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ORIGINAL PAPER

Why are larvae of the social parasite wasp *Polistes sulcifer* not removed from the host nest?

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Abstract A challenge for parasites is how to evade the sophisticated detection and rejection abilities of potential hosts. Many studies have shown how insect social parasites overcome host recognition systems and successfully enter host colonies. However, once a social parasite has successfully usurped an alien nest, its brood still face the challenge of avoiding host recognition. How immature stages of parasites fool the hosts has been little studied in social insects, though this has been deeply investigated in birds. We look at how larvae of the paper wasp obligate social parasite *Polistes sulcifer* fool their hosts. We focus on cuticular hydrocarbons (CHCs), which are keys for adult recognition, and use behavioral recognition assays. Parasite larvae might camouflage themselves either by underproducing CHCs (odorless hypothesis) or by acquiring a chemical profile that matches that of their hosts. GC/MS analyses show that parasite larvae do not have lower levels of CHCs and that their CHCs profile is similar to the host larval profile but shows a reduced colony specificity. Behavioral tests show that the hosts discriminate against alien conspecific larvae from different colonies but are more tolerant towards parasite larvae. Our results demonstrate that parasite larvae have evolved a host larval profile, which overcomes the host colony recognition system probably because of the lower proportion of branched compounds compared to host larvae. In some ways, this is a similar hypothesis to the odorless hypothesis, but it assumes that the parasite larvae are covered by a chemical blend that is not meaningful to the host.

Keywords *Polistes* wasps · Obligate social parasites · Nestmate recognition · Parasite integration · Larval recognition

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Introduction

Brood parasitism has always attracted the attention of evolutionary biologists for the evolutionary puzzle it poses: Why do hosts accept and care for the parasitic brood of other species? A lot of studies have investigated this topic in avian brood parasites. On the contrary, the means used by the immature brood of insect social parasites to fool hosts about their identity have received scarce attention.

A fundamental requirement for social groups is clear boundaries that keep non-group members from exploiting group resources. *Polistes* wasp colonies are closed social systems whose members keep intruders out. This gatekeeping is essential for the integrity of the colony, so altruism is not dispensed towards non-relatives. Each wasp at emergence learns the odor of its own natal nest, and this odor is used as a template to compare the chemical phenotypes of individuals that each wasp encounters (Gamboa 1996; Singer et al. 1998; Gamboa 2004). This odor is a mixture of hydrocarbons that each wasp bears on its cuticle and uses as an acceptance pass (Singer and Espelie 1992, 1996). This system allows efficient

nestmate discrimination, so that a colony is a fortress that is well-defended against intruders. A recognition system for alien immature brood seems unnecessary as alien individuals are normally prevented from entering the fortress-colony. However, females of *Polistes* intra- and inter-specific social parasites breach colony boundaries, take over as queens, and use the workers of the host to rear their own brood (Cervo and Dani 1996; Cervo 2006). Interspecific obligate social parasites—*Polistes sulcifer*, *Polistes semenowi*, and *Polistes atrimandibularis*—are often successful in the host colony, both at invading and at obtaining care for their larvae (Cervo 1990; Lorenzi et al. 1992; Cervo 2006). Having lost both the capacity to build a nest and the worker caste, they must exploit congeneric social species to rear their reproductives. Numerous studies have shown that these social parasites use a chemical strategy (lack of odor or/and chemical mimicry) to fool the hosts as to their real identity as cheaters (Bagnères et al. 1996; Turillazzi et al. 2000; Sledge et al. 2001; Lorenzi and Bagnères 2002; Dapporto et al. 2004; Lorenzi et al. 2004; Lorenzi 2006). But eluding the nestmate recognition system by queens is only the first step for successful invasion. Once an adult parasite has successfully usurped a colony, her immature brood also have to be tolerated and reared by the host workers. A recent paper (Cervo et al. 2004) has shown that the larvae of *P. sulcifer*, the obligate social parasite of *Polistes dominulus*, grow very quickly in host nests, probably because of their capacity to monopolize host parental care. These larvae are not only accepted into the alien nest but are also fed at an increased rate compared to host larvae (Cervo et al. 2004). This indicates that parasite larvae are well integrated into the host colonies and that they play an active role in host exploitation, soliciting even more food from workers than do host larvae.

Although *Polistes* represents one of the best-known models for the study of nestmate recognition in social insects, little is known about the capacity of adult females to discriminate between alien and domestic immature brood (Gamboa 1996, 2004). Panek and Gamboa (2000) suggested that *Polistes fuscatus* queens are able to discriminate between their own and alien conspecific larvae and hypothesized that the cue for larval recognition is probably the same hydrocarbon blend that mediates adult recognition. Studying intracolonial larval recognition, Strassmann et al. (2000) found no evidence for preferential brood feeding in *Polistes carolina*: The queen was no more likely to feed her own larvae than she was to feed the larvae of her sisters. More recently, some of us (Cotoneschi et al. 2007) have investigated the epicuticular hydrocarbon blend of *P. dominulus* larvae and found a characteristic larval chemical profile distinct from the adult profile.

The aim of this study is to investigate how larvae of the social parasite *P. sulcifer* avoid rejection by their hosts, *P. dominulus*. First, we investigated whether parasite larvae have chemical profiles that fool the host as to their real identity. We tested whether parasite larvae possess the same cuticular chemical profiles as the host larvae (chemical mimicry hypothesis, Dettner and Liepert 1994) and/or if they have low levels of hydrocarbons, which might give them a sort of chemical invisibility within the host colony (chemical insignificance hypothesis, Lenoir et al. 2001). We also performed behavioral experiments to test the ability of hosts from both unparasitized and parasitized colonies to recognize alien conspecific and parasite larvae.

Materials and methods

Collection sites

P. sulcifer is restricted to countries around the Mediterranean and Caspian basins where it is patchily distributed (Cervo and Dani 1996; Cervo 2006). This means that there are areas with abundant parasites and areas without parasites (Cervo 2006). We collected *P. dominulus* colonies for behavioral experiments and for chemical analysis both from a parasitized population (on the slopes of the Lessini mountains—North East Italy) and from completely unparasitized populations (various sites around Florence—Central Italy).

Colony collections for larva transplantation experiments

At the end of May 2002, we collected 20 single-foundress *P. dominulus* colonies in the pre-worker phase near Florence (Central Italy). At the beginning of June 2002, we collected 25 single-foundress *P. dominulus* colonies in the pre-worker phase, 11 unparasitized and 14 parasitized, in the Lessini mountains. Four of the latter parasitized colonies were used as sources of parasite larvae. Parasitized colonies had recently been invaded by a *P. sulcifer* female who was still present and laying eggs.

Colony collections for larva recognition tests

In June 2003, we collected 11 worker-phase *P. dominulus* colonies near Florence to obtain workers (five colonies) and host larvae (six colonies) for use in alien larva recognition tests. In June 2003, we collected four worker-phase-parasitized colonies in the Lessini mountains to obtain parasite larvae for use in these experiments.

