Imperfect chemical mimicry explains the imperfect social integration of the inquiline ant *Ectatomma parasiticum* (Hymenoptera: Formicidae: Ectatomminae)

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Abstract

Inquilinism is an extreme form of social parasitism where the parasite permanently depends on its host. This parasitism is widespread among Formicinae and Myrmicinae ants but rare in other subfamilies, like Ectatomminae where *Ectatomma parasiticum* Feitosa & Fresneau, 2008 is the sole inquiline described so far. This species is genetically and morphologically very similar to its host, *E. tuberculatum* (Olivier, 1792), and uses its worker force to produce exclusively sexuals. During their life cycle, *E. parasiticum* queens enter into established host colonies and cohabit with the resident queens over an extended period of time. However, previous experiments in the laboratory have shown that some parasites are attacked by host workers, suggesting that their social integration into host colonies is incomplete or unstable. We thus investigate how the chemical cues of the parasites relate to the host's recognition system. For this, we compare the cuticular hydrocarbon profiles of parasites, host queens and host workers using solid-phase microextraction. Although overlapping, the chemical profiles of both species are distinct. Parasites have no specific compounds but a reduced total amount of cuticular hydrocarbons compared with hosts. We suggest that *E. parasiticum* uses an imperfect chemical mimicry strategy as it is well-discriminated by its host species.

Key words: Chemical strategy, cuticular hydrocarbons, inquiline ants, *Ectatomma parasiticum*, *Ectatomma tuberculatum*.

Introduction

Social insects have evolved finely tuned recognition systems to discriminate nestmates from non-nestmates. This ability is primarily based on chemical compounds borne on the insects’ cuticle and shared by all colony members (D’ETTORRE & LENOIR 2009). Among cuticular lipids, hydrocarbons seem to be involved in nestmate recognition (“Hydrocarbon hypothesis”, HEFETZ 2007, BLOMQUIST & BAGNÈRES 2010) as shown by direct evidences (e.g., LAHAV & al. 1999, AKINO & al. 2004, OZAKI & al. 2005, BRANDSTAETTER & al. 2008, MARTIN & al. 2008) and suggested by correlative studies (see MARTIN & DRIJFHOUT 2009a). The mechanisms involved in their production, transfer and perception have been partly elucidated (VANDER MEER & MOREL 1998, LENOIR & al. 2001, HOWARD & BLOMQUIST 2005). Cuticular hydrocarbons (CHC) are also known to differ in quality between species and in quantity within species (i.e., colony, caste, age and sometimes individual), thus forming a multi-component signal (D’ETTORRE 2008). However, which compounds or groups of compounds encode each specific level of recognition is far from clear. Social parasites which are able to bypass host-colony specific barriers provide good opportunities to disentangle the chemical cues involved at each level, and thus give new insights on recognition systems in insects.

Social parasites entirely depend on another social species for survival and reproduction. They take advantage of the host-colony resources through chemical strategies and behavioral adaptations. Social parasites may either express no identification cues, or produce the host-specific chemical cues, or alternatively acquire them directly from host individuals and nest materials (LENOIR & al. 2001, AKINO 2008, NASH & BOOMSMA 2008). These tactics are referred to as chemical insignificance, chemical mimicry and chemical camouflage respectively in chemical ecology (but see VON BEEREN & al. 2012 for a critical review of the terminology). In addition, parasites may lower the production of some classes of CHCs, such as branched alkanes and alkenes, which are considered to be involved in nestmate recognition (CHÂLINE & al. 2005, MARTIN & al. 2008, MARTIN & DRIJFHOUT 2009b). They may also use specific chemicals, such as appeasing or propaganda signals (see AKINO 2008).

In the ultimate form of social parasitism, i.e., inquilinism, parasite species have lost the worker caste and produce only sexuals. They emerge in their native colony and have to overcome the host-recognition system to either stay therein or to gain entry into another established colony to be adopted permanently (BUSCHINGER 1990, BOURKE & FRANKS 1991, BUSCHINGER 2009). Consequently, they are
expected to adjust their strategies to the different phases of the host-exploitation process. As documented in wasps (Lorenzi & Bagnères 2002) and bumblebees (Dronnet & al. 2005 but see Martin & al. 2010), parasites expressed a weak chemical signature at the beginning of the process and then acquired the host chemical compounds to facilitate their social integration. From an evolutionary point of view, chemical insignificance is considered to precede chemical mimicry (Lambardi & al. 2007), and therefore chemical mechanisms should give information on the origin of the divergence of the parasite and host species. Indeed, inquiline ants and their host are supposed to derive from a common ancestor (Emery’s rule; Busching 1990, Bourke & Franks 1991, Busching 2009).

Inquiline ants are widespread in Formicinae and Myrmicinae, although only in a few genera, but rare in other subfamilies. Ectatomma parasiticum Feitosa & Fresneau, 2008 is the first and, until now, the only inquiline species described in the Ectatomminae subfamily (Busching 2009). It possesses several parasitic life-history traits: rarity in the field, local distribution, quasi-exclusive production of sexuals, queen miniaturization, and intra-colