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Nectar-inhabiting microorganisms influence nectar volatile composition and attractiveness to a generalist pollinator

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Summary

• The plant microbiome can influence plant phenotype in diverse ways, yet microbial contribution to plant volatile phenotype remains poorly understood. We examine the presence of fungi and bacteria in the nectar of a coflowering plant community, characterize the volatiles produced by common nectar microbes and examine their influence on pollinator preference.

• Nectar was sampled for the presence of nectar-inhabiting microbes. We characterized the headspace of four common fungi and bacteria in a nectar analog. We examined electrophysiological and behavioral responses of honey bees to microbial volatiles. Floral headspace samples collected in the field were surveyed for the presence of microbial volatiles.

• Microbes commonly inhabit floral nectar and the common species differ in volatile profiles. Honey bees detected most microbial volatiles tested and distinguished among solutions based on volatiles only. Floral headspace samples contained microbial-associated volatiles, with 2-ethyl-1-hexanol and 2-nonanone – both detected by bees – more often detected when fungi were abundant.

• Nectar-inhabiting microorganisms produce volatile compounds, which can differentially affect honey bee preference. The yeast *Metschnikowia reukaufii* produced distinctive compounds and was the most attractive of all microbes compared. The variable presence of microbes may provide volatile cues that influence plant–pollinator interactions.

Introduction

Plant volatile emissions are a key phenotype that mediate a myriad of ecological interactions in natural and managed systems (Paré & Tumlinson, 1999; Kessler & Baldwin, 2001; Pichersky & Gershenzon, 2002; Junker & Tholl, 2013), including pollination (Dobson, 1994; Raguso, 2001). Indeed, floral volatile emission can mediate pollinator choice among (Byers *et al.*, 2014) and within (Galen & Newport, 1988; Kessler *et al.*, 2011) plant species, and is often a target of pollinator-mediated selection (Parachnowitsch *et al.*, 2012; Gervasi & Schiestl, 2017).

Microorganisms can contribute to plant phenotype in diverse ways (Friesen, 2013) including by changing plant volatile emissions (Pineda *et al.*, 2010; Shapiro *et al.*, 2012). In some cases, microbial pathogens alter the host plant's volatile profile to attract the pathogen's insect vector (Jiménez-Martínez *et al.*, 2004; Mann *et al.*, 2012). That microbial volatiles contribute to plant–pollinator interactions has been hypothesized (Raguso, 2004, 2008; Pozo *et al.*, 2009), and implicated (Golonka *et al.*, 2014; Peñuelas *et al.*, 2014; Schaeffer *et al.*, 2017). However, whether volatiles produced by microbes, rather than their plant hosts, can alter ecological interactions remains poorly understood (but see Davis *et al.*, 2013), particularly for plant–pollinator interactions. If microorganisms directly modify a plant's chemical phenotype (chemotype), their influence could extend not only to pollination, but possibly other plant–insect interactions as well (Beck & Vannette, 2017).

Yeasts and bacteria are common and abundant inhabitants of floral nectar (Herrera *et al.*, 2008, 2009), and often rely on pollinators to disperse among individual flowers (Brysch-Herzberg, 2004; Canto *et al.*, 2008; Herrera *et al.*, 2008; Vannette & Fukami, 2017). The microorganisms that specialize on the nectar environment must rapidly and repeatedly disperse and re-establish themselves in new flowers, which in some cases may require multiple pollinator visits (Mittelbach *et al.*, 2016a,b). Yeasts that inhabit other ephemeral habitats, including decaying fruit or plant material, tend to rely on other organisms for dispersal to specific habitats (reviewed by Mittelbach & Vannette, 2017) in a process called phoresis. For example, volatile compounds emitted by yeasts are attractive to insect vectors including *Drosophila* (Buser *et al.*, 2014). In nectar, microbial metabolism can influence sugar composition and concentration (Herrera *et al.*, 2013; Vannette *et al.*, 2013; Good *et al.*, 2014; Schaeffer *et al.*, 2014, 2015), ethanol concentration, amino acid

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composition (Peay *et al.*, 2012) and other metabolic products that contribute to flavor and scent of nectar (Vannette & Fukami, 2016). Pollinators may respond innately or learn to associate such products with nectar availability or quality (Knauer & Schiestl, 2015). Volatile chemicals produced by microbes are suspected to play a role in pollination: while nectar-inhabiting yeasts have been reported to emit volatile compounds (Golonka *et al.*, 2014), volatile profiles of nectar-inhabiting microorganisms have not been compared and the influence of microbial volatiles on pollinator preference has not been previously examined.

Here, we test the hypothesis that nectar-inhabiting microorganisms vary in volatile emission and differentially influence attractiveness to a generalist insect pollinator. First, to establish the ecological relevance of microbes in nectar, we examined the presence of bacteria and fungi in the nectar of 28 species of coflowering plants in California. Second, we characterized volatile headspace of four commonly isolated microbial species in a synthetic nectar analog at multiple time points. Third, we used electroanntenographic (EAG) bioassays to examine which microbial volatiles were detectable to honey bees (*Apis mellifera*), and proboscis extension response (PER) bioassays to determine honey bee response to microorganisms inoculated in a nectar analog. We also determined whether microbe-associated volatiles could be detected in floral headspace samples taken from the field. Our results provide evidence that nectar-inhabiting microorganisms are common but variable inhabitants of nectar, and that different microbial species produce distinct volatile blends detectable in field samples, which may influence pollinator detection and preference.

