BACTERIA MAY CONTRIBUTE TO DISTANT SPECIES RECOGNITION IN ANT-APHID MUTUALISTIC RELATIONSHIPS

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Abstract

Mutualistic interactions between ant and aphid species have been the subject of considerable historical and contemporary investigations, the primary benefits being cleaning and protection for the aphids and carbohydrate-rich honeydew for the ants. Questions remained, however, as to the volatile semiochemical factor influencing this relationship. A recent study highlighted the role of bacterial honeydew volatile compounds in ant attraction. Here, ant's ability to distantly discriminate two aphid species was investigated based on bacterial honeydew semiochemicals emissions using a two-way olfactometer. Both the mutualistic black bean aphid (*Aphis fabae* L.) and the non-myrmecophilous pea aphid (*Acyrthosiphon pisum* Harris) were found to be attractive for the black garden ant (*Lasius niger* L.). The level of attraction was similar in both assays (control versus one of the aphid species). However, when given a choice between these two aphid species, ants showed a significant preference for *Aphis fabae*. Honeydew volatiles, mostly from bacterial origins, are known to be a key element in ant attraction. Using the same olfactometry protocol, the relative attractiveness of volatiles emitted by honeydews collected from each aphid species and by bacteria isolated from each honeydew was in investigated. Again, ants significantly preferred volatiles released by *Aphis fabae* honeydew and bacteria. This information suggests that microbial honeydew volatiles enable ants to distantly discriminate aphid species. These results emphasize the importance of investigating the presence and potential effects of microbes in insect symbioses.

Keywords: 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 and 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 and 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 and 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 a climate-controlled room (16 h light-8h dark photoperiod; 20 ± 2°C), black bean aphids, *Aphis fabae*, and pea aphids, *Acyrthosiphon pisum*, were reared for several generations on broad beans, *Vicia faba*, cultivated on a 1/1 mix of perlite and vermiculite substrate. Plants used in experiments were about 15 cm high. All substrates used in behavioral assays were previously sterilized. *Lasius niger* colonies were collected in Gembloux (Belgium), and kept under the same climatic conditions, but in separate chambers. Nests were placed in plastic containers coated with polytetrafluoroethylene (Fluon®,Whitford, U.K.) to prevent escape. Test tubes covered with a red transparent foil were used as laboratory rearing nests; a water and aqueous brown sugar solution (342 g/L) was provided *ad libitum*, and dead insects (fruit flies and mealworms) were provided weekly as an additional food source. All nests used in the bioassays were comprised of a queen, brood, and a minimum of 500 foragers.

Choice tests with two-way olfactometer

The level of attraction on ants was assessed for different samples by using a protocol previously successfully used for similar tests with ants (Fischer et al. 2015a). The two-way olfactometer consisted of a Y-shaped glass tube (diameter: 1.5 cm, entrance length: 20 cm; length of each arm: 30 cm). Samples and controls were placed in 4 L glass jars. Filtered air was forced into the jars at 200 mL/min and delivered to the olfactometer's branches via Teflon® tubing.

Ants were starved for three days prior to an assay. The olfactometer's entrance was placed in the ant's rearing container, allowing the ants to enter the system. Only one ant worker at a time was allowed in the olfactometer; the entrance was closed to prevent additional ants from entering after the first ant went in. The choice, *i.e.* the branch selected to reach the end of the olfactometer arm, and the average linear speed while passing through the branch were recorded for each tested ant. The test ended when the ant reached a point located 25 cm from where the two arms branched ("choice point"). The linear speed was calculated by measuring the time spent to pass through an olfactometer branch, and was expressed in cm/s. The attractiveness of

a sample was expressed by the relative number of ants (%) choosing the particular sample side as their final choice.

All assays were conducted at 20°C in a dark-walled chamber presenting no visual cues that may influence ant choices. To prevent ants from laying trails, they were never allowed to reach the actual samples. Moreover, in order to palliate any potential bias induced by the environment or by any marking of the substrate by exploring ants, sample and control sides were switched every five ants. The olfactometer was completely cleaned every 20 ants.

