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ORIGINAL PAPER

The interplay between genetic and environmental effects on colony insularity in the clonal invasive little fire ant *Wasmannia auropunctata*

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Abstract The little fire ant, *Wasmannia auropunctata*, constitutes one clonal supercolony throughout Israel, providing an opportunity to examine the effects of genotype versus environment on nestmate recognition. Intraspecific encounters among field-collected or among laboratory-maintained colonies were nonaggressive, but encounters between freshly collected and laboratory-maintained colonies were highly aggressive. Analyses of cuticular hydrocarbons revealed that freshly field-collected colonies had distinguishable profiles. Moreover, freshly collected colonies had profiles disparate from those of the same colonies after 4 months in the laboratory. These results indicate a strong interplay between genetic-based and environmentally based effects on the recognition cues. We propose that in the field the ants' diet breadth is broad and consequently the incorporation of diet-borne substances is insufficient to mask the genetically determined cues. In the laboratory, however, the restricted diet promoted the incorporation of alien hydrocarbons at high levels, thus altering the genetically based cues to the point of alienation. These results shed a new light on the mechanisms by which environmental cues may affect label and/or template formation in ants.

Keywords Invasive species · *Wasmannia auropunctata* · Cuticular hydrocarbons · Nestmate recognition

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Introduction

Discriminating between nestmates and nonnestmates is crucial for the functioning of social insect colonies; nestmate recognition allows them to reject alien conspecifics and thus prevent them from taking advantage of the colony's wealth (Hölldobler and Wilson 1990). Cuticular hydrocarbons (CHC) were shown directly or indirectly to serve as nestmate recognition cues in several ant species (reviewed by Hefetz 2007). The composition of ant CHCs is generally dynamic (Lahav et al. 2001) and is affected both by the genetic makeup of the colony and environmental influences such as diet (Liang and Silverman 2000; Richard et al. 2004; Silverman and Liang 2001), habitat (Heinze et al. 1996; Nielsen et al. 1999), or time of year (Nielsen et al. 1999). Novel hydrocarbons are rapidly integrated into the recognition system by means of continuous exchanges between nestmates (Lenoir et al. 2001; Soroker et al. 1995).

The relative contribution of environmental factors *vs.* innate factors on nestmate recognition can vary according to species. Zinck et al. (2008) suggested that both genetic and environmental cues are involved in nestmate recognition in *Ectatomma tuberculatum*. In *Temnothorax longispinosus*, however, genetic relatedness was found to be a more important contributor to aggressive behavior than geographic distances (Stuart and Herbers 2000). In contrast, Chen and Nonacs (2000) claimed that nestmate recognition

in *Linepithema humile* is based primarily on environmental cues in the introduced populations since intraspecific aggression disappeared after 2 months under uniform conditions in the laboratory. Similarly, Obin et al. (1993) found that, in *Solenopsis invicta*, despite the fact that field-collected colonies show low intraspecific aggression, this becomes elevated in laboratory-maintained colonies of polygynous ants and is determined by the ants' diet rather than their collection locality. Workers of polygyne nests of *S. invicta* showed aggression towards other colonies that were fed on a different diet, regardless of their collection locality, but not when fed on the same diet.

Environment can also affect the broadness of the template. In the fire ant *S. invicta*, it was suggested that, when colonies are reared in the laboratory under uniform conditions, newborn workers develop with a template that accepts only a narrow range of nestmate recognition cues. Thus, when encountering a field-born worker, the odds are that it will not fit the template and will therefore be recognized as alien (Tschinkel 2006).

Unicolonial ants form supercolonies, in which there are no colony boundaries and no aggression between colonies (Hölldobler and Wilson 1977). It is assumed that these ants are free of the need to protect their territory against conspecifics and might hence divert their energy towards interspecific competition and high reproduction rates, achieving high population densities and a numerical advantage (Abbott 2005; Giraud et al. 2002; Hölldobler and Wilson 1977; Holway et al. 1998; Holway and Suarez 2004; Porter et al. 1997; Suarez et al. 1999; Tsutsui et al. 2000). Unicoloniality is overrepresented in invasive and pest ant species, compared to its rate among the Formicidae (Holway et al. 2002). The question of whether unicolonial species have lost the ability to discriminate between nestmates and nonnestmates is still unresolved. In the unicolonial ant *Formica paralugubris*, it seems that although workers were able to discriminate nestmate from nonnestmate and despite significant genetic differences between colonies, there was no intercolonial aggression. However, the nestmate recognition cues were not studied (Chapuisat et al. 2005; Holzer et al. 2006). In fact, the role of CHCs as nestmate recognition cues in unicolonial ants is little understood.

Wasmannia auropunctata, the little fire ant, is a world-wide invasive species. Native to South and Central America, it has invaded some Pacific and Caribbean Ocean islands, West Africa, and Australia (DPI&F 2006; Holway et al. 2002; Wetterer and Porter 2003). First discovered in Israel at the end of 2005, we assume that it had arrived in the north of Israel about 8 years earlier and since then it has been discovered in 23 localities throughout the country. Because of the unique clonal reproduction system identified in at least some populations (Fournier et al. 2005a; Foucaud et al. 2006, 2007), it is especially interesting to study this species' social structure. In the current study, we determined the reproduction system and population genetic structure of *W. auropunctata* in the only nontropical area of its introduction range, Israel. Inferring from other invasive populations of this species, we then capitalized on the presumed clonal reproduction system of the species in Israel in order to obtain further insight into the potential impact of environmental factors on nestmate recognition. This was achieved by conducting aggression tests and analyzing CHC profiles of field-collected and laboratory-reared colonies. We more specifically addressed the following questions: (1) what is the genetic structure of *W. auropunctata* population in Israel and is this the consequence of a single introduction event or multiple introductions? (2) Does *W. auropunctata* behave as a unicolonial species in Israel? And (3) what is the role of environment *vs.* genotype in shaping the nestmate recognition cues in this species and, specifically, how does a narrow diet affect the workers' patterns of intraspecific aggression?

Materials and methods

Sampling

Two hundred eighty-five colonies of *W. auropunctata* were collected from discrete nests in Israel between December 2005 and August 2007 in 13 out of 23 known infestation localities. Each colony contained a few hundred to a few thousand workers and brood and one to 60 queens. Some colonies also had gynes and males. The colonies were kept in the laboratory in artificial nests with a moistened plaster floor that also served as foraging arena. The colonies were supplied with an identical diet consisting of crickets (*Acheta domestica*) and sugar water (1:1 *w*/*v*) twice a week. Water was supplied ad libitum, and all colonies were kept at a constant temperature of 25°C, 70% relative humidity, and 14:10 L–D regime.

Colonies of *Paratrechina longicornis*, *Pheidole teneriffana*, and *Monomorium subopacum* used for the interspecific aggression tests were collected either in Tel Aviv or in the Jordan Valley, where they are sympatric with *W. auropunctata*. The colonies were kept in the laboratory under the conditions described above.

