How to escape from the host nest: Imperfect chemical mimicry in eucharitid parasitoids and exploitation of the ants' hygienic behavior

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ABSTRACT

Communication in ants is based to a great extent on chemical compounds. Recognition of intruders is primarily based on cuticular hydrocarbon (CHC) profile matching but is prone to being cheated. Eucharitid wasps are specific parasitoids of the brood of ants; the immature stages are either well integrated within the colony or are protected within the host cocoons, whereas adult wasps at emergence must leave their host nest to reproduce and need to circumvent the ant recognition system to escape unscathed. The behavioral interactions between eucharitid wasps and workers of their host, the Neotropical ant *Ectatomma tuberculatum*, are characterized. In experimental bioassays, newly emerged parasitoids were not violently aggressed. They remained still and were grabbed by ants upon contact and transported outside the nest; host workers were even observed struggling to reject them. Parasitoids were removed from the nest within five minutes, and most were unharmed, although two wasps (out of 30) were killed during the interaction with the ants. We analyzed the CHCs of the ant and its two parasitoids, *Dilocantha lachaudii* and *Isomerala coronata*, and found that although wasps shared all of their compounds with the ants, each wasp species had typical blends and hydrocarbon abundance was also species specific. Furthermore, the wasps had relatively few CHCs compared to *E. tuberculatum* (22–44% of the host components), and these were present in low amounts. Wasps, only partially mimicking the host CHC profile, were immediately recognized as alien and actively removed from the nest by the ants. Hexane-washed wasps were also transported to the refuse piles, but only after being thoroughly inspected and after most of the workers had initially ignored them. Being recognized as intruder may be to the parasitoids' advantage, allowing them to quickly leave the natal nest, and therefore enhancing the fitness of these very short lived parasitoids. We suggest that eucharitids take advantage of the hygienic behavior of ants to quickly escape from their host nests.

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1. Introduction

Like all social Hymenoptera, ants possess highly developed recognition mechanisms which allow them to accept group-members (nestmates) and reject strangers (Hölldobler and Wilson, 1990; Nash and Boomsma, 2008; Sturgis and Gordon, 2012; Vander Meer and Morel, 1998; van Zweden and d'Ettorre, 2010). An efficient system to discriminate between nestmates and non-nestmates is essential to prevent the exploitation of colony resources by intruders. Nestmate recognition is thus of fundamental significance to maintain the integrity and cohesion of societies and to ensure their functioning (Hölldobler and Wilson, 1990), but it is prone to deception.

Nestmate recognition in social insects is largely based on cuticular hydrocarbons (CHCs) (d'Ettorre and Lenoir, 2010; Howard and Blomquist, 1982, 2005; Lenoir et al., 1999, 2001; Liu et al., 2000). CHC profiles, detected through antennal contact or over a very short distance (Brandstaetter et al., 2008; Cuvillier-Hot et al., 2005; Ozaki et al., 2005), are thought to be compared to a template that defines group membership. Differences from the template can lead to a behavioral recognition response such as aggression, and the workers of many species of ants are known to attack and reject or kill non-nestmates that try to enter their nests (d'Ettorre and Lenoir, 2010; Vander Meer and Morel, 1998).

Because of the protection provided by their environmentally buffered nests, their stored resources and the abundant mass of immature brood and adults, ants are attractive targets for a wide range of other organisms (Ayasse and Paxton, 2002; Hölldobler and Wilson, 1990; Hughes et al., 2008). Numerous and diverse organisms, the so-called myrmecophiles, exploit ant colonies and their social resources through a complex interaction network (see Pérez-Lachaud and Lachaud, 2014), establishing facultative or obligate associations with ants and spending variable proportions of their lives within ant colonies (Hölldobler and Wilson, 1990; Hughes et al., 2008; Kistner, 1982; Lachaud et al., 2012, 2013; Schmid-Hempel, 1998).

Intruders survive inside ant nests if they succeed at either preventing resident ants from attacking them or defending themselves against ant attack (Eisner et al., 1978). Myrmecophiles are known to overcome ant recognition systems and defenses through a combination of morphological, chemical or acoustic mimicry, defensive chemicals, appeasement secretions, and/or behavioral strategies (Akino, 2008; Bagnères and Lorenzi, 2010; Barbero et al., 2012; Dettner and Liepert, 1994; Haynes and Yeargan, 1999; Kistner, 1979; Lenoir et al., 2001, 2012; Rödel et al., 2013). The strategies deployed to infiltrate ant colonies vary according to the various degrees of integration of the intruder, its life history traits (Sala et al., 2014), and the life stage involved.

Eucharitidae (Insecta: Hymenoptera: Chalcidoidea) are specific ectoparasitoids of ants with a highly specialized life cycle (Clausen, 1941; Heraty, 1994; Heraty et al., 2004; reviewed in Lachaud and Pérez-Lachaud, 2012). Females lay eggs in or on plant tissue. The small first-instar larvae (planidia) are active in finding their hosts, attaching to ant foragers or to potential prey in order to gain access to the ant nest where they initially parasitize ant larvae, completing their development when the host pupates (Clausen, 1940; Heraty, 2000; Pérez-Lachaud et al., 2006a). Adult wasps emerge inside ant nests, but mating occurs outside, on neighboring plants (Clausen, 1941; Heraty, 1994; Pérez-Lachaud et al., 2006a). The extremely small size of the planidia is supposed to facilitate both entrance into the host colony and initial parasitism (Vander Meer et al., 1989), but adult parasitoids face the challenge of avoiding the host recognition system after hatching. Several past reports have stated that adult wasps are not treated aggressively by ants (Ayre, 1962; Clausen, 1923; Vander Meer et al., 1989; Wheeler, 1907). In laboratory conditions, wasps were observed to be carried and removed from the nest without damage, but were killed and dismembered in closed containers (Clausen, 1923; Howard et al., 2001; Lachaud et al., 1998). Furthermore, in the field, fortuitous observations have shown that parasitized cocoons containing wasps ready to emerge (Buys et al., 2010) and those containing the wasp's remains (Pérez-Lachaud et al., 2006a; Electronic Supplementary Material, Video S1) are, on some occasions, discarded. Since recently hatched wasps have CHC profiles that resemble those of their ant hosts, it was previously proposed that adults of eucharitids used chemical mimicry to deceive their hosts (Howard et al., 2001; Vander Meer et al., 1989). Active removal of parasitoids from the nest suggests, however, that ants may recognize eucharitids as alien, but as yet the behavioral interactions between ants and eucharitid wasps have not been studied in detail.

A population of the Neotropical ant *Ectatomma tuberculatum* (Olivier), parasitized simultaneously by two eucharitid wasps, *Dilocantha lachaudii* Heraty and *Isomerala coronata* (Westwood), was studied in a coffee-cocoa plantation in southern Mexico. Both parasitoid wasps attack the same host developmental stage, share the same life history traits, and individuals from both species frequently infest the same nest and on occasions the same individual host (Pérez-Lachaud et al., 2006b, 2010). Here we quantitatively characterize the behavioral interactions between adult eucharitids and their host ants and analyze their cuticular hydrocarbon profiles. We demonstrate that wasps, in general, not only survive encounters with ants but are also quickly transported outside unscathed. We propose that only partial matching of the host chemical profile is sufficient to both placate ant aggressiveness and allow wasps being recognized as intruders, which might eventually be to the parasitoid's advantage.

2. Material and methods

2.1. Study organisms

D. lachaudii, *I. coronata* and *E. tuberculatum* individuals used for CHC analysis were obtained during a larger survey that evaluated the impact of eucharitids on a population of their ant host (Pérez-Lachaud et al., 2010). In brief, ant nests were excavated from a population located in a coffee-cocoa plantation at the Instituto Nacional de Investigaciones Forestales, Agrícolas y Pecuarias (INIFAP) Experimental Station, at Rosario Izapa, Chiapas, Mexico (14°58′25″ N, 92°09′19″ W, 430 m asl). Cocoons (pupae) from each nest were kept separately in glass vials under controlled conditions (28 °C ± 2 °C and 75% ± 5% RH). Vials were checked twice a day to collect adult parasitoids upon emergence. Supplementary nests were collected from the same site in 2009 and in 2010 to verify the identification of the compounds. A sample of ants and parasitoids was preserved in 70% ethanol, and voucher specimens were deposited in the Entomological collection of El Colegio de la Frontera Sur (Tapachula, Chiapas, Mexico). Wasps were identified by JM Heraty (Department of Entomology, UCR); and all material was revised by one of us (G P-L).

2.2. Behavioral assays

The study of the interaction of eucharitids with their ant hosts presents many challenges, particularly as these parasitoids are rare. Two preliminary observations with *D. lachaudii* wasps were carried out in 2005 and bioassays with this same wasp species were set up in 2006 and in 2009 using different *E. tuberculatum* colonies, which demonstrated that the observed behavior could be replicated (see Section 3, Table 1). Wasps from two treatments were observed. The first comprised live, freshly hatched, untreated wasps with no previous contact with ants and the second was composed of dead, hexane-washed wasps (the wasps were killed by exposure to cold temperatures, washed with hexane during 10 min to wash off or at least substantially reduce the amounts of CHCs, and then air dried for 2 h prior to the bioassays). Hexane-washed wasps were different from those used for trials with live wasps. We recorded the behavior of the ants when faced with adult parasitoids inside the nest by individually introducing parasitoids into experimental nests (a round plastic box: 24 cm diameter, 10 cm height), connected *via* a glass tube (20 cm length, 1 cm diameter) to a foraging arena (a plastic container: 22 × 30 × 10 cm). The side walls of the nesting space and of the foraging arena were treated with FLUON (Whitford GmbH). Experimental colonies were standardized to 30 workers, the queen, and some of their own larvae or pupae. Ants were provided with water *ad libitum* and fed every two days with apple, diluted honey, and weevil larvae (*Caulophilus oryzae*). Large cocoa leaves placed in the nest box provided shelter for the ants.

Bioassays were conducted after a 1-week period of acclimatization to laboratory conditions, by which time the ants had already established a refuse heap in the foraging area and usually another one in the nest box. The protocol for observations was as follows: a parasitoid was introduced into the experimental nest with the aid of fine forceps, and gently deposited in the middle of the plastic

Table 1

Summary of the number of interactions between *Ectatomma tuberculatum* ants and newly emerged *Dilocantha lachaudii* parasitoids observed from 2005 to 2009, and their output. Each year a different, recently excavated *E. tuberculatum* nest was used. Records from focal nests #2 (2006) and G#1 (2009) were used for behavior description and statistical analyses in this study.

Year	Sex of the parasitoid	Nest ID	Number of observations	Wasp removed from nest	Wasp transported to the internal refuse pile	Wasp final condition	
						Alive	Dead
2005	Female	Nest#1	1	1	0	1	
	Male	Nest#1	1	1	0	1	
2006	Female	Nest#2	11	8	3	11	
	Male	Nest#2	2	1	1	1	1
2007	Female	Nest#3	4	1	3	4	
	Male	Nest#3	1	0	1	1	
2009	Female	NestJP#2	3	2	1	3	
	Male	NestJP#2	0	–	–	–	
2009	Female	Nest G#1	10	8	2	9	1
	Male	Nest G#1	7	5	2	7	

box between the cocoa leaves and the exit tube. Ant behavior was video recorded for subsequent analysis using a color video camera (Sony camcorder CCD TRV94, Sony, USA) placed above the experimental nest. We used focal sampling as the method of recording under natural light and at room temperature. Each recording began when the parasitoid was introduced, and was monitored for up to 15 min or less if the parasitoid was deposited on a refuse pile. "Latency" (from the introduction of the parasitoid until contact by a worker ant), "latency to seizure" (from the introduction of the parasitoid to the moment a worker seized the parasitoid between its mandibles), and "handling plus transport" times were calculated (from seizure to abandonment on a refuse heap). The total duration of the interaction (from first contact to abandonment) was also analyzed. The outcome of the interaction was either "rejected outside the nest" (parasitoid transported to the refuse heap in the foraging arena), or "rejected inside" (parasitoid transported to the refuse pile inside the nest). The physical condition of the parasitoids was also noted: "wasp unharmed" or "wasp injured" (bitten, stung).

The interactions were recorded on six separate days in 2006 and on five days in 2009. All of the observations were carried out between 9:00 a.m. and 2:00 p.m. and each parasitoid individual (whether alive or hexane-washed) was tested only once. Thirteen trials with newly hatched parasitoids were performed in 2006 (0–1 day-old *D. lachaudii;* 2 males, 11 females) and 17 in 2009 (7 males and 10 females). Three trials with hexane-washed wasps were performed in 2006 and 15 in 2009.

2.3. Sample preparation and CHCs extraction

Samples from *D. lachaudii* (males: $n = 10$; females: $n = 8$) and *I. coronata* (males: $n = 3$; females: $n = 4$) were analyzed. Extracted wasps were different from those used for the behavioral experiments. Wasps from different nests were extracted separately. Newly emerged wasps that did not have any contact to adult workers yet, were placed individually (for the very uncommon *I. coronata* males) or in groups of three individuals of the same species/sex (for *D. lachaudii* and for *I. coronata* females) into 4-ml glass vials that contained 1 ml HPLC-grade n-hexane (J.T. Baker, 95%) and shaken slightly for 1 min. The hexane was transferred using a Pasteur pipette to a clean 4-ml vial, and the washing procedure was repeated two more times. The combined portions from each replicate were concentrated under a gentle stream of N_2 almost to dryness. Ant workers originating from the same colonies as adult eucharitids were also extracted using the same procedure (five workers per replicate, $n = 10$).

2.4. Chemical analyses

Samples were analyzed by using combined gas chromatography (Varian CP 3800, Palo Alto, California) – mass spectrometry (Varian, Saturn 4D 2200, Palo Alto, California). Aliquots (2 µl) were introduced by splitless injection onto a non-polar capillary column (DB-5 MS, 30 m long, 0.25 µm i.d.; J and W Scientific Folsom, California). Helium was the carrier gas, flowing at 1 ml/min. The temperature was 100 °C during injection (2 min), and was then increased to 300 °C at 20 °C/min, followed by a constant 300 °C for 3 min. Ionization was achieved by Electron Impact at 70 eV. The solvent was checked for purity by GC–MS prior to use. The identification of the substances was verified using a GC–MS VGM250Q system (Perkin-Elmer) operating at 70 eV with the same DB-5 fused silica capillary column. Temperature was kept at 150 °C during the initial two minutes, then raised from 150 °C to 300 °C at 5 °C/min and held at 300 °C for the last 10 min.

Retention times for each hydrocarbon component and equivalent chain length values (ECL) were obtained by comparison with known *n*-alkane standards (mixtures C_8–C_{30}, Sigma Aldrich, Toluca, Mex. and C_{12}–C_{60}, Supelco, Toluca, Mex.). Individual components in the total ion scanning mode were identified from their characteristic EI-MS fragmentation patterns (Jackson and Blomquist, 1976; Nelson, 1978). The relative abundance of each compound was estimated as the proportional peak area from total ion chromatograms. Quantitative analyses were conducted using the total ion scanning mode (Saturn GC/MS Workstation ver. 90.0.552.0, Varian, Inc.).

2.5. Statistical analyses

Latency, latency to seizure, time for handling and transport and the total duration of interactions were compared using a two-way Anova (unbalanced factorial design), with colony (each year a different colony was observed) and treatment (live and treated wasps) as factors. Data were log transformed to meet the prerequisite of homoscedasticity.

