

# Movers and shakers: Bumble bee foraging behavior shapes the dispersal of microbes among and within flowers

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**Abstract.** Dispersal is central to the ecology and evolution of spatially structured communities. While flower microbial communities are spatially structured among floral organs, how dispersal vectors distribute microbes among floral organs is unknown. Pollinators are recognized as key microbial vectors, but effects of their different foraging behaviors on transfer dynamics among flowers or different floral organs are not known. We asked how foraging behaviors of a model pollinator (*Bombus impatiens*) affect acquisition and dispersal of microbes among flower organs. We used monkeyflowers (*Mimulus guttatus*) to examine dispersal within a natural context and artificial flowers to test how common bee foraging behaviors (nectaring, buzzing, or scrabbling) shaped dispersal of a green fluorescent protein-labeled bacteria, *Pseudomonas fluorescens*. Bees acquired 1% of a flower's microbes and dispersed 31% of acquired microbes to the next flower. All bees acquired microbes, and 85% and 76% of bees dispersed microbes to live and artificial flowers, respectively. Microbes acquired from the corolla were mainly deposited on the corolla, followed by the stamens, and least on the nectary/pistil. Bee foraging behavior affected acquisition, with scrabbling for pollen resulting in 23% more microbes acquired than nectaring, and with buzzing for pollen resulting in a 79% slower rate of microbial acquisition relative to scrabbling. Bee foraging behavior also affected deposition but depended on the floral organ: Scrabbling and buzzing for pollen led to greater deposition than nectaring for corolla and stamen but not nectary. Our results have implications for transmission of beneficial and pathogenic microbes among plants and pollinators, and thus the ecology and evolution of floral microbial communities.

**Key words:** behavior; dispersal; floral organs; flower microbes; foraging; microbial communities; microbial transmission; movement ecology; pollination; vectors.

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## INTRODUCTION

Dispersal is a fundamental biological process that profoundly shapes the ecology and evolution of communities, and microbial communities are no exception (reviewed in Büchi and Vuilleumier 2012, Henriques-Silva et al. 2015, Albright and Martiny 2018). Microbial dispersal is also key in models of disease dynamics and biocontrol, such as for wild and agricultural plant species

(Alexandrova et al. 2002, reviewed in Kevan et al. 2007, Nadarasah and Stavrinides 2010). Flowers are critical to plant reproduction and are also nutrient-rich environments that act as habitats for abundant and diverse microbes (Aleklett et al. 2014, Junker and Keller 2015). Flower visitation by pollinators can alter the floral microbial community (Ushio et al. 2015, Vannette and Fukami 2017; M. Rebolleda-Gómez and T.-L. Ashman, *unpublished manuscript*), and flower-pollinator

interactions can be critical to the spread of disease among plants and pollinators (reviewed in Kevan et al. 2007, McArt et al. 2014, Adler et al. 2018). Surprisingly, however, the rate that pollinators acquire microbes from flowers and subsequently disperse those microbes to other flowers has been barely explored (Graystock et al. 2015, Hausmann et al. 2017).

The dispersal of floral microbes has been considered nearly exclusively in the context of pollinators visiting flowers for nectar (Brysch-Herzberg 2004, Herrera et al. 2008, Hausmann et al. 2017 and references within). Yet flowers are complex structures composed of multiple organs that differ in morphology, function, and suitability as habitats for floral microbes (Alekklett et al. 2014, Steven et al. 2018). Indeed, different flower organs (e.g., corolla, pistil, stamens, and nectary) can possess distinct microbial communities (Alekklett et al. 2014, Junker and Keller 2015, Steven et al. 2018; M. Rebolleda-Gómez and T.-L. Ashman, *unpublished manuscript*), but it is not known whether this is due to differences in dispersal mediated via interactions with pollinators or differences in the strength of floral organs as environmental filters (Allard et al. 2018; M. Rebolleda-Gómez and T.-L. Ashman, *unpublished manuscript*). Pollinators physically interact with different floral organs to varying degrees (Lavery 1980), which should mediate patterns of microbial dispersal among floral organs, and, subsequently, microbial community assembly. Nonetheless, whether pollinators disperse microbes among multiple floral organs and how microbes are apportioned among the floral organs via dispersal are unknown.

Pollinators exhibit different foraging behaviors at flowers that could result in different patterns of microbial dispersal (Zemenick et al. 2018). Pollinators such as bees must extract pollen and nectar from flowers, which necessitates use of highly distinct behaviors, even on the same flower (Free 1968, Corbet et al. 1988, Westerkamp 1999). For example, to extract nectar a bee must insert its proboscis and possibly other body parts into the corolla tube to probe the nectary (nectaring) while pushing aside the pistil and stamens, while a bee collecting pollen typically uses vigorous leg movements (scrabbling) or powerful thoracic vibrations (buzzing; used by >58% of bee species; Cardinal et al. 2018) to dislodge pollen from the

stamens (Lavery 1980, 1994, Russell et al. 2017a). While pollinators using different foraging behaviors manipulate floral organs differently, no study has examined the effects of foraging behavior on microbial dispersal among flowers and among floral organs.

In this laboratory study, we examined how generalist bumble bee (*Bombus impatiens*) foraging behavior shaped the dispersal of a green fluorescent protein (GFP)-labeled common plant-associated microbe (*Pseudomonas fluorescens*) among flowers and floral organs. We first used monkeyflowers (*Mimulus guttatus*; Phrymaceae) as a model to characterize the extent to which foraging bumble bees acquired microbes from the corolla of live flowers and dispersed those microbes to flowers and among the corolla, stamens, and pistil. We selected these flower organs because their different roles in pollinator attraction and interactions with foragers within the flower (Lunau 1992, Connolly and Anderson 2003, Russell et al. 2018) could plausibly affect microbe dispersal. We next used artificial flowers to precisely manipulate bumble bee foraging behavior (scrabbling, buzzing, or nectaring) and to test how acquisition of microbes from flowers and the dispersal of those microbes among floral organs were affected.

## METHODS

### Microbes

To study pollinator-mediated microbial dispersal among flowers, we used *P. fluorescens*, a bacterium commonly found on plants, related to *Pseudomonas* spp. isolated from flowers and pollinators, used in managed apivectoring to flowers, and readily transformable according to established literature (Johnson et al. 1993, Ganeshan and Kumar 2005, Pusey et al. 2009, McFrederick et al. 2012, Alekklett et al. 2014, Melvin et al. 2017). To track microbial dispersal, we transformed *P. fluorescens* SBW25 with a plasmid (pSMC21) that constitutively expresses GFP through electroporation following Bloemberg et al. (1997). For electroporation, we mixed 10 g of plasmid with 100 µL of 10% glycerol-suspended cells and electroporated at 1.8 kV. This plasmid contains a *rhIA::gfp* transcriptional fusion and confers resistance against carbenicillin (the selective marker). The plasmid is stable for at

least several days in *P. fluorescens* in the absence of antibiotic selection (Bloemberg et al. 1997, Melvin et al. 2017). We cultured the strain in sterile tryptic soy broth and 100 mg/L carbenicillin in a shaker at 28°C and 200 rpm for 24 h. The strain was thereafter transferred to 1.5-mL sterile microcentrifuge tubes (Fisher Scientific, Hampton, New Hampshire, USA) and put through two rounds of centrifuging (9.6g for 5 min) and sterile saline (8.5% NaCl) substitution and elimination to purge media. Green fluorescent protein microbes were stored at 4°C for up to five days for behavioral trials and regrown weekly from parent stock stored at –80°C in 25% glycerol.

### Flowers

To study microbial dispersal among live flowers, we used the monkeyflower *M. guttatus* (synonym *Erythranthe guttata*; Phrymaceae; Fig. 1a). Flowers of *M. guttatus* are zygomorphic and tubular (1.9 cm long, 1 cm wide at the mouth) with pollen-bearing stamens and variable amounts of nectar concealed within the tube, at the mouth or base, respectively (Martin 2004, Arceo-Gómez and Ashman 2014, Carr et al. 2015). One hundred plants were grown from seed (collected from 38°52' N, 122°24' W in the McLaughlin Natural Reserve) in a greenhouse with supplemental halogen lights to extend day length to a 14:10-h cycle and were fertilized weekly (PlantTone, NPK 5:3:3, Espoma, Millville, New Jersey, USA).

To test how a given foraging behavior affected microbe dispersal, we used sterile artificial flowers resembling *M. guttatus* in size and form. Flowers had a flared mouth, a corolla tube ( $L \times W$ , 4 × 1 cm), a stamen close to the mouth, and a nectary deep within the tube (Fig. 1b). Each flower was created from a 2-mL snap-cap microcentrifuge tube (Fischer Scientific) with the tip of a pipette tip cut and hot-glued into the microcentrifuge tube (0.1- to 100-μL micropipette tip; Fisher Scientific; corolla), a foam cuboid (0.2 × 0.2 × 2 cm; Fibrecraft, Artist and Craftsman Supply, Pittsburgh, Pennsylvania, USA) pinned along its length (enameled insect pin, size 1; Carolina Biological Supply, Burlington, North Carolina, USA; stamen) into the pipette tip, and the 1-cm conical end of a PCR tube (0.2-mL PCR Tubes; Cole-Parmer, Vernon Hills, Illinois, USA; nectary) inserted into the end of the microcentrifuge tube. To attract test bees, blue plastic (Index Dividers; Target,

Pittsburgh, Pennsylvania, USA) was fit around the mouths of the microcentrifuge tubes (Fig. 1b).

### Bees and housing

As the microbial vector, we used the generalist and widespread bumble bee, *B. impatiens* (Ploewright and Lavery 1984). Bumble bees (*Bombus* spp.) are assumed to be the key pollinators of *M. guttatus* (Carr et al. 2015). We maintained three commercially obtained (Koppert Biological Systems, Howell, Michigan, USA) captive colonies on 2 mol/L sucrose solution scented with 10 PPM peppermint oil (Nielsen-Massey Vanillas, Waukegan, Illinois, USA) and pulverized honey bee-collected pollen (Koppert Biological Systems). Artificial feeders provided food within an enclosed training arena ( $L \times W \times H$ : 82 × 60 × 60 cm) set to a 14-h:10-h light:dark cycle. Pollen feeders were constructed following Russell and Papaj (2016). To familiarize bees to foraging on flowers in experiments, sucrose solution was offered by six artificial training flowers created from 2-mL microcentrifuge tubes, a purple foam cuboid glued lengthwise in each microcentrifuge tube, and blue plastic fit around the mouth of the microcentrifuge tubes (Fig. 1c). To supply sucrose solution, the microcentrifuge tubes were perforated at their tips and glued horizontally into sealed reservoirs with cotton dental wicks inserted (Fig. 1c).

