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Cuticular Hydrocarbons Rather Than Peptides Are Responsible for Nestmate Recognition in *Polistes dominulus*

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Abstract

A colony of social insects is like a fortress where access is allowed only to colony members. The epicuticular mixture of hydrocarbons has been widely reported to be involved in nestmate recognition in insects. However, recent studies have shown that polar compounds (mainly peptides) are also present, mixed with hydrocarbons, on the cuticle of various insects, including the paper wasps of the genus *Polistes*. As these polar compounds are variable among *Polistes* species and are perceived by the wasps, this cuticular fraction could also be involved in nestmate recognition. Through MALDI-TOF (Matrix-Assisted Laser Desorption Ionization Time of Flight) mass spectrometry analysis, we assessed, for the first time, the intercolonial variability of the cuticular polar fraction of *Polistes dominulus* in order to evaluate its reliability as source of nestmate recognition cues. We then tested through behavioral assays the importance of the 2 isolated fractions (apolar and polar) in nestmate recognition by presenting them separately to colonies of *P. dominulus*. Our results showed that the cuticular polar compounds are not colony specific and they are not used by paper wasps to discriminate nestmates from non-colony members. On the contrary, we confirmed that the isolated cuticular hydrocarbons are the chemical mediators prompting nestmate recognition in paper wasps.

Key words: chemical communication, cuticular lipids, MALDI-TOF Mass Spectrometry , paper wasps , polar compounds, social insects

Introduction

At the end of the 1970s, some landmark publications shed light on the non-volatile cuticular lipids of insects (Howard et al. 1978; Blomquist et al. 1979) and led up to more and more studies showing the involvement of these compounds as cues in nestmate recognition in many social insects (Breed 1998; Clément and Bagnères 1998; Vander Meer and Morel 1998; Gamboa 2004; Howard and Blomquist 2005). Several studies on cuticular lipids (mainly saturated and unsaturated hydrocarbons) confirmed that although their primary and main function is to reduce both internal water loss and parasites and pathogens attacks (Blomquist and Dillwith 1985), they also form a very complex and variable mixture able to mediate communication. Thus, a highly sophisticated system based on these ''contact pheromones'' allows the individuals to identify a non-colony member among large numbers of colony mates.

The increasing attention given in the last decades to hydrocarbons of social insects and the striking evidence of their involvement in communication, boosted by the exponential development of more sensitive analytical techniques, could have masked the potential role of additional cuticular compounds as recognition pheromones. Peptides and proteins, for example, have been detected on the bodies of various insects, such as locusts, honeybees, cockroaches, paper wasps, and termites (Zupko et al. 1993; Korchi et al. 1998; Cornette et al. 2002; Turillazzi, Mastrobuoni, et al. 2006; Hanus et al. 2010), and they have been reported to play a possible role as pheromones in some insects (Kubli 1992; Cornette et al. 2002, 2003; Turillazzi, Dapporto, et al. 2006).

In the social wasps of the *Polistes* genus, this cuticular blend of polar substances, analogously to the cuticular hydrocarbons (CHCs), is formed by numerous compounds

(ranging between 900 and 3000 Da) highly variable among species (Turillazzi et al. 2007). Moreover, this blend, once passively deposited on the substrate by congeneric individuals, can be detected by young mated *Polistes* foundresses of the following season and used to locate suitable hibernation sites (Turillazzi, Dapporto, et al. 2006).

Due to the presence of this detectable and variable mixture of polar compounds on the cuticle of paper wasps, their putative role as semiochemicals in other contexts of the colonial life seems reasonable. The role of CHCs in *Polistes* wasps as nestmate recognition cues has been clearly demonstrated by registering the colony wasps' reactions in hydrocarbons removal–reapplication bioassays (by removing hydrocarbons of an alien wasp in apolar solvents and deposing them on a dead washed individual) (Dani et al. 1996; Lorenzi et al. 1997; Sledge, Dani, et al. 2001). Supplementation experiments (by augmenting single compounds on the cuticle of alive wasps) have also been used to show the importance of these compounds in recognition processes (Dani et al. 2001; Lorenzi et al. 2004).

However, chemical analyses recently carried out by our group have unexpectedly shown that pentane, an apolar solvent, commonly used for CHCs removal, is also able to remove part of the peptides fraction from the wasp's cuticle (Figure 1). Some of these peptides, which represent the low molecular fraction of the venom, are probably spread on the cuticular outermost layer of the wasps by grooming (Turillazzi and Bruschini 2010). Even if they are not soluble in apolar solvents, they are probably removed together with the hydrocarbons during the full body wash in apolar solvents.

