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RESEARCH ARTICLE

The chemical basis of host nest detection and chemical integration in a cuckoo paper wasp

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SUMMARY

Insect social life is governed by chemicals. A great number of studies have demonstrated that the blend of hydrocarbons present on the cuticle (CHCs) plays a pivotal role in intra- and inter-specific communication. It is not surprising, therefore, that social parasites, specialized in exploiting the costly parental care provided by host workers, exploit the host chemical communication system too. Throughout their life cycle, social parasites intercept and break this CHC-based code. Recently, however, several polar compounds (mainly peptides) have been found in addition to CHCs both on the cuticle and on the comb surface of social insects, and their semiochemical role has been demonstrated in some circumstances. In the present study, we used the paper wasp social parasite–host system *Polistes sulcifer* (Zimmerman)–*Polistes dominulus* (Christ) to evaluate the relative importance of the CHCs and polar compounds in two different steps of the host exploitation process: host nest detection by the pre-usurping parasite and parasite chemical integration into the host colony. After separating the polar and apolar fractions of the host nest as well as those of pre- and post-usurpation parasites, we carried out laboratory assays based on the binary choice model. Our results show that nest polar compounds neither are used by the parasite to detect the host's nest nor play a role in parasite chemical integration into the host colony. In contrast, we demonstrate that CHCs are fundamental in both steps, thus confirming their primary role in social insect life and consequently in social parasite–host interactions.

Key words: social parasitism, host finding, parasite recognition, chemical mimicry, hydrocarbons, peptides.

INTRODUCTION

Social parasites are specialized in exploiting the resources gathered inside social insect colonies, mainly the costly parental care provided by workers. The exploitation of social insect brood care has evolved independently several times in different insect orders, from Coleoptera to Lepidoptera (Hölldobler and Wilson, 1990), and is especially widespread in social Hymenoptera such as bees, ants and wasps (Wilson, 1971). In the most extreme social parasitism stage, i.e. permanent obligate social parasitism, parasite females are completely dependent on the host resources, having lost the capacity for nest building and the worker caste. The obligate parasite's fitness thus entirely depends on its ability to get into the host colony and reproduce. Three main challenges must be faced by the parasite to achieve its goal: finding the host colony, conquering it and exploiting its resources.

Insect social life is mainly governed by chemicals, and about 30 years of research has provided overwhelming evidence that the blend of lipids present on the cuticle of social insects (mainly hydrocarbons, hereafter CHCs) plays a pivotal role in intra- and inter-specific communication (Howard and Blomquist, 2005). Therefore, it is not surprising that social parasites exploit the host chemical communication system throughout their life cycle. The parasite can 'eavesdrop' on the host's CHCs to find the colony to usurp (Tengö et al., 1992; Bunk et al., 2010; Kreuter et al., 2010) or cheat the host to gain access to the nest and integrate into the colony using sensory deception tricks either by matching the host CHC profile or by approaching the colony with low amounts of CHCs (reviewed in Lenoir et al., 2001; Howard and Blomquist, 2005; Lorenzi, 2006; Bagnères and Lorenzi, 2010).

Recently, however, the paradigm of an exclusive role of CHCs in social insect communication has been challenged by the discovery of several proteinaceous and peptidic compounds on the insect cuticle (Korchi et al., 1998; Cornette et al., 2002; Turillazzi et al., 2006a; Hanus et al., 2010). Moreover, their role as semiochemicals has in some cases been demonstrated (Kubli, 1992; Cornette et al., 2002; Cornette et al., 2003; Turillazzi et al., 2006b). However, virtually nothing is known about the importance that cuticular polar compounds (CPCs) may have in the different contexts of host–parasite interactions.

In the present study, we used the paper wasp social parasite–host system *Polistes sulcifer–Polistes dominulus* to evaluate the relative importance of the two fractions – apolar compounds (HCs) and polar compounds (PCs) – in two different but fundamental steps of the host colony usurpation process: host nest detection by the pre-usurping parasite and parasite chemical integration into the host colony. This parasite–host pair represents a particularly suitable model system to address this topic. The presence of HCs (Dani et al., 1996) and PCs (Turillazzi et al., 2006a) on both the body and the comb surface has been demonstrated in the host species. Moreover, it has been shown that hosts are able to perceive and use these PCs in specific contexts (Turillazzi et al., 2006b). However, while CHCs have received extensive attention during the last few decades as cues for recognition processes [for social wasps see review (Bruschini et al., 2010)], CPCs have only recently been investigated (Bruschini et al., 2011).

Polistes sulcifer is the obligate permanent social parasite of *P. dominulus*. In spring, after overwintering on mountaintops (Cervo, 2006), *P. sulcifer* females migrate to the lowland plains, where they seek a host colony just around the emergence of host offspring (Cervo and Turillazzi, 1996). During the host-finding process, parasites could use their sight to locate their hosts and follow them back home (Cervo et al., 1996). However, once in the vicinity of the nest, parasites are able to detect its presence by chemical cues alone (Cervo et al., 1996) but the nature of the chemical cues underlying this detection mechanism remains unknown. Chemical analyses have revealed that the paper nest of several species of *Polistes* is covered with the same HC mixture as on the wasp cuticle (Espelie and Hermann, 1990; Espelie et al., 1990; Singer et al., 1992; Lorenzi, 1992; Cotoneschi et al., 2007); it has been suggested that this HC blend could represent the main cue used by parasites to locate the host colony (Cervo et al., 1996). However, peptides have also been found on the paper nest (Turillazzi et al., 2006a), suggesting that HCs might not be the exclusive compounds mediating the host nest-detection process.

We performed laboratory bioassays to assess which chemical compounds were responsible for triggering the detection of the host nest by the approaching parasites. We first separated the HC and PC fractions of the host nest and afterwards presented them separately but simultaneously to the parasites during the usurpation period.

Once they have detected the host nest, the parasites adopt a violent strategy to usurp the colony (Turillazzi et al., 1990), aided by their larger size (Cini et al., 2011) and distinctive morphological modifications (e.g. mandibles and anterior legs) (Cervo, 1994). After the conquest of the host colony, the parasite female chemically integrates into the colony by matching the CHC profile of the host species (Turillazzi et al., 2000), as well as the specific profile of the usurped colony (Sledge et al., 2001) and of the dominant female (Dapporto et al., 2004), in order to elude the host recognition system and to be accepted as a colony nestmate. Here again, the putative role of PCs in nestmate recognition has never been investigated. Recently, we have shown that CPCs are not used by the host to discriminate between colony members and conspecific intruders, probably because of the absence of a colonial profile of the polar cuticular blend (Bruschini et al., 2011). At the species level, however, polar profiles have been demonstrated to differ among several *Polistes* species (Turillazzi et al., 2007). PCs could thus be informative for the host in order to detect heterospecific intruders, like social parasites. *Polistes sulcifer* represents an important threat to *P. dominulus* colonies, as in some host populations the parasitic infectivity can reach 50% (Ortolani and Cervo, 2010). In order to understand the role played by the cuticular polar blend in host colony defence against social parasites we evaluated: (a) whether the CPC profile could be used by the host to recognize the parasite, i.e. we evaluated whether the parasite CPC profile differs from that of the host in the pre-usurpation period, and (b) whether the host uses these cues to recognize and attack the parasite, i.e. we performed intruder recognition bioassays under laboratory conditions.

Moreover, if CPCs play a role in the host recognition system, we would expect that, in addition to matching the CHC profile of the host, the parasite would benefit from a resemblance to the polar cuticular blend of the host species. As the CPCs of the host species do not show a colonial profile (Bruschini et al., 2011), we expect that the resemblance would be at the specific level. If CPCs are not involved in the host recognition system, we would not expect the CPC profile of the parasite to mimic the host's specific profile after usurpation. We thus carried out chemical analyses to compare the chemical polar profiles of parasites before and after usurpation with those of the host species.

MATERIALS AND METHODS

Host nest detection experiment

Twenty *P. sulcifer* females were collected from their hibernation sites at the end of April in Central Italy (Sibillini Mountains) and kept under laboratory conditions mimicking overwintering (4°C, L:D cycle 08.00 h:16.00 h) until the end of May, when usurpation usually takes place in the wild (Cervo and Turillazzi, 1996). Parasites were then activated for a period ranging from 4 to 16 days by a 'warming treatment' [as defined previously (Ortolani et al., 2008); i.e. natural L:D conditions with additional artificial lighting from 08.00 h to 20.00 h]. This treatment simulates the field condition during which parasites usurp the nests in the wild (Ortolani et al., 2008; Ortolani and Cervo, 2009). Twenty colonies of *P. dominulus* were collected before the emergence of workers in early spring (April) in the area around Florence (Central Italy). A ~1 cm^2 piece of nest material (paper) was removed from each nest and used for the extraction of the apolar (mainly hydrocarbons) and polar fraction (mainly peptides) according to the following procedure.

The piece of nest material was washed in a mixture of pentane:water (1:1) for 15 min. Several dilution steps were performed to obtain two purified aliquots: a pentane fraction containing HCs and a water fraction containing PCs (see 'HC and PC extraction procedure' below). The two aliquots were checked for purity through gas chromatography coupled to mass spectrometry (GC-MS) and MALDI-TOF mass spectrometry, respectively (for details, see Bruschini et al., 2011). Then the pentane fraction and the water fraction were completely dried out and re-suspended in 50 µl of pentane and 50 µl of water, respectively, in order to be presented as stimuli in the behavioural bioassays.

Each host nest detection bioassay consisted in the simultaneous presentation of 50 µl of each of four chemical stimuli (two controls and two treatments) to one *P. sulcifer* female during the period corresponding to the host nest search in the field. The stimuli consisted of the polar and apolar fractions extracted from the nest material of a *P. dominulus* nest as treatments and the polar (water) and apolar (pentane) solvents as controls. Each stimulus was randomly applied in one of four separate and equally spaced spots on filter paper inside a Petri dish (9 cm in diameter). A single parasite was introduced into each Petri dish and its behaviour was video-recorded for 10 min. The video was then watched by one observer blind to the experimental conditions. Antennation by the parasite is a typical and easily recognized behavioural pattern, i.e. a soft antennation with the tips of the antennae repeatedly rubbed forward and backward on the substratum, which is performed by the parasite to a great extent when in the vicinity of a host nest, in both field and laboratory conditions (Turillazzi et al., 1990) (A.C. and R.C., personal observations). Following Cervo et al. (Cervo et al., 1996), we considered this particular antennation behaviour as a 'chemical cues detection index'. We thus recorded the time each female spent antennating the substratum on each stimulus.

We used the non-parametric Friedman test to compare the time spent antennating at each of the four spots and *post hoc* tests for multiple comparisons [as reported elsewhere (Field, 2005)] were carried out to assess whether significant differences exist between pairs of treatments ($P<0.05$). Twelve out of the 20 tested parasites showed the typical antennation response and were thus considered for the analysis. The remaining 8 parasites did not show any reaction to the stimuli, staying motionless during the entire test period. As a similar fraction of non-responding parasites represents the normal

percentage of failure after the warming protocol under laboratory conditions (A.C. and R.C., unpublished results), we considered them to be not physiologically ready to usurp and excluded them from the analysis.

Pre-usurpation parasite recognition experiment

For this experiment we collected N=20 additional parasites and N=20 additional host colonies using the above-explained procedure (see 'Host nest detection experiment'). The host colonies all had multiple foundresses and were at the same developmental stage (with regard to both the number of adults and the immature brood) when tested (mean colony population ± s.d., 10.3±4.6 wasps; mean colony size ± s.d., 59.8±24.1 cells). Hosts were reared in laboratory conditions in glass chambers (15×15×15 cm) and supplied with *ad libitum* water, sugar and fly maggots daily.

In order to test the possible involvement of the CPC fraction in parasite recognition by the host, we evaluated the response of the colony members to the simultaneous presentation of the pure CPC extract fraction of a parasite female ('pre-usurpation parasite') and the solvent (water) as a control. We did the same for the CHC extract fraction, using pentane as a control. CHCs are known to mediate the aggressive response towards intruders; therefore, we compared the aggressive response elicited by CPCs with that elicited by CHCs. The CHC and CPC extracts were obtained following the same procedure used for the piece of the paper nest (see the 'HC and PC extraction procedure' below). A 30 cm long stick with a fork at one end was then used to present two filter papers (1 cm^2; placed 2 cm apart from one another), to which 50 µl of the extract and of the respective solvent were randomly applied either on the left or on the right side. The fork device was slowly introduced into the colony glass chamber 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 two chemical stimuli were switched after 30 s). Two randomized and subsequent presentations, 2 h apart, were performed for each nest (N=20 trials for each treatment) to test CPCs and CHCs separately. All experiments were performed blindly by a first experimenter and video-recorded by a second experimenter. One CHC trial was not correctly video-recorded and was thus excluded from the analysis. The videos (N=20 for CPCs and N=19 for CHCs) were then watched by two different independent observers. The total number of bites and the total amount of time spent biting the two filter papers by all the individuals of the colony was counted and considered as an estimate of the colonial aggressive response.

The behavioural data (number of bites and total time spent biting) were analysed with Wilcoxon non-parametric tests between pairs of treatments.

HC and PC extraction procedure

Each piece of nest (for the host nest detection experiment) and each wasp (for the pre-usurpation parasite recognition experiment, after being killed by freezing) were individually placed in a 2 ml glass vial containing 600 µl mixture of *n*-pentane:water (1:1, v:v) for 15 min. The piece of nest and the body of the wasp were then removed from the vials. The two fractions were clearly visible: the pentane fraction was withdrawn with the aid of a micropipette and transferred in a new 2 ml glass vial. The remaining aqueous phase was extracted with 3×200 µl aliquots of *n*-pentane: each time, the recovered *n*-pentane was added to the previously collected aliquots. A total of ~900 µl of *n*-pentane was collected.

The remained aqueous fraction was withdrawn with the aid of a microsyringe and transferred into a new 2 ml vial, leaving a minimum volume of residual *n*-pentane and water in the original vial. Then, 200 µl of water were added, vortex mixed, recovered and added to the previous water aliquot, resulting in a total of ~400 µl of water.

Chemical analyses

We evaluated (a) the differences in the CPC profile between host and parasite species by analysing 15 host foundresses and 15 parasites in the pre-usurping phase, and (b) the degree of parasite chemical resemblance with the host polar profile after usurpation.

Pre-usurpation parasite females were collected in their hibernation sites at the end of April in Central Italy and activated as already explained (see 'Host nest detection experiment'). Host foundresses were collected either in flight or on flowers at the beginning of the season when workers were not yet present. Post-usurpation parasites and host workers were collected from 8 colonies, 10 days after the colonies were usurped by a parasite female in the laboratory (3 workers per colony for 6 colonies and 2 workers per colony for two colonies).

These specimens were killed by freezing and kept at –20°C until analysis. The epicuticular chemicals were then extracted as described above to obtain the pure apolar and polar extracts separately. The polar fraction was analysed using a MALDI Ultraflex TOF mass spectrometer (for details, see Bruschini et al., 2011).

CPC calibrated spectra were imported into ClinProTools™ (CPT) software and processed [CPT parameters used for model generation were: peak width, 0.01; smoothing (width, 5 Da, cycles, 1); average peak list calculation (relative threshold base peak, 0.01, signal-to-noise threshold, 5; limit peak number, 50/false); area calculation (integration type, zero level); and peak selection (use all peaks: true, sort mode: *P*-value tta)]. The values obtained by the CPT were used to calculate the percentage of the area of any single peak in each spectrum with respect to the total area of the peaks in order to compare differences among various groups of individuals. The data were then 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. A cross-validation test (leave-one-out), where each specimen is blindly attributed to one of the *a priori* determined groups, was performed.

Euclidian distances are measures of dissimilarity in the quantity of individual compounds between pairs of individuals and were used to estimate the chemical distances between the three groups (pre-usurpation parasites, post-usurpation parasites and host workers), and the differences among groups were analysed by the non-parametric Mann–Whitney *U*-test, verified with the Monte Carlo method.

RESULTS

Host nest chemical cue detection experiment

Parasites did not spend equal time antennating each stimulus spot (Friedman χ^2=24.486, d.f.=3, N=12, P<0.001); they antennated the spot with the CHC stimulus for 82% of the total antennation time. Parasites thus spent significantly more time antennating the CHC spot than the PC spot (*post hoc* test P<0.05) and the two controls (*post hoc* test P<0.05). The PC spot, however, did not evoke a longer response compared with the solvent spot (*post hoc* test P>0.05) (Fig. 1).

Pre-usurpation parasite recognition

The colony members spent significantly more time biting the filter with the CHC extract of the pre-usurping parasite than the filter with solvent only (pentane) (Wilcoxon paired test, Z=–2.817, P=0.005, 19 colonies; Fig. 2), whilst there was no difference

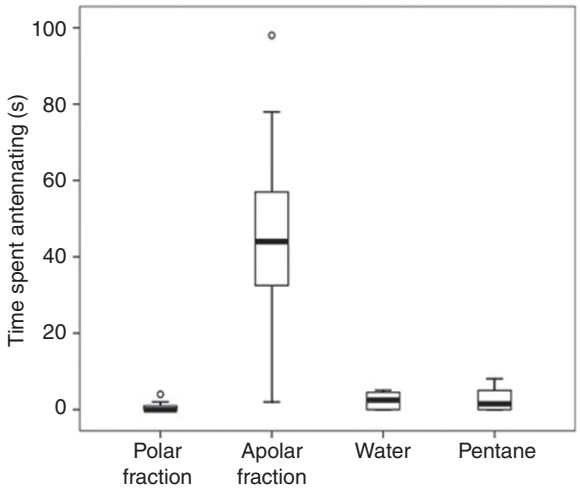

Fig. 1. Host nest detection response in the parasite is evoked by the nest hydrocarbons (apolar fraction). Parasites spent significantly more time antennating the apolar fraction than the polar fraction and the two controls (water and pentane). Box plots show medians (bold lines), the 75th and 25th percentiles (boxes), the extremes (vertical lines) and the outliers (circles).

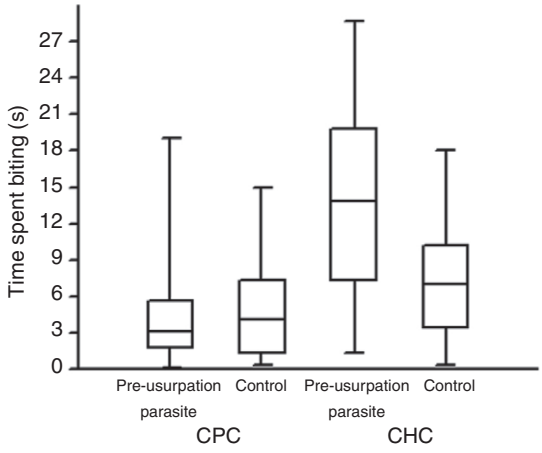

Fig. 2. Time spent biting the filter with the cuticular polar compound (CPC) extract of pre-usurpation parasites and control (solvent) and the cuticular hydrocarbon (CHC) extract of pre-usurpation parasites and control (solvent). Box plots show medians (bold lines), the 75th and 25th percentiles (boxes), the extremes (vertical lines) and the outliers (circles).

between the time spent biting the CPC extract and the control (water) (Z=–0.262, P=0.794, 20 colonies; Fig. 2). The same results were obtained when considering the number of bites (Z=–3.340, P=0.001 and Z=–0.153, P=0.879, respectively).

Chemical analyses

Parasite and host CPC profiles were markedly different (Fig. 3). The DA performed on the 15 pre-usurpation parasites and 15 host foundresses using 30 CPCs showed that they were fully discriminated, correctly assigning 100% of individuals to their original groups (function 1 explained variance=100%; Wilks' lambda=0.032, χ^2=88.040, d.f.=5, P<0.001). The cross-validation attribution of specimens revealed that all parasites and host foundresses could be correctly attributed to their groups (100% of original grouped cases correctly classified).

The parasite CPC profile approaches that of the host after usurpation (Fig. 4). By setting as a baseline the chemical distance between two random hosts (host–host in Fig. 4), we were able to compare the mean distance between parasites and hosts before (pre-host) and after (post-host) usurpation. While before usurpation parasite–host chemical distance was on average 14% greater than that of the host–host (Mann–Whitney, U=104100.0, P<0.001, N=510 and N=561, respectively), after usurpation this distance decreased by up to 6% on average. Even though it was still significantly different from (bigger than) the host–host distance (Mann–Whitney, U=102254.0, P=0.004, N=408 and N=561, respectively), this difference is significantly smaller than the pre-usurpation difference (Mann–Whitney, U=75755.0, P<0.001, N=510 and N=408, respectively), which shows that the CPC profile of the parasite approached that of the host.

DISCUSSION

Our results show that PCs are not involved in either of the two fundamental steps of the host colony usurpation process: host nest detection by the pre-usurping parasite and chemical integration into the host colony. Moreover, we have shown that the host nest HCs evoke the host detection response in the parasite and confirm that hosts use CHCs to recognize and attack individuals belonging to the parasitic species.

For highly specialized parasites with few potential host species, the ability to detect and recognize specific host chemical cues allows them to reduce the risk of usurping the nests of unprofitable species. Indeed, while the visual appearance of different colonies may be similar, chemical cues could give the parasite essential information about potentially suitable colonies before attempting the conquest. *Polistes sulcifer* is specialized on a single host species (*P. dominulus*) and is known to use olfaction both to choose its host's nest and to select the most developed colonies (Cervo et al., 1996). The quantity and quality of chemical compounds may inform the parasite about nest size as well as the developmental stage and amount of immature brood. Here, we demonstrated that, during the usurpation period, the HCs extracted from the nest material are the cues that drive the parasite to detect the host nest, which suggests that they are also responsible for mediating the discrimination processes performed by the parasite. Moreover, our results show that the nest PCs are not involved in host nest detection by the parasite. Both the HC and the PC fractions are known to vary among different *Polistes* species (Bruschini et al., 2010): therefore, both might represent informative cues for finding and choosing the potential host nests. Indeed, the hydrocarbons as well as the peptides present on the nest paper mainly derive from the wasps' cuticle by physical transfer (Bruschini et al., 2010). However, even though no specific studies have been carried out, CHCs have been suggested to be more abundant than polar compounds on the adult body (and indirectly on the comb surface) as well as on all immature stages (Bruschini et al., 2011). Indeed, CHCs have the fundamental function of protecting the wasps against desiccation and pathogen/parasite entrance (Blomquist and Dillwith, 1985). In contrast, polar compounds are primarily produced in the venom and are then probably spread in small amounts through self-grooming on the wasp cuticle, where they play an antimicrobial role (Turillazzi et al., 2006a; Lambardi et al., 2007; Turillazzi and Bruschini, 2010). The supposed low amount of PCs on the nest may prevent their reliability and usefulness as cues for finding and selecting host nests by the parasite.

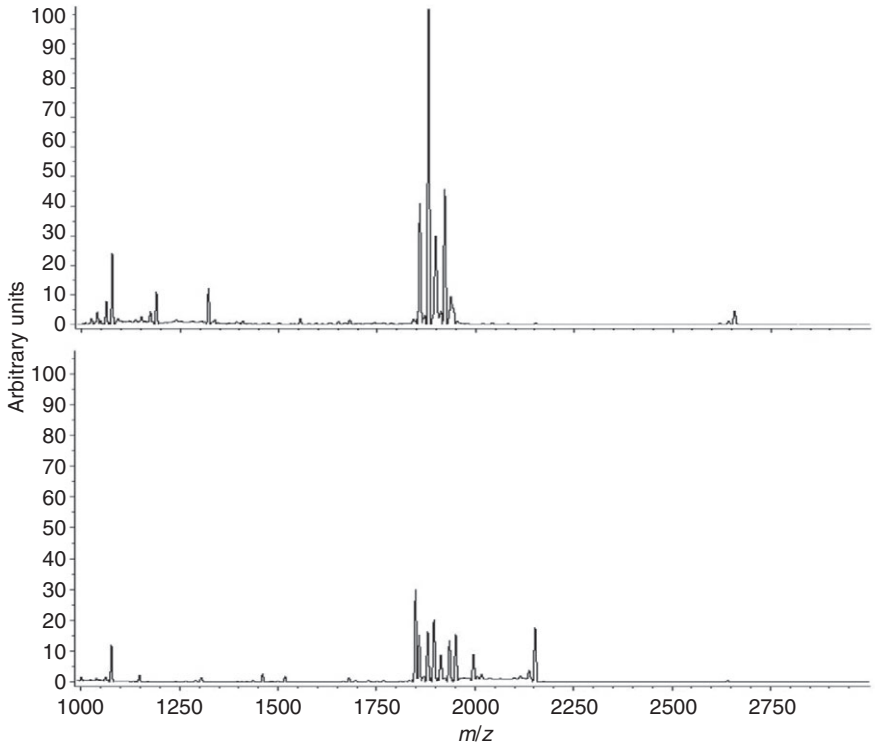

Fig. 3. CPC profiles of parasites (top) and host foundresses (bottom) in the pre-usurpation phase (two randomly chosen spectra are shown).

