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ORIGINAL PAPER

Oviposition deterring infochemicals in ladybirds: the role of phylogeny

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Abstract Faced with an ephemeral prey, aphidophagous ladybirds rely on the hydrocarbons present in the tracks of their larvae to choose an unoccupied patch for egg laying. Although both conspecific and heterospecific larval tracks might deter females from oviposition, the response to the later is often less striking. Several explanations have been suggested to account for this. In this paper we tested the phylogeny hypothesis, which predicts that the chemical composition of the tracks of closely related species of ladybirds will be more similar to one another than to those of more distantly related species. Qualitative and quantitative information on the chemical nature of the larval tracks and a molecular phylogeny of seven species belonging to three different genera are provided, and the congruence between these two sets of results assessed. The results confirm the phylogeny hypothesis and infer a gradual mode of evolution of these infochemicals.

Keywords Oviposition deterring infochemicals · Phylogeny · Coccinellidae

Introduction

Aphidophagous ladybirds are faced with difficult decisions when looking for an oviposition site. Although the number of aphids in a colony may often reach high levels, each colony only exists for a relatively short period of time (Dixon 1998). This abundant but

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ephemeral resource might attract other consumers, both conspecific and heterospecific. In order to maximise their fitness, ladybirds therefore need to be able to evaluate the quality of potential oviposition sites in terms of the risk to their progeny of starvation, cannibalism and/or intraguild predation.

Many species of ladybird respond to cues associated with the presence of conspecific or heterospecific individuals and refrain from laying eggs in patches of prey already occupied by predators (Růžička 1997, 2001, 2003, 2006; Doumbia et al. 1998; Yasuda et al. 2000; Agarwala et al. 2003; Oliver et al. 2006; Magro et al. 2007), which is a common strategy among insects (Nufio and Papaj 2001). In the majority of the cases, female ladybirds respond to chemical tracks that larvae produce while walking, which in the case of *Adalia bipunctata* (L.), *A. decempunctata* (L.) and *Coccinella septempunctata* L. are mainly composed of alkanes (Hemptinne et al. 2001; Magro et al. 2007). Ladybirds respond similarly to conspecific faeces (Agarwala et al. 2003), and, in the case of *Cheilomenes sexmaculata* (F.), to (Z)-Pentacos-12-ene extracted from the cuticule of its larvae (Klewer et al. 2007).

Although females benefit from recognising and responding to both conspecific and heterospecific larval tracks, refraining from oviposition in the presence of the former would appear to be more advantageous. The rationale is as follows. Many species of ladybirds are restricted to a specific habitat (Hodek and Honěk 1996). Furthermore, aphid colonies are exploited by a sequence of ladybirds, with small species laying eggs at lower aphid population densities than large species (Dixon 2007). That is, the probability of meeting a conspecific is much greater than of meeting a heterospecific individual. As expected, the recognition of the tracks of other species is less striking than the response to the tracks of their own species. Several explanations, based on geographical distribution, habitat similarity/overlap, defence mechanisms, low risk of predation or phylogeny have been suggested to account for this (e.g. Yasuda et al. 2000; Růžička 2001; Oliver et al. 2006; Magro et al. 2007).

The objective of this paper is to test a prediction of the phylogeny hypothesis. This prediction is that the chemical composition of the tracks of closely related species of ladybirds will be more similar to one another than to those of more distantly related species, which is to be expected if the mode of evolution was gradual (Symonds and Elgar 2008). Qualitative and quantitative information on the chemical nature of the larval tracks and a molecular phylogeny of seven species belonging to three different genera are provided, and the congruence between these two sets of results assessed.

Materials and methods

Seven species belonging to three genera were studied: *A. bipunctata*, *A. decempunctata*, *C. septempunctata*, *Coccinella undecimpunctata* L., *Coccinella quinquepunctata* L., *Harmonia axyridis* (Pallas) and *Harmonia quadripunctata* (Pontoppidan).

The *C. undecimpunctata* and *C. quinquepunctata* used to initiate the cultures were collected in the United Kingdom, *A. bipunctata*, *A. decempunctata*, *C. septempunctata* and *H. quadripunctata* in France and *H. axyridis* in Japan.

The chemical analyses of the tracks of *A. bipunctata*, *A. decempunctata* and *C. septempunctata* are those previously published by Magro et al. (2007).

Ladybird culture

Stock cultures of each species were maintained in the laboratory. These consisted of adults reared at 20 ± 1°C, LD 16:8, in 5-l plastic boxes with a piece of corrugated filter paper, on

which the females laid eggs. Three times a week the ladybirds were fed an excess of pea aphids, *Acyrthosiphon pisum* Harris. Two stems of broad bean, *Vicia faba* L. (variety "Primabel"), were added to each box to improve the survival of the aphids.

Eggs were taken from the stock cultures and incubated in 175 cm^3 plastic boxes kept under the same conditions as the stock cultures. After hatching, larvae were fed excess pea aphids three times a week.

Phylogeny

In order to clarify their phylogenetic relationships, which are traditionally based on morphological characters, the seven species were analysed at the molecular level.

DNA extraction, amplification and sequencing

Total genomic DNA was extracted from entire individuals (minus elytra) using DNeasy Blood and tissue Kit from QIAGEN with PBS protocol according to the manufacturer's instructions. Partial sequences of the 12S rDNA mitochondrial region were amplified by PCR using the primers SR-J-14233 5′-AAGAGCGACGGGCGATGTGT-3′ and SR-N-14588 5′-AAACTAGGATTAGATACCCTATTAT-3′ (Kergoat et al. 2004). The amplification protocol (touch down PCR) started with denaturation for 5 min at 95°C followed by 5 cycles of 1 min at 95°C, 1 min at 55–50°C, 1 min at 72°C and 30 cycles (1 min at 95°C, 1 min at 50°C, 1 min at 72°C), and a 10 min final extension at 72°C. The 28S rDNA gene sequences (299 bp) were obtained using the primers 28Sa 5′-GACCCGTCTTGAAA CACGGA-3′ 28Sb 5′-TCGGAGGGAACCAGCTACTA-3′ (Whiting et al. 1997). PCR used the following thermal cycling parameters: 5 min at 95°C, 35 cycles (1 min at 95°C, 1 min at 50°C, 1 min at 72°C), and a 10 min final extension at 72°C. Polymerase chain reactions were performed with 50 ng of DNA in 25 or 50 µl volumes containing a final concentration of 1X PCR buffer, 0.2 µM of each primer, 0.2 mM of each dNTPs, 1.5 mM of $MgCl_2$ and 1U of Taq polymerase. All PCR amplified products were sequenced directly by an automated sequencer. Sequences were deposited in GenBank (see Table 2, in the "Appendix", for GenBank accession nos.).

Phylogenetic analyses

Sequences were manually aligned using Bioedit software (Hall 1999). Gaps were coded as missing data. The program Modeltest version 3.7 (Posada and Crandall 1998), was used to determine the sequence evolution model that best fits our data using the Akaike Information Criterion (AIC). Phylogenetic reconstructions were performed by maximum likelihood (ML) using PHYML v2.4.4 (Guindon and Gascuel 2003) injecting model parameters selected by Modeltest. The robustness of nodes was estimated by ML bootstrap (BP) percentages (1,000 replicates). Bayesian analysis was performed with MrBayes [version 3.1.2] (Ronquist and Huelsenbeck 2003) using the same model of sequence evolution and two partitions, one for each gene. Two independent analyses using four chains were run for 1,000,000 generations. We sampled trees (and parameters) every 100 generations. Convergence was assessed by examining the average standard deviation of split frequencies and the Potential Scale Reduction Factor. For each run, the first 25% of sampled trees were discarded as burn-in. A 50% majority rule consensus of the sampled trees was constructed to calculate the posterior probabilities (PP) of the tree nodes.

The genera *Bruchidius* and *Diacantha*, from the Bruchidae and Chrysomelidae families respectively [closely related to the Coccinellidae] (Hunt et al. 2007), were used as outgroups. Chimeric data (i.e. different sequences derived from more than one species of a genus) were used only for the two outgroup taxa, based on the sequences available (Table 2, in the "Appendix"): *Bruchidius* (*B.* sp. and *B. terrenus*) and *Diacantha* (*D. unifasciata* and *D. collaris*).

Chemical nature of the larval tracks

Production and extraction of larval tracks

Second instar larvae were taken from the stock cultures and isolated in 5 mm Petri dishes, fed three times a week and checked daily for moulting. Freshly emerged L4 larvae (1–24 h old) were isolated in 5 mm Petri dishes, and deprived of food for 24 h. Each larva was then carefully introduced into a glass tube (12 mm diameter; 75 mm long). These tubes were sealed with a cotton plug and kept at 20 ± 1°C, LD 16:8. As the larvae were hungry, they spent most of the time walking. After 24 h the larvae were removed and the tubes were stored at −18°C. For each species, three batches of 30 tubes were produced. Tubes with no larvae were handled similarly and used as a control. The fasting period imposed on the larvae prior to the experiment, and the careful introduction of these larvae into the tubes, reduced the likelihood of the tracks being contaminated by faeces or reflex bleeding, respectively.

To extract the larval tracks, the 30 tubes of each batch were successively washed with 1 ml of hexane (hexane Merck, HPLC grade for liquid chromatography). Then, they were washed a second time with 1 ml of hexane for maximum extraction. Afterwards, the 2 ml of extract for each batch of tubes were transferred to a small vial and evaporated under a gentle stream of nitrogen. Finally, the dry residue was re-dissolved in 40 µl of hexane. The samples were kept at 4°C until analysed using Gas Chromatography-Mass Spectrometry (GC-MS). The control tubes were similarly treated.

It should be noted that the techniques used for the production and extraction of larval tracks are the same as those used by Magro et al. (2007), and hence results are fully comparable.

Chemical analyses

The GC-MS analyses were performed on a Finnigan Trace 2000 chromatograph directly coupled to a mass spectrometer quadrupole detector (electron impact at 70 eV). The temperature source was set at 200°C, the interface between GC and MS modules at 250°C and the splitless injector at 280°C. Helium was the carrier gas and the flow rate was 1.2 ml/min. Samples of 1 µl were injected in an apolar capillary column (Restek RTX-5MS 30 m × 0.25 mm, 0.25 µm film thickness, 5% diphenyl and 95% dimethylpolysiloxane). The chromatograph oven was programmed as follows: 50°C for 1 min, then from 50 to 140°C at 20°C per min, from 140 to 300°C at 3°C per min and finally held at 300°C for 3 min. The mass spectra were scanned from 60 to 500 m/z. The whole system was controlled by a Xcalibur data system, 1.2 version. Detection limits of chromatographic peaks were automatically established by the Xcalibur software, which only considered peaks to be significant if larger than a noise factor fixed at 10.

In order to identify the compounds present in the larval tracks, a selected ion monitoring at m/z = 85 value was initially carried out (Lockey 1988; Nelson 1993; Fortes and Baugh 1999) in order to find the peaks corresponding to hydrocarbons. Then, the identification of the compound's structure was determined using the mass spectral fragmentation patterns, comparison of the retention times with those of injected known compounds and NIST library spectra. A standard mixture of alkanes, from n-C12 to n-C60 (Supelco, Sigma-Aldrich, 0.01% w/w each component), was used as a qualitative reference. The nonadecane (Sigma-Aldrich) was used as internal standard. In order to quantify each compound, 5 µl of extract were mixed with 5 µl of a nonadecane solution at 0.0781 mg per litre in n-hexane). Then, the quantification in mg per litre and per batch (30 tubes) of each compound was carried out using a correlation between the area of the peaks and concentrations of the linear alkanes.

In order to abbreviate the names of the identified compounds, we use the IUPAC nomenclature (International Union of Pure and Applied Chemistry). This nomenclature uses a descriptor (XX) for the total number of carbons in the hydrocarbon component (C_{XX}) [i.e., nonacosane becomes *n*-C_{29}], the number of double bonds (Y) follows a colon ($C_{XX:Y}$) [i.e., heneicos-6-ene becomes $C_{21:6}$] and the location of methyl groups uses the descriptor (X-Me) [i.e., 3-methylheptacosane becomes 3-MeC_{27} and 7,12-dimethyloctacosane becomes 7,12-diMeC_{28}].

Congruence between phylogeny and chemical nature of larval tracks

To study the congruence between phylogeny and chemical nature of larval tracks, different analyses were performed.

Firstly, the chemical compositions of the larval tracks of the seven species were compared using a stepwise backward discriminant (SBD) analysis (Systat 9.0 software), following Chouteau et al. (2008). The seven species were separated into three grouping variables corresponding to the genera: *Adalia* (*A. decempunctata* and *A. bipunctata*), *Coccinella* (*C. septempunctata*, *C. undecimpunctata*, *C. quinquepunctata*) and *Harmonia* (*H. axyridis*, *H. quadripunctata*). The chemical compounds of the larval tracks were the independent variables. The data was analysed qualitatively (absence = 0 or presence = 1) with unidentified compounds being omitted from the analysis. In the case of the 2 *Adalia* and *C. septempunctata*, results corresponded to one trial each (data from Magro et al. 2007); for *C. undecimpunctata*, *C. quinquepunctata* and the two species of *Harmonia* it was three trials.

Secondly, a phylogeny of the species was reconstructed based on the chemical compounds in the tracks. The presence or absence of each chemical compound was coded as binary characters. Unidentified compounds were not considered in the analyses. The data set comprises 114 characters including 60 parsimony-informative ones. All characters are treated as unordered and equally weighted. Maximum parsimony (MP) analyses were performed by PAUP (Swofford 1998), using exhaustive search (ACCTRAN optimization). Bootstrap support for MP trees was calculated using 10,000 bootstrap replicates. Additionally, a distance matrix was built using the same data: the distance between each pair of species is calculated as the number of chemical compounds by which they differ over the total number of identified compounds in the group and expressed in percents.

