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Cuticular hydrocarbon profiles in velvet ants (Hymenoptera: Mutillidae) are highly complex and do not chemically mimic their hosts

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Abstract

Although recognition using cuticular chemistry is important for host–parasite interactions within aculeate Hymenoptera, cuticular hydrocarbon (CHC) profiles of only a few host–parasite pairs were characterized and compared. One largely neglected family in this context is the Mutillidae (velvet ants), whose species are ectoparasitoids of bees and wasps. In our study, we characterized and compared the CHC profiles of five species of Mutillidae and seven host species. The CHC profile of velvet ants differed among species and included large proportions of *n*-alkanes and methyl-branched alkanes. Alkenes were much less abundant in the CHC profiles of three species of velvet ants compared with their hosts, while the other two species possess a much lower abundance of methyl-branched alkanes than their hosts. Both the number of peaks and compound diversity were generally higher in velvet ants compared with their hosts. Thus, CHC profiles of parasitoids did not show signs of mimicry when compared with their hosts. In dyadic encounters between one species of velvet ant and its host bee species, the parasitoid mainly avoided interacting, while aggression by the host was rare. Our results suggest that velvet ants did not evolve chemical mimicry, perhaps in accordance with their wide host spectrum which would limit chemical specialization. However, the reduction of alkenes in social bee-attacking species and the reduction of methyl-branched alkanes in social wasp-attacking species may favour host nest invasion, since these two CHC classes are known to be important in nestmate recognition for social bees and wasps, respectively. A larger, phylogeny-corrected comparison of Mutillidae and hosts may help clarifying the evolution of the CHC profile of these parasitoids.

Keywords Cuticular hydrocarbon profile · Mutillidae · Bee hosts · Wasp hosts · Chemical mimicry

Introduction

Brood parasitism, i.e. invasion of a host's nest to exploit its resources, is widespread in insects and fascinated natural historians for a long time (Buschinger 2009; Cini et al. 2019;

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Litman 2019). Brood parasites can be subdivided into three following categories: parasitoids (whose brood feeds on the host immatures) (O'Neill 2001), cleptoparasites (whose brood feeds on the host's food stores) (Michener 2007), and social parasites (reproductive females occupy and exploit the worker force of the host colony to raise their own offspring) (Lorenzi 2006). These brood parasites have to sneak into the host nests without being detected by the adult hosts. In doing so, females of insect brood parasites easily leave chemical traces in the nest during invasion, potentially provoking a defensive response by the host, including the destruction of the parasites' egg or by attacking the adult parasite (Rosenheim 1988; Ballesteros et al. 2012; Polidori et al. 2009). Thus, brood parasites evolved strategies to reduce the probability of being detected by their hosts or, alternatively, to reduce damages in case of direct fights. In turn, hosts evolved mechanisms to detect brood parasites, to avoid parasitism, or to physically defend themselves better in case of

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direct fight (Poulin et al. 2000). This leads to an evolutionary arms race between hosts and parasites (Dawkins and Krebs 1979; Schmid-Hempel 1998) involving reciprocal adaptations in behaviour (e.g. Polidori et al. 2010; Foitzik et al. 2003), morphology (e.g. Ortolani and Cervo 2010) or physiology (e.g. Brandt et al. 2005; Wurdack et al. 2015; Barbero et al. 2009). The latter often alter communication channels that allow parasites to enter the host's nest undetected.

Among such physiological adaptations in brood parasitic insects, many are linked to strategies that prevent recognition by hosts via chemical deception and are especially studied in aculeate Hymenoptera (bees, wasps and ants), a diverse insect group in which parasitism evolved independently several times (O'Neill 2001; Lorenzi 2006; Michener 2007). Chemical deception exploits chemical recognition and communication in intra- and interspecific interactions in insects (Howard and Blomquist 2005). Three strategies are known, all of them are essentially based on modifications of the cuticular hydrocarbon (CHC) profile, i.e. a thin layer of non-polar substances that covers the entire surface of insects. Besides their primary function in reducing desiccation, abrasion, and infection (e.g. Menzel et al. 2017), CHCs act as semiochemicals in various contexts of communication (Howard and Blomquist 2005; Blomquist and Bagnères 2010). One strategy is chemical mimicry, where the parasite synthetises de novo a CHC profile matching that of the host (Strohm et al. 2008; Wurdack et al. 2015; Polidori et al. 2020a). A second strategy is chemical insignificance, where the parasites have a quantitatively reduced CHC profile and/or a simpler composition of the profile, so that they are poorly perceived by the hosts (Johnson et al. 2001; Kroiss et al. 2009; Polidori et al. 2020b). A third strategy is chemical camouflage, which seems to be restricted to social parasites, where the parasite's CHC profile is acquired from the host through extended contacts with nest material or the hosts themselves (Lenoir et al. 2001; Cini et al. 2011; Johnson et al. 2001; Lorenzi 2006). For chemical camouflage, the selective pressure acts on the behavioural traits of the parasite (i.e. fear response, social behaviour) while for the de novo synthesis of CHC profile (mimicry and insignificance), the selection pressure acts on the biosynthetic pathway of the CHCs.

CHC profiles and their relation with those of the hosts were characterized for species belonging to only few families within aculeate Hymenoptera (Vespidae, Apidae, Halictidae, Formicidae, Chrysididae) and, among them, most often in social parasites compared with solitary parasitoids or kleptoparasites. For the latter two groups, data are available mainly for cuckoo wasps (Chrysididae) (Strohm et al. 2008; Wurdack et al. 2015; Polidori et al. 2020a; Soon et al. 2021; Fröhlich et al. 2022; Castillo

et al. 2022) and for cuckoo bees (Halictidae) (Polidori et al. 2020b). However, brood parasitism evolved in many other aculeate families, such as Sapygidae, Pompilidae, Scoliidae, Apidae, and Megachilidae (Danforth et al. 2019; O'Neill 2001; Branstetter et al. 2017). Hence, we are just at the beginning to understand the diversity and evolution of CHC profiles in solitary aculeates and to evaluate if natural enemies employ chemical deception strategies to increase the success of host attack. One of the neglected families whose CHC profile remains largely not studied are the Mutillidae. Investigations were made only on *Mutilla europea* L., a parasitoid of bumblebees and a kleptoparasite of the eusocial paper wasp *Polistes biglumis* L. (Uboni et al. 2012, Uboni and Lorenzi 2013) and recently on *Myrmilla capitata* (Lucas), a parasitoid of digger bees (Ronchetti et al. 2023). In *M. europea*, the CHC profile includes more hydrocarbons compared with its host, but the total amount of CHCs on the cuticle is significantly lower than the amount found on the *Polistes* host, suggesting an insignificance strategy. The *M. capitata* CHC profile also diverges from that of the host bees and shows a complex composition.

Mutillidae is a large wasp family including around 4600 species from 220 genera (Brothers and Lelej 2017; Pagliano et al. 2019). They are known as velvet ants because of their well-visible and often dense pilosity all over their body, and because their females are apterous (Mickel 1928). These wasps are ectoparasitoids and attack post-defecated larvae or pupae of other insects, most often other aculeate Hymenoptera, both solitary and social and both ground-nesters and aerial-nesters (reviewed in Ronchetti and Polidori 2020). Despite being elusive insects, velvet ants can be still observed patrolling the nesting areas of their hosts, especially in the early morning and late afternoon (Manley and Spangler 1983; Schmidt and Buchmann 1986; Polidori et al. 2009, 2010; Lienhard et al. 2010). Host use, probably known for only about 2–3% of all described mutillid species (Brothers 1989; Ronchetti and Polidori 2020), varies across mutillid lineages. Mutillids specialize on hosts with different ecological traits (nest type, larval diet and sociality). Therefore, they can be defined as ecological specialists rather than taxonomically specialist in their host use (Ronchetti and Polidori 2020). Velvet ants have remarkably interested zoologists for a long time because of the wealth of their defence strategies. These include the ability to stridulate by rubbing a scraper on a file on their gaster (Polidori et al. 2013), a variation of aposematic coloration which might lead to large Müllerian mimicry complexes (Wilson et al. 2015; Hines et al. 2017), the longest sting relative to body size among aculeate Hymenoptera (Sadler et al. 2018), a remarkably strong exoskeleton (Schmidt and Blum 1977), Zn-enriched

mandibles (Jorge et al. 2017), and a powerful venom (Starr 1985; Schmidt 1990; Jensen et al. 2021). All these traits were shown or at least strongly suggested to primarily serve as deterrent to the attack of predators (Schmidt and Blum 1977; Masters 1979; Gall et al. 2018; Schmidt et al. 2021), but some of them may also favour host nest invasions (e.g. cuticle robustness, metals in mandibles).

In our study, we analysed the composition of CHC profiles of velvet ants and their hosts. Because velvet ant species are often generalist in their host choice, we hypothesize that chemical mimicry did not evolve in these parasitoids. Indeed, chemical mimicry was essentially associated with hymenopteran brood parasites with a narrow host specialization (Strohm et al. 2008; Wurdack et al. 2015; Polidori et al. 2020a). However, velvet ants may still have evolved chemical traits favouring host nest invasion, such as reduction of CHC profile complexity, or reduction in the abundance of CHC classes involved in host chemical communication, that would make them less detectable by the hosts.

Material and methods

Sample collection and species synopsis

The collection of velvet ants was performed between 1st and 30th of June 2018 at three localities in Southern Europe: Alberese (Tuscany, Italy: 42°40′5″N, 11°6′23″E), El Saler (Valencia, Spain: 39°22′57″N 0°19′57″W) and Almarail (Soria, Spain: (41°34′ 50″N, 2°22′52″W). Five mutillid species were collected (Fig. 1A–B). Hosts were collected either at the nesting sites where their associated mutillids were found or in other locations (see below). Only female velvet ants and females of their host species were collected. *Myrmilla capitata* ($n = 10$) (Myrmillinae) and workers of its host bee *Lasioglossum malachurum* (Kirby) ($n = 10$)
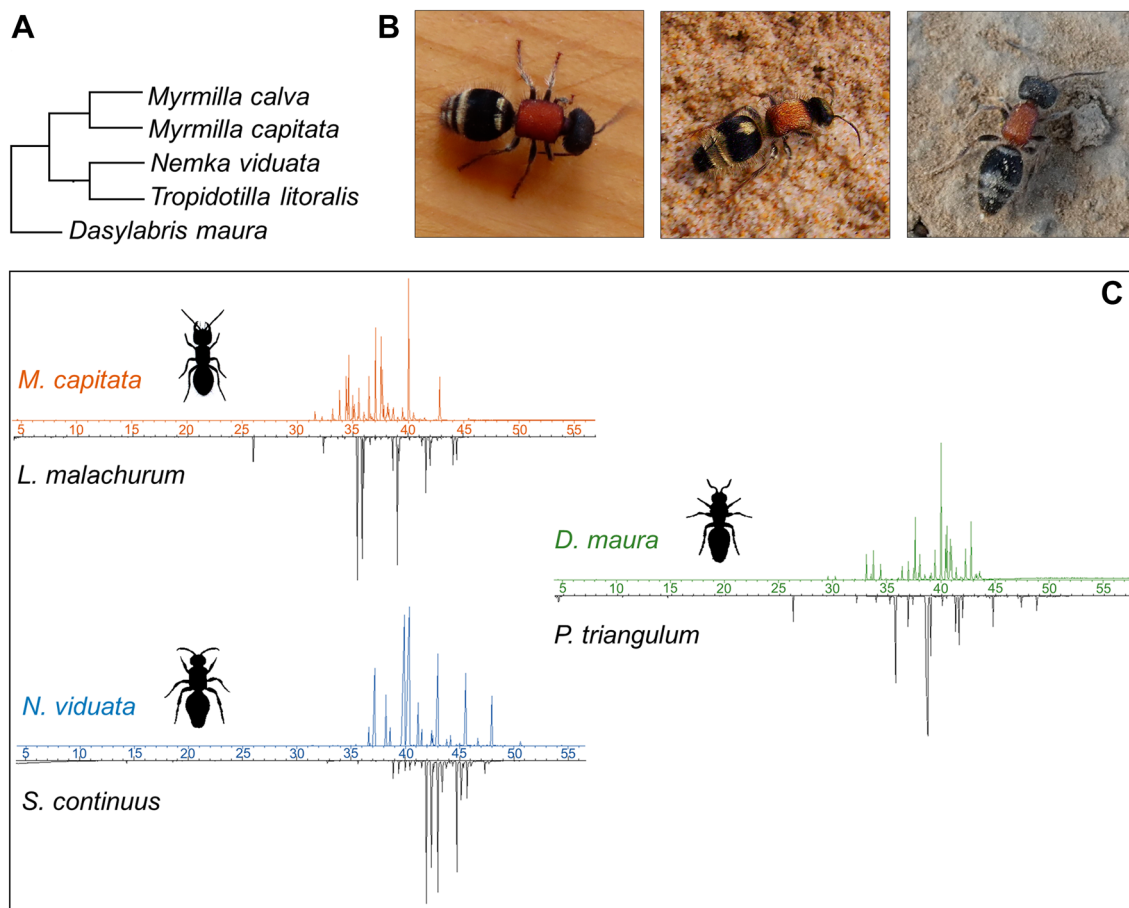

Fig. 1 **A** phylogenetic relationships among the five studied mutillid species, following Brothers and Lelej (2017). **B** pictures taken on field of adult females of three of the studied species of velvet ants (left to right: *T. litoralis*, *N. viduata*, *M. capitata*). **C** representative chromatograms of the CHC profiles of three species of velvet ants (above, coloured) and their respective hosts (below, in grey)

(Halictidae) were sampled in Alberese, at a nesting site of the latter, located in a trail bounded in part by cultivated fields (wheat or alfa-alfa, depending on the year), nearby the Ombrone River. *Myrmilla calva* ($n = 6$) and its host bee *L. malachurum* were collected at the same location, while a second host bee species, *Lasioglossum calceatum* (Scopoli) ($n = 7$), was collected at southern German localities in Baden-Württemberg (Freiburg: 47°59′41′′N, 7°50′59′′E). *Nemka viduata* (Pallas) ($n = 7$) (Smicromyrmini) and its host wasps *Stizus continuus* (Klug) ($n = 13$) and *Bembecinus tridens* (Fabricius) ($n = 5$) (Crabronidae) were collected at El Saler, at one nesting site of both hosts. *Dasylabris maura* (L.) ($n = 4$) (Dasylabrini) and its host wasp *Ammophila laevicollis* (Ed. André) ($n = 6$) (Sphecidae) were collected at a host nesting site at Almarail, while a second host wasp species, *Philanthus triangulum* (Fabricius) ($n = 10$), was collected at a nesting site in Würzburg (Germany: 49°47′40″N 9°55′46″E). *Tropidotilla litoralis* (Petagna) ($n = 11$) (Mutillini) was collected at Alberese, while its host wasp *Polistes gallicus* (L.) ($n = 3$) (Vespidae) was collected on Pag, an island of Croatia (44°30′27″N 14°56′26″E). *Stizus continuus*, *B. tridens*, *P. triangulum* and *A. laevicollis* are solitary species specialized in hunting grasshoppers, homopterans, honeybees and lepidopteran larvae, respectively, to feed the brood (O'Neill 2001; Evans and O'Neill 2007), while *L. malachurum*, *L. calceatum* and *P. gallicus* are eusocial species with a generalist pollen (the former two) or arthropod (the latter) larval diet (Michener 2007; Hunt 2007). All hosts are ground-nesting species except *P. gallicus*, which builds aerial paper nests (Hunt 2007).