Colony collection for larval odor presentation experiments

In June 2004, we collected 40 worker-phase *P. dominulus* colonies near Florence for use in larval odor presentation experiments. In June 2004, we collected four worker-phase *Polistes gallicus* colonies and four worker-phase *P. domi-*

nulus colonies to obtain larvae for odor presentation experiments. In June 2004, we collected three worker-phase-parasitized colonies in the Lessini mountains to obtain parasite larvae for use in these experiments.

Larva collection and cuticular lipid extraction for chemical analysis

We removed larvae of different developmental stages from five parasitized and from three unparasitized nests collected in June 2002 in the Lessini Mountains using soft tweezers cleaned with pentane between removals (to avoid chemical contamination across larvae). We defined small larvae as those in the first three instars and large larvae as those in the last two instars. We collected 56 large larvae (41 from parasitized colonies and 15 from unparasitized colonies) and 30 small larvae (15 from parasitized colonies and 15 from unparasitized colonies). The larvae were immediately stored at −20°C in individual tubes.

We extracted cuticular hydrocarbon compounds (CHCs) from the cuticle of larvae by immersing each of them in heptane (50 μl for small larvae, 100 μl for large larvae) for 2 min in a sonic bath. For each larva, 3 μl of extract was injected using an HP 7673 autoinjector into a Hewlett Packard 5890 A gas chromatograph coupled to a HP 5971A (Palo Alto, CA, USA) mass selective detector (using 70-eV electron impact ionization). We used a fused silica capillary column (30 m×0.25 mm×0.5 μm) coated with 5% diphenyl-95% dimethyl polysiloxane (Rtx-5MS, Restek, Bellefonte, PA, USA). The injector port and transfer line were set at 280°C, and the carrier gas was helium (at 12 psi, 83 kPa). The temperature protocol for the solvent injections was as follows: 70–150°C at a rate of 30°C min^{-1} (held for 5 min) and 150–320° C at 5°C min^{-1} (held for 13 min). The extracts of specimens of the two species were analyzed in a random order. Therefore, any possible change in either instrument performance or in variation of sample solvent volume (although the room was air conditioned to 20°C) should have equally affected the samples of the two species.

Laboratory rearing

We placed collected colonies in glass cages (15×15×15 cm) and provided water, sugar, and fly larvae as food, and paper for nest building, until they were used for experiments or for larval collections. We maintained the cages under natural light and temperature conditions with additional artificial light from 8:00 a.m. to 8:00 p.m.

Genetic analysis for species and sex assignment

We genotyped 13 adults of the host species and 16 adults of the parasite species at up to six polymorphic microsatellite loci developed for the host species *P. dominulus*: Pdom 2, Pdom7, Pdom20, Pdom117, Pdom122, and Pdom140 (Henshaw 2000). These data allowed us to create a molecular profile that distinguished larvae of the two species because they are not morphologically distinguishable. After cuticular lipid extraction, we preserved each larva in ethanol and then genotyped them at up to six loci. DNA of each larva and adult was extracted, amplified, and scored according to standard techniques of Strassmann et al. (1996). The genetic analysis allowed us to confirm larva species assignment made on the basis of length of egg hatching period (see below) and to determine sex.

Recognition experiments

Experimental procedure for alien larva transplant experiments: The general experimental procedure was the same as that used by Lorenzi and Filippone (2000) to test for egg recognition in *Polistes biglumis*. First, we temporarily removed the adult wasps so we could work with the larvae. We removed about ten larvae from each experimental nest with clean, soft tweezers. Then, half of the larvae were reintroduced into their own nests (control larvae), while the other half were substituted with larvae of the same stage extracted from alien nests (experimental larvae). The alien larvae came from either another *P. dominulus* nest (conspecific) or from parasitized nests that we checked daily for parasite–host larval discrimination (see below).

In a parasitized colony, there are larvae of both host and parasite intermingled. To make sure that we collected parasite larvae, we checked five parasitized colonies daily to determine their immature brood content. A previous study (Cervo et al. 2004) has shown that in parasitized colonies, eggs hatching in fewer than 5 days always belong to the parasite *P. sulcifer*, while eggs taking longer than 8 days always belong to the host *P. dominulus.* Eggs hatching between 5 and 8 days cannot be reliably assigned to either species on development time alone.

We transferred larvae quickly to minimize any harm or change in larval condition. We noted the precise cell into which we introduced larvae and recorded any absent or dead larvae 24 and 48 h after insertion. A further check for transplanted larvae fate was performed after 5 days.

Using this procedure, we performed experiments to investigate the following predictions.

Prediction 1: *P. dominulus* wasps are unable to recognize alien conspecific larvae

To test this null prediction, we introduced alien conspecific larvae into 20 single-foundress colonies before worker emergence and into 10 single-foundress colonies after worker emergence. The transplantation experiments per-

formed on pre-worker colonies were carried out to test the recognition of larvae destined to become workers (in *Polistes* the early spring larvae become workers), while those performed on post-emergence phase colonies (late July) were carried out to test larvae destined to become reproductives (the larvae produced later in the colonial cycle become reproductives). The alien conspecific larvae came from colonies collected far away from the tested colony (more than 10 km). This eliminates any complications from relatedness between introduced alien conspecific larvae and colony inhabitants.

Prediction 2: *P. dominulus* wasps are unable to recognize parasite larvae

To test this prediction, we introduced parasite larvae into 11 unparasitized *P. dominulus* colonies in the post-emergence phase**.** We obtained *P. sulcifer* larvae from five naturally usurped *P. dominulus* colonies and verified that the larvae used were *P. sulcifer* (see above). Since *P. sulcifer* does not have a worker caste, all its larvae are reproductives. In this transplantation experiment, we simulated the natural situation by testing parasite larvae on early host colonies to compare them with control host larvae belonging to the worker caste.

Prediction 3: the physical presence of the *P. sulcifer* queen is necessary for the maintenance of parasite larvae

We tested whether or not the hosts changed their tolerance towards parasite larvae in the presence of the parasite queen using ten natural colonies of *P. dominulus* usurped by *P. sulcifer*.

Prediction 4: the recognition system of parasitized colonies is less restrictive

Lorenzi (2003) suggested that in a similar host–parasite system (*P. atrimandibularis–P. biglumis*), the contemporaneous presence on the colony of host and parasite affect the nestmate recognition ability of the hosts. To test the recognition ability of host workers in parasitized nests, we transferred both alien *P. dominulus* and alien *P. sulcifer* larvae into parasitized colonies after we removed the parasite queen. The parasite removal was performed to limit the test to the recognition ability of host workers. This manipulation was performed on the same parasitized colonies used in the previous experiment.

