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Response of Wild Spotted Wing Drosophila (*Drosophila suzukii*) to Microbial Volatiles

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Abstract

The olfactory cues used by various animals to detect and identify food items often include volatile organic compounds (VOCs) produced by food-associated microorganisms. Microbial VOCs have potential as lures to trap animal pests, including insect crop pests. This study investigated microorganisms whose VOCs are attractive to natural populations of the spotted wing drosophila (SWD), an invasive insect pest of ripening fruits. The microorganisms readily cultured from wild SWD and SWD-infested fruits included yeasts, especially *Hanseniaspora* species, and various bacteria, including Proteobacteria (especially Acetobacteraceae and Enterobacteriaceae) and Actinobacteria. Traps in a raspberry planting that were baited with cultures of *Hanseniaspora uvarum*, *H. opuntiae* and the commercial lure Scentry trapped relatively high numbers of both SWD and non-target drosophilids. The VOCs associated with these baits were dominated by ethyl acetate and, for yeasts, other esters. By contrast, *Gluconobacter* species (Acetobacteraceae), whose VOCs were dominated by acetic acid and acetoin and lacked detectable ethyl acetate, trapped 60–75% fewer SWD but with very high selectivity for SWD. VOCs of two other taxa tested, the yeast *Pichia* sp. and *Curtobacterium* sp. (Actinobacteria), trapped very few SWD or other insects. Our demonstration of among-microbial variation in VOCs and their attractiveness to SWD and non-pest insects under field conditions provides the basis for improved design of lures for SWD management. Further research is required to establish how different microbial VOC profiles may function as reliable cues of habitat suitability for fly feeding and oviposition, and how this variation maps onto among-insect species differences in habitat preference.

Keywords Attract-and-kill · Acetobacteraceae · *Drosophila suzukii* · *Hanseniaspora* · Microbial volatiles · Spotted wing Drosophila

Introduction

Many animals use olfactory cues to detect and locate their food, and there is increasing evidence that volatiles produced by microorganisms associated with potential food items can contribute to animal decisions whether to approach and consume the food. For example, the products of microbial fermentation of animal cadavers can be a reliable cue of a protein-rich food source for necrophagous insects and vertebrate scavengers (Verheggen et al. 2017). Similarly, ethanol and other fermentation products of sugar-rich fruits and nectar can be indicative of abundant, accessible calories to frugivorous and nectarivorous animals (Dominy 2004; Dudley 2000; Wiens et al. 2008), while specific microbial products or blends of products, especially at high concentrations, can be repellant, indicating a hazardous level of microbial contamination (Adler 2000; Stensmyr et al. 2012; Tasin et al. 2012).

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The basis for this study is the abundant evidence that dipteran insects that feed and oviposit on fleshy fruits are attracted to volatiles produced by microorganisms associated with fruits. These microorganisms are also regularly found in the insect gut, suggesting that they may contribute to insect nutrition. For example, the olive fly *Bactrocera oleae* is attracted to volatiles, particularly methyl thiolacetate, released from a strain of *Pseudomonas putida* isolated from its olive host plant (Liscia et al. 2013). Similarly, the apple maggot fly *Rhagoletis pomonella* is attracted to a strain of the bacterium *Enterobacter agglomerans* originally isolated from this insect (MacCollum et al. 1992), with 3-hydroxy-2-butanone (acetoin) implicated as a key attractant (Robacker et al. 2004). In addition, the attraction of *Drosophila melanogaster* to fermenting fruit has been attributed primarily to yeast-derived volatiles, especially ethanol, acetic acid, acetoin, 2-phenyl ethanol and 3-methyl-1-butanol (Becher et al. 2012). These findings illustrate the complexity of ecological and evolutionary processes that shape the trajectory of fruit ripening and rotting (Dudley 2000; Janzen 1977; Ruxton et al. 2014), and they also provide the opportunity for novel behavioral pest control strategies based on microbial volatiles to trap pests for monitoring and suppression of populations (Beck and Vannette 2017; Davis et al. 2013).

The specific focus of this study on the interaction between microbial volatiles and fruit-infesting insects is the invasive "spotted-wing drosophila" *Drosophila suzukii* (SWD), originating from South East Asia. Unlike most other fruit-feeding drosophilid flies which lay their eggs into overripe fruit, SWD oviposits into ripening soft-skinned fruit such as berries and cherries (Atallah et al. 2014). Combined with its high rate of population increase and lack of natural enemies in introduced regions, SWD is now a major pest in North America and Europe, with chemical insecticide sprays as the dominant control strategy (Asplen et al. 2015). Nevertheless, there is strong demand for alternative control strategies in conventional and especially organic fruit production (Schetelig et al. 2017).

Several lines of evidence suggest that SWD is attracted to microbial volatiles. Under field conditions, apple cider vinegar and baker's yeast with sugar are widely used as baits for SWD (Iglesias et al. 2014; Knight et al. 2015; Swoboda-Bhattarai et al. 2017), suggesting that SWD is attracted to fermentative yeast odors. Furthermore, laboratory olfactometer assays reveal that SWD are attracted to the volatiles produced by the yeast *Hanseniaspora uvarum* (Mori et al. 2017) and some acetic acid bacteria (Acetobacteraceae) (Mazzetto et al. 2016), both of which have high prevalence in SWD (Bing et al. 2018; Hamby et al. 2012). Complementary analyses of SWD response to specific volatiles have identified several attractive blends, e.g. a quaternary blend of ethanol, acetoin, acetic acid and methionol (Cha et al. 2018), and a 5-compound blend derived from fermented apple juice (acetoin, ethyl octanoate, acetic acid, phenethyl alcohol and ethyl acetate) (Feng et al. 2018), as well as various commercial formulations, e.g. Scentry (Scentry Inc.), Suzukii Trap bait (BioIberica) and SPLAT SWD (Cloonan et al. 2018). However, the utility of these strategies is constrained by two issues: their relatively low selectivity for SWD, and variability in efficacy, both between laboratory and field trials and across different field trials (Asplen et al. 2015; Cloonan et al. 2018; Schetelig et al. 2017).