In all cases, both the velvet ant and host individuals were collected on sunny days, at hours of their foraging activity peak in the morning (9.00–12.00) (Polidori, et al. 2009, 2010). Female mutillid wasps were sampled using large plastic tubes with sponge caps directly on the ground, while hosts were sampled with an entomological net while approaching their nests or while exiting from their nests or while foraging on flowers. Once collected, the specimens were frozen at −20 °C. The morphology of the individuals was observed under a stereomicroscope to confirm identification of species and sex.

Chemical analysis

The samples were immerged in hexane in glass vials for 10 min to extract their cuticular profile. The extracts were stored at −20 °C. The specimens were stored in 99% ethanol to preserve the DNA. By using a gentle stream of nitrogen, the volume of the extracts was reduced to about 200 µl. The gas chromatography/mass spectrometry analysis was performed using an Agilent 6890 gas chromatograph (GC) coupled with an Agilent 5975 Mass Selective Detector (MS) (Agilent, Waldbronn, Germany). The GC (split/splitless injector in splitless mode for 1 min, injected volume: 1 µl at 300 °C) was equipped with a DB-5 Fused Silica capillary column (30 m × 0.25 mm ID, df = 0.25 µm, J&W Scientific, Folsom, USA). We used helium as carrier gas at constant flow of 1 ml per minute. The following temperature program was used: start temperature 60 °C, temperature increase by 5 °C per minute up to 300 °C and isotherm at 300 °C for 10 min. The electron ionization mass spectra (EIMS) were acquired at an ionization voltage of 70 eV (source temperature: 230 °C). For recording and the analysis of chromatograms and mass spectra (Fig. 1C), we used the software HP Enhanced ChemStation G1701AA Version A.03.00. The compounds were identified by using retention indices and diagnostic ions. The retention index of each peak was calculated according to the formula of McNaught and Wilkinson (1997).

Behavioural assays

Behavioural interactions were studied between females of *M. capitata* and females (workers) of its host bee *L. malachurum* at their nesting site in Alberese in July 2021 (9.00–15.00). Staged dyadic encounters in a circle-tube apparatus (e.g. Pabalan et al. 2000; Boesi and Polidori 2011; Polidori et al. 2020b) were used to test if a host bee reacts differently when encounters a velvet ant or a conspecific. We recorded the interactions between *M. capitata* and *L. malachurum*; data on conspecific interactions in *L. malachurum* from the same nest aggregations come from a previous work (Polidori et al. 2020b). The circle-tube apparatus consisted of a 22 cm-long piece of clear sterile plastic tube of 0.7 cm inner diameter fashioned into circles. Pairs of *M. capitata* and *L. malachurum* were collected at the host nest aggregation and kept for a maximum of 15 min in a shaded place before to be used for the experiments. We first introduced the host bee in the circle-tube. After two minutes we introduced the parasitoid to resemble the situation of a nest invasion attempt. Each trial lasted 10 min, which was sufficient to detect behavioural differences (Pabalan et al. 2000). After each experiment, the used arena was discarded to avoid odour contaminations in subsequent trials (Smith and Weller 1989). A total of 26 trials, 19 host–host and 7 mutillid–host trails, were performed.

The recorded behaviours were classified as one of the three categories following previous circle-tube studies: tolerant, aggressive, and avoidant interactions (Pabalan et al. 2000; Packer 2006; Boesi and Polidori 2011; Polidori et al. 2020b). Tolerant behaviours include "mutual passing" (individuals accommodate each other while they pass in opposite directions), "following" (a forward movement by an individual toward the other which walks backward through the circle tube), and "stop in contact" (individuals in a frontal

encounter stop in contact and touch each other slowly with antennae and mandibles). Aggressive behaviours include "C-posture" (an individual curls her abdomen under the thorax with the intention to sting the other one), "mandibular hold" (an individual clamps the mandibles around the neck, limbs or antenna of the other one), "mandibular flare" (an individual quickly approaches the other one with open mandibles) and "push and lunge" (an individual lunges forward, often with a short forceful push, against the other one, usually with mandibles open). Avoidant behaviours include "withdrawing" (an individual makes a $180° \pm$ turn away from the other one and backs moves away from it) and "stop without contact" (the two individuals stop in front to each other but without touching) (Supplementary file BehavDATA).

Statistical analysis

We first deleted all the compounds which added less than 0.01% to the overall relative amount within each group. If a compound shows up to more than 0.01% in a single group, we kept it in all investigated groups for the comparative analysis. In a second step we eliminated all compounds which did not occur at least in 50% of all individuals within a group. The final matrix, including both velvet ants and hosts, included a total of 153 peaks (Tables S1–S2). Prior to the statistical analysis, since relative peak areas of a sample are not statistically independent, we transformed all the % peak values following the modified Aitchison (1986)'s method used by Strohm et al. (2008). With such transformation (log10((% peak area/geometric mean of % peak area) + 1)), we did not exclude compounds that do not occur in all samples, which is more adequate in case of parasite-host CHC comparisons (since the exclusion of peaks that are not present in all samples may erroneously increase the similarity between the groups). Transformed relative values used in the subsequent statistical analyses are provided in the Supplementary file ChemDATA.

We tested for chemical differences between groups by performing non-parametric comparisons and multivariate analyses. We tested for differences in the abundances of CHC classes between parasitoids and their hosts with Mann–Whitney test in case of systems composed by one parasitoid and one host or with Kruskal–Wallis test followed by Dunn's paired comparisons in case of systems composed by one parasitoid and two hosts. Bonferroni correction for multiple comparisons was applied to P-values in the latter case. We compared the total number of peaks and the peak diversity among groups as an overall indication of CHC profile complexity (Torres et al. 2018, and references therein). The peak diversity was estimated with the Shannon–Wiener index, with higher values indicating greater chemical diversity; this index depends on both the

number of substances and on the relative abundances of the substances. We used permutation tests (2000 permutations) to test for differences in peak numbers and Shannon–Wiener diversity in parasitoid–host paired comparisons. In addition, we measured the chain-length as the number of carbon atoms in the backbone of each molecule, and we calculated, for each species, a quantitative chain length distribution as the sum of CHC abundances separately for each chain-length (Menzel and Schmitt 2012). Then, we compared the distributions of compound abundances across chain-lengths between velvet ants and hosts, with a Kolmogorov–Smirnov test (one test per each of the parasitoid–host pair). We also repeated this latter analysis using only n-alkanes, since n-alkanes aggregate more tightly, especially molecules with higher chain-length, compared with methyl-branched alkanes and alkenes, thus making them less volatile (Gibbs and Pomonis 1995; Gibbs 2002).

The multivariate analyses were all based on Bray–Curtis dissimilarity matrices, which are suitable for zero-inflated datasets (Leyer and Wesche 2007). These analyses do not require a priori grouping of species, meaning that these methods allow pattern formation that are exclusively based on CHC similarities. We first performed an agglomerative cluster analysis based on the unweighted pair group method using arithmetic means of Bray–Curtis dissimilarities. Second, Bray–Curtis dissimilarities were used for ordinations using non-metric multidimensional scaling analysis (NMDS), which is a non-parametric method that avoids assuming linearity among variables (McCune et al. 2002) and whose resulting plot shows the spatial distances between individuals (i.e. their chemical distances). In the NMDS, deviations are expressed in terms of "stress", for which values ≤ 0.15 indicate a good fit of ordination (Kruskal and Carroll 1969).

PERMANOVA (Non-Parametric MANOVA) was employed to test for differences among the studied species (Anderson 2001). The significance is computed by permutation of group membership (9999 replicates). Pairwise PERMANOVA between all pairs of groups was also computed as a post-hoc test. To correct error rates for multiple comparisons, we used the Bonferroni—Holm procedure (Holm 1979) (or sequential Bonferroni significance), a procedure which adjusts the rejection criteria for each hypothesis (Giacalone et al. 2018). Similarity percentages (SIMPER) were calculated to identify the compounds that predominantly contributed to the Bray–Curtis dissimilarities among pairs of species (Clarke 1993). We used the Kruskal–Wallis test to compare the average SIMPER dissimilarity among host–parasitoid, parasitoid–parasitoid, and host–host species pairs.

Count data from the behavioural experiments were not always normally distributed (Shapiro–Wilk test:

$W = 0.46$–0.93, $0.00001 < P < 0.6$) and contain many zeros. Thus, as in previous studies with similar behavioural data (e.g. Legendre et al. 2008; Smith et al. 2019; Polidori et al. 2020a, b,) we used the non-parametric Kruskal–Wallis test (followed by paired comparisons through Dunn's procedure) to test for differences in the total number of interactions and in the number of aggressive, tolerant, and avoidance interactions observed across the three groups of heterospecific and conspecific trials (*M. capitata* towards *L. malachurum*, *L. malachurum* towards *M. capitata* and in *L. malachurum*-L. *malachurum* pairs).

In the text and tables, mean values are expressed with standard error. The statistical analyses were performed in PAST 3.04 (Paleontological Statistics Software Package) (Hammer et al. 2001).

Results

Cuticular hydrocarbon profiles

The CHC profile of the velvet ants was composed of 37–59 substances (median = 49) and showed a Shannon–Wiener diversity ranging from 2.55 to 3.12 (median = 2.87), depending on the species (Table 1). The CHC profile of the hosts was composed of 22 to 60 substances (median = 41) and showed a Shannon–Wiener diversity ranging from 1.99 to 2.99 (median = 2.42), depending on the species (Table 1). In paired permutation tests, parasitoids had both, higher compound richness and diversity than their hosts in four cases, while richness only was higher in a parasitoid than in its host in an additional case. In the remaining paired comparisons, parasitoids and hosts either did not differ in both, compound richness and diversity (2 cases), or parasitoids had both, lower compound richness and diversity than their hosts (1 case: *N. viduata* vs. *S. continuus*) (Table 1).

The CHC profiles of velvet ants spanned the following five substance classes: *n*-alkanes, monomethyl-branched alkanes, dimethyl-branched alkanes, trimethyl-branched alkanes, and alkenes (Fig. 2A, Table S1–S2). All substance classes were represented in all species, except for trimethyl-branched alkanes, which lacked in *N. viduata*. *n*-Alkanes were abundant in all species (38.5%–72.4%), followed by monomethyl-branched alkanes (9.8%–45.3%). Dimethyl-branched alkanes were less abundant, reaching maximum 16.1% and in one case being < 1%. Trimethyl-branched alkanes, when present, never exceeded 2% in abundance. Alkenes were abundant only in *N. viduata* (33.3%), being

Table 1 Compound richness (number of peaks) and peak diversity (Shannon–Wiener index) of the five species of velvet ants and their seven species of hosts, together with the *P* values from permutation tests of parasitoid–host comparisons

|	Richness	Diversity
Parasitoids		
M. calva	37	2.85
M. capitata	49	3.12
D. maura	59	2.55
N. viduata	40	2.59
T. litoralis	57	2.86
Hosts		
L. malachurum	22	1.99
L. calceatum	41	2.82
P. triangulum	35	2.06
A. laevicollis	48	2.42
S. continuus	57	2.99
B. tridens	32	2.27
P. gallicus	60	2.95
Contrasts	*P*	*P*
M. calva vs. *L. calceatum*	0.294	0.9817
M. calva vs. *L. malachurum*	0.0001	0.0001
M. capitata vs. *L. malachurum*	0.0001	0.0001
N. viduata vs. *S. continuus*	0.0001	0.0001
N. viduata vs. *B. tridens*	0.0003	0.0082
D. maura vs. *A. laevicollis*	0.0007	0.1698
D. maura vs. *P. triangulum*	0.0001	0.0003
T. litoralis vs. *P. gallicus*	0.5097	0.8567
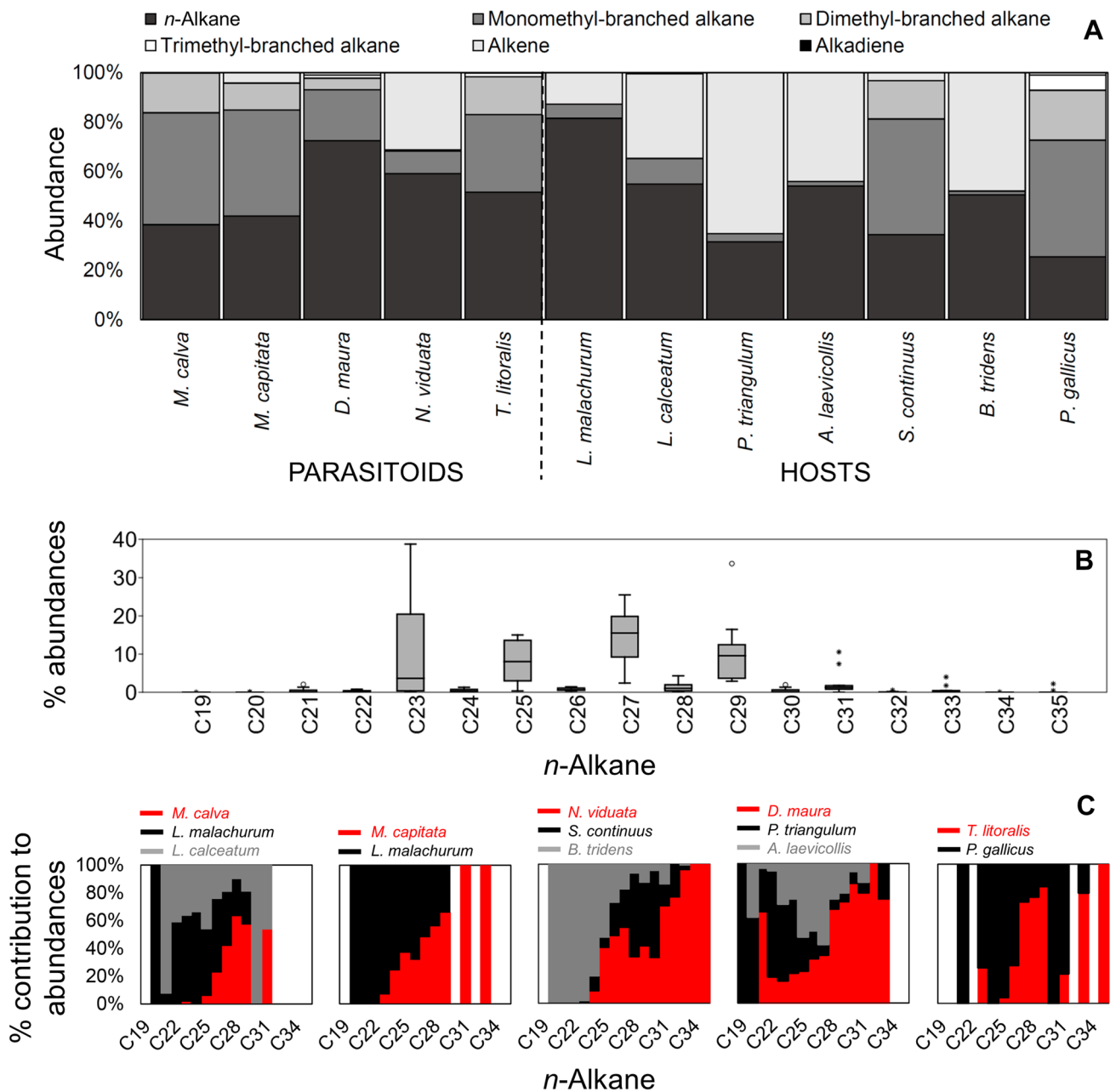
Fig. 2 **A** relative abundance of the hydrocarbons substance classes in the CHC profiles of the five studied species of velvet ants and of their seven host species. **B** box-and-whisker plots showing medians (horizontal lines within boxes), 1° and 3° quartiles (horizontal lines closing the boxes) and maximum and minimum values (ends of the whiskers) for relative abundance of the 17 detected n-alkanes. Outliers with a value more than 1.5 times the interquartile range are shown as circles, values with more than three times the interquartile range are shown as stars. C, contribution to abundance of the of the 17 detected n-alkanes in all the studied parasitoid-host systems