Finally, to estimate the congruence between the chemical composition of tracks and phylogenetic relationships of the species, the informative characters were mapped on the molecular phylogenetic tree.

Results

Phylogeny

The results of the molecular analysis were used to establish the cladogram presented in Fig. 1.

The final alignment included 634 sites and 9 taxa for the concatenated dataset (335 sites for the 12S gene and 299 sites for the 28S gene). The best-fitting model for the sequence evolution was the general time-reversible model (GTR) using a gamma shape (G). Analysis of the combined dataset produced a single ML tree (Fig. 1, lnL = −2,120.533104). Our molecular analyses were fully congruent whatever the method used (see Fig. 1 for the ML topology) and all unambiguously retrieved two robust (BP =>90%; PP > 0.90; see Fig. 1) monophyletic groups. One corresponds to the genus *Harmonia* (*H. axyridis* and *H. quadripunctata*), and the other to the cluster *Adalia* + *Coccinella*. *Adalia* appears to be monophyletic (100% BP, PP = 1.0) and *Coccinella* paraphyletic, with *C. undecimpuntata* clustering with *Adalia*, although lacking statistical support. Although lacking bootstrap support the two other *Coccinella* species, *C. quinquepunctata* and *C. semptempunctata*, appear to be sister species.

Chemical nature of the larval tracks

The hexane extracts of the seven species' larval tracks consisted mainly of hydrocarbons [90%] (Fig. 2, 3, 4). The results of the qualitative and quantitative analyses are presented in Table 3 (in the "Appendix"). A total of 142 compounds were detected, 28 of which are unidentified. 104 of the hydrocarbons are alkanes and 10 are alkenes, although the identification of the latter needs to be confirmed using derivatization (see Francis and Veland 1981; Vicenti et al. 1987 for details).

The total weight of track material produced by 30 larvae varied greatly among the species and is not related to species size: 7.819 µg for *A. bipunctata*, 10.734 µg for *A. decempunctata*, 14.581 µg for *C. septempunctata*, 20.415 µg for *C. undecimpunctata*, 0.779 µg for

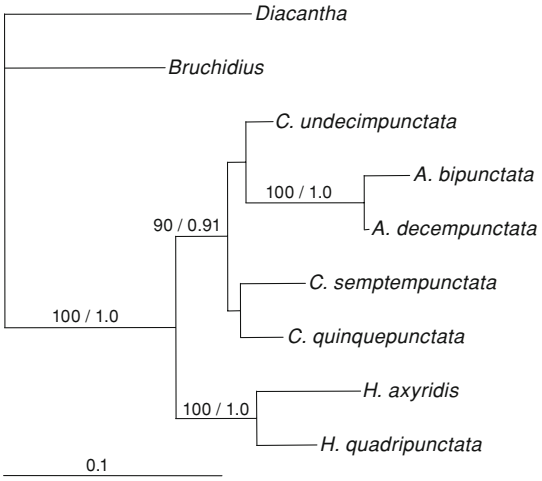

Fig. 1 Maximum likelihood tree obtained using the combined dataset (12S and 28S genes). Bootstrap (*BP*) values (%) obtained from the ML analyses, as well as Bayesian posterior probabilities (*PP*) are indicated as BP/PP above branches for BP > 50% and PP > 0.5

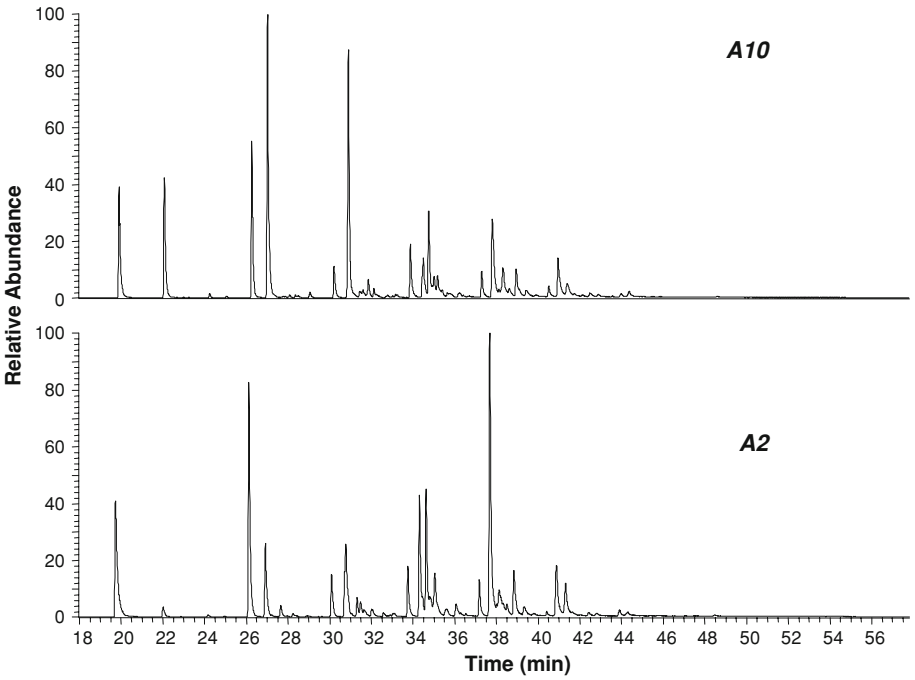

Fig. 2 Chromatographic profiles of the larval tracks of *A. decempunctata* (*A10*) and *A. bipunctata* (*A2*), obtained after selected ion monitoring (Xcalibur data system) at m/z = 85 value

C. quinquepunctata, 12.803 μg for *H. axyridis* and 42.218 μg for *H. quadripunctata*. The species that produced most is *H. quadripunctata* and that which produced least *C. quinquepunctata*.

In terms of the diversity of compounds, *H. axyridis* produced most (48 compounds) and *C. septempunctata* least (32). Only eight compounds are produced by all the ladybirds: nC_{25}, nC_{26}, nC_{27}, $5MeC_{27}$, nC_{28}, nC_{29}, nC_{31} and $13\text{-}MeC_{31}$. In contrast, each species has a few specific hydrocarbons; for the seven species there are 72 of these molecules and *C. quinquepunctata* has the highest number of species specific compounds (22).

Congruence between phylogeny and chemical nature of larval tracks

In the SBD analysis, the jackknifed classification matrix resulted in a total of 89% of the data being correctly classified [100% for *Adalia*, 89% for *Coccinella* and 88% for *Harmonia*] (Fig. 5). It held back six variables ($C_{19:1}$, $C_{19:1}$, $C_{21:1}$, nC_{21}, $10,12\text{-}diMeC_{24}$, $11\text{-}MeC_{31}$). The eigenvalues for the two axes were 12.38 and 3.32 respectively, with the corresponding canonical correlations of 0.97 and 0.88. Wilks' lambda test was significant (Wilks' lambda = 0.017, $P < 10^{-3}$).

The distance matrix built on binary chemical qualitative data is presented on Table 1. The chemical signature of the genus *Coccinella* is more similar to that of *Adalia* than of *Harmonia*, with component differences of 39.5 and 44.6%, respectively. Of the *Coccinella*, *C. septempunctata* is more similar to *Adalia* spp (25.4% difference) than the other two

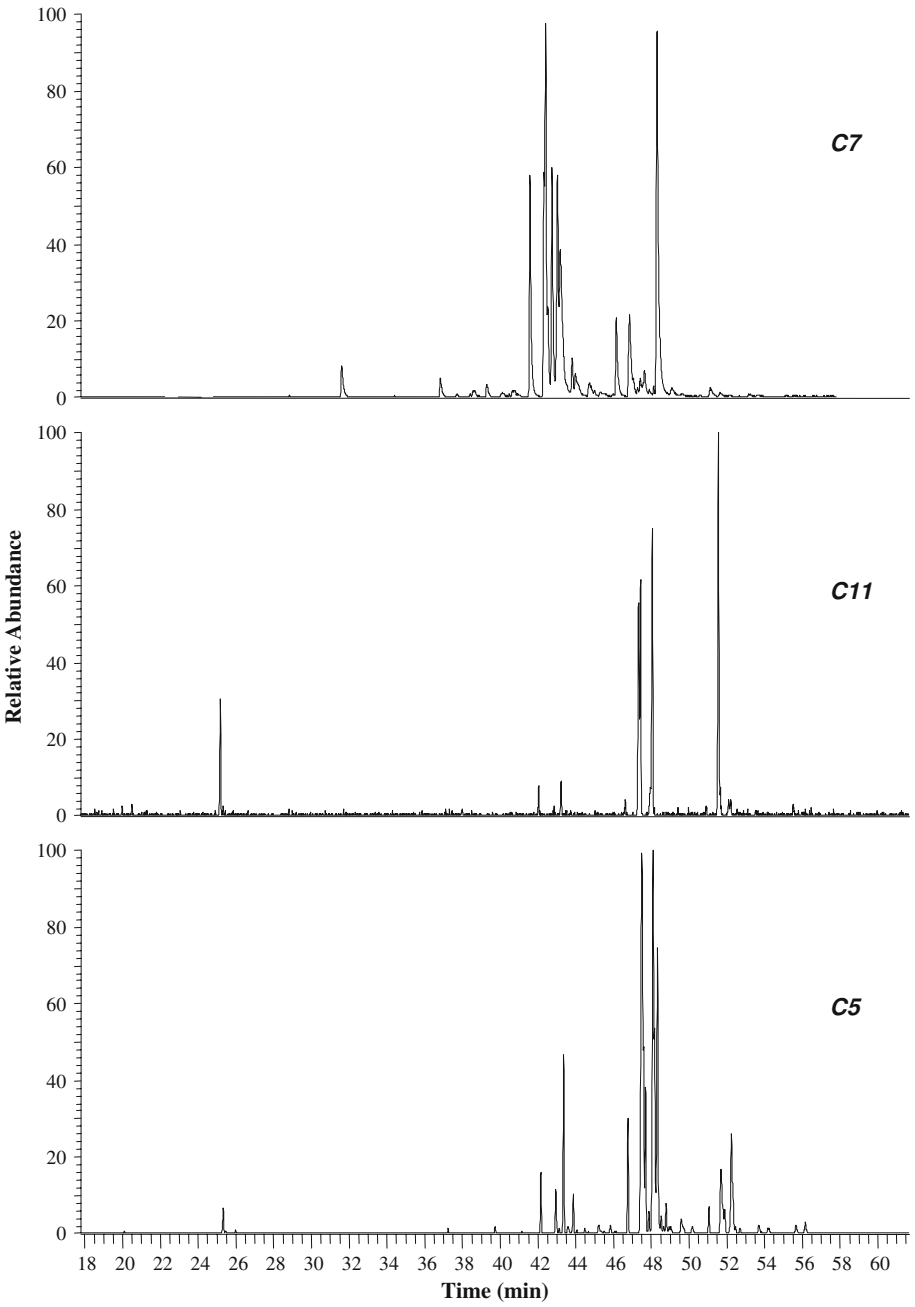

Fig. 3 Chromatographic profiles of the larval tracks of *C. septempunctata* (*C7*), *C. undecimpunctata* (*C11*) and *C. quinquepunctata* (*C5*), obtained after selected ion monitoring (Xcalibur data system) at m/z = 85 value

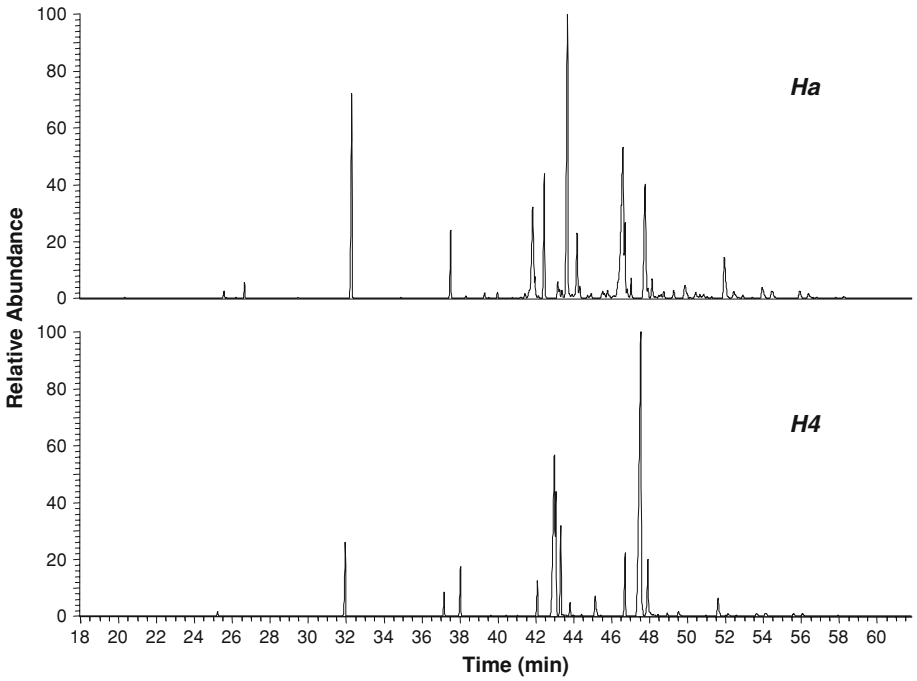

Fig. 4 Chromatographic profiles of the larval tracks of *H. axyridis* (*Ha*) and *H. quadripunctata* (*H4*), obtained after selected ion monitoring (Xcalibur data system) at m/z = 85 value