### Experiment 1: Do bees disperse microbes to live flowers readily and differentially among flower organs?

Here, we tested acquisition and dispersal of epiphytic GFP microbes from and to monkeyflowers by bumble bees. We used 23 bees from three colonies. To initiate a behavioral trial, we set up a single horizontally displayed freshly clipped flower on the arena wall in a cleaned test arena. To prevent desiccation, flowers were placed into custom water tubes (Russell et al. 2017a). From the training arena, a single worker bee naïve to monkeyflowers was gently captured using a 40-dram vial (BioQuip, Compton, California, USA) and immediately released in the test arena. We allowed each test bee to forage twice on each of the three monkeyflowers presented sequentially and individually such that bees only had a single flower to forage on at a time (Fig. 2a). We used the first flower to reduce

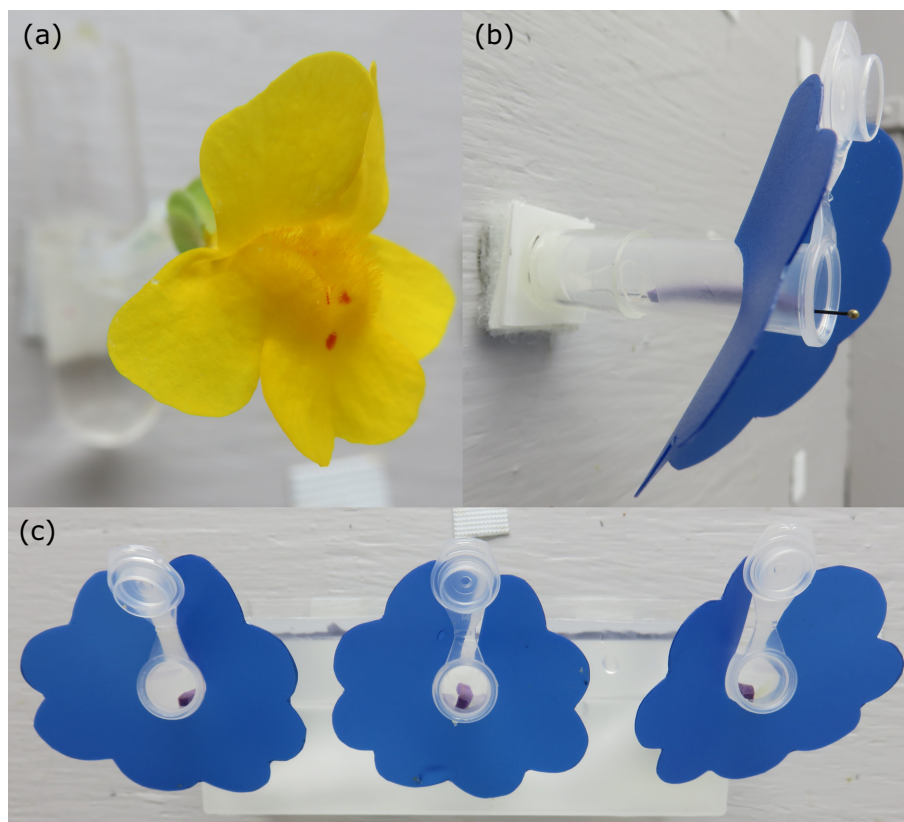


Fig. 1. Types of flowers used to study dispersal of microbes (*Pseudomonas fluorescens*) by bumble bees (*Bombus impatiens*). (a) Live monkeyflower (*Mimulus guttatus*), (b) artificial flowers similar in size and morphology to monkeyflowers, and (c) artificial training flowers.

variation in how bees interacted with live flowers while learning to forage (training flower). We used the second flower to dispense GFP microbes (donor flower). We used the third flower to receive GFP microbes (recipient flower) from the bee inoculated by the donor flower. To add microbes to donor flowers, we used a pipette tip to spread a 10  $\mu$ L solution of 200,000 GFP microbes within the corolla tube, placing flowers in a laminar flow hood for 30 min to dry to simulate natural floral conditions (drying does not result in noticeable microbial mortality; e.g., Rhodes and Fisher 1950, Leach et al. 1959). This is an ecologically realistic quantity of epiphytic microbes found on live flowers (Russell and Ashman 2019). Each flower was replaced with the next flower after having received two foraging visits, using jumbo forceps (BioQuip Products), while the bee was in flight, and bees did not exhibit signs of being disturbed by our activity, such

as aggressive behavior or attempts to escape from the arena. To precisely control how many microbes we applied to flowers, we created a standard curve by plating GFP microbes of known density in tryptic soy with 15% agar (TSA) and 100 mg/L carbenicillin and related the number of colonies to optical density using a NanoDrop spectrophotometer (ThermoFisher Scientific, Waltham, Massachusetts, USA). Each day, before running behavioral trials we adjusted saline-stored GFP microbes to the correct optical density. We never reused flowers or bees across trials.

We defined a foraging visit as the duration a bee was on a flower, when it collected nectar and/or pollen (we could not control what the bee foraged for using live flowers). We defined nectar collection (nectaring) as bees that had crawled past the stamens, with proboscis extended into the nectary and abdomen pumping (indicating



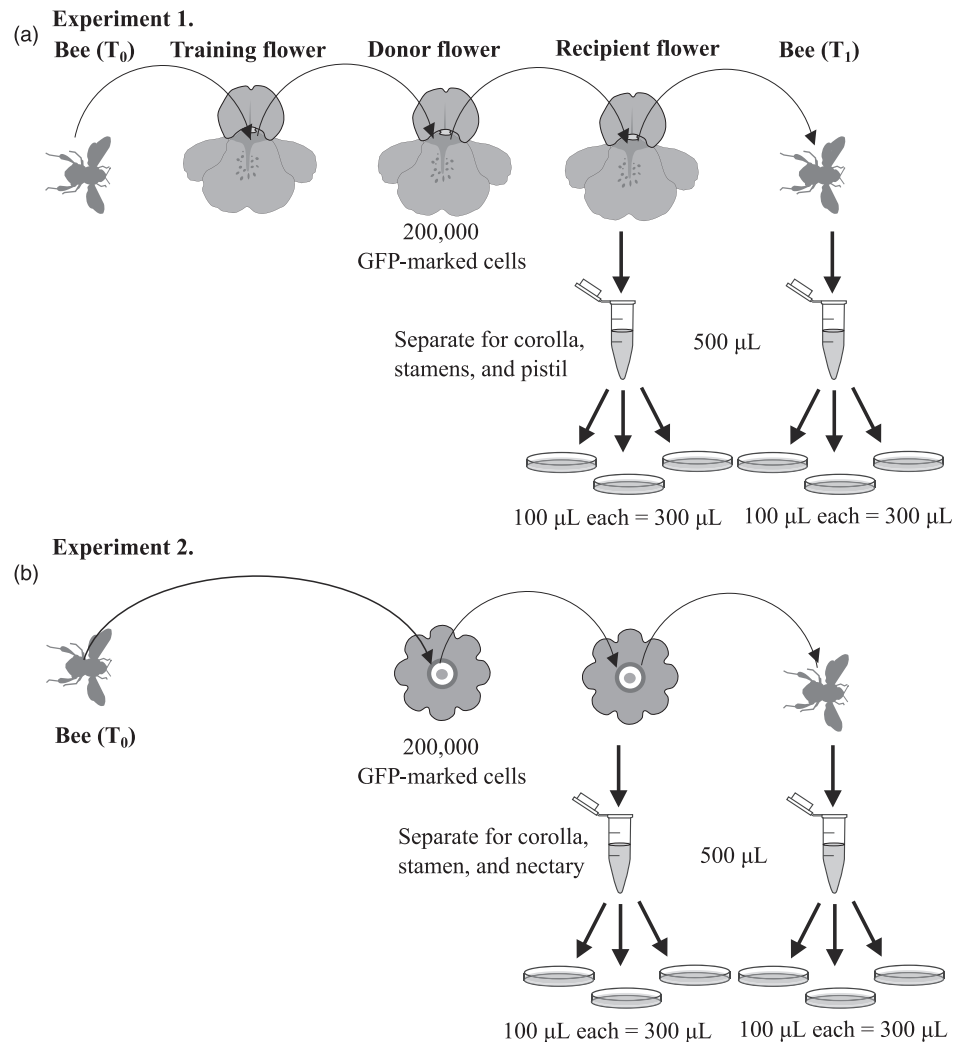


Fig. 2. Schematic of the two experiments. (a) In Experiment 1, bees foraged twice on each of the three mon-keyflowers, in sequence. We used the first flower to train bees (training flower). On the second flower (donor flower), bees acquired green fluorescent protein (GFP)-marked microbes (*Pseudomonas fluorescens*). Bees deposited acquired microbes by visiting the third flower (recipient flower). (b) In Experiment 2, bees foraged twice on an artificial donor flower and immediately thereafter foraged twice on an artificial recipient flower. Bees were split into three groups differing in the foraging behavior they could use: either scrabbling for pollen, buzzing for pollen, or nectaring. For both experiments, we plated 3/5 of the wash for each bee and flower organ.

nectar uptake). Bees collected pollen either by scrabbling with their legs on the stamens (scrabbling; see Russell and Papaj 2016) or by buzzing the stamens (Russell et al. 2017a). Because foraging duration might affect microbial dispersal, to allow precise quantification we recorded trials with a 1080P HD Sports Action Camera (LD6000; Lightdow, Guangdong Shenzhen, China).

To count microbes acquired by bees and dispersed to recipient flowers, once the bee foraged twice on each of the three flower types (training, donor, and recipient), we captured it in a sterile 90-mL container (Wide-Mouth Bio-Tite; Thomas Scientific, Swedesboro, New Jersey, USA), cold-anesthetized it (to avoid loss of microbial cells), and placed the bee, recipient corolla, recipient

stamens, and recipient pistil (samples) into separate sterile 2-mL microcentrifuge tubes with 500  $\mu$ L of saline each within 15 min. Since dispersal patterns might reflect differences in surface area, we flattened and photographed flower organs from 10 flowers (each from a different plant) not used in Experiment 1, using ImageJ (National Institutes of Health, Bethesda, Maryland; <http://imagej.nih.gov/ij/>) to measure the internal surface area of the corolla and the external surface area of both sides of the flattened stamens and pistil.