This unexpected finding, coupled with the variability of the peptides mixture and the wasps' ability to detect them, raises the question whether the results of the bioassays, carried out in the past to demonstrate the role of CHCs as cues for nestmate recognition in social insects, could have neglected a possible and additional involvement of the polar substances in this mechanism.

In the present paper, we addressed this topic using a framework analogous to that used in experiments performed to demonstrate the role of CHCs for nestmate recognition. First, we investigated, through mass spectrometry analyses, the intercolonial variability of the cuticular polar fraction of *Polistes dominulus* (after separating it from the CHCs fraction) in order to evaluate their reliability as nestmate recognition cues. Second, we tested, through laboratory bioassays, whether this isolated blend of polar cuticular compounds is involved in nestmate recognition by evaluating the capacity of colony members to discriminate nestmates from non-nestmates by presenting the 2 separate fractions (polar and apolar) applied to lures to colonies of *P. dominulus*.

Materials and methods

Studied species

Polistes dominulus (Christ) is the most common species of the genus *Polistes* in Mediterranean and Caspian countries (Pardi 1996). The colony cycle of *P. dominulus* starts in springtime (March–April) when one or more inseminated females (foundresses) emerge from hibernacula and found a nest (solitary or associative foundation). In this period, only the foundresses are present on the nests ("pre-emergence phase"). The first workers start to emerge ("worker phase") at the end of May, whereas male and female reproductives emerge in late summer (July–August) (Pardi 1996). The chemistry of the epicuticular lipids and the mechanisms behind nestmate recognition have been deeply investigated in the paper wasps of the genus *Polistes* (reviewed by Gamboa 1996; Dani 2006; Bruschini et al. 2010), and the involvement of cuticular lipids in nestmate recognition has been demonstrated in *P. dominulus* (Dani et al. 1996, 2001). Moreover, CHCs of this species

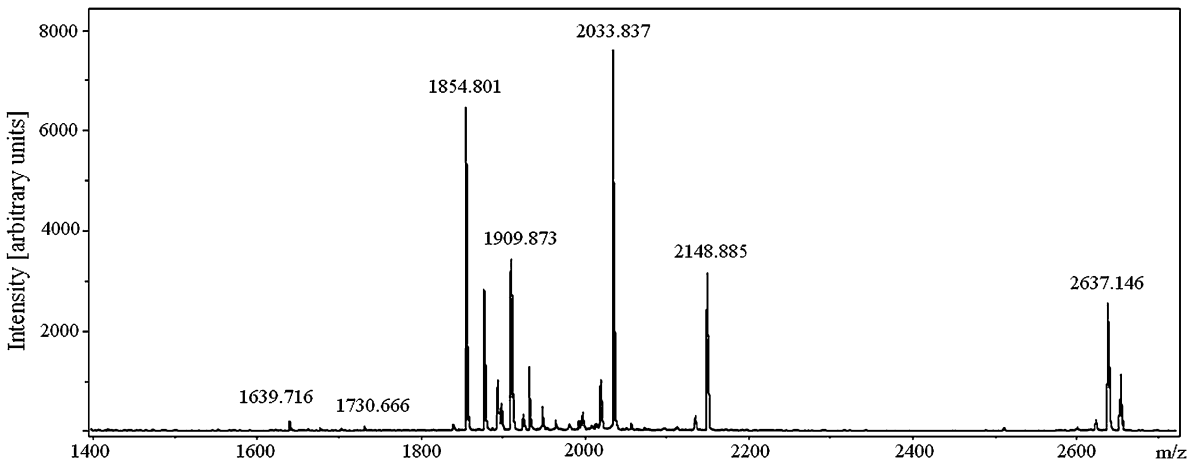

Figure 1 Polar cuticular compounds profile of a *Polistes dominulus* female removed by pentane extraction. This apolar solvent unexpectedly removes also the polar fraction in addition to the apolar one. For a list of *P. dominulus* polar cuticular compounds, see Turillazzi et al. 2007 and Dapporto et al. (2008).

differ among colonies (Sledge, Dani, et al. 2001) and within the same colony between queens and workers and between dominants and subordinates (Bonavita-Cougourdan et al. 1991; Sledge, Boscaro, and Turillazzi 2001; Dapporto et al. 2007; Dapporto, Bruschini, Cervo, Dani, et al. 2010; Dapporto, Bruschini, Cervo, Petrocelli, and Turillazzi 2010; Izzo et al. 2010). The cuticular polar fraction has been found to be highly variable in this species as foundresses and workers show clear differences in polar compounds (Dapporto et al. 2008).

Extraction and separation of epicuticular fractions

Each wasp used for chemical analyses and bioassays was individually placed in a 2-mL glass vial containing 600 μL mixture of *n*-pentane: water (1:1, v:v) for 15 min. The body was then removed from the vial and the 2 separated fractions, pentane at the top and water at the bottom, were withdrawn with the aid of a micropipette and a microsyringe, respectively, and placed into 2 different vials after several dilution steps (for the complete extraction protocol, see Supplementary Material). The 2 aliquots, pentane fraction containing CHCs and water fraction containing cuticular peptides (CPs), were checked for purity through gas chromatography coupled to mass spectrometry (GC–MS) and Matrix-Assisted Laser Desorption Ionization Time of Flight (MALDI-TOF) mass spectrometry, respectively (for details on the chemical analyses, see Supplementary Material). This simultaneous extraction of each wasp allowed us to obtain 2 aliquots: pentane with pure CHCs and water with pure CPs.

Chemical analyses of CPs and CHCs profiles of workers

To assess the colonial nature of the cuticular polar compounds, 3 workers (at least 3 days old to allow the development of a complete chemical epicuticular profile, Lorenzi et al. 2004) were collected from each of 10 nests coming from the same locality in Tuscany (Central Italy) and killed by freezing and kept at –20 °C until analysis. The epicuticular chemicals of these specimens were extracted as reported above to obtain the 2 different fractions of cuticular compounds.

The polar fraction was analyzed using an Ultraflex III MALDI-TOF/TOF (for details on the chemical analysis, see Supplementary Material). CPs calibrated spectra were imported into the ClinProTools (CPT) software and processed (for details, see Supplementary Material). The program calculates the areas of the most important peaks that account for statistical differences between the CPs profiles of the various specimens (Zhang et al. 2004; Turillazzi, Mastrobuoni, et al. 2006; Turillazzi et al. 2007). The values furnished by the CPT were used to calculate the percentage of the area of any single peak in each spectrum to the total peaks area in order to compare differences between various groups of individuals.

For comparison, we assessed the colonial nature of CHCs by analyzing the GC–MS data reported in a previous study of our group (Cotoneschi et al. 2007) performed on 37 workers from 8 colonies (mean number of adults per nest 4.6 ± 0.5) (for extraction and chemical analysis details, see Cotoneschi et al. 2007). The peak areas of the CHCs total ion chromatogram of each wasp were transformed into percentages. All compounds present either in less than 75% of samples or in less than 75% of the individuals belonging to the same group were excluded from the analysis to reduce the number of variables for multivariate analysis.

Then both CPs and CHCs data were subjected to stepwise discriminant analysis (DA). The significance of Wilks' lambda and the percentage of correct assignments were used to estimate the validity of the discriminant function and a cross-validation test (leave-one-out) was performed. This additional analysis is more conservative and reliable as it blindly attributes each specimen to one of a priori determined groups.

Bioassays

Colonies and specimens collection for bioassays

Associative foundations of *P. dominulus* ($n = 29$) were collected in April in different localities of Tuscany (Italy). Colonies were transported to the laboratory and transplanted in glass boxes (15 × 15 × 15 cm) and reared with sugar, larvae of *Tenebrio molitor*, and water ad libitum; blotting paper was supplied for nest construction. Foundresses were marked to be discriminated after the emergence of workers; behavioral observations were performed to determine the position of the individuals in the dominance hierarchy of each colony. Dominance behaviors observed include rapid beating of the antennae ("antennal boxing") by dominants, whereas subordinate behaviors include unilateral lowering of the body and offering of regurgitated liquid droplets to dominants (Pardi 1942; Röseler 1991). The dominant foundress (own colony foundress) from 20 of the 29 collected colonies was removed the day before the experiment. Alien foundresses ($n = 20$) were collected either on nests or in flight during the pre-emergence phase to assure that they belong to the foundress category. The collection site of foundresses was located distant from where the experimental colonies were gathered to avoid any possibility of previous encounters between wasps. Workers ($n = 29$) were gathered at the very beginning of the worker phase on laboratory colonies where foundresses had been previously marked. All these specimens were killed by freezing immediately after collection and kept at –20 °C until extraction.

General procedure for laboratory bioassays

Each bioassay consisted in the simultaneous presentation of 2 chemical stimuli to each multiple foundresses colony of *P. dominulus*. The 2 aliquots, pentane and water, obtained from the simultaneous extraction of each wasp were completely evaporated and resuspended with 50 μL of pentane and 50 μL of water, respectively. A 30-cm-long stick with a fork at one end was used to present 2 lures consisting of filter papers (1 cm^2) (placed 2 cm apart from each other), to which the different extracts or solvents used (see the

following experiment sections) were randomly applied on the left or on the right 30 min before experiments (in order to ensure the evaporation of the solvents). The fork device was slowly introduced into the colony glass cage and held 1 cm from the nest for 1 min after the first interaction between the colony members and the presented object. To avoid position bias, the 2 chemical stimuli were switched after 30 s. All experiments were performed blind by a first experimenter and video recorded by a second experimenter. The videos were then blindly watched by 2 other independent observers. The total number of bites and the total amount of time spent biting the 2 filter papers by all the individuals of the colony were counted.

Preliminary experiment. To test the effectiveness of the presentation device, we presented 50 μL of pentane extract of a foreign wasp versus 50 μL of solvent only (pentane), applied on the filter papers held by the fork device, to 17 *P. dominulus* colonies. The pentane cuticular extract induced on average a significantly greater number of bites (12.94 ± 7.04) compared with the control (6.56 ± 10.91, Wilcoxon test for paired data, $Z = -2.381$, $P = 0.017$) as reported in the literature on the same species using dead wasp lures (Dani et al. 1996). This confirmed the efficacy of our device using filter paper lures instead of dead wasps as in the previous works.

Epicuticular fractions extracts of foreign versus colony foundresses experiment. The same procedure reported above was performed to separate and to present the 2 epicuticular fractions of foreign foundresses and colony foundresses of 20 laboratory colonies. In fact, in order to test the possible involvement of the cuticular polar fraction (CPs) in nestmate recognition, we evaluated the response of each of the 20 colonies to the simultaneous presentation of the pure CPs extract of a nestmate (own colony foundress) and of an alien individual (foreign foundress). Moreover, to confirm the role of the CHCs in nestmate recognition, we evaluated the response of the same 20 colonies to the simultaneous presentation of the pure CHCs extract of the same individuals used for CPs extraction. The 2 presentations were performed to each nest 2 h apart in a randomized order.

Epicuticular fractions extracts of foreign workers versus solvents-only experiment. Finally, as we know that CPs are semiochemicals during the overwintering period (Turillazzi, Dapporto, et al. 2006), in order to assess whether the sole CPs extract of an alien individual (worker) evokes any aggressive response in a colony context, we presented it simultaneously with the solvent only (water) to a *P. dominulus* colony ($n = 29$). The same procedure was performed to each colony for the pure CHCs fraction of an alien individual (worker) versus pentane. The 2 presentations were performed to each nest 2 h apart in a randomized order.

Behavioral data statistical analysis

The behavioral data (total number of bites and the total amount of time spent biting by the members of the colony) were analyzed with Wilcoxon nonparametric tests between pairs of treatments.

Results

Chemical analyses of CPs and CHCs profiles of workers

Stepwise DA was performed on 39 cuticular polar compounds generated by the CPT of 29 workers. Although the DA showed that 79.3% of the samples were correctly assigned to their respective colonies ($n = 10$), as only 3 individuals were misclassified (Wilk's lambda = 0.001, $P < 0.001$), the more conservative and reliable cross-validation analysis revealed a scarce assignment capability (55.2% of original grouped cases correctly classified), misclassifying 13 workers of 29 (Figure 2a).

On the contrary, DA performed on the 35 selected compounds of 37 workers showed that they can be fully attributed to their respective colonies ($n = 8$) on the basis of their CHCs, correctly assigning 100% of the individuals to their original groups (Wilk's lambda = 0.001, $P < 0.001$). The cross-validation attribution of specimens revealed that most workers can be correctly attributed to their colonies (91.9% of original grouped cases correctly classified), misclassifying only 2 workers (Figure 2b).