Genetic analysis

We collected *W. auropunctata* samples for DNA analysis from 29 colonies in 13 localities. Distances between colonies in each locality ranged from a few meters to 660 m, according to the size of the infested village. For each colony, we analyzed six to eight workers and zero to three queens and, when available, males and gynes. The

fertilized queens were dissected under a binocular microscope in order to obtain sperm samples from their spermathecae. In total, we genotyped 230 workers, 53 queens, nine males, three gynes, and sperm from 35 spermathecae (all 53 wingless queens were dissected, but the spermatheca in 18 of these queens was either not found or damaged during the dissection).

We extracted individual DNA using cetyltrimethylammonium-bromide-based protocol (Doyle and Doyle 1987). Following Fournier et al. (2005b), 12 microsatellite loci were amplified using polymerase chain reaction and genotyped using a MegaBACE DNA sequencer (Amersham Biosciences, Uppsala, Sweden). We analyzed the results using a personal computer program programmed in Pascal object programming language, identifying identical multilocus genotypes (see Foucaud et al. 2006; inquiries about this program should be sent to JF or AE). Genetic diversity measures, *F* statistics (Weir and Cockerham 1984), and corrected or uncorrected relatedness coefficients (as defined in Pamilo 1985) were computed using FSTAT 2.9.3 (Goudet 2001).

Behavioral assays

The behavioral experiments constituted group aggression tests of three types: intraspecific same locality; intraspecific different localities; and interspecific (with each of the three ant species listed above). For the intraspecific same-locality tests, we used nine colonies from three different localities (three colonies per locality). Using these nine colonies, we constructed 11 different group–encounter pairs, for each of which we performed two to three replicate encounters. Replicates of each pair were averaged and constituted a single case (n=11). In addition, we conducted six tests using different colonies without per group–pair replicas (n=17 in total). Intracolonial tests served as control (ten colonies in total). For the intraspecific tests among localities (total of 38 tests), we used different colonies from the same locality as replicates since there was no aggression between such colonies (see "Results"). All the above intraspecific tests were performed with colonies kept in the laboratory for no longer than 30 days; all colonies used were in good condition and contained a few queens, brood, and at least a few hundred workers. Colonies from the same locality were collected at a distance of between 10 and 660 m from one another. Interspecific aggression tests included encounters between *W. auropunctata* and *P. longicornis* (n=6), *P. teneriffana* (n=8), or *M. subopacum* (n=9).

To confirm the effect of laboratory-rearing conditions on ant aggression, we performed a series of intraspecific encounters between the above freshly collected *W. auropunctata* colonies from the field and workers reared in the laboratory for 40–140 days (total of 53 experiments, 40 from different localities and 13 from the same locality). We also performed encounters between colonies that were reared in the laboratory for over 40 days (20 experiments, 19 of them between colonies from different localities).

Each aggression test consisted of an encounter between two groups of ten workers (intraspecific or interspecific) in a neutral arena, i.e., in a Fluon-coated Petri dish (9-cm diameter, 1.5 cm high). Prior to testing, each group was confined in an upright Fluon-coated glass tube for 20 min to acclimate. The experiment started by removing the glass tubes and allowing the two groups to interact for 15 min. The number of aggressive interactions (number of events in which at least one worker was observed biting another worker) was monitored by spot checks every 30 s (Errard et al. 2005). Because of the extremely small size of the ants (1.5 mm), we could not mark them individually without seriously hampering their behavior (and certainly interfering with the perception of CHCs). Therefore, we could not determine the colony origin of the attacking or attacked ants. Data are expressed as cumulative acts of aggression per test. The interspecific encounters were conducted as above. In all cases, workers were used only once for each test.

Kruskal–Wallis analysis of variance by ranks, followed by multiple comparisons of mean rank post hoc test, was used to compare cumulative numbers of aggressive interactions in the intraspecific and interspecific aggression tests. All analyses were performed using Statistica 7.1 (StatSoft Inc., OK, USA).

Chemical analysis

For comparison of CHC profiles, colonies of *W. auropunctata* were collected throughout their introduced range in Israel. A total of 16 nests from six of the most infested localities, separated by distances of 20 to 660 m in each locality, were used in the analyses. To assess whether the CHC profiles change under standard laboratory conditions, five nests were analyzed twice: at 0–2 days after collection (t_0) and after 90–105 days in the laboratory (t_1). Two additional nests were field-sampled at the same time as the second analysis (t_1) adjacent to the above localities and analyzed immediately after collection from the field for additional comparison.

For extraction, whole freshly frozen ants (−20°C for a few min) were immersed in pools of 50 workers from each nest (five replicates per nest) in 400-µl pentane for 1 h, after which the solution was withdrawn into a separate vial and stored at −20°C until analysis. Each sample was supplemented with octadecane and triacontane as internal standards (625 ng/20 µl and 750 ng/20 µl, respectively). Because we assumed that change in CHC profiles of the ants in the laboratory could be attributed to their diet, we studied the CHC composition of the domestic cricket *A. domestica*.

Abdominal cuticle of five adults was extracted as above, but the extract was further purified (for eliminating polar cuticular components) using an ethyl-acetate-prewashed Florisil column (Merck, Germany) and eluted with pentane.

Primary identification of the total body wash was performed by combined gas chromatography/mass spectrometry (GC/MS at the EI mode, 70 eV) using a DB-1 capillary column that was temperature-programmed from 90°C (1 min hold) to 280°C at 5°C/min. Compound identification was done by their mass fragmentation and in comparison with available synthetic compounds.

Quantification of the cuticular hydrocarbons was done by GC (Varian CP 3900) using a DB-1 fused silica column (30 m long, 0.25-mm inner diameter) that was temperature-programmed from 90°C (1 min hold) to 300°C at 5°C/min with a final hold of 15 min. Compound quantification was done by peak integration (Galaxie Chromatography Data System 1.8).

CHC profiles of 16 colonies from the above-mentioned six localities were compared by standard stepwise discriminant function analysis. CHC profiles of laboratory *vs.* field colonies were compared by principal components analysis (PCA). For each of the above two types of analyses, 21 compounds responsible for more than 1% of the total peak area were used. In order to analyze the change in the CHC profile of *W. auropunctata* in the laboratory, we calculated the ratios of the two cricket compounds, 4-methyloctacosane and 4-methyltriacontane (Fig. S 2c) to the ant compound 3-methylheptacosane. As control, we also calculated the ratio of 5-methylheptacosane to 3-methylheptacosane, which are both ant compounds. 4-Methyloctacosane and 4-methyltriacontane were used in the PCA but not in the discriminant function analysis because in field colonies they were either missing or only in trace amounts, which made their quantification unreliable and therefore were not taken into consideration in the discriminant function analysis. In laboratory-reared colonies, on the other hand, they were present in considerable and quantifiable amounts. The average ratio per nest was arcsine transformed and compared by dependent *t* test. All analyses were performed using Statistica 7.1 (StatSoft Inc., OK, USA).