All samples were handled with sterile equipment. We vortexed samples for 20 s and plated three 100- $\mu$ L aliquots per sample. Bees were euthanized after this step. We incubated plates at 30°C for 48 h and photographed them using a transilluminator (Amersham Imager 600; GE Healthcare Life Sciences [Marlborough, Massachusetts, USA]; settings: Epi-RGB, 460 nm, Cyy2 filter, 0.1 s exposure). We counted all fluorescing colonies (indicating GFP expression), or, when colony density was exceptionally high, we estimated total colonies per plate by subsampling the plate. We analyzed and report the mean count of the three aliquots (equivalent to 1/5 of a sample's total microbes). We measured body size of each test bee (head width in mm) using a stereoscope and ImageJ (National Institutes of Health) following Russell et al. (2017b).

#### **Experiment 2: Does bee foraging behavior shape the acquisition and dispersal of microbes?**

We tested how three bee foraging behaviors affected acquisition and dispersal of microbes to the three flower organs using artificial flowers, which allowed precise control of bee foraging behavior. We used 68 bees from three colonies. We systematically alternated assignment of bees to one of three treatments (scrabbling, buzzing, or nectaring) to control for effects of bee, time, and day on behavior. In the scrabbling treatment, we evenly spread 4 mg of commercially available cherry pollen (*Prunus avium* pollen; Pollen Collection and Sales, Lemon Cove, California, USA) on the stamens to compel bees to scrabble. In the buzzing treatment, we added a *Solanum houstonii* stamen extract (Russell et al. 2017a) to the stamens to compel bees to buzz, as well as 2 mg of cherry pollen to collect while buzzing. In the nectaring treatment, to compel bees to forage for nectar we filled the nectary with 20  $\mu$ L of

sterile peppermint-scented sucrose solution. Using methods described above, bees in each treatment were allowed two visits to a donor flower followed by two visits to a recipient flower (Fig. 2b). Before assembling donor flowers (with nectary and stamen), we pipetted microbes into the microcentrifuge tubes (the corolla), evenly spread microbes within by shaking, and placed corollas in a laminar flow hood for 30 min to dry. We plated and counted microbes from the bee, recipient corolla, recipient stamen, and recipient nectary as above.

Control assays comparing the survival of GFP microbes exposed to cherry pollen, cherry pollen and anther extract, or scented sucrose solution confirmed that these treatments did not affect GFP microbe survival (Appendix S1).

#### **Data analyses**

All data (Data S1) were analyzed using R v.3.5.0 (R Development Core Team 2016). We excluded 12 of 91 trials from analyses because either all corresponding bee or corolla plates were overgrown.

*Experiment 1.*—To determine whether there was unequal dispersal of microbes to live flower organs, we used a zero-inflated negative binomial generalized linear mixed model (GLMM), using the `glmmTMB()` function in the `glmmTMB` package (Magnusson et al. 2018). We checked model assumptions for all GLMMs using the `DHARMA` package (Hartig 2018). The response variable was GFP microbe quantity, the explanatory variables were flower organ and body size (head width), and we included bee as a random factor (bee within colony would not converge). We used the `two-argument ANOVA()` function in R to examine overall effects. In cases of significant effects, we determined which pairs were significant using Tukey's post hoc test via the `lsmeans()` function in the `lsmeans` package (Lenth and Love 2018). We also used linear models (LMs) to determine whether foraging duration (either total or only on donor flowers) was correlated with microbe acquisition or dispersal, respectively. We added 0.1 to the independent variable and log-transformed it and thereby normalized the residuals.

*Experiment 2.*—To determine whether bee foraging behavior (scrabbling, buzzing, and nectaring) affected total acquisition (sum of flower organs and bee) or acquisition rate (total acquisition/duration foraging on donor) of GFP

microbes from artificial flowers, we used one-way ANOVA using the `aov()` function in R. We log-transformed the independent variable and thereby normalized the residuals. We ran Tukey's post hoc test, using the `TukeyHSD()` function in R, to determine which pairs were significant. To determine whether bee foraging behavior affected the dispersal of microbes to flower organs, we used a GLMM as above. The explanatory variables were flower organ and behavior. Exactly as in Experiment 1, we also used LMs to determine whether foraging duration (without regard to type of foraging behavior) was correlated with total microbe acquisition or dispersal. To determine whether foraging behavior affected the proportion of bees that dispersed microbes to a given flower organ, we used Fisher's exact tests (FET), using the `fisher.test()` function in R, with pairwise comparisons adjusted for multiple comparisons (via `fdr`; Benjamini and Hochberg 1995) and performed using the `fisher.multcomp()` function in the `RVAideMemoire` package (Hervé 2018).