Materials and Methods

Study system and microbial isolation

Nectar standing crop was collected from 1170 individual flowers of 28 nectar-producing plant species native to California in October 2015 and March–June 2016 (Supporting Information Table S1). All available flowering plant species at Stebbins Cold Canyon that produced at least 0.1 μl of nectar during the sampling period were sampled to examine frequency of bacterial and fungal colonization of nectar. Honey bees, used in later experiments, are common pollinators of many of the plant species sampled here, and also very common generalist pollinators of native plants and agricultural crops in California and world-wide.

Briefly, open flowers were collected, nectar was extracted in the laboratory and dilutions were plated. Yeasts and yeast-like fungi were cultured on yeast media agar (YMA: 0.3% malt extract, 0.5-% peptone, 1% glucose, 0.3% yeast extract, 2% agar) and a subset of representative colony morphotypes were identified from over 1200 plates by sequencing the D1/D2 domain of the large subunit nuclear ribosomal RNA with primers NL1/NL4 (O'Donnell, 1993). Bacteria were cultured on R2A media supplemented with 20% sucrose (BD, Franklin Lakes, NJ, USA) and colonies identified by sequencing the 16S rRNA gene using primers 27F/1492R (Lane, 1991; Turner *et al.*, 1999). The presence of fungi or bacterial colonies on plates was recorded for each individual flower, with lower detection limits of *c.* 5 colony forming units (CFU) for fungi and 50 CFU for bacteria. Isolates of identified species were stored at −80°C as glycerol stocks until use, and propagated on the media described above until inoculation into synthetic nectar solutions.

For this study, four commonly isolated microorganisms were selected for further analyses: the fungi *Metschnikowia reukaufii* and *Aureobasidium pullulans*, and the bacteria *Neokomagataea* sp. and *Asaia astilbes* (GenBank IDs: MF319536, MF325803, MF340296 and KC677740). Isolates were taken from the nectar of plant species *Epilobium canum* and *Mimulus aurantiacus*, which have a diverse group of flower visitors including honey bees, hummingbirds and other native insects. These microbes are all commonly isolated from flowers in the current and previous studies, so are all ecologically relevant to the nectar environment. However, these taxa are also likely to vary in their ecological affinity to flowers and pollinators. Previous work has found that *M. reukaufii* is dependent on pollinators for transmission among flowers (e.g. Belisle *et al.*, 2012; Vannette & Fukami, 2017), while *A. pullulans* has a broader habitat range (e.g. Andrews *et al.*, 1994; Wehner *et al.*, 2017). The ecological affinities of the bacterial taxa profiled here are unknown, but strains from these genera have been found in floral nectar in other studies previously (Vannette *et al.*, 2013; Good *et al.*, 2014).

Microbial growth conditions

Sterile synthetic nectar (0.3% w/v sucrose; 0.6% w/v each of glucose and fructose; 0.1 mM each of glycine, L-alanine, L-asparagine, L-aspartic acid, L-glutamic acid, L-proline and L-serine), designed to mimic floral nectar of bee-pollinated flowers (Baker & Baker, 1982; Gardener & Gillman, 2001), was inoculated with 20 ml of 10^3 cells μl^{-1} from actively growing subcultures and incubated at 29°C under aerobic conditions in sealed 118 ml Mason jars ($n = 3$ replicates of each microbial species: *M. reukaufii*, *A. pulluans*, *Neokomagataea* and *Asaia*). Mason jar lids remained hermetically sealed for the duration of each experiment, except for just before volatile collections times when the lids were gently lifted under sterile conditions to allow venting of accumulated volatiles. Lids were modified to accommodate two sampling ports by fitting GC septa (high temperature low bleed septa, 11 mm; Agilent, Santa Clara, CA, USA) into pre-drilled holes. Glassware was autoclaved immediately before use and preparations were carried out under sterile conditions. Microbial growth was confirmed visually, by pH decrease (Vannette *et al.*, 2013) and optical density (for bacteria) or cell count (for yeast) increase.

Volatile collection, analysis and identification

Headspace volatiles from inoculated microbes were collected onto solid-phase microextraction (SPME) fibers (Supelco, Bellafonte, PA, USA; 50/30 μm, 2 cm, divinylbenzene/carboxen/polydimethylsiloxane) at intervals of 0, 48 and 96 h (± 0.4 h). Two identical SPME fiber types were simultaneously inserted into the sampling ports installed on jar lids. Volatile collections used the following fiber parameters (Beck *et al.*, 2008): accumulation of volatiles in the freshly sealed container (see above for venting procedure to allow for collection of volatiles at the specific time

point), 15 min; exposure of fiber to adsorb volatiles, 15 min; storage time of volatiles on fiber, ≤ 1 min; and thermal desorption of volatiles in injector ports, 6 min. The adsorbed volatiles were thermally desorbed in splitless mode onto an Agilent 7890A gas chromatograph coupled to a quadrupole 5975C MSD detector in electron ionization mode (Palo Alto, CA, USA) outfitted with a J&W Scientific (Folsom, CA, USA) DB-Wax column (60 m × 320 μm × 0.25 μm), and an Agilent 7890B gas chromatograph coupled to a quadrupole 5977B MSD detector in electron ionization mode and equipped with a J&W Scientific DB-1 column (60 m × 320 μm × 0.25 μm). Volatiles were analyzed using parameters identical to those previously described (Beck *et al.*, 2016) with the following modifications: DB-1 had a final temperature of 190°C, and the DB-Wax had an adapted flow of 3 ml min^{-1}. Data from the GC MSD device fitted with a DB-Wax column provided superior peak shape for polar analytes and was used for the qualitative comparison of compounds. Data from the DB-1-equipped instrument were used for additional identification and retention index (RI) calculations. RIs were calculated using a homologous series of *n*-alkanes on both the DB-1 and the DB-Wax columns. RI values from both columns were used to assist with initial identification, and identities were further confirmed by comparison to retention times and fragmentation patterns of standards. Compound identities not verified on both instruments with a commercial or other available standard were marked as tentatively identified. Additionally, if peaks could not be authenticated and the library matches were poor, their identities were labeled as unknown (see Table S2). The most abundant ions for unknowns are tabulated in Table S2. Peaks identified as background from the containers, fibers, columns and synthetic nectar that were found in media controls and blanks were removed before statistical analysis.

Electroantennographic bioassays

To examine if honey bee antennae respond to microbial volatiles, we tested all available and identified compounds that were produced by microorganisms (21 compounds, see Table 1). Honey

Table 1 Volatile compounds produced by microorganisms grown in synthetic nectar

		Retention indices[a]		Normalized EAG response (%; $n = 6$ bees)		Peak area[i] at 96 h ($\times 10^5$; mean ± SE, $n = 3$ jars)			
Class	Chemical	DB-Wax	DB-1	40 μmol	0.4 μmol	*Asaia*	*Neok*	*Metsch*	*Aureo*
1° Alcohol	ethanol[c]	933	–	1 ± 3	1 ± 3	23 ± 8	18 ± 3	6800 ± 200	3000 ± 100
	n-propanol[c]	1038	–	17 ± 4**	4 ± 6	0	0	30 ± 2	103 ± 4
	isobutanol[e]	1092	613	24 ± 14	−4 ± 4	1.5 ± 0.8	2.2 ± 0.2	614 ± 3	730 ± 30
	2-methyl-1-butanol[c,g]	1209	720	99 ± 16**	−6 ± 8	44 ± 2[g]	46 ± 1[g]	6990 ± 80[g]	1500 ± 100[g]
	3-methyl-1-butanol[c]	1209	718	143 ± 11**	1 ± 3				
	3-methyl-3-buten-1-ol[c]	1250	713	104 ± 34*	8 ± 12	–[h]	0	5.5 ± 0.2	3 ± 0.1
	4-penten-1-ol[c]	1304	735	69 ± 10**	0 ± 3	0	0	8.9 ± 0.6	8.9 ± 0.4
	***n*-hexanol**[c]	1355	853	160 ± 16**	62 ± 21*	5.1 ± 0.3	3.38 ± 0.08	6 ± 2	4.6 ± 0.3
	3-ethoxy-1-propanol[c]	1380	817	30 ± 7**	−4 ± 2	0	0	1.8 ± 0.4	0
	2-ethyl-1-hexanol[c]	1493	1014	60 ± 9**	59 ± 15**	77 ± 6	60 ± 2	29 ± 2	90 ± 60
	2-phenylethanol[c]	1913	1082	62 ± 5**	50 ± 9**	4.7 ± 0.5	4.6 ± 0.2	260 ± 20	16 ± 8
2° Alcohol	2-butanol[d]	1024	–	465 ± 86**	−3 ± 6	0	0	10 ± 1	0
Aldehyde	acetaldehyde[b]	–	–	–	–	3 ± 2	1.7 ± 0.4	96 ± 7	130 ± 40
Ester	ethyl acetate[c]	885	–	4 ± 8	−2 ± 4	0	0	130 ± 10	18 ± 3
	2-methylpropyl acetate[c]	1012	756	133 ± 26**	−24 ± 16	0	0	5.3 ± 0.6	0
	ethyl butyrate[c]	1035	783	68 ± 19**	−4 ± 3	0	0	6 ± 1	0
	3-methylbutyl acetate[c]	1122	861	81 ± 16**	6 ± 7	0	0	41 ± 2	0
Isoprenoid	Isoprene[b]	–	–	9 ± 7	9 ± 8	9 ± 1	10 ± 9	0	0
Ketone	4-methyl-2-pentanone[c]	717	1008	127 ± 20*	−7 ± 8	0	0	0	20 ± 10
	4-methyl-3-penten-2-one[f]	1132	776	–	–	1 ± 0.5	0	0	–[h]
	3-hydroxy-2-butanone[c]	1284	677	177 ± 93	21 ± 13	15 ± 1	12.3 ± 0.3	53 ± 0.9	20 ± 3
	2-nonanone[c]	1389	1072	87 ± 4**	76 ± 10**	0	0	0	6 ± 1
Misc.	**2,5-dimethylfuran**[c]	950	696	10 ± 5	−13 ± 13	16 ± 4	9 ± 1	0	0
	Unknown 1	1087	727	–	–	0	0	0	–[h]
	Unknown 2	1197	1022	–	–	1 ± 0.5	1.7 ± 0.2	20 ± 10	70 ± 70
	Unknown 3	1278	719	–	–	–[h]	0	19 ± 1	9 ± 1
	Unknown 4	1430	1094	–	–	0	0	0	5 ± 4

Fungal species included *Metschnikowia reukaufii* (*Metsch*) and *Aureobasidium pullulans* (*Aureo*); bacterial species included strains from the genera *Asaia* and *Neokomagataea* (*Neok*.). Full sampling details are found in the Materials and Methods section.

