Original Article

Associative learning of flowers by generalist bumble bees can be mediated by microbes on the petals

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Communication is often vital to the maintenance of mutualisms. In plant-pollinator mutualisms, plants signal pollinators via floral displays, composed of olfactory, visual, and other plant-derived cues. While plants are understood to be associated with microbes, only recently has the role of microbial (yeast and bacteria) inhabitants of flowers as intermediaries of plant-pollinator communication been recognized. Animals frequently use microbial cues to find resources, yet no study has examined whether microbes directly mediate learned and innate pollinator responses. Here, we asked whether microbes on the flower surface, independent of their modification of floral rewards, can mediate these key components of pollinator preference. In the field, we characterized flower and bumble bee microbial abundance, and in laboratory assays we tested whether bumble bees (Bombus impatiens) discriminated flowers on the basis of an experimental floral microbial community on the petals and whether microbe-derived chemicals were effective cues. Learning of microbial community cues was associative and reward context-dependent and mediated by microbial chemicals. Deconstructing the experimental microbial community showed bees innately avoided flowers with bacteria, but were undeterred by yeast. Microbial cues thus potentially facilitate dynamic communication between plants and pollinators such as bumble bees, especially as pollinator visitation can change flower microbiota. We suggest that the study of communication in mutualism generally would benefit by considering not only the multicellular eukaryote partners, but their microbial associates.

Key words: bumble bee, communication, floral cues, flower microbes, learning, microbial volatiles.

INTRODUCTION

Microbes commonly mediate species interactions between eukaryotes, such as by producing cues used in antagonistic interactions (e.g., squid antipredator defense McFall-Ngai 1990; plant anti-herbivore defense Ray et al. 2015, and parasitoid host plant selection; Tao et al. 2017), but their role in mutualistic interactions remains poorly understood. Furthermore, animals are frequently sensitive to microbial cues (Janzen 1977; Schulz and Dickschat 2007; Davis et al. 2013) and can rely on microbial cues such as odor and even color to find resources in diverse environments (Tosi and Sola 1993; Dillon et al. 2000; Archie and Theis 2011; Leroy et al. 2011; Hendry et al. 2018). Mutualism has been extensively studied in the context of plant-pollinator interactions, with relatively little attention paid to how microbial associates of flowers might mediate plant-pollinator communication and how pollinators might use microbial cues to forage. Animal-pollinated flowering plant species advertise their presence to pollinators via a diversity of floral rewards and entice pollinators to return by offering rewards, such as pollen and nectar (Kevan and Baker 1983; Kitaoka and Nich 2009). Indeed, pollinator preferences for floral visual, olfactory, tactile, gustatory, and even electrical cues are thought to be a key selective agent in the evolution of floral displays (Chittka and Thomson 2005; Leonard et al. 2011; Clarke et al. 2013; Schiestl and Johnson 2013). Yet while floral cues mediating pollinator preference have previously been considered to be produced primarily by the flower (Raguso 2008), the role of microbes living on flowers in shaping the floral display and the resultant pollinator preference has only recently begun to be examined in detail (e.g., Kevan et al. 1988; Good et al. 2014; Junker et al. 2014; Schaeffer et al. 2017; Rering et al. 2018).

The preferences of diverse pollinator taxa are typically shaped by both innate biases and learning (Schiestl and Johnson 2013) and have been especially well-documented in generalist bees (Chittka and Thomson 2005). Indeed, the foraging preferences of generalist bees have been so well studied that they are considered a model system for the study of learning (Giurfa 2007; Menzel 2012;
Leonard and Masek 2014). Because floral microbes (and pollen) often depend on pollinators such as bees and hummingbirds for dispersal (Eisikowitch et al. 1990; Brysch-Herzberg 2004; Pozo et al. 2012; McFrederick et al. 2012), both microbe and flower could benefit when microbes modify the floral display in a manner innately preferred by such pollinators. However, it might also benefit plant and pollinator when pollinators innately avoid previously recently visited flowers, which are likely to be depleted of rewards (including pollen), to be vectors of pathogens, and to be already pollinated (Jennersten 1988; McArt 2014; Graystock et al. 2013; although, multiple visits typically enhance mate fitness, e.g. Ashman 2000). In contrast to innate (fixed) preference, learning enables pollinators to rapidly and flexibly alter preference as floral conditions change, thereby maximizing foraging success (Papaj and Prokopy 1989; Giurfa 2007; Raine and Chittka 2006). Because floral microbiota change rapidly as a consequence of flower visitation (e.g., de Vega and Herrera 2012; Good et al. 2014; Ushio et al. 2015), it could benefit pollinators to respond flexibly to floral microbial cues via learning, particularly when microbial cues are associated with the presence or absence of a floral reward. Yet learning in this context has not been studied and the relative contribution of innate bias versus learning of microbial cues to pollinator preference remains unknown.

Flowers possess diverse epiphytic yeasts and bacteria whose populations often reach high densities on flower surfaces such as the corolla, styles, and anthers that comprise the floral display (reviewed in Aeklett et al. 2014; Junker and Keller 2015; Wei and Ashman 2018; Supplementary Figure S1). Yet research on how floral microbes mediate the responses of pollinators such as bees to date has exclusively concerned effects of microbes on nectar rewards (i.e., changes to nectar chemistry). This is a significant oversight because bees and other pollinators visit angiosperms for diverse floral rewards (e.g., pollen, oils, and shelter; Simpson and Neff 1981; Kitaoka and Nich 2009), and even when nectar is available, nectar and cues to its presence are often concealed from bees (LaVerty and Flowright 1980; Lewis 1993; Hansen et al. 2012). Thus, microbes living unconcealed on flower surfaces are potentially a key and common source of cues to bees.

For flower microbes to act as a cue mediating learned and innate floral preferences, these microbes must modify the floral display. A variety of sensory modalities could be involved. For instance, floral microbes potentially alter the gustatory (e.g., via excretion of metabolic compounds; Ehlers and Olesen 1997; Wiens et al. 2008; Rering et al. 2018) and/or scent profile of the floral display. Floral yeasts and bacteria can add scent compounds to flowers and may also alter floral scent emission by inducing, reducing, and/or even potentially catabolizing various scent compounds or other floral chemistry (Peachuelas et al. 2014; Helletsgruber et al. 2017; Burdon et al. 2018). Generalist bees perceive at least some of these scents (Rering et al. 2018), and while bees find bacteria-colonized nectar to be distasteful (Vannette et al. 2013; Good et al. 2014; Junker et al. 2014), bees respond positively or neutrally to yeast-colonized nectar (Kevan et al. 1988; Junker et al. 2014; Rering et al. 2018; Schaeffer et al. 2017). Furthermore, generalist bees are sensitive to a wide range of odors and are capable of quickly learning odors associated with floral nectar (Giurfa 2007; Lawson et al. 2018), suggesting they could also potentially learn microbial chemical cues. However, how a microbial community on flower surfaces affects bee preference, whether yeast versus bacteria affect preference divergently, and the nature of microbial cues mediating preference remains unknown.

In this study, we hypothesized that floral microbes, rather than microbial changes to rewards, could mediate both innate and learned components of preference for the generalist bumblebee Bombus impatiens, and that innate preference would differ with microbial type (yeast versus bacteria). Furthermore, we hypothesized that microbial chemical cues could mediate bumblebee preferences. To test bumble bee perception of ecologically realistic floral microbial cues, we first characterized in the field the extent of epiphytic microbes on diverse flowers offering pollen and/or nectar rewards and on the putative microbial vectors, generalist bumble bees (Bombus spp.). To test how floral microbes mediated innate and learned components of preference, we trained free-foraging captive bumble bees (B. impatiens) under controlled laboratory conditions to discriminate artificial flowers with and without an experimental floral microbial community, composed of 2 yeast strains and 2 bacterial strains commonly found on bees and animal-pollinated flowers. We next used Evacum affine (“Persian violet,” Gentianaceae) as a model to test whether bees learned to discriminate flowers with or without supplementary yeast and bacteria when in the context of a natural floral background and microbiome. We also evaluated whether innate preferences of bumble bees on live flowers were due to the supplementary bacteria or yeast. Finally, we assessed whether the chemicals that the experimental microbial community produced mediated learning, independent of the microbes themselves.