Results

Genetic analysis

We found that all queens and gynes shared the same genotype to all microsatellite loci, the few observed differences corresponding to rare mutational or conversion events (i.e., the conversion of a heterozygous locus into a locus homozygous for one of the two parental alleles; see Foucaud et al. 2006). Therefore, our results are compatible with the clonal reproduction of queens through thelytokous parthenogenesis and the introduction of a single queen genotype in Israel. All males and spermathecae contents had the same multilocus genotype, with alleles differing from those of the queen genotype at ten of the 12 loci (Table 1). Finally, the multiple genotypes obtained for workers were all compatible with a sexual mix of the male and queen genotypes (Table 1). Therefore, the Israeli population originates from one queen and one male genotype, which both reproduce clonally, while workers are produced through sexual reproduction between queens and males (see Foucaud et al. 2006, 2007; Fournier et al. 2005a, for further details regarding this original reproduction system).

The introduction of a single clonal male genotype and a single clonal queen genotype in Israel and the sexual production of worker offspring translate into unusual but coherent population genetic statistics for workers. The worker allelic richness per locus over all sampled localities always equals two (when the introduced pair shares one allele) or three (when the introduced pair does not share any allele; Table 1). For the same reasons, the observed heterozygosities per locus equal either one or 0.5 (Table 1). The F_{st} value computed taking localities as unit is null, indicating an absence of spatial genetic structure in Israel (Table S1). This result is in accord with both a single introduction event and the above-mentioned reproduction system. The F_{is} value is negative, indicating a strong outbreeding signal at the worker level. This result simply reflects the fact that the clonal male and queen genotypes generating the worker genotypes through sexual reproduction include different alleles at most loci. When computed without taking into account the above outbreeding signal, the relatedness coefficient between workers of the same locality is null (*r* in Table S1). This is expected because all Israeli workers are the offspring of a single genotypic pair so that workers from different localities are genetically similar. On the other hand, when taking into account the outbreeding signal within the Israeli population (see Pamilo 1985 for details), the relatedness coefficient measure between workers becomes very close to 0.75 (*r** in Table S1). The latter value, which corresponds to the relatedness level theoretically expected between full sisters in haplodiploids, conforms with the presence of a single clonal male genotype and a single clonal queen genotype in Israel. Similar results were obtained when considering different spatial scales and treatment units (e.g., nest instead of locality; results not shown).

Aggression tests

The results of the aggression tests were significantly affected by the treatment (colony origin, species, and rearing conditions. Fig. 1; Kruskal–Wallis test for all

Table 1 Individual genotypes at 12 microsatellite loci of queens, gynes, spermathecae contents, males, and workers of three colonies (KI1-3) collected in Kinneret, in the Jordan Valley in Israel (as an example). Individual allelic patterns clearly show clonal reproduction of males and queens and production of workers through sexual reproduction.

	1/166		1/gam		2/164		2/25		2/75		3/176		4/18		5/21		5/66		6/80		7/16		7/30	
Queens																								
KI1Q1	91	99	282	307	280	282	221	223	105	117	222	222	98	104	201	197	257	269	153	153	188	188	177	179
KI1Q2	91	99	282	307	280	282	221	223	105	117	222	222	98	104	201	197	257	269	153	153	188	188	177	179
KI2Q1	91	99	282	307	280	282	221	223	105	117	222	222	98	104	201	197	257	269	153	153	188	188	177	179
KI3Q1	91	99	282	307	280	282	221	223	105	117	222	222	98	104	201	197	257	269	153	153	188	188	177	179
KI3Q2	91	99	282	307	280	282	221	223	105	117	222	222	98	104	201	197	257	269	153	153	188	188	177	179
Gynes																								
KI1G2	91	99	282	307	280	282	221	223	105	117	222	222	98	104	201	197	257	269	153	153	188	188	177	179
KI2G1	91	99	282	307	280	282	221	223	105	117	222	222	98	104	201	197	257	269	153	153	188	188	177	179
KI2G2	91	99	282	307	280	282	221	223	105	117	222	222	98	104	201	197	257	269	153	153	188	188	177	179
Spermateca																								
KI1S1	87		302		280		225		103		224		112		201		265		165		192		199	
KI1S2	87		302		280		225		103		224		112		201		265		165		192		199	
KI3S2	87		302		280		225		103		224		112		201		265		165		192		199	
Males																								
KI2M3	87	302	280	225	103	224	112	201	265	165	192	199												
Workers																								
KI1W1	87	91	302	282	280	280	225	221	103	105	224	222	112	98	201	201	265	257	165	153	192	188	199	179
KI1W2	87	91	302	307	280	280	225	223	103	117	224	222	112	98	201	197	265	269	165	153	192	192	199	179
KI1W3	87	91	302	307	280	280	225	223	103	117	224	222	112	98	201	197	265	269	165	153	192	192	199	179
KI2W1	87	99	302	282	280	282	225	223	103	117	224	222	112	104	201	201	265	269	165	153	192	188	199	179
KI2W2	87	99	302	307	280	282	225	223	103	105	224	222	112	98	201	197	265	257	165	153	192	192	199	179
KI2W3	87	99	302	282	280	280	225	221	103	105	224	222	112	104	201	201	265	257	165	153	192	188	199	179
KI3W1	87	99	302	282	280	282	225	223	103	117	224	222	112	104	201	197	265	269	165	153	192	188	199	177
KI3W2	87	91	302	307	280	280	225	223	103	117	224	222	112	98	201	197	265	269	165	153	192	192	199	179
KI3W3	87	99	302	307	280	280	225	223	103	117	224	222	112	104	201	197	265	269	165	153	192	192	199	179

treatments together: H=99.87, $p<0.0001$). The tests comprising encounters between nestmates revealed, as expected, zero aggression ($n=10$). Likewise, the intraspecific encounters involving different nests that were all freshly field-collected exhibited very low aggression levels, irrespective of whether involving workers from the same locality or from different localities (multiple comparison [two-tailed], $p>0.99$). Low aggression also characterized the intraspecific encounters involving colonies that were reared in the laboratory for over 40 days (multiple comparison [two-tailed], $p>0.99$). In contrast, the interspecific encounters exhibited a significantly higher aggression than the above-mentioned intraspecific encounters (in Fig. 1, the three species were pooled together since there were no significant differences between them, Kruskal–Wallis $H=2.53$, $p=0.28$). Interestingly, encounters between freshly field-collected colonies and colonies that were maintained in the laboratory for over 40 days were highly aggressive, significantly more than the intraspecific encounters (multiple comparison [two-tailed], $p<0.0001$) and not different from the interspecific encounters (multiple comparison [two-tailed], $p>0.99$).