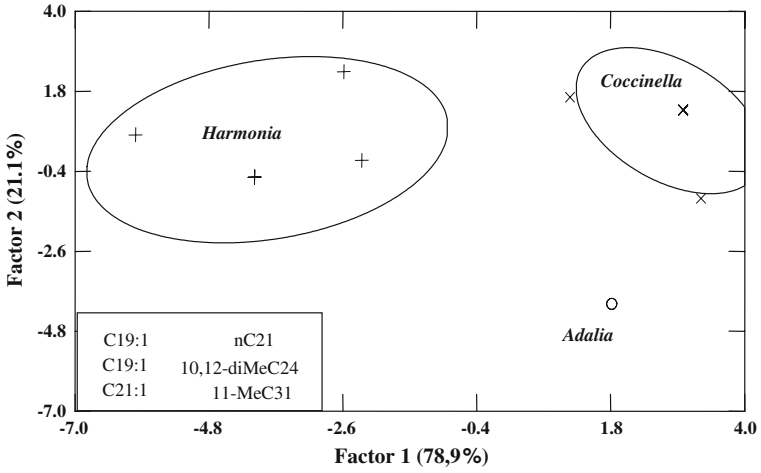

Fig. 5 Qualitative multivariate analysis of larval tracks, based on the presence or absence of compounds. Analysed species are: *A. bipunctata*, *A. decempunctata* and *C. septempunctata*, each based on one trial (results from Magro et al. 2007) and *C. undecimpunctata*, *C. quinquepunctata*, *H. axyridis* and *H. quadripunctata*, each based on three trials. Chemical names indicate components that contributed most to the separation of clusters. *Ellipses* are drawn by the program; some *dots* overlap because the results are qualitatively identical

Table 1 Distances between species based on the presence/absence of chemical compounds

	A2	A10	C7	C11	C5	Ha	H4 (%)
A2	–	0	25.4%	47.4%	45.6%	46.5%	42.1
A10	0	–	25.4%	47.4%	45.6%	46.5%	42.1
C7	29	29	–	43.0%	39.5%	43.9%	39.5
C11	54	54	49	–	31.6%	46.5%	43.9
C5	52	52	45	36	–	48.2%	45.6
Ha	53	53	50	53	55	–	20.2
H4	48	48	45	50	52	23	–

The above diagonal values correspond to relative character differences (%), the below diagonal values to total character differences. The species are: *Adalia bipunctata* (A2), *A. decempunctata* (A10), *Coccinella septempunctata* (C7), *C. undecimpunctata*, (C11) *C. quinquepunctata* (C5), *Harmonia axyridis* (Ha) and *H. quadripunctata* (H4)

Coccinella species are to *Adalia* spp (41.25%). The chemical signatures of the two *Adalia* species are identical. *Harmonia* is clearly different from *Adalia* and *Coccinella* (differences of 44.3–44.6%).

The maximum parsimony analyses based on the chemical compounds in the larval tracks produced a single most parsimonious tree, presented in Fig. 6 (129 steps, CI = 0.8217, RI = 0.7356). The chemical composition of the tracks of *C. septempunctata* is closely related to that of *Adalia* (bootstrap support = 95%). *C. undecimpunctata* and *C. quinquepunctata* are sister species in this chemical tree, as are the two *Harmonia* species. While the genera *Adalia* and *Harmonia* are each characterised by 10 specific chemical compounds, only one substance (8MeC28) is specific to *Coccinella* (Fig. 7). There are more steps between the cluster *C. septempunctata–Adalia* and the genus *Harmonia* (14 changes) than the cluster *C. undecimpunctata–C. quinquepunctata* (10 changes, cf. Fig. 6).

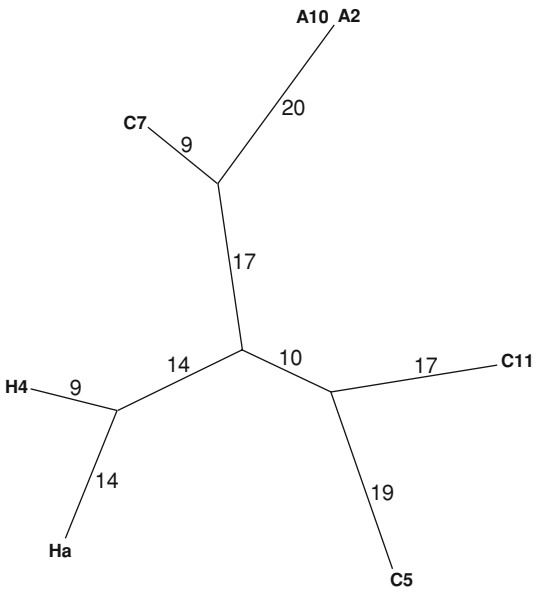

Fig. 6 Most parsimonious unrooted tree (129 steps, CI = 0.8217, RI = 0.7356) based on the chemical compounds (presence/absence) in the larval tracks of *Adalia bipunctata* (*A2*), *A. decempunctata* (*A10*), *Harmonia axyridis* (*Ha*), *H. quadripunctata* (*H4*), *Coccinella septempunctata* (*C7*), *C. undecimpunctata* (*C11*) and *C. quinquepunctata* (*C5*). The *values along branches* correspond to the number of character changes

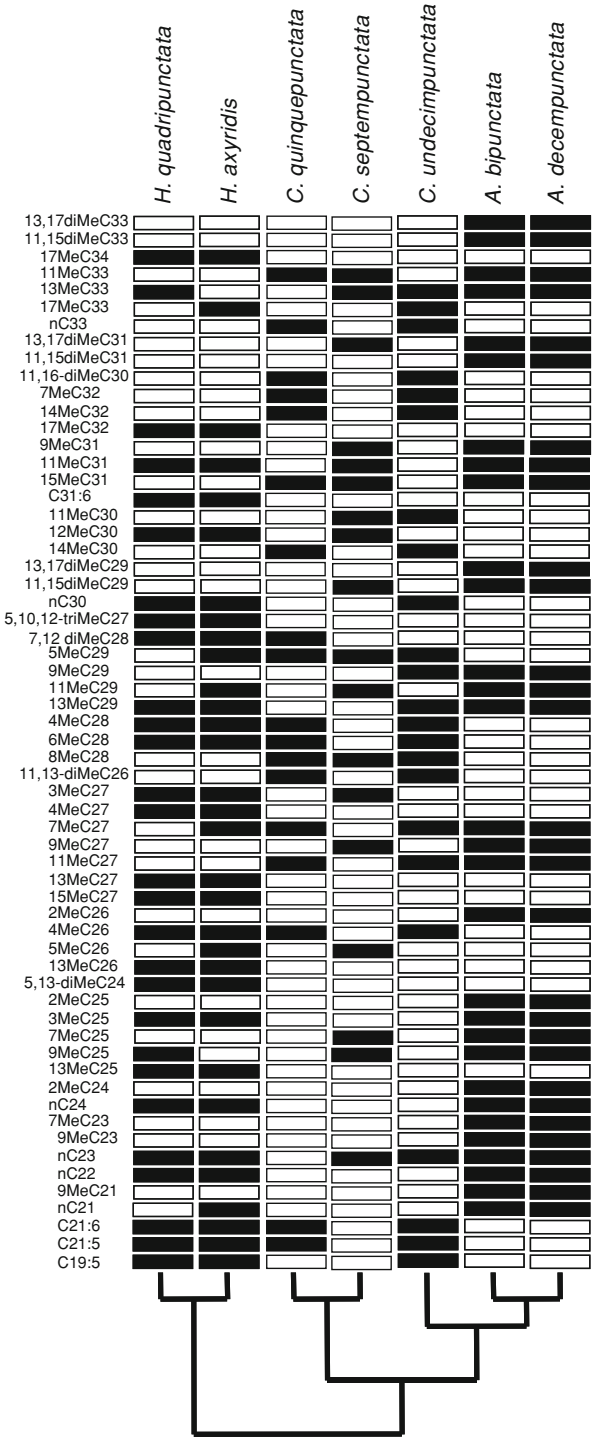

Fig. 7 Molecular phylogeny of the species of Coccinellidae included in this study, with the chemical compounds superimposed. *Filled squares* indicate the presence of a chemical compound; *white squares* indicate its absence