**METHODS**

**Field survey**

To test pollinator perception of ecologically realistic floral microbial cues we characterized the extent of epiphytic microbes on morphologically diverse flowers and foraging bumble bees along 2 transects in Pittsburgh, PA (flowers were collected in July 2017, along a 4.3 km transect: 40°28′39.8″N 80°00′52.7″W to 40°27′15.5″N 80°00′22.7″W; bees were collected in August 2017, along a 4.6 km transect (40°29′05.6″N 79°54′53.6″W to 40°27′12.9″N 79°55′53.0″W). Randomly selected intact flowers (Supplementary Figure S1) were cut just under the sepals from 5 plant species with floral displays of similar size to greenhouse E. affine (also sampled) used in behavioral experiments, and which offered pollen and nectar rewards, or only pollen (Commelina communis and E. affine). Most samples were collected directly into 50 mL Falcon tubes (Fisher Scientific) and stored on ice; some bees were netted first (nets sterilized with 70% ethanol after each use). To determine background contamination, every 10th sample a sterile Falcon tube was opened for 1 min. To capture the full range of microbial communities, we characterized bees with and without pollen in their pollen baskets (corbiculae) as pollen and nectar foragers, respectively, though the former may have also collected nectar. Prior to counting microbes we removed corpicular pollen from bees. For each sample we added 2 mL of sterile saline, sonicated 10 min, and then transferred 1 mL to sterile 1.5 mL micro-centrifuge tubes with 10 uL of 2.5% safranin stain. Safranin stains cell walls and pollen grains are distinctly shaped and typically much larger than microbes, thereby enabling us to distinguish between microbes and non-microbes. For each sample, we counted microbes in a 10 uL aliquot using a hemocytometer (Hausser Scientific, Horsham, PA) at 400 x or 100 x (Leica DM 500) and subtracted the mean number of microbes in controls to arrive at an estimate for each sample.

**Bees and housing**

We maintained 4 commercially obtained (Koppert Biological Systems, Howell, MI) captive colonies of the bumble bee B. impatiens following Russel et al. (2017). In brief, we allowed colonies
to forage freely on 2 M sucrose solution and pulverized honeybee-collected pollen (Koppert Biological Systems) from artificial feeders within enclosed test arenas (LWH: 82 x 60 x 60 cm) set to a 14:10 h light:dark cycle.

Plants and flowers

So that experimental microbes could not modify nectar and thereby mediate bee responses, we used only pollen-rewarding flowers in experiments. In natural flower experiments we used the nectarless E. affine, which offers pollen within tube-like poricidal anthers, reasoning that this morphology isolated the experimental microbes on the corolla (see later methods) from the pollen reward. Sixty-five plants were grown in a greenhouse with supplemental halogen lights to extend day length to a 14:10 h light:dark cycle, and were fertilized weekly (Miracle Gro, NPK 10:30:20) and handled with sterile equipment. Artificial flowers were of comparable size to E. affine and created from laminated paper (corolla) and chenille stem (anther; see Russell and Papaj 2016) (Supplementary Figure S2).

Microbes

We cultured 2 epiphytic yeast (Metschnikowia gruessii Y-17809, Candida rancensis Y-40759), and 2 bacteria (Leuconostoc fructosum B-2041, Lactobacillus micheneri HV6) commonly isolated from bees and flowers (Brysch-Herzberg et al. 2004; McFrederick et al. 2012; Pozo et al. 2012; McFrederick et al. 2018). These were acquired from the USDA-NRRL (ARS Culture Collection National Center for Agricultural Utilization Research, Peoria, IL) or provided by Q. McFrederick (L. micheneri).

Yeast and bacteria strains were grown separately in sterile YM (in 1 L water: 3 g each yeast and malt extracts, 5 g peptone, 10 g glucose) and YM + 2% fructose broth, respectively, in a shaker at 30 °C and 200 RPM. To achieve similar cell densities, we grew C. rancensis and L. fructosum for 24 h and M. gruessii and L. micheneri for 48 h and counted microbes via hemocytometer as above. Strains were thereafter transferred to separate 1.5 mL sterile microcentrifuge tubes, and put through 2 rounds of centrifuging (10 000 RPM for 5 min) and sterile saline (8.5% NaCl) substitution and elimination to purge media. Strains were stored at 4 °C for up to 5 days for behavioral trials, and regrown weekly from parent stock stored at −80 °C in 25% glycerol.

Experiments

General experimental protocol

Behavioral trials were conducted in a cleaned test arena attached to an enclosed bumble bee colony following Russell et al. (2016). In brief, flowers were displayed horizontally on the arena wall opposite the nest entrance. Flowers (number varying with experiment, see below) were spaced 7 cm apart in Cartesian grid design. When trials offered 2 types of flowers, types were systematically alternated by position in the grid.