[a]Retention indices relative to *n*-alkanes on DB-1 and DB-Wax columns for compound identification. A dash indicates retention time was too low to calculate the retention index. [b]Sources were Fisher Scientific. [c]Sources were Sigma-Aldrich. [d]Sources were Mallinckrodt Baker. [e]Sources were J. T. Baker. [f]Compounds were tentatively identified due to lack of commercial standard. [g]Peak areas for the isomers 2-methyl-1-butanol and 3-methyl-1-butanol are summed as a result of co-elution. [h]Compound observed in only one replicate on day 4. [i]Peak areas gathered from DB-Wax-equipped GC-MS. **Normalized electroanntenographic (EAG) response is significantly different from 0 (false discovery rate < 0.05; *$P < 0.05$, **$P < 0.01$). *Normalized EAG response is significantly different from 0 (false discovery rate < 0.05). Compounds in bold type could be detected using *in situ* methods.

bees ($n = 6$ bees tested for each identified compound) were collected each day from outside hives housed in an apiary located at the USDA-ARS Center for Medical, Agricultural and Veterinary Entomology campus in Gainesville (FL, USA). Before experiments, honey bees were fed a sugar solution (1 : 1 water/sucrose) and stored in the dark. Immediately before bioassays, bees were placed in a trimmed 15 ml centrifuge tube and secured from behind with cotton. Under a low power stereo-microscope, both antennae were excised at the scape using micro-scissors and mounted on a forked probe (Syntech, Kirchzarten, Germany; internal gain 10×) with electrode gel (Parker Laboratories, Fairfield, NJ, USA). The prepared probe was mounted in the humidified constant air and volatile sample tube and allowed to equilibrate to the air flow for 3–5 min (until signal reached *c.* 0 μV). Sample puffs were delivered at 1-min intervals to allow antennae to re-equilibrate after exposure. Antennal responses, which indicate detection rather than attraction or repulsion, were recorded with the Autospike software (Syntech, v.3.9).

Odor samples were prepared in similar fashion to Beck *et al.* (2014) by loading 7 mm bioassay discs with 20 μl of pentane containing each compound at each of two loading doses: 40 or 0.4 μmol. Pilot experiments revealed these loading doses provided consistent antennal responses to known honey bee pheromones and have been used previously (Bhagavan & Smith, 1997). Slightly higher compound loadings were used to accommodate the 'wind tunnel' design of the EAG bioassay system used (Fig. S1). The pentane was allowed to evaporate for 2 min and then the filter paper was placed within a Pasteur pipette trimmed at the tapered end to a final length of 6.5 cm and sealed with Parafilm. A 0.5 s pulse flow (300 ml min^{-1}) and a humidified continuous flow (125 ml min^{-1}) directed odors through an air and volatile tube (1.5 cm diameter) containing the mounted antennae and probe. A Faraday cage protected against ambient electrical interference. To account for variability in response among individuals, responses to blanks (20 μl pentane and bioassay disc) were subtracted from each sample and antennal response values were normalized to a standard stimulus (0.4 μmol citral, a component of bee pheromone produced by the Nasanov gland), which was set at 100%.

Proboscis extension response assay

To assay the effect of microbial scent on honey bee feeding preference, we used a PER assay, which has been previously used to examine how nectar solutes influence acceptability of a nectar source (Scheiner *et al.*, 2004) based on odor or solute detection by honey bee antennae. Synthetic nectar solutions, including a sterile control, were prepared as above, incubated for 68–74 h, then used to assess bee preference. Foraging bees were collected at the entrance of three hives at UC Davis Laidlaw Honey Bee Research Facility. Bees were restrained, screened for responsiveness (~75% success) and allowed to feed on the control solution, then starved for 2 h before initiation of the assay. Microcapillary tubes containing 2 μl of the solution were wafted 5 mm from an individual bee's antennae for 6 s and proboscis extension was recorded. Solutions were introduced in two different sequences.

In situ floral headspace analysis

Volatiles were collected from the floral headspace of five plant species (Table S3), a subset of those surveyed above, at the UC Davis Stebbins Cold Canyon Reserve (Winters, CA, USA) in May and June 2016 (a total of 24 samples across three sampling dates). The plant species *Delphinium nudicaule*, *M. aurantiacus*, *Clarkia unguiculata*, *Collinsia heterophylla* and *Delphinium hesperium* were chosen because all were found to host bacteria or fungi in floral nectar, often at high abundance. Pairs of flowers or an entire floral cluster were enclosed in a nylon oven bag (406 × 444 mm; Reynolds, Richmond, VA, USA) secured using metal clips to minimize total headspace. A PDMS Twister bar (Gerstel Inc., Linthicum, MD, USA) was enclosed within each bag to collect volatiles for 60 min between 09:00 and 10:00 h. After headspace analysis, flowers were collected and culturable microorganisms were plated as described above for sampled flowers, and colonies counted to quantify microbial abundance (CFU). No-flower controls were collected by bagging a Twister bar in the field. Twisters were kept on ice for no more than 48 h, then were thermally desorbed in splitless mode using a thermal desorption unit (Gerstel), which ramped from 30 to 250°C, holding for 3 min. Desorbed volatiles were cryofocused at −80°C, then heated to 260 C for 3 min in a cooled injection unit (Gerstel), where they were splitlessly introduced to the GC column. Separation occurred on an Agilent 7890B gas chromatograph coupled to a single quadrupole 5977A MSD device outfitted with an HP-5MS column (30 m × 250 μm × 0.25 μm; Agilent) at a constant flow of 1.5 ml min^{-1} with a temperature program as follows: initial temperature, 40°C; 4°C min^{-1} ramp to 200°C; 30°C min^{-1} ramp to 300 C; 4 min hold. MS detection scanned from 33 to 300 *m/z*. A series of commercially available chemical standards (subset of the microbial volatiles identified above) was compared to identify compounds in floral headspace samples by similarity in retention times and mass spectrum fragmentation patterns. Samples were scored blind (without identifying sample information). Volatile presence in a sample was scored based on similarity in retention times and fragmentation patterns or a match to the NIST mass spectrum library (Table S2).

This analysis was intended to qualitatively report compounds that were detected in both floral microbes and the headspace of the same flower. Because of its affinity to apolar compounds, our Twister-DB5 system may have not recovered all of the polar compounds found in the headspace above the flowers (Table 1). Thus, we may be underreporting the number of compounds found in both isolated microbes and flower samples. Still, the experiment as executed allowed us to report that some volatiles produced by floral microbes were also found in the headspace of wild flowers.

