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7   ORIGINAL ARTICLE

8   **Bacteria may contribute to distant species recognition in ant–aphid mutualistic relationships**

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19   **Abstract**

20   Mutualistic interactions between ant and aphid species have been the subject of considerable historical and

21   contemporary investigations, the primary benefits being cleaning and protection for the aphids and

22   carbohydrate-rich honeydew for the ants. Questions remained, however, as to the volatile semiochemical

23   factor influencing this relationship. A recent study highlighted the role of bacterial honeydew volatile

24   compounds in ant attraction. Here, ant's ability to distantly discriminate two aphid species was

25   investigated based on bacterial honeydew semiochemicals emissions using a two-way olfactometer. Both

26   the mutualistic aphid *Aphis fabae* L. and the non-myrmecophilous aphid *Acyrthosiphon pisum* Harris were

27   found to be attractive for the ant *Lasius niger* L. The level of attraction was similar in both assays (control

28   versus one of the aphid species). However, when given a choice between these two aphid species, ants

29   showed a significant preference for *Aphis fabae*. Honeydew volatiles, mostly from bacterial origins, are

30   known to be a key element in ant attraction. Using the same olfactometry protocol, the relative

31   attractiveness of volatiles emitted by honeydews collected from each aphid species and by bacteria isolated

32   from each honeydew was in investigated. Again, ants significantly preferred volatiles released by *Aphis*

33   *fabae* honeydew and bacteria. This information suggests that microbial honeydew volatiles enable ants to

34   distantly discriminate aphid species. These results strengthen the interest of studying the occurrence and

35   potential impact of microorganisms in insect symbioses.

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37   **Key words** ant; aphid; bacteria; honeydew; mutualism; recognition; VOC

Introduction

Ant–aphid interactions, as a major model of mutualistic relationships, have been the subject of considerable historical and contemporary investigations. Small and defenseless aphids are easy prey for numerous predators and parasitoids. However, some aphid species are frequently found in association with ants that tend and protect the aphids in exchange of honeydew, which is a reliable and abundant carbohydrate source. Other aphid species do not develop such partnerships with ants and are rather considered as preys (Dixon, 1985; Bristow, 1991; Stadler & Dixon, 2005).

In Europe, it has been estimated that myrmecophily, *i.e.* ants tending, is observed for two thirds of aphid species (Stadler, 1997). Three ant genera appear to be primarily involved in these mutualistic interactions: *Lasius, Myrmica* and *Formica* (Stadler & Dixon, 1999; Guénard *et al.*, 2007), among which the black garden ant, *Lasius niger* L. (Hymenoptera, Formicidae), is well known to tend several aphid species, including the black bean aphid, *Aphis fabae* Scopoli (Homoptera, Aphididae) (El-Ziady & Kennedy, 1956). These two species are frequently used in studies on mutualistic interactions. Besides, the pea aphid, *Acyrthosiphon pisum* Harris can be reared on the same host plant, *Vicia faba* L., as the bean aphid but is never observed in association with ants. For these reasons, these three insect species, widespread in their natural environment, were selected as model taxa for this research (Wilson, 1955; Holman, 2009).

While aphids do not seem to actively search for ant partners, ants are known to search for their aphid partners and are able to use different volatile cues to orientate this search, namely volatile organic compounds emitted by honeydew bacteria (mVOCs) and E-*β*-farnesene (EBF), an aphid pheromone involved in alarm and social behaviors (Verheggen *et al.*, 2012; Fischer *et al.*, 2015a; Fischer *et al.*, 2015b). This last molecule is a major component of the alarm pheromone of numerous aphid species, and is thus not likely to constitute alone a suitable species recognition cue (Francis *et al.*, 2005). In contrast, honeydew VOCs emission profiles vary between aphid species, depending on various factors including the aphid species and its gut microflora, and could thus contribute to distant aphid discrimination. This paper describes a study designed to investigate the role of honeydew mVOCs in distant aphid discrimination by ant partners.

Material and methods

Plants and insects

In climate-controlled rooms (16 h light-8h dark photoperiod; 20 ± 2°C), *Aphis fabae* and *Acyrthosiphon pisum*, were reared for several generations on *Vicia faba* plants cultivated on a 1/1 mix of perlite and vermiculite substrate. Plants used in experiments were about 15 cm high. All substrates used in biological tests were previously sterilized. *Lasius niger* colonies were collected in Gembloux (Belgium), and kept in separate chambers under the same conditions. To prevent escape, the nests (test tubes covered with a red transparent foil) were placed in plastic containers coated with polytetrafluoroethylene (Fluon®, Whitford, UK). Water and aqueous brown sugar solution (342 g/L) were provided *ad libitum*. Dead fruit flies and mealworms were provided weekly as a complementary food source. All nests were composed by a queen, brood, and a minimum of 500 foragers.

Biological tests and chemical investigations

Choice tests with two-way olfactometer as well as characterization of honeydew's and bacterial VOCs emission profiles by SPME-GC-MS were undertaken according to Fischer *et al.* (2015a).

Samples and controls used in the behavioral assays are summarized in Table 1. For each modality, samples and controls presented to ants were used to test 20 ants and then renewed. The first samples were aphid-infested plants (substrate with nine *V. faba* infested for three days either by 50 myrmecophilous *Aphis fabae* or by 50 non-myrmecophilous *Acyrthosiphon pisum* respectively); their attractiveness were compared to that of a healthy plants without aphids.

The following test assessed the relative attractiveness of the two first samples, plants infested by one of these two species being presented at each side of the olfactometer.

The global attractiveness of an aphid-infested plant relies mostly on volatile organic compounds (VOCs) released by the honeydew accumulating around aphid colonies, and more specifically by aphid-associated bacteria present in honeydew (Fischer *et al.*, 2015a). The relative attractiveness of honeydews was thus assessed for the two tested aphid species. Sample consisted of *Aphis fabae* honeydew that was collected for three days from a heavily infested plant onto wet substrate (perlite : vermiculite 1 : 1) to avoid

91 desiccation, while control was *Acyrthosiphon pisum* honeydew (collected the same way). The relative

92 attractiveness of two taxonomically close honeydew bacteria known to be involved in aphid interactions

93 with other insect species was also tested. The first one, *Staphylococcus xylosus*, is found in *Aphis fabae* gut

94 and honeydew and is known to produce mVOCs attractive for *L. niger* (Fischer *et al.*, 2015a); the second

95 one, *Staphylococcus sciuri*, is found in *Acyrthosiphon pisum* gut and honeydew and is known to attract

96 *Episyrphus balteatus* (De Geer), an aphid enemy (Leroy *et al.*, 2011). Both these bacteria were found only

97 in one of the two studied aphid species. Sample and control consisted in 60mL of 868 culture medium (20

98 g of glucose and 10 g of both yeast extract and casein peptone per liter of distilled water) inoculated with

99 *S. xylosus* and *S. sciuri* respectively and incubated for 2 days at 20°C.

100 Binomial tests were carried out to assess the significance of the ant preferences. The significances

101 of differences of attractiveness observed between tests were assessed by χ^2 test. Average linear speeds of

102 ants in the two branches were compared, assay by assay, with *t*-tests. Differences were considered

103 significant at $P < 0.05$. Statistical analyses were performed using Minitab 15.1 (State College,

104 Pennsylvania, USA).

105 **Results**

106 **VOCs and ant attraction**

107 Plants infested by myrmecophilous and non-myrmecophilous aphid species significantly attracted

108 ants when tested against healthy plants; respectively 68% and 65% of the tested foragers were attracted

109 towards *Aphis fabae* and *Acyrthosiphon pisum* infested plants (binomial tests, $n = 100$, $P < 0.001$ and $P =$

110 0.004, respectively). These attraction percentage towards these two aphid species, tested separately against

111 non-infested plants, are not statistically different (χ^2 test, $P = 0.520$).

112 However, when plants infested with these two aphid species were tested against each other, plants

113 infested by *Aphis fabae* attracted significantly more ant foragers (61%, binomial test, $n = 100$, $P = 0.035$).

114 The same tendency is also observed for honeydews of these two species, and for cultures of bacteria found

115 in these honeydews. When presented with honeydews of the two species, 65% of ant foragers chose the

116 *Aphis fabae* honeydew branch (binomial test, $n = 60$, $P = 0.027$). Facing a choice between cultures of *S.*

117   *xylosus* and *S. sciuri*, 65% of ant foragers selected the *S. xylosus* branch (binomial test, $n = 60$, $P = 0.027$).

118   These attraction levels towards *Aphis fabae*, its honeydew, and a bacterium from its honeydew, tested

119   against *Acyrthosiphon pisum*, its honeydew, and a bacterium from its honeydew respectively, are

120   statistically not different from the attractiveness observed in the first assay (χ^2 tests, $P = 0.133$, $P = 0.618$

121   and $P = 0.618$, respectively).

122   No significant differences in speed were observed between branches of the olfactometer in any

123   assay (*t*-tests, equality of variances verified, all $P > 0.099$).

124   **Characterization of honeydew's and bacterial VOCs emission profiles**

125   Twenty-eight volatile chemicals, including esters, alcohols, acids, aldehydes and ketones, were

126   identified from honeydew and bacterial cultures. A comparison with other data from our former

127   investigations is shown in Table 2.

128   Among the 28 compounds observed in honeydews, 9 were observed in honeydews of both species.

129   However, the relative amount of these compounds may vary strongly between species. For example,

130   benzenethanol constitutes 53% of total peak area for *Aphis fabae* honeydew VOC, and only 1.7% of total

131   peak area for *Acyrthosiphon pisum*.

132   Sixteen of the 28 compounds identified from honeydews were also observed in *Staphylococcus*

133   cultures. The mVOCs emitted by both *Staphylococcus* species are qualitatively nearly identical, the only

134   difference being linalool solely observed for *S. xylosus*. However, strong quantitative differences are also

135   observed (Table 2).

136   **Discussion**

137   In order to establish and maintain a mutualistic relationship, ant foragers have first to find aphid colonies

138   and asses their suitability as partners based on various parameters including aphid morphology and

139   behavior, honeydew quality and quantity…. However, in order to asses these criteria, ant foragers must

140   already have found the aphid colony. Previous studies already showed ants ability to orient their search for

141   aphid colonies using volatile cues (Verheggen *et al.*, 2012; Fischer *et al.*, 2015a). The data presented here

142   is the first evidence of distant aphid species discrimination by ants that is driven by aphid honeydew and

143   mediated by the associated microflora through release of VOCs, in laboratory conditions.

144   Using a methodology previously applied to highlight honeydew mVOCs effect on ant behavior

145   (Fischer *et al.*, 2015a), we observed similar ant attraction toward both myrmecophilous and non-

146   myrmecophilous aphid species when the aphids were presented alone. However, when given a choice

147   between the two aphid species, ant showed a significant preference for the myrmecophilous *Aphis fabae*

148   with an attraction level similar to the attraction observed for this aphid presented alone. Moreover, this

149   tendency is supported by the relative attractiveness of honeydews and bacterial cultures issued of these two

150   aphid species, confirming both ants ability to distantly discriminate between two aphid species, and

151   honeydew VOCs involvement in that discrimination. Even though mVOCs emitted from honeydew have

152   been reported to attract aphid enemies and potential ant partners (Leroy *et al.*, 2011; Fischer *et al.*, 2015a),

153   this is the first evidence of ant ability to use such volatile cues to distantly discriminate two aphid species.

154   Furthermore, the attractiveness shown by *Acyrthosiphon pisum* when presented alone, strongly reduced

155   when *Aphis fabae* is added in the possible choices, suggests that ants might adapt their response depending

156   on available resources.

157   Foraging behavior of ant foragers is influenced by the colony needs, depending on various

158   parameters including population, brood presence … (Portha *et al.*, 2004; Buffin *et al.*, 2011; Oliver *et al.*,

159   2012). For example, in laboratory conditions, ant larval presence significantly reduces the growth rate of

160   tended aphid colonies, suggesting that ant colonies balance the flow of two separate resources from aphid

161   colonies, renewable sugars or a protein-rich meal, depending on demand from ant larvae within the nest

162   (Oliver *et al.*, 2012). In this context, ant ability to distantly recognize aphid species and discriminate

163   between potential mutualistic partners, providing both a stable sugar source and potential preys, and non-

164   myrmecophilous species, constituting only preys, would be an advantage.

165   The volatile compounds released by both aphid honeydews and bacterial cultures are qualitatively

166   very close. They originate mostly from bacterial degradation of diverse compounds. Potential origins and

167   biosynthetic pathways of each compounds, as well as their involvement in different ant species

168   communication, have been previously discussed (Fischer *et al.*, 2015a). Although qualitative VOCs

169 profiles detected are very close, even nearly identical for the two tested bacterial cultures, ants still shown

170 a preference for *Aphis fabae*-related samples, suggesting recognition based on ratios between compounds

171 instead of the attractiveness of a single molecule or group of molecules. The impact of compounds ratios

172 on insects communication is a well-known phenomenon which has already been highlighted for numerous

173 insect species, including ant and aphid species (Castracani *et al.*, 2008; Cardé & Millar, 2009; Byers *et al.*,

174 2013; Han *et al.*, 2014).

175 Honeydew VOCs seem to play a role in distant aphid discrimination, and their microbial origins are

176 interesting. Indeed, this signal depends on microbial populations growing in aphid gut and honeydew,

177 which is shaped by various parameters among which microbe's ability to survive in that kind of hostile

178 environment (low oxygen, high osmotic pressure, potential antimicrobial compounds…) and affinity for

179 the host constitute key factors (González-Teuber *et al.*, 2009; Herrera *et al.*, 2010; Álvarez-Pérez *et al.*,

180 2012; Kirzinger & Stavrinides, 2012). Thus, while honeydew composition and potential aphid

181 antimicrobial secretion contribute to select microbial populations and modulate their emissions, they

182 constitute an aphid-presence signal mostly independent from the aphid itself, which is thus less likely to be

183 altered by the aphid (mimicry, dissimulation…). It constitutes a reliable "honest" cue for ant foragers.

184 The data presented here indicate that, in addition to attraction towards potential food sources,

185 honeydew microbial volatile compounds enable ant foragers to distantly discriminate between aphid

186 species. This underlines once more the key role of honeydew in ant–aphid interactions, adding distant

187 discrimination in its already multiple known effects on this mutualism. Moreover, these results emphasize

188 the importance of investigating the presence and potential effects of microbes in insect symbioses.

193 **Disclosure**

194 The authors declare no conflicts of interest, or specific financial interests, relationships or affiliations.

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255   **Fig. 1** Behavioral response of *L. niger* to VOCs related to two different aphid species. Five preference tests

256   of *L. niger* foragers in a two way-olfactometer presented with VOCs originating from (1) and (3) *Aphis*

257   *fabae* infested plants ($n = 100$), (2) *Acyrthosiphon pisum* infested plants ($n = 100$), (4) *Aphis fabae*

258   honeydew ($n = 60$), (5) 863 medium inoculated with *S. xylosus* ($n = 60$). Controls for these five treatments

259   were (1) and (2) healthy plants, (3) *Acyrthosiphon pisum* infested plants, (4) *Acyrthosiphon pisum*

260   honeydew, and (5) 863 medium inoculated with *S. sciuri*. (A) Ants choice between sample and control

261   branches as their final destination in the olfactometer (%). (B) Linear speed (cm/s, $X \pm SEM$) of ants in

262   each olfactometer branch. * and ** indicate significant differences from the control at $P < 0.05$ and $P <$

263   0.01, respectively.

264 **Table 1** Summary of samples and controls used in the behavioral assays.