Multivariate statistics were used to estimate the similarity of the chemical profiles of ants and parasitoids. To overcome statistical problems associated with GC-derived data, a standard procedure including selection of peaks and normalization of peak areas was applied. For the quantitative analysis, we included only the peaks present in all of the individuals from the three species. The relative areas of the six selected peaks were standardized to 100% and transformed according to Reyment's formula ($Z_{ij} = \ln[Y_{ij}/g(Y_j)]$; Aitchison, 1986), where Z_{ij} is the standardized peak area i for individual j, Y_{ij} is the peak area i for individual j,

and g(Y$_j$) is the geometric mean of the areas of all peaks for individual j. The transformed areas were used as variables in a principal components analysis.

We also carried out a discriminant analysis on the transformed/standardized peak areas to determine whether species could be discriminated on the basis of their chemical profiles, and to assess the degree of similarity between groups (species). To obtain a simple representation of chemical distances between groups, we conducted a cluster analysis (single linkage, Euclidian distance, Ward method) using the mean proportions of all of the peaks. Finally, the proportions of the different structural classes of hydrocarbons (calculated as the ratio of n-alkanes, methyl-branched alkanes, or alkenes to the total number of hydrocarbons of each species) were compared between species using a Fisher's exact test. All statistics were carried out with Statistica 6 for Windows (Statsoft) or with XLSTATS v. 2008 (Addinsoft).

3. Results

3.1. Behavioral assays

A summary of the number of interactions between *E. tuberculatum* ants and newly emerged parasitoids observed from 2005 to 2009, and their outcome, is presented in Table 1. Each year, a different focal colony was observed. The following description of behavior is based on a total of 30 encounters set up in 2006 and in 2009 (9 *D. lachaudii* males and 21 females). Upon encountering a wasp inside the artificial nest, *E. tuberculatum* workers touched it with their antennae and, upon contact, snapped their open mandibles in its direction, quickly grasping the parasitoid. Ants carried parasitoids between their mandibles, walking rapidly. At times, the ants stopped and flexed their gaster forward while trying to put the wasp in a more suitable position for transport or in an attempt at stinging the wasps (the sting protruded at the tip of the worker's abdomen), and then continued their course (Electronic Supplementary Material, Video S2). Attempts at stinging were sometimes observed but wasps were rarely stung or bitten (2/30, 6.7%). Wasps were usually transported outside to the foraging arena with the majority being transported to the refuse heap located the furthest away (22/30, 73.3%). Eight parasitoids (26.7%) were transported to the inside refuse pile and left there after the workers failed to gain access to the exit tube since it was blocked by the activities of several workers. Most of the time, live parasitoids were grabbed by the first worker that detected them (25/30, 83.3%) and were then either transported to the refuse pile by the same worker (9/30, 30%) or, more frequently, by successive workers (21/30, 70%) that struggled severely to take over the transport of the wasp (Electronic Supplementary Material, Video S3). On average, 2.25 ± 0.27 workers (mean ± SEM; n = 28) interacted with live parasitoids per trial. For their part, the eucharitids froze immediately upon contact with an ant and most remained still while being transported even if other ants attempted to seize them. They were finally dumped into refuse piles where they remained motionless for a few seconds, and then flew off or ran away, unharmed (28/30, 93.3%). On two occasions, however, parasitoids were killed: a very large female parasitoid elicited a great number of aggressive behaviors (repeated snapping and stinging), and a male was pulled to pieces by two workers. The response of the ants did not vary based on the sex of the wasp (Fisher's exact test, two-tailed, p = 0.66). Of a total of 9 males and 21 females observed, 6 males and 16 females were immediately removed from the nest, and 3 males and 5 females were deposited in the colony refuse. Ten other trials with live wasps were performed in 2005, 2007 and 2009 (Table 1). The behavior of ants and the output of the interaction followed the same trend, with half the wasps directly removed from the nests and the others first deposited in the colony refuse, and later removed. All wasps were alive and dispersed.

Most of the ants confronted with hexane-washed wasps touched the parasitoid with their antennae, and then went away without any sign of behavioral modification. In 14 out of the 18 trials (77.8%) with hexane-washed wasps, several ants came in contact with the wasp without displaying any reaction (range: 1–7, mean 2.8 ± 0.5 workers, n = 14), before one ant finally lifted the wasp after prolonged antennation (mean antennation time: 7.1 ± 1 s; range: 2–12 s; n = 11). In only four trials hexane-washed wasps were seized by the first worker that came into contact with them (22.2%), and then only after a thoroughly inspection of the wasps (mean antennation time: 11.5 ± 4.1 s; range: 4–22 s; n = 4). Grasping and attempts at stinging were not observed with hexane-washed wasps. The number of wasps "ignored" by the first ant that came into contact with them differed significantly
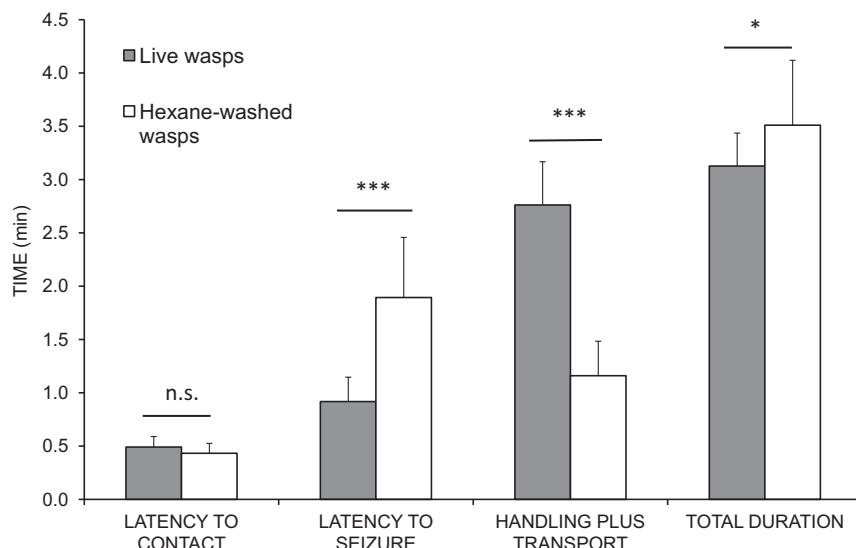

Fig. 1. Behavioral interaction between *Dilocantha lachaudii* wasps and workers of *Ectatomma tuberculatum*. Mean (+SEM) latency to contact, latency to seizure, handling plus transport time and total duration of interactions of ants with live, newly emerged wasps (n = 30, gray bars) and hexane-washed wasps (n = 18, white bars). No difference between colonies was found for all the behavioral categories (see text). Means represent pooled data for the two colonies observed. Differences between treatments are derived from a two-way Anova (n.s. not significant, ***p < 0.01, *p < 0.05).

between treatments: 5/30 in live parasitoids (16.7%); 14/18 for hexane-washed parasitoids (77.8%) (Fisher's exact test, two-tailed, $p < 0.001$).

Three hexane-washed wasps (16.7%) were not seized or transported during the observation period. The others were transported to the refuse heaps: 55.6% (10/18) were removed from the nest and discarded outside; and 27.8% (5/18) were discarded on the refuse pile inside the nest. Ants carrying hexane-washed wasps did not elicit the interest of the other ants which did not attempt to seize the eucharitids or struggle to hold them. All of the hexane-washed wasps transported by ants were discarded by the same worker that had initially seized them.