Statistical analyses

To compare volatile blends among microbial species and days following inoculation, peak areas were log-transformed. Microbial volatile composition was visualized using principal

coordinates analysis (PCoA) based on Bray–Curtis dissimilarities and the interactive effects of microbial strain and day were assessed using PerMANOVA implemented using the adonis function in the R package 'VEGAN' (Oksanen *et al.*, 2012). DESEQ2 (Love *et al.*, 2013) was used to assess which compounds differed in relative abundance between prespecified groups. First, we examined which compounds differentiated bacteria from fungi (days 2 and 4 only) and, second, which differentiated the nectar-specialist yeast *M. reukaufii* from the generalist yeast *A. pullulans*. Because bacterial species did not differ significantly in volatile composition, we did not examine their differences further. We examined if microbial species differed in variance in volatile composition at each timepoint (days 2 and 4 separately) using the betadisper function in VEGAN.

To examine which compounds were detected by honey bees, we used a *t*-test to examine if normalized EAG responses were significantly different from zero (no detectable response), and used a false discovery rate (FDR) correction to control for multiple comparisons.

To examine if microbial species in nectar influenced honey bee proboscis extension (PER), we used a binomial regression to examine if the proportion of positive responses by honey bees varied among microbial species. The sequence of solution introduction was included in the model, but was not significant ($P > 0.10$), so was dropped from the model.

For field volatile data, we examined if the presence of each validated microbial-associated volatile (Table 1) was predicted by fungal CFU abundance, bacterial CFU abundance or plant species identity of the sampled flowers using Pearson chi-squared tests.

Results

Floral nectar often contained culturable nectar-inhabiting microorganisms, including bacteria and fungi (Fig. 1). Among plant species, the proportion of individual flowers that contained detectable microorganisms ranged from 20% to 86% of flowers sampled.

All focal microorganisms produced detectable volatile compounds when grown in artificial nectar, and species differed in the composition of the volatile blend emitted ($F_{3,28} = 40.22$, $P < 0.001$; Fig. 2). Surprisingly, species differences in volatile composition were detectable immediately after inoculation (Day = 0, 30 min after inoculation into synthetic nectar with 15 min each permeation and exposure time), and volatile blends further diverged after 2 and 4 d of growth (Species × Day $F_{3,28} = 6.86$, $P < 0.001$; Fig. 2). Alcohols, esters and ketones were more abundant in fungal solutions, while the volatile 2,5-dimethylfuran was characteristic of bacteria (Table 1). All four microbes produced *n*-hexanol. When we compared which compounds differentiated the fungal taxa, the generalist fungi *A. pullulans* emitted the ketones 2-nonanone and 4-methyl-2-pentanone, the short chain alcohols isobutanol and *n*-propanol, and two additional unidentified compounds (Table 1, unknowns 1 and 4). By contrast, the specialist nectar yeast *M. reukaufii* was characterized by esters, including ethyl butyrate, 2-methylpropyl acetate and 3-methylbutyl acetate, the alcohols 2-butanol and 3-ethoxy-1-propanol, and a relatively greater abundance of carbon dioxide, ethyl acetate, and the alcohols 3-methyl-1-butanol, 2-methyl-1-butanol, 3-methyl-3-buten-1-ol, ethanol and 2-phenylethanol. Across all microbial species, the replicates of *M. reukaufii* showed the least variance in volatile composition (Fig. 2, betadisper Day 2 $P = 0.02$; Day 4 $P = 0.01$).

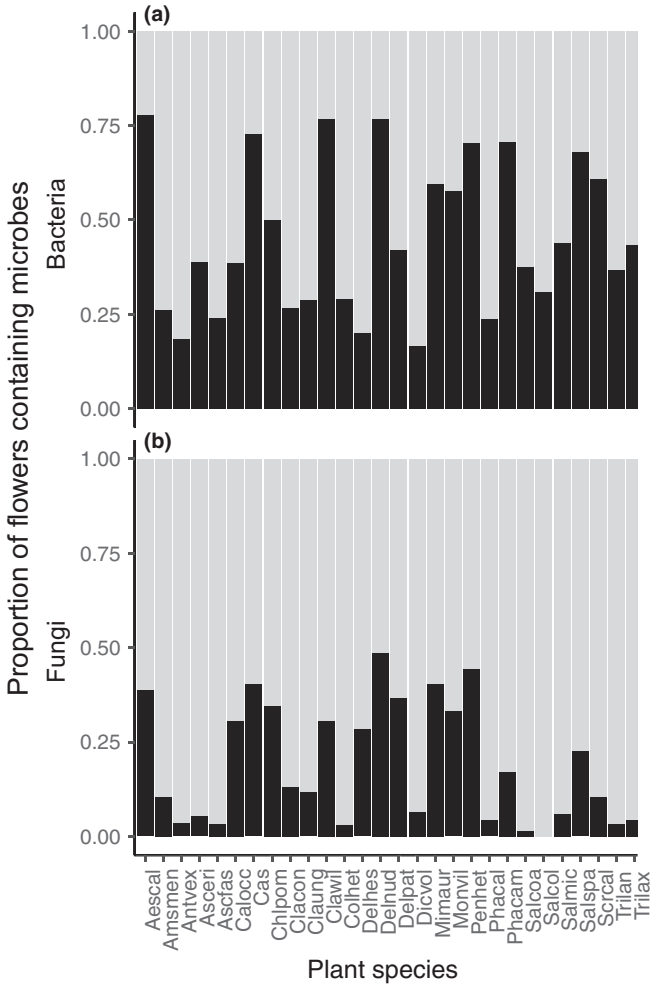

Fig. 1 Proportion of individual nectar samples from plant species hosting culturable (a) bacteria or (b) fungi (black) or no detectable microorganisms (light gray) Thresholds for detection were *c.* 5 colony-forming units (CFU) for fungi and 50 CFUs for bacteria. Some flowers contained both bacteria and fungi. Flower abbreviation indicates the first three letters of the genus and species for each plant species sampled with $n = 1170$ individual flowers sampled with $n = 13$–133 samples per plant species. Microbial density within individual flowers ranged from 0 to 10^6 CFUs μl^{-1} nectar.