Despite the great diversity, the results of the different analyses of the composition of the larval tracks of the seven species strongly support the conclusion inferred from the chromatograms (Fig. 2, 3, 4), i.e., the chemical compositions of the intrageneric larval tracks are more similar than the intergeneric tracks. The difference between genera also conforms to the phylogeny: *Adalia* and *Coccinella* are closer to each other than to the *Harmonia*. That is, there is a strong congruence between the chemical composition of the larval tracks and the phylogeny of the beetles.

Discussion

Many species of ladybird refrain from ovipositing in the presence of conspecific larval tracks (e.g. Růžička 1997, 2001, 2003, 2006; Doumbia et al. 1998; Yasuda et al. 2000; Agarwala et al. 2003; Oliver et al. 2006; Magro et al. 2007). This is considered to be adaptive as it prevents the overpopulation of aphid patches. Too many predators in colonies would result in starvation and cannibalism, reducing fitness.

The oviposition behaviour of several ladybird species in the presence of heterospecific larval tracks has also been studied. Indeed, individuals of several species are frequently observed attacking the same aphid colonies and might therefore compete for food, which could result in intraguild predation. The oviposition deterring effect of heterospecific larval tracks has been recorded, although less frequently than that of conspecific larval tracks (e.g. Doumbia et al. 1998; Yasuda et al. 2000; Růžička 2001; Oliver et al. 2006). In a previous paper, Magro et al. (2007) suggested that the degree of phylogenetic relatedness could be an important factor contributing to explain the differences in the oviposition response of females of one species to larval tracks of another species.

The results of this study indicate that the composition of the larval tracks is more similar within genera than between genera. Symonds and Elgar (2008) claim that this is often the case because members of the same genus share similar biosynthetic pathways. However, we also found that the chemical distances between genera are congruent with the phylogenetic distances: *Adalia* and *Coccinella* chemical signatures are closer to each other than to those of *Harmonia*, the same appears to be true for phylogenetic relatedness. In addition, although more species need to be studied, these results provide strong evidence that more distantly related species differ more in the chemical composition of their larval tracks than closely related species. This, therefore, supports the phylogeny hypothesis. It is, however, important to mention that, despite the very low bootstrap support for the molecular phylogeny, it indicated that *C. undecimpunctata* is more closely related to *Adalia*, which contrasts with the relationship based on the chemistry of the larval tracks, which indicates that *C. septempunctata* is closely related to *Adalia*.

Our results indicate a gradual evolution (Roelofs and Brown 1982) of ladybird larval tracks, in which species accumulate small changes in chemical components as they diverge, resulting in phylogenetic conservatism (Symonds and Elgar 2008). In contrast, when there is a sudden substantial or complete change in the chemical components, that generates a very different blend, then evolution is said to occur by saltational shifts (Baker 2002; Symonds and Elgar 2008).

In pheromone communication systems, gradual evolution, driven by genetic drift, is expected when the information conveyed by the cues is not species-specific (Symonds and Wertheim 2005). This heritable variation does not contribute to the reproductive isolation of the species, which is ensured by other mechanisms. It is, for example, the case of the evolution of the *Drosophila* aggregation pheromones (Symonds and Wertheim 2005).

Aggregation of adults of different species of *Drosophila* at a resource and subsequent aggregated oviposition and increased larval densities, enhances the quality of the larval substrate (Wertheim et al. 2002). This is achieved through the interaction among adults, larvae and micro-organisms, which inhibits fungal growth, detrimental to larval development (Wertheim et al. 2002). In this case the mutual attraction of adults of different species to a resource benefits them all (Symonds and Wertheim 2005). In contrast, the pheromones used in *Drosophila* mate attraction, where species specificity is important and therefore selection against mixed messages is strong, are likely to have evolved by saltational shifts (Symonds and Elgar 2008), which give rise to strong pheromone divergence.

We do not know enough about the interaction between ladybird larvae and females to be able to identify the selective pressures that might drive what seems to be a gradual evolution of the larval tracks. In the *Drosophila* example, cited above, and in parasitoids, the senders and receivers have the same interests. For ladybirds, chemical communication is probably more complex. Larvae produce a substance that is likely to be an adhesive or a surfactant enabling them to walk upside down on a smooth surface (Laubertie et al. 2006); for females this substance is a cue indicating already occupied patches. In this case, larvae are not "senders" of a message but simply producers of an infochemical substance.

Finally, our results will add to the general knowledge of oviposition deterring infochemicals' composition, as only a few have been chemically characterised so far (Anderson 2002).

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Appendix

See Table 2 and 3.

Table 2 Taxa analysed and GenBank accession numbers for DNA sequences

Species	Origin	GenBank accession no.	
		12S	28S
Adalia bipunctata	France	FJ621318	FJ621325
Adalia decempunctata	France	FJ621317	FJ621324
Coccinella quinquepunctata	United Kingdom	FJ621320	FJ621326
Coccinella septempunctata	France	FJ621321	FJ621328
Coccinella undecimpunctata	United Kingdom	FJ621319	FJ621327
Harmonia axyridis	Japan	FJ621323	FJ621330
Harmonia quadripunctata	France	FJ621322	FJ621329
Bruchidius terrenus[a]		DQ524351	–
Bruchidius sp.[a]		–	AJ841542
Diacantha collaris[a]		EF421574	–
Diacantha unifasciata[a]		–	AJ841614

[a] Sequences obtained from GenBank

Table 3 Results of the qualitative and quantitative (μg per 30 larvae) analysis of the compounds present in the larval tracks of *A. bipunctata* (A2), *A. decempunctata* (A10), *C. septempunctata* (C7) (from Magro et al. 2007), *C. undecimpunctata* (C11), *C. quinquepunctata* (C5), *H. axyridis* (Ha) and *H. quadripunctata* (H4) [each the mean of 3 trials]