Prediction 5: parasite larvae are distinguished from alien conspecific larvae (arena choice test)

As another test on whether parasite larvae are distinguished from alien conspecific larvae, we employed the same experimental design used by Panek and Gamboa (2000). We presented each host worker with both an alien conspecific larva and an alien parasite larva. The presentation was performed in a Petri dish, so only larval characteristics were involved in recognition. We used 19 host larvae from six unparasitized colonies and 19 parasite larvae from four usurped colonies. Each larva was extracted from its cell with clean, soft tweezers. Each parasite larva was paired with a host larva of similar size and then placed at opposite sides of a 9-cm diameter Petri dish. Nineteen workers belonging to five unparasitized colonies were chilled for 10 min in a refrigerator at 5°C. Then, each worker was placed in the center of the Petri dish with the two larvae, and its behavior was videotaped for 15 min. Larval species identification was unknown to the videotape observers, so the observations were blind. We noted the number and duration of aggressive behaviors (bites) and the duration of pacific behaviors (antennations, licking) towards the two types of larvae. No worker or larva was used more than a single time in any experiment.

Prediction 6: parasite larvae chemical profiles are the pass for parasite larvae acceptance (odor presentation experiment)

Finally, we tested whether cuticular chemical profiles of parasite larvae alone are sufficient to elicit an aggressive response. To collect larval cuticular compounds, we removed a large larva from its cell, then, gently rubbed its body for 2 min with a rounded point glass capillary. Some analyses, using GC/MS, showed that a distinct cuticular CHCs larva pattern is present on a capillary after rubbing the larva, but we cannot exclude the possibility that other cuticular substances were collected. We prepared 80 capillaries by rubbing the bodies of 40 *P. dominulus* larvae from eight unparasitized nests, 20 *P. sulcifer* larvae from four parasitized nests, and 20 *P. gallicus* larvae from four colonies.

In the first set of experiments, we tested whether the wasps were more aggressive towards conspecific alien larval odor than towards the parasite larval odor. Two capillaries inserted into a forked rod were presented simultaneously for 3 min to each of 20 *P. dominulus* colonies; one of the capillaries had been rubbed on the body of a parasite larva, while the other had been rubbed on a conspecific larva.

In the second set of experiments, we tested whether the reaction of wasps towards parasite larvae is simply because they belong to a different species. So we compared reactions to capillaries rubbed on the body of a non-parasitic species—*P. gallicus*—to that to a capillary rubbed on an alien conspecific larva. As *P. gallicus* larvae are smaller than *P. dominulus* ones, we rubbed a smaller

surface of the *P. dominulus* larva body to collect an analogous quantity of epicuticular compounds.

The capillary presentations were video-recorded, and then an observer ignorant of capillary treatment watched the video to note the number of aggressive behaviors (bites) and duration of aggressive and explorative ones (antennations) that wasps performed towards the capillaries.

Data analyses

Genetic data For determining larval species identity, we used a Bayesian clustering analysis with the program STRUCTURE (Pritchard et al. 2000; Falush et al. 2003). This is a Markov Chain Monte Carlo method that assigns individuals to populations and simultaneously estimates allele frequencies of each population. We assumed no admixture because the host and parasite are different species, and under these conditions, the program estimates the probability each individual originated from a given species. Using all the loci, we were able to definitively assign all but four larvae to host or parasite with a posterior probability of at least 99%. We excluded the four uncertain larvae from chemical distance analyses.

Any larva that was heterozygous at one or more loci we called a female because heterozygosity indicated she was diploid. We ran three more loci to be sure individuals homozygous at three loci were consistently homozygous and therefore male and actually hemizygous because they are haploid. The additional loci we ran for these individuals identified six more females out of a total of 41 that were homozygous at the first three loci.

Chemical data First, we ascertained that larval size did not affect our analysis of total quantity of CHCs by comparing the mass of 19 host larvae and 13 parasite larvae at the same stage of development. Then, we calculated the area of each peak (representing one or more cuticular compounds) and summed all the peak areas to compare the total quantity of hydrocarbons between different larva samples.

We identified CHCs on the basis of their mass spectra and then, to compare the chemical profiles of the two species, we calculated the relative percentage of each peak for each larva. Only 23 peaks were used in a Stepwise DA to determine whether the pre-defined groups could be discriminated on the basis of their cuticular composition. We eliminated three compounds from the analysis because they were present in only one species. Wilks' lambda probability significance and the percentage of correct assignments were used to evaluate the validity of the discriminant function. In addition, we employed a multidimensional scaling (MDS) analysis for assessing differences in chemical composition between larvae of parasite and host species without any a prori indication of group membership. We tested three different classes of chemical compounds to see if they were differentially involved in the composition of cuticular mixture of parasite and host larvae. We compared the average relative percentages of linear alkanes, of branched alkanes (mono- and dimethyl-branched alkanes), and of alkenes both for host larvae (n=16) and for parasite larvae (n=32) belonging to parasitized colonies.

Moreover, we performed Stepwise DA to verify if larvae of host and parasite species belonging to different colonies possess different cuticular hydrocarbon profiles.

The same chemical data (for 29 host larvae from unparasitized nests and 32 parasite larvae) were used to calculate the Euclidean distances (Turillazzi et al. 2000). Euclidian distances were used to estimate the chemical distances between pairs of parasite or host larvae by standardizing peak percentages with z scores (Turillazzi et al. 2000). The differences among groups (larvae from the same or different colonies) were analyzed with non-parametric Mann–Whitney U tests, verified using a Monte Carlo simulation. All statistical analyses were performed using SPSS 11.5 for Windows.

Behavioral data In alien larva transplantation experiments, we calculated the percentage of living larvae out of the total number of manipulated larvae after 24 h and after 48 h for each tested colony. We did not count the very few cases where larvae pupated before the final check. We used the Wilcoxon-signed rank test for comparing control and experimental larvae from the same nest (paired data) using an exact means of estimating p when samples were small instead of the asymptotic one usually employed in programs (Mundry and Fischer 1998). When multiple comparisons were done, we performed Bonferroni correction to adjust significance level dividing α by the number of comparisons (0.05/3=0.0167).

We analyzed both kinds of behavioral data (arena choice test and odor presentation experiments) with Wilcoxon-signed rank tests.

Results

Genetic analysis

Genetic analysis allowed us to divide the 56 larvae collected from parasitized colonies into three groups: 34 larvae belonging to *P. sulcifer* and 18 larvae belonging to *P. dominulus*, and 4 larvae of uncertain assignment that were not used further. The genetic assignments to species matched those based on egg development time in 47 out of 52 cases.

Genetic analysis also allowed us to assign the sex of each larva. From parasitized colonies, we had 32 female

larvae (14 of *P. sulcifer* and 18 of *P. dominulus*) and 20 male larvae (20 of *P. sulcifer* and 0 of *P. dominulus*). From unparasitized colonies, we had 14 female and 16 male larvae.

Chemical analysis

Cuticular compounds For four out of 82 larvae, the total ion chromatogram was too weak to allow for compound identification. The compounds found on the cuticle of both *P. dominulus* and *P. sulcifer* larvae were not as numerous as the compounds found on the adults (for the adult profiles, see Bonavita-Cougourdan et al. 1991; Dani et al. 1996; Turillazzi et al. 2000; Dapporto et al. 2004; Cotoneschi et al. 2007). On the cuticle of *P. dominulus* larvae (n=46), we detected 26 different compounds (Fig. 1): n-alkanes, methylalkanes, and alkenes with chains from 23 to 31 carbon atoms. All of them (Fig. 1) were also present on the cuticle of *P. sulcifer* larvae (n=32) except for an alkene and 7-methyl C_{31}. However, 7-methyl C_{31} was found to be absent not only on the cuticle of all analyzed parasite larvae but also on the cuticle of many host larvae (36 out of 46 analyzed host larvae). Similarly, the alkene was absent on the cuticle of nearly all host larvae (44 out of 46 analyzed host larvae). So these compounds are not definitive for the host species.

Comparison of hydrocarbon quantity between species Combining males and females, the mean total quantity of cuticular hydrocarbons of large larvae did not differ between the two species (mean ± SE: parasite larvae 14.8 counts±2.8 n=22, host larvae 19.0 counts±2.7 n=29; Mann–Whitney test, U=239, p=0.128), and the large larvae of the two species are not different in weight (parasite larvae 0.108±0.009 g n=13, host larvae 0.098± 0.008 g n=19, U=103, p=0.431). Small larvae of the parasite had significantly higher mean levels of cuticular hydrocarbons than did host larvae (4.9 counts±0.9 n=10 and 1.9 counts±0.5 n=16, respectively, U=26, p=0.003). No statistical differences emerged between parasite and

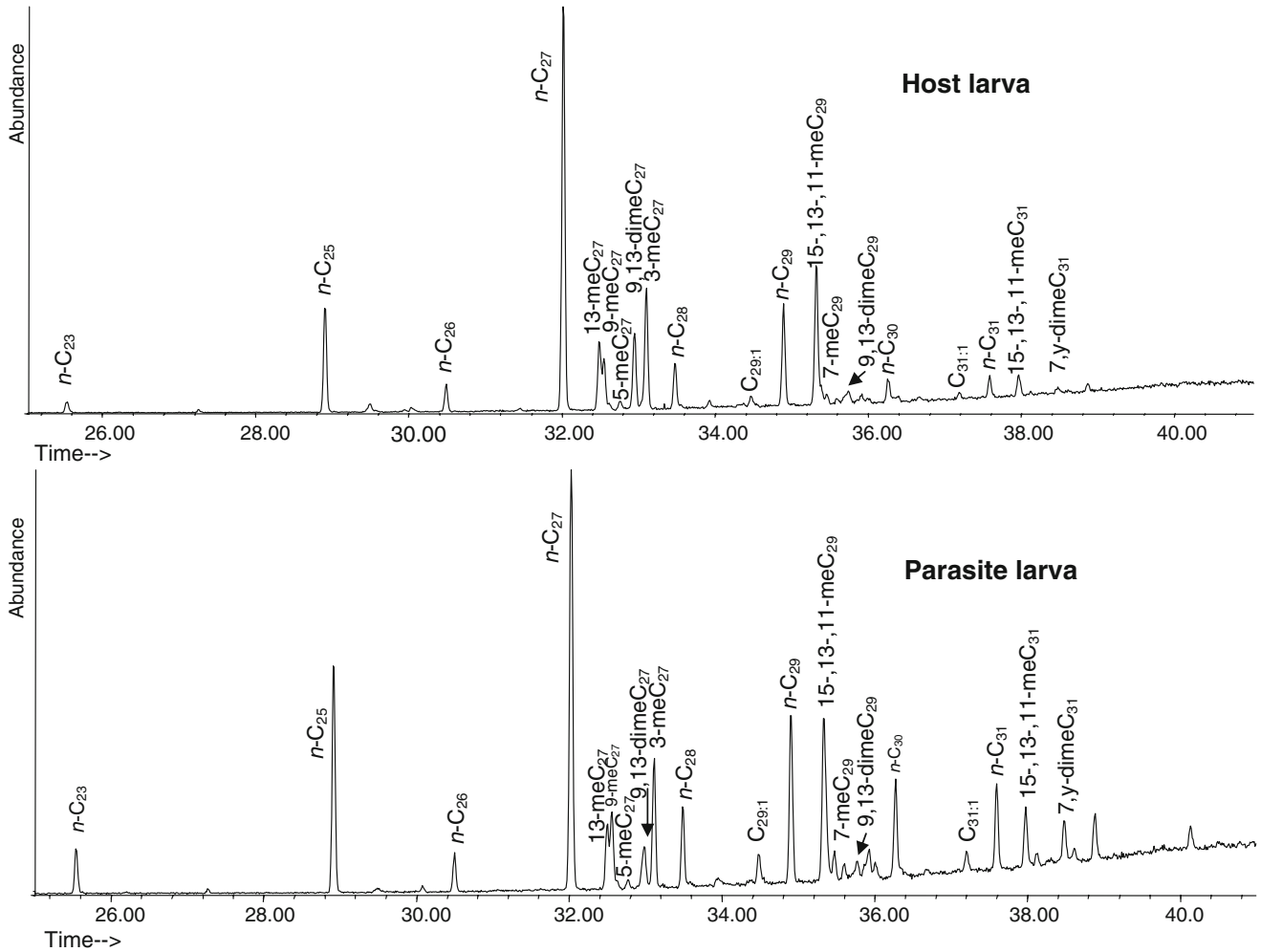

Fig. 1 Chemical profiles of two larvae belonging to the same parasitized colony. The names of the principal compounds are reported. The *arrows* connect the name of the CHC with the respective peak where there are too many peaks in the chromatogram

host (combining large and small larvae) when male (n=19 parasites, n=13 hosts) and female larvae (n=13 parasites, n=28 hosts) were compared separately (U=105, p=0.495; U=181, p=0.989). These results do not support the hypothesis that the identity of parasite larvae is concealed by their reduced quantity of cuticular hydrocarbons.

Comparison of cuticular hydrocarbon profiles between species Unfortunately, our parasitized host larva sample was made up of only females; however, as no differences in the chemical profile between males and female larvae belonging to the parasite species were revealed by DA (canonical correlation=0.850, Wilks' λ=0.278 χ^2=24,3, p=0.332), in the following comparisons, we investigated only differences between species. Stepwise DA revealed that the larvae of the two species in parasitized colonies differed in their cuticular hydrocarbon profiles. In all, 91.7% of the larvae were classified as their correct species (canonical correlation=0.821, Wilks' λ=0.327, χ^2=49,79, df=3 p=0.001). But considering only large larvae (Fig. 2) to compare more homogeneous samples, 97.2% of the larvae were classified to their correct species (canonical correlation=0.93, Wilks' λ=0.134, χ^2=65.23, df=3 p=0.001). Only one *P. dominulus* larva was misclassified. The