## RESULTS

### *Bumble bees disperse microbes to monkeyflowers readily and differentially among flower organs*

Foraging bumble bees acquired 1.2% of the GFP microbes from donor monkeyflowers (estimated mean total no. microbes  $\pm$  standard error [SE]:  $2367 \pm 881$ ,  $N = 20$  bees), but transferred  $31.2\% \pm 6.4\%$  (mean  $\pm$  SE) of acquired microbes to recipient flowers within two visits. Bees dispersed microbes unequally among flower organs; that is, the corolla received significantly more microbes than the stamens, which received significantly more microbes than the pistil (Fig. 3a; GLMM: overall effect:  $\chi^2_1 = 41.31$ ,  $P < 0.0001$ ). Furthermore, while all bees acquired microbes, only 85%, 65%, and 35% of bees transferred microbes to the corolla, stamens, and pistil, respectively. There was no correlation between foraging duration and how many microbes were acquired by bees or dispersed to the recipient flower (LMs: effect of time foraging on donor on acquisition:  $F_{1,18} = 0.52$ ,  $P = 0.48$ ,  $R^2 = 0.03$ ; effect of total foraging duration on dispersal:  $F_{1,18} = 2.32$ ,  $P = 0.14$ ,  $R^2 = 0.11$ ). Microbe dispersal was also not affected by bee body size (GLMM:  $\chi^2_1 = 1.28$ ,  $P = 0.257$ ), but followed

expectations based on the surface area of recipient flower organ (percentage of microbes  $\pm$  SE: corolla:  $81 \pm 7$ ; stamens:  $17 \pm 7$ ; pistil:  $12 \pm 1$ ; percentage of flower surface area  $\pm$  SE: corolla:  $87 \pm 1$ ; stamens:  $8 \pm 1$ ; pistil:  $4 \pm 0$ ;  $N = 10$  flowers).

### *Bee foraging behavior shapes the acquisition and differential transfer of microbes to artificial flowers*

Foraging behavior significantly affected the quantity of GFP microbes bees acquired from artificial flowers (Fig. 3b; ANOVA:  $F_2 = 3.77$ ,  $P = 0.029$ ). Specifically, bees scrabbling for pollen acquired significantly more microbes than nectaring bees (23.3% more), though microbe acquisition for neither scrabbling nor nectaring bees was different from that of bees buzzing for pollen. Interestingly, buzzing behavior resulted in a significantly slower rate of microbial acquisition (78.5% slower) relative to scrabbling, though not relative to nectaring behavior (ANOVA:  $F_2 = 5.14$ ,  $P = 0.009$ ; mean microbes per s  $\pm$  SE: scrabbling:  $7 \pm 2$ ; buzzing:  $2 \pm 0$ ; nectaring:  $8 \pm 5$ ). As with bees foraging on monkeyflowers, there was no correlation between foraging duration and how many microbes were acquired by bees or dispersed to artificial flowers (LMs: effect of time foraging on donor on acquisition:  $F_{1,57} = 0.55$ ,  $P = 0.46$ ,  $R^2 = 0.01$ ; effect of total foraging duration on dispersal:  $F_{1,57} = 0.28$ ,  $P = 0.60$ ,  $R^2 = 0.005$ ).

Bees overall dispersed microbes unequally among artificial flower organs, with the corolla receiving significantly more microbes than the stamen, which received significantly more microbes than the nectary (Fig. 3c; GLMM:  $\chi^2_2 = 18.79$ ,  $P < 0.0001$ ). The effect of foraging behavior depended on the flower organ, being strongest for the corolla and weakest for the nectary (Fig. 3c; GLMM: flower organ  $\times$  behavior:  $\chi^2_4 = 20.30$ ,  $P < 0.0005$ ). For the corolla, bees scrabbling or buzzing for pollen deposited significantly more microbes than nectaring bees. Likewise, for the stamen, scrabbling deposited more microbes than nectaring. In contrast, for the nectary, all foraging behaviors resulted in a similar low quantity of microbes transferred.

Additionally, foraging behavior determined the likelihood that a bee dispersed microbes to the corolla and the stamen, but not the nectary. A