	A2	A10	C7	C11	C5	Ha	H4
C19:1					0.0002		
C19:1				0.0160		0.0022	0.0010
C21:1				0.1922	0.0033	0.0118	0.0574
C21:1				0.0157	0.0002	0.0019	0.0008
nC21	0.4482	0.0706				0.0941	
9-MeC21	0.0052	0.0036					
nC22	0.0113	0.0092				0.0105	0.0009
nC23	0.8432	1.0111	0.0999	0.0052		1.1741	0.9990
9-MeC23	1.0812	0.3728					
7-MeC23	0.0077	0.0037					
6-MeC23							0.0008
nC24	0.1819	0.0618				0.0103	0.0012
2-MeC24	0.0153	0.0043					
C25:1						0.0025	
nC25	0.2797	0.2918	0.0902	0.0162	0.0005	0.4616	0.3433
13-MeC25						0.0231	0.9190
11-MeC25				0.0497			
9-MeC25	1.3265	0.5332	0.0065				0.0014
7-MeC25	0.0332	0.0826	0.0199				
3-MeC25	0.0440	0.1070				0.0449	0.0112
2-MeC25	0.0010	0.0559					
10,12-diMeC24				0.0011			
5,13-diMeC24						0.0030	0.0015
nC26	0.0788	0.0328	0.0547	0.0122	0.0008	0.0508	0.0172

Table 3 continued

	A2	A10	C7	C11	C5	Ha	H4
12-MeC26						0.0023	
13-MeC26						0.0023	0.0280
9-MeC26			0.0100				
8-MeC26			0.0118				
5-MeC26			0.0214			0.0128	
NI							0.0009
4-MeC26				0.0016	0.0003	0.0479	0.0144
2-MeC26	0.0596	0.0296					
C27:1						1.0237	
nC27	0.8319	0.4624	1.7351	0.2964	0.0083	0.7787	0.7206
15-MeC27						0.0093	4.6301
13-MeC27						0.1556	4.6301
11-MeC27	0.6558	1.9705		0.0618	0.0084		
9-MeC27	0.9361	1.2124	4.1839				
7-MeC27	0.2042	0.5074		0.0055	0.0007	0.0468	
5-MeC27	0.0445	0.1167	1.4955	0.3321	0.0317	2.0174	2.0865
4-MeC27						0.0142	0.0420
3-MeC27			1.7487			0.4617	0.3328
NI				0.0014			
NI							0.0233
2-MeC27			1.5008				
7,13-diMeC26					0.0065		
11,13-diMeC26				0.0056	0.0015		
NI					0.0006		
12,15-di-MeC26				0.0620			
C28:1						0.0552	

Table 3 continued

	A2	A10	C7	C11	C5	Ha	H4
nC28	0.0578	0.1501	0.2608	0.0061	0.0011	0.0156	0.0585
13-MeC28							0.4496
14-MeC28							0.4496
12-MeC28			0.2416				
10-MeC28						0.0886	
NI						0.0364	
8-MeC28			0.1831	0.0025	0.0027		
NI				0.0265			
6-MeC28				0.0035	0.0004	0.0635	0.0219
NI					0.0004		
4-MeC28				0.0039	0.0017	0.0198	0.0202
NI					0.0003		
NI				0.0049			
C29:1						2.5325	
NI						0.2321	
nC29	0.0493	0.0418	0.8328	0.3397	0.0248	0.0697	2.0213
15-MeC29					0.1802		
13-MeC29	0.2325	0.6872		2.8101		0.2497	18.0205
11-MeC29	0.0508	0.0830	1.2638			0.2497	
9-MeC29	0.0069	0.0151		2.2175			
7-MeC29					0.0313		
5-MeC29			0.1237	0.0283	0.0043	0.6710	
13,18-diMeC28							2.4295
9,13-diMeC28				3.8903			
7,12 diMeC28					0.0764	0.0676	0.0239
11,15-diMeC28					0.1612		

Table 3 continued

	A2	A10	C7	C11	C5	Ha	H4
NI					0.0012		
6,11-diMeC28						0.0163	
NI							0.0152
6,12-diMeC28					0.0035		
5,11,12-triMeC27				0.0140			
7,12,15-triMeC27					0.0059		
5,10,12-triMeC27						0.0285	0.0647
NI					0.0026		
nC30				0.0275		0.0438	0.1493
NI			0.0280				
11,15-diMeC29	0.0130	0.1909	0.2530				
13,17-diMeC29	0.0086	0.0212					
7,12-diMeC29			0.0158				
15-MeC30				0.1157			
14-MeC30				0.1157	0.0065		
12-MeC30			0.0879			0.1166	0.3304
11-MeC30			0.0641	0.0202			
9-MeC30				0.0207			
NI					0.0030		
8-MeC30							0.0256
C31:1						0.1226	
C31:1						0.0552	0.0041
nC31	0.0208	0.0287	0.0750	0.3415	0.0092	0.0138	0.0505
15-MeC31	0.0586	0.9736	0.1050		0.0221		
13-MeC31	0.0072	0.5040	0.0249	3.1196	0.0221	0.6124	1.4204
11-MeC31	0.0040	0.0173	0.0078			0.0920	0.0679

Table 3 continued

	A2	A10	C7	C11	C5	Ha	H4
9-MeC31	0.0024	0.0528	0.0078				
7-MeC31					0.0038		
nC32				0.0787			
12-MeC32				0.1020			
17-MeC32						0.1848	0.4117
14-MeC32				0.1020	0.0039		
NI					0.0024		
NI						0.0262	
NI							0.0381
13-MeC32					0.0039		
15-MeC32				3.1196			
NI						0.2560	
NI							0.3490
7-MeC32				0.0848	0.0078		
11,16-diMeC30				0.7034	0.0618		
9,15-diMeC30				0.8080			
11,15-diMeC31	0.0169	0.1343					
13,17-diMeC31	0.0318	0.0974	0.0091				
nC33				0.0164	0.0171		
NI				0.0501			
17-MeC33				0.3033		0.1848	
16-MeC33						0.2273	
15-MeC33				0.3033			
13-MeC33	0.0236	0.2235	0.0091	0.3033			0.3872
NI							0.4133
NI					0.0027		

Table 3 continued

	A2	A10	C7	C11	C5	Ha	H4
5-MeC33							0.0117
11-MeC33	0.0288	0.1173	0.0091		0.0241		
NI				0.0242			
NI					0.0037		
nC34					0.0083		
NI				0.0449			
17-MeC34						0.0424	0.1199
NI						0.1506	
7-MeC33				0.1875			
11,15-diMeC33	0.0828	0.3251					
13,17-diMeC33	0.0341	0.1314					
nC35					0.0063		
10-MeC35					0.0073		
NI					0.0022		
Total mass (μg/30 larvae)	7.8194	10.7343	14.5806	20.4145 (SD 24.9162)	0.7793 (SD 0.5074)	12.8031 (SD 3.8491)	42.2175 (SD 29.2292)

NI not identified

References

Agarwala BK, Yasuda H, Kajita Y (2003) Effect of conspecific and heterospecific feces on foraging and oviposition of two predatory ladybirds: role of fecal cues in predator avoidance. J Chem Ecol 29(2):357–376. doi:10.1023/A:1022681928142