To initiate a trial we set up flowers and allowed one flower-naive worker bee (hereafter “naive bee”) into the arena. Each bee visited most flowers in its trial at least once. To ensure trials were comparable, we terminated a trial after the bee reached a predetermined criterion (see experiments for details) or, rarely, if the bee did not approach any flower for a period of 5 min, whichever came first. Bees never depleted pollen from rewarding flowers during trials and rarely filled their corbiculae during trials. After a bee completed its trials, the bee was euthanized. Across all laboratory experiments, we used 203 workers from the 4 colonies. Experiments that used live flowers used freshly clipped flowers for each trial and were never reused (4680 flowers altogether). Each plant was represented at most once per trial. Chenille stems of artificial flowers were discarded after each trial, and the corollas were soaked and scrubbed in 70% ethanol and reused. We systematically alternated treatments in a given experiment to control for effects of day and time on behavior.

Experiment 1: do bees learn to discriminate artificial flowers with and without floral microbes?

Here we tested initial (innate) preference and trained bees to discriminate between artificial flowers with and without an experimental floral microbial community (Figure 1a). We used 38 bees from 2 colonies. We tested initial preference by presenting naive bees with equal numbers of 2 types of rewarding artificial flowers in a 3 × 4 array. Each bee was allowed to make up to 40 flower visits in its single trial. A rewarding artificial flower’s anther was always loaded with 4 mg of commercially available cherry pollen (Prunus avium pollen, Pollen Collection and Sales; Lemon Cove, CA), and the corolla of one flower type received a microbial community (“Microbe flowers”) composed of 2 bacterial strains (8.09 × 10^4 L. fructosum cells and 2.01 × 10^5 L. micheneri cells) and 2 yeast strains (3.86 × 10^4 C. rancensis cells and 3.19 × 10^4 M. gruessii cells). We reasoned that by adding an order of magnitude fewer epiphytic microbes (1.72 × 10^5 cells) than found on flowers (Supplementary Figure S1) we tested pollinator perception under realistic conditions. The corolla of the other flower type received a sterile control solution (“Control flowers”), with both sterile culture media mixes and diluted following methods described above. To inoculate flowers, 1 h prior to a behavioral trial each microbe strain and the control solution was vortexed and incubated at 28 °C. After incubation, we pipetted and evenly spread four 5 µL droplets per flower corolla: each Microbe flower received a single droplet of each strain.

Bees that had their initial preference tested were painted with unique color combinations using non-toxic oil markers (Sharpie, CA) and returned to their colony. Twenty-four to 48 h later, marked bees were randomly assigned to 1 of 2 differential conditioning (to test associative learning; see Giurfa 2007) training treatments and presented with a 5 × 4 array of Microbe and Control flowers. Treatments differed in which flower type was rewarding, and bees were allowed to visit flowers until they had met the learning criterion (8 of the last 10 visits made to the rewarding flower type). Unrewarding flowers were pollen-scented to preclude possible discrimination via pollen scent, following Muth et al. (2016). If a bee did not meet the learning criterion in a single training trial (5 bees), it was returned to its colony to unload its pollen and allowed to visit a new set of flowers in a second training trial. Eight additional bees failed to complete the microbe training treatment and 6 other bees would not visit flowers after the initial preference test.

To test whether bees retained the learned preference, each trained bee was given a single retention test 30–60 min after training, using a 4 × 5 array of Microbe and Control flowers. We allowed each bee to make up to 40 flower visits. Flowers in the retention test were unrewarding to test whether learning of microbes was associative.

To train and assess preference, we recorded bee behavior during trials following Muth et al. (2016). In brief, we recorded flower “visits,” defined as a bee touching the scented or pollen-covered...
anther. When bees collected pollen from rewarding flowers (scraped from anthers by “scrabbling”; see Russell and Papaj 2016), the visit was recorded as “rewarded”. When bees visited unrewarding flowers, the visit was recorded as “unrewarded”. In the rare instances when bees visited, but did not collect pollen from rewarding flowers, we excluded the visit from analyses because we could not be sure whether the visit reinforced or inhibited learning.

**Experiment 2: do bees learn to discriminate live flowers with and without supplemental floral microbes?**

Here we examined innate preference and trained bees to discriminate live *E. affine* flowers—which already possess a microbiome and a suite of potentially competing floral cues—on the basis of an experimental floral microbial community (Figure 1b). We used 53 bees from 3 colonies.