Chemical analysis

Figure 2 depicts the CHC composition of field-collected *W. auropunctata* workers. It consisted of at least 50 compounds, ranging from heneicosane to dimethyl tritriacontane, and was dominated by four peaks representing pentacosane, 5-methylpentacosane, heptacosane, and a mixture of 11-, 13-, and 15-methylheptacosane, comprising 53.98%±3.99 of the total extract (average±SD, $n=125$; calculated from relative peak area). Oleic and palmitic acid were sporadically detected in the chromatograms and were excluded from the analyses.

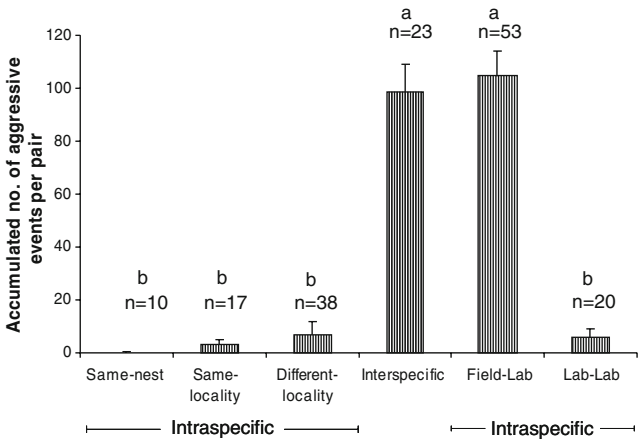

Fig. 1 Cumulative number of aggressive events per experiment in group encounters. Intraspecific encounters comprised workers from: the same nest; nests from the same locality; nests from different localities; nests reared in the laboratory for over 40 days *vs.* freshly collected nests ("Field–Lab"); and two groups of laboratory-reared nests ("Lab–Lab"). Interspecific tests were conducted with three species found within *W. auropunctata*'s invasive range (see text for further details). *Different letters* indicate significant differences (Kruskal–Wallis followed by multiple-comparison test)

Discriminant analyses based on colonial CHC profiles (Fig. 3a) showed colony specificity, with each colony having a distinct profile as all five replicas for each clustered very closely together, irrespective of their collection site. Nonetheless, the analysis based on localities showed that nests from the same locality (three or two nests per locality) clustered together and were distinct from those of other localities (Fig. 3b). No direct relationship was found between geographic distance and CHC profile resemblance, neither the six nests presented in Fig. 3a, collected 260–930 m from one another in each village, nor the six localities presented in Fig. 3b, clustered according to geographic distance: although Maabarot and Menahemya are separated by 70 km compared to 5.5 km separating Menahemya and Kinneret, they were all separated by similar distances in the discriminant analysis.

Worker CHC profiles were also affected by time and rearing conditions (Fig. S 2a and 2b). Profile comparison by principal components analysis of five freshly collected nests (t_0) and the same nests after 90–105 days under laboratory conditions (t_1) revealed that the profiles of each of them had not only changed but that field-collected colonies were also clearly separated from the laboratory-reared colonies by the first axis (Fig. 4). Furthermore, the five major compounds responsible for the observed variance between freshly collected nests and laboratory nests explained by PC1 were major compounds of the crickets. To verify that profile deviation of the colonies was due to laboratory conditions, we compared two additional nests that were collected at the same place from the field at t_1 and immediately analyzed. These clustered together with t_0 (the freshly collected nests) and were separated from the laboratory nests (Fig. 4).

To verify the impact of a narrow homogenous cricket diet on worker CHC composition of laboratory-maintained colonies, we also analyzed cricket CHCs (Fig. S 2c). Two of the dominant cricket compounds, 4-methyloctacosane and 4-methyltriacontane, increased significantly in laboratory-reared *W. auropunctata* workers between t_0 and t_1 (dependent *t* test, $t=-15.75$, $df=4$, $p<0.0001$ and $t=-11.17$, $df=4$, $p<0.001$, respectively, Fig. 5). In comparison, the amount of 5-methylheptacosane, which appears in *W. auropunctata* in a similar amount to the above compounds but is present only in a small amount in crickets, did not change (dependent *t* test, $t=-0.33$, $df=4$, $p=0.75$, Fig. 5).

