Cuticular lipids as trail pheromone in a social wasp

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We investigated the origin and composition of the chemical trail of the common yellow jacket *Vespula vulgaris* L. (Vespidae) and found that an artificial trail made from an extract of cuticular lipids from *V. vulgaris* foragers was biologically as active as a trail laid naturally by the foragers. Chemical analysis of natural trail extracts and the behaviourally active cuticular extracts by coupled gas chromatography–mass spectrometry revealed that the majority of cuticular hydrocarbons were also present in the trail extract at similar ratios. Thus, bioassay data and chemical analysis provide strong evidence that these cuticular hydrocarbons act as a trail pheromone in *V. vulgaris*.

Keywords: Vespidae; *Vespula vulgaris*; trail pheromone; cuticular hydrocarbons

1. INTRODUCTION

Trail pheromones play an important part in communication of social insects, allowing optimal exploitation of food sources or occupation of new nest sites (Wilson 1971; Chapman 1982). Orientation along chemical trails was mainly demonstrated in species that forage exclusively by walking, namely ants (Bradshaw & Howse 1984; Attygalle & Morgan 1985; Hölldobler & Wilson 1990; Jackson & Morgan 1993; Vander Meer & Alonso 1998) and termites (Wilson 1971; Pasteels & Bordereau 1998). The use of continuous terrestrial trails in hymenopteran species with flying foragers was demonstrated before in *Bombus* sp. (Cederberg 1977; Cameron *et al.* 1999), and most recently in the vespine species *Vespa crabro* L. and *Vespula vulgaris* L. (Steinmetz *et al.* 2002), which are all characterized by cavity breeding (Heinrich 1979; Greene 1991; Matsuura 1991). The foragers usually walk through dark tunnels between nest and cavity entrance, using the chemical trail for home orientation. However, until now nothing was known about the chemical composition and the origin of trail pheromones in these taxa.

The main role of the lipid layer covering the cuticle of most insects is to minimize transpiration and to protect terrestrial insects from desiccation (Lockey 1988). Furthermore, in numerous studies during the past decades one class of these lipids, the cuticular hydrocarbons, was supposed to play a part in nestmate recognition of social insects (Clément & Bagnères 1998; Vander Meer & Morel 1998; Lahav *et al.* 1999). Behaviour-modifying properties of synthetic hydrocarbons were demonstrated only recently in social wasps (Dani *et al.* 2001; Ruther *et al.* 2002). In several wasps the hydrocarbon profiles of the cuticle match those of the nest-paper surface (Espelie & Hermann 1990; Espelie *et al.* 1990), and there is evidence that hydrocarbons are used for nest recognition (Singer *et al.* 1998). In this study, we present results strongly indi-

cating that cuticular hydrocarbons are also involved in trail-following behaviour of the social wasp *V. vulgaris* L.

2. MATERIAL AND METHODS

(a) Insects

Experiments were carried out between July and September 2001 with three colonies of *V. vulgaris* (A, B and C) which were relocated a few weeks before from their natural nest sites (cavities in buildings) to a laboratory located in the garden of the Institute of Biology in Berlin-Dahlem. Colonies A, B and C consisted of about 600, 800 and 1400 foragers, respectively (number estimated according to Malham *et al.* (1991)). Colony A was used for the bioassays, gas chromatography–mass spectrometry (GC–MS) analyses of the cuticle were performed with foragers of all three colonies, and GC–MS analysis of the trail components was done with colonies B and C.

(b) Preparation of extracts

(i) Cuticular extract for bioassay

Previous work (Steinmetz *et al.* 2002) had shown that in *V. vulgaris* the trail does not transmit colony-specific information. Hence, foragers used for extraction were collected from all three colonies in order to reduce the disturbance of the social structure of the colonies to a minimum. The foragers were stored at -70°C and were extracted in 1 ml of hexane (for 60 s) on the next day. Bioassays were carried out with an extract from 18 foragers and with an extract from 78 foragers.