Our strategy to study the response of SWD to microbial volatiles comprised three steps: first, to isolate microbial taxa from SWD and fruits infested by SWD; second to quantify the efficacy of these isolates as bait for trapping SWD and other drosophilids from natural populations; and finally to quantify the volatile organic compounds emitted by these microbial taxa. This approach enabled us to correlate specific VOCs and blends with both trapping efficacy and selectivity for SWD, although our exclusive focus on culturable forms precludes global analysis of the microbiota associated with the flies and fruits. Our results are discussed in the context of the ecology of SWD and related fruit-feeding flies and improved design of microbial volatile-based lures for monitoring and control of SWD populations.

Materials and Methods

Cultivation of Microorganisms The microorganisms were isolated from wild SWD and associated fruits collected from two sites on 6 September 2016: a plot of fruiting primocane raspberry on The Research North Farm as part of Cornell AgriTech, New York State Agricultural Experiment Station in Geneva, NY (42 degrees 52′ 03.50" N by 77 degrees 02' 21.09" W); and a bank of fruiting pokeweed *Phytolacca americana* on a commercial farm near Geneva, NY (42 degrees 47′ 38.03" N by 76 degrees 59' 58.97" W). At each site, 40 fruits and 10 adult SWD of each sex were sampled. Individual fruits were transferred using sterile forceps to individual 50 ml sterile plastic tubes (Falcon, Corning, NY). The flies were collected live overnight in traps (Bost et al. 2018; detailed design provided in Supplementary Methods) designed to permit entry but restrict escape and to preclude access to the bait of crushed raspberry fruit, and sorted on return to the laboratory. Indications that the SWD populations were interacting with the fruits came from the presence of *Drosophila* larvae in many of the collected fruits (Fig. S1), and of fruit-colored material in the crop of many of the collected flies.

To cultivate bacteria and fungi, 20 fruits and 5 flies of each sex from each site were inoculated onto 9 cm diam. plates, using sterile technique in a laminar flow cabinet. In pilot experiments we assessed the microbial growth on four media: nutrient broth (#CM0001, Oxoid Ltd. Hampshire, UK), potato dextrose (#CM013, Oxoid), yeast-peptone-dextrose

((#CM0920, Oxoid) and modified de Man, Rogosa, and Sharpe medium [mMRS 1.25% proteose peptone (Becton Dickinson), 0.75% yeast extract, 2% glucose, 0.5% sodium acetate, 0.2% dipotassium hydrogen phosphate, 0.2% triammonium citrate, 0.02% magnesium sulfate heptahydrate,0.005% manganese sulfate tetrahydrate and 1.2% agar (all constituents from Sigma-Aldrich, St Louis, MO, USA except agar from Apex, San Diego, CA, USA)]. We obtained greatest morphological diversity of colonies with mMRS and nutrient broth, and so the definitive isolations were conducted on these two media. Each fruit was crushed into a pulp on the agar plate and the juices were spread using a sterile glass rod. The gut was dissected from each fly with sterile forceps, and then aseptically transferred to the agar plate, teased open with sterile forceps and spread with 50 µl sterile phosphate buffered saline (PBS) across the plate. Plates were incubated in the dark at 30 °C under aerobic conditions for up to one week.

Microorganisms of diverse colony morphologies grew on all the plates. Each of multiple colonies from each plate was streaked individually onto a fresh plate of the same medium, to obtain pure clonal cultures. Representative colonies of each morphotype were inoculated into 5 ml broth of the same composition and grown at 30 °C to turbidity. Each culture was identified as bacterial or yeast by light microscopy; then, 20% and 10% glycerol stocks, respectively, were prepared and stored at -80 °C.

Identification of Microorganisms A sample from each glycerol stock (obtained above) was streaked onto an agar plate, and a single colony was grown in 5 ml broth, as above. A 1 ml sample of turbid culture was centrifuged, and the cells were resuspended in 678 µl cell lysis buffer (108 mM Tris-HCl pH 8.0, 1.5 M NaCl, 21.6 mM EDTA) with 30 µl 1 mm diam. Glass beads (Scientific Industries, Vernon Hills, IL, USA) 16 U proteinase K (Qiagen, Hilden, Germany). The homogenate was incubated at 56 °C for 2 h, mixed with 35 U RNaseA (Qiagen), and incubated at 37 °C overnight. DNA was extracted with 750 µl phenol:chloroform:isoamyl alcohol (25:24:1) (Thermo Fisher Scientific, Waltham, MA) with centrifugation at 19,000×*g* for 15 min at 4 °C. DNA was then precipitated from 550 µl aqueous phase by overnight incubation at −20 °C with 45 µl 3 M sodium acetate (pH 5.2) and 900 µl ethanol. Following centrifugation at 7000×*g* for 15 min at 4 °C, the pellet was washed in 750 µl cold 75% ethanol, air-dried, resuspended in 50 µl sterile endonuclease-free water and stored at −20 °C.

Microbial samples were identified by Sanger sequencing of PCR amplicons of bacterial 16S rRNA gene using the primers 16SA1 (Forward: 5′-AGAGTTTGATCMTGGCTCAG-3′) and 16SB1 (Reverse: 5′-TACGGYTACCTTGTTACGACTT-3′) of Fukatsu and Nikoh (1998) and the fungal ITS/5.8S rRNA gene regions using the primers ITS1 (forward: 5’-TCCCTACCTGAACCTGCGG-3′) and ITS4 (reverse: 5’-TCCTCCGCITATTGATATGC-3′) of White et al. (1990). The PCR reactions contained 0.2 µM of either bacterial or fungal primers, 1 U One*Taq*® 2X Master Mix with Standard Buffer (New England BioLabs, Ipswich, MA) with 94 °C for 30 s, 30 amplification cycles of 94 °C for 30 s, 60 s annealing temperature at 55 °C for bacteria or 55.3 °C for fungi, 68 °C for 60 s, with final extension for 5 min at 68 °C. PCR products were purified with ExoSAP-IT™ PCR Product Clean Up Reagent (Thermo Fisher Scientific) following manufacturer's protocols, and Sanger sequencing was conducted for both directions on Applied Biosystems 3730xl at the Cornell University Genomics Facility. Sequences were assembled de novo on Geneious Prime® 2019.0.4 and taxonomic identity was assigned by querying against the NCBI nucleotide database using BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The sequence data are available at GenBank, NCBI (Accession Numbers MN378407-MN378470).