Laboratory bioassays

Foreign versus own foundresses epicuticular fractions extracts experiment

The members of 20 tested colonies spent significantly more time biting the filter paper with CHCs extracts of foreign foundresses compared with the filter paper with CHCs extracts of their own foundress (Wilcoxon paired test, $Z = -2.698$, $P = 0.007$, Figure 3a). By contrast, there was no difference in the time spent biting the filter paper with CPs extracts of foreign foundresses compared with the filter paper with CPs extracts of their own foundress ($Z = -1.490$, $P = 0.136$, 20 colonies, Figure 3b). The same results were obtained when considering the number of bites ($Z = -2.637$, $P = 0.008$, Figure 3c and $Z = -1.128$, $P = 0.259$, Figure 3d, respectively).

Foreign workers epicuticular fractions extracts versus solvents-only experiment

The colony members of 27 tested colonies spent significantly more time biting the filter paper with CHCs extracts of foreign workers compared with the filter paper with the solvent (pentane) (Wilcoxon paired test, $Z = -2.487$, $P = 0.013$, Figure 4a), whereas there was no difference in the time spent by the members of 29 tested colonies biting the filter paper with CPs extracts compared with the filter paper with the control (water) ($Z = -0.096$, $P = 0.923$, Figure 4b). The same

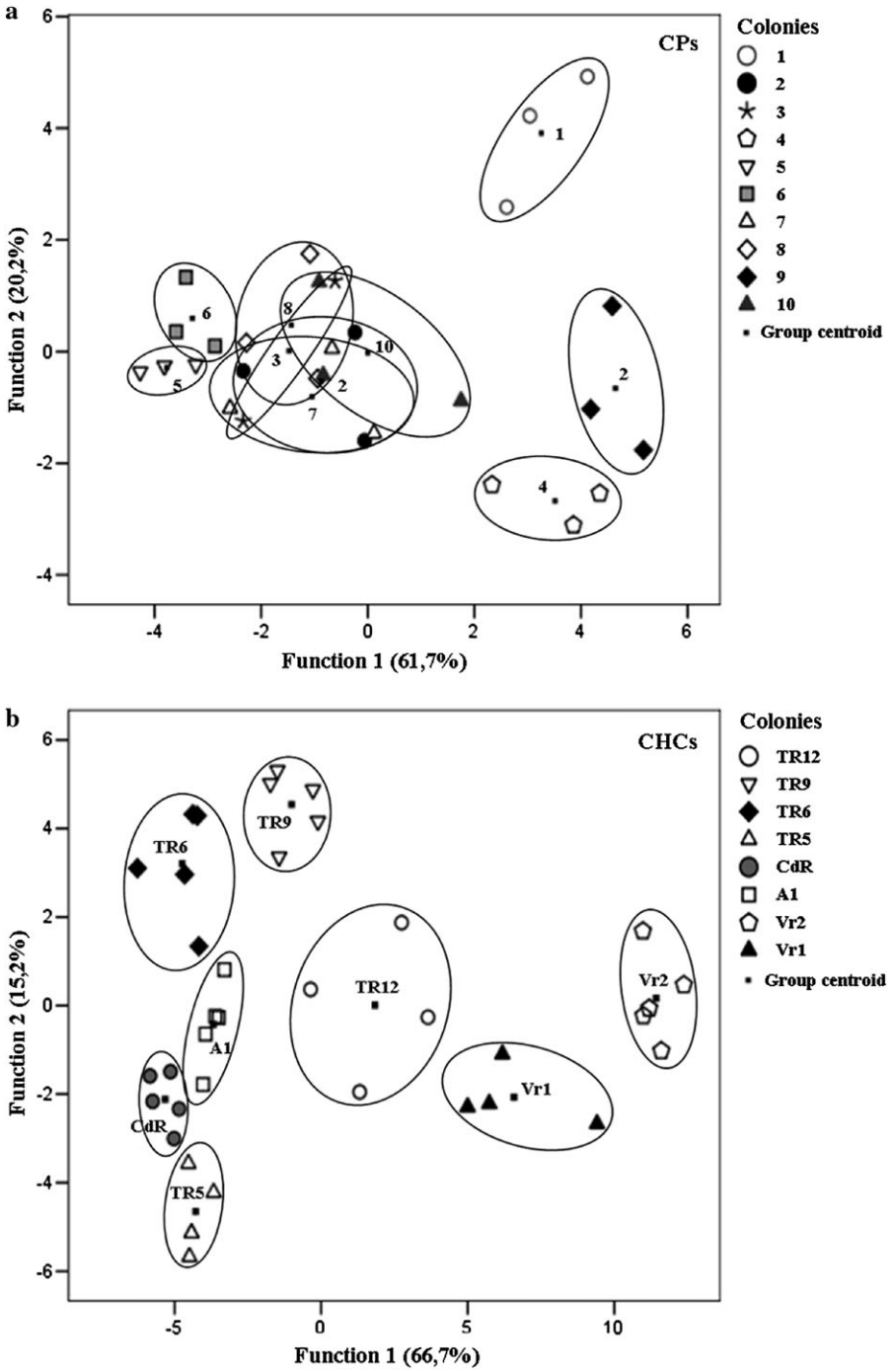

Figure 2 Stepwise DA of **(a)** polar CPs of 29 workers of 10 colonies and **(b)** CHCs of 37 workers of 8 colonies. A clear colonial separation of the individuals is shown only for CHCs and not for CPs.

results were obtained when considering the number of bites ($Z = –2.363$, $P = 0.018$, Figure 4c and $Z = –0.801$, $P = 0.423$, Figure 4d, respectively).

Discussion

Our results show that the MALDI-TOF spectral profiles of medium molecular weight polar compounds (ranging from 900 to 3000 Da) of *P. dominulus* workers do not strongly differ among colonies and that paper wasps do not use the pure cuticular polar fraction to distinguish between a colony mate and a non-colony individual. Our bioassays also confirm the role of purified CHCs in nestmate recognition.

Nestmate recognition mechanism involves 3 steps: the production of unique phenotypic cues (expression), the perception of these labels, and the action taken by an animal in