Several sample-control couples were tested following this protocol (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 (a pot holding 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 plant 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 desiccation, while control was *Acyrthosiphon pisum* honeydew (collected the same way). The relative attractiveness of two taxonomically close honeydew bacteria known to be involved in aphid interactions with other insect species was also tested. The first one, *Staphylococcus xylosus*, is found in *Aphis fabae* gut and honeydew and is known to produce mVOCs attractive for *L. niger* (Fischer et al. 2015a); the second one, *Staphylococcus sciuri*, is found in *Acyrthosiphon pisum* gut and honeydew and is known to attract aphid enemies like the hoverfly *Episyrphus balteatus* (De Geer)(Leroy et al. 2011). Both these bacteria were found only in one of the two studied aphid species. Sample and control consisted in 60mL of 868 culture medium (20 g of glucose and 10 g of both yeast extract and casein peptone per liter of distilled water) inoculated with *S. xylosus* and *S. sciuri* respectively and incubated for 2 days at 20°C.

The significance of the ant preferences was assessed with binomial tests. The significances of differences of attractiveness observed between tests were assessed by χ^2 test. Average linear speeds of ants in the two branches were compared, assay by assay, with t-tests. Differences were considered significant at $P < 0.05$. Statistical analyses were conducted using Minitab 15.1 (State College, Pennsylvania, USA).

Characterization of honeydew's and bacterial VOCs emission profiles

The volatile organic compounds (VOCs) of honeydew were sampled using 250 µL glass inserts and solid-phase micro-extraction (SPME, 10 mm fiber with a 50/30 µm carboxen-divinylbenzene-polydimethylsiloxane coating – Supelco). For each analysis, the same fibers were conditioned at 250°C for 1 h in a split-splitless injector before sampling. *Aphis fabae* honeydew, dripping from aphid-infested *V. faba* plants, was immediately collected on sterilized plastic foil under sterile conditions using 2 µL microcapillaries. For each analysis, 10 µL of honeydew were sampled at 25°C for 24 h. Empty inserts were used for controls. Four replicates were conducted.

In order to determine the bacteria contribution in the emission of honeydew volatile compounds, analyses were also performed on bacterial cultures using a protocol proposed by Leroy *et al.* (Leroy et al. 2011). *Staphylococcus xylosus* and *S. sciuri* were grown in 863 liquid culture medium at 20°C for 48 h. A 1 mL aliquot of the culture medium was placed in a 20 mL SPME vial. Control samples were 1 mL aliquots of sterile medium placed in similar vials. Volatile compounds were sampled for 3 h at 25°C with the same SPME fibers than previously. Three replicates were conducted.

Desorption and GC-MS analyses were conducted on a Thermo Trace GC coupled with a Trace MS (Thermo Electron Corporation, Interscience, Louvain-la-Neuve, Belgium) equipped with an Optima 5 Accent (Macherey-Nagel, Düren, Germany) capillary column (30 m x 0.25 mm I.D.; 0.25 µm film thickness) under the following conditions: splitless injector at 230°C; vector gas was helium, at a 1 mL/min flow rate; oven temperature program: 40°C held for 2 min, raised at 5°C/min to 150°C, then at 10°C/min to 210°C, and finally at 120°C/min to 280°C held for 1 min; transfer line was at 250°C. Mass spectra were acquired at 70eV; the scanned mass ranged from m/z 35 to 450 amu; results were analyzed using the NIST05 and Wiley8 libraries. Identifications were confirmed either by comparison with retention times of synthetic standards (Sigma-Aldrich, Steinheim, Germany) or by determination of retention indices. The relative proportions of each of the identified components are expressed in percent of total sample-related peak area.

Results

VOCs and ant attraction

Plants infested by myrmecophilous and non-myrmecophilous aphid species significantly attracted ants when tested against healthy plants; respectively 68 % and 65 % of the tested foragers were attracted towards *Aphis fabae* and *Acyrthosiphon pisum* infested plants (binomial tests, $n = 100$, $p < 0.001$ and $p = 0.004$ respectively). These attraction percentage towards these two aphid species, tested separately against non-infested plants, are not statistically different (χ^2 test, $p = 0.520$).