Sample	Control	Number of tested ants
Aphis fabae infested plant	Healthy plant	100
Acyrthosiphon pisum infested plant	Healthy plant	100
Aphis fabae infested plant	*Acyrthosiphon pisum* infested plant	100
Aphis fabae honeydew, collected on wet substrate	*Acyrthosiphon pisum* honeydew, collected on wet substrate	60
Staphylococcus xylosus-inoculated 863 medium	*Staphylococcus sciuri*-inoculated 863 medium	60

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Table 2| Volatile organic compounds (VOCs) found in aphid-secreted honeydew and bacteria-inoculated medium				
Retention time (min)	VOC	A.fabae honeydew[†]	S. xylosus-inoculated 863 liquid medium[†]	S. sciuri-inoculated 863 liquid medium	A. pisum honeydew[‡]
1.65	Propanone	0.85±0.14	0.98±0.02	3.65±2.53	9.25 ± 2.99
1.78	Methyl acetate	6.75±3.83			
2.05	2,3-Butanedione	0.45±0.17	0.27±0.03	0.15±0.03	2.31 ± 1.26
2.22	Ethyl acetate	19.95±15.11			
2.39	2-Methylpropanol	0.67±0.25			
2.61	3-Methylbutanal		1.54±0.32	0.32±0.01	14.01 ± 3.24
2.71	2-Methylbutanal		1.91±0.02	0.57±0.01	12.92 ± 1.33
2.97	Ethanoic acid	1.47±0.82	46.36±0.73	15.73±1.02	
3.50	1-Methylethyl acetate	0.10±0.03			
3.57	3-Hydroxy-2-butanone	0.05±0.05			0.78 ± 0.24
3.70	3-Methyl-3-buten-1-ol	0.28±0.13	0.05±0.01	0.03±0.00	0.89 ± 0.39
3.80	3-Methyl-1-butanol	5.40±3.41	16.45±1.03	37.93±3.14	12.32 ± 5.58
3.87	2-Methyl-1-butanol	1.73±0.35	4.43±0.12	3.44±0.13	
4.52	2-Methylpropanoic acid		1.20±0.04	1.67±0.01	
4.57	2-Methylpropyl acetate	0.38±0.20			
5.51	Butanoic acid	0.05±0.03	5.43±0.17	1.85±0.22	6.24 ± 3.45
7.06	3-Methylbutanoic acid	1.93±1.13	7.88±0.10	22.30±0.65	4.56 ± 0.45
7.18	2-Methylbutanoic acid	0.99±0.70	0.93±0.00	5.79±0.39	6.73 ± 5.55
7.28	3-Methyl-1-butyl acetate	1.03±0.44	0.18±0.02	0.45±0.10	
7.36	2-Methyl-1-butyl acetate	0.46±0.15	< 0.01	< 0.01	
8.32	2,5-Dimethylpyrazine		1.41±0.03	0.34±0.01	0.31 ± 0.16
9.83	Benzaldehyde	0.14±0.07	5.18±0.19	3.15±0.14	
10.58	6-Methyl-5-heptene-2-one		4.00±0.49	0.58±0.09	
12.04	2-ethyl-1-hexanol	0.17±0.02			
12.42	Benzeneacetaldehyde	0.40±0.13	0.63±0.10	0.19±0.02	
14.18	Linalool		0.37±0.02		
14.56	Benzeneethanol	53.12±16.40	0.82±0.15	1.85±0.14	1.73 ± 0.50
17.81	Phenylethyl acetate	3.65±1.89			
	2-Methyl-2-buten-1-ol				14.41 ± 1.39
	3-Methyl-2-butenal				10.73 ± 2.71
	Limonene				2.81 ± 0.17

Relative proportions (%±s.e.m.; honeydew: n=4 ; cultures: n=3) of the volatile compounds collected by solid-phase microextraction and analyzed by gas chromatography-mass spectrometry
[†] data from Fischer *et al.* (2015a) ; [‡] data from Leroy *et al.* (2011)

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