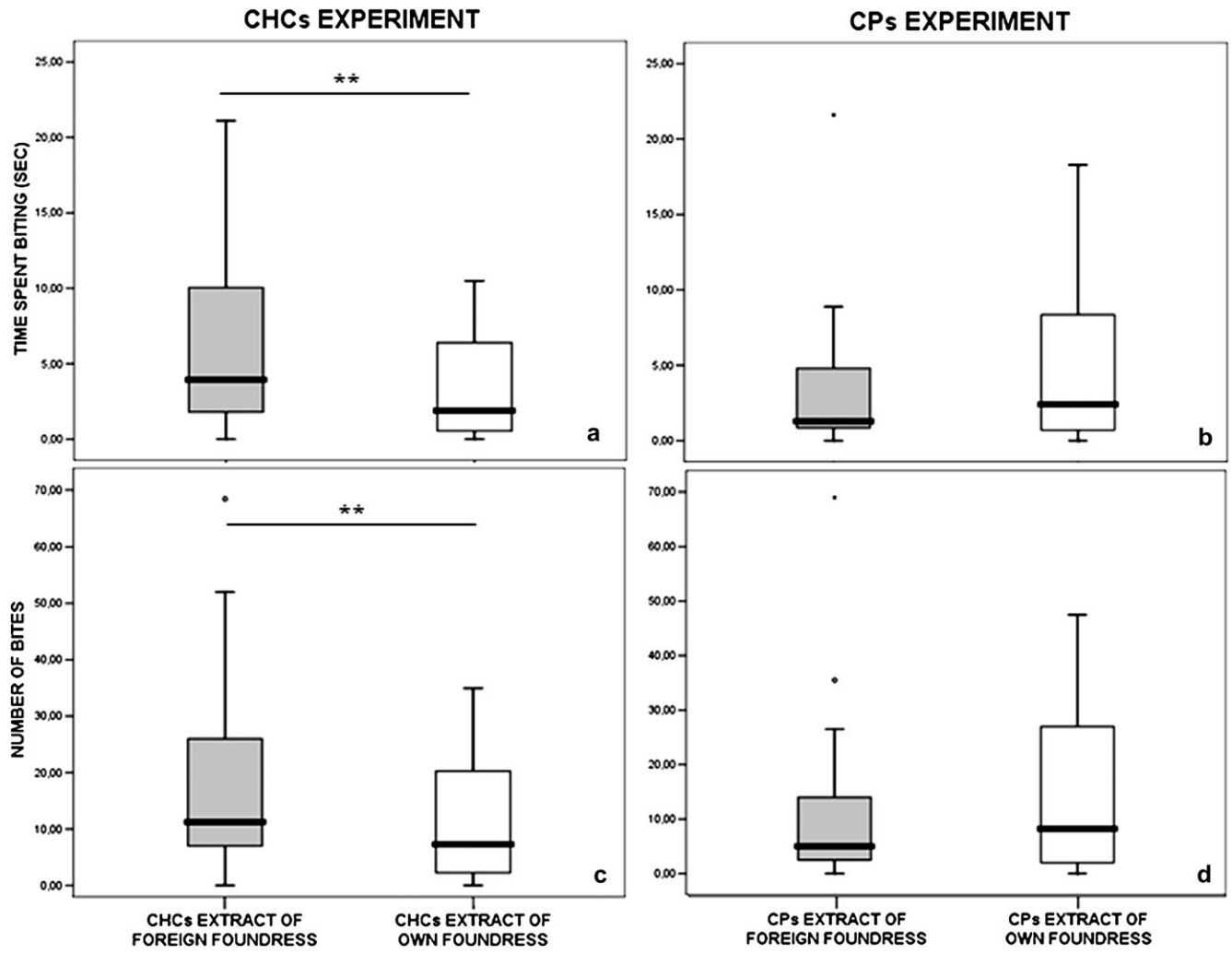

Figure 3 Aggressive response (time spent biting and number of bites) of the individuals of 20 colonies of *Polistes dominulus* toward the CHCs **(a, c)** or CPs **(b, d)** extracts of foreign and own foundresses. The wasps are significantly more aggressive toward the foreign foundress only when presenting CHCs extracts. Box plots show the 75th and 25th percentiles as the box, the median as the line in the box, and the extremes as the vertical lines. $^{**}P < 0.01$.

response to the perceived similarity between its template and an encountered phenotype (Mateo 2004). CHCs represent better recognition cues, compared with CPs, as they support all 3 steps of the nestmate recognition process. CPs, even if they are present on the cuticle and can be detected by wasps (Turillazzi, Dapporto, et al. 2006), do not evoke a differential treatment of conspecifics, and they cannot be used alone as templates in nestmate recognition. However, our experiments do not exclude that CPs contribute in some way to the recognition when mixed to the CHCs. Recognition labels and the ability to perceive them provide proximate or mechanistic explanations for social recognition, whereas the action component provides functional or adaptive explanations for recognition (Mateo 2004). Therefore, it is legitimate to question which may be the possible explanations accounting for the unreliability of CPs as cues for nestmate recognition. First, peptides are present in the venom and they are most probably spread on the cuticle by grooming. As a consequence, the mixture of polar cuticular compounds could be extremely variable both quantitatively and qualitatively as it is mainly context dependent. It is plausible that during the colonial period (spring–summer), workers are heavily involved in colonial tasks (foraging, brood caring, defense, etc.), thus reducing the time spent grooming. The mated future foundresses, instead, could increase the grooming behavior during the overwintering period to take advantage of the antimicrobial activity of the CPs on their body and on the hibernation sites (Turillazzi, Dapporto, et al. 2006). This makes CPs good cues to indicate suitable hibernation sites for the next generations. Furthermore, because these polar compounds are easily removed by an aqueous solvent from the wasps bodies and from exposed surfaces, they do not seem suitable to constitute a ''permanent chemical uniform'' to communicate among colony members.

On the contrary, CHCs are synthesized by cells associated with the epidermis (Nelson and Blomquist 1995), and their

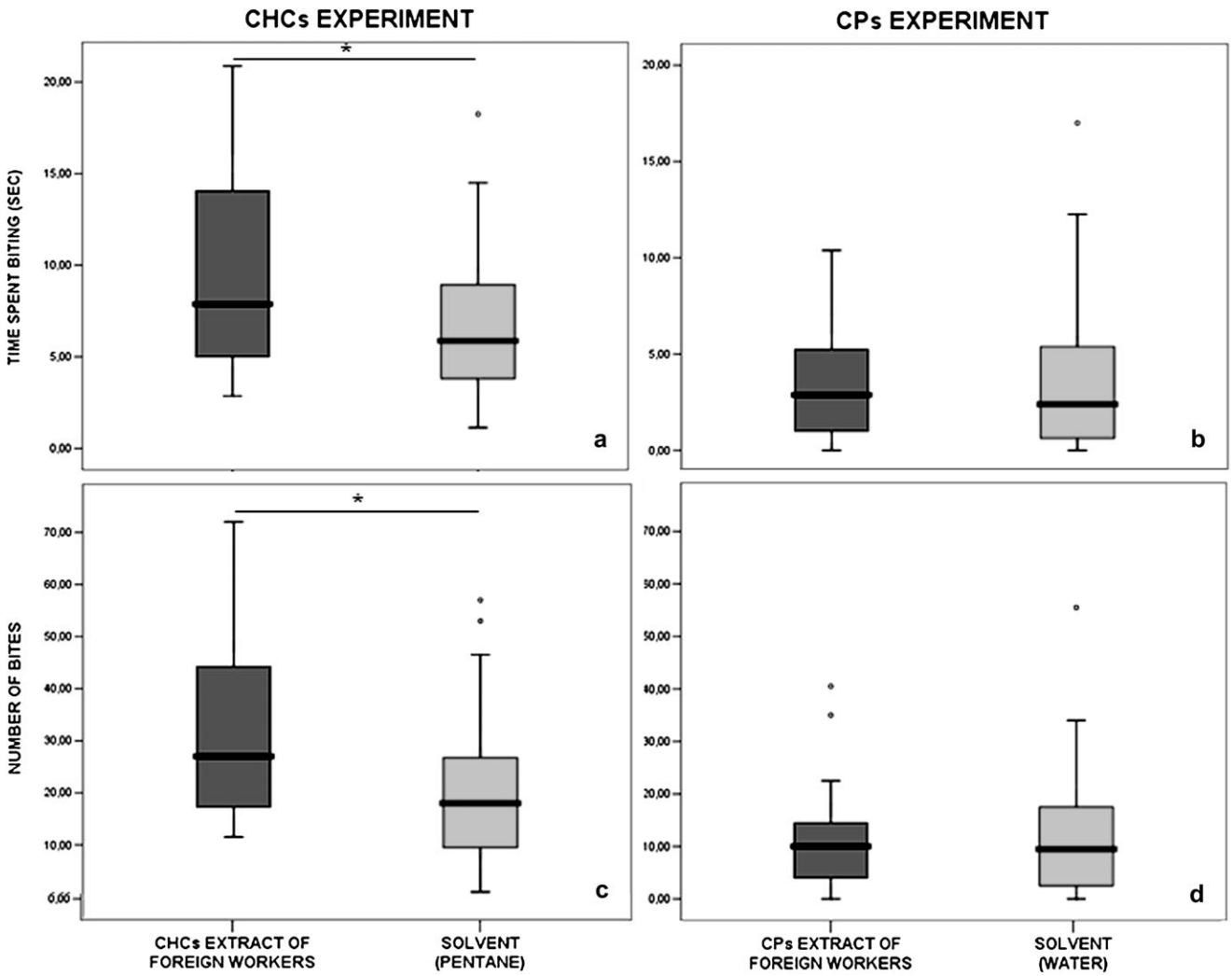

Figure 4 Aggressive response (time spent biting and number of bites) of the individuals of *Polistes dominulus* colonies toward the CHCs extracts of foreign workers and solvent (pentane) **(a**, **c)** or CPs extracts of foreign workers and solvent (water) **(b**, **d)**. The wasps are significantly more aggressive toward the foreign workers only when presenting CHCs extracts. Box plots show the 75th and 25th percentiles as the box, the median as the line in the box, and the extremes as the vertical lines. $*P < 0.05$.

primary role is to reduce water loss from the insect body and pathogens/parasite attacks. Therefore, a minimum threshold quantity on the insects' cuticle is required for them to be effective barriers. As a consequence, CHCs could represent a more reliable cue for nestmate recognition and most probably, from an evolutionary point of view, the presence of this layer of lipids on the cuticle prevented the development of other existing cuticular compounds as cues used for recognition. This hypothesis seems to be supported by our chemical analyses that show a poor capability to discriminate the colony members from their CPs profiles compared with the CHCs.

However, we cannot completely exclude that the polar compounds fraction is, somehow, involved in the nestmate recognition system. More tests are, in fact, needed to verify possible synergic effects of the CPs and CHCs which could enhance the discrimination power of the epicuticular mixture. Thereafter, as well as the CHCs are evolved for other tasks (mechanic defense against pathogens and dissection) and then used as cues for recognition during evolution by insects, peptides could bring essential information to reinforce the hydrocarbons' message.

In conclusion, we demonstrated, for the first time, that CPs are not colony specific compared with CHCs. We also provided evidence that nestmate recognition in *P. dominulus* is based on the purified CHCs and that the isolated polar cuticular compounds, despite their presence and variability, do not play a role in this context.

Finally, we confirmed the role of CHCs as cues for nestmate recognition even when tested isolated from the cuticular polar compounds, corroborating all the previous studies reported in the past to evaluate the role of these compounds in the recognition process in which the 2 fractions had been tested together.

Supplementary material

Supplementary material can be found at http://www.chemse.oxfordjournals.org/.

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