However, when plants infested with these two aphid species were tested against each other, plants infested by *Aphis fabae* attracted significantly more ant foragers (61 %, binomial test, $n = 100$, $p = 0.035$). The same tendency is also observed for honeydews of these two species, and for cultures of bacteria found in these honeydews. When presented with honeydews of the two species, 65% of ant foragers chose the *Aphis fabae* honeydew branch (binomial test, $n = 60$, $p = 0.027$). Facing a choice between cultures of *S. xylosus* and *S. sciuri*, 65% of ant foragers selected the *S. xylosus* branch (binomial test, $n = 60$, $p = 0.027$). These attraction levels towards *Aphis fabae*, its honeydew, and a bacterium from its honeydew, tested against *Acyrthosiphon pisum*, its honeydew, and a bacterium from its honeydew respectively, are statistically not different from the attractiveness observed in the first assay (χ^2 tests, $p = 0.133$, $p = 0.618$ and $p = 0.618$ respectively).

No significant differences in speed were observed between branches of the olfactometer in any assay (t-tests, equality of variances verified, all $p > 0.099$).

Characterization of honeydew's and bacterial VOCs emission profiles

Twenty-eight volatile chemicals, including esters, alcohols, acids, aldehydes and ketones, were identified from honeydew and bacterial cultures (Table 2).

Among the 28 compounds observed in honeydews, 9 were observed in honeydews of both species. However, the relative amount of these compounds may vary strongly between species. For example, benzenethanol constitutes 53% of total peak area for *Aphis fabae* honeydew VOC, and only 1.7% of total peak area for *Acyrthosiphon pisum*.

Sixteen of the 28 compounds identified from honeydews were also observed in *Staphylococcus* cultures. The mVOCs emitted by both *Staphylococcus* species are qualitatively nearly identical, the only difference being linalool solely observed for *S. xylosus*. However, strong quantitative differences are also observed (Table 2).

Discussion

In order to establish and maintain a mutualistic relationship, ant foragers have first to find aphid colonies and asses their suitability as partners based on various parameters including aphid morphology and behavior, honeydew quality and quantity …. However, in order to asses these criteria, ant foragers must already have found the aphid colony. Previous studies already showed ants ability to orient their search for aphid colonies using volatile cues (Verheggen et al. 2012; Fischer et al. 2015a). The data presented here represent the first evidence of distant aphid species discrimination by ants that is driven by aphid honeydew and mediated by the associated microflora through release of VOCs, in laboratory conditions.

Using a methodology previously applied to highlight honeydew mVOCs effect on ant behavior (Fischer et al. 2015a), we observed similar ant attraction toward both myrmecophilous and non-myrmecophilous aphid species when the aphids were presented alone. However, when given a choice between the two aphid species, ant showed a significant preference for the myrmecophilous *Aphis fabae* with an attraction level similar to the attraction observed for this aphid presented alone. Moreover, this tendency is supported by the relative attractiveness of honeydews and bacterial cultures issued of these two aphid species, confirming both ants ability to distantly discriminate between two aphid species, and honeydew VOCs involvement in that discrimination. Even though mVOCs emitted from honeydew have been reported to attract aphid enemies and potential ant partners (Leroy et al. 2011; Fischer et al. 2015a), this is the first evidence of ant ability to use such volatile cues to distantly discriminate two aphid species. Furthermore, the attractiveness shown by *Acyrthosiphon pisum* when presented alone, strongly reduced when *Aphis fabae* is added in the possible choices, suggests that ants might adapt their response depending on available resources.

Foraging behavior of ant foragers is influenced by the colony needs, depending on various parameters including population, brood presence … (Portha et al. 2004; Buffin et al. 2011; Oliver et al. 2012). For example, in laboratory conditions, ant larval presence significantly reduces the

growth rate of tended aphid colonies, suggesting that ant colonies balance the flow of two separate resources from aphid colonies, renewable sugars or a protein-rich meal, depending on demand from ant larvae within the nest (Oliver et al. 2012). In this context, ant ability to distantly recognize aphid species and discriminate between potential mutualistic partners, providing both a stable sugar source and potential preys, and non-myrmecophilous species, constituting only preys, would be an advantage.