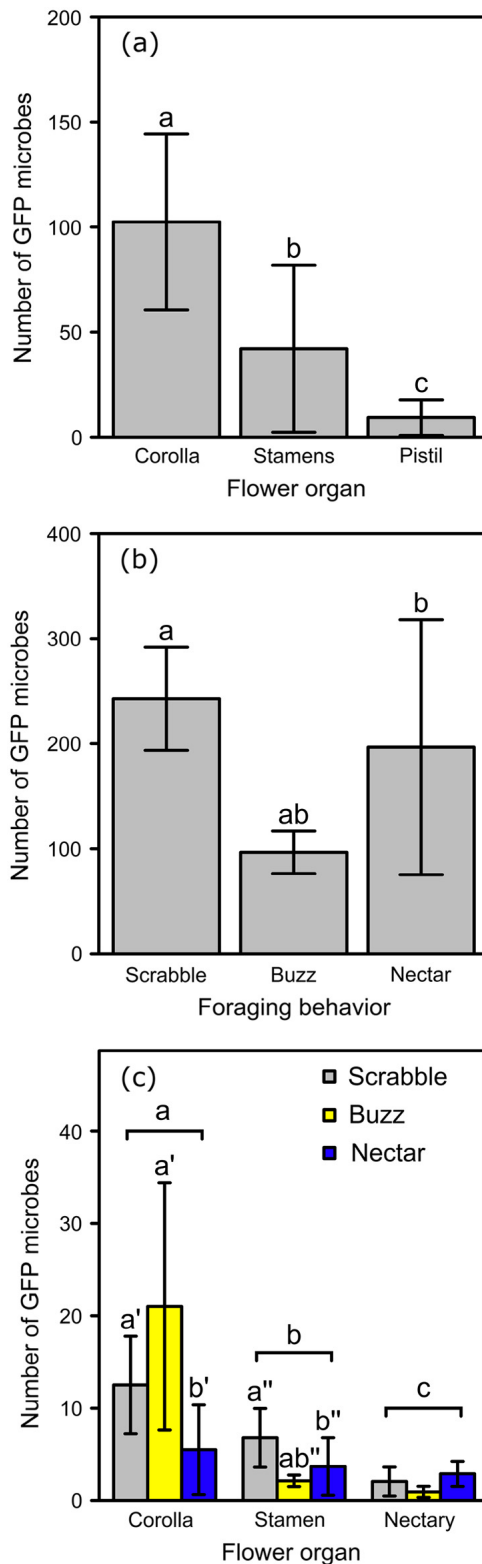


Fig. 3. Mean number of green fluorescent protein

significantly greater proportion of scrabbling bees dispersed microbes to the corolla, relative to buzzing or nectaring bees (FET:  $P < 0.0001$ ; 100%, 79%, and 44% of scrabbling, buzzing, and nectaring bees, respectively). A significantly greater proportion of scrabbling bees dispersed microbes to the stamen, relative to nectaring bees, but not buzzing bees (FET:  $P < 0.0004$ ; 95%, 74%, and 50% of scrabbling, buzzing, and nectaring bees, respectively). The proportion of bees that dispersed microbes to the nectary was unaffected by foraging behavior (FET:  $P = 0.076$ ; 56%, 21%, and 50% of scrabbling, buzzing, and nectaring bees, respectively).

## DISCUSSION

We demonstrate that microbial transmission among flowers by a generalist bumble bee can depend on both the foraging behavior of the bee and the organ on the recipient flower. Both the amount of microbes transmitted and the proportion of bees transmitting microbes were affected by behavior and organ, as well as their interaction. In addition, we found that foraging behavior affected microbial acquisition from flowers, with bees scrabbling for pollen acquiring 23% more microbes than bees foraging for nectar. Bumble bees acquired microbes first from the corolla and readily dispersed those microbes among the corolla, stamen, and nectary or pistil of other flowers. Assuming our results are representative of pollinator–flower–microbe interactions, pollinators

(Fig. 3. Continued)

(GFP) microbes (± standard error [SE]) acquired by foraging bees and deposited on different flower organs. (a) Mean number of microbes (*Pseudomonas fluorescens*) (±SE) deposited on the corolla, stamens, and pistil of live monkeyflowers. N = 20 bees. (b) Mean number of microbes (±SE) acquired by bees foraging via scrabbling, buzzing, or nectaring on artificial flowers (log-transformed data were analyzed; raw data are plotted). N = 22, 19, and 18 bees for the scrabbling, buzzing, and nectaring treatments, respectively. (c) Mean number of microbes (±SE) deposited on the corolla, stamens, and nectary of artificial flowers by bees foraging via scrabbling, buzzing, or nectaring. Letters above bars indicate significant differences at  $P < 0.05$  via Tukey's post hoc tests.



could contribute to the maintenance of a core microbiome among floral organs (Junker and Keller 2015, Allard et al. 2018; M. Rebolleda-Gómez and T.-L. Ashman, *unpublished manuscript*). Given that bees can also transmit microbes to and from artificial nectar (Hausmann et al. 2017), future work will be required to characterize the extent to which microbes acquired from floral organs other than the corolla are dispersed among floral organs.

Microbial community structure can differ distinctly among floral organs, and such differences have been attributed to environmental variation among floral organs filtering microbial growth or dispersal, although few studies have addressed these mechanisms directly (Herrera et al. 2010, Junker et al. 2011, Pozo et al. 2012, Aleklett et al. 2014, Junker and Keller 2015). Our results provide direct evidence that pollinators can contribute to differences in microbial abundance among and within flowers through inoculation and that their foraging behaviors can mediate these differences (Pozo et al. 2012). Specifically, we found that bumble bees foraging on live flowers or foraging for pollen (scrabbling or buzzing) on artificial flowers deposited most microbes on the corolla, consistent with observations in nature (Pozo et al. 2012). Likewise, less than 40% of our bees deposited any microbes into the nectary, suggesting that low richness of nectar microbial communities observed in nature may potentially be driven by low dispersal (though this could also be a consequence of acquisition from the corolla vs. other floral organs). While our understanding of how dispersal affects flower microbial communities has been nearly entirely informed by a floral nectar context (Tucker and Fukami 2014, Toju et al. 2017, Vannette and Fukami 2017), our results broaden this understanding by indicating that the relative importance of dispersal may depend on the floral organ. Similarly, microbial placement on bees via interaction with specific floral organs (similar to pollen placement; e.g., Tong and Huang 2018) may be an important factor mediating microbial dispersal and warrants future study. For instance, our study suggests dispersal may occur via bee cuticle or proboscis (used to collect nectar and groom pollen in part), and Hausmann et al. (2017) demonstrate the importance of proboscis-mediated dispersal.