We assigned naive bees to 1 of 3 treatments: 1 no-experience treatment, and 2 flower-experience treatments. In the no-experience treatment, naive bees had their innate preference for flowers with an experimental microbial community tested, by presenting them with equal numbers of rewarding Microbe and Control flowers in a 4 × 5 array. In the 2 flower-experience treatments, we evaluated effects of experience with 2 separate groups of naive bees. Following Russell et al. 2016, via absolute conditioning (Giurfa 2007) bees were trained in a single trial with a 4 × 5 array of Microbe or Control flowers. Twenty to 60 min after training, we gave trained bees a single retention test, using an array identical to that used in the no-experience treatment. We allowed each bee up to 40 pollen collecting flower visits in each training trial and each retention test. From analyses, we excluded 5 bees, which had made fewer than 40 pollen collecting flower visits during their training trial and would not visit retention test flowers.

To train and test learned preference with the live flowers, which always presented a reward, we recorded landings, defined as the bee touching the flower with at least 3 of its legs simultaneously, and whether the bee collected pollen (via floral sonication of the anthers; see Russell et al. 2017). A bee occasionally visited the same flower more than once in a row (landing, hovering within 3 cm of the same flower, and then relanding). For retention tests, we discarded these landings (mean = 10.2% of all visits), as the bee may not have had the opportunity to actively assess the other flowers. We kept landings for training trials, because landings might reinforce learning. To determine whether bees perceived microbial cues prior to touching flowers, we also recorded approaches, defined as the bee greatly reducing its velocity while facing the flower, while within 3 cm of the flower. All landings were preceded by an approach (“acceptance”), although not all approaches were followed by landing (“rejection”).

**Experiment 3: do bacteria or yeast strains contribute to bee innate preferences?**

We determined whether patterns of innate preference could be explained by bacteria or yeast of the experimental microbial community, using 28 bees from 2 colonies (Figure 1c). Naive bees were assigned to either of 2 no-experience treatments and again allowed to make up to 40 pollen collecting visits in their trial. Both treatments used 2 types of live *E. affine* flowers. In one treatment, naive bees had their innate preference for supplemental floral bacteria tested. For this treatment, 2 flower types were created: to the corollas of one flower type we added two 5 uL drops of each bacterial strain (“Bacteria flowers”); 2.02 × 10^7 cells added). To the other flower type, we added a sterile control solution (“Control flowers”), composed of the diluted YM + 2% fructose broth. In the other treatment, instead of Bacteria flowers, we made “Yeast flowers” (1.41 × 10^8 cells added). Control flowers in this treatment received the diluted YM broth.

**Experiment 4: do microbial chemical cues mediate learned preferences?**

Here we tested whether bees could learn to discriminate flowers based only on chemicals produced by the experimental microbial community (Figure 1d). We incubated and centrifuged strains in saline solution to isolate the microbes from their chemicals suspended within the supernatant. We confirmed using a
hemocytometer that centrifuging eliminated most microbes from the supernatants (mean remaining cells in the supernatants: 4333 \( L. \) fructosum; 5167 \( L. \) micheneri; 4167 \( C. \) rancensis; 3500 \( M. \) gruissii).

We assigned naïve bees to 1 of 4 treatments using live \( E. \) affine flowers: 2 no-experience treatments, and 2 flower-experience treatments. Treatment design followed experiment 2, but differed in the flower types used. In one no-experience treatment, we tested innate preference for the experimental floral microbial community, using the 2 types of formerly described flowers (Control and Microbe flowers). We used this no-experience treatment to compare to a second no-experience treatment in which we tested a separate group of bees’ innate preference for the chemicals of the experimental microbial community. We created 2 flower types for this treatment: to the corolla of one flower type we added two 5 μl drops of microbial chemicals (“Supernatant flowers”). To the other flower type we added the sterile control solution (“Control flowers”). The 2 flower-experience treatments used absolute conditioning to evaluate effects of experience with Supernatant flowers versus Control flowers. We used 51 bees from 2 colonies and excluded 5 bees from analyses, because they made fewer than 40 pollen collecting flower visits during the training trial and would not visit retention test flowers.

Data analyses

All data were analyzed using R v3.3.2 (\cite{R} Development Core Team 2016).

Experiment 1

To determine whether with successive visits bees made more visits to the rewarding flowers (i.e., learned which flower type was rewarding), we used generalized linear mixed effects models with a binomial distribution (generalized linear mixed models [GLMMs]) using the glmer() function in the lme4 package (\cite{Bates} et al. 2015), specifying type II Wald chi-square (\( \chi^2 \)) tests via the Anova() function in the car package (\cite{Fox} 2015). We checked model assumptions for all GLMMs using the DHARMa package (\cite{Hartig} 2018). The response variable was “visit type” (rewarded or unrewarded) and the explanatory variables were “treatment” and “visit number”. We included “bee” and “colony” as random factors, with “visit number” as repeated measures within bee, within colony. To determine whether bees learned to associate the presence or absence of floral microbes with the pollen reward, we compared initial preference to retention test preference, using paired \( t \)-tests.

