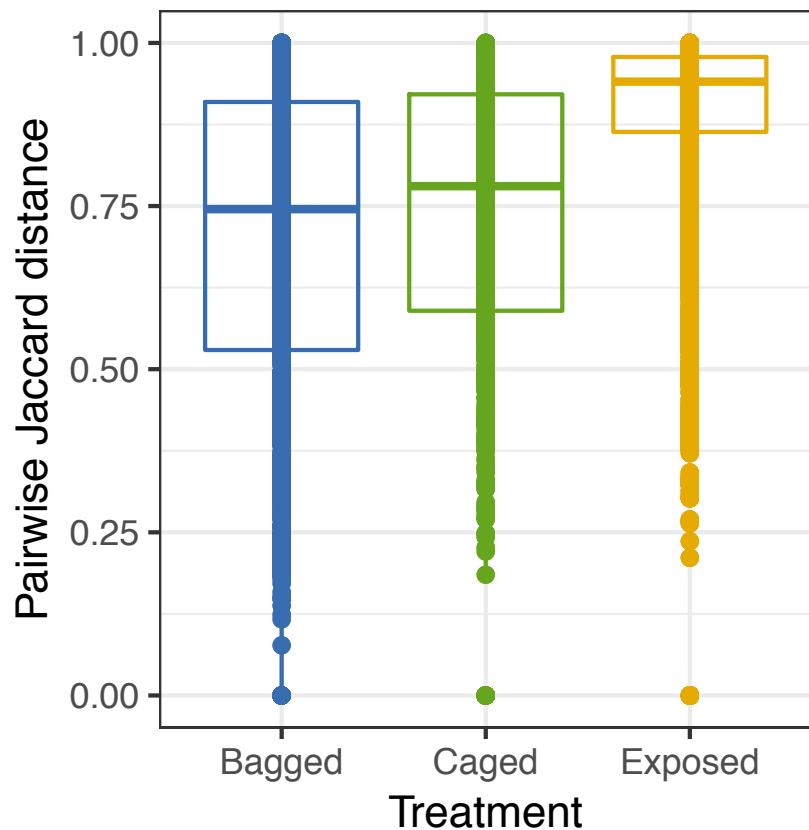
Supplementary Figure 5.

Number of sequences of bacterial taxa *Acinetobacter* and *Neokomagataea* from bills of individual *Calypte anna* hummingbirds (out of 500 sequences/sample).



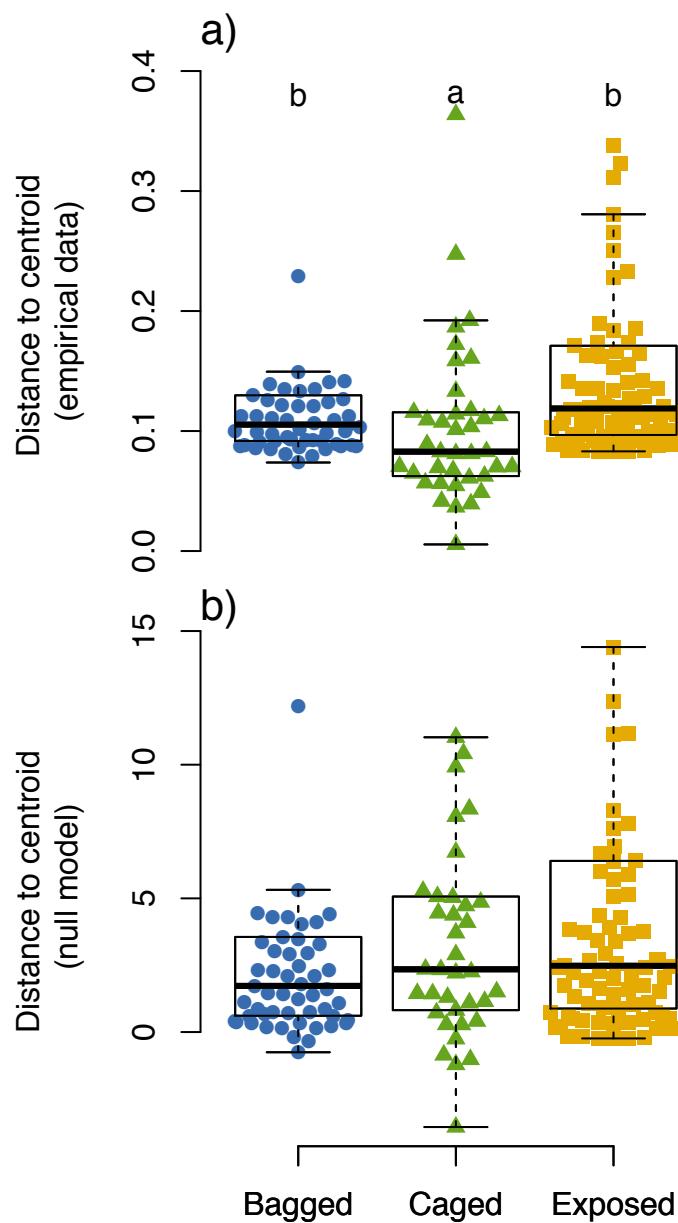
Supplementary Figure 6.