Honey bee antennae detected 14 of the 20 microbial volatiles tested using EAG at a loading of 40 µmol, but only three compounds at 0.4 µmol. Compounds detected at low concentrations included *n*-hexanol, 2-ethyl-1-hexanol and 2-phenylethanol, which were produced by all surveyed microbes but most abundantly by yeasts (Table 1). Additionally, 2-nonanone was also detected by antennae but was produced only by the fungi *A. pullulans*. Antennal response was strongest in response to the short chain secondary alcohol 2-butanol, which was only

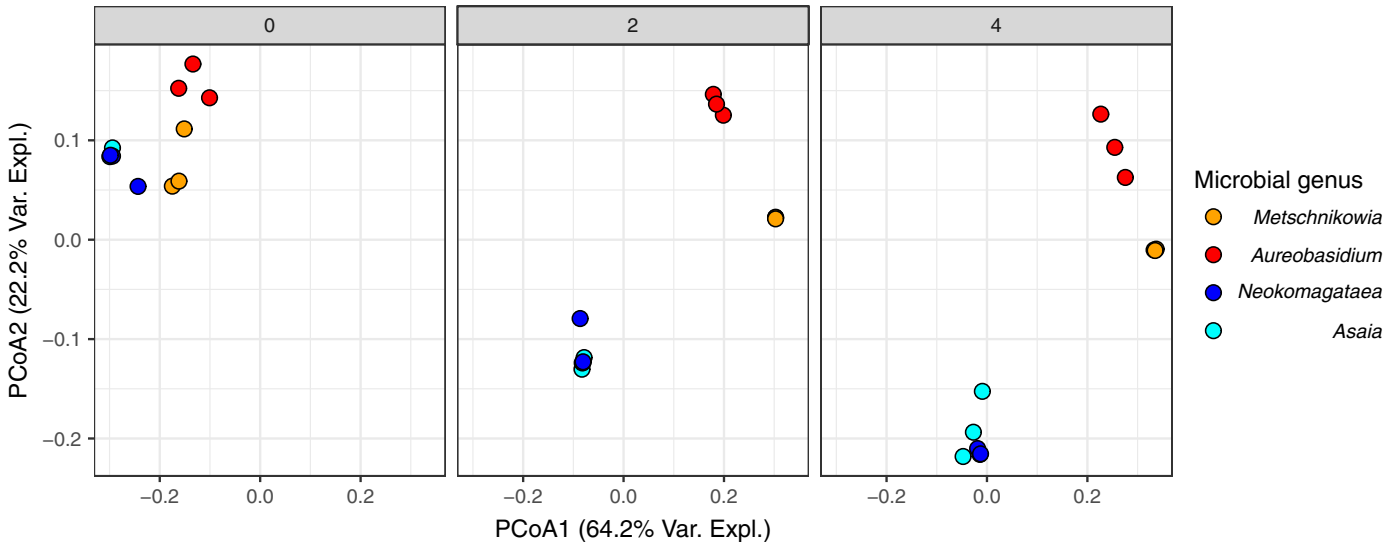

Fig. 2 Principal coordinates analysis showing variation in microbial volatile composition across microbial taxa and sampling days (days 0, 2, and 4) based on Bray–Curtis dissimilarity. Microbial species differ from each other (Species $P < 0.001$) and diverge from each other over time (Species × Day $P < 0.001$). In panels for days 2 and 4, replicates of *Metschnikowia reukaufii* are largely overlapping.

produced by the yeast *M. reukaufii* (465 ± 86% normalized antennal response at 40 µmol exposure dose, Table 1), while its structural isomer and primary alcohol isobutanol, produced by all microbes, did not elicit a significant response (24 ± 14% normalized antennal response at 40 µmol).

Honey bees varied in acceptance of microbial solutions based solely on volatile exposure: control and *M. reukaufii* solutions were accepted in over 70% of trials, whereas *A. pullulans* and *Asaia* were accepted 36% and 48% of the time, respectively. *Neokomagataea* received the fewest positive responses, with only 17% of bees accepting this solution (Fig. 3, $P < 0.001$).

A subset of compounds that characterized microbial volatile emission in synthetic nectar were identified in floral headspace of naturally occurring plants. Specifically, *n*-hexanol, 2,5-dimethyl furan, 2-ethyl-1-hexanol, 3-hexen-1-ol and 2-nonanone were detected and either absent or present at low abundance in the no-flower controls (Table S3). Notably, floral samples with abundant fungal colony-forming units in nectar were more likely to contain some microbial-associated volatiles (Fig. 4), including 2-ethyl-1-hexanol ($\chi^2 = 9.98$, $P = 0.001$) and 2-nonanone ($\chi^2 = 5.83$, $P = 0.01$; Table S4) in the headspace sample. Plant species varied in the presence of *n*-hexanol ($\chi^2 = 13.10$, $P = 0.02$), but species identity did not predict the presence of other volatiles and no apparent relationship was found between bacterial abundance and focal volatiles detected in the field analyses (Table S4).