Ants rapidly detected live and hexane-washed parasitoids. Latency to first contact did not differ between treatments (two-way Anova; $F_{1,44} = 0.95$, $p = 0.33$, Fig. 1) or colonies ($F_{1,44} = 0.07$, $p = 0.79$). Latency to seizure was significantly longer for hexane-washed parasitoids than for live parasitoids ($F_{1,44} = 11.47$,

$p = 0.001$) and did not differ between colonies ($F_{1,44} = 0.13$, $p = 0.73$); however, there was an interaction between colony and treatment ($F_{1,44} = 12.61$, $p = 0.001$). Handling plus transport took significantly longer for live parasitoids than for hexane-washed ones ($F_{1,44} = 10.8$, $p = 0.001$), a consequence of wasp transport slowing down when several workers tried to take over carrying the live wasps, but did not differ between colonies ($F_{1,44} = 3.08$, $p = 0.09$), and there was no interaction between colony and treatment ($F_{1,44} = 0.41$, $p = 0.52$). In general, interactions with live wasps lasted less than interactions with hexane-washed wasps ($F_{1,44} = 4.42$, $p = 0.04$; Fig. 1).

3.2. Chemical analyses

The CHC profile of *E. tuberculatum* workers was composed of forty-eight peaks, which corresponded to a total of 89 identified hydrocarbons (Table 2; Electronic Supplementary Material,

Table 2
Comparative chemical composition of the major CHC peaks for *E. tuberculatum* and for its two eucharitid parasitoids (mean ± SEM).[a]

Peak No.	Compound[b]	Ectatomma tuberculatum Workers (x5) $n = 10$	Dilocantha lachaudii Females (x3) $n = 8$	Dilocantha lachaudii Males (x3) $n = 10$	Isomerala coronata Females (x3) $n = 4$	Isomerala coronata Male (x1) $n = 3$
1	$C_{21:1}$	1.86 ± 0.80				
2	C_{21}	0.06 ± 0.05				
3	$C_{22:1}$	0.07 ± 0.03				
4	8-, 10-Me C_{22}	0.04 ± 0.03				
5	8,12-DiMe C_{22}	0.05 ± 0.02				
6	$C_{23:1}$	0.97 ± 0.38				
7	C_{23}	0.28 ± 0.10				
8	9-, 11-Me C_{23}	0.37 ± 0.12				
9	9,13-DiMe C_{23} + 3-Me C_{23}	0.37 ± 0.16				
10	3,7-, 3,9-DiMe C_{23}	0.91 ± 0.29				
11	10-, 11-, 12-Me C_{24}	0.32 ± 0.10				
12	4-Me C_{24}	0.10 ± 0.03				
13	$C_{25:1}$	2.20 ± 0.77				
14	C_{25}	0.82 ± 0.26				
15	9-, 11-, 13-Me C_{25}	0.76 ± 0.20				
16	5-Me C_{25}	0.32 ± 0.09				
17	3-Me C_{25}	0.81 ± 0.30				
18	3,9-DiMe C_{26}	1.44 ± 0.39				
19	10-, 11-, 12-, 13-Me C_{26}	0.59 ± 0.22				
20	4-Me C_{26}	0.37 ± 0.15				
21	$C_{27:1}$	4.96 ± 1.73				
22	C_{27}	2.13 ± 0.55	–	1.6 ± 0.7	1.0 ± 0.7	–
23	7-, 9-, 11-, 13-Me C_{27}	3.18 ± 0.89	0.9 ± 0.2	1.1 ± 0.5	–	–
24	5-Me C_{27}	1.27 ± 0.41				
25	5,9-, 5,11-DiMe C_{27} + 3-Me C_{27}	3.64 ± 1.11	1.2 ± 0.3	2.1 ± 1.0	–	–
26	3,9-, 3,11-DiMe C_{27}	7.58 ± 2.17	0.9 ± 0.3			
27	10-, 11-, 12-, 13-, 14-Me C_{28}	2.98 ± 0.92	–	2.8 ± 1.0	–	–
28	4-Me C_{28}	t[c]	–	2.2 ± 0.9	–	–
29	$C_{29:1}$	8.99 ± 3.02	–	1.6 ± 0.5	11.5 ± 5.7	–
30	**C_{29}**	5.24 ± 1.42	3.0 ± 1.0	9.2 ± 3.2	17.8 ± 2.5	12.9 ± 8.5
31	**7-, 9-, 11-, 13-Me C_{29}**	4.24 ± 1.11	9.3 ± 2.8	28.4 ± 8.0	12.3 ± 4.6	44.5 ± 23.7
32	5-Me C_{29}	2.64 ± 0.83	–	–	4.2 ± 2.4	–
33	**3-Me C_{29}**	8.86 ± 3.04	17.9 ± 6.1	20.4 ± 6.0	16.9 ± 4.6	22.8 ± 12.7
34	**10-, 11-, 12-, 13-, 14-, 15-Me C_{30}**	10.50 ± 3.17	6.6 ± 2.5	7.8 ± 1.6	2.5 ± 1.0	7.4 ± 5.2
35	**8,12-, 8,14-, 10,12-, 10,14-DiMe C_{30}**	3.04 ± 1.00	4.5 ± 1.5	6.6 ± 1.2	3.4 ± 3.3	7.4 ± 4.4
36	5-Me C_{30}	0.63 ± 0.22	1.8 ± 0.4			
37	4-Me C_{30}	0.59 ± 0.14				
38	$C_{31:1}$	1.23 ± 0.35	7.8 ± 2.6	–	16.4 ± 12.6	–
39	C_{31}	1.84 ± 0.54	7.1 ± 2.8		7.8 ± 7.1	
40	**9-, 11-, 13-, 15-Me C_{31}**	1.51 ± 0.31	14.9 ± 5.2	6.1 ± 2.8	6.3 ± 5.4	4.9 ± 3.3
41	5-Me C_{31}	1.16 ± 0.26		3.6 ± 0.7		
42	11,13-, 13, 15-DiMe C_{31}	1.24 ± 0.33	9.1 ± 3.6			
43	3-Me C_{31}	4.65 ± 1.55	13.7 ± 5.7	4.7 ± 1.5		
44	5,9-, 5,11-DiMe C_{31}	2.83 ± 0.90		1.8 ± 0.6		
45	13-, 15-Me C_{32}	0.97 ± 0.31	1.2 ± 0.3			
46	$C_{33:1}$	0.34 ± 0.19				
47	C_{33}	0.39 ± 0.28				
48	11-, 13-, 15-, 17-Me C_{33}	0.68 ± 0.39				

[a] Means based on n samples of five ants and three wasps each, except for *I. coronata* males which were individually extracted.
[b] Compounds in bold were used for multivariate analysis.
[c] Traces.

Fig. S1). These were composed of homologous series (C_{21}–C_{33}) of saturated *n*-alkanes (10.8% ± 1.21; mean ± SEM, *n* = 10), alkenes (monoenes only, 20.6% ± 1.56), and methyl-branched alkanes: monomethyl alkanes (47.5% ± 0.92) and dimethyl alkanes (22.1% ± 0.72). Methyl alkanes and dimethyl alkanes were usually present as a mixture of several compounds.

Forty-nine of the hydrocarbons of the highest molecular weight (C_{27}–C_{32}) found on the host cuticle (55.1% of the compounds from the ant, 22 peaks, accounting for 79.1% of their total chromatogram peak area [TPA], Table 2), constituted the eucharitid chemical profiles. All of the CHCs found on *D. lachaudii* and *I. coronata* were also present on the host cuticle (peak 28, 4-Me C_{28} representing 2.2% in *D. lachaudii* males was present as traces in the host; Table 2).

The chemical profiles of *D. lachaudii* males and females were composed of 15 peaks each (39 and 37 hydrocarbons, respectively, ranging from C_{27} to C_{32} for females and from C_{27} to C_{31} for males). Of these, nine peaks (representing 28 hydrocarbons, >70% of CHCs) were actually shared by both sexes (Table 2), each one having six gender-specific peaks accounting for 27.9% of the female CHCs and 13.6% of the male CHCs. By comparison, *I. coronata* wasps had considerably fewer peaks and hydrocarbons, which ranged from C_{27} to C_{31}. Males presented only six peaks (i.e., 20 hydrocarbons ranging from C_{29} to C_{31}), which were all shared with females; additionally, *I. coronata* females had five peaks representing five sex-specific hydrocarbons accounting for 40.9% of their CHCs.