The volatile compounds released by both aphid honeydews and bacterial cultures are qualitatively very close. They originate mostly from bacterial degradation of diverse compounds. Potential origins and biosynthetic pathways of each compounds, as well as their involvement in different ant species communication, have been previously discussed (Fischer et al. 2015a). Although qualitative VOCs profiles detected are very close, even nearly identical for the two tested bacterial cultures, ants still shown a preference for *Aphis fabae*-related samples, suggesting recognition based on ratios between compounds instead of the attractiveness of a single molecule or group of molecules. The impact of compounds ratios on insects communication is a well-known phenomenon which has already been highlighted for numerous insect species, including ant and aphid species (Castracani et al. 2008; Cardé and Millar 2009; Byers et al. 2013; Han et al. 2014).

Honeydew VOCs seem to play a role in distant aphid discrimination, and their microbial origins are interesting. Indeed, this signal depends on microbial populations growing in aphid gut and honeydew, which is shaped by various parameters among which microbe's ability to survive in that kind of hostile environment (low oxygen, high osmotic pressure, potential antimicrobial compounds…) and affinity for the host constitute key factors (González-Teuber et al. 2009; Herrera et al. 2010; Álvarez-Pérez et al. 2012; Kirzinger and Stavrinides 2012). Thus, while honeydew composition and potential aphid antimicrobial secretion contribute to select microbial populations and modulate their emissions, they constitute an aphid-presence signal mostly independent from the aphid itself, which is thus less likely to be altered by the aphid (mimicry, dissimulation…). It constitutes a reliable "honest" cue for ant foragers.

The data presented here indicate that, in addition to attraction towards potential food sources, honeydew microbial volatile compounds enable ant foragers to distantly discriminate between aphid species. This underlines once more the key role of honeydew in ant-aphid interactions, adding distant discrimination in its already multiple known effects on this

mutualism. Moreover, these results emphasize the importance of investigating the presence and potential effects of microbes in insect symbioses.

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Disclosure

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

References

Álvarez-Pérez, S., Herrera, C.M., de Vega, C. (2012) Zooming-in on floral nectar: A first exploration of nectar-associated bacteria in wild plant communities. *FEMS Microbiol Ecol*, 80, 591–602.

Bristow C.M. (1991) Why are so few aphids ant-tended? *Ant/Plant Interactions* (eds. D.F. Cutler and C. Huxley), pp 104–119, Oxford University Press, Oxford.

Buffin, A., Mailleux, A.C., Detrain, C., Deneubourg, J.L. (2011) Trophallaxis in Lasius niger: A variable frequency and constant duration for three food types. *Insectes Soc*, 58, 177–183.

Byers, J.A., Fefer, D., Levi-Zada, A. (2013) Sex pheromone component ratios and mating isolation among three Lygus plant bug species of North America. *Naturwissenschaften,* 100, 1115–1123.

Cardé, R.T. and Millar, J.G. (2009) Pheromones. *Encyclopedia of Insects* (eds. V.H. Rush & R.T. Carde), pp 766–772, Academic Press, San Diego.

Castracani, C., Tamarri, V., Grasso, D.A., et al (2008) Chemical communication in mating behaviour of the slave-making ant Polyergus rufescens (Hymenoptera, Formicidae): 3-Ethyl-4-methylpentanol as a critical component of the queen sex pheromone. *Insectes Soc,* 55, 137–143.

Dixon, A.F.G. (1985) *Aphid ecology*. Blackie, Glasgow

El-Ziady, S. and Kennedy, J.S. (1956) Beneficial effects of the common garden ant, Lasius niger L., on the black bean aphid, Aphis fabae Scopoli. *Proc R Ent Soc Lond,* 31:5.