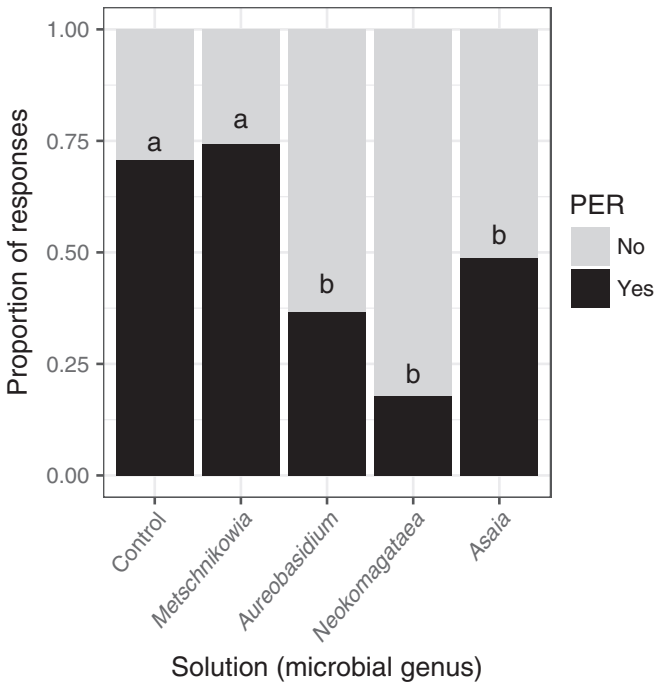

Fig. 3 Proportion of positive proboscis extension responses by honey bee (*Apis mellifera*) following exposure to solutions inoculated with microorganisms. Acceptance differed among microbial species ($P < 0.001$). Proboscis extension response (PER) indicates proboscis extension when bee was exposed to focal solution. Letters indicate treatments that differ at $P < 0.05$ with Tukey's honest significant difference test. Number of bees tested for each treatment ranged between 40 and 82.

Discussion

Here, we demonstrate that common microbial inhabitants of floral nectar differ in the volatile profiles emitted, and can influence acceptability of nectar to a generalist pollinator. The chemical compounds produced by the microorganisms assayed have been previously described in the floral headspace of plant species (Lemfack *et al.*, 2014). Some compounds, including 2-phenylethanol (Knudsen *et al.*, 2006; Galen *et al.*, 2011), are commonly documented and produced by many plant species, while others such as ethanol, 3-methyl-1-butanol, 3-hydroxy-2-butanone (acetoin) or ethyl acetate are described as major components of 'fetid' smelling flowers, including those that attract flies

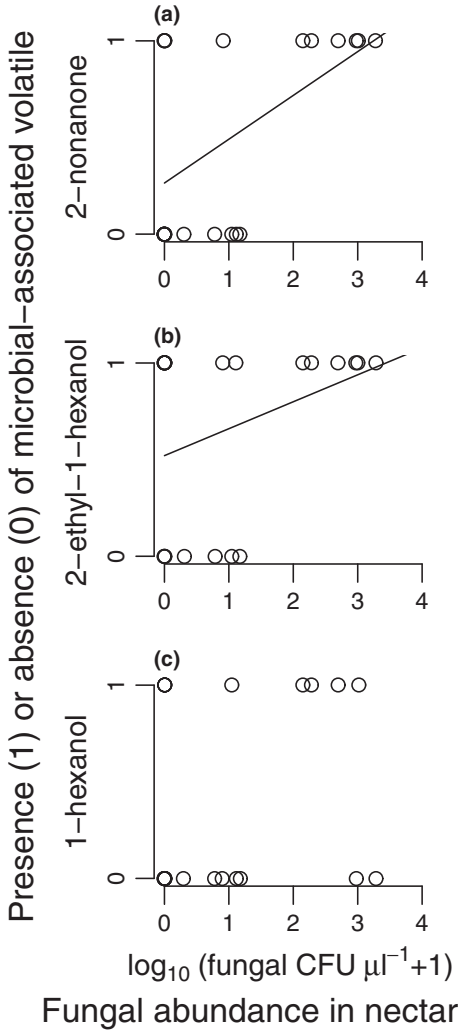

Fig. 4 Relationship between fungal abundance and the presence of microbially produced volatiles (Table 1) from floral headspace samples collected in the field. Points indicate individual samples containing flowers sampled from the field. A list of plant species sampled is provided in Supporting Information Table S3. Fungal abundance was quantified by plating nectar on YMA media. The line on each plot indicates the best-fit binomial regression model between fungal abundance (fungal colony forming units (CFU)) and presence of each individual compound ($P < 0.05$). See also full results and statistics in Table S4.

or beetles (Dobson, 2006; Goodrich *et al.*, 2006). For example, the nectar of *Agave palmeri*, which smells of rotting fruit, overlaps to some degree with the chemicals described here, containing short-chain and aromatic alcohols including 3-methyl-1-butanol, *n*-hexanol and 2-phenylethanol (Raguso, 2004). By contrast, bee- or butterfly-pollinated species are typically not characterized by those fatty acid derivatives or fermentation volatiles (Dobson, 2006). Instead, these compounds are minor components of the volatile profile in some bee-pollinated plants, including field-collected clover (Buttery *et al.*, 1984), *Silene caroliniana* (Golonka *et al.*, 2014), and the current study. Although some compounds may be produced by both plants and microorganisms, if yeasts or other microbes contribute to floral scent, the presence of these components of the volatile profiles should be dynamic over time, and vary with microbial presence and abundance in nectar, as our field survey suggests (Fig. 4). A recent meta-analysis indicated that floral volatile profiles are, on average, the most variable of all plant or animal volatile blends examined (Junker *et al.*, 2018). Our data suggest that nectar microbes can contribute to this variability.