As in *E. tuberculatum* workers, the CHC profile of both wasp species contained *n*-alkanes, mono- and dimethyl-branched alkanes, and alkenes, but in different proportions. Most of the hydrocarbons present on the wasp and ant cuticles were methyl-branched alkanes accounting for 45.6–87.6% of their TPA (Table 2). By comparison, *n*-alkanes represented 10.1–26.6% of the TPA for both the host ant and the eucharitids. Alkenes were absent from *Isomerala* males, but represented 27.9% of the TPA for the females, and constituted 1.6% and 7.8% of the TPA for *D. lachaudii* males and females, respectively, and 20.6% of that for ants. The proportions of the different classes of hydrocarbons did not differ significantly between the three species (Electronic Supplementary Material, Table S1; Fisher's exact test, two-tailed, *p* = 0.69).

An initial inspection of quantitative data using principal components analysis based on the six selected peaks (peaks 30, 31, 33, 34, 35, and 40 as presented in Table 2) suggested that the ants differed from the parasitoids in CHCs relative abundance. A plot of the first and second components showed that individuals belonging to the same species clustered together forming three, clearly separated groups (Fig. 2). Within wasp species, there was an evident grouping of individuals by sex. In addition, each species showed characteristic proportions of these CHCs (discriminant analysis, Wilks's λ = 0.147, $F_{4,62}$ = 24.88, *p* = 0.0001; Electronic Supplementary Material, Fig. S2). The dendrogram obtained considering all the peaks confirmed that parasitoid females are closest to the host workers, while males present distinct differences (Electronic Supplementary Material, Fig. S3).

4. Discussion

Qualitative partial descriptions of the behavior of ants against their eucharitid parasitoids have already been published by some authors (e.g., Clausen, 1923), including for species in the genus *Ectatomma* (*E. ruidum*: Howard et al., 2001; *E. tuberculatum*: Lachaud et al., 1998; Rocha et al., 2014). However, quantitative data is lacking. Here, we provide a detailed quantitative characterization of the behavioral interactions between workers of *E. tuberculatum* and adults of the eucharitid wasp, *D. lachaudii*, and analyze the chemical strategy used by two eucharitid parasitoids to evade worker hostility. Based on our observations, interactions
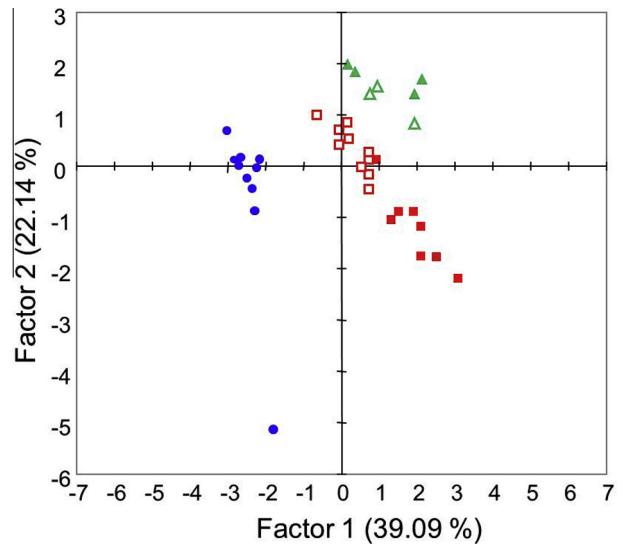

Fig. 2. Principal components analysis of the cuticular hydrocarbon profiles of ants and parasitoid wasps. *Ectatomma tuberculatum* workers (solid circles); *Dilocantha lachaudii* (squares); *Isomerala coronata* (triangles). Open symbols: males, filled symbols: females.

between *E. tuberculatum* ants and *D. lachaudii* parasitoids can be classified as moderately aggressive (mandible opening, grasping, and flexing of the gaster), confirming previous reports. This contrasts with the violent attacks (i.e., repeated stinging and biting) exhibited when workers of this species were presented with ants from another colony/species or with potential prey (Dejean and Lachaud, 1992; Fénéron, 1996; GP-L and J-PL, unpublished results; but see Zinck et al., 2008). Our results also indicate that the chemical profiles of the two eucharitid parasitoid species differ from one another and that they only partially mimic the chemical profile of their ant host, though compounds detected on the wasps are also principle components of the ant profiles.

Most of the literature on deception deals with arthropod species that, at some point in their life cycle, enter ant colonies and become integrated, spending much of their lifetime inside nests, interacting with workers on a daily basis, as is the case for obligate brood predators and specialized social parasites (Akino, 2008; Bagnères and Lorenzi, 2010; Guillem et al., 2014; Lenoir et al., 2001). The general picture that emerges from these studies is the relatively high degree of chemical similarity between the host and the intruder and the fact that the latter is not only tolerated, but, in some cases, considered a nestmate and transported when the ants are disturbed or the colonies moved (Akre et al., 1988; Orivel et al., 2004). The intruder can even receive a special treatment similar to that of individuals of high status in the host colony hierarchy (such as the colony's queen) (Barbero et al., 2009; Maurizi et al., 2012). A few well-integrated myrmecophiles such as the larvae of *Microdon albicomatus* Novak and *Microdon piperi* Knab (Syrphidae), obligate predators of ant brood, attain perfect chemical mimicry by biosynthesizing *de novo* all of the CHCs of their hosts (Howard et al., 1990a,b) ("innate chemical mimicry" *sensu* von Beeren et al., 2012b). However, it has recently been found that the chemical profile of *M. myrmicae* Schönrogge et al. which parasitizes several species of *Myrmica*, is composed of few CHCs, all at low concentrations, suggesting different strategies of social integration even in closely related species (Witek et al., 2013). Some other myrmecophiles vary in the level of social integration attained, in the aggressiveness they elicit in their ant hosts, and in their level of chemical mimicry (Lohman et al., 2006; Maruyama et al., 2009; von Beeren et al., 2011a). All these traits seem to be correlated with the encounter rate with ants

(i.e., the level of interaction with the host), whether inside the nest or in its vicinity (Lohman et al., 2006; Stoeffler et al., 2011), and with the pressure they impose on their host (i.e., their ecological niche: brood predator, cleptoparasite, or other) (Stoeffler et al., 2011; von Beeren et al., 2011a,b).

By contrast, studies on the strategies used by the intruders (or their offspring) to leave the natal nests are still rather uncommon (Lhomme et al., 2012), and very little is known about those species with a parasitoid life-style, such as eucharitids and some diapriid wasps or syrphid flies (see Fernández-Marín et al., 2006; Lachaud and Pérez-Lachaud, 2012; Pérez-Lachaud et al., 2014), or about lycaenid butterflies (Pierce et al., 2002), whose adults emerge inside ant nests.

The immature stages of the eucharitid species studied here are well protected inside the cocoons of the host ant. Only the newly emerged adults really have to circumvent the ant recognition system (or otherwise cope with ant aggressiveness) during a short time window, and must manage to escape from the nest to reproduce (but see Vander Meer et al., 1989 and Wheeler, 1907 for contrasting examples where eucharitid immatures lay among the naked larvae and pupae of their host). Adult eucharitids are very short lived (about 5–6 days) (Pérez-Lachaud et al., 2006a, 2010; Varone and Briano, 2009; Vásquez-Ordóñez et al., 2012); they emerge inside ant nests which can consist of a complex network of galleries and brood chambers patrolled by workers (see Pérez-Lachaud and Lachaud, 2014). Under such circumstances, the quicker the adult wasps leave the nest, the better and, in an evolutionary perspective, any strategy reducing the time spent inside host nests would be expected to be favored.