Experiments 2–4

We analyzed naïve bees’ innate preference on their very first flower visit for each treatment using G-tests. To analyze naïve bees’ overall preference for one or the other flower type in each treatment, we performed paired \( t \)-tests on the mean proportion of each bees’ rewarded visits to both flower types, if assumptions of normality and equal variance were met (using Shapiro-Wilk and \( F \) tests, respectively, in the mgcv package: \cite{Wood} 2015) or, otherwise, Wilcoxon signed-ranks tests.

Experiments 2 and 4

To analyze how experience affected flower preference, we used GLMMs as above. The response variable was “flower type landed on,” the explanatory variable was “treatment,” and we specified random factors as above. We also used GLMMs to analyze the effect of experience on whether bees approached, but did not land on a flower type. The response variable was “approach type” (rejection vs. acceptance), the explanatory variable was “treatment,” and we specified random factors as above. In cases of significant effects, we ran Tukey’s post hoc test using the glht() function in the multcomp package (\cite{Hothorn} et al. 2015) to determine which pairs were significant.

RESULTS

Number of microbial cells on bee and flower surfaces

All field flowers had abundant epiphytic microbes (Supplementary Figure S1): mean microbes ± SE: 1.02 × 10^6 ± 1.09 × 10^5; 5 plant species, \( N = \sim 10 \) flowers/species). Field-collected bumble bees had on average 4.26 × 10^6 ± 9.97 × 10^5 cuticular microbial cells (Supplementary Figure S1; \( N = 49 \) bees), suggesting they could be potent vectors of microbes (see Supplementary Table S1 for \( B. \) impatiens). Relative to flowers in the field survey and as expected of unvisited flowers, greenhouse-grown \( E. \) affine flowers had 73.4% fewer epiphytic microbes (Supplementary Figure S1: 2.50 × 10^6 ± 8.13 × 10^5).

Bees learn to discriminate flowers with and without microbes

In experiment 1, bumble bees (\( B. \) impatiens) visiting artificial flowers rapidly learned via differential conditioning whether an experimental floral microbial community composed of yeast and bacteria on the corolla was associated with the pollen reward (Figure 2a). Bees in both training treatments made more flower visits across consecutive visits to the rewarding flower type (GLMM: visit number effect: \( \chi^2_1 = 20.897, P < 0.0001 \); treatment effect: \( \chi^2_1 = 0.538, P = 0.463 \)). However, bees trained to associate flowers without microbes (Control flowers) with a pollen reward learned significantly faster than bees trained to associate Microbe flowers with a pollen reward (GLMM: treatment * visit number interaction: \( \chi^2_1 = 8.450, P = 0.0037 \)). This difference corresponds to a 22% better performance in the mean number of flower visits to reach the learning criterion (mean no. visits ± SE: trained to Control flowers: 45.3 ± 2.2; trained to Microbe flowers: 57.9 ± 5.8, \( N = 12 \) bees in each treatment).

Learned preferences were retained for at least 1 h: in unrewarded retention tests bees significantly preferred the artificial flower type they had been trained to relative to their initial preference (Figure 2b,c): trained to Microbe flowers: Wilcoxon signed-rank test: \( V = 77, P < 0.001 \); trained to Control flowers: paired \( t \)-test: \( t_9 = -6.352, P < 0.0001 \); \( N = 12 \) bees in each treatment)

Furthermore, supplementary microbial cues were learned against a live flower background and its established microbiome (Figure 3a). Bees trained via absolute conditioning to collect pollen from pollen-rewarding \( E. \) affine flowers with or without an experimental microbial community in experiment 2 significantly preferred the experienced flower type in the retention test 1 h later (GLMM: overall treatment effect: \( \chi^2_1 = 51.807, P < 0.0001 \)).

Naïve bees prefer live flowers without supplemental bacteria

Pooling equivalent treatments from experiments 2 and 4, naïve bees expressed a strong preference for live flowers without an experimental microbial community on both their first landing and across all landings (Figure 4a,b: first landing: G-test: \( G = 7.366, P = 0.0066 \); all landings: Wilcoxon signed-rank test: \( V = 473, P < 0.0001 \),
Naïve bees visiting artificial flowers however showed no such preferences on their first visit or across all visits (Figure 4a, b: first l: G-test: $G = 0.027$, $P = 0.869$; all visits: paired $t$-test: $t_{36} = −0.422$, $P = 0.676$; $N = 37$ bees).