rarely represented (0.1–4.2%) in the other species. Overall, the relative abundances of the substance classes differed across species (Fig. 2A, Table S1–S2). Host species also show significant variation in the relative abundance of the CHC classes. Hosts possessed substances belonging to the same substance classes detected in velvet ants, *plus* alkadiens, which were rare (0.5%) and only present in *L. calceatum* (Fig. 2A, Table S1–S2). *n*-Alkanes were the most abundant

substance class in the hosts, while methyl-branched alkanes were abundant in *S. continuus* and *P. gallicus* (>61%) but much rarer in other host species (<6%); similar patterns appeared while considering monomethyl- and dimethyl-branched alkanes separately. Alkenes were abundant in all hosts (>12%) apart from *S. continuus* and *P. gallicus*, in which this compound class is much rarer (<4%) (Fig. 2A,

Table 2 Mean value ± standard error of the relative abundances of five CHC classes found on the cuticle of the five species of velvet ants and their seven species of hosts, together with the parasitoid-host statistical comparisons

	n-Alkanes	Monomethyl-branched alkanes	Dimethyl-branched Alkanes	Trimethyl-branched Alkanes	Alkenes
Parasitoids					
M. calva	38.48 ± 4.11	45.26 ± 2.76	16.05 ± 2.80	0.12 ± 0.08	0.09 ± 0.04
M. capitata	41.88 ± 2.72	42.98 ± 1.76	10.85 ± 1.54	0.06 ± 0.02	4.22 ± 0.48
D. maura	72.44 ± 4.87	20.60 ± 3.80	4.73 ± 0.73	1.18 ± 0.12	1.06 ± 0.25
N. viduata	56.38 ± 2.53	9.77 ± 1.84	0.53 ± 0.05	0.00	33.32 ± 1.14
T. litoralis	51.61 ± 6.26	31.40 ± 4.84	15.33 ± 3.98	1.55 ± 0.21	0.11 ± 0.03
Hosts					
L. malachurum	81.48 ± 1.09	5.79 ± 0.75	0.00	0.00	12.73 ± 0.96
L. calceatum	54.93 ± 3.90	10.34 ± 5.95	0.08 ± 0.02	0.00	34.14 ± 2.86
P. triangulum	31.50 ± 1.58	3.32 ± 0.49	0.00	0.00	65.18 ± 1.75
A. laevicollis	54.09 ± 3.69	1.92 ± 0.51	0.00	0.00	43.99 ± 3.80
S. continuus	34.41 ± 1.76	46.78 ± 2.11	15.61 ± 0.17	0.00	3.20 ± 0.28
B. tridens	50.48 ± 0.98	1.55 ± 0.12	0.05 ± 0.02	0.00	47.91 ± 1.01
P. gallicus	25.48 ± 4.78	47.18 ± 3.16	20.18 ± 4.92	6.09 ± 3.36	1.08 ± 0.54
Contrasts					
M. calva-hosts system	$\chi^2 = 17.9, df = 2, P = 0.00013$	$\chi^2 = 11.8, df = 2, P = 0.002$	$\chi^2 = 16.1, df = 2, P < 0.0001$	$\chi^2 = 3.2, df = 2, P = 0.01$	$\chi^2 = 18.9, df = 2, P < 0.0001$
M. calva vs. *L. calceatum*	$P = 0.56$	$P = 0.005$	$P = 0.005$	$P = 0.03$	$P < 0.0001$
M. calva vs. *L. malachurum*	$P = 0.00016$	$P = 0.008$	$P < 0.0001$	$P = 0.015$	$P = 0.05$
M. capitata vs. *L. malachurum*	$U = 0, N = 20, P = 0.0001$	$U = 0, N = 20, P = 0.0001$	$U = 0, N = 20, P < 0.0001$	$U = 20, N = 20, P = 0.010$	$U = 0, N = 20, P < 0.0001$
N. viduata-host system	$\chi^2 = 18.5, df = 2, P < 0.0001$	$\chi^2 = 19.9, df = 2, P < 0.0001$	$\chi^2 = 19.9, df = 2, P < 0.0001$	–	$\chi^2 = 19.9, df = 2, P < 0.0001$
N. viduata vs. *S. continuus*	$P = 0.0001$	$P = 0.011$	$P = 0.011$	–	$P = 0.011$
N. viduata vs. *B. tridens*	$P = 1$	$P = 0.49$	$P = 0.49$	–	$P = 0.49$
D. maura-host system	$\chi^2 = 15.5, df = 2, P = 0.0004$	$\chi^2 = 13.5, df = 2, P = 0.0011$	$\chi^2 = 9.1, df = 2, P < 0.0001$	$\chi^2 = 9.1, df = 2, P < 0.0001$	$\chi^2 = 16.0, df = 2, P = 0.0003$
D. maura vs. *A. laevicollis*	$P = 0.82$	$P = 0.0007$	$P < 0.0001$	$P < 0.0001$	$P = 0.57$
D. maura vs. *P. triangulum*	$P = 0.001$	$P = 0.08$	$P < 0.0001$	$P < 0.0001$	$P = 0.0006$
T. litoralis vs. *P. gallicus*	$U = 5, N = 14, P = 0.09$	$U = 3, N = 14, P = 0.03$	$U = 7, N = 14, P = 0.17$	$U = 11, N = 14, P = 0.45$	$U = 9, N = 14, P = 0.28$

The two-species parasitoid-host systems were analysed with Mann–Whitney test, while for the three-species parasitoid-host systems, both the overall test for differences (Kruskal–Wallis test) and the paired tests (Dunn's procedure) are included. "- " indicates that the CHC class was absent in all species within the system. Alkadiens were only found at small proportions (0.5% on average) in *L. calceatum* and are not shown here (see text)

Table S1–S2). Trimethyl-branched alkanes only occurred in *P. gallicus* (Fig. 2A, Table S1).