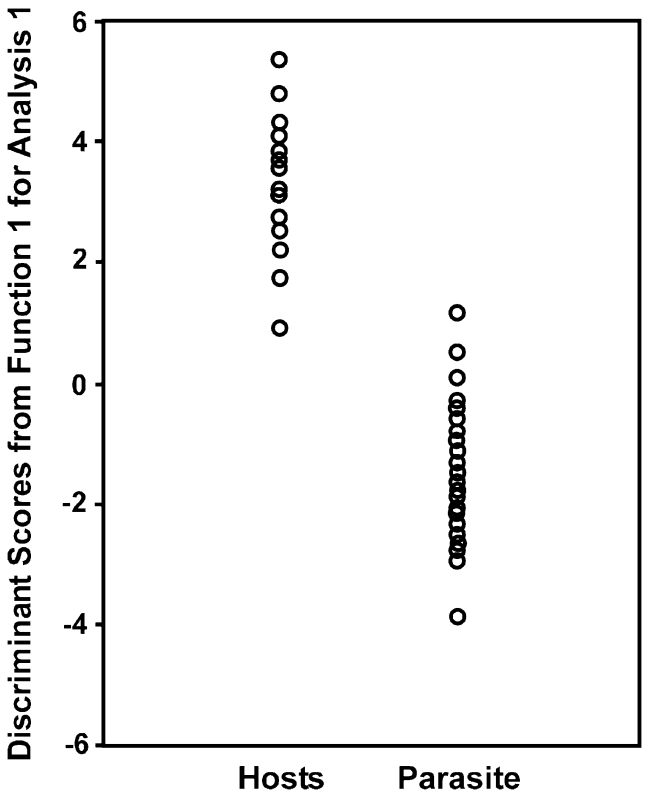

Fig. 2 Discriminant scores obtained by discriminant analysis between large larvae of *P. dominulus* (n=14) and *P. sulcifer* (n=22) in the usurped colonies

constructed "map" of MDS (Fig. 3) between larvae of the two species showed host larvae to be well grouped while parasite larvae were more dispersed. The stress value for this analysis is 0.14.

The cuticular mixture of parasite larvae (n=32) has, on average, a higher relative proportion of linear alkanes (U test coupled with Monte Carlo method, U=156, p=0.029) but a lower proportion of branched compounds and unsaturated hydrocarbons (U=152, p=0.022 and U=164, p=0.039) than the cuticular mixture of host larvae (n=16; Fig. 4).

Stepwise DA performed on host larvae of three unparasitized colonies revealed a 100% correct assignment to the colony of origin (n=29, Function 1: Wilks' λ=0.002, p<0.0001, explaining 87.7% of variance; Function 2: Wilks' λ=0.123, p<0.0001, explaining 12.3% of variance), as was just reported by Cotoneschi et al. (2007) for this species. By contrast, only 40.6% of parasite larvae (n=32) were correctly assigned to their own colonies.

To further investigate whether parasite larva odor is more homogeneous than that of host larvae, we compared the chemical distances of parasite larvae belonging to different and to the same parasitized colonies with those of host larvae belonging to different and to the same colonies. Euclidean distances (Fig. 5) showed that the chemical profiles of host larvae from different unparasitized colonies differed more strongly than host larvae did from the same colony (U=3053, p=0.002). On the contrary, the chemical profile distances did not differ within parasite larvae according to colony. Moreover, the chemical distances between larvae within an unparasitized host colony were significantly less than those between parasite larvae within a parasitized colony (U=1194, p=0.03).

Recognition experiments

Prediction 1: P. dominulus are unable to recognize alien conspecific larvae. In our alien larvae transplantation experiments, *P. dominulus* solitary foundresses (n=20) eliminated significantly more alien conspecific larvae than control larvae introduced into their nests after both 24 and 48 h (Wilcoxon test, Z=−2.9 p=0.004, Z=−3.1 p=0.002; Table 1, experiment 1a). The percentage of alien conspecific larvae decreased further the following day and 5 days after the experiment (after 72 h only 24.6% of transplanted larvae were still alive, after 5 days no alien larvae were present). On the contrary, the same percentage of control larvae were still alive both 72 h and 5 days after the experiment. The same results were obtained when the experiment was performed on colonies (n=10) that had been begun by a single female but were currently at the advanced stage when most of the tested larvae were destined to become reproductives. Significantly more alien

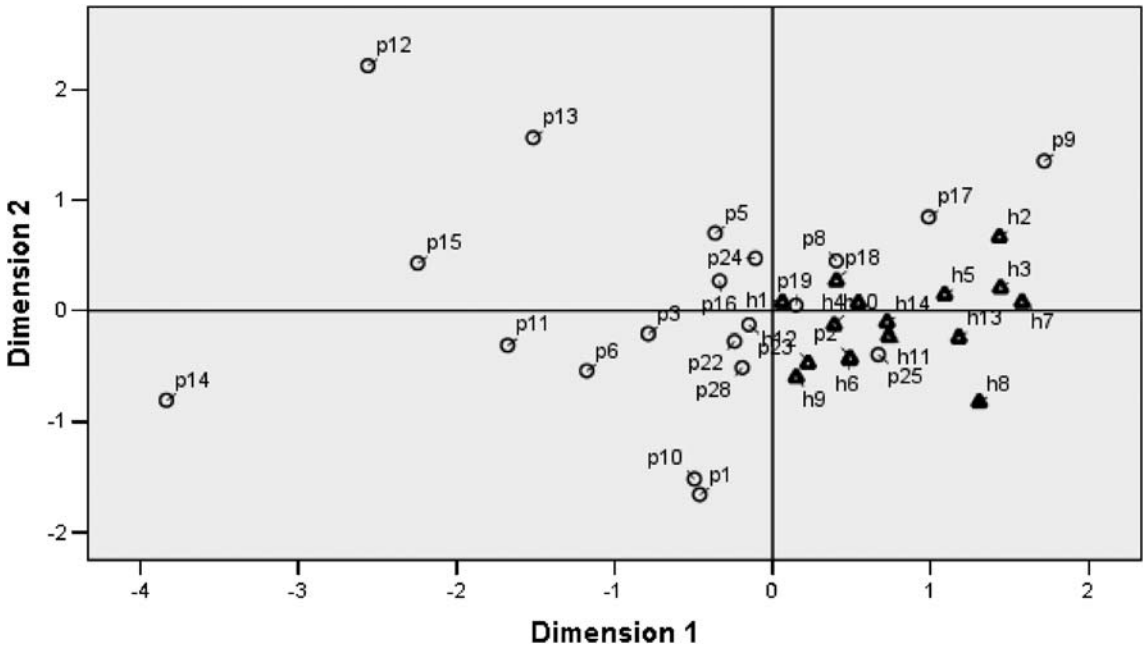

Fig. 3 Multidimensional scaling of parasite (*circle*) and host larvae (*triangle*). *Dimension 1* displays relative positions of larvae by species

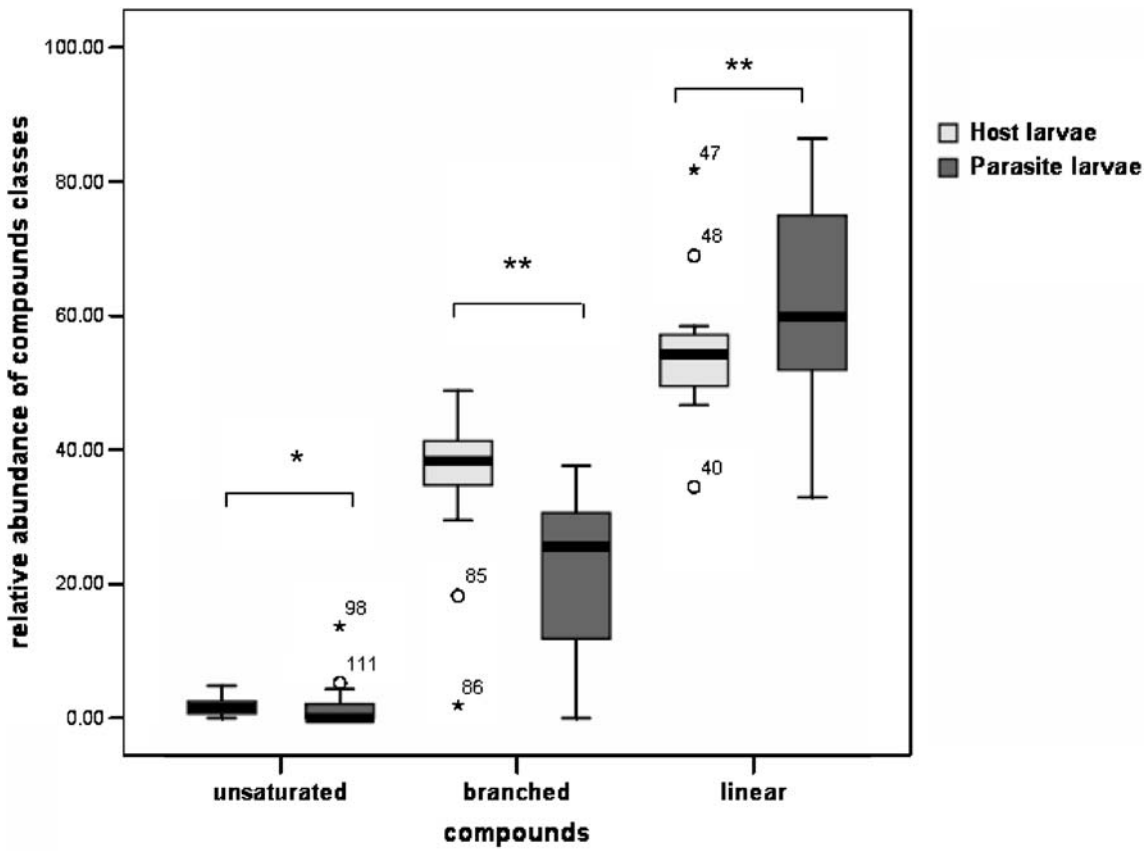

Fig. 4 Relative abundance of different classes of hydrocarbons (unsaturated, branched and linear alkanes) present on the cuticular mixture of parasite (n=32) and host larvae (n=16) belonging to parasitized nests. *Box plots* show 75th and 25th percentiles as the *box*, the median as the *line in the box*, and the extremes as the *vertical lines*. *Asterisks* indicate significant differences: *single asterisk* $p<0.04$, *double asterisk* $p<0.03$

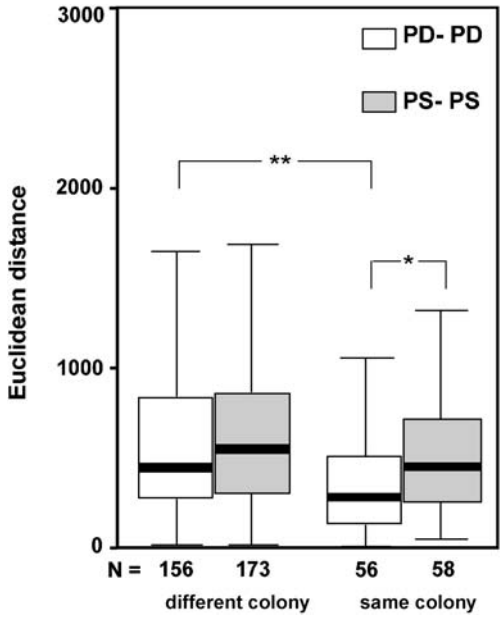

Fig. 5 Chemical distance between pairs of host larvae (*PD*) and parasite larvae (*PS*) belonging to different nests or to the same nests. *Box plots* show 75th and 25th percentiles as the *box*, the median as the *line in the box*, and the extremes as the *vertical lines*. *Asterisks* indicate significant differences: *double asterisk* $p=0.002$, *single asterisk* $p=0.03$

larvae were eliminated than control larvae after both 24 and 48 h (Wilcoxon test, exact version, $p=0.004$ and $p=0.002$, respectively; Table 1, experiment 1b).

These experiments show that *P. dominulus* foundresses and workers can recognize and discriminate against alien conspecific larvae destined to become both workers and reproductives.

Prediction 2: P. dominulus are unable to recognize parasite larvae The parasite larvae experimentally introduced into unparasitized colonies ($n=11$) were not eliminated significantly more frequently than control larvae after either 24 or 48 h (Wilcoxon test, exact version, $p=0.065$ and $p=0.109$, respectively; Table 1, experiment 2). Eight days after the experiment, we checked the colonies again and found no further elimination of parasite larvae. Moreover, on two of these nests, two parasite males had emerged and had been accepted by host workers. These results suggest that hosts do not discriminate against parasite larvae.

Prediction 3: the physical presence of a P. sulcifer usurper queen is necessary for maintaining parasite larvae There was not one significant difference in larval elimination before or after the experimental removal of the parasite queen from the colony she usurped ($n=10$; Wilcoxon test, exact version, $p=1.00$, both after 24 and 48 h; significance level after Bonferroni correction $0.05/3=0.0167$; Table 1, experiment 3). An additional check for larval presence carried out after 5 days from parasite queen removal showed the same percentage of larvae still alive in parasitized nests. These results show that the host workers did not change their tolerance towards parasite larvae in the absence of the parasite queen, suggesting that her physical presence is not important for the tolerance of parasite larvae in the host nest.

Prediction 4: the recognition system of parasitized colonies is less restrictive In parasitized colonies ($n=10$), the conspecific alien larvae were eliminated significantly more by host workers than were the control larvae after either 24 or 48 h (Wilcoxon test, exact version, $p=0.001$, after either 24 or 48 h; significance level after Bonferroni correction $0.05/3=0.0167$; Table 1, experiments 4a and 4b). Moreover, 5 days after the experiment, no alien conspecific larvae were still alive. These results show that *P. dominulus* workers were able to discriminate alien conspecific larvae even in parasitized nests where larvae of the two species cohabit. In the same colonies, the parasite alien larvae were not eliminated significantly more than the control larvae after either 24 h or 48 h (Wilcoxon test, exact version, $p=0.65$ and $p=0.024$, significance level after Bonferroni correction $0.05/3=0.0167$). A further check performed 5 days after the experiment showed no changes in alien parasite larvae presence. Thus, the workers seem to discriminate against alien conspecific larvae but not against alien larvae of the parasite species.