Pairwise dissimilarity among points within a treatment based on Jaccard distance (Bennett and Gilbert 2016). All treatments are statistically different from each other (Overall $P<0.001$, Tukey HSD $P<0.001$).



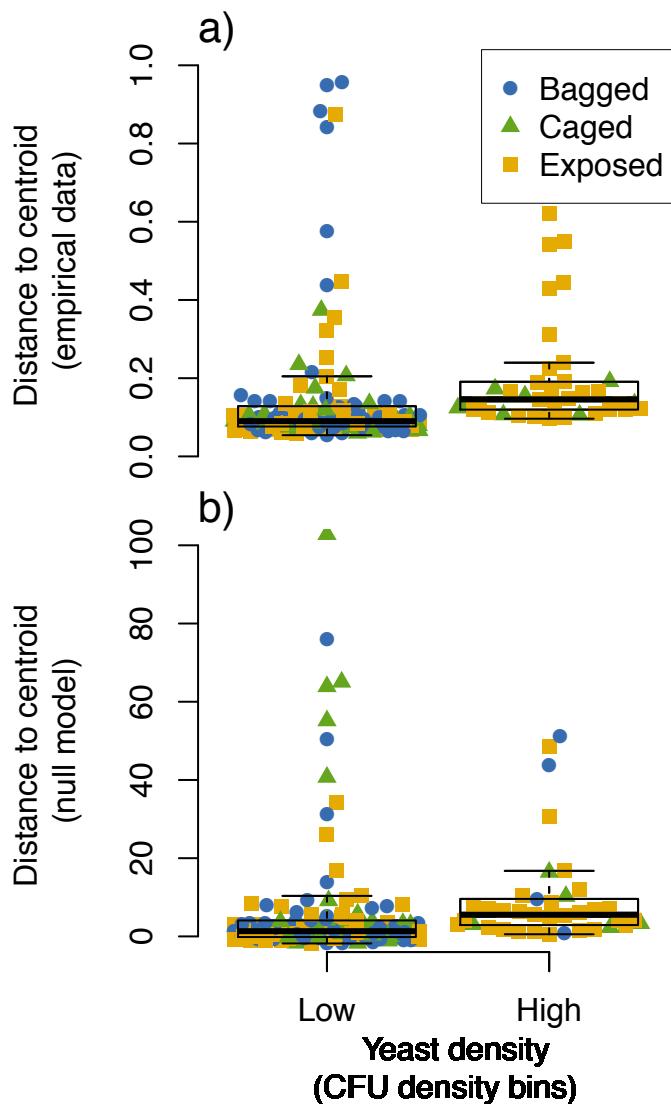
Supplementary Figure 7.

Effect of floral dispersal treatment on β diversity in fungal communities using a) empirical data and b) deviation from null model expectations. β diversity was calculated using distance to centroid using the betadisper function in vegan based on Bray-Curtis dissimilarities. Null model analyses were conducted as described in the Methods section using 999 permutations. Nectar samples from all months are included in both panels. Letters indicate treatments that differ significantly at $P < 0.05$. Boxplot hinges represent first and third quartiles, error bars indicate 95% confidence intervals, and center bar represents the median.



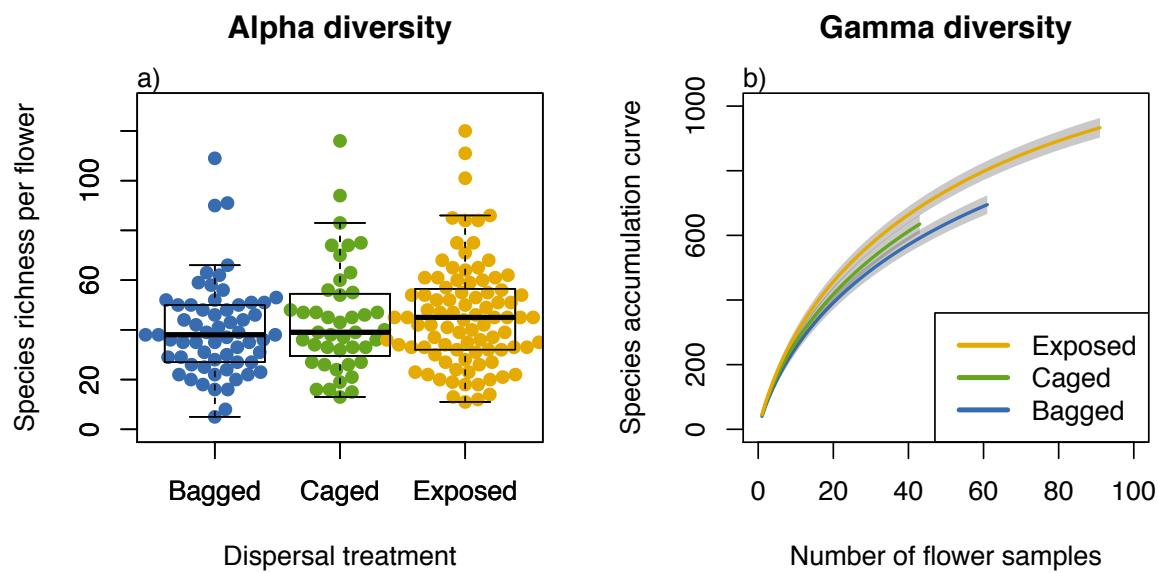
Supplementary Figure 8.