Volatiles produced by nectar microbes are likely to be byproducts of microbial metabolism or fermentation, but may have diverse ecological functions. Nectar-inhabiting microbes often suppress the growth of late-arriving species in nectar (Peay *et al.*, 2012; Vannette & Fukami, 2014; Mittelbach *et al.*, 2016b), and the volatiles ethyl acetate, 2-butanol, isobutanol, ethanol, 2-ethyl-1-hexanol and 2-phenylethanol have been shown to inhibit microbial growth (Cruz *et al.*, 2012; Hua *et al.*, 2014; Pereira *et al.*, 2016). Alternatively, for microbes that rely on pollinator-mediated phoresis, volatiles may be attractive or aid in dispersal. The yeast *M. reukaufii* is a nectar specialist (Brysch-Herzberg, 2004; Pozo *et al.*, 2011) and is largely reliant on floral visitors for dispersal among flowers (Belisle *et al.*, 2012). Notably, *M. reukaufii* produced the volatile blend most attractive to honey bees among all microbes tested (Fig. 3). In field trials and feeding assays, *M. reukaufii* has been found to be either attractive (Herrera *et al.*, 2013; Schaeffer & Irwin, 2014; Schaeffer *et al.*, 2017) or not deterrent to bee pollinators (Good *et al.*, 2014). By contrast, the other microbes tested in this experiment have been isolated from a broad range of habitats, including plant surfaces, rotting fruits or pollinator-associated habitats (Swings & De Ley, 1981; Andrews *et al.*, 1994), so may be less reliant on pollinators for dispersal to appropriate habitats. Differences in honey bee attraction to microbial species support the hypothesis that microbes vary in their dependence on insect vectors or differ in the identity of dispersal vectors (Davis & Landolt, 2013; Vannette *et al.*, 2013).

Our study suggests that microbially produced volatiles have differential effects on honey bee physiology and behavior. For example, 2-butanol, a compound only emitted by *M. reukaufii*, elicited the strongest antennal response, over 400× greater than that of the 0.4 µmol citral control (e.g. Table 1). While EAG cannot reveal if this compound is attractive or deterrent and further studies should verify the ecological validity of chosen concentrations, PER assays indicated that the blend of compounds including 2-butanol was not deterrent. Yet other compounds emitted by *M. reukaufii* have been shown to act as insect honey bee semiochemicals. For example, 3-methylbutyl acetate (isoamyl acetate or isopentyl acetate) is the principal component of the honey bee alarm pheromone (Free, 1987; Hunt, 2007) and can influence *A. mellifera* behavior (Pastor & Seeley, 2005; Nieh, 2010; Urlacher *et al.*, 2010). Alternatively, it is possible that yeast-emitted volatiles could function, either individually or as a blend, as 'honest signals' of nectar rewards (Knauer & Schiestl, 2015). Our data cannot address if the identified microbial volatiles are attractive, honest signals or if *M. reukaufii* simply lacks deterrent volatile cues that may be produced by other microbes (Mittelbach *et al.*, 2016a). For example, scent produced by nectar-inhabiting microorganisms may indicate the presence of floral nectar and provide foraging cues for some pollinators.

Nevertheless, given that individual flowers vary in the presence and abundance of microorganisms (Fig. 1 and Herrera *et al.*, 2009), it is possible that pollinators could use these varying cues to distinguish among flowers depending on the presence or abundance of particular microorganisms.

The consequences of altered bee visitation or behavior for microbial dispersal and pollination remain unclear. If bacteria inhibit visitation by pollinators, their presence could reduce microbial dispersal and pollination, whereas decreased visit duration could instead increase dispersal and potential for outcrossing. Our results support the former (reduced attraction), but cannot resolve this question. Moreover, we only examined responses of the generalist pollinator *A. mellifera* in the current study so it is possible that other floral visitors respond either more or less strongly to the presence of microbial volatiles or differ in attraction to specific microbial taxa (Davis *et al.*, 2013), so the consequences for plant reproduction and microbial dispersal may depend on the specific combination of plant, microbe and floral visitor.

More generally, the finding that microbial volatiles can contribute to plant phenotype suggests a novel mechanism of microbial influence on ecological interactions between plants and animals. In addition to previously documented microbial effects on phenotype mediated by changes in nutrition, production of defensive compounds and altered hormonal signaling (Friesen, 2013), we present evidence that microbes can directly contribute to plant volatile chemotype, an understudied phenomenon. The fitness consequences for plants and microorganisms were not examined in this study, but our results suggest that when they align (as may be the case for *M. reukaufii* and plant reproduction), microbial volatile emission may enhance fitness benefits for both partners. Although more research is necessary to fully examine the prevalence, magnitude and consequences of microbial contribution to host semiochemicals, our results imply that microbial contribution to host volatile signaling may be an important but largely overlooked effect of the microbiome on host phenotype and subsequent ecological interactions.

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Author contributions

R.L.V. and J.J.B. conceived the study, R.L.V. and G.W.H. performed field work, including nectar volatile collections and microbial isolations. G.W.H. performed PER assays. C.C.R. performed volatile analyses of single strain inocula and EAG analyses. M.M.M. assisted with methods and GC-MS analyses of floral headspace samples. All authors contributed to writing and manuscript editing.

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information tab for this article:

Fig. S1 The 'wind tunnel' volatile introduction system used for the electroantennographic bioassays.

Table S1 Full names of plant species at Stebbins Cold Canyon reserve sampled for fungi and bacteria

Table S2 Unknown compound retention indices, principal ions and peak areas in *ex situ* microbial headspace

Table S3 Plant species names and other metadata for floral headspace samples

Table S4 Statistical results of generalized linear model examining fungal abundance, bacterial abundance and plant species on the presence of microbially associated volatiles in floral headspace collected in the field

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