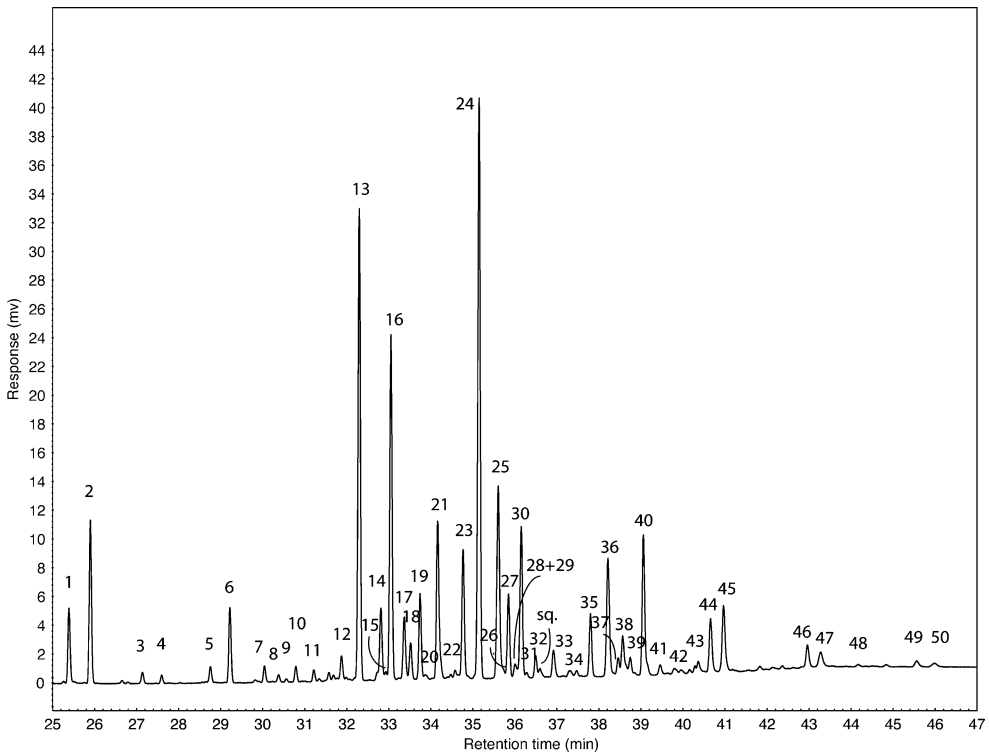

Fig. 2 Gas chromatogram of total body wash of *W. auropunctata* workers. Peak identity (*sq.*=squalene. Compounds marked with * were used for the discriminant analyses; compounds marked with § were used for the principal components analysis): (*1*) heneicosene; (*2*) heneicosane*§; (*3*) 3-methylheneicosane*; (*4*) docosane; (*5*) tricosene; (*6*) tricosane*§; (*7*) 5-methyltricosane; (*8*) 3-methyltricosane; (*9*) 5, 9-dimethyltricosane; (*10*) tetracosane; (*11*) 10-+12-+14-methyltetracosane; (*12*) pentacosene*; (*13*) pentacosane*§; (*14*) 9-+11-+13-methylpentacosane*§; (*15*) 7-methylpentacosane; (*16*) 5-methylpentacosane*§; (*17*) 3-methylpentacosane*§; (*18*) 5, 9-dimethylpentacosane*§; (*19*) hexacosane*§; (*20*) 10-+12-+14-methylhexacosane; (*21*) 12,14-dimethylhexacosane; (*22*) 4-methylhexacosane; (*23*) heptacosene*§; (*24*) heptacosane*§; (*25*) 11-+13-+15-methylheptacosane*§; (*26*) 7-methylheptacosane; (*27*) 5-methylheptacosane*§; (*28*) 11,15-dimethylheptacosane; (*29*) 9, 13-dimethylheptacosane; (*30*) 3-methylheptacosane*§; (*31*) 3,9-dimethylheptacosane; (*32*) octacosane; (*33*) 12-+14-+16-methyloctacosane*; (*34*) 4-methyloctacosane§; (*35*) nonacosane*§; (*36*) 11-+13-+15-methylnonacosane*§; (*37*) 5-methylnonacosane; (*38*) 11,15-+13, 15-dimethylnonacosane*§; (*39*) 3-methylnonacosane; (*40*) 12-methyltriacontane; (*41*) 12,14-dimethyltriacontane; (*42*) 4-methyltriacontane§; (*43*) hentriacontane; (*44*) 11-+13-+15-methylhentriacontane*§; (*45*) 11,15-+13,15-dimethylhentriacontane*§; (*46*) 11-+13-+15-methyltritriacontane§; (*47*) 11,15-+13,15-dimethyltritriacontane; (*48*) pentatriacontane; (*49*) 11-methylpentatriacontane; (*50*) 15,19-dimethylpentatriacontane

Discussion

Biological invasions provide a unique opportunity to study basic processes in population biology (Sakai et al. 2001). The invasive ant *W. auropunctata* exhibits a particular mode of reproduction in at least some populations of its native and invasive ranges (Foucaud et al. 2006, 2007; Fournier et al. 2005a). The queens and males reproduce clonally, while workers are the product of regular sexual reproduction, presenting very low genetic variation (Fournier et al. 2005a). This special reproduction system creates genetically uniform populations across large-scale regions, providing the opportunity to differentiate genetic variations from other factors influencing biological phenomena, e.g., nestmate recognition. In the present study, we explored the significance of environmental impact on nestmate recognition in the clonal population of *W. auropunctata* in Israel. While many studies have examined either the behavior (i.e., intraspecific aggression), the chemistry (i.e., CHC composition), or the genetic aspects of nestmate recognition in ant species that form supercolonies (e.g., Giraud et al. 2002; Martin et al. 2008; Rosset et al. 2007; Thomas et al. 2006; Tsutsui et al. 2000; Van Wilgenburg et al. 2007), only a few studies have encompassed all these in the same study, as we present here (e.g., Foitzik et al. 2007; Steiner et al. 2007; Stuart and Herbers 2000). Moreover, unlike the *Wasmannia* case, all these other studies have dealt with species with standard sexual reproduction systems.

Our microsatellite-based survey revealed that the *W. auropunctata* population in Israel is genetically homogenous

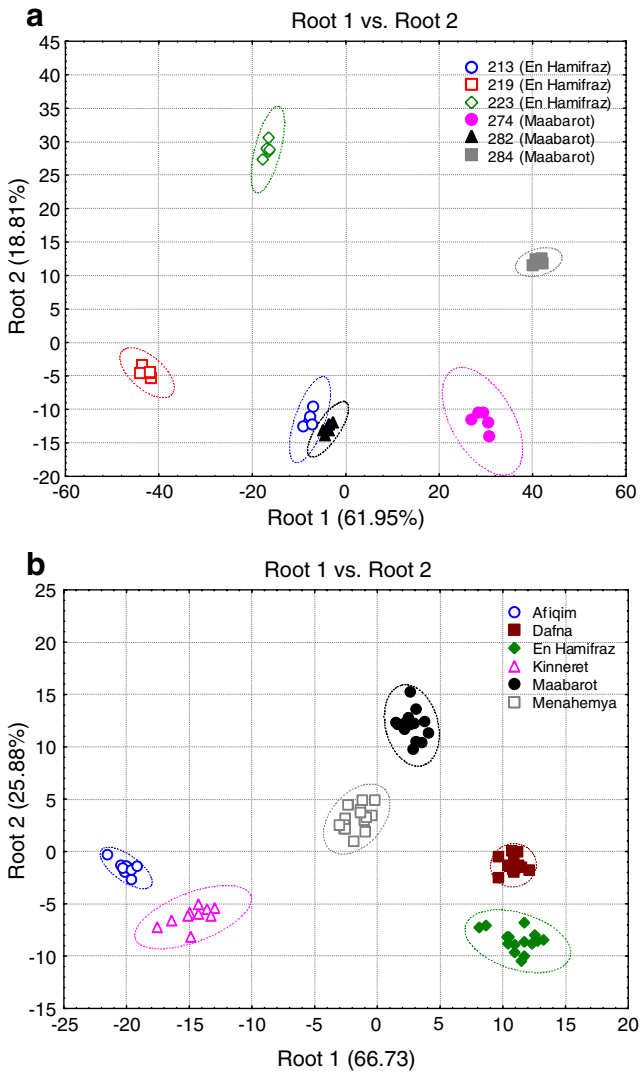

Fig. 3 Discriminant analyses based on cuticular hydrocarbon profiles of *W. auropunctata* workers. Each colony is represented by five replicates of body washes of 50 workers; **a** separation according to colonies (the locality of each nest is indicated in *brackets*); **b** separation according to localities, each of which comprised two or three colonies. *Ellipses* represent 95% confidence levels

and that all the ants of this population are descendants of a single male genotype and a single queen genotype, with both sexes reproducing clonally. This pattern is similar to that found for another invaded area, New Caledonia (Foucaud et al. 2006), albeit substantially different genotypes having been identified in the two populations. The lack of intraspecific aggression among *W. auropunctata* workers in the nine localities tested (out of 23 known infested villages) indicates that this species in Israel constitutes a single supercolony. This is not the result of a general reduction in the species' aggression as a consequence of the invasion because, irrespective of collection site, the workers remained highly aggressive towards other ant species. *W. auropunctata* workers bit

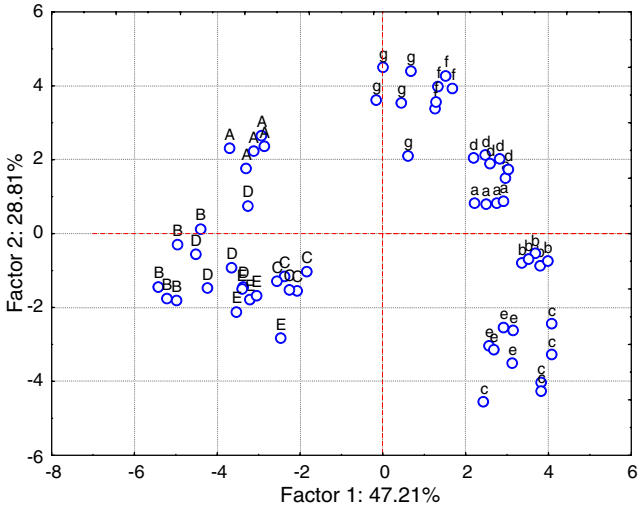

Fig. 4 Principal components analysis based on cuticular hydrocarbon profiles of *W. auropunctata* workers (five body wash replicates of 50 pooled workers per colony). Five colonies (*a*–*e*) were sampled twice: first at 0–2 days from collection (*lowercase*) and second after being maintained in the laboratory for 90–105 days (*uppercase*). Samples *f* and *g* correspond to fresh extracts (1 to 2 days after collection) of colonies from the same villages as *d* and *a*, respectively, made at the same time as the second analyses of the samples *D* and *A*, respectively

and stung the opponent ants, causing high casualties, regardless of which side had initiated the aggression. These results conform with earlier findings regarding patterns of intraspecific and interspecific aggression in another population of *W. auropunctata* (i.e., New Caledonia), which also forms a single supercolony (Errard et al. 2005; Le Breton et al. 2004).

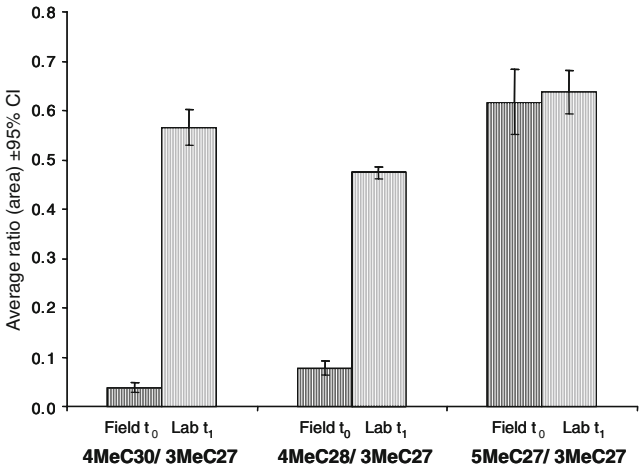

Fig. 5 Ratios between 4-methyltriacontane, 4-methyloctacosane (cricket major CHC), 5-metylheptacosane (*W. auropunctata* component), and 3-methylheptacosane (*W. auropunctata* component) in the CHC profile of five nests (a–e, see Fig. 4). Extractions from each nest were made twice—0–2 days from collection (field t_0) and after 90–105 days in the laboratory (lab t_1)

However, the results obtained in our study also indicate that there is a strong phenotypic or environmentally derived component to *W. auropunctata* nestmate recognition. While confrontations between freshly collected colonies irrespective of nest location were never aggressive, confrontations between laboratory-reared colonies and freshly collected colonies were always aggressive. This can be attributed to changes in nestmate recognition cues, as reflected in the changes in CHC profiles, the postulated nestmate recognition cues in this species (Errard et al. 2005). We indeed found that the CHC profiles of five sampled nests changed considerably after 90–105 days under laboratory conditions and that these changes occurred in the same direction. In addition, five of the major cricket compounds were responsible for the variance between the two time intervals, as shown in the PCA. Since the two field-collected colonies at t_1 were still similar to the field-collected colonies at t_0, we attribute the changes in *W. auropunctata* CHC profiles to environmental influence (i.e., laboratory-rearing conditions) rather than to changes in colony demography over time. Moreover, due to the clonal reproduction system, genetic drift can be excluded in this species, and any genetic variance in the sexually produced workers is minimized by the large worker sample (n=50) for each extract. Inspection of the CHC profiles of laboratory ants revealed that they had incorporated some of the major cricket hydrocarbons, the addition of which made the profiles of laboratory-reared colonies quite disparate from the corresponding freshly field-collected colonies.

Nestmate recognition is often influenced by environmental factors that affect CHC compositions through a diet effect (Liang and Silverman 2000; Richard et al. 2004; Silverman and Liang 2001; Sorvari et al. 2008), habitat odors (Katzav-Gozansky et al. 2004; Nielsen et al. 1999), or seasonality (Nielsen et al. 1999). However, we cannot exclude the possibility of additional phenotypic plasticity, which is not necessarily directly linked to environmental factors. Marshall and Jain (1968) hypothesized an inverse relationship between phenotypic plasticity and genetic variance since these represent two alternative strategies for dealing with heterogeneous environments.

If diet indeed affects nestmate recognition in *W. auropunctata*, why are distant populations that are bound to have different diet compositions not aggressive towards each other? We suggest that at least in this species there is an interplay between genetic background and diet in shaping nestmate recognition. In the field, the ants' diet breadth is probably very large, and consequently the incorporation of diet-borne substances may be small per substance and not sufficient to mask the genetically determined cues. Thus, the ants rely on innate recognition cues as the basis for acceptance/rejection of conspecifics. This would explain the lack of aggression between freshly collected colonies even from remote localities. In the laboratory, the diet breadth of the ants was very restricted, and this might have facilitated the incorporation of diet-borne hydrocarbons (confirmed by our chemical analyses) into the recognition label to the point of masking or overriding the innate cues. Such colonies were recognized by freshly collected colonies as alien (and vice versa), resulting in the unusual intraspecific aggression.

The case of the invasive ant *L. humile* may also be explained by the diet breadth hypothesis, in which there was no aggression between populations of a large European supercolony spread over hundreds of kilometers (Giraud et al. 2002), whereas members of a Californian supercolony that were fed in the laboratory with different hydrocarbon-rich diets became alien towards fragments of the same supercolony (Silverman and Liang 2001). Although the former study lacks the chemical analyses of the CHCs, we can assume that, as in *W. auropunctata*, a natural diet composed of diverse arthropods will have little impact on the recognition cues, leaving the innate recognition cues as the discriminatory means. In contrast, a narrow diet like that used for the Californian laboratory population of Silverman and Liang (2001) could have a great impact on CHC composition and mask the innate recognition cues.