Relationship between the density of colony-forming units (CFUs) on YM plates in nectar of *Mimulus aurantiacus* and a) β diversity estimated from yeast empirical data ANOVA $P=0.11$ b) β deviation (Standardized effect size) from null model predictions. ANOVA $P=0.03$, Kruskal-Wallis $P=0.0007$. Boxplot hinges represent first and third quartiles, error bars indicate 95% confidence intervals, and center bar represents the median.



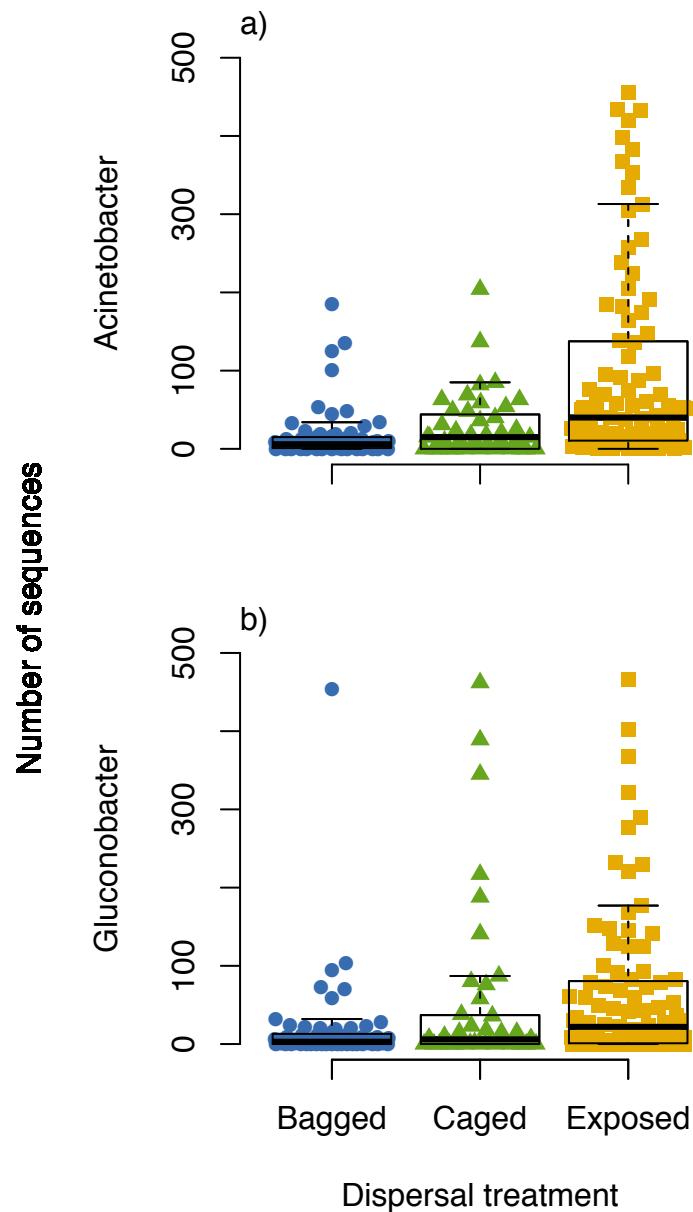
Supplementary Figure 9.

Estimates of bacterial a) alpha diversity, based on the number of OTUs found within an individual flower and b) gamma diversity, estimated using resampling and unconditioned estimates (Colwell et al. 2012). In the boxplot in a, hinges represent first and third quartiles, error bars indicate 95% confidence intervals, and center bar represents the median. In b, lines represent the mean of resampling draws, and shaded regions represent 95% confidence intervals around the mean. Non-overlapping confidence intervals indicate significantly different estimates.



Supplementary Figure 10.

Total sequence abundance of a) all OTUs assigned to the genus *Acinetobacter* (39) or b) all OTUs assigned to the genus *Neokomagataea* (1 OTU), previously *Gluconobacter*. Sequence pools were rarefied to 500 sequences per sample from *Mimulus aurantiacus* flowers. Boxplot hinges represent first and third quartiles, error bars indicate 95% confidence intervals, and center bar represents the median.



Supplementary Figure 11.

Bivariate correlations between overall bacterial community composition (PC axis 3) and the number of sequences from nectar of *Mimulus aurantiacus* assigned to a) *Acinetobacter* sp. or b) *Neokomagataea* sp. (previously known as *Gluconobacter*). In c) the relationship between sequence abundance of *Acinetobacter* and *Neokomagataea* is shown. Flower samples from all dispersal treatments were included in the analyses.