(ii) Cuticular extract for chemical analyses

For the analysis of the cuticular hydrocarbon profile of *V. vulgaris*, 15 foragers (5 per colony) were extracted for 60 s each in 1 ml of hexane. Five hundred microlitres of the extract were evaporated to dryness in a gentle stream of nitrogen. The residue was re-dissolved in 50 μl of dichloromethane, containing 10 $\text{ng } \mu\text{l}^{-1}$ docosane as an internal standard (IS). One microlitre of each extract was used for chemical analyses by GC–MS.

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Figure 1. Scheme of the experimental set-up, viewed from the top. Arrows mark the pathway of homecoming *Vespula vulgaris* foragers through the boxes. The arrows correspond to the position of the trail. By exchanging the glass plates of box no. 1 and no. 2 the direction of the trail was varied. Thick lines mark opened tubes.

(iii) Extraction of trail components

Trail components from colonies B and C were extracted from microscope slides (75 mm × 25 mm, six per colony) on which foragers had walked and laid a trail for two weeks. The trail of each colony was eluted with 0.3 ml of hexane. The solvent was removed and the residue was redissolved in 150 µl of dichloromethane, containing 10 ng µl⁻¹ docosane as an IS. One micro-litre of the extract was used for GC-MS.

(c) Bioassays

When leaving for foraging flights and returning to their nest boxes, *V. vulgaris* foragers had to pass through an experimental system (length ca. 1.5 m) consisting of two glass boxes, connected by two transparent plastic tubes (figure 1). By closing one of the tubes, only one possible path was offered to the workers for passing the experimental system on the removable glass plates (19 cm × 25 cm) at the bottom of the boxes. After 5 days, a biologically active trail (about 2 cm wide and 20 cm long on each glass plate) was laid by the foragers along this path. The trails on the glass plates were visible as a smeary film. Box 2 (figure 1) was used as test box. Under training and control conditions the trail ran to the left for the returning foragers in the test box and nearly all wasps (90%) followed this trail.

For the experiments the original glass plate was replaced with differently treated glass plates:

- (i) a glass plate with the own, naturally laid trail, but arranged in a novel direction to the right (this was achieved by exchanging plate no. 1 and no. 2 after turning them by 180°, see figure 1);
- (ii) a glass plate with an artificial trail (2 cm wide and 20 cm long) to the right laid by applying the 18-forager-extract using an Eppendorf pipette;
- (iii) a glass plate with an artificial trail (2 cm wide and 20 cm long) to the right laid by applying the 78-forager-extract;
- (iv) a glass plate with an artificial trail (2 cm wide and 20 cm long) to the right with hexane (control I);
- (v) an untreated glass plate (control II).

Subsequently, the reaction of the forager was recorded for 5 min defining the following behavioural categories:

Figure 2. Number of *Vespula vulgaris* foragers (colony A) shown as percentages that walked to the right-hand side on differently treated glass plates in the bioassay. con1, untreated glass plate (control 1); con2, hexane trail (control 2); F18, application of a cuticular extract from 18 foragers; F78, application of a cuticular extract from 78 foragers; nat, naturally laid trail. Different lowercase letters indicate significant differences at $p < 0.05$ (Bonferroni-corrected multiple χ^2 test).

- (i) the forager crossed the test box to the right-hand exit tube, following the chemical trail;
- (ii) the forager still used the left-hand exit tube experienced during the training situation;
- (iii) the forager was disorientated and did not choose any exit-tube.

The behavioural categories 'walking to the right' and 'walking to the left', respectively, were defined as walking straight on at least three-quarters of the glass plate (this distance was marked on the underside of the glass plate in test box no. 2) without stopping or flying. Social influences on forager orientation during testing periods were ruled out by using microscope-slide gates to allow individual observations. After each test period the training situation was maintained for at least 15 min to avoid habituation of the wasps to the test conditions. In the training situation, the naturally laid trail was in the training position and only the left tube was left open.

Because trail-following rate in *V. vulgaris* is higher at low luminous intensity compared with brightness (I. Steinmetz and E. Schmolz, unpublished data), all experiments were carried out at a maximal value of 5 lux.