The diet breadth hypothesis is also supported by our chemical analysis of CHCs, although the picture appears to be more complex. Despite the clonal nature of the queens and males in the *W. auropunctata* population, we could detect a nonnegligible variation in CHC compositions. This seems to be graded because, although nests from specific localities could be distinguished by our discriminant analysis, they still clustered according to locality. Such gradation may suggest that environmental impact combines with cue exchange between colony members to create the colony gestalt. However, since these differences did not translate into aggressive behavior, they may be too subtle and therefore ignored, consistent with the discrimination threshold hypothesis (Le Conte and Hefetz 2008). We cannot exclude the possibility that CHCs are not used as nestmate recognition cues in *W. auropunctata*, but, in view of the strong association between aggressive behavior and CHC profile differences for the laboratory *vs.* field reared colonies, we tend to refute this possibility.

Invasive species are expected to benefit from the loss of intraspecific aggression given their generally explosive demography (Giraud et al. 2002). This is exemplified in the clonal population of *W. auropunctata* in Israel: although workers have possibly retained their discriminatory ability, they show no intraspecific aggression, forming a single supercolony. As evident from other parts of its invasive range, this trait could be highly significant for their territorial expansion and their impact on other organisms.

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Conflict of interest The authors declare that they have no conflict of interest.

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Electronic Supplementary Material

Table S1 F-statistics (F_{st} and F_{is}; Weir and Cockerham 1984) and relatedness statistics (r and r*; Pamilo 1985) computed taking localities as unit. NC: not computable.

	F_{st}	F_{is}	r	r*
Estimation over all loci	0.000	-0.588	0.002	0.740
S.E.	0.001	0.054	0.007	NC

Caption:

Fig. S2 Gas chromatograms of total body wash of *Wasmannia auropunctata* workers (a and b) and crickets (c); **a** total body wash made three days after collection; **b** wash made 120 days after collection (nest B); **c** abdomen cuticle wash of five adult crickets (*Acheta domestica*). The peak numbers are the same as in Fig. 3. Peak identity (roman numbering refers to compounds found only in the crickets. sq. = squalene): (1) Heneicosene; (2) Heneicosane*§; (3) 3-Methylheneicosane*; (4) Docosane; (5) Tricosene; (6) Tricosane*§; (7) 5-Methyltricosane; (8) 3-Methyltricosane; (9) 5, 9-Dimethyltricosane; (10) Tetracosane; (11) 10-+12-+14-Methyltetracosane; (12) Pentacosene*; (13) Pentacosane*§; (14) 9-+11-+13-Methylpentacosane*§; (15) 7-Methylpentacosane; (16) 5-Methylpentacosane*§; (17) 3-Methylpentacosane*§; (18) 5,9-Dimethylpentacosane*§; (19) Hexacosane*§; (20) 10-+12-+14-Methylhexacosane; (21) 12,14-Dimethylhexacosane; (22) 4-Methylhexacosane; (23) Heptacosene*§; (24) Heptacosane*§; (25) 11-+13-+15-Methylheptacosane*§; (26) 7-Methylheptacosane; (27) 5-Methylheptacosane*§; (28) 11,15-Dimethylheptacosane; (29) 9,13-Dimethylheptacosane; (30) 3-Methylheptacosane*§; (31) 3,9-Dimethylheptacosane; (32) Octacosane; (33) 12-+14-+16-Methyloctacosane*; (34) 4-Methyloctacosane§; (I) Nonacosene; (35) Nonacosane*§; (36) 11-+13-+15-Methylnonacosane*§; (37) 5-Methylnonacosane; (II) 4-Methylnonacosane; (38) 11,15-+13,15-Dimethylnonacosane*§; (39) 3-Methylnonacosane; (III) Triacontane; (40) 12-Methyltriacontane; (41) 12,14-Dimethyltriacontane; (42) 4-Methyltriacontane§; (IV) 2-Methyltriacontane; (V) Hentriacontene; (43) Hentriacontane; (44) 11-+13-+15-Methylhentriacontane*§; (VI) 13,17-Dimethylhentriacontane; (45) 11,15-+13,15-Dimethylhentriacontane*§; (VII) 5,13-Dimethylhentriacontane; (VIII) Dotriacontane; (IX) 4-Methyldotriacontane; (46) 11-+13-+15-Methyltritriacontane§; (47) 11,15-+13,15-Dimethyltritriacontane; (X) 15,17-Dimethyltritriacontane; (XI) 12-+14-+16-Methyltetratriacontane; (XII) 13,15,17-+15,17,19-Trimethyltritriacontane; (48) Pentatriacontane; (XIII) 13-+15-Methylpentatriacontane; (49) 11-Methylpentatriacontane; (50) 15,19-Dimethylpentatriacontane.