Anderson P (2002) Oviposition pheromones in herbivorous and carnivorous insects. In: Hilker M, Meiners T (eds) Chemoecology of insect eggs and egg deposition. Blackwell Publishing, Berlin, pp 235–263

Baker TC (2002) Mechanism for saltational shifts in pheromone communication systems. Proc Natl Acad Sci USA 99:13368–13370. doi:10.1073/pnas.222539799

Chouteau M, Gibernau M, Barabé D (2008) Relationships between floral characters, pollination mechanisms, life forms, and habitats in Araceae. Bot J Linn Soc 156:29–42

Dixon AFG (1998) Aphid ecology, 2nd edn. Chapman and Hall, London

Dixon AFG (2007) Body size and resource partitioning in ladybirds. Popul Ecol 49:45–50. doi:10.1007/s10144-006-0019-z

Doumbia M, Hemptinne J-L, Dixon AFG (1998) Assessment of patch quality by ladybirds: role of larval tracks. Oecologia 113:197–202. doi:10.1007/s004420050368

Fortes ICP, Baugh PJ (1999) Study of analytical on-line pyrolysis of oils from macauba fruit (*Acrocomia sclerocarpa* M) via GC/MS. J Braz Chem Soc 10:469–477. doi:10.1590/S0103-50531999000600009

Francis GW, Veland K (1981) Alkylthiolation for the determination of double-bond positions in linear alkenes. J Chromatogr A 219:379–384. doi:10.1016/S0021-9673(00)80381-7

Guindon S, Gascuel O (2003) A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. Syst Biol 52:696–704. doi:10.1080/10635150390235520

Hall TA (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp Ser 1999(41):95–98

Hemptinne J-L, Lognay G, Doumbia M et al (2001) Chemical nature and persistence of the oviposition deterring pheromone in the tracks of the larvae of the two spot ladybird. Chemoecology 11:43–47. doi:10.1007/PL00001831

Hodek I, Honěk A (1996) Ecology of Coccinellidae. Kluwer, The Netherlands

Hunt T, Bergsten J, Levkanicova Z et al (2007) A comprehensive phylogeny of beetles reveals the evolutionary origins of a superradiation. Science 318:1913–1916. doi:10.1126/science.1146954

Kergoat G, Delobel A, Silvain J-F (2004) Phylogeny and host specificity of European seed beetles (Coleoptera, Bruchidae), new insights from molecular and ecological data. Mol Phyl Evol 32:855–865. doi:10.1016/j.ympev.2004.02.019

Klewer N, Růžička Z, Schulz S (2007) (Z)-Pentacos-12-ene, an oviposition-deterring pheromone of *Cheilomenes sexmaculata*. J Chem Ecol 33:2167–2170. doi:10.1007/s10886-007-9372-4

Laubertie E, Martini X, Cadena C et al (2006) The immediate source of oviposition-deterring pheromone produced by larvae of *Adalia bipunctata* (L.) (Coleoptera, Coccinellidae). J Insect Behav 19(2):231–240. doi:10.1007/s10905-006-9018-3

Lockey H (1988) Lipids of the insect cuticle: origin, composition and function. Comp Biochem Physiol B 89:595–645. doi:10.1016/0305-0491(88)90305-7

Magro A, Téné JN, Bastin N et al (2007) Assessment of patch quality by ladybirds: relative response to conspecific and heterospecific larval tracks a consequence of habitat similarity? Chemoecology 17:37–45. doi:10.1007/s00049-006-0357-5

Nelson DR (1993) Methyl-branched lipids in insects. In: Stanley-Samuelson DW, Nelson DR (eds) Insect lipids. Chemistry, biochemistry and biology. University of Nebraska Press, Lincoln, pp 271–315

Nufio CR, Papaj DR (2001) Host marking behavior in phytophagous insects and parasitoids. Entomol Exp Appl 99:273–293. doi:10.1023/A:1019204817341

Oliver TH, Timms JEL, Taylor A et al (2006) Oviposition responses to patch quality in the larch ladybird *Aphidecta obliterata* (Coleoptera: Coccinellidae): effects of aphid density, and con-and heterospecific tracks. Bull Entomol Res 96:25–34. doi:10.1079/BER2005395

Posada D, Crandall KA (1998) Modeltest: testing the model of DNA substitution. Bioinformatics 14:817–818. doi:10.1093/bioinformatics/14.9.817

Roelofs WL, Brown RL (1982) Pheromones and the evolutionary relationships of Tortricidae. Annu Rev Ecol Syst 13:395–422. doi:10.1146/annurev.es.13.110182.002143

Ronquist F, Huelsenbeck JP (2003) MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19:1572–1574. doi:10.1093/bioinformatics/btg180

Růžička Z (1997) Recognition of oviposition-deterring allomones by aphidophagous predators (Neuroptera: Chrysopidae, Coleoptera: Coccinellidae). Eur J Entomol 94:431–434

Růžička Z (2001) Oviposition responses of aphidophagous coccinellids to tracks of ladybird (Coleoptera: Coccinellidae) and lacewing (Neuroptera: Chrysopidae) larvae. Eur J Entomol 98:183–188

Růžička Z (2003) Perception of oviposition-deterring larval tracks in aphidophagous coccinellids *Cycloneda limbifer* and *Ceratomegilla undecimnotata* (Coleoptera: Coccinellidae). Eur J Entomol 100:345–350

Růžička Z (2006) Oviposition-deterring effects of conspecific and heterospecific larval tracks *Cheilomenes sexmaculata* (Coleoptera: Coccinellidae). Eur J Entomol 103:757–763

Swofford DL (1998) PAUP*. Phylogenetic analysis using parsimony (*and other methods). Version 4. Sinauer Associates, Sunderland

Symonds MRE, Elgar MA (2008) The evolution of pheromone diversity. Trends Ecol Evol 23(4):220–228. doi:10.1016/j.tree.2007.11.009

Symonds MRE, Wertheim B (2005) The mode of evolution of aggregation pheromones in *Drosophila* species. J Evol Biol 18:1253–1263. doi:10.1111/j.1420-9101.2005.00971.x

Vicenti M, Guiglielmetti G, Cassani G et al (1987) Determination of double bond position in diunsaturated compounds by mass spectrometry of dimethyl disulfide derivatives. Anal Chem 59:694–699. doi:10.1021/ac00132a003

Wertheim B, Marchais J, Vet LEM et al (2002) Allee effect in larval resource exploitation in *Drosophila*: an interaction among density of adults, larvae and micro-organisms. Ecol Entomol 27:608–617. doi:10.1046/j.1365-2311.2002.00449.x

Whiting M, Carpenter J, Wheeler Q et al (1997) The strepsiptera problem: phylogeny of the holometabolous insect orders inferred from 18S and 28S ribosomal DNA sequences and morphology. Syst Biol 46:1–68. doi:10.2307/2413635

Yasuda H, Takagi T, Kogi K (2000) Effects of conspecific and heterospecific larval tracks on the oviposition behaviour of the predatory ladybird, *Harmonia axyridis* (Coleoptera: Coccinellidae). Eur J Entomol 97:551–553