Fischer, C.Y., Lognay, G.C., Detrain, C., et al (2015a) Bacteria may enhance species association in an ant–aphid mutualistic relationship. *Chemoecology,* 1–10.

Fischer, C.Y., Vanderplanck, M., Lognay, G.C., et al (2015b) Do aphids actively search for ant partners? *Insect Sci,* 22, 283–288.

Francis, F., Vandermoten, S., Verheggen, F., et al (2005) Is the (E)-β-farnesene only volatile terpenoid in aphids? *J Appl Entomol*, 129, 6–11.

González-Teuber, M., Eilmus, S., Muck, A., et al (2009) Pathogenesis-related proteins protect extrafloral nectar from microbial infestation. *Plant J,* 58, 464–473.

Guénard, B., Lucas, É., Francoeur, A. (2007) Mutualisme fourmis pucerons et guilde aphidiphage associée : le cas de la prédation furtive. *Université du Québec à Montréal*

Han, B.-Y., Wang, M.-X., Zheng, Y.-C., et al (2014) Sex pheromone of the tea aphid, Toxoptera aurantii (Boyer de Fonscolombe) (Hemiptera: Aphididae). *Chemoecology*, 24, 179–187.

Herrera, C.M., Canto, A., Pozo, M.I., Bazaga, P. (2010) Inhospitable sweetness: Nectar filtering of pollinator-borne inocula leads to impoverished, phylogenetically clustered yeast communities. *Proc R Soc B Biol Sci*, 277, 747–754.

Holman, J. (2009) The Aphids and their Host Plants. *Host Plant Catalog of Aphids*. pp 7–651Springer Netherlands.

Kirzinger, M.W.B. and Stavrinides, J. (2012) Host specificity determinants as a genetic continuum. *Trends Microbiol,* 20, 88–93.

Leroy, P.D., Sabri, A., Heuskin, S., et al (2011) Microorganisms from aphid honeydew attract and enhance the efficacy of natural enemies. *Nat Commun,* 2:7.

Oliver, T.H., Leather, S.R., Cook, J.M. (2012) Ant larval demand reduces aphid colony growth rates in an ant-aphid interaction. *Insects* 3, 120–130.

Portha, S., Deneubourg, J.L., Detrain, C. (2004) How food type and brood influence foraging decisions of Lasius niger scouts. *Anim Behav,* 68, 115–122.

Stadler, B. (1997) The relative importance of host plants, natural enemies and ants in the evolution of life-history characters in aphids. *Vertical food web interactions : evolutionary patterns and driving forces* (eds. K. Dettner, G. Bauer , W. Völkl), pp 241–256, Springer - Verlag.

Stadler, B. and Dixon, A.F.G. (2005) Ecology and evolution of aphid-ant interactions. *Annu Rev Ecol Evol Syst* 36, 345–372.

Stadler, B. and Dixon, A.F.G. (1999) Ant attendance in aphids: why different degrees of myrmecophily? *Ecol Entomol*, 24, 363–369.

Verheggen, F.J., Diez, L., Sablon, L., et al (2012) Aphid Alarm Pheromone as a Cue for Ants to Locate Aphid Partners. *PLoS One* , 7

Wilson, E.O. (1955) A Monographic Revision of the Ant Genus Lasius. *Bull. Mus. Comp. Zool.*, 113, 59–77.