(d) Chemical analysis

Analytical separations were performed on a Fisons model 8060 gas chromatograph, mass spectra were obtained on a MD800 quadrupole mass spectrometer (Thermo Finnigan, Egelsbach, Germany), and analytical conditions as follows: injector temperature: 240 °C; column: 30 m × 0.32 mm ID DB-5 ms fused silica column, film thickness 0.25 µm (J & W); carrier gas, helium; inlet pressure 10 kPa. The temperature programme was started at 150 °C, increasing at a rate of 3 °C min⁻¹ to 280 °C. Identification of the straight-chain hydrocarbons was carried out by comparisons of mass spectra and retention times with those of reference compounds. Branched hydrocarbons were identified by diagnostic ions resulting from the favoured fragmentation at the branching points (Nelson 1993). The positions of the double bonds of unsaturated hydrocarbons were determined by iodine-catalysed methylthiolation using dimethyl disulphide (Francis & Veland 1981). Peak areas for each compound were calculated and related to the total peak area for each run. Relative retention indices of branched and unsaturated hydrocarbons were calculated by using the retention times of

Table 1. Relative composition of cuticular extracts from workers and trails laid by workers of *Vespula vulgaris*. (IS, internal standard; sym, symmetric molecule leading to only one pair of diagnostic ions; DMDS, diagnostic ions after derivatization with dimethyl disulphide.)

peak number	linear retention index	compound	diagnostic ions	relative amount in worker (%)		relative amount in trail (%)	
				mean	s.d.	mean	s.d.
IS	2200	C22	310				
1	2300	C23	324	1.72	0.81	3.73	1.24
2	2335	11-MeC23 + 9-MeC23	168/169, 196/197 140/141, 224/225	1.47	1.99	0.75	2.35
3	2341	7-MeC23	112/113, 252/253	0.37	0.63	0.54	1.87
4	2349	5-MeC23	84/85, 280/281	0.54	0.82	0.30	0.48
5	2372	3-MeC23	309	1.89	2.52	1.08	1.56
6	2380	5,11-DiMeC23 + 5,9-DiMeC23	337, 84/85, 294/295, 182/183, 196/197 337, 84/85, 294/295, 154/155, 224/225	0.41	0.74	0.24	0.47
7	2400	C24	338	1.38	1.14	2.26	0.97
