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Response of the Aphid Parasitoid *Aphidius funebris* to Volatiles from Undamaged and Aphid-infested *Centaurea nigra*

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Abstract Two issues have hindered the understanding of the ecology and evolution of volatile-mediated tritrophic interactions: few studies have addressed noncrop systems; and few statistical techniques have been applied that are suitable for the analysis of complex volatile blends. In this paper, we addressed both of these issues by studying the noncrop system involving the plant *Centaurea nigra*, the specialist aphid *Uroleucon jaceae*, and the parasitoid *Aphidius funebris*. In a Y-tube olfactometer, *A. funebris* was attracted to the odor from undamaged *C. nigra*, but preferred the plant–host complex (PHC) after 3 d of feeding by 200 *U. jaceae* over the undamaged plant, but not after three or 5 d of feeding by 50 *U. jaceae*. When aphids were removed, the initial preference for the damaged plant remained, but the final preference was not greater than for the undamaged plant. No qualitative differences were detected between the headspaces of *C. nigra* and the *C. nigra–U. jaceae* PHC. For quantitative analysis, we used a compositional approach, which treats each compound produced as part of a blend, and not as a compound released in isolation, thus allowing analysis of the relative contribution of each compound to the blend as a whole. With this approach, subtle increases and decreases of some green leaf volatiles and monoterpenoids on the third day of aphid infestation were detected. Mechanically damaged *C. nigra* had a volatile profile that differed from undamaged *C. nigra* and the PHC. One and 10 ng of (*Z*)-3-hexenyl acetate, and 10 or 100 ng of 6-methyl-5-hepten-2-one were attractive to the parasitoid when placed in solution on filter paper. *A. funebris* appears to be using a combination of chemical cues to locate host-infested plants.

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Introduction

Many parasitoids respond to plant odors (Turlings et al., 1991; Powell et al., 1998), and many can also distinguish undamaged from host-infested or previously host-damaged plants (Guerrieri et al., 1999; Lo Pinto et al., 2004; Moraes et al., 2005). Changes in volatile profiles that lead to increased parasitoid attraction have been characterized in many plant species (De Moraes et al., 1998; Röse et al., 1998; Bukovinszky et al., 2005), and it is apparent that volatiles released from storage immediately after damage, as well as those resulting from induction of biosynthetic pathways after herbivory, are important for attraction of parasitoids (Mattiacci et al., 2001; Hoballah and Turlings, 2005). Some responses are host-herbivore specific (Du et al., 1996; De Moraes et al., 1998; Shiojiri et al., 2001; Moraes et al., 2005), and attempts to characterize individual compounds that confer specificity to parasitoid responses have revealed 6-methyl-5-hepten-2-one as important as a specific indicator of the presence of *Acyrthosiphon pisum* for the parasitoid *Aphidius ervi* (Du et al., 1998), and the green-leaf volatiles (*E*)-2-hexenal and (*Z*)-3-hexenyl acetate for the parasitoid *Cotesia glomerata* (Shiojiri et al., 2006). However, this specificity is not universal (e.g., Shiojiri et al., 2001; van Poecke et al., 2003).

Although the effects described above have been found for a wide range of parasitoids of pest insects, there is little information on the occurrence of plant volatile-mediated effects in noncrop systems. In noncrop systems, plants have not been modified by artificial selection, which could have had effects on plant metabolism that affect their volatile profiles. There is large variability in volatile production among closely related species in the maize genus *Zea* (Gouinguené et al., 2001) as well as among maize (*Z. mays*) cultivars (Turlings et al., 1998), and some domesticated species have less attractive blends than wild relatives (Bukovinszky et al., 2005). This indicates that artificial selection has led to changes in the metabolic pathways involved in the production of plant volatiles, and genetic uniformity in crops is likely to result in the loss of variability in volatile production. Thus, the identity and amount of volatiles released is likely to vary less among individuals in crop plants compared to noncrop plants. Crops come from a reduced subset of plants, mainly fast growing annual ruderals from early successional habitats (Feeny, 1976), so generalizations drawn from these studies might not always apply to natural ecosystems, for which noncrop plants need to be studied.

The aim of this study was: (a) to elucidate the olfactory responses of the parasitoid *Aphidius funebris* Mackauer (Hymenoptera, Braconidae) to the plant *Centaurea nigra* (L.) (Asteraceae, Cardueae) when it is undamaged, and when it is infested by the aphid *Uroleucon jaceae* (L.) (Aphididae, Aphidinae); (b) to determine the changes in *C. nigra* volatile chemistry that occur after attack by *U. jaceae* that might lead to increased attraction of *A. funebris* by using a compositional approach; and (c) to determine the importance of (*Z*)-3-hexenyl acetate and 6-methyl-5-hepten-2-one as attractants of *A. funebris* because these compounds have electrophysiological activity in *A. ervi* (Du et al., 1998). This system provides a good contrast to crop systems studied because *C. nigra* is a perennial plant, which spends most of the year as a rosette and then bolts for flowering in early summer, and the aphid *U. jaceae* is highly specialized on *Centaurea* species, unlike most pest aphids

of herbaceous crops, which tend to be more polyphagous than their nonpest relatives (Blackman and Eastop, 2000). *A. funebris* is a specialist on *Uroleucon* spp., and attacks its hosts from outside the colony (Völkl et al., 1995), with greater success when attacking instars L2, L3, and L4, as compared to L1 and adults (Weisser, 1995).

Methods and Materials

Plants *Centaurea nigra* seeds were purchased from Herbiseed (Twyford, UK) and Emorsgate Wild Seeds (Norfolk, UK). They were germinated on damp paper, and plants were grown in a glasshouse under a 16:8 light:dark (L:D) regime and were aged between 35 and 45 d old, but of similar size (6–8 leaf stage) when used in experiments. None of the plants used had flowered.

Insect Rearing and Preparation for Bioassays *Uroleucon jaceae* were collected from the field at Rothamsted Farm, Herts, UK, between May and July 2002, and reared at 18°C and 16:8 L:D on *C. nigra*.

Aphidius funebris cultures were set up on *U. jaceae* feeding on *C. nigra* from mummies collected during June 2002 from *U. jaceae* (also feeding on *C. nigra*) in fields on Rothamsted Farm, Herts, UK, at 18°C and 16:8 L:D. Mummies were removed from culture plants and placed in an emergence cage. On the day of the bioassay, adult females were collected in glass vials and placed in the bioassay room to acclimatize for 1 hr. All females were less than 3 d old and were assumed to have mated before collection.

