1 **Microbial metabolites mediate bumble bee attraction and feeding**

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3 Robert N. Schaeffer[1,2,4,†], Caitlin C. Rering[3,†], Isabelle Maalouf[2], John J. Beck[3], Rachel L.

4 Vannette[2]

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6 [1]Department of Entomology, Washington State University, Pullman, WA 99164

7 [2]Department of Entomology and Nematology, University of California Davis, Davis, CA 95616

8 [3]Chemistry Research Unit, Center for Medical, Agricultural and Veterinary Entomology,

9   Agricultural Research Service, United State Department of Agriculture, Gainesville, FL

10 32608

11

12 [4]Corresponding author and current address: Department of Biology, Utah State University,

13 Logan, UT 84321, robert.schaeffer@usu.edu

14 [†]Equally-contributing authors

15

16 **Abstract**

17   Animals such as bumble bees use chemosensory cues to localize and evaluate essential

18 resources. Increasingly, it is recognized that microbes can alter the quality of foraged resources

19 and produce metabolites that act as foraging cues. The distinct nature of these sensory cues

20 however and their use in animal foraging remain poorly understood. Here, we test the hypothesis

21 that species of nectar-inhabiting microbes differentially influence pollinator attraction and

22 feeding via microbial metabolites in nectar. We examined electrophysiological potential of

23 bumble bee antennae to respond to volatile microbial metabolites, followed by behavioral

24 responses using choice assays. We assessed gustatory responses through both no-choice and

25 choice feeding assays. Antennae responded to some microbial volatiles, and bees chose *Asaia*

26 bacterial solutions compared to *Metschnikowia* yeast based on volatiles alone. However, *B.*

27 *impatiens* consumed significantly more *Metschnikowia*-inoculated nectar, suggesting distinct

28 roles for volatile and non-volatile microbial metabolites in mediating feeding decisions, with

29 potential to affect associative learning and future foraging. Our results suggest that microbial

30 metabolites may represent non-reinforcing cues with potential consequences for forager learning,

31 economics and floral host reproduction.

32

35

36   **Introduction**

37   To successfully persist in a chemosensory environment, animals must receive and

38   interpret cues and signals of ecologically-important information, such as the quantity and quality

39   of resources potentially available to them [1]. This is especially true of pollinators such as

40   bumble bees, which integrate multi-modal signals, including form, color, and scent, to accurately

41   identify rewarding flowers [2]. Like other food resources, flowers host varied microbial species

42   and communities [3,4], which produce metabolites that may act as cues of resource availability

43   and quality, with consequences for pollinator foraging [5,6]. Indeed, insect pollinators are highly

44   sensitive to shifts in volatile abundance and identity [7–9], with scents being known to influence

45   learned foraging preferences [10]. However, the role of volatile and non-volatile microbial

46   metabolites in mediating pollinator attraction and foraging decisions still remains largely unclear.

47   In standing crop nectar, bacteria and fungi colonize between 20-70% of individual

48   flowers, attain densities exceeding 10^5 and 10^7 cells/µL respectively [3,4] and metabolize sugars

49   and amino acids [5,11], affecting pollinator foraging and plant reproduction [5,12,13]. Intense

50   competition between microbes in nectar often results in flowers that are dominated by either

51   yeast or bacteria [14]. Yeasts and bacteria differ in volatile composition and acceptance to

52   pollinators [6], but also differentially influence non-volatile nectar traits (Vannette & Fukami

53   2018) and shift pollinator perceptions of nectar quality [15]. Predicting microbial effects on

54   pollinator foraging and behavior requires examining responses to olfactory (headspace volatiles)

55   and gustatory (dissolved chemicals) cues.

56   Here, we test the hypothesis that yeast and bacteria differentially influence bumblebee

57   attraction and feeding. Bumble bees (*Bombus impatiens*) are an ideal system, due to their close

58   ecological relationships with yeasts [16,17] and bacteria [18,19]. We examined antennal

59   responses to microbial metabolites using electroanntenographic (EAG) bioassays, bee choice

60   using olfactometer (Y-tube) bioassays, and gustatory preferences using choice and no-choice

61   feeding assays. We found bumble bees show distinct responses to volatile vs gustatory microbial

62   cues to inform foraging decisions, indicating the potential for associative learning, where bumble

63   bees may adjust behavioral responses to volatile blends after exposure to gustatory microbial

64   cues.

65

66   **Materials and methods**

67   *Study system*

68   We used three colonies of the generalist bumble bee *Bombus impatiens* (Koppert

69   Biological Systems, Inc.; Howell, MI, USA) and strains of the nectar-inhabiting yeast

70   *Metschnikowia reukaufii* (Metschnikowiaceae; GenBank ID: MF319536) and bacteria *Asaia*

71   *astilbes* (Acetobacteraceae; GenBank ID: KC677740). Both *M. reukaufii*, and *A. astibles* are

72   commonly isolated from floral nectar [20] and pollinators (Good *et al.* 2014), but differentially

73   influence nectar chemistry and scent [21].