8	2405	3,13-DiMeC23 + 3,11-DiMeC23 + 3,9-DiMeC23 + 3,7-DiMeC23	337, 56/57, 323, 168/169, 210/211 337, 56/57, 323, 196/197, 182/183 337, 56/57, 323, 154/155, 224/225 337, 56/57, 323, 126/127, 252/253	1.05	2.00	0.51	0.97
9	2434	12-MeC24 + 10-MeC24 + 8-MeC24	182/183, 196/197 154/155, 224/225 126/127, 252/253	1.61	2.47	0.67	0.66
10	2444	3,5-DiMeC23	337, 56/57, 322/323, 98/99, 280/281	0.21	0.34	0.17	0.11
11	2456	2-MeC24	309, 337	0.85	1.22	0.56	0.58
12	2471	C25:9	444, 173, 271 (DMDS)	1.09	1.17	0.97	0.51
13	2479	C25:7	444, 145, 299 (DMDS)	0.30	0.27	0.97	3.49
14	2488	4,12-DiMeC24 + 4,10-DiMeC24 + 4,8-DiMeC24	351, 70/71, 323, 196/197 (sym) 351, 70/71, 323, 168/169, 224/225 351, 70/71, 323, 140/141, 252/253	0.79	1.47	0.41	0.68
15	2500	C25	352	12.64	7.43	15.33	7.69
16	2533	13-MeC25 + 11-MeC25 + 9-MeC25	196/197 (sym) 168/169, 224/225 140/141, 250/251	12.18	11.37	5.95	6.53
17	2540	7-MeC25	112/113, 280/281	1.51	1.85	0.77	0.96
18	2549	5-MeC25	84/85, 308/309	2.55	2.41	1.75	1.72
19	2562	9,15-DiMeC25 + 9,13-DiMeC25	365, 140/141, 266/267, 168/169, 238/239 365, 140/141, 266/267, 196/197, 210/211	0.51	1.18	0.31	0.24
20	2572	3-MeC25	337	6.55	7.32	5.91	4.51
21	2579	5,13-DiMeC25 + 5,11-DiMeC25 + 5,9-DiMeC25	365, 84/85, 323, 196/197, 210/211 365, 84/85, 323, 182/183, 224/225 365, 84/85, 323, 154/155, 252/253	2.34	3.16	1.38	1.91
22	2600	C26	366	1.89	1.59	2.99	2.50
23	2604	3,13-DiMeC25 + 3,11-DiMeC25 + 3,9-DiMeC25 + 3,7-DiMeC25	365, 56/57, 351, 196/197, 210/211 365, 56/57, 351, 182/183, 224/225 365, 56/57, 351, 154/155, 252/253 365, 56/57, 351, 126/127, 280/281	4.02	5.96	2.24	3.44
24	2631	13-MeC26 + 12-MeC26 + 10-MeC26	196/197, 210/211 182/183, 224/225 154/155, 252/253	2.20	1.77	1.34	1.30
25	2643	3,5-DiMeC25	365, 56/57, 351, 98/99, 308/309	0.19	0.17	0.20	0.15
26	2656	2-MeC26	337	0.65	0.49	0.72	0.71
27	2673	C27:9	472, 173, 299 (DMDS)	1.00	0.80	0.76	0.77
28	2681	C27:7	472, 145, 327 (DMDS)	0.11	0.16	0.05	0.13
29	2687	4,12-DiMeC26 + 4,10-DiMeC26 + 4,8-DiMeC26	379, 70/71, 351, 196/197, 224/225 379, 70/71, 351, 168/169, 252/253 379, 70/71, 351, 140/141, 280/281	0.71	0.89	0.52	0.64
30	2700	C27	380	6.31	4.41	13.35	11.83
31	2731	13-MeC27 + 11-MeC27 + 9-MeC27	196/197, 224/225 168/169, 252/253 140/141, 280/281	6.13	4.75	3.97	3.25