Field Experiments The attractive characteristics of volatile compounds produced from microbes associated with SWD and its fruit hosts were evaluated in the field using a mixed variety raspberry planting at Cornell AgriTech, Geneva, NY (42°52′5.69“N, 77° 2’19.91”W) during the late summer of 2018. The assessment was based on the number of captures of adult SWD in traps containing a 60 mm diam. Petri dish with 6 ml autoclaved *Drosophila* food (negative control) comprising 10% Brewer's yeast (inactive; MP Biomedicals, Santa Ana, CA), 10% glucose (Sigma-Aldrich, St Louis, MO), 1.2% agar (Apex Bio, Houston, TX), each of the six microbial strains (Table 1) and the commercial monitoring lure (Scentry, Billing, MT) as positive control.

To prepare the plates for the field trials, a sample of the glycerol stock was streaked onto solid medium and incubated at 30 °C for 24–30 h for colony growth. A single colony was transferred to 5 ml broth culture and grown to mid-exponential phase. A sample (100–500 µl) was transferred to a 1.5 ml microtube, washed with centrifugation in sterile PBS, quantified by optical density (OD), and then diluted to 10^8 cells ml^{-1}

Table 1 Microbial strains selected for detailed analysis

Microorganism[1]	Source
Curtobacterium sp. EB2016–150	Pokeweed Fruit
Gluconobacter cerinus EB2016–59	Pokeweed Fruit
Gluconobacter oxydans EB2016–84	Pokeweed Female SWD
Hanseniaspora opuntiae EB2016–35	Raspberry Fruit
Hanseniaspora uvarum EB2016–122	Raspberry Male SWD
Pichia sp. EB2016–32	Raspberry Fruit

[1] All the microorganisms were grown in mMRS, apart from *Curtobacterium* sp., which was grown in nutrient broth (#CM0001, Oxoid Ltd. Hampshire, UK)

PBS using OD/cell number calibration curves constructed for each species. Then, a 50 µl sample was transferred to each of 5 replicate 60 mm diam. × 15 mm height tight-fit lid Petri dishes (VWR) containing 6 ml autoclaved *Drosophila* food. The microbial cell suspensions were spread using 5–6 4 mm sterile glass beads (Merck KGaA, Darmstadt, Germany) that had been cleaned with hexane (Sigma-Aldrich).

The custom-made traps (Fig. 1; see supplementary methods for details of construction) were suspended in the fruiting zone of raspberry canes. Treatments were deployed in a randomized complete block design with 6 replicates, and one replicate of each treatment per block, with order randomly determined. The trial was repeated three times, 27-August, 5-September and 17-September, 2018, with order of treatments re-randomized for each experiment. Trap counts were conducted at 24 h and 48 h by filtering, and then replacing the trap drowning solution. Male and female SWD and non-target drosophilids were identified by reference to Werner et al. (2018) and quantified for each replicate trap.

Volatile Analysis Five replicates of each test microorganism were prepared by the procedure used for the field experiments, but using 50 mm diam. × 9 mm height Petri dishes (Falcon) with 4 ml *Drosophila* food. The lid was laid gently on top of each Petri dish, which was then sealed in 9 × 9 cm oven bags (Reynolds Kitchens™, Lake Forest, Illinois), transferred into an air-tight Snapware container (World Kitchen LLC, Rosemont, Illinois) and incubated at 24.5 °C for 48 h. Volatile compounds equilibrated within the oven bags were sampled using customized micro-adsorbent traps filled with 5 mg Tenax TA (60/80 mesh size) as described by Arguello et al. (2013). Volatiles were collected for 10 min from each sample replicate (see Supplementary Methods). Three replicate headspace samples were taken from purchased Scentry lures in a similar manner, but sampling time was limited to 10 s. to prevent saturation and/or breakthrough of odorants on the micro-adsorbent traps.

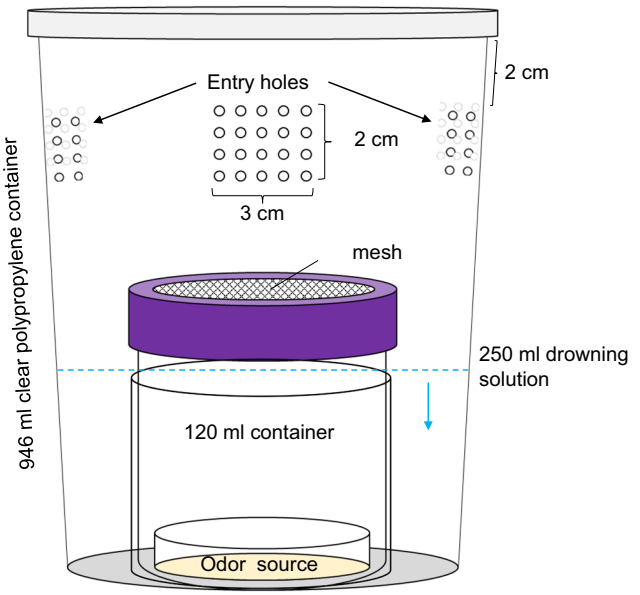

Fig. 1 The SWD trap. The Petri dish bearing the microbial cells is placed in a 120 mL specimen cup covered with insect-proof mesh. Insects gain access to the trap via 3 mm diameter entry holes, and are collected in drowning solution comprising water with a drop of unscented soap. Full details of trap construction are provided in supplementary methods

Trapped volatiles were analyzed using direct thermal desorption (TD) coupled with gas chromatography-mass spectrometry (GC-MS; GC2010+, Shimadzu Scientific Instruments, Inc., Kyoto, Japan) using an Optic 3 high performance GC injector, (ATAS GL, International BV, Eindhoven, The Netherlands), as described by Arguello et al. (2013) (see supplemental methods). Control sample chromatograms collected from un-inoculated growth medium (*Drosophila* food plus PBS) were subtracted from those representing microbial cultures, allowing us to exclude (as background volatiles) any compounds present in microbial cultures that were not at least 3-fold greater in total ion current (TIC) peak area than the corresponding peak in the control samples. Identification of volatile compounds was accomplished by co-injection with authentic standards and by comparison with mass spectral libraries and published chromatographic data available on the NIST Chemistry WebBook (https://webbook.nist.gov/) and websites provided by PubChem (https://pubchem.ncbi.nlm.nih.gov/) and PheroBase (www.pherobase.com/). Peak-integrated chromatographic data have been archived digitally at the eCommons site at Cornell University (https://ecommons.cornell.edu/handle/1813/2162?) using the link https://doi.org/10.7298/4dqz-0k28.