74

75   *Experiment 1: Electroantennographic bioassay*

76   We examined antennal response (n=6 /metabolite) to volatiles produced by

77   *Metschnikowia* and *Asaia* (Table 1) by puffing each metabolite (0.4 µmol) over excised *B.*

78   *impatiens* antennae. Antennal responses were recorded and corrected by responses to blanks and

79   positive control stimuli (0.4 µmol geraniol), see electronic supplementary material S1 Methods.

80

81   *Experiment 2: Olfactory response of bumble bees to nectar-inhabiting microbes*

82   To assess whether bumble bees exhibit innate preferences when exposed to volatile

83   microbial metabolites, we used an olfactometer assay (Y-tube; Fig. S1) under red light. Naïve

84   bumble bees housed at the University of California Davis were starved for 6 hours, then released

85   individually into the Y-tube. For each bee, initial choice and the time spent in each arm was

86   recorded, and the assay was repeated twice for each bee, with treatment direction reversed. These

87   bees were both fed and treated similarly to those used for the EAG assays and a total of 32 bees

88   were tested in this assay from two source colonies. For details, see electronic supplementary

89   material S1 Methods.

90

91   *Experiment 3: Gustatory responses of bumble bees to nectar-inhabiting microbes*

92   To assess gustatory responses of bumble bees (n=42 bees from two colonies) to nectar

93   colonized by microbial taxa, we used both no-choice and choice feeding assays. For the no-

3

94   choice assay, bees were housed in individual vials with modified lids that accommodated a

95   feeding apparatus (Fig. S2). Vials were filled with 1 mL of either *Asaia-* or *Metschnikowia-*

96   treated nectar, weighed, and bees were allowed to feed for 24 hr, after which tubes were re-

97   weighed to determine consumption. For details, see electronic supplementary material S1

98   Methods.

99

100   *Experiment 4: Effects of volatile and gustatory microbial cues on associative learning*

101   Because bees exhibited marked differences in response to volatile and gustatory

102   microbial cues (see Results below), we also assessed how exposure to gustatory cues influenced

103   bee preference for volatiles (n=24 bees from two colonies). Individual foragers were subjected to

104   the olfactometer assay (above), then a gustatory choice assay where individual bees were housed

105   in a feeding chamber, consisting of ~9 cm of perforated tubing, with feeding vials on either end

106   of the chamber (Fig. S3) for 24 hr. Vials were weighed to determine nectar consumption. Bees

107   were then subjected to a second olfactometer assay.

108

109   *Statistical analyses*

110   To assess which compounds were detected by bumble bees (*Experiment 1*), we used

111   *t*-tests with false discovery rate correction to examine if normalized EAG responses were

112   significantly different from zero (i.e., no detectable response). To determine if bee preference

113   differed between microbes, data from *Experiment 2* were analyzed using a binomial test for first

114   choice. A linear mixed-effect (LME) model [22] was used for time spent in each arm, with

115   microbial treatment as a fixed effect, and bee individual as a random effect. For gustatory cues

116   (*Experiment 3*), we used a *t*-test to assess how nectar consumption was affected by the nectar

117   treatment. For *Experiment 4*, we fit a LME model with proportion of time spent in olfactometer

118   arms as the response variable, nectar treatment, choice test order, and their interaction as fixed

119   effects, and bee individual as a random effect. Bumble bee feeding responses were also analyzed

120   with a LME model, with amount consumed as the response variable, nectar treatment as a fixed

121   effect, and bee individual as a random effect. All analyses were performed in R (v. 3.5.2) [23].

122

123   **Results**

124   Bumble bee antennae responded to a subset (4/20) of volatile metabolites tested through

125 EAG (*Experiment 1*; Table 1) at 0.4 µmol, including 1-hexanol, 2-ethyl-1-hexanol, 2-

126 phenylethanol, and 3-methylbutyl acetate (i.e., isoamyl acetate, isopentyl acetate). The alcohol 2-

127 ethyl-1-hexanol elicited the strongest EAG depolarization response, surpassing that of the

128 positive control (0.4 µmol geraniol).

129   Volatile blends emitted by nectar-inhabiting microbes also influenced bee behavior.

130 Naïve bees on average spent ~two-thirds of their time in Y-tube arms containing *Asaia*-produced

131 volatiles (Figure 1A; $F_{1,64}$=21.52, P<0.0001), although no difference was found for first choice

132 (P=0.67). In contrast, bees consumed approximately 50% less *Asaia*-conditioned nectar than

133 *Metschnikowia* nectar (Figure 1B; $t_{29.5}$=-2.70, P=0.011) in a no-choice assay (*Experiment 3*).

134   Mirroring earlier results, bumble bees across both choice tests performed in *Experiment 4*

135 spent ~15% more of their time in the Y-tube arm assigned to *Asaia* compared to that of

136 *Metschnikowia* ($F_{1,163}$=9.09, P=0.003). During the feeding assay, bees again consumed on

137 average nearly double the amount of *Metschnikowia*-conditioned nectar compared to *Asaia*

138 ($F_{1,46}$=12.29, P=0.001). After experiencing gustatory cues in the feeding assay, bees reduced the

139 frequency with which they chose the *Asaia* volatile blend, increasing both the proportion of 'no

140 choice' and that for the yeast arm, as well as the amount of time spent (albeit not significant) in

141 the *Metschnikowia* arm of the olfactometer.