(Continued.)

Table 1. (Continued.)

peak number	linear retention index	compound	diagnostic ions	relative amount in worker (%)		relative amount in trail (%)	
				mean	s.d.	mean	s.d.
32	2739	7-MeC27	112/113, 308/309	0.40	0.38	0.37	0.50
33	2747	5-MeC27	84/85, 336/337	0.64	0.49	1.79	4.40
34	2757	11,15-DiMeC27	393, 168/169, 266/267, 196/197, 238/239	1.14	0.97	0.77	0.76
		+ 9,13-DiMeC27	393, 140/141, 294/295, 210/211, 224/225				
35	2771	3-MeC27	336/337, 364/365	3.16	3.74	4.11	2.55
36	2776	5,13-DiMeC27	393, 84/85, 351, 210/211, 224/225	0.96	0.84	0.76	0.79
		+ 5,9-DiMeC27	393, 84/85, 351, 154/155, 280/281				
37	2800	C28	394	3.96	2.81	3.56	2.77
		+ 3,13-DiMeC27	393, 56/57, 379, 210/211, 224/225				
		+ 3,11-diMeC27	393, 56/57, 379, 182/183, 252/253				
		+ 3,9-DiMeC27	393, 56/57, 379, 154/155, 280/281				
		+ 3,7-DiMeC27	393, 56/57, 379, 126/127, 308/309				
38	2829	14-MeC28	210/211, 224/225	0.67	0.58	0.59	0.27
		+ 12-MeC28	182/183, 252/253				
		+ 10-MeC28	154/155, 280/281				
		+ 8-MeC28	126/127, 308/309				
39	2857	2-MeC28	365	0.28	0.18	0.51	0.19
40	2875	C29:9	500, 173, 327 (DMDS)	0.81	0.77	0.56	1.59
41	2886	4,14-DiMeC28	407, 70/71, 379, 224/225 (sym)	0.30	0.29	0.30	0.13
		+ 4,12-DiMeC28	407, 70/71, 379, 196/197, 252/253				
		+ 4,10-DiMeC28	407, 70/71, 379, 168/169, 280/281				
		+ 4,8-DiMeC28	407, 70/71, 379, 140/141, 308/309				
42	2900	C29	408	1.77	2.02	5.86	8.43
43	2929	15-MeC29	224/225 (sym)	1.14	1.13	1.17	1.11
		+ 13-MeC29	196/197, 252/253				
		+ 11-MeC29	168/169, 280/281				
44	2955	13,17-DiMeC29	421, 196/197, 266/267 (sym)	0.34	0.27	0.31	0.21
		+ 11,15-DiMeC29	421, 168/169, 294/295, 224/225, 238/239				
		+ 9,13-DiMeC29	421, 140/141, 322/323, 210/211, 252/253				
45	2972	3-MeC29	393	0.38	0.99	0.51	0.44
46	2976	5,13-DiMeC29	421, 84/85, 378/379, 154/155, 308/309	0.39	0.39	0.27	0.09
		+ 5,9-DiMeC29	421, 84/85, 378/379, 210/211, 252/253				
47	3001	3,13-DiMeC29	421, 56/57, 407, 210/211, 252/253	1.94	2.11	2.37	0.54
		+ 3,11-DiMeC29	421, 56/56, 407, 182/183, 280/281				
		+ 3,9-DiMeC29	421, 56/56, 407, 154/155, 308/309				
48		cholesterol		3.26	3.00	0.00	0.00
49	3100	C31	436	1.10	1.98	2.98	5.14
50	3200	C32	450	0.96	1.43	0.53	1.43
51	3300	C33	464	0.55	0.99	0.55	1.54
52	3400	C34	478	0.38	0.71	0.00	0.00
53	3500	C35	492	0.30	0.61	0.00	0.00

the straight-chain hydrocarbons present in the extracts (Van den Dool & Kratz 1963).

(e) *Statistical analysis*

Bioassay data were analysed by means of the Bonferroni-corrected χ^2 test (Zar 1984) using SIGMASTAT, v. 2.03 (Jandel Scientific, Erkrath, Germany).

3. RESULTS

(a) *Bioassay*

After changing the direction of the naturally laid trail by exchanging the glass plates of boxes 1 and 2, 46% of

the foragers followed the new direction (figure 2). Twenty-seven per cent of the foragers followed the old direction they had learned during the training situation, and 27% were disorientated. By contrast, when the control plates were exposed to the foragers, only 5% (untreated glass plate: old direction 25%, disorientated 70%) and 4% (hexane-treated glass plate: old direction 30%, disorientated 66%), respectively, changed the direction. This demonstrates that *V. vulgaris* foragers orient by chemical trail following. Both cuticular extracts from 18 foragers (28% trail following, 19% old direction, 53% disorientated) as well as from 78 foragers (47% trail following, 15% old direction, 38% disorientated) induced

Figure 3. Reconstructed ion chromatograms of (a) a cuticle extract and (b) a trail extract from *Vespula vulgaris* colony C. Peak numbers correspond to table 1. Abbreviations: IS, internal standard; c, contaminant; rt, retention time.

trail following in *V. vulgaris* foragers. The artificial trail obtained by applying the 78-forager extract was as active as the naturally laid trail.

(b) Chemical analyses

In total 95 straight-chain, methyl-branched or mono-unsaturated hydrocarbons were identified in the cuticular extracts from *V. vulgaris* foragers (table 1). Methyl-branched hydrocarbons were most abundant (64%), followed by saturated (29%) and monounsaturated straight-chain hydrocarbons (3%). In the cuticular extracts cholesterol (3%) was found, probably originating from the wasps' cell membranes. The chain length of the hydrocarbons varied between 23 and 35 carbon units. Chemical analysis of the trail extracts from colonies B and C revealed that the majority of these hydrocarbons were also present there at similar ratios (figure 3).