Statistical Analysis Total adult SWD captured in each 48 h experiment (sum of captures at 24 h and 48 h) were analyzed using generalized linear mixed models (Proc glmmix, SAS 2009) with experiment and block as fixed effects and, using a Poisson distribution with log link, specifying a random effect at the residual level (odor treatment * block * experiment #) to account for overdispersion. Means were compared using the Tukey test. A beta regression was implemented to assess the specificity of SWD captures compared to all *Drosophila* species identified in the traps using the 'betareg' package (Cribari-Neto and Zeileis 2010) in R (R Core Team 2015). The six replicates in each block were summed to give a single estimate per experiment, generating three replicates per treatment. Replicates that resulted in proportions equal to 1 (only SWD captured) or 0 (no SWD captured) were changed to 0.99 or 0.01 to stabilize model results (this occurred for all three replicates of *Curtobacterium sp*, *G. cerinus*, and no microbe-free control, and two replicates *G. oxydans*). Microbial treatments were included as a categorical fixed effect, and replicates were weighted by the total number of flies captured. Residuals were visually assessed for homoscedasticity. A likelihood ratio test was used to identify significance of the fixed

effect (compared fixed effect model to intercept-only model), and a post hoc Tukey test was conducted to generate pairwise comparisons between all treatments.

Variation in volatile chemical composition between replicate microbial samples was explored with multivariate statistical methods using the 'vegan: Community Ecology Package' (Oksanen et al. 2019) in R. Wisconsin double standardized TIC peak area data for all detected compounds were used to calculate a Bray–Curtis similarity index, which was further analyzed using two alternative statistical tests. First, differences in VOC composition were analyzed using analysis of similarity (ANOSIM (Clarke and Gorley 2006), with the significance of R determined by 999 random permutations to generate an empirical distribution of R under the null model. Second, Permutational Multivariate Analysis of Variance (PERMANOVA) tested for volatile differences within vs. between microbial accessions (species, kingdom, and species nested within kingdom). The calculated F-statistic was compared to 999 random permutations, employing a false discovery rate correction for multiple comparisons (Benjamini and Hochberg 1995). Further exploration of chemical differences used Similarity Percentage Analysis (SIMPER) (Clarke and Gorley 2006), which calculates the average contributions of specific variables (VOCs) to Bray-Curtis dissimilarity in pairwise group comparisons. To visualize the variation in VOC composition among microbial isolates, volatile and microbial species were grouped, and an associated dendrogram constructed, via hierarchical cluster analysis of Euclidean distances (default parameters for the heatmap.2 function in package 'gplots' in R).

A probability cutoff of alpha = 0.05 was applied for tests of significance in all statistical analyses.

Results

The Microorganisms Cultured from SWD and Fruits In total, 217 microorganisms were isolated into culture (Table S1). Of these, 162 (75%) yielded 16S rRNA gene or ITS sequence PCR amplification products (for bacteria and fungi, respectively), comprising 93 isolates of bacteria and 69 isolates of fungi (Table 2). The bacterial isolates could be assigned to 20 taxa in three phyla, Proteobacteria (α-proteobacteria and γ-proteobacteria), Actinobacteria and Firmicutes. Just three taxa, *Gluconobacter cerinus* (α-proteobacteria), *Curtobacterium* sp. (Actinobacteria) and *Leuconostoc pseudomesenteroides* (Firmicutes), accounted for 53 (57%) of the bacterial isolates. The fungal isolates were identified to 10 taxa, comprising 8 taxa of the order Saccharomycetales (Ascomycotina) and just one taxon in each of Dothideales (Ascomycota) and Basidiomycota. Two species of *Hanseniaspora* (Saccharomycetales) accounted for 52 (75%) of the fungal isolates. Overall, 23 (77%) of the 30 microbial taxa identified were obtained from just one of the four sample types, i.e. from either SWD or fruit (but not both) at one of the two sites. Just three taxa were recovered from both fruits and SWD: the bacterium *Curtobacterium* sp. and the yeasts *Hanseniaspora uvarum* and *Pichia* sp.

Our selection of six microbial strains for detailed analysis comprised taxa that grew well on the Drosophila food used for field traps and were prevalent in our collection (Table 1).

Field Experiments Over the 48 h test period, up to 35 SWD from natural populations were recovered from each trap set out in a raspberry planting, with 594 SWD trapped in total (Dataset S1). The numbers trapped varied significantly with odor source ($F_{7,119} = 8.79$, $P < 0.0001$) (Fig. 2a), with no significant effect of either experiment ($F_{2,119} = 0.20$, $P = 0.81$) or block nested within experiment ($F_{15,119} = 0.73$, $P = 0.75$). The negative control of autoclaved *Drosophila* food trapped 0–3 SWD, and significantly higher capture than the negative control was obtained for the commercial lure Scentry and two yeasts, *Hanseniaspora uvarum* and *H. opuntiae*, but not for the yeast *Pichia* sp. or any of the three bacterial species tested (Fig. 2a). The number of male and female SWD recovered from the traps did not differ significantly (Mann Whitney U: W = 20,142, $p = 0.315$).

Several drosophilid species other than SWD were recovered from the traps: *Drosophila melanogaster* and species in the *D. obscura* group, with an occasional capture of *D. tripunctata*, *D. falleni* and flies of the genus *Chymomyza,* closely related to *Drosophila* (Dataset S1). Visual inspection of the data revealed that, although the total numbers of flies trapped by baits with *Gluconobacter* was considerably lower than with baits with *Hanseniaspora*, the specificity of the *Gluconobacter* baits was high, with SWD accounting for all but one of the 87 drosophilid flies trapped. The proportion of SWD varied significantly with trap bait ($\chi^2_{\ 7} = 1810.4$, $p < 0.001$), and Tukey's post hoc test yielded significantly greater proportion of SWD in traps baited with *Gluconobacter* species than other treatments (Fig. 2b).