142

143 **Discussion**

144   Microbes commonly inhabit food resources, contributing both volatile and non-volatile

145 metabolites that can function to inform foragers as to their location and quality. We found

146 distinct effects of olfactory vs. gustatory cues produced by two common, nectar-inhabiting

147 microbes on bumble bee behavior and nectar consumption. The difference in bee response to

148 olfactory vs. gustatory cues suggests that these cues are not reinforcing, which may complicate

149 pollinator foraging and learning based on microbial metabolites [24]. Further, bee preference for

150 *Asaia* volatiles decreased after exposure to gustatory cues and feeding, suggesting behavior

151 modification. We suspect that acetic acid produced by *Asaia* (but not *Metschnikowia)*, although

152 not detectable in our volatile screening, may be aversive to bees. In natural systems, bees likely

153 develop associations between microbial chemosensory cues through repeated exposure to the

154 scent and taste of yeast or bacterial-colonized nectar. However, our findings, and recent

5

155 experimental results [24], suggest that microbial signals may be more difficult to learn than other

156 sensory combinations. Such difficulties may manifest to affect learned preferences, floral

157 constancy and the quantity and quality of benefits exchanged in these mutualistic interactions.

158   Collectively, our results indicate that volatile and non-volatile microbial metabolites have

159 significant potential to shape interspecific, plant-pollinator signaling. In remains to be

160 determined whether pollinators benefit from microbial-derived cues can translate to improved

161 foraging efficiency, or whether such cues may be more exploitative, and benefit microbes that

162 rely upon pollinator dispersal to reach new floral habitats [25]. Such outcomes may hinge on

163 both the identity and density of the microbial species encountered, where varied immigration

164 histories can give rise to divergent microbial communities both within flowers of a host and

165 among other species. Our results demonstrate that future investigations on the evolutionary

166 ecology of floral signaling should consider multiple ways in which microbes influence host

167 phenotype and the innate and learned response of pollinators.

168

169 **Authors' contributions.** R.S., C.R., J.B., and R.V. conceived the study. R.S., C.R., and I.M.

170 collected data, while R.S. and C.R. performed statistical analyses and drafted the manuscript. All

171 authors contributed to manuscript editing, gave final approval for publication, and agree to be

172 held accountable for the worked performed therein.

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Figure legends

Figure 1 Behavioral (A) and gustatory (B) responses of bumble bees to artificial nectar colonized by nectar-inhabiting microbes and the volatile organic compounds they emit.

Table 1 - Volatile organic compounds produced by nectar-inhabiting microorganisms and their respective normalized mean bumble bee electroantennogram (EAG) response ± standard error (n=6) and corresponding false discovery rate corrected p-values.

Class	Chemical	Presence in nectar headspace[a]		Normalized EAG response[b] (%;n = 6 bees)	P-value
		A. astilbes	*M. reukaufii*		
1º Alcohol	ethanol	++	++++	-12 ±14	0.72
	1-propanol	-	++	-2 ± 4	0.80
	isobutanol	+	+++	-8 ± 6	0.67
	3-methyl-1-butanol	++	++++	-7 ± 9	0.72
	3-methyl-3-buten-1-ol	-	+	-9 ± 9	0.72
	4-penten-1-ol	-	+	-5 ± 5	0.72
	1-hexanol	+	+	66 ± 42 *	0.047
	3-ethoxy-1-propanol	-	+	-4 ± 14	0.80
	2-ethyl-1-hexanol	++	+	144 ± 8 ***	0.00025
	2-methyl-1-butanol	++	++++	-12 ± 14	0.72
	2-phenylethanol	+	+++	73 ± 13 *	0.022
2º Alcohol	2-butanol	-	+	-5 ± 8	0.72
Aldehyde	acetaldehyde	+	++	23 ± 7 [†]	0.07
Ester	ethyl acetate	-	+++	-5 ± 7	0.72
	2-methylpropyl acetate	-	+	20 ± 7	0.11
	ethyl butyrate	-	+	-10 ± 18	0.76
	3-methylbutyl acetate	-	+	24 ± 6 *	0.047
Isoprenoid	isoprene	+	-	-1 ± 9	0.93
Ketone	3-hydroxy-2-butanone	++	++	54 ± 35	0.52
Misc.	2,5-dimethylfuran	++	-	-5 ±15	0.80

[a]Relative abundance of volatiles are represented by +/- symbols where - indicates a chemical was not or infrequently detected (one replicate or less) and + symbols correspond to the relative peak areas orders of magnitude in microbial headspace after 96 h growth in synthetic nectar as reported in [5]. [b]Normalized mean response is significantly different from 0 (false discovery rate † P < 0.1, * P < 0.05, ** P < 0.01, *** P < 0.001).

265 **Figure 1**

266