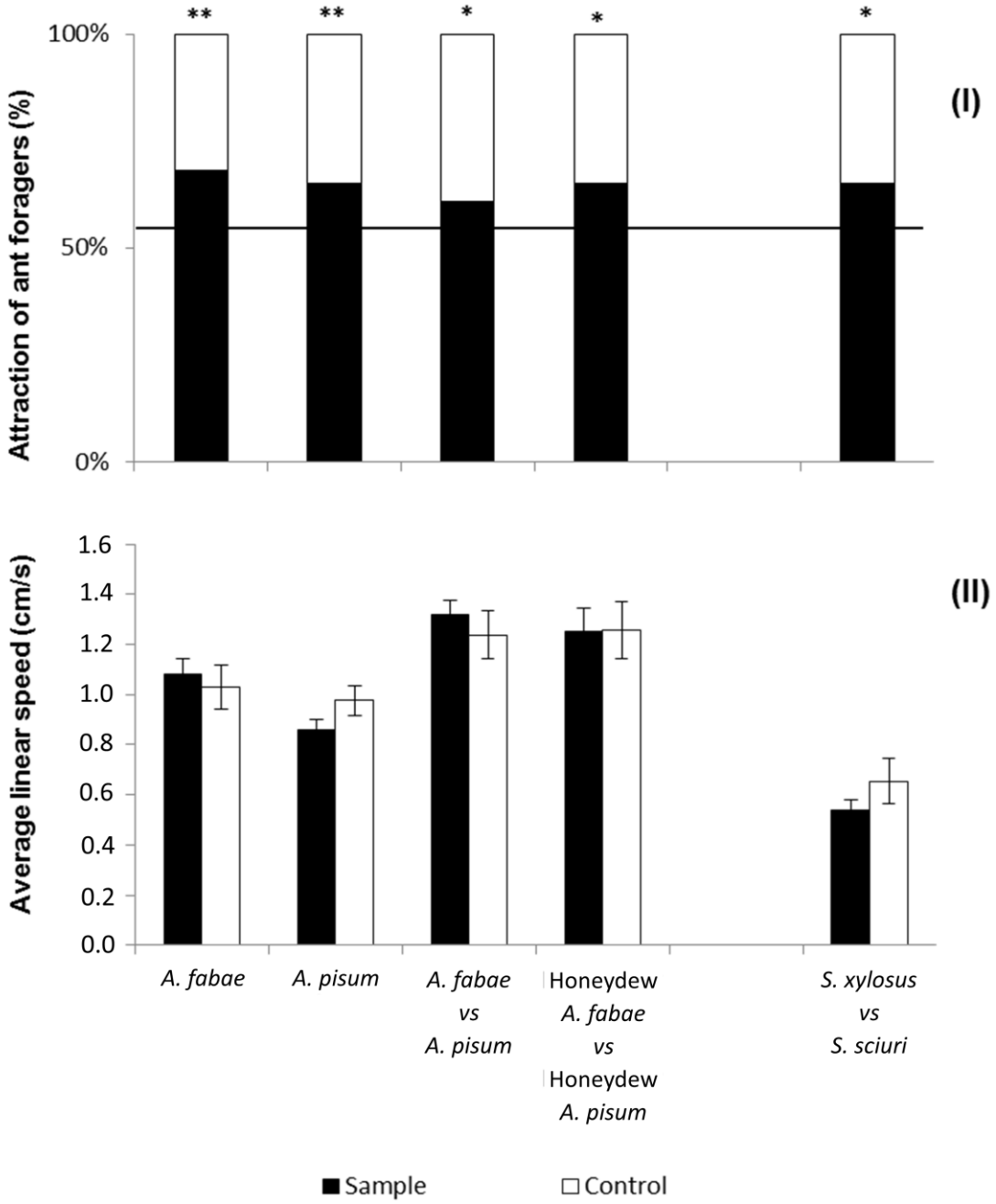

Fig. 1 - Behavioral response of *L. niger* to VOCs related to two different aphid species

Five preference tests of *L. niger* foragers in a two way-olfactometer presented with VOCs originating from (1) and (3) *Aphis fabae* infested plants (n=100), (2) *Acyrthosiphon pisum* infested plants (n=100), (4) *Aphis fabae* honeydew (n=60), (5) 863 medium inoculated with *S. xylosus* (n=60). Controls for these five treatments were (1) and (2) healthy plants, (3) *Acyrthosiphon pisum* infested plants, (4)) *Acyrthosiphon pisum* honeydew, and (5) 863 medium inoculated with *S. sciuri*. **(I)** Ants choice between sample and control branches as their final destination in the olfactometer (%). **(II)** Linear speed (cm/s, X ± SEM) of ants in each olfactometer branch. * and ** indicate significant differences from the control at $P<0.05$ and $P<0.01$, respectively.

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

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 Leroy *et al.* (2011)