Assuming our results hold across epiphytic microbial species to some degree, bee-mediated dispersal would also affect microbial diversity (Allard et al. 2018, Miller et al. 2018). Indeed, given that nearly all bumble bees dispersed microbes to the corolla and the corolla received the most microbes, our results are consistent with studies showing that the corolla typically hosts more microbial diversity than the pistil and stamens (Junker et al. 2011, Pozo et al. 2012, Junker and Keller 2015). We should note however that floral organ size likely shapes microbial receipt (larger flower organs received more microbes in our study) and that bumble bees foraging for nectar on artificial flowers deposited microbes equally among floral organs (though we could have found no effect due to low microbial dispersal by nectar foragers). Given that floral organ size varies among plant species and that pollinators may use multiple foraging behaviors on a single flower (Laverty 1980, Corbet et al. 1988), patterns of microbial community structure in nature are likely more diverse than our study implies, though they might still reflect the dominant pollinator foraging behavior (e.g., nectaring, scrabbling, buzzing; see also Zemenick et al. 2018). Additionally, while artificial flowers compared to live flowers likely differ in physical properties that affect microbial dispersal, in the present study dispersal patterns were qualitatively similar between live and artificial flowers.

While it has long been acknowledged that pollinator behaviors affect the efficiency of pollen transfer and contribute to the evolution of pollen packaging strategies (Castellanos et al. 2006, Hargreaves et al. 2009), our results suggest that collateral transfer of microbes should be considered when assessing floral trait evolution. We found that bumble bees foraging for nectar dispersed 54% fewer microbes to flowers than bees foraging for pollen. Differences in microbial dispersal were not due to differences in time spent foraging (see also Adler et al. 2018). However, bees foraged much more vigorously within flowers for pollen than for nectar (A. Russell, *personal observation*), likely explaining differences in dispersal. Thus, additional to the cost of offering their gametes (pollen) as a reward (Hargreaves et al. 2009, Nicholls and Hempel de Ibarra 2016), our results suggest that flowering plants offering mainly pollen to pollinators may typically

contend with more (or more diverse) epiphytic microbes than plants that offer mainly nectar. Consistent with this, for several dioecious species, male flowers had more (or more diverse) microbes than female flowers (which have no pollen to offer to pollinators; Tsuji and Fukami 2018, Wei and Ashman 2018). Assuming greater microbial transmission translates to more pathogens received, this pattern could in part account for male flowers generally being shorter lived than female flowers, as higher pathogen transmission is predicted to select for reduced flower longevity (Ashman and Schoen 1994, 1996, Shykoff et al. 1996). Likewise, our results suggest that pollen-offering plants may potentially evolve traits that manipulate bee behavior and thereby reduce microbial load. For instance, we found that bees buzzing for pollen on artificial flowers acquired floral microbes 79% slower than bees scrabbling for pollen. We speculate that perhaps poricidal floral morphology, which protects pollen from the environment (Gottsberger and Silberbauer-Gottsberger 1988, Zhang et al. 2014), and which requires bees to buzz and has evolved in 6% of plant species (22,000 species across >80 plant families; Buchmann 1983, Russell et al. 2017a; D. D. Jolles, et al., *unpublished data*), evolved in part as a means to reduce pathogenic microbial transmission. However, while direct selection by pollen transfer on flower morphology can often be strong (Gervasi and Schiestl 2017), the strength of selection by floral microbes is unknown. Much work remains to determine how diverse floral traits, beyond the single general floral morphology we tested, affect and evolve in response to acquisition and transmission of potentially deleterious microbes.

Our work lays the foundation for studying characteristics that affect dispersal of microbes among flowers and their pollinators by quantifying microbial acquisition and deposition by bees. Although bumble bees acquired relatively few microbes with each flower visit, individual flowers can receive hundreds of visits and pollinators may likewise need to make hundreds of floral visits per foraging trip (Laverty 1994, Williams and Thomson 1998, Balfour et al. 2015). Low acquisition could also have been a result of using a microbe not necessarily adapted for dispersal by pollinators, though future work is required to determine whether microbes have

such adaptations (Brysch-Herzberg 2004). Further, bees in our study retained 69% of floral microbes across visits, suggesting that microbes acquired from even a single flower visit may be vectored to many subsequent flowers. Similarly, these results suggest pollinators might be persistently exposed to microbes by flowers (see also Graystock et al. 2015, Adler et al. 2018, McFrederick and Rehan 2019). How quickly animal-pollinated flowers in nature acquire a floral microbial community is unknown, but our study suggests these rates may be considerable and could contribute to the evolution of floral traits such as flower longevity (Ashman and Schoen 1994, 1996, Shykoff et al. 1996). Given increasing awareness of the importance of plant–pollinator–microbe interactions and of flowers as hotspots of microbial transmission in natural and agricultural systems (Kevan et al. 2007, Rering et al. 2017, Adler et al. 2018, McFrederick and Rehan 2019, Russell and Ashman 2019), consequences of pollinator behavior extend beyond plant fertilization to patterns of microbial transmission.

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