Y-tube Olfactometer Bioassays A glass Y-tube of 1 cm internal diameter, a 7 cm "trunk" and 5 cm arms was used as the bioassay arena. A pump provided an air stream that was passed through an activated charcoal filter and a flow meter before being split in two. Each stream was passed through a 5-l glass vessel containing an odor source and then through a flow meter set at 400 ml/min before entering an arm of the Y-tube. All connections between the parts described were 1 mm ID Teflon (PTFE) tubing. Twenty-four hours before each bioassay, the Y-tube, glass vessels, Teflon tubing, and glass fittings were all washed and rinsed with acetone and placed in an oven at 160°C.

All bioassays were carried out at 20°C on a white Formica bench under artificial lighting. On each arm of the Y-tube, a *choice line* was drawn halfway up the arm prior to the start of the bioassay. During the bioassay, each parasitoid was introduced individually into the base of the Y-tube. A first choice was recorded if the parasitoid crossed one of the choice lines drawn on the arms. A final choice was recorded if either the parasitoid spent more than 15 sec in motion beyond the choice line without crossing back out again, or if the parasitoid was not continuously in motion, if it spent 1 min beyond the choice line without crossing back out. To control for directional bias the arms of the Y-tube through which the odor sources were presented were swapped after every three parasitoids tested. Each parasitoid was used only once.

For testing the response of *A. funebris* to undamaged *C. nigra*, a single potted plant was taken directly from the glasshouse and tested against a pot filled with soil only. To test the response of *A. funebris* to the plant–host complex (PHC) relative to an undamaged plant, a set number of aphids of mixed ages (initial aphid density) were placed on a plant 3 d before the bioassay. Both the infested plant and the undamaged control plant were individually placed in porous Cryovac bread packing bags, which prevent aphid escape but do not accumulate humidity and allow air exchange. They were left in an 18°C controlled temperature room at

16:8 L:D. The responses of *A. funebris* to the following choices were compared: (1) PHC (initial aphid density of 50) vs. undamaged plant; (2) PHC (initial aphid density of 200) vs. undamaged plant. Additionally, the response to the PHC (initial aphid density of 50) after 5 d of aphid feeding vs. an undamaged plant was tested. Finally, to confirm that any responses to the PHC were due to the plant and not to semiochemicals from the aphid, the response of *A. funebris* to aphid-damaged plants (ADP) was tested. To do this, *C. nigra* plants were treated as described above for (2), and immediately before the bioassay was started all aphids were removed, and every leaf was swabbed with warm distilled water to remove aphid traces. The plant was then rinsed with distilled water, and any excess water remaining was removed with clean tissue paper. The same process was carried out for the undamaged plant to control for any effect of the cleaning process. Each of the bioassays described above was carried out on several (2–6) different days. On each day, a single plant or combination of plants was used, and between 10 and 40 parasitoids were tested on any given day.

The dose responses of *A. funebris* to (*Z*)-3-hexenyl acetate and 6-methyl-5-hepten-2-one were measured in the Y-tube olfactometer because the related parasitoid *A. ervi* had previously shown electrophysiological responses to these compounds (Du et al., 1998). Solutions were prepared from synthetic commercial (*Z*)-3-hexenyl acetate (Aldrich, Milwaukee, WI, USA) and 6-methyl-5-hepten-2-one (Aldrich, St. Louis, MO, USA). For the bioassays, either 10 μl of the bioassay solution or 10 μl hexane (control) were placed on a 7-cm (diameter) circular piece of clean filter paper at the bottom of each of two 500 ml glass vessels, which were left for 1 min before starting the bioassay to allow the solvent to evaporate. Throughout the bioassay, the arms delivering the test and control odors were alternated after every three parasitoids tested. The amounts of (*Z*)-3-hexenyl acetate tested were 0.1, 1, 10, and 100 ng. For 6-methyl-5-hepten-2-one the amounts tested were 1, 10, 100, and 1,000 ng.

Volatile Collection by Air Entrainment Super Q 80/100 mesh (Alltech, PA, USA) was used to adsorb the volatiles and was placed in small glass tubes secured with glass wool. The adsorbent was conditioned with hexane and diethyl ether, and heated at 135°C with a flow of nitrogen for 24 hr. Between uses, the adsorbent tubes were rinsed with 1 ml ether and heated (135°C) with a nitrogen flow for at least 3 hr.

Plant volatiles were collected by placing the aerial parts of the plant in a 2.2 l glass vessel, closed at the base with two metal plates. Because the plates do not seal the vessel, the soil in which the plant was growing was covered with aluminium foil to minimize volatiles from the soil seeping into the air entrainment vessel. The adsorbent tube was secured in one of the apertures at the top of the vessel, and through another aperture a Teflon tube carrying charcoal-filtered air was inserted into the vessel. Air was pumped into the vessels at a rate of 1.2 l/min and sucked through the adsorbent tube at 1 l/min for 24 hr. Before every air entrainment, all glass, metal and nondisposable Teflon material was washed, rinsed with acetone, and heated at 160°C for 24 hr. The adsorbed compounds were eluted with 500 μl of redistilled ether, and each sample was concentrated to 100 μl under a gentle flow of filtered nitrogen gas. Blanks were regularly carried out to test the purified air for impurities.

Air Entrainments Immediately before the start of the air entrainment, 200 *U. jaceae* of mixed ages were placed on one of the two plants (randomly selected), while the other plant was left undamaged. Fourteen replicate air entrainments were carried out over 5 d, pairing undamaged *C. nigra* with the PHC in a randomized block design. The adsorbent tube was changed every 24 hr to obtain daily profiles (ignoring variation in volatile production due to the light cycle).

Air entrainments with mechanically damaged *C. nigra* were carried out by cutting 1 cm off every leaf tip of a plant to determine the compounds released upon mechanical damage (not to mimic aphid damage). Six paired (undamaged and damaged) air entrainments were carried out for 3 d, with a sample taken every 24 hr.

Compound Identification Volatile samples were injected into a coupled gas chromatography–mass spectrometry (GC–MS) system for identification (Thermo-Finnigan MAT95XP magnetic sector mass spectrometer), equipped with an ion trap analyzer, which was directly coupled to a Finnigan Trace 2000 GC with a non-polar 50 m HP-1 column (methyl siloxane, 0.52 μm film thickness, 250 μm ID, J&W Scientific, Folsom, CA, USA) with helium as carrier gas. Ionization was by electron impact (70 eV, source temperature 200°C). Two microliters were injected for each sample in a temperature ramp program (30°C/5 min, 5°C/min to 250°C/21 min).