4. DISCUSSION

The present study is the first, to our knowledge, providing evidence that cuticular lipids act as a trail pheromone in a social wasp. Extracts of the naturally laid trail as well as the behaviourally active cuticular extracts from *V. vulgaris* consisted largely of the same hydrocarbons at similar ratios. This strongly indicates that these hydrocarbons are the cues responsible for the observed chemical orientation of *V. vulgaris* foragers. It has to be mentioned here that the ultimate proof about the behavioural activity of cuticular hydrocarbons can only be provided by performing experiments with synthetic chemicals. This has hardly ever been done in studies addressing the role of cuticular hydrocarbons in communication of social insects, as cuticular hydrocarbon mixtures of insects are very complex and may contain numerous unsaturated and methyl-branched

components that are not commercially available. Furthermore, many methyl-branched hydrocarbons are chiral, and gas chromatographic columns to separate enantiomers of such large molecules are not available at present. Therefore, tremendous efforts are necessary in the future to elucidate the stereochemistry and to synthesize these compounds before this problem can be solved satisfactorily.

The finding that the trail of *V. vulgaris* simply consists of cuticular hydrocarbons may be a simple explanation for the presence of chemical terrestrial trails in flying hymenopterans. Studies with several social wasps (Espelie & Hermann 1990, 1988; Espelie *et al.* 1990) showed the similarity of cuticular and nest-paper hydrocarbon profiles. According to the 'Gestalt model' these molecules can be transferred between the workers, from workers to the nest substrate and vice versa (Crozier & Dix 1979; Pfennig *et al.* 1983; Singer *et al.* 1998). Originally, these molecules were supposed to contribute to the common nest odour and serve for nest and nestmate recognition in several taxa of social hymenopterans (e.g. Hangartner *et al.* 1970; Espelie *et al.* 1990; Breed & Stiller 1992; Layton & Espelie 1995; Singer *et al.* 1998; Vander Meer & Morel 1998). In species with exposed nests, the majority of the Vespinae (Wenzel 1991), foragers perceive the nest odour molecules when approaching the nest by flying. *V. vulgaris* is characterized by cavity breeding (Greene 1991), which appears to be a derived character within the Vespinae when mapped on a phylogenetic tree of this group (Carpenter 1991; Wenzel 1991). *V. vulgaris* foragers usually walk through tunnels between nest and cavity entrance. We hypothesize that hydrocarbons are transferred deliberately from exocrine glands or passively from the insects' cuticle to the substrate when the wasps walk in the vicinity of the nest. In this way the nest odour is expanded as a trail, which is used for homing orientation. Hence, our findings may give an idea about the evolution of trail-following behaviour in social wasps. In the present study, nest odour substances, serving primarily for nest recognition, are also used for chemical orientation outside the nest in a cavity-breeding wasp. Thus, nest odour molecules can be rated as a preadaptation for the evolution of breeding in dark and narrow cavities with changed demands on orientation mechanisms, compared with nest orientation towards exposed nests.

Previous examination showed that the degree of trail-following behaviour of *V. vulgaris* foragers was the same on trails laid by nestmates and by non-nestmates (Steinmetz *et al.* 2002). This indicates that in *V. vulgaris* colony-specific information is not important in the context of trail orientation. Thus, the chemical trail in *V. vulgaris* seems to play a part only for orientation and does not serve as a home range marker, as known, for example, for ant trails (Hölldobler & Wilson 1990).

The fact that between 15% and 27% of the foragers chose the left-hand exit tube they had experienced during the training situation instead of the chemical trail demonstrates that cues other than the trail pheromone are also involved in orientation of the wasps. The number of wasps following the chemical trail can be increased dramatically by performing the experiments in the dark (I. Steinmetz and E. Schmolz, unpublished data). This demonstrates that optical cues contribute significantly to wasp orien-

tation during the laboratory bioassays. However, chemical orientation should dominate in the natural situation where nests of *V. vulgaris* are often located in the soil, being connected to the ambience by tunnels.

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