The abundance of *n*-alkanes differed in most host–parasitoid systems. Exceptions include *M. calva-L. calceatum*, *N. viduata-B. tridens* and *T. litoralis-P. gallicus* parasite-host pair (Table 2, Fig. S1). *M. capitata* and *M. calva* had lower abundances of *n*-alkanes than their host *L. malachurum* but did not differ from its host *L. calceatum*. *Dasylabris maura*

had a higher abundance of *n*-alkanes than its host *P. triangulum*, but a similar abundance compared to the other host *A. laevicollis*. *Nemka viduata* had a higher abundance of *n*-alkanes than its host *S. continuus*, but a similar abundance compared to the other host *B. tridens* (Table 2, Fig. S1). Monomethyl-branched alkanes were always more abundant in parasitoids than in their hosts, with exception of *N. viduata* and *T. litoralis*, which had lower abundances than their

hosts or a similar abundance in the case of *N. viduata* compared to *B. tridens* (Table 2, Fig. S1). Dimethyl-branched alkanes followed a similar pattern, being always more abundant in parasitoids than in their hosts, with exception of *N. viduata* and *T. litoralis*, which had lower (*N. viduata* to *S. continuus*) or similar (*N. viduata* to *B. tridens* and *T. litoralis* to *P. gallicus*) abundances than their hosts (Table 2, Fig. S1). Trimethyl-branched alkanes were much rarer, being absent in seven out of 12 studied species (Table 2, Fig. S1). At last, alkenes were always less abundant in parasitoids than in their hosts, with exception of *N. viduata* and *T. litoralis*, which had higher (*N. viduata* to *S. continuus*) or similar (*N. viduata* to *B. tridens* and *T. litoralis* to *P. gallicus*) abundances than their hosts (Table 2, Fig. S1).

The carbon chain length of the CHC profiles ranged from 19 to 35 C atoms, with highest abundances recorded in all species for compounds with 23–31 C atoms (Fig. 2B). Few differences appeared among species. For example, compounds with 19 C atoms were only found in one velvet ant and one host species, while compounds with 35 C atoms only occurred on the cuticle of two velvet ant and one host species (Table S1-S2). However, overall, velvet ants and their hosts had similar total chain lengths in their CHC profiles. This was true, considering all compounds (Kolmogorov–Smirnov test: $0.17 < D < 0.29$, $N = 17$, $P > 0.3$) and *n*-alkanes alone (Kolmogorov–Smirnov test: $0.12 < D < 0.24$, $N = 17$, $P > 0.6$) (Fig. 2C).

The CHC profiles of the five velvet ant species, as well as that of the seven host species, were species-specific. In the dendrogram produced by the hierarchical cluster analysis, individuals of each species formed clusters (Fig. 3A). The two main groups of clusters included six species each. In one, five species were hosts and one was a parasitoid, while, in the other one, four species were parasitoids and two were hosts (Fig. 3A). There may be preliminary indications for a possible effect of phylogeny on chemical distances in velvet ants: the two species of *Myrmilla* fall close to each other in a sub-cluster, while the other sub-cluster included *N. viduata* and *T. litoralis*, and the last species, *D. maura*, was more distant. This pattern (*Dasylabris* + ((*Myrmilla*) + (*Nemka* + *Tropidotilla*))) reasonably reflects the current known phylogenetic relationships in velvet ants (Fig. 1A).

Fig. 3 A dendrogram based on the agglomerative cluster analysis (Bray-Curtis distances) of all individual CHC profiles from the 12 studied species. Colours identify species as depicted in (**B**), grey arrows link each velvet ant species with its host(s). **B** non-metric multidimensional scaling (NMDS) based on Bray-Curtis distances of all individual CHC profiles from the 12 studied species. Triangles represent mutillids and circles represent host species
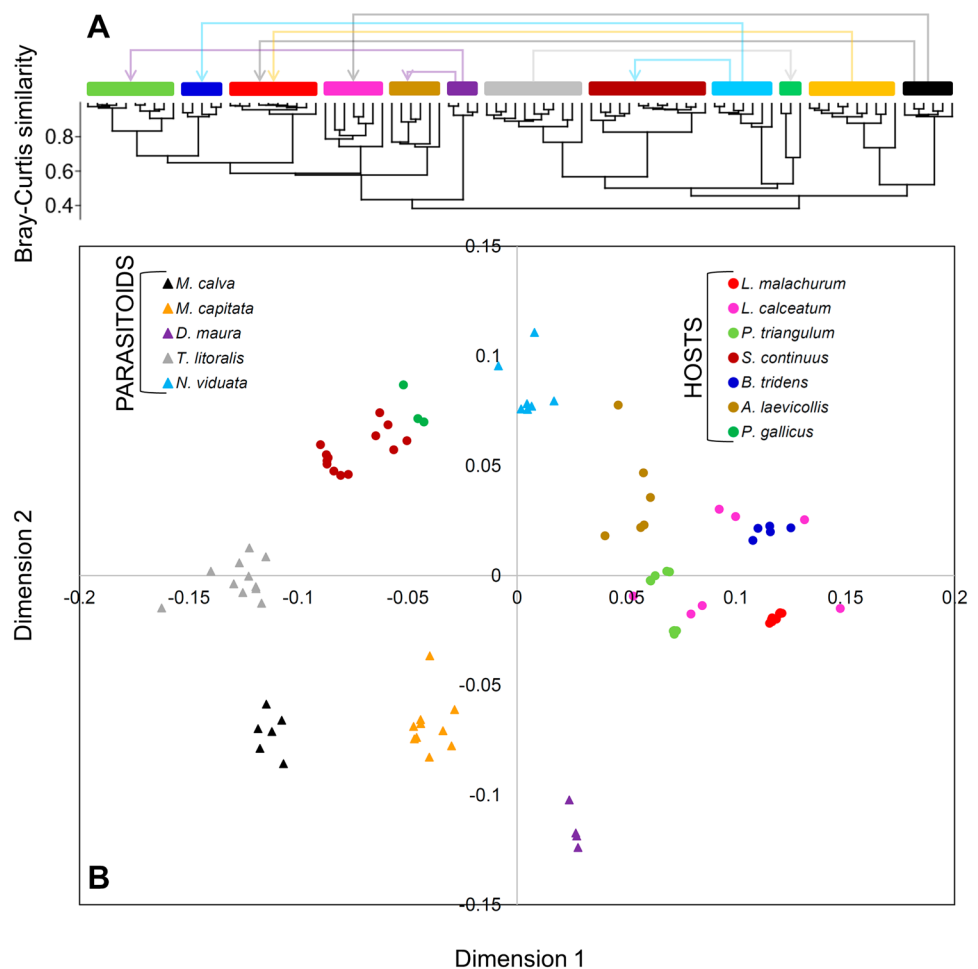
In accordance with the cluster analysis, the 12 studied species resulted in significantly separated clusters in the NMDS multivariate space (stress = 0.13, PERMANOVA: total sum of square = 13.39, within-group sum of square = 0.74, $n = 92$, $F = 124.3$, $P = 0.0001$), despite partial overlap of *L. calceatum* with three other host species. In all cases, all paired species differences were significant (Pairwise test: $0.0001 \leq P \leq 0.03$) (Fig. 3B). The SIMPER analysis showed that the overall average chemical distance ranged between 30.8 and 74.5 and did not differ among host-parasitoid (median = 56.4), parasitoid-parasitoid (median = 58.6) and host-host (median = 48.0) species pairs (Kruskal–Wallis test: $\chi^2 = 3.83$, $df = 2$, $P = 0.15$). In host–parasitoid contrasts, the lowest values of distance were recorded between a parasitoid and its host only in the cases of *D. maura* vs. *P. triangulum* and *N. viduata* vs. *S. continuus* (Fig. S2). In all other cases, the velvet ant species showed the lowest distance to a non-host species (Fig. S2). Especially bee hosts (*Lasioglossum* spp.) had very high distances to their *Myrmilla* parasitoids (Fig. S2). The SIMPER analysis revealed that the compounds contributing to more than 1% of CHC distances among species (36 substances for a total of 46.2% of contribution) were monomethyl-branched alkanes (21 substances) and alkenes (9 substances). Alkenes were the substance class most contributing to chemical distances (9%–15%) in all cases except in *T. litoralis-P. gallicus* in which the most important substance class was dimethyl-branched alkanes (8.1%) (Fig. S3). Monomethyl-branched alkanes also importantly accounted for distances in all cases except in *N. viduata-B. tridens* and in *M. calva-L. calceatum* in which *n*-alkanes were important (Fig. S3).