Prediction 5: parasite larvae are distinguished from alien conspecific larvae (choice test in arena) Host workers ($n=19$) behaved differently towards conspecific alien larvae compared to parasite larvae (Table 2, a). They were significantly more aggressive towards alien conspecific larvae than towards parasite larvae both in number of bites and in time spent in aggressive contacts (Wilcoxon test, $Z=-2.5$, $p=0.012$ and $Z=-2.6$, $p=0.008$). In two trials, workers killed conspecific larvae and transported them away from their original positions, while no parasite larvae were ever killed. We found no significant differences in time spent by workers in pacific contacts (lickings and antennations) between the two types of larvae ($Z=-8.5$, $p=0.395$). However, workers licked parasite larvae more than they licked conspecific larvae, though this difference is not statistically significant.

Prediction 6: chemical profiles are the pass for parasite larval acceptance in the host colony (odor presentation experiment) P. dominulus wasps were significantly less aggressive (time spent in aggressive contacts) towards capillaries that had been rubbed on parasite larvae (Wilcoxon test, $Z=-2.37$; $p=0.017$, $n=20$) than towards capillaries that had been rubbed on larvae of an alien

Table 1 Tolerance (mean percentage of live larvae) of *P. dominulus* foundresses and/or workers towards alien conspecific or parasite larvae experimentally introduced in their own colonies (sample size is given in the 2nd column) parasitized or not by the social parasite *P. sulcifer*

Experiment to test the recognition of:	Tested colonies of *P. dominulus*	Tested individuals	Experimental larvae	Control larvae	Mean percentage (±SE) of live larvae (experiment vs control), *p* value	
					After 24 h	After 48 h
(1a) Alien conspecific larvae by host foundresses	Unparasitized colonies in pre-worker phase (n=20)	Solitary foundresses	Alien conspecific larvae	Same colony larvae	60.4±4.4 vs 83.2±4.1, p=0.004	42.6±5.8 vs 72.1±5.4, p=0.002
(1b) Alien conspecific larvae by host foundresses and workers	Unparasitized colonies in post-worker phase (n=10)	Foundresses and workers	Alien conspecific larvae	Same colony larvae	51.8±4.9 vs 80.3±4.7, p=0.004	19.3±5.7 vs 74.2±3.6, p=0.002
(2) Parasite larvae by unparasitized hosts	Unparasitized colonies in post-worker phase (n=11)	Foundresses and workers	Parasite larvae	Same colony larvae	27.3±9.2 vs 51.4±6.4, p=0.065	18.2±6.8 vs 45.7±6.5, p=0.109
(3) Parasite larvae in the absence of the parasite queen	Parasitized colonies in post-worker phase (n=10)	Host workers only	Parasite larvae in the absence of parasite queen	Parasite larvae in the presence of parasite queen	63.6±8.9 vs 69.1±6.2, p=1.00[a]	58.1±7.8 vs 67.3±6.2, p=1.00[a]
(4a) Alien conspecific larvae by parasitized hosts	Parasitized colonies in post-worker phase (n=10)	Host workers only	Alien conspecific (*P. dominulus*) larvae	Same colony larvae in the absence of the parasite queen	20.4±6.0 vs 69.1±6.2, p=0.001[a]	9.5±5.1 vs 67.3±6.2, p=0.001[a]
(4b) Alien parasite larvae by parasitized host	Parasitized colonies in post-worker phase (n=10)	Host workers only	Alien parasite (*P. sulcifer*) larvae	Same colony larvae in the absence of parasite queen	55.5±7.5 vs 69.1±6.2, p=0.65[a]	38.2±7.8 vs 67.3±6.2, p=0.024[a]

[a] The significance level after Bonferroni correction is 0.05/3=0.0167

conspecific colony (Table 2, b_1). However, we found no difference in time spent in exploratory contacts with the capillaries rubbed on an alien conspecific larva compared to those rubbed on a parasite larva (Z=− 0.8, p=0.422, n=20). Furthermore, we found no differences in time spent in aggressive contacts (Z=− 0.36, p=0.717, n=20) or in duration of exploratory contacts (Z=− 0.56, p=0.572, n=20) when *P. dominulus* colonies were tested with capillaries rubbed on an alien consecific larva and a larva of a different non-parasitic species, *P. gallicus.* The lower response towards capillaries with *P. dominulus* odor in this set of experiments (see Table 2) with respect to the previous one is probably due to the smaller larval surface sampled (see "Materials and methods").

Discussion

The results of our larval transplantation experiments into host nests suggest that larval recognition and discrimination occur in this species, but social parasite larvae escape detection.

Moreover, our odor presentation experiment shows that the chemical profile is responsible for larva recognition. We focus on cuticular hydrocarbons, which are central to the *Polistes* nestmate recognition system (see Gamboa 2004), though we cannot exclude the additional involvement of other substances. The recognition of alien conspecific larvae agrees with the findings of a recent study (Cotoneschi et al. 2007), where we demonstrated that *P. dominulus* larvae show a characteristic colony-specific pattern of CHCs. Moreover, in this paper, we show that *P. sulcifer* larvae do not have lower levels of cuticular hydrocarbons as would be predicted by the chemical insignificance hypothesis (Lenoir et al. 2001). The total quantity of hydrocarbons on the epicuticles of *P. sulcifer* parasite larvae is not lower than that on the cuticles of host larvae. The original function of the insect cuticular lipid layer is to provide a barrier against desiccation and attack by pathogens and toxins. Since the lipid layer cannot be below the survival threshold, the very low level of cuticular lipids, which might be necessary for parasite larvae acceptance via the odorless hypothesis, might compromise larval survival.

We expected that the parasite larvae would have a chemical camouflage process similar to that of the parasite queen after host nest usurpation. The invading parasite queens of *P. sulcifer* acquire the cuticular hydrocarbon profiles of the adult hosts within a few hours of colony invasion (Turillazzi et al. 2000; Sledge et al. 2001; Dapporto et al. 2004). This quick cuticular change facilitates host colony acceptance of the parasite queen and is important because workers strongly repel any individual arriving at the nest with an alien cuticular

Table 2 Behavioral responses

	Mean ± ES		
	P. sulcifer	*P. dominulus*	*P. gallicus*
a Larval choice test in arena (n=19)			
No. of bites	0.37±0.14	1.05±0.27*	
Time (s) spent in aggressive contacts	0.68±0.28	14.26±8.36**	
Time (s) spent in pacific contacts	9.21±1.84	7.31±1.51 (n.s.)	
b Larval odor presentation experiment (n=20)			
b_1			
Time spent (s) in aggressive contacts	3.6±0.67	6.75±0.94*	
Time spent (s) in explorative contacts	25.9±2.88	23.0±2.45 (n.s.)	
b_2			
Time (s) spent in aggressive contacts		5.0±0.87 (n.s.)	5.95±1.15
Time (s) spent in explorative contacts		17.8±4.42 (n.s.)	17.9±3.87

a Behavioral responses of *P. dominulus* workers (n=19) towards parasite larvae vs conspecific alien larvae tested in a small arena, b_1 behavioral responses of *P. dominulus* colonies (n=20) towards paired glass capillaries rubbed on either a parasite larva or rubbed on an alien conspecific larva, b_2 behavioral responses of *P. dominulus* colonies (n=20) towards paired glass capillaries rubbed on either a larva of a different non-parasitic species (*P. gallicus*) or rubbed on an alien conspecific larva

n.s. not significant

*p<0.05; **p<0.01 denote significance of the Wilcoxon test.

hydrocarbon profile (see Singer et al. 1998; Gamboa 2004). It has been proposed that a parasite queen acquires the host colonial profile by vigorously stroking her abdomen on the surface of the host nest (Turillazzi et al. 2000; Cervo and Dani 1996). The blend of hydrocarbons that covers the surface of a *Polistes* comb is similar to that of the adult wasps that live on it (Lorenzi et al. 1996; Singer et al. 1998).