Volatile Analysis Volatile collections coupled with TD-GC/MS analysis resulted in the detection of 83 VOCs from three yeast and three bacterial accessions (Fig. 3; Table S2). These compounds included 36 aliphatic esters and 9 alcohols (e.g. ethyl acetate, ethanol, isobutyl acetate, 3-methylbutanol), five aliphatic ketones (including acetoin), five aromatic compounds, one alkane and a series of six carboxylic acids ranging from acetic to hexanoic acid. Additional VOCs included nine putative isoprenoids, six compounds whose mass spectra suggested the presence of substituted cyclohexane rings and six compounds not assignable to a structural or biosynthetic class. Yeast accessions were marked by high total emissions (summed TIC peak areas; Table S2), producing 20- to 1000-fold greater TIC peak areas than bacterial accessions and

Table 2 Microorganisms cultured from swd and fruits

Phylum	Family	Species	Total no. isolates	No. isolates			
				Site-1 (raspberry)		Site-2 (pokeweed)	
				SWD	Fruit	SWD	Fruit
(a) Bacteria							
Proteobacteria							
α-proteobacteria	Acetobacteriaceae	*Gluconobacter cerinus*	23		8		15[a]
		Gluconobacter oxydans	7			7[a]	
		Gluconobacter sp.	1				1
		Acetobacter orientalis	1	1			
		Asaia lannensis	1		1		
γ-proteobacteria	Enterobacteriaceae	*Brenneria nigrifluens*	7			7	
		Brenneria sp.	7			7	
		Enterobacter sp.	1	1			
		Rouxiella sp.	1			1	
		Tatumella ptyseos	2			2	
	Xanthomonadaceae	*Stenotrophomonas* sp.	1			1	
Firmicutes	Leuconostocaceae	*Leuconostoc pseudomesenteroides*	11				11
	Planococcaceae	*Kurthia* sp.	1	1			
Actinobacteria	Microbacteriaceae	*Curtobacterium* sp.	19		9[a]	5	5
		Frigoribacterium sp.	1	1			
		Microbacterium sp.	5				5
		Herbiconiux sp.	1			1	
		Unclassified	1			1	
	Micrococcaceae	*Micrococcus* sp.	1	1			
	Corynebacteriaceae	R*hodococcus* sp.	1	1			
(b) Fungi							
Ascomycota Saccharomycetales	Saccharomycodaceae	*Hanseniaspora opuntiae*	28		12[a]		16
		Hanseniaspora uvarum	24	6[a]	7		11
	Saccharomycetaceae	*Kluyveromyces dobzhanskii*	1		1		
		Candida railenensis	2	1			1
		Pichia kudriazevii	3	1	1	1	
		Pichia membranifaciens	1			1	
		Pichia sp.	3		3[a]		
	Metschnikowiaceae	*Metschnikowia* sp.	2			2	
Dothideales	Dothioraceae	*Aureobasidium pullulans*	1		1		
		Aureobasidium sp.	3		3		
Basidiomycota Sporidiales	Incertae sedis	*Rhodotorula* sp.	1		1		

[a] Includes the strain selected for detailed analysis (see Table 1)

three-fold higher chemical richness (mean ± SEM number of VOCs from yeasts 49.3 ± 2.6 but only 15.7 ± 4.7 compounds from bacteria). Replicate samples taken from Scentry lures were dominated by ethyl acetate, with much smaller amounts (in decreasing order of abundance) of acetic acid, methyl acetate, ethanol, acetoin, vinyl acetate and methionol (Table S2). Three of these compounds (methyl acetate, vinyl acetate and methionol) were undetectable in the volatile headspace of the microbial accessions.

ANOSIM revealed significant variance structure for VOC data collected from yeast and bacterial accessions within the Bray-Curtis dissimilarity matrix, both by kingdom ($R = 0.5238$, $P = 0.001$) and species ($R = 0.9972$, $P = 0.001$). PERMANOVA performed on the same data set revealed similar outcomes for comparisons between kingdom ($R^2 = 0.456$, $P = 0.001$, DF = 1) and species nested within kingdom ($R^2 = 0.506$, $P = 0.001$, DF = 4). Pairwise PERMANOVA between all microbial pairs yielded significant pairwise differences (all