Experiment 3 showed that naïve bees foraging for pollen on live flowers with or without supplemental bacteria strongly preferred flowers without supplemental bacteria on their first landing and across all landings (Figure 4a, b: first landing: G-test: $G = 7.925$, $P = 0.0049$; all landings: paired $t$-test: $t_{13} = 2.503$, $P = 0.0264$, $N = 14$ bees); however, this was not the case for supplemental yeast (Figure 4a, b: first landing: G-test: $G = 0.287$, $P = 0.592$; all landings: paired $t$-test: $t_{13} = 0.168$, $P = 0.869$, $N = 14$ bees).

Learned preferences are mediated by microbe chemicals

Indicating that microbial cues acted at a distance, bees discriminated flower types before touching flowers: experienced bees visiting live flowers in the retention tests of experiment 2 rejected (i.e., approached without landing on) the experienced flower type significantly less frequently (Figure 3c, d; GLMMs: trained to Control flowers: $\chi^2_{10} = 25.916$, $P < 0.0001$; trained to Microbe flowers: $\chi^2_{10} = 105.27$, $P < 0.0001$).

Further, in experiment 4, bees trained via absolute conditioning to collect pollen from live flowers with or without microbe chemical cues had learned to prefer the experienced flower type in the retention test 1 h later (Figure 3b: GLMM: overall treatment effect: $\chi^2_{1} = 51.274$, $P < 0.0001$).

DISCUSSION

Our results demonstrate that floral microbes can directly mediate plant-bumble bee communication, and thus their potential to influence this and possibly other plant-pollinator mutualisms goes well beyond microbial effects on nectar chemistry. We determine that alone, an epiphytic microbial community that includes both yeasts and bacteria and their chemicals can mediate both learned and innate components of bumble bee floral preference. As bees discriminated flowers prior to landing or touching the flower, their preferences were likely mediated by microbial scent (see also Rering et al. 2018). Our results thus significantly extend prior findings (e.g., Vannette et al. 2013; Pozo et al. 2014; Vannette and Fukami et al. 2015).
In showing that regardless of the floral reward (e.g., pollen, nectar), plant-bumble bee communication can be mediated by epiphytic microbes commonly found on flowers and/or transported by bees. Because floral signal evolution is often driven by pollinator preferences and especially learned preferences (Leonard et al. 2011; Schiestl and Johnson 2013), our results imply pollinator preference could also drive the evolution of floral microbial associates that mediate floral signals. For instance, bumble bees should more consistently visit flowers with microbial cues that are innately more attractive and/or can be learned. Since flower microbes are often dispersed by pollinators such as bumble bees (Brysch-Herzberg 2004; Hausmann et al. 2017; Russell et al. in review), microbes that evolve more attractive and/or stronger cues would have higher dispersal and survival.

 Associative learning can rapidly increase pollinator preference for floral cues associated with floral rewards and thus learning of floral cues is generally considered to benefit pollinators (Leonard et al. 2011; Schiestl and Johnson 2013). We showed that learning of microbial cues was associative and reward context-dependent, and we expect such learning benefits bumble bees by enabling them to forage preferentially on the most rewarding flowers. Learning of microbial cues by bees is thus likely to be highly flexible, similar to learning of flower cues like color, size, and pattern (Chittka and Thomson 2005; Giurfa 2007; Muth et al. 2016). Yet we also found evidence for a bias in learning of microbial cues. While learning in both reward contexts resulted in equally strong preferences, bees learned to associate floral microbes with a pollen reward significantly more slowly than when they had to learn to associate absence of floral microbes with a pollen reward. Furthermore, fewer bees completed training when they had to associate floral microbes with a pollen reward, possibly due to a reduced motivation to Forage on flowers with microbes. Together, these results suggest bees must overcome an innate avoidance of offensive microbial cues when such cues are paired with a floral reward.