Mass spectra for the compounds were compared against a NIST library. A tentative identification was made if the library match was greater than 75% and the KI matched when compared to a database. GC analyses (Hewlett Packard 6980) were carried out by cool-on-column injection of standards and samples in temperature ramps in a polar 25 m DB-Wax column (polyethylene glycol, 0.50 μm film thickness, 320 μm internal diameter, J&W Scientific, Folsom, CA, USA; 4.00 psi, 30°C/0.10 min, 2°C/min to 100°C/0.10 min, 3°C/min to 150°C/0.10 min, 10°C/min to 250°C/8 min), and a nonpolar HP-1 column (dimensions as above; 10.00 psi, 30°C/0.10 min, 5°C/min to 150°C/0.10 min, 10°C/min to 230°C/20 min), with an inlet temperature of 30°C and hydrogen as carrier gas. Detection was by a flame ionization detector (FID) at 250°C. Fragmentation of the compounds from the sample was compared to the fragmentation of standards when available. Peak enhancement coinjection was carried out with authentic standards to confirm the identities of compounds. The sources of authentic compounds were as follows: (*Z*)-3-hexenyl acetate (98+%): Aldrich, Milwaukee, WI, USA; alkane series, hexanal (98%), butyl acetate (99+%), (*E*)-2-hexenal (98%), 6-methyl-5-hepten-2-one (99%), α-pinene (98%), (1*S*)-(−)-β-pinene (99%), 3-carene (90%), (+)-longifolene (98+%): Aldrich, St. Louis, MO, USA; (−)-camphene (85%), sabinene (98.5%), myrcene (90%), (*R*)-(−)-α-phellandrene (50%), (*R*)-(+)-limonene (96%), γ-terpinene (97%), (+,−)-camphor (95%), (+)-cyclosativene, (−)-α-copaene (90%), β-caryophyllene (98.5%), α-humulene (98%): Fluka, Buchs., Switzerland; (*E,E*)-α-farnesene and (*E*)-β-farnesene: provided by J. Aldrich (USDA, Beltsville, MD, USA).

Volatile Quantification Volatiles from undamaged *C. nigra* and the PHC were quantified by using GC analysis with heneicosane (25 ng/μl) as the internal standard (IS). Samples were injected into an HP-1 column as described above. The total amount of each compound released in the 24 hr of each air entrainment was calculated.

Design and Statistical Analyses For the Y-tube bioassay, data analyses were carried out for first and final choices of the parasitoid. The *responsiveness* (*r*) refers to the percentage of parasitoids making a choice for one of the odors in that bioassay and only these parasitoids were used in the analyses.

The first and final choices of all responding insects were summarized into daily 2×2 contingency tables of counts classified by the arm chosen by the parasitoid (left or right) and the side the test odor was presented (left or right arm). The treatment effect on each day is hence represented by the interaction (diagonal) in each table. The counts from all days for first or final choice were then analyzed by using a generalized linear model (GLM) with Poisson errors and logarithm link function. Main effects of day (replicates) were removed

before fitting other terms. The significance of the treatment effect was assessed by comparing the deviance for the interaction term against tabulated χ^2 values with 1 *df*.

Three approaches were used for the analysis of volatile compounds. First, the mean total amounts of volatiles (transformed to natural logarithms) produced in each 24 hr period were compared between plant treatments (either aphid-infected or undamaged) by using a split-plot repeated measures analysis of variance (ANOVA), as implemented in the AREPMEASUREMENTS procedure of GenStat (Payne et al., 2005). Blocks represented groups of entrainments carried out simultaneously, and repeated measures over time were treated as split-plots within each entrainment. Degrees of freedom in the lowest (time) stratum were corrected by using Greenhouse and Geisser's (1959) epsilon statistic to account for patterns of unequal correlation among days (a violation of ANOVA split-plot assumptions). Confidence limits (95%) for means were calculated on the logarithmic scale and back-transformed for presentation.

To assess the changes in different classes of compounds, each of the compounds identified and quantified was allocated to one of five classes: (1) aldehydes, ketones, and esters (AKE); (2) hydrocarbons; (3) monoterpenoids; (4) sesquiterpenoids; and (5) *unknowns*. For each class separately, the total amount of volatiles summed over all allocated compounds was converted to logarithms and analyzed by using repeated measures ANOVA as described above for the 5 d of entrainment. Confidence limits (95%) were calculated on the logarithmic scale and back-transformed for presentation.

Finally, because the volatiles emitted are part of a blend of compounds produced by one plant, and hence are not independent, multivariate log-ratio analyses (Aitchison, 1986) were used to analyze amounts of all compounds simultaneously, both at the individual compound level within each of the five classes separately and for the class totals. This analysis considers the contribution of each component relative to the rest of the components. Thus, it is suitable for comparing multivariate data that are intrinsically interrelated, as is the case for volatile compounds produced by plants. At the compound level the data for each replicate were first standardized by expressing the amount of each compound produced as a proportion of the total amount of volatiles in that class. Similarly, at the class level, the total amount of volatiles of each type was expressed as a proportion of the total volatiles produced. One component (compound or class) was used as a baseline (see below), and the amounts of all other components were expressed as a ratio of the amount of the baseline components. These ratios were then logged (natural) and analyzed with multivariate analysis of variance (MANOVA). If one or more components were absent for any plant, a proportional correction was applied (see Aitchison, 1986).

When analyzed at the class level, the baseline was the *unknowns* class. This allowed an assessment of the differences in contribution of each class of compounds to the blend in the undamaged *C. nigra* treatment compared to the PHC treatment. When analyzing at the compound level within each class separately, the following compounds were used as the baseline: hexanal in the AKE class; octane in the hydrocarbon class; α-pinene in the monoterpenoid class; and cyclosativene in the sesquiterpenoid class.

Within each MANOVA, the overall effect of treatment was assessed by using an *F* test, but, to determine which variables contributed to any observed treatment differences, a canonical variate analysis (CVA) was also used to search for a linear combination of the variables that best discriminates between the treatments. With two treatments, there is only one possible canonical variate, and the CVA loadings are one dimensional, and represent the contribution of each particular log-ratio variable to the discrimination between treatments. Biplots of the CVA scores (for individual plants) and loadings enabled visual inspection of

which variables contributed to the separation of the undamaged *C. nigra* and PHC treatments.

Results

Full Plant Bioassays Aphidius funebris preferred the odor of *C. nigra* over clean air on first and final choice (Fig. 1a, b). The parasitoid showed no significant preference for the 3-d-50-aphid plant–host complex (PHC) odor, or for the 5-d-50-aphid PHC odor over that of undamaged *C. nigra*. However, the 3-d-200-aphid PHC odor was preferred over undamaged *C. nigra* by *A. funebris* on both first and final choice. Also, the aphid-damaged plant (ADP) odor was preferred over that from undamaged *C. nigra* on first choice, although the final choice preference was not statistically significant (Fig. 1a, b).

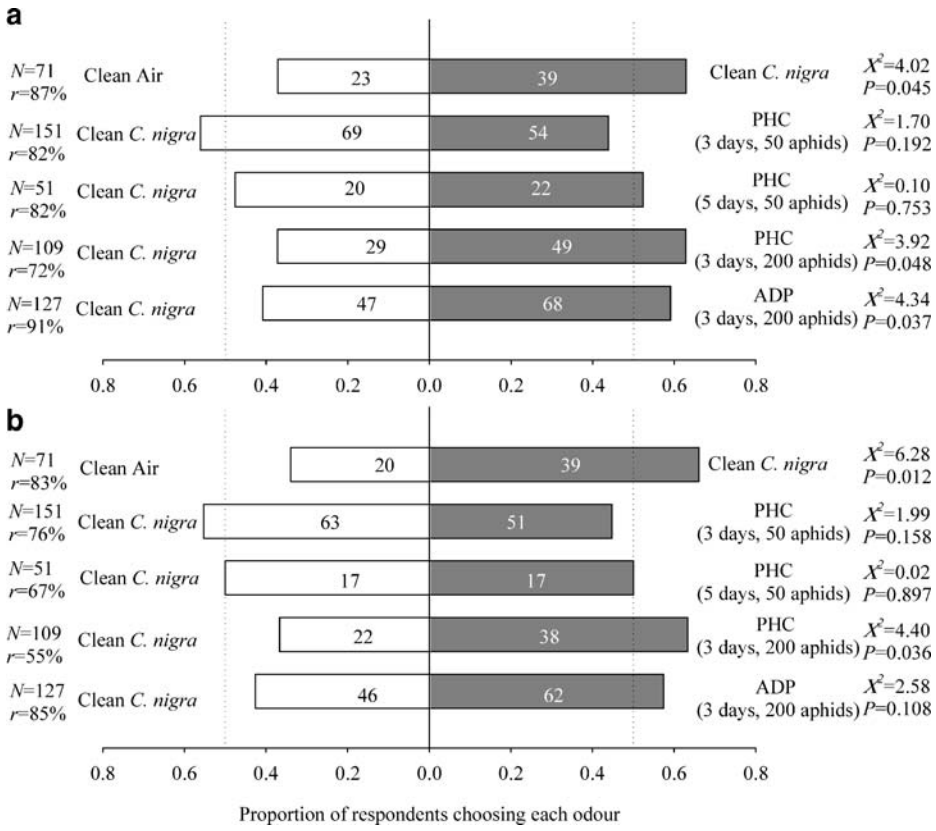

Fig. 1 First (**a**) and final (**b**) responses of *A. funebris* to *C. nigra* odors in a Y-tube olfactometer. Clean *C. nigra*=undamaged plant. For the plant–host complexes (PHC) and the aphid-damaged plant (ADP), the time they were infested before the bioassay and the initial number of aphids are indicated in brackets. *N* is the total number of parasitoids tested and *r* is the responsiveness. Within the bar is the number choosing each odor. The *horizontal axis* gives the proportion of responding insects that chose each odor, and the *dotted line* indicates no preference (50% responses in each arm). X^2 values are obtained from the change in deviance of the GLM interaction term upon deletion from the model (see text for detail)

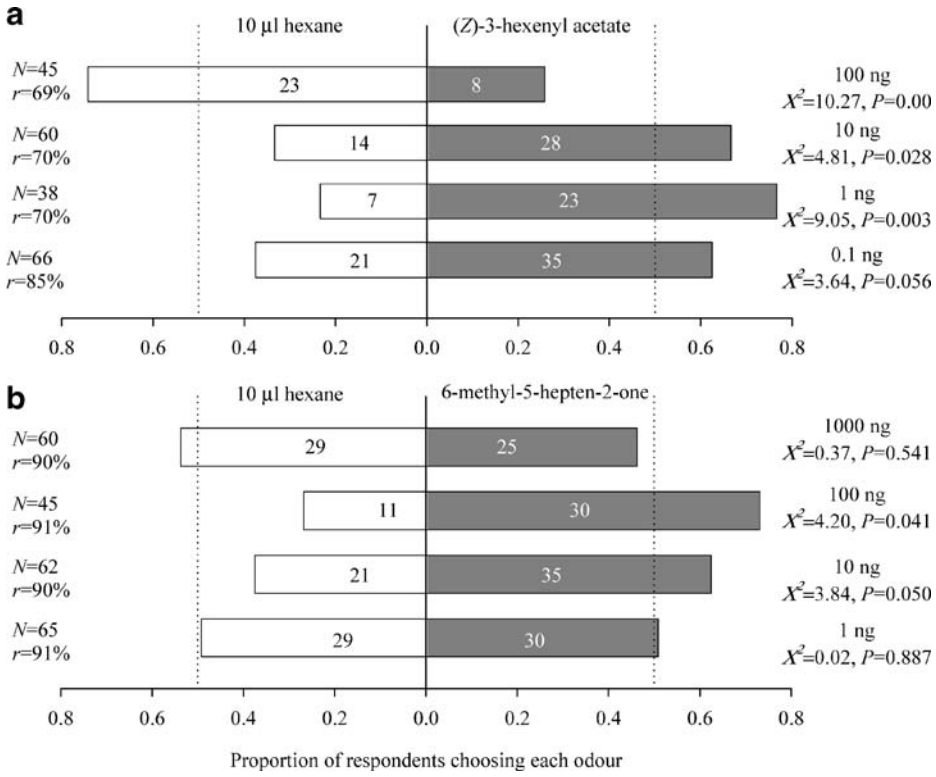

Fig. 2 Dose response of *A. funebris* to (**a**) (*Z*)-3-hexenyl acetate and (**b**) 6-methyl-5-hepten-2-one in a Y-tube olfactometer. *N* is the total number of parasitoids tested and *r* is the responsiveness and within the bar are the number choosing each odor. The *horizontal axis* gives the proportion of responding insects that chose each odor, and the *dotted line* indicates no preference. X^2 values are obtained from the change in deviance of the GLM interaction term upon deletion from the model (see text for detail)

Dose Response Curves Only the first choice responses are presented here. *Aphidius funebris* significantly avoided (*Z*)-3-hexenyl acetate at the highest dose of 100 ng, but showed a positive response to 10 and 1 ng of (*Z*)-3-hexenyl acetate. The response to the lowest dose of 0.1 ng also appeared to be positive, but was not statistically significant (Fig. 2a). The parasitoid showed a positive response to 6-methyl-5-hepten-2-one at 100 and 10 ng doses, but not at the highest (1,000 ng) and lowest (1 ng) doses (Fig. 2b).

Volatile Profiles A total of 40 compounds were identified (of which nine tentatively) (Table 1). The volatile profile of *C. nigra* and the *C. nigra–U. jaceae* PHC did not differ qualitatively.

Repeated Measures Analyses of Volatile Profiles The total amount of volatiles produced in 24 hr was neither affected by aphid infestation ($F_{1,14}$=0.40, P=0.535) nor by time ($F_{2.93,74.65}$= 1.96, P=0.128), and the effect of treatment did not change with time (no significant interaction: $F_{2.93,74.65}$=1.55, P=0.210) (Table 1).

Of the four classes of identified compounds, hydrocarbons, AKEs (aldehydes, ketones, and esters), monoterpenoids, and sesquiterpenoids, none was affected by treatment (P>

Table 1 Compounds identified from *C. nigra* and volatile amounts produced by undamaged *C. nigra* and the *C. nigra–U. jaceae* plant–host complex (PHC)

Compounds[a]	KI		Mean (±SE) Ln Amount Released (In[ng/24 hr])									
	HP-1	DB-Wax	Undamaged *C. nigra*					*C. nigra–U. jaceae* PHC				
			Day 1	Day 2	Day 3	Day 4	Day 5	Day 1	Day 2	Day 3	Day 4	Day 5
Hydrocarbons												
1. Octane	800	800	2.43±0.61	2.56±0.52	2.66±52	2.54±0.56	2.72±0.60	1.78±0.44	2.98±0.68	3.08±0.610	3.14±0.65	3.23±0.61
2. Nonane	900	900	1.79±0.44	1.63±0.38	1.27±0.40	1.62±0.41	1.39±0.41	1.38±0.41	2.05±0.38	1.97±0.39	1.26±0.45	1.94±0.38
3. Decane	1,000	1,000	2.10±0.49	2.28±0.47	1.90±0.47	2.17±0.56	1.90±0.47	2.01±0.51	2.39±0.50	2.32±0.51	2.07±0.50	2.59±0.43
4. Undecane	1,100	1,100	2.31±0.47	2.23±0.47	1.59±0.50	2.07±0.48	2.17±0.49	2.03±0.51	2.47±0.51	2.68±0.48	2.18±0.51	2.33±0.51
5. Dodecane	1,200	1,200	2.23±0.47	2.15±0.51	1.51±0.52	2.17±0.49	2.09±0.48	2.10±0.51	2.35±0.52	2.73±0.46	1.96±0.52	2.26±0.50
6. Tridecane	1,300	1,300	2.05±0.50	1.83±0.50	1.38±0.49	2.05±0.53	1.82±0.48	1.94±0.50	2.22±0.48	2.56±0.45	1.86±0.49	2.20±0.45
7. Tetradecane	1,400	1,400	2.58±0.47	2.29±0.50	1.99±0.50	2.27±0.54	2.69±0.45	2.37±0.50	2.54±0.45	2.39±0.48	2.05±0.51	2.24±0.50
8. 1-Pentadecene (T)	1,491	–[a]	Nq	0.56±0.39	Nq	Nq	Nq	1.31±0.54	Nq	1.57±0.65	1.11±0.59	0.78±0.53
9. Pentadecane	1,500	1,500	2.77±0.36	2.60±0.37	2.29±0.42	2.45±0.47	2.58±0.48	1.95±0.47	3.16±0.33	2.82±0.40	1.91±0.51	2.36±0.43
10. 1-Hexadecene (T)	1,591	–[a]	2.24±0.59	Nq	Nq	Nq	Nq	Nq	2.19±0.60	0.88±0.46	Nq	2.24±0.44
11. Hexadecane	1,600	1,600	1.60±0.29	1.55±0.31	1.02±0.32	1.38±0.31	1.14±0.34	1.65±0.33	1.80±0.28	1.17±0.34	0.99±0.35	1.09±0.34
12. 3-Heptadecene (T)	1,691	–[a]	1.41±0.46	1.93±0.55	1.74±0.51	1.92±0.50	1.63±0.47	2.13±0.26	2.26±0.48	2.16±0.39	1.92±0.50	
Aldehydes												
13. Hexanal	775	1,054	1.92±0.48	1.78±0.48	1.22±0.43	1.59±0.39	1.86±0.36	1.46±0.40	2.17±0.44	2.36±0.52	2.36±0.46	2.28±0.41
14. (*E*)-2-Hexenal	827	1,179	1.97±0.41	2.22±0.34	1.26±0.36	1.38±0.32	1.23±0.28	1.21±0.36	2.10±0.47	1.85±0.37	1.49±0.30	1.48±0.27
Ketones												
15. 6-Methyl-5-hepten-2-one	965	1,298	1.34±0.47	1.14±0.50	0.93±0.42	0.68±0.37	0.97±0.37	1.46±0.45	1.79±0.50	1.63±0.54	1.28±0.52	1.43±0.54
Esters												
16. Butyl acetate	797	–[a]	Nq	0.53±0.31	0.25±0.17	0.56±0.32	0.65±0.32	Nq	0.59±0.27	0.99±0.37	0.93±0.35	0.51±0.24
17. (*Z*)-3-Hexenyl acetate	988	1,283	5.97±0.63	2.78±0.84	2.84±0.68	1.65±0.55	1.84±0.49	4.21±0.90	4.29±0.95	3.98±0.78	2.78±0.75	2.36±0.69
Monoterpenoids												
18. Tricyclene (T)	921	–[a]	1.15±0.37	0.84±0.38	0.67±0.34	0.59±0.31	0.59±0.32	0.89±0.32	0.72±0.40	1.27±0.43	0.71±0.39	0.87±0.39
19. α-Thujene (T)	928	–[a]	0.75±0.33	0.59±0.33	0.67±0.33	0.81±0.56	0.49±0.27	0.62±0.32	0.83±0.38	1.42±0.51	0.75±0.46	1.41±0.49
20. α-Pinene	931	1,006	6.24±0.49	6.21±0.47	5.97±0.53	5.63±0.58	5.92±0.52	5.65±0.67	6.44±0.49	6.67±0.43	6.41±0.38	6.45±0.45
21. α-Fenchene (T)	944	–[a]	2.49±0.43	1.75±0.56	1.89±0.54	1.84±0.51	2.08±0.55	2.05±0.53	2.59±0.56	2.74±0.50	2.19±0.54	2.54±0.49
22. Camphene	944	1,047	2.82±0.46	3.09±0.41	2.71±0.49	2.69±0.47	2.65±0.51	2.58±0.48	3.00±0.52	3.09±0.48	2.86±0.43	3.08±0.40
23. Sabinene	967	1,099	1.93±0.49	1.32±0.50	1.86±0.53	1.83±0.52	1.69±0.49	1.81±0.54	1.27±0.47	2.05±0.51	1.82±0.53	1.66±0.48
24. β-Pinene	970	1,085	3.24±0.48	3.21±0.45	2.90±0.54	2.83±0.48	3.08±0.47	3.02±0.53	3.30±0.51	3.67±0.45	3.33±0.44	3.55±0.45
25. Myrcene	983	1,145	2.68±0.52	1.50±0.50	1.22±0.49	1.22±0.49	1.48±0.50	2.41±0.56	1.64±0.55	2.23±0.46	1.64±0.49	2.02±0.50

Table 1 (continued)

Compounds[a]	KI		Mean (±SE) Ln Amount Released (ln[ng/24 hr])									
	HP-1	DB-Wax	Undamaged *C. nigra*					*C. nigra–U. jaceae* PHC				
			Day 1	Day 2	Day 3	Day 4	Day 5	Day 1	Day 2	Day 3	Day 4	Day 5
26. α-Phellandrene	998	1,145	0.39±0.27	0.40±0.27	0.43±0.29	Nq	Nq	Nq	Nq	0.81±0.44	Nq	0.46±0.31
27. 3-Carene	1,003	1,128	5.42±0.36	5.34±0.40	5.07±0.42	4.56±0.59	4.66±0.63	4.76±0.57	5.39±0.48	5.32±0.59	5.21±0.47	5.34±0.48
28. Limonene	1,021	1,176	4.66±0.56	4.46±0.56	4.18±0.51	3.81±0.62	3.60±0.68	3.93±0.65	4.96±0.59	4.60±0.57	4.40±0.46	4.18±0.54
29. γ-Terpinene	1,050	1,222	0.39±0.27	0.62±0.34	0.43±0.29	Nq	0.39±0.28	0.35±0.22	0.45±0.32	0.58±0.40	0.63±0.43	0.86±0.35
30. Camphor	1,118	1,469	Nq	0.28±0.19	0.42±0.31	0.40±0.28	Nq	Nq	0.51±0.36	0.68±0.37	0.40±0.28	0.39±0.20
Sesquiterpenoids												
31. Cyclosativene	1,372	1,464	1.20±0.31	1.45±0.35	1.58±0.37	1.30±0.31	1.54±0.38	1.05±0.31	1.64±0.39	1.87±0.38	1.30±0.39	1.52±0.38
32. α-Copaene	1,379	1,478	1.35±0.35	1.30±0.30	1.43±0.27	1.74±0.28	1.94±0.26	1.14±0.38	1.41±0.36	2.10±0.36	1.92±0.37	1.95±0.35
33. β-Cubebene (T)	1,394	–[a]	1.24±0.42	2.28±0.46	1.53±0.45	1.61±0.43	1.98±0.43	1.78±0.43	1.87±0.46	2.11±0.43	2.04±0.48	2.08±0.42
34. Longifolene	1,406	1,543	1.10±0.37	1.17±0.32	1.11±0.25	1.35±0.35	1.55±0.34	0.83±0.28	1.31±0.38	1.68±0.40	2.06±0.45	1.79±0.31
35. β-Caryophyllene	1,420	1,570	0.51±0.24	0.36±0.20	0.54±0.19	0.91±0.28	1.07±0.25	0.74±0.26	0.86±0.31	1.13±0.34	1.16±0.29	1.24±0.31
36. α-Bergamotene (T)	1,439	–[a]	0.89±0.33	0.45±0.26	0.72±0.31	0.41±0.21	1.20±0.33	0.79±0.31	1.23±0.37	0.89±0.36	1.10±0.30	1.03±0.27
37. (*E*)-β-Farnesene	1,449	1,639	0.79±0.29	0.61±0.27	0.95±0.30	0.98±0.26	1.27±0.27	2.07±0.38	2.34±0.32	2.42±0.33	2.45±0.28	2.44±0.36
38. α-Humulene	1,455	1,698	2.52±0.54	1.51±0.51	1.89±0.51	1.93±0.46	0.93±0.35	1.14±0.30	1.91±0.56	2.17±0.40	1.62±0.38	1.32±0.32
39. Germacrene D (T)	1,488	–[a]	0.52±0.27	0.31±0.19	0.16±0.11	0.48±0.19	1.01±0.35	0.64±0.23	0.31±0.15	1.06±0.33	1.37±0.36	1.46±0.39
40. (*E,E*)-α-Farnesene	1,495	1,718	0.83±0.38	1.00±0.41	1.01±0.40	0.88±0.30	1.36±0.42	1.58±0.47	0.43±0.30	1.22±0.41	1.12±0.38	1.15±0.41
Total amount			8.98±0.16	8.81±0.16	8.62±0.16	8.62±0.16	8.59±0.16	8.80±0.16	8.97±0.16	8.93±0.16	8.73±0.16	8.78±0.16

KI values were calculated on HP-1 and DB-Wax columns from standards (see text for detail). The amounts presented are the mean and SE of the natural logarithm of the amount produced in 24 hr of entrainment (N=14), calculated relative to the area of the internal standard.

(*T*) indicates tentative identifications where no peak enhancement was carried out with authentic standards; *Nq* not quantifiable

[a] KI calculated from assumed compound (from fragmentation pattern) on HP-1 column in the GC-MS and no standard available for calculation on DB-Wax.

0.05), and only the AKEs showed an effect of time ($F_{2.27,57.94}$=7.26, P<0.001) and a time by treatment interaction ($F_{2.27,57.94}$=3.88, P=0.022). For this group, there was a sharp decrease in the amount produced by undamaged *C. nigra* from the first to subsequent days, but the amount produced by the PHC increased (from a level lower than in the undamaged *C. nigra*) and then decreased.

Compositional Analyses of Volatile Profiles During the 1st d (0–24 hr) of entrainment, the contribution of the different classes to the volatile profiles of *C. nigra* plants differed between treatments ($F_{4,10}$=3.57, P=0.047). The AKE class (loading=0.42) was associated with undamaged *C. nigra*, while the sesquiterpenoids (loading=−0.42) were associated with the PHC, and these contributed to separating treatments more than the monoterpenoid or hydrocarbon classes (loadings=0.11 and 0.09, respectively) (Fig. 3a).