Larvae differ substantially from adults in the following two contexts: They are unarmed grubs living inside cells and they completely shed their cuticle five times as they go through the five larval instars. Their movements are restricted, and behaviors like the adult nest stroking are impossible. This poor capacity for hydrocarbon acquisition from nest paper is confirmed by the recent finding (Cotoneschi et al. 2007) showing that, in *P. dominulus*, adults are chemically more similar to the nest paper than to their larvae. Parasite larvae may simply not be able to achieve chemical camouflage by acquiring hydrocarbons from the nest.

Moreover, though chemical camouflage is a rapid process, it is not instantaneous. During the acquisition period, the parasite is detectable as alien and can be attacked by the hosts. Parasite queens are larger than hosts (Cervo 1994) and so can repel attacks in ways that would be impossible for larvae. If a larva had an acquired cuticular chemical camouflage, it would need to be renewed after every moult. As the larval stage is very short in this parasite species (Cervo et al. 2004)—it lasts only about 10 days—the camouflage process would be necessary every other day. Finally, it is difficult to believe that parasite larvae chemically integrate themselves into the host colony by mimicry (sensu Howard et al. 1990), as they would have to be able to synthesize the chemical blend characteristic for the specific colony where they are born. Our chemical analyses show that the larval CHCs profiles of the two species are qualitatively similar, suggesting that the parasite larvae have evolved a genetic system to match the larval host profile. However, having the CHCs profile of the larvae of the host species is not sufficient to overcome the colonial recognition system of the host as our alien conspecific host larva transplantation experiments have shown. Stepwise DA of chemical data separates parasite and host larvae according to their CHCs profiles. Moreover, what differentiates parasite larval profiles from host profiles is their reduced colony specificity. Host larvae possess a CHCs colony-specific pattern (Cotoneschi et al. 2007; this paper) but parasite larvae do not. While the cuticular hydrocarbon profiles of host larvae vary among colonies, those of the social parasite are largely similar among colonies. This confirms that parasite larvae do not acquire the host larval colony pattern from the nest paper but actively produce their own hydrocarbons.

Our analysis shows that the relative abundance of branched and unsaturated hydrocarbons is lower in parasite than in host larval cuticular mixtures, while that of the linear alkanes is higher in parasite larvae. It has been suggested (Dani et al. 2001) that in *P. dominulus*, linear alkanes are not used as recognition cues, whereas branched alkanes are more easily detectable by wasp receptors because of their conformation. This could allow parasite larvae to escape host detection. This lower proportion of branched alkanes is probably also the cause of the reduced colony specificity of the parasite larvae. In fact, in a recent

study by our group, we found that the chemical differences of larvae belonging to different host colonies are mainly based on branched compounds (cf. the results of Stepwise DA on larval cuticle; Cotoneschi et al. 2007).

We propose that parasite larvae have been selected for a profile, which is neutral to hosts, and does not trigger rejection. In some ways, this is a similar hypothesis to the odorless hypothesis, but it assumes that the parasites are covered by a chemical blend that is not perceived by the host workers or not meaningful for the host and that it does not trigger rejection (neutral odor hypothesis).

Our behavioral transplantation experiments show that neither parasitized colonies nor unparasitized colonies removed parasite larvae significantly more often than they removed control larvae. In choice tests in an arena away from the nest, host workers attack alien conspecific larvae significantly more often than they attack parasite larvae. Yet parasite larvae are nevertheless tolerated in the host colony, whether or not the host colony has had experience with parasites. Probably, parasite larvae are favored over alien conspecific larvae because they possess a "neutral" larval host profile that does not trigger rejection. Host workers are not aggressive towards capillaries with parasite larval odors, while they are aggressive towards those with larval odors of a different non-parasitic species or of conspecific alien colonies. These findings indicate that the chemical signature of parasite larvae is the key for understanding their acceptance in host nests and agree with the hypothesis that it is neutral for host nestmate recognition.

The acceptance of parasite immature brood in the few species where it has been studied is based on chemical mimicry (sensu Howard et al. 1990). For example, larvae of the lycaenid butterfly *Maculinea reblei* closely mimic the hydrocarbon blend of the larvae of *Myrmica* ants, inducing the ant workers to bring them into the host nest where they are socially accepted (Akino et al. 1999; Schonrogge et al. 2004). The deception of ants that forage outside of colonies can be attained only through secreting chemicals because they have no previous contact with hosts. Similar results are reported for the larvae of *Microdon mutabilis*, a syrphid fly (Elmes et al. 1999) that exploits ant social systems; individuals of the larval stage, which inhabit the ant nest, are protected by a thick dorsal cuticle covered by biosynthesized mimetic cuticular hydrocarbons (Howard et al. 1990; Dettner and Liepert 1994). A recent chemical analysis of eggs of the slave-making ant *Polyergus breviceps* even reports convergences of chemical profiles of parasite eggs with the egg profiles of their local host species (Johnson et al. 2005) facilitating the adoption of parasite eggs by the host.

Taken together, our results demonstrate that the way the larvae of *P. sulcifer* promote their social acceptance in a colony of *P. dominulus* is different from that used by usurping queens that perfectly match the specific profile (Sledge et al. 2001) of the colony they usurp probably via a camouflage process (Turillazzi et al. 2000). *P. sulcifer* larvae produce a host larva cuticular profile with a reduced relative abundance of branched compounds that could facilitate their acceptance into a host colony as their cuticular mixture is not meaningful for the hosts. As we have previously discussed, the chemical strategy adopted by *P. sulcifer* larvae seems to be ideal for successful integration by a motionless, frequently molting larva into the nest of its host species.

In the evolutionary battle between host and parasite, each partner evolves better tricks or better defenses and they co-evolve together. However, as every parasite encounters a host, but not every host encounters a parasite, selection will be stronger on the parasite than on the host, favoring the parasite that is one step ahead of its host. *P. sulcifer* larvae seem to have evolved an efficient strategy to overcome the host recognition system because this trait is crucial for parasite success.

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