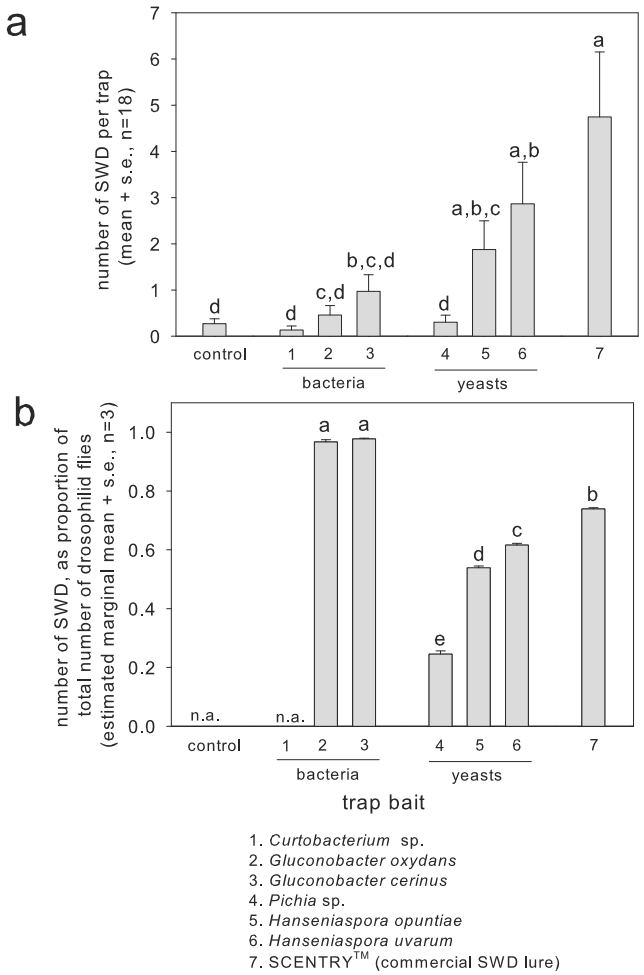

Fig. 2 Capture of drosophilid flies in traps with different odor sources, in a raspberry planting and the negative control comprising autoclaved *Drosophila* food. **a**). Number of SWD captured over 48 h. Mean values (back transformed from LSmeans generated in SAS) are for 18 replicates (6 replicate traps in each of 3 experiments) for each treatment, following nonsignificant effects of block and experiment (see text). *, significantly different from the negative control by Dunnett's test ($p < 0.05$). **b**) Number of SWD captured expressed as proportion of the total drosophilid flies trapped over 48 h. The estimated marginal means and standard errors are plotted from beta regression. Different letters indicate statistical significance as determined by Tukey's test. n.a., not analyzed: the control and *Curtobacterium* sp. baits were excluded from Tukey's test because the total numbers of flies captured was very low

$P < 0.02$) after applying corrections for multiple comparisons. Follow-up analysis of specific volatiles contributing to differences between accessions using SIMPER (Table S3) showed that VOC composition among the three yeast accessions differed significantly primarily through the exclusive detection of ethanol and higher emissions of isoamyl acetate and 2-phenylethyl acetate from *Pichia* sp. than from *H. opuntiae* and *H. uvarum*. Bacterial volatile blends lacked various VOCs, including ethyl acetate, associated with the yeasts (Table S3). *Gluconobacter oxydans* and *G. cerinus* had very similar chemical composition dominated by acetoin, acetic acid and other carboxylic acids (heat map, Fig. 3). The headspace of the *Curtobacterium* accession differed from all other accessions (Fig. 3), consisting of short chain alcohols (including 3-methyl butanol, hexanol) and the ketone nonanone (Table S2).

Discussion

In this study, we demonstrate that different microorganisms isolated from SWD and SWD-infested fruits vary in their attractiveness to wild SWD populations. Because the traps were designed to prevent direct contact between the insects and microbial cultures, these effects can be attributed to insect olfactory responses to microbial volatiles. Our parallel analysis of VOC profiles of the microorganisms in the laboratory has yielded candidate volatiles contributing to this variation. However, these associations should be treated with due caution because we cannot exclude the possibility that the VOC profile of the microorganisms differed between field and laboratory conditions.

Our data confirm and extend current understanding of microbial VOCs as a determinant of insect behavior and ecology, as well as informing strategies for improved control of SWD. In particular, our demonstration that natural populations of SWD are reliably attracted to single-taxon cultures of *Hanseniaspora* yeasts and *Gluconobacter* bacteria in the complex field environment of a mixed raspberry planting reinforces the evidence, primarily from laboratory studies, that SWD is responsive to olfactory cues of microbial origin (Cloonan et al. 2018). For example, there is published evidence that laboratory cultures of SWD significantly prefer *H. uvarum* cultures relative to sterile medium in two-choice assays and to other yeasts in multi-choice assays (Scheidler et al. 2015). Similarly, the two-arm olfactometer trials of Mazzetto et al. (2016) yielded significant attraction of SWD to one of the *Gluconobacter* species, *G. oxydans*, used in our study. Mazzetto et al. (2016) did not investigate *G. cerinus*, the second species used in this study.

In principle, the microbial taxa tested for attractiveness to SWD might be expected to fall into four functional groups: those that attract both SWD and other drosophilid flies, those that attract either exclusively SWD or various drosophilids other than SWD and, finally, those that fail to attract any insects. We obtained representatives of three of these groups, but no microbial taxa that only attracted non-SWD insects. This absence could have arisen by chance, a consequence of the relatively small number of microbial taxa tested, or from bias introduced by our selection of readily-culturable microorganisms isolated from SWD and SWD-infested fruits. Nevertheless, these data are consistent with the conclusion of Kleiber et al. (2014) that SWD is responsive to a wider range of volatiles than other drosophilid flies.

Fig. 3 Heat map of volatile data for all microbial samples, organized by species (columns), with numbered replicates and the 83-compound data set (rows; see Table S2). The scaleindicates ln transforms of TIC peak areas for all verified compounds

The selective attraction of SWD, and not other drosophilids, to the VOCs of some microorganisms is trait may, in turn, be correlated to the generalist ecology of SWD. Specifically, although both SWD and other drosophilids feed extensively from over-ripe fruits and other fermenting plant material, SWD also feed on macrofungi and oviposit into ripening fruits and also, in non-choice conditions in the laboratory, into mushroom substrate (Atallah et al. 2014; Keesey et al. 2015; Stockton et al. 2019; Werner et al. 2018). Consistent with this evidence that SWD is likely less specialized than some other fruit-feeding drosophilids, GC/EAD studies reveal that SWD is significantly more responsive to leaf odors and various fruit odors compared to *D. melanogaster*, which utilizes over-ripe fruits (Keesey et al. 2015). However, SWD does display some specificity in its response to microbial odors. Notably, SWD responded significantly in two-arm olfactometer trials to just three of the six species of Acetobacteraceae tested by Mazzetto et al. (2016), who identified ethanol as a likely key component of the VOCs from the three attractive species. As for animal-microbial interactions generally (Douglas 2018), the interactions between SWD and microorganisms are also likely influenced by the life history traits of the microbial taxa.

The evidence that SWD but no other drosophilid flies in the habitat were attracted to *Gluconobacter* species tested in this study offers a potential route to develop lures of greater specificity for SWD. Future strategies to improve SWD capture can include methods to increase total VOC production by *Gluconobacter* (which is considerably lower than the emissions from yeasts in our laboratory studies, Table S2) and to test a wider panel of *Gluconobacter* isolates, following the evidence of among-isolate variation in SWD responses (Mazzetto et al. 2016).