During the fourth day, there was a marginally nonsignificant effect of treatment ($F_{4,11}$= 3.24, P=0.055) on the contributions by the different classes of compounds, and the main contribution was by hydrocarbons (loading=0.79), which were associated with the undamaged *C. nigra*. This was opposed by the AKE class (loading=−0.62), which was associated with the PHC. The monoterpenoid and sesquiterpenoid classes were more associated with the PHC, but their loadings were lower (Fig. 3b). On the second day ($F_{4,10}$=1.71, P=0.224), third day ($F_{4,11}$=2.12, P=0.146), and fifth day ($F_{4,11}$=0.92, P=0.488), no difference between treatments were detected in the contribution of the four classes.

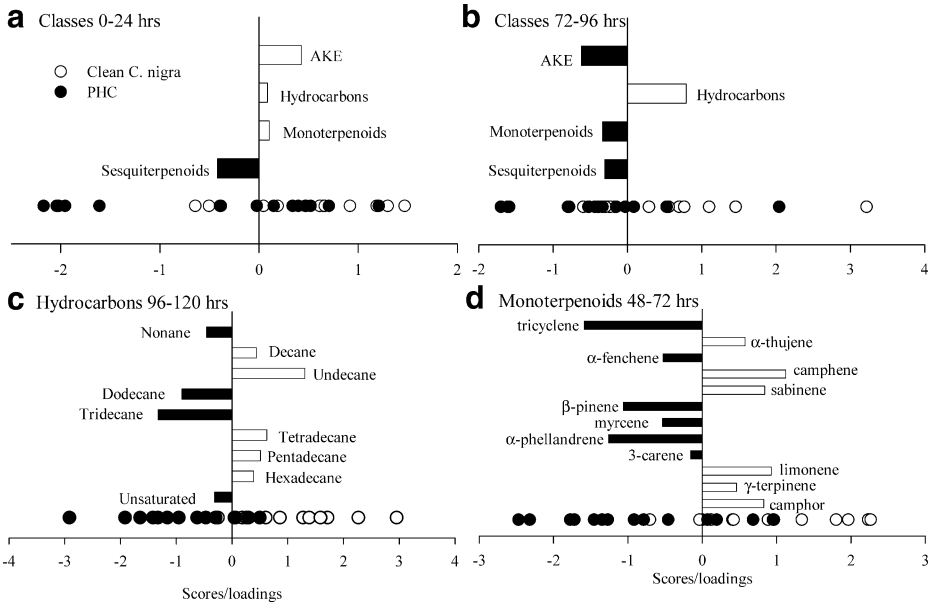

Fig. 3 Canonical variate analysis (CVA) biplots from the comparisons between undamaged (clean) *C. nigra* and the *C. nigra–U. jaceae* plant–host complex (PHC) air entrainments. **a** Compositional analysis for the classes of compounds (acetates, ketones, and esters (AKE), hydrocarbons, monoterpenoids, and sesquiterpenoids) on the first day (0–24 hr) of aphid damage. **b** Class compositional analysis for the fourth day (72–96 hr). **c** Hydrocarbon compositional analysis for the fifth day (96–120 hr). **d** Monoterpenoid compositional analysis for the third day (48–72 hr). The *points* represent the CVA scores for each individual replicate and the *bars* are the CVA loadings, indicating the contribution of each variable towards separating the treatments

Of the hydrocarbons listed in Table 1, the three unsaturated hydrocarbons (1-pentadecene, hexadecene, and 3-heptadecene) were grouped together for the analysis, and the others were included individually. Within this group, there was a marginal effect on the fifth day ($F_{9,6}$= 3.27, P=0.081), and the greatest contribution to separating the treatments came from tridecane, which was associated with the PHC, and undecane, which was associated with undamaged *C. nigra* (Fig. 3c). Dodecane was associated with the PHC. Days 1–4 revealed no differences between the treatments ($F_{9,5}$=0.34, P=0.926; $F_{9,5}$=0.41, P=0.883; $F_{9,3}$= 0.61, P=0.749; $F_{9,6}$=2.29, P=0.162, respectively).

The AKE class consisted of hexanal, (*E*)-2-hexenal, 6-methyl-5-hepten-2-one, butyl acetate, and (*Z*)-3-hexenyl acetate. The composition of the compounds within this class did not vary between the treatments on any of the days of entrainment (in order: $F_{5,8}$=1.18, P= 0.396; $F_{5,9}$=0.31, P=0.896; $F_{5,10}$=0.18, P=0.965; $F_{5,10}$=0.24, P=0.936; $F_{5,9}$=0.57, P= 0.721).

For the compounds within the monoterpenoid class, a marginally nonsignificant effect on the third day of entrainment ($F_{12,3}$=5.68, P=0.089) was observed. On this day, three compounds (tricyclene, β-pinene, and α-phellandrene) were strongly associated with the PHC, while four compounds (camphene, sabinene, limonene, and camphor) were associated with undamaged *C. nigra* (Fig. 3d).

Within the sesquiterpenoids, none of the compounds changed in its contribution to the volatile blend over the 5 d of entrainment (for the 5 d in order: $F_{10,3}$=1.48, P=0.414; $F_{10,4}$= 1.68, P=0.326; $F_{10,5}$=2.34, P=0.181; $F_{10,4}$=1.40, P=0.398; $F_{10,5}$=2.16, P=0.205).

Mechanical Damage Tests Changes due to mechanical damage trials were not quantified, but it led to an apparent increase in 1-pentadecene, 1-hexadecene, germacrene D, (*E,E*)-α-farnesene, and β-caryophyllene. This increase in volatiles occurred during the first 24 hr after damage, and then decreased back to the same levels as the undamaged plant on the second and third day of entrainment.

Discussion

We have demonstrated that the parasitoid *A. funebris* responds to odors from undamaged *C. nigra* and the *C. nigra–U. jaceae* PHC. A high initial aphid density is needed for increased parasitoid attraction, which contrasts with the *Vicia faba–A. pisum* system, where 40 aphids feeding for 3 d were enough to trigger changes in parasitoid behavior (Guerrieri et al., 1999). The species studied have important ecological differences compared to other plant–aphid systems studied in a tritrophic context. In particular, the plant is perennial and the aphid highly specialized, and such a plant-aphid relationship is likely to be tightly coupled. The aphid is likely to overcome host plant defenses efficiently, with minimal effects on plant physiology, leading to small changes in volatile chemistry. Herbivore-induced changes in plant volatile chemistry probably evolved primarily as a direct result of plant–aphid interactions, and predators and parasitoids subsequently evolved responses to the subtle changes in plant volatile profiles resulting from these interactions. This suggests that "plant signalling" was originally nonadaptive in the tritrophic context, and is a function acquired after the natural enemies evolved responses to plant volatiles (Bronstein and Barbosa, 2002).