Fig. S2a

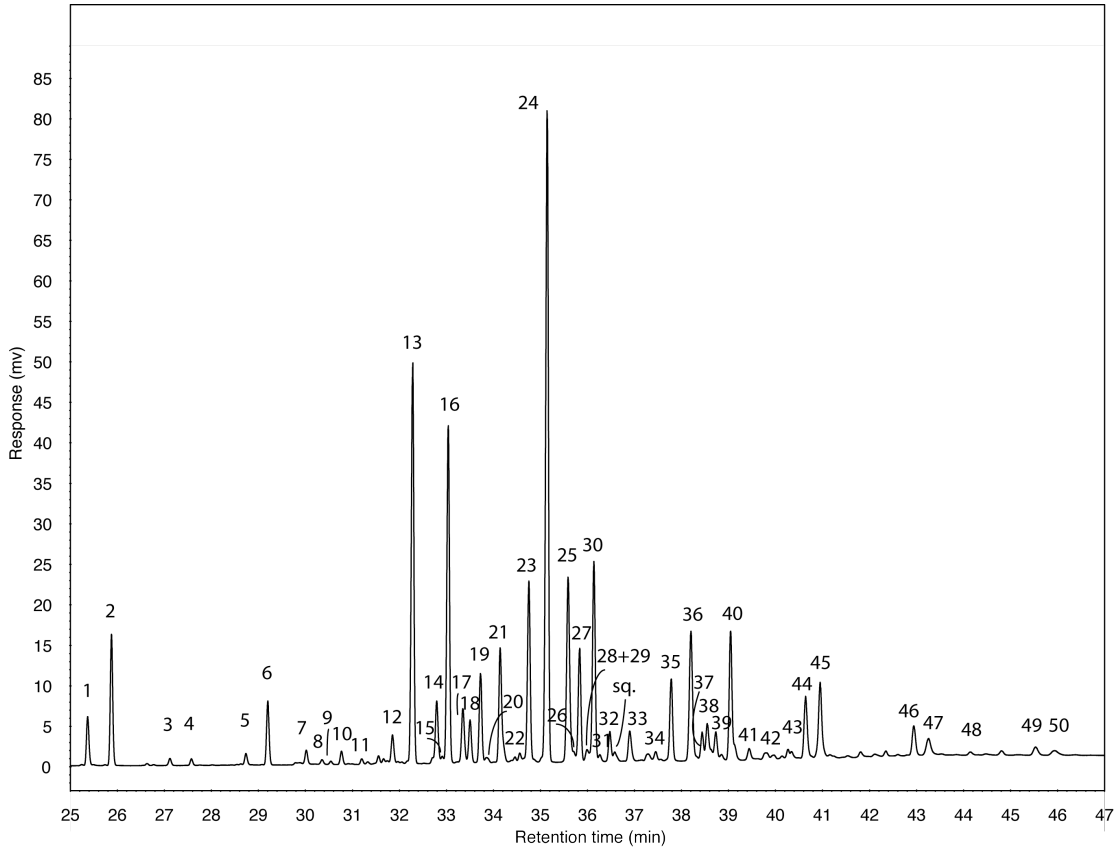

Fig. S2b

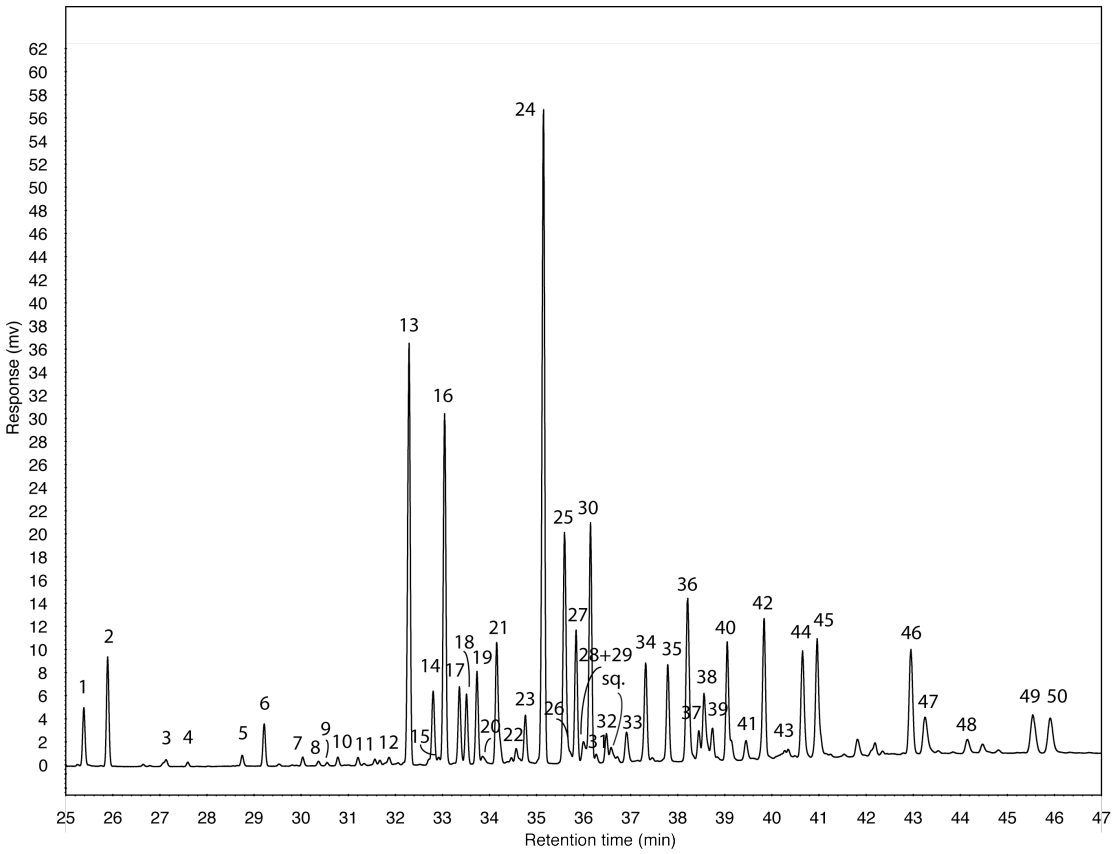
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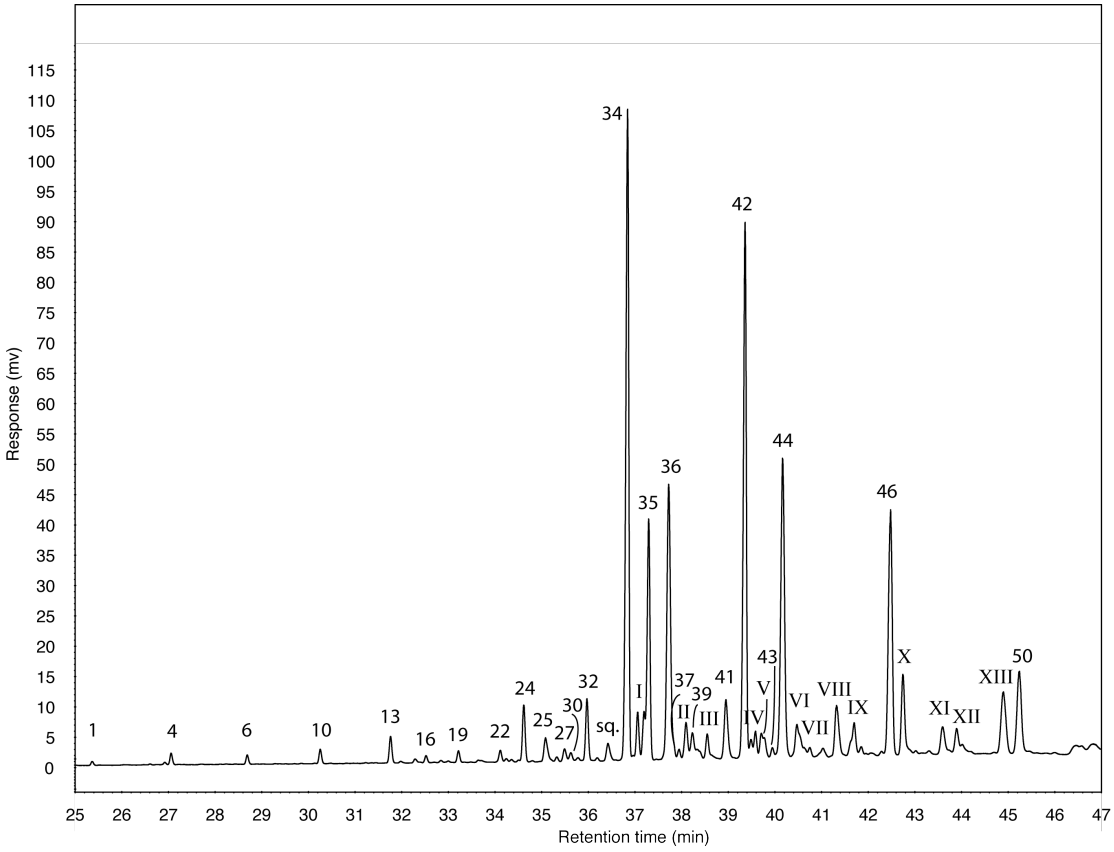
Fig. S2c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