 When we examined innate preferences specifically, we found that naive bees visiting live flowers significantly preferred flowers without an experimental microbial community. Innate avoidance is likely to have functional significance: previously visited flowers, which presumably have more microbes than unvisited flowers (Aizenberg-Gershtein et al. 2013; Schaeffer et al. 2015), should have less rewards. Further, remaining rewards may be of lower quality due

**Figure 3**

Top panels: Mean percentage of landings (±SE) on (a) microbe-supplemented or (b) microbial supernatant-supplemented live flowers in retention tests (experiment 2 and 4, respectively). Bottom panels: Mean percentage of rejections (approaches that do not end in landing) (±SE) on live flowers (c) with and (d) without a supplemental microbial community (experiment 2). In each experiment, the 3 treatments differed in terms of training: naïve bees were either given no experience (naïve) before being tested, or given experience foraging for pollen from either of 2 flower types (absolute conditioning: +). Experiment 2: either flowers with or without an experimental microbial community. Experiment 4: either flowers with or without an experimental microbial supernatant. N = 12 bees in each experience treatment and 24 bees for the no-experience treatment (aside from experiment 4, where N = 22 bees). Letters above bars indicate significant differences at P < 0.05 according to Tukey’s post hoc tests.
to pollinator-mediated microbial contamination. For instance, nectar colonized by pollinator-dispersed bacteria or yeast often has less sugar and amino acids (e.g., Herrera et al. 2008; Vannette et al. 2013; Good et al. 2014; Vannette and Fukami 2018). Such changes have been proposed to mediate pollinator preference, and our results significantly extend these findings in demonstrating that microbes and their chemicals need not be consumed to strongly affect the preference of free-foraging pollinators such as bumble bees.

Prior studies have found that bee preference for nectar colonized by microbes is strongly dependent on microbial type. Consistent with those studies, we found that bees were strongly innately repelled by floral bacteria on the corolla (e.g., Vannette et al. 2013; Good et al. 2014; Junker et al. 2014), but were indifferent to floral yeast on the corolla (e.g., Kevan et al. 1988; Rering et al. 2018). Accordingly, our work bolsters prior evidence that pollinator preference, at least in part, could drive selection for plants to evolve antibacterial traits. Selection for such traits could be reduced, however, if floral bacteria mediated bee learning. Learned preferences not only benefit pollinators such as bees (Gregear and Laverty 1995; Raine and Chittka 2008; Schiestl and Johnson 2013), but because learning generally increases pollinator fidelity to a given flower type, learned preferences can provide direct benefits to the plant by increasing conspecific pollen transfer and by reducing interference by foreign pollen (rev. Lewis 1993; Arceo-Gómez and Ashman 2011; Brosi and Briggs 2013). Future work will be required to determine whether both floral bacteria and yeast contribute to learned preferences.

Flower microbial associates have the potential to be an especially common and dynamic channel for plant-pollinator communication. Our results indicate that microbes frequently occur in high abundance on flower surfaces (Supplementary Figure S1), and microbial scents are thought to be common in flowers (e.g., Kevan et al. 1988; Goodrich et al. 2006; Rering et al. 2018); microbial cues perceived by pollinators are likely common. Furthermore, as all live flowers in our assays had a microbiome, our results demonstrate bees could discriminate even modest differences in microbial abundance, and suggest that bees can discriminate differences in microbial community composition. Consequently, microbial cues might conceivably be used by generalist bumble bees and probably other pollinators in a variety of common foraging contexts. For instance, a pollinator might potentially use such cues to assess flower age, phase (corresponding to reward type: e.g., Morris 1996), and prior visitation. Because bees disperse microbes while collecting floral rewards (Hausmann et al. 2017; Russell et al. in review), microbial cues may be an especially reliable cue, perhaps comparable with well-studied social cues such as scent marks (e.g., Stout and Goulson 2001; Witjes and Eltz 2009) and conspecifics (e.g., Baude et al. 2008; Dunlap et al. 2016).

In conclusion, we demonstrate that plant-bumble bee communication can be directly mediated by floral yeast and bacteria and their chemicals. Most importantly, microbial cues can mediate both learned and innate components of bumble bee preference, and because epiphytic microbes occur in high abundance on flowers and their pollinators, microbes could be a key player mediating plant-pollinator mutualisms generally. Microbial cues are likely to act in concert with plant-derived cues, such as flower patterns and colors and could therefore be an important component of the complex floral display (Giger and Srinivasan 1995; Leonard et al. 2011; Leonard et al. 2012; Lawson et al. 2018). We suggest future work should not only assess the variability and ontogeny of microbial cues and their mechanistic basis, but also taxonomic (and individual level) variability in pollinator response to these cues. Furthermore, we assert that study of mutualisms generally would benefit from an increased focus on microbial associates and their role in mediating communication between mutualist eukaryote partners.

**SUPPLEMENTARY MATERIAL**

Supplementary data are available at Behavioral Ecology online.

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