A further feature of this study was the minimal numbers of flies of any species that were attracted to our isolate of the yeast *Pichia* sp., even though this isolate was more volatile-rich, yielding 30–50% greater TIC peak area than the *Hanseniaspora* yeasts, which attracted SWD (Table S2). Interestingly, 3-methylbutyl acetate (also known as isoamyl acetate), which is significantly elevated in the unattractive *Pichia* relative to the attractive *Hanseniaspora* species (Table S3), has been reported to decrease the attraction of SWD to acetic acid and ethanol under laboratory and field conditions (Cha et al. 2012; Cha et al. 2014). Taken together, the results of this study provide the basis for further research on microbial volatiles and volatile blends that are specific attractants and deterrents under field conditions.

Immediately relevant to these considerations is the evidence that SWD is also highly responsive to visual cues, especially red and black (Basoalto et al. 2013; Kirkpatrick et al. 2016; Rice et al. 2016) and spectral contrasts between fruit and background vegetation (Little et al. 2018). The importance of vision in SWD is reflected in the anatomy of the insect. Compared with *D. melanogaster*, which is more dependent on olfactory cues, adult SWD have relatively larger compound eyes with more ommatidia, fewer trichoid sensilla on their antennae and, in the brain, larger optic lobes and smaller antennal lobes (Keesey et al. 2019). This difference offers the opportunity to improve the specificity of SWD trapping strategies by combining visual cues, e.g. colored spheres, with specific microbial odors and, for lure-and-kill strategies, insecticidal formulations (Rice et al. 2017), More generally, SWD offers the opportunity for multi-modal investigations of how insects integrate information from volatiles from microbial and other sources, together with visual cues as they navigate the natural environment to identify food and sites for mating and oviposition.

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References

Adler LS (2000) The ecological significance of toxic nectar. Oikos 91: 409–420

Arguello JR, Sellanes C, Lou YR, Raguso RA (2013) Can yeast (*S. cerevisiae*) metabolic volatiles provide polymorphic signaling? PLoS One 8:e70219

Asplen MK, Anfora G, Biondi A, Choi DS, Chu D, Daane KM, Gibert P, Gutierrez AP, Hoelmer KA, Hutchison WD et al (2015) Invasion biology of spotted wing drosophila (*Drosophila suzukii*): a global perspective and future priorities. Pest Manag Sci 88:469–494

Atallah J, Teixeira L, Salazar R, Zaragoza G, Kopp A (2014) The making of a pest: the evolution of a fruit-penetrating ovipositor in *Drosophila suzukii* and related species. Proc Biol Sci 281: 20132840

Basoalto E, Hilton R, Knight A (2013) Factors affecting the efficacy of a vinegar grap for *Drosophila suzukii*. J Appl Entomol 137:561–570

Becher PG, Flick G, Rozpedowska E, Schmidt A, Hagman A, Lebreton S, Larsson MC, Hansson BS, Piskur J, Witzgall P, Bengtsson M (2012) Yeast, not fruit volatiles mediate *Drosophila melanogaster* attraction, oviposition and development. Funct Ecol 26:822–828

Beck JJ, Vannette RL (2017) Harnessing insect-microbe chemical communications to control insect pests of agricultural systems. J Agric Food Chem 65:23–28

Benjamini Y, Hochberg Y (1995) Controlling the false discovery rate: a practical and powerful approach to multiple testing. J Roy Statist Soc B 57:289–300

Bing X, Gerlach J, Loeb G, Buchon N (2018) Nutrient-dependent impact of microbes on *Drosophila suzukii* development. mBio 9: 02199–02117

Bost A, Franzenburg S, Adair KL, Martinson VG, Loeb G, Douglas AE (2018) How gut transcriptional function of *Drosophila*

melanogaster varies with the presence and composition of the gut microbiota. Mol Ecol 27:1848–1859

Cha DH, Adams T, Rogg H, Landolt PJ (2012) Identification and field evaluation of fermentation volatiles from wine and vinegar that mediate attraction of spotted wing *Drosophila, Drosophila suzukii*. J Chem Ecol 38:1419–1431

Cha DH, Adams T, Werle CT, Sampson BJ, Adamczyk JJ, Rogg H, Landolt PJ (2014) A four-component synthetic attractant for *Drosophila suzukii* (Diptera: Drosophilidae) isolated from fermented bait headspace. Pest Manag Sci 70:324–331

Cha DH, Loeb GM, Linn CE, Hesler SP, Landolt PJ (2018) A multiple-choice bioassay approach for rapid screening of key attractant volatiles. Environ Entomol 47:946–960

Clarke KR, Gorley RN (2006) PRIMER v6: user manual/tutorial. In: PRIMER-e. https://www.primer-e.com/

Cloonan KR, Abraham J, Angeli S, Syed A, Rodriguez-Saona C (2018) Advances in the chemical ecology of the spotted wing Drosophila (*Drosophila suzukii*) and its applications. J Chem Ecol 44:922–939

Cribari-Neto F, Zeileis A (2010) Beta Regression in R. J of Stat Softw 34(2):1–24 http://www.jstatsoft.org/v34/i02/

Davis TS, Crippen TL, Hofstetter RW, Tomberlin JK (2013) Microbial volatile emissions as insect semiochemicals. J Chem Ecol 39:840–859

Dominy NJ (2004) Fruits, fingers, and fermentation: the sensory cues available to foraging primates. Integr Comp Biol 44:295–303

Douglas AE (2018) What will it take to understand the ecology of symbiotic microorganisms. Env Microbiol 20:1920–1924

Dudley R (2000) Evolutionary origins of human alcoholism in primate frugivory. Q Rev Biol 75:3–15

Feng Y, Bruton R, Park A, Zhang A (2018) Identification of attractive blend for spotted wing Drosophila, *Drosophila suzukii*, from apple juice. J Pest Sci 91:1251–1267

Fukatsu T, Nikoh N (1998) Two intracellular symbiotic bacteria from the mulberry psyllid *Anomoneura mori* (Insecta, Homoptera). Appl Environ Microbiol 64:3599–3606

Hamby KA, Hernandez A, Boundy-Mills K, Zalom FG (2012) Associations of yeasts with spotted-wing Drosophila (*Drosophila suzukii*; Diptera: Drosophilidae) in cherries and raspberries. Appl Environ Microbiol 78:4869–4873