As our data show, interactions between newly emerged eucharitid adults and ants lasted less than 5 min and were characterized by the ants' eagerness to transport live eucharitids outside the nest, including some worker-worker disputes to take over transportation of the wasps. Interestingly, interactions with hexane-washed wasps (likely bearing no chemical cues or in a reduced amount) lasted longer than interactions with live wasps. The fact that all the wasps are removed from the nest combined with the agonistic behavior of the workers seems to indicate that *E. tuberculatum* workers do recognize the parasitoids as not being part of the colony. As illustrated by the fate of two individuals in this study and by previous observations on experimental nests where the wasps were not given the means to escape (Lachaud et al., 1998), interactions with ants may be fatal if they last a long time. However, as our behavioral observations showed, most parasitoid wasps were not injured and dispersed once discarded on the refuse pile. Newly emerged eucharitids are able to walk and jump inside artificial nests but cannot fly in the narrow galleries (GP-L, unpublished results); furthermore, the entrance to *E. tuberculatum* nests is always guarded and any parasitoid walking near the chimney-like entrance is likely to be noticed. Although the eucharitids are probably able to find the nest exit on their own, the fact that the ants readily transport them outside is likely to reduce both the time spent inside the nest and the potential risks linked to this situation, maximizing the probability for parasitoids to disperse and ultimately enhancing their fitness.

"Acquired chemical mimicry" (*sensu* von Beeren et al., 2012b) through physical contact with the host has been proposed as a low cost mechanism responsible for chemical similarity in various myrmecophile/ant interactions (Akino et al., 1996; Vander Meer and Wojcik, 1982; von Beeren et al., 2011b; Witte et al., 2009). The results of the only two studies on eucharitid CHCs published so far led the authors to suggest that these wasps used chemical mimicry to avoid being attacked by ants. In the case of *Orasema xanthopus* (Cameron), wasp pupae and newly emerged adults possessed CHC profiles 75% identical to that of their host, *Solenopsis invicta* Buren, but those of the adults collected outside were only

14% similar to that of the ants (Vander Meer et al., 1989). In the case of *Kapala izapa* neonates (referred to as *Kapala sulcifacies*), the CHCs shared with their host, *Ectatomma ruidum* (Roger), represented 92.6% of all CHCs for female wasps, and 84.3% for males, but only 67.7% for the ants (Howard et al., 2001). The results obtained here regarding the association between *D. lachaudii* or *I. coronata* and *E. tuberculatum* globally reflect a pattern similar to that observed between *K. izapa* and *E. ruidum*. However, in the present study, the chemical differences between the parasitoids and their host ants were more pronounced, and major differences between both parasitoids were also noted. From a qualitative point of view, the adults of both eucharitid species shared all of the compounds in their CHC profile with their host. However, wasps had relatively few compounds in low amounts, when compared with those of *E. tuberculatum* (between 22% and 44% of the host components according to the parasitoid species and sex of the individuals). In addition, the relative abundance of individual compounds on the cuticle of both the wasps and the ant differed significantly. Both qualitatively and quantitatively, the profiles of the two parasitoid wasp species differed markedly from one another, and also between the sexes within species, contrary to what might be expected under the acquired chemical mimicry hypothesis, i.e., not only a match of the major host compounds, but also a high level of similarity between the CHC profiles of both wasp species whose biology is identical. Our data thereby suggest that the CHCs of *D. lachaudii* and *I. coronata* may have been biosynthesized, but a mixed strategy would also be possible (biosynthesizing few compounds and acquiring the others from the host pupae). However, further studies are needed because in the lower concentrated samples of wasps, some compounds may be more likely to fall below the detection threshold.

It is recognized that CHCs are used in ant nestmate recognition, but the mechanisms underlying discrimination remain substantially unknown (Bos and d'Ettorre, 2012; Sturgis and Gordon, 2012). It has long been thought that discrimination was based on complex blends of all the CHCs, making the CHC profile resemble a blurred barcode (Boomsma and Franks, 2006). However, recent investigations suggest that nestmate discrimination in social insects has evolved to rely upon highly sensitive responses to relatively few compounds (Martin et al., 2008b); for example, ants readily attack nestmates bearing experimentally added odor cues that are novel to their own colony CHC profile (Guerrieri et al., 2009). In the present study, parasitoids sharing all of their CHC compounds with their ant hosts, but only partially mimicking its chemical signature, were not overtly attacked, although they elicited a weak aggressive response, and were likely not recognized as nestmates. Hexane-washed parasitoids, likely with at least substantially reduced CHC amounts, did not elicit any agonistic behavior and were initially ignored by ants. Furthermore, the ants did not struggle to handle and transport hexane-washed wasps, which contrasted with the ants' readiness to transport live wasps. Overall, these observations suggest that some chemical cues present on the cuticle of live parasitoids that are apparently detected upon contact, do convey information that allows discrimination between nestmate and intruder, but the nature of these cues, whether hydrocarbons, proteins (Matsuura et al., 2007), or other compounds, and the proximate causes of the observed rejection behavior remain elusive.

Apart from partial chemical mimicry, other explanations could account for the limited aggressiveness of *E. tuberculatum* towards eucharitids, such as (i) "chemical insignificance" (Lenoir et al., 2001), i.e., the presence of very low quantities of hydrocarbons on the cuticle of an individual wasp; (ii) "chemical transparency" (Martin et al., 2008a), i.e., the intruder bears mainly saturated hydrocarbons which are not involved in recognition; or (iii) could be a by-product of inner-nest CHC saturation as proposed by

Lenoir et al. (2009) to explain the absence of alien rejection in *Lasius niger* (Linnaeus). Due to the high amount of inner-nest marking with CHCs, the likely saturation of the ants' antennal receptors, and the lack of colony-specificity of this marking, any alien individual will be considered a fellow nestmate provided it has succeeded in entering the nest. However, eucharitid wasps in our study elicited great interest in the ants suggesting that they were not chemically insignificant or transparent, and inner-nest CHC saturation does not explain wasp transport and rejection from the colony. Furthermore, a chemical resemblance does not necessarily mean that the host is deceived by the myrmecophile (von Beeren et al., 2012a). Once detected, animals may survive encounters with potential predators through a variety of behavioral, chemical and morphological defenses that facilitate their escape (Langerhans, 2007). Interestingly, most wasps in our study showed a calm behavior when ants seized them. From a eucharitid's perspective, attack deterrence through calm behavior and/or death feigning may increase the likelihood in escaping as calm behavior of the wasp might be an important trigger eliciting more calm behavior in the ants. Such strategy has been suggested to facilitate social integration in the case of *Gamasomorpha maschwitzi* (Wunderlich), a cleptoparasitic spider in the colonies of the army ant *Leptogenys distinguenda* (Emery). Similarly to the eucharitids reported here, no spider specific compound was found and the spider also showed a rather weak resemblance to the host CHCs (von Beeren et al., 2012a). Incidentally, other explanations may contribute in elucidating ant deception in eucharitid/ant associations such as, for example, the possible presence of fatty acids (especially oleic acid) that triggers necrophoric behavior in some ant species (Gordon, 1983; Haskins and Haskins, 1974; Wilson et al., 1958). Nevertheless, we consider this unlikely because *E. tuberculatum* workers thoroughly examine dead conspecifics before rejecting them from the nest (GP-L et al., unpublished results; see also Renucci et al., 2011), which differs markedly from the prompt seizure of eucharitids. Further investigation aimed at elucidating the stimuli that promote wasp rejection is in progress.

Pressure from parasitoids is expected to induce selection for host defenses and counter measures in parasites (Boomsma et al., 2005; Dawkins and Krebs, 1979; Nash and Boomsma, 2008). As parasitoids, eucharitid wasps may have considerable impact on host fitness, though prevalence among *E. tuberculatum* nests is very variable (Pérez-Lachaud et al., 2010). However, wasps may be sufficiently rarely encountered by individual workers within the nest to prevent selection for specific defensive traits in their ant hosts, which then only rely on their general methods of defense to cope with uncertain situations.

Social insects have evolved a suite of physiological, immunological and behavioral defensive responses to counter exploitation by micro- and macro-parasites both at the individual and the colony level (Boomsma et al., 2005; Cremer and Sixt, 2009; Hughes, 2008). The first line of defense is avoidance, but once a parasite is inside the nest (or hatches inside the nest), hygienic behavior and waste management practices are the most general and flexible forms of behavioral defenses of social insects to minimize damage without incurring high costs (reviewed in Boomsma et al., 2005). This is seemingly the case for pathogens, and may be a plausible low cost mechanism to cope with small, macroscopic, parasitic myrmecophiles that emerge in the host nest and exhibit a calm behavior, such as eucharitid wasps (Rocha et al., 2014). However, larger parasitic myrmecophiles, trying to penetrate into the host colony might be best defended by aggression (Hölldobler, 1978; Hölldobler et al., 1981; von Beeren et al., 2011a; Witte et al., 2009). Ants are known to transport, displace and aggregate many kinds of objects and remove any debris and refuse they encounter inside their nests. Responses like this to particular stimuli, that are general and predictable (sensory traps), may be open to

exploitation. We suggest that eucharitids exploit the general hygienic behavior of ants to quickly escape from their host nests. Other holometabolous myrmecophiles which are obligate predators or parasitoids of ant brood with free-living adults hatching within the host nests (see Fernández-Marín et al., 2006; Fiedler, 2012; Pérez-Lachaud et al., 2014) must face the same selective pressures. Interactions between diapriid or syrphid parasitoids and ants, for example, are poorly known, but these species might also exploit the general cleaning defensive behavior of ants to escape from their natal nests quickly. We propose that this strategy may be widespread not only among ant parasitoids but also among parasitoids of other social insects as may be the case of the adults of the very rare stingless bee parasitoid *Plega hagenella* (Neuroptera: Mantispidae), which was recently reported attacking *Melipona subnitida* (Maia-Silva et al., 2013). Distinct taxa of macroparasites might have evolved similar mechanisms to exploit social insects' hygienic behavior. However, whether such mechanisms represent a manipulation by the wasp, a general defensive strategy of the hosts, or a by-bore result (sensu Hughes, 2008) of cleaning activities remains to be explored.

5. Conclusions

Adult eucharitid parasitoids only partially mimicked their host CHC profile and were likely recognized as alien and quickly removed from the nest by their hosts. Hexane-washed wasps did not elicit the prompt seizure response of ants, but were still removed from the nest. We propose that the general, defensive hygienic behavior of ants may be exploited by eucharitids to quickly escape from their natal nest unscathed. Rapid ejection of intruders likely benefits the eucharitids since it allows the wasps to quickly exit from the host nest to find a partner and reproduce. Furthermore, by removal of parasitoids ants get rid of these intruders at a very low energetic cost compared with escalated aggression.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jinsphys.2015.03.003.

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How to escape from the host nest: imperfect chemical mimicry in eucharitid parasitoids and exploitation of the ants' hygienic behavior.

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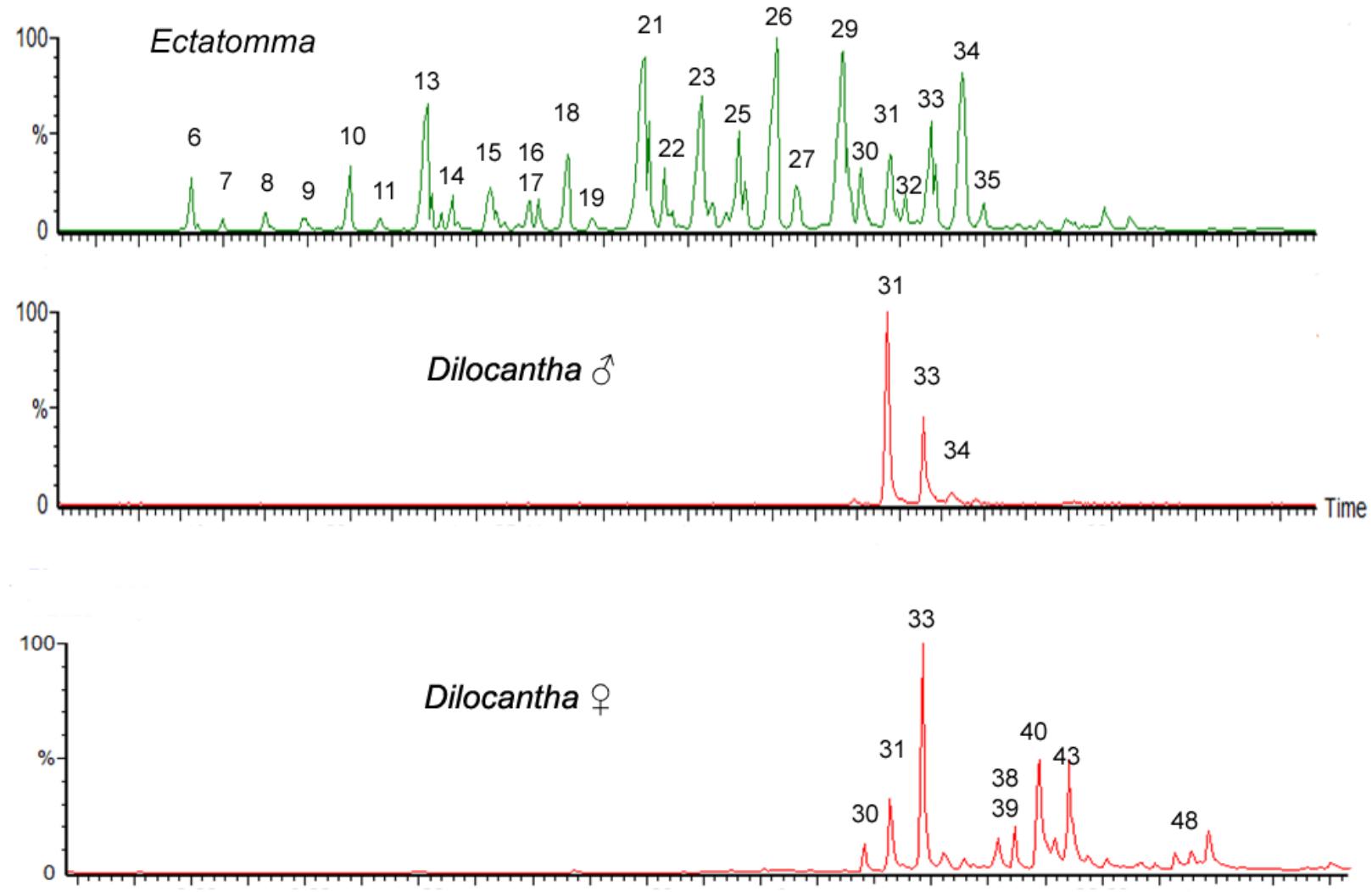
ELECTRONIC SUPPLEMENTARY MATERIAL

Figure S1
Representative gas chromatograms of the cuticular hydrocarbons of *E. tuberculatum* and its parasitoids. The numbers correspond to the peaks for *E. tuberculatum* and follow peak numbers in Table 2.
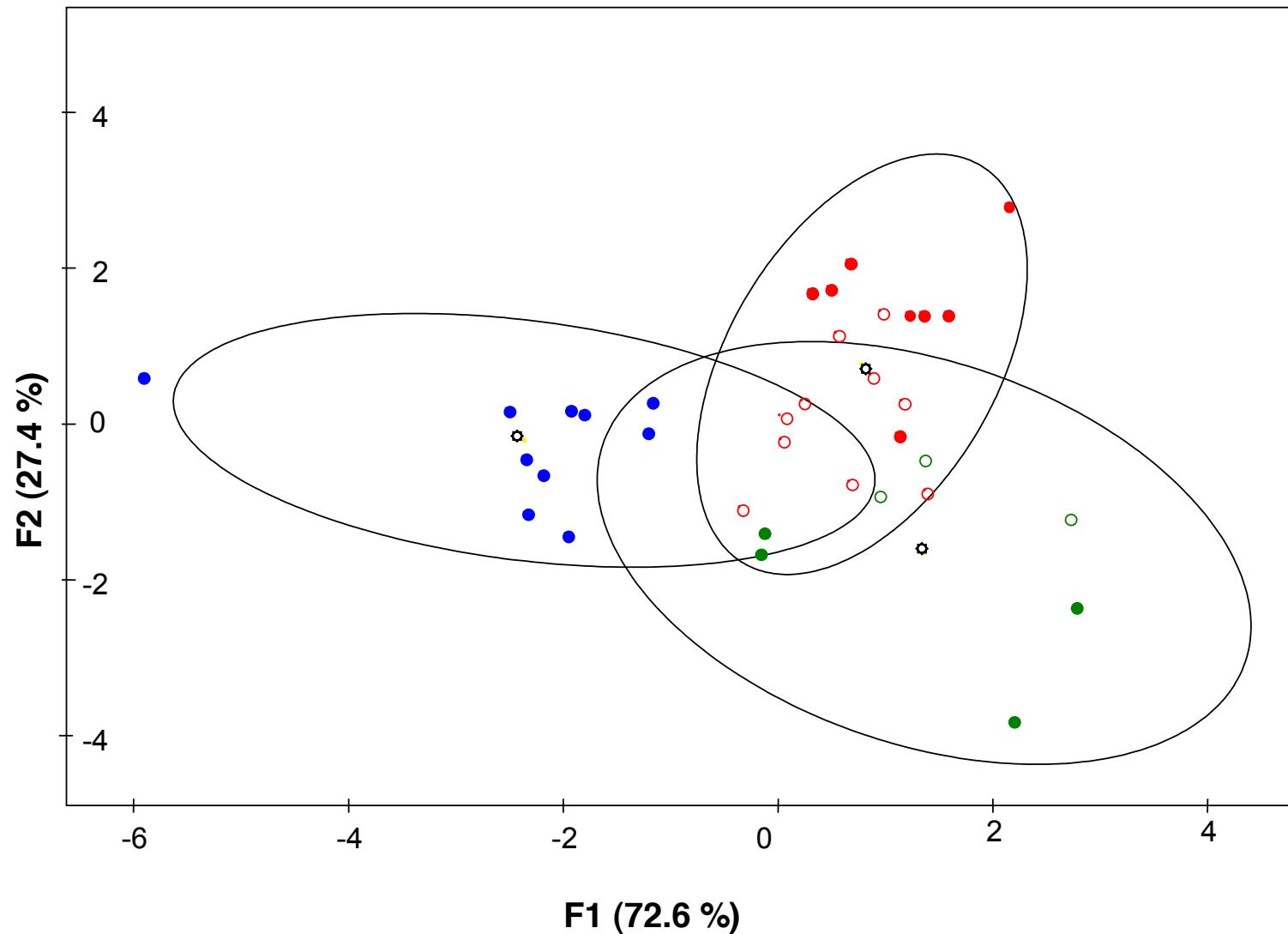
Figure S2
Discriminant analysis based on the cuticular hydrocarbons of ants and parasitoids. *E. tuberculatum* workers (blue); *D. lachaudii* (red); *I. coronata* (green). The percentage of the variance explained by each function is presented in parentheses. Ellipses are 95% confidence intervals around centroids (+). Open symbols: males, filled symbols: females. The model permitted the correct classification of 91.4% of the individuals. Males from the two parasitoid species were more similar to each other than to females of their own species.
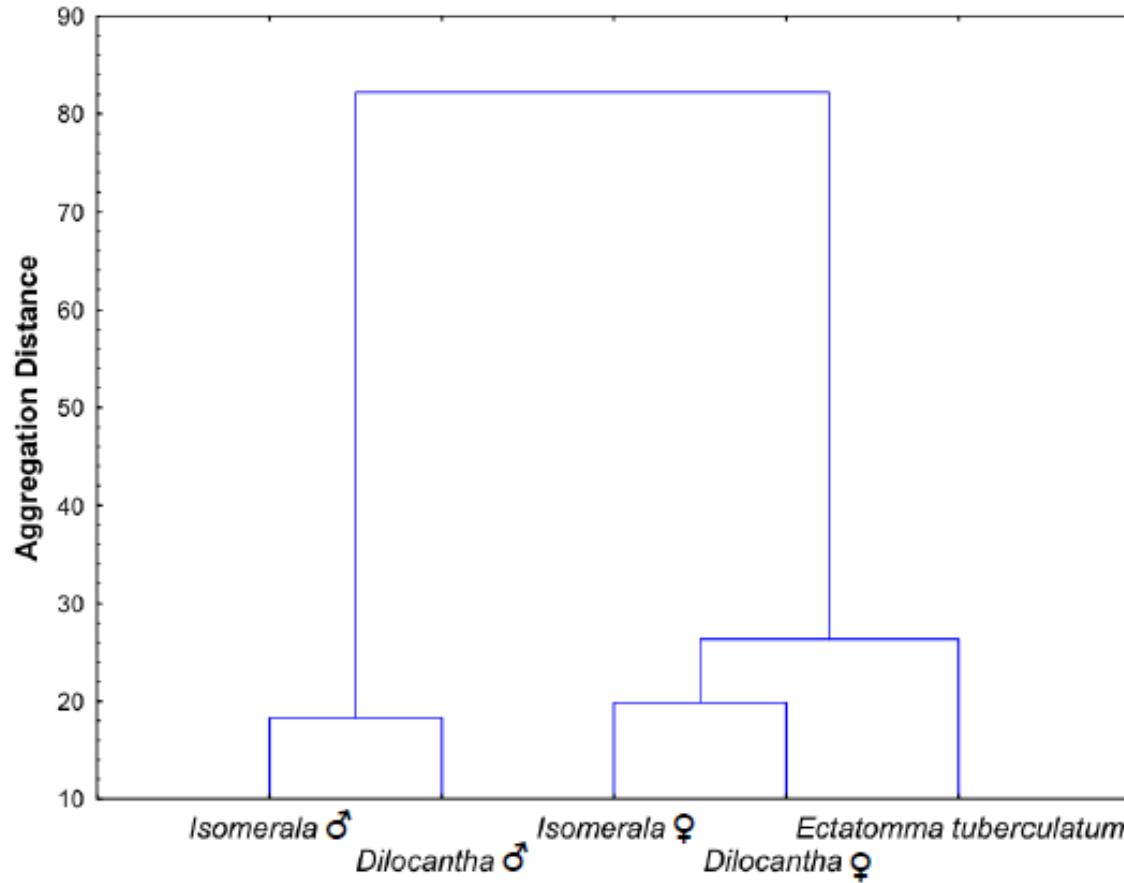
Figure S3.
Hierarchical cluster analysis of the CHCs of *E. tuberculatum* workers and of their eucharitid parasitoids, *D. lachaudii* and *I. coronata*. Analysis based on the relative proportions of all compounds, single linkage, Euclidian distances, Ward's method.

Table S1. Comparison of percent composition by class of CHCs between *Ectatomma tuberculatum* and its

parasitoid wasps

CHC Class	*Ectatomma tuberculatum* workers	*Dilocantha lachaudii*		*Isomerala coronata*	
		female	male	female	male
n-alkanes	7.9% (7/89)	5.4% (2/37)	5.1% (2/39)	12% (3/25)	5% (1/20)
methyl-branched alkanes	83.1% (74/89)	91.9% (34/37)	92.3% (36/39)	80% (20/25)	95% (19/20)
alkenes	9% (8/89)	2.7% (1/37)	2.6% (1/39)	8% (2/25)	0