Behaviour

Individuals placed in the circle-tubes started to interact within 1 min from the start of the experiments. Overall, we have recorded 579 interactions, out of which most were tolerant (350), followed by avoidant (204) and by aggressive behaviours (25). *Lasioglossum malachurum* interacted much more with a conspecific (11–64 interactions/trial, median = 21) than with *M. capitata* (3–19 interactions/trial, median = 7), and the velvet ants also tended to weakly interact with the host bee (1–11 interactions/trial, median = 7) (Fig. 4A) (Kruskal–Wallis test: $\chi^2 = 20.1$, $df = 2$, $P < 0.0001$). Dunn's test for paired comparison revealed that overall differences are due essentially to the behaviour recorded in the *L. malachurum-L. malachurum* trials. Tolerant behaviours, expressed mainly with "mutual passing" (58% of cases) were very common in host bee intra-specific trails (5–45 interactions/trial, median = 14) but they were very rare or null in the heterospecific experiments (*L. malachurum-M. capitata*: median = 3, *M. capitata- L. malachurum*: no records)
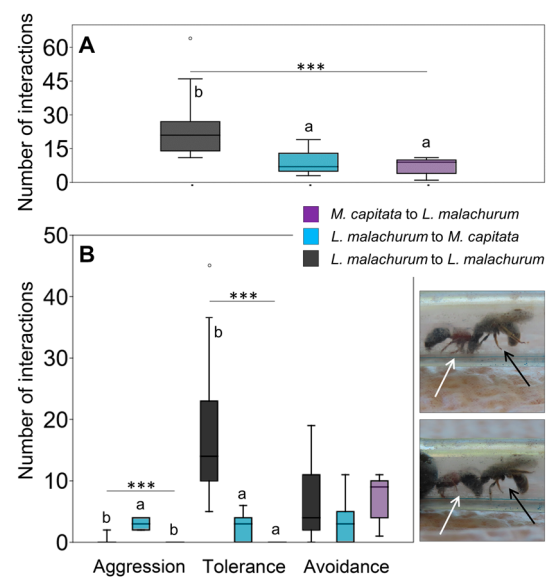

Fig. 4 Box-and-whisker plots showing medians (horizontal lines within boxes), 1° and 3° quartile (horizontal lines closing the boxes), and maximum and minimum values (ends of the whiskers) for the number of behavioural interactions recorded in circle-tube experiments. **A** total number of interactions. **B** number of aggressive, tolerant and avoidant interactions. Outliers with a value more than 1.5 times, the interquartile range are shown as circles. *** means that differences among types of interactions are significant at $p < 0.001$; letters identify pairwise differences (Dunn's procedure). Pictures on the left show a female *L. malachurum* "holding with mandibles" (above) and "pushing and lunging" (below) a female *M. capitata* (both aggressive behaviours)

(Kruskal–Wallis test: $\chi^2 = 24.1$, $df = 2$, $P < 0.0001$). Differences occurred only between conspecific trials and heterospecific trials (Dunn's test: $P < 0.001$) (Fig. 4B). Aggressive interactions, most often expressed by "mandibular hold" and "push and lunge" (72% of cases) (Fig. 4B) were overall rarely recorded in the experiments: while the host bee performed 0–2 aggressions towards a conspecific (median = 0) and no aggressions were observed in *M. capitata* towards the host bee, the former showed a significantly higher number of aggressions towards the parasitoid (2–4 interactions/trial, median = 3) (Kruskal–Wallis test: $\chi^2 = 15.9$, $df = 2$, $P < 0.0001$; Dunn's test: $P < 0.001$) (Fig. 4B). Avoidant behaviours, essentially expressed by "withdrawing" (92% of cases), were highly common in both host bee intraspecific trials (0–19 interactions/trial, median = 4) and in heterospecific trials (*L. malachurum* towards *M. capitata*: 0–11 interactions/trial, median = 3; *M. capitata* towards *L. malachurum*: 1–11 interactions/trial, median = 9) (Kruskal–Wallis test: $\chi^2 = 3.8$, $df = 2$, $P = 0.15$) (Fig. 4B).

Discussion

Enlarging the spectrum of studied lineages is fundamental to reconstruct the evolution of CHC in a large group of Hymenoptera, the Aculeata, spanning very diverse parasitism strategies. In this study, we explored some aspects of the chemical ecology of Mutillidae, by characterizing the CHC profiles of five species and evaluating the chemical differences between them and those of their bee and wasp hosts. Chemical strategies by parasitoid aculeates which attack bees and wasps were previously investigated only in chrysidid wasps, which are in general more specialized in host choice compared with velvet ants. Hence, we investigated whether the more generalist mutillids evolved chemical strategies to parasitize their hosts.

Species-specificity is a common feature of CHC profiles in insects, including Hymenoptera (e.g. Kather and Martin 2012; Caderón-Fernández et al. 2012; Moore et al. 2021) and was confirmed here for velvet ants. The inter-specific differences among the five studied velvet ant species seem to harbour a possible effect of common ancestry, since the placement of species in the clusters seem to follow the known phylogeny (Brothers and Lelej 2017, Waldren et al. 2023). However, we must investigate CHC profiles of more velvet ant species to further test this hypothesis. Indeed, since CHC profiles in Hymenoptera are usually under strong selection by intra- and inter-specific communication and/or abiotic factors, changes in CHC profiles generally do not show a phylogenetic signal (Hefetz 1993).