Iglesias LE, Nyoike TW, Liburd OE (2014) Effect of trap design, bait type, and age on captures of Drosophila suzukii (Diptera: Drosophilidae) in berry crops. J Econ Entomol 107:1508–1518

Janzen DH (1977) Why fruits rot, seed mold, and meat spoils. Am Nat 111:691–713

Keesey IW, Knaden M, Hansson BS (2015) Olfactory specialization in *Drosophila suzukii* supports an ecological shift in host preference from rotten to fresh fruit. J Chem Ecol 41:121–128

Keesey IW, Grabe V, Gruber L, Koerte S, Obiero GF, Bolton G, Khallaf MA, Kunert G, Lavista-Llanos S, Valenzano DR et al (2019) Inverse resource allocation between vision and olfaction across the genus *Drosophila*. Nat Commun 10:1162

Kirkpatrick DM, McGhee PS, Hermann SL, Gut LJ, Miller JR (2016) Alightment of spotted wing Drosophila (Diptera: Drosophilidae) on odorless disks varying in color. Environ Entomol 45:185–191

Kleiber JR, Unelius CR, Lee JC, Suckling DM, Qian MC, Bruck DJ (2014) Attractiveness of fermentation and related products to spotted wing Drosophila (Diptera: drosophilidae). Environ Entomol 43:439–447

Knight AL, Basoalto E, Yee W, Hilton R, Kurtzman CP (2015) Adding yeasts with sugar to increase the number of effective insecticide classes to manage *Drosophila suzukii* (Matsumura) (Diptera: Drosophilidae) in cherry. Pest Manag Sci 72:1482–1490

Liscia A, Angioni P, Sacchetti P, Poddighe S, Granchietti A, Setzu MD, Belcari A (2013) Characterization of olfactory sensilla of the olive fly: behavioral and electrophysiological responses to volatile organic compounds from the host plant and bacterial filtrate. J Insect Physiol 59:705–716

Little CM, Chapman TW, Hillier NK (2018) Effect of color and contrast on highbush blueberries to host-finding behavior by *Drosophila suzukii* (Diptera: Drosophilidae). Environ Entomol 47:1242–1251

MacCollum GB, Lauzon CR, Weires RW, Rutowski AA (1992) Attraction of adult apple maggot (Diptera: Tephritidae) to microbial isolates. J Econ Entomol 85:83–87

Mazzetto F, Gonella E, Crotti E, Vacchini V, Syrpas M, Pontini M, Mangelinckx S, Daffonchio D, Alma A (2016) Olfactory attraction of *Drosophila suzukii* by symbiotic acetic acid bacteria. J Chem Ecol 89:783–792

Mori BA, Whitener AB, Leinweber Y, Revadi S, Beers EH, Witzgall P, Becher PG (2017) Enhanced yeast feeding following mating facilitates control of the invasive fruit pest *Drosophila suzukii*. J Appl Ecol 54:170–177

Oksanen J, Blanchet FG, Friendly M, Kindt R, Legendre P, McGlinn D, Minchin PR, O'Hara RB, Simpson GL, Solymos P, et al. (2019) Vegan: community ecology package. (R package version 2.5-4). https://CRAN.R-project.org/package=vegan

R Core Team (2015) R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria

Rice KB, Short BD, Jones SK, Leskey TC (2016) Behavioral responses of *Drosophila suzukii* (Diptera: Drosophilidae) to visual stimuli under laboratory, semifield, andfField conditions. Environ Entomol 45:1480–1488

Rice KB, Short BD, Leskey TC (2017) Development of an attract-and-kill strategy for *Drosophila suzukii* (Diptera: Drosophilidae): evaluation of attracticidal spheres under laboratory and field conditions. J Econ Entomol 110:535–542

Robacker DC, Lauzon CR, He X (2004) Volatiles production and attractiveness to the Mexican fruit fly of *Enterobacter agglomerans* isolated from apple maggot and Mexican fruit flies. J Chem Ecol 30:1329–1347

Ruxton GD, Wilkinson DM, Schaefer HM, Sherratt TN (2014) Why fruit rots: theoretical support for Janzen's theory of microbe-macrobe competition. Proc Biol Sci 281:20133320

Scheidler NH, Liu C, Hamby KA, Zalom FG, Syed Z (2015) Volatile codes: correlation of olfactory signals and reception in Drosophila-yeast chemical communication. Sci Rep 5:14059

Schetelig MF, Lee K-Z, Otto S, Talmann L, Stokl J, Degenkolb T, Vilcinskas A, Halitschke R (2017) Environmentally sustainable pest control options for *Drosophila suzukii*. J Appl Entomol 142:3–17

Stensmyr MC, Dweck HK, Farhan A, Ibba I, Strutz A, Mukunda L, Linz J, Grabe V, Steck K, Lavista-Llanos S, Wicher D, Sachse S, Knaden M, Becher PG, Seki Y, Hansson BS (2012) A conserved dedicated olfactory circuit for detecting harmful microbes in *Drosophila*. Cell 151:1345–1357

Swoboda-Bhattarai KA, McPhie DR, Burrack HJ (2017) Reproductive status of D*rosophila suzukii* (Diptera: Drosophilidae) females influences attraction to fermentation-based baits and ripe fruits. J Econ Entomol 110:1648–1652

Tasin M, Knudsen GK, Pertot I (2012) Smelling a diseased host: grapevine moth responses to healthy and fungus-infected grapes. Anim Behav 83:552–562

Verheggen F, Perrault KA, Megido RC, Dubois LM, Fancis F, Haubruge E, Forbes SL, Focant J-F, Stefanuto P-H (2017) The odor of death:

an overview of current knowledge on characterization and applications. BioScience 67:600–613

Werner T, Steenwinkel T, Jaenike J (2018) Drosophilids of the Midwest and northeast. Open Access Books, Michigan Tech University, MI

White TJ, Bruns T, Lee S, Taylor J (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ (eds) PCR protocols: a guide to methods and applications. Academic Press, New York, pp 315–322

Wiens F, Zitzmann A, Lachance MA, Yegles M, Pragst F, Wurst FM, von Holst D, Guan SL, Spanagel R (2008) Chronic intake of fermented floral nectar by wild treeshrews. Proc Natl Acad Sci U S A 105: 10426–10431