Host plant specialization by aphids leads to morphological adaptation to those host plants (Moran, 1986), and this might be reflected in the adaptation of feeding structures to the host plant because there is within-species variation in the stylet movement in aphids (Bernays

and Funk, 2000). Such physical adaptation would be important in reducing defensive metabolic changes in the plant, and the stylet path is known to play a role in reducing plant responses to injury (Will and van Bel, 2006) via the stylet sheath, which might have a function in dampening the wound responses of the plant (Miles, 1999) and as a "plug" preventing the influx of calcium ions, which are important in activating defense proteins in the phloem (Tjallingii, 2006; Will and van Bel, 2006).

The lack of induced terpenoids observed in *C. nigra* could be due to low jasmonic acid (JA) levels, which could be a result of these reduced wound responses (Fäldt et al., 2003; Pauw and Memelink, 2004), but could also be due to inhibition by salicylic acid (SA)-dependent gene expression, which is activated by many phloem feeders, including several aphid species (Walling, 2000; Moran and Thompson, 2001; Martinez de Ilarduya et al., 2003). The interaction between JA and SA dependent responses can be antagonistic, though there are also cases of simultaneous activation of both defense pathways (reviewed by Bostock, 2005). This interaction can be important in determining the quantity and identity of induced volatiles, and can provide a mechanism of species-specific volatile emission (Kessler and Baldwin, 2002; Dicke et al., 2003; Heidel and Baldwin, 2004; Kaloshian and Walling, 2005).

The parasitoid showed a significant response to the ADP on first choice, but not on final choice. It is possible that the presence of aphids provides cues that reinforce its attraction to the plant. As it gets closer to the host it encounters and uses more reliable cues (Vet and Dicke, 1992), and if it fails to perceive these more specific cues, it might give up that particular search. It is likely that *A. funebris* uses a sequence of cues, each reinforcing the previous, to find its hosts (Vinson, 1976). The lack of qualitative changes in the volatile profile of *C. nigra* detectable by GC-FID suggests that quantitative changes in several compounds of different classes are responsible for attraction of *A. funebris* to aphid-infested plants, and this is likely due to the interactions between signalling pathways and aphid-specific responses discussed above. The small relative changes in "minor" and abundant monoterpenoids may serve as cues that *A. funebris* can exploit. In particular, the changes in relative contributions of tricyclene, β-pinene, myrcene, and α-phellandrene (greater relative presence after herbivory), and camphene, sabinene, limonene, and camphor (greater relative presence in undamaged plants) might be important. To determine this, electroantennography (EAG) and bioassays with different ratios of compounds are needed. Changes in volatile composition are often quantitative (e.g., Colazza et al., 2004b; Moraes et al., 2005), and use of ratios can provide specific information on herbivore presence and identity for some species of parasitoid (De Moraes et al., 1998; Smid et al., 2002). In individual parasitoid antennae, many different compounds show EAG activity (Du et al., 1998; Smid et al., 2002; Gouinguené et al., 2005). Specific types of damage are often needed for maximum attraction of parasitoids (Mattiacci et al., 2001; Colazza et al., 2004a; Hoballah and Turlings, 2005), and individual compounds with EAG activity are not always attractive in isolation (Ngi-Song et al., 2000), suggesting that the use of a combination of compounds might be a common strategy. *Aphidius funebris* therefore differs from *A. ervi*, which seems to use 6-methyl-5-hepten-2-one as a specific indicator of the presence of a suitable aphid (Du et al., 1998), and we agree with the conclusion by Bukovinszky et al. (2005) that subtle changes are responsible for attraction.

Aphidius funebris responds to (*Z*)-3-hexenyl acetate, a ubiquitous green leaf volatile. This has been shown for many parasitoid species (Takabayashi et al., 1991; Whitman and Eller, 1992; Röse et al., 1998; Hoballah and Turlings, 2005) and might provide a means of finding vegetation, but specificity is probably due to components present in relatively low amounts in the volatile blend (Gouinguené et al., 2005). The responses to cues of increasing reliability as

the parasitoid gets nearer to its host is likely to be highly influenced by heterogeneity at different spatial scales, many of which are absent in crop systems because the patch is usually a field of a single plant, highlighting the importance of studying noncrop systems.

The PHC showed an increased emission of sesquiterpenoids on the first day after aphid infestation (Fig. 3a). This is probably due to minor mechanical damage by the aphids because the mechanical damage tests showed an increase in several sesquiterpenoids during the first day. These compounds are probably released from storage upon damage, possibly due to the systemic JA-mediated wound response (Paré and Tumlinson, 1997), and probably indicate general damage.

This study has shown that semiochemical-mediated parasitoid attraction occurs in this noncrop system, and could help explain patterns of parasitism in the field because it has been demonstrated (Pareja, 2006) that individuals in larger colonies are at greater risk of parasitism than those in smaller colonies in the field (i.e., parasitism is directly density dependent). This suggests that response to volatiles could mediate processes such as density dependence that affect community structuring and population dynamics in the field, and are likely to be highly scale-dependent. This has been suggested in the past (Vet, 1996, 1999), but the studies that have demonstrated the potential for semiochemical interactions mediating host-parasitoid population and community processes are few (see e.g., Geervliet et al., 2000; Roßbach et al., 2005).

Effects in this system are subtle, and the parasitoid *A. funebris* is sensitive to minor changes in plant volatile profiles. The statistical technique we present for analyzing volatile data has a firm base outside ecology, but has never been utilized in chemical ecology. We believe that it is suitable for dealing with the complexity of volatile production by plants because it treats volatile compounds as parts of a blend, and not as unrelated compounds released in isolation, which makes intuitive sense when we consider the interrelated metabolic processes that give rise to these compounds. Ignoring the correlations between compounds due to these metabolic interactions could lead to erroneous conclusions on patterns of volatile release upon herbivory. There is potential for combining this analysis with EAG because this will indicate physiologically active compounds that should be included in the compositional analysis. We have also introduced the use of GLMs for the analysis of olfactometer data. Though GLM techniques are well established in ecology, they have not been widely used in behavior, despite their large potential. The introduction of these methods into chemical ecology could prove to be useful tools in the study of volatile-mediated interactions.

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