We found large chemical distances between CHC profiles of velvet ants and those of their hosts, except for *D. maura* which had the shortest chemical distance with its host *P. triangulum* and *N. viduata* which had the shortest distance with its host *S. continuus*. These two cases may suggest at first that weak chemical mimicry evolved in such systems. However, it is also true that both *D. maura* and *N. viduata* had larger chemical distances to other hosts, making this scenario unlikely. Not even a weak mimicry in these two cases are likely to be the result of local adaptation. Indeed, both hosts of *N. viduata* nested in close sympatry in a single mixed nesting area of about 100 m^2, while *D. maura* was collected at the same nesting site of its more chemically distant host species (*A. laevicollis*) and around 2000 km from the nesting site of its chemically closest host. The same is true for *P. gallicus*, which exhibit a more similar CHC to its parasitoid *T. litoralis*, the latter having been collected thousands of km far from its host. CHC distances did not point to even a weak chemical mimicry in any other velvet ant-host systems. This is especially visible in the two *Myrmilla* species attacking bees, which had much different CHC profiles. Hence, overall, chemical mimicry seems to essentially lack in velvet ants, although most of the species of this family

remain to be studied to generalize this conclusion with more confidence. In any case, our findings are in accordance with the hypothesis outlined before. Since these parasitoids are known to be taxonomically generalists in host use, each species often attacking host species from different genera or even families, it is not possible to match the CHC profiles of all of the exploited host species. Differences in the CHCs profile between mutillids and hosts were previously reported for *M. europea* and for previously studied individuals of *M. capitata* (Uboni et al. 2012; Ronchetti et al. 2023), and hence they are probably common for these parasitoid-host systems.

In previous studies on brood parasitic aculeate Hymenoptera, the evolution of chemical mimicry seems indeed associated with the degree of taxonomic host specialization. In chrysidid wasps, host specificity is often high (generally one cuckoo wasp species parasitizes one or two host species of a single genus) and accordingly chemical mimicry is more common (Strohm et al. 2008; Wurdack et al. 2015; Castillo et al. 2022; Polidori et al. 2020a). On the other hand, *Sphecodes* cuckoo bees, which are more generalist (each species attack host bee species from different genera and sometimes families) do not show chemical mimicry at all. Instead, these cuckoo bees evolved an alternative chemical insignificance strategy by reducing the CHC profile complexity and lacking CHC classes relevant in host nestmate recognition (Polidori et al. 2020b). Also in the only velvet ant species studied before our investigation, which attacks disparate hosts (bumblebees and paper wasps), insignificance is achieved by reducing the number of compounds as well as through the reduction of the total amount of hydrocarbons on the cuticle (Uboni et al. 2012). Chemical insignificance based on CHC amount reduction and/or profile simplification was also previously reported among many socially parasitic Hymenoptera at the invasion stage, i.e., prior to colony integration (and hence camouflage) (Lorenzi 2006; Johnson et al. 2001; Dronnet et al. 2005). In our study, velvet ants showed a complex CHC profile, often more diverse and richer in compounds than their hosts. However, we did not study if velvet ants possessed smaller amounts of hydrocarbons than their hosts. This strategy has to be tested in a future study.

On the other hand, we found patterns of alkene and methyl-branched alkane abundances that could perhaps suggest an adaptation to decrease CHC profile detectability by the hosts. There is evidence that these two substance classes are more important than *n*-alkanes in the recognition process, though not in all Hymenoptera (review in van Zweden and d'Ettorre 2010, see also below). Concerning alkenes, they were found to be less abundant in velvet ants compared with their hosts in most of the studied systems. On the other hand, monomethyl-branched alkanes were linked to nest mate recognition in some eusocial wasps (Dani et al. 1996; Dapporto et al. 2006; Lorenzi et al. 2004; Ruther et al. 2002; Tannure-Nascimento et al. 2007), and they were found

in lower abundance in a velvet ant species attacking *Polistes* social wasps. Interestingly, methyl-branched alkanes were also found in lower proportions in *M. europea* compared with its *Polistes* host (Uboni et al. 2012). In addition to these two substance classes, bee-attacking mutillids were the only species having lower abundances of *n*-alkanes compared with their hosts, and kin-based odour differences in *L. malachurum* were seen to be mainly based on *n*-alkanes (Soro et al. 2011). Chemically insignificant profiles have often a reduced number of substances or even lack entire substance classes important for host recognition (Johnson et al. 2001; Martin et al. 2008; Polidori et al. 2020b). This could be the case for velvet ants. However, the actual importance of these hydrocarbon classes in detecting velvet ants during nest invasion has to be evaluated in future studies. Furthermore, it may be important, to achieve chemical insignificance, to have both a simpler CHC profile and a reduction of CHCs amount, and this remains to be verified in our studied mutillid species.

The CHC profile's chain length was similar between the parasitoids and their hosts. This is interesting, since mutillid females, being wingless and thus being constantly in contact with the high temperatures of soil in summer, would be expected to have greater abundances of compounds with long chain length, which are known to reduce the effects of desiccation by having higher melting temperatures (Gibbs 1998; Gibbs and Pomonis 1995; Gibbs et al. 2003). An increase of unsaturated compounds (alkenes), on the other hand, are known to decrease CHC profile melting temperatures and can cause increased rates of water loss (Gibbs 1998). Indeed, alkenes were found to be poorly represented in velvet ants, except for *N. viduata*. Also, methyl-branched alkanes seem to protect from water loss (Gibbs 1998), though to minor extent compared with *n*-alkanes, and were found to be generally abundant in velvet ants.

Our behavioural data revealed that at least in the studied system composed of *M. capitata* and the bee *L. malachurum*, velvet ants are poorly recognized as a foe by the host. Indeed, while aggressive behaviours were expressed by the host at slightly higher frequencies in heterospecific than in conspecific encounters, these behaviours remain rare, compared with avoidance behaviours. Such results were similar to what was observed in aculeate brood parasites that either employ chemical mimicry (e.g. Chrysididae: Strohm et al. 2008; Polidori et al. 2020a) or chemical insignificance (Polidori et al. 2020b; Uboni et al. 2012; Uboni and Lorenzi 2013) to reduce host aggression. In *M. capitata-L. malachurum*, perhaps the very low abundance of alkenes may help parasitoids to hide from their host bees. Interestingly, *Sphecodes* cuckoo bees attacking *Lasioglossum* hosts lack alkenes and are not attacked by the hosts in similar behavioural experiments (Polidori et al. 2020b). Despite the clearly aggressive

behaviours recorded in our experiments (e.g. biting with mandibles), it was previous highlighted that true quantitative behavioural experiments should record the behavioural sequence and then use unbiased learning approaches to categorize similar pattern which then get interpreted, because the biological meaning of some behaviours (e.g. those falling in the "avoidance" category) may not necessarily be the same for all species (Dew et al. 2014). Hence, further experiments carried out with high-resolution cameras and machine learning tools will give more insights into the interactions between mutillid wasps and their hosts.

In conclusion, velvet ants did not evolve chemical mimicry. On the other hand, the possible role of some hydrocarbons-related chemical traits (reduced abundance of certain CHC classes), which may help them to invade the host nest by reducing host detection, need to be formally tested in future studies. However, morphological adaptations, including a robust, heavily sclerotized body, seem to be widespread as alternative/additional strategy to chemical strategies in brood parasitic insects (Kistner 1979; Cervo 1994; Tishechkin et al. 2017; von Beeren et al. 2021) and could be important traits favouring nest invasion in velvet ants.

Declarations

Conflict of interest The authors declare no competing interests.

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