After finding and choosing the right colony, a parasite needs to conquer it by overcoming colony defences. Heterospecific intruders like social parasites are recognized by the host on the basis of visual and chemical stimuli. In particular, intruders (conspecific and heterospecific) are recognized and attacked by *P. dominulus* on the basis of their CHC profile (Dani et al., 1996; Ortolani et al., 2010). Here, we showed that the parasite's CPC profile is strikingly different from that of the host in the pre-usurpation period, thus representing an additional cue for parasite recognition. Our behavioural bioassay, however, showed that hosts do not use this information during parasite invasion, as the parasite's CPCs did not evoke any aggressive response in the host. In contrast, pre-usurping parasite CHCs evoked the host aggressive response, thus confirming the important role these cues play in colony defence (Ortolani et al., 2010).

The lack of importance of PCs in both host nest detection and colony defence in this parasite–host system could be interpreted from an evolutionary perspective. The social life of free-living species of the *Polistes* genus is largely regulated by HCs; variable blends of these compounds have important communicative functions mainly linked to nest and brood recognition (for reviews, see Gamboa, 2004; Dani, 2006; Bruschini et al., 2010). The existence of reliable and informative cues such as HCs could have prevented the use in the host of a different and less reliable set of cues. In particular, as the parasite can easily be detected by the host on the basis of its HC profile (Ortolani et al., 2010), the selective pressure favouring the evolution of PCs as recognition cues during parasite invasion might have been very weak or even absent. Moreover, the benefits associated with the evolution of a recognition system based on PCs could be outweighed by the associated costs (developmental and recognition error costs).

Polistes obligate permanent social parasites are generally considered to be derived from *Polistes* free-living species *via* facultative intra- and inter-specific parasitic species (see Taylor, 1939; Cervo and Dani, 1996; Cervo, 2006). In the *P. sulcifer–P. dominulus* system, phylogenetic relationships are very close, as the two species belong to two sister clades (Choudary et al., 1994). The ancestral social parasites may have detected the hosts using the same chemical cues used for communicative aims by their hosts, instead of relying on different available cues, i.e. PCs.

Given that the host nestmate recognition system is not based on the CPC fraction [see this study for heterospecific intruders and Bruschini et al. (Bruschini et al., 2011) for conspecific intruders], we would expect the *P. sulcifer* polar profile not to mimic that of the host species after usurpation. Our chemical analyses, however, demonstrate that the parasite's CPC profile approaches that of the host species after the colony conquest. Before usurpation, the

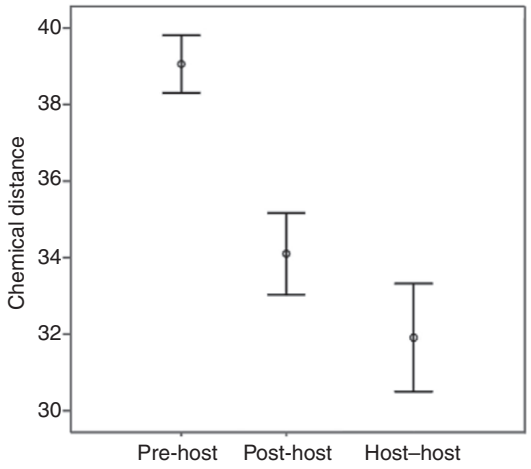

Fig. 4. The parasite CPC profile approaches that of the host after usurpation. The mean and the 95% confidence interval of the chemical distances between parasites and hosts before (pre-host) and after usurpation (post-host) and between two random hosts (host–host) are shown.

parasite and host profiles are markedly different, while after usurpation the two profiles become more similar. This polar profile resemblance at the species level could be explained as a by-product of the parasite and the host sharing the same environment once in the nest rather than an attempt at chemical mimicry.

The chemical resemblance, despite its uselessness in terms of acceptance by the host colony members, suggests that chemical mimicry should not always be explained as an adaptation in order to fool the host recognition system, even though this strategy represents the most likely explanation for many sophisticated examples of chemical mimicry. Behavioural bioassays are fundamental for testing whether the involved cues actually play a role in the host recognition system and whether, consequently, the chemical mimicry of these cues is really needed by the parasite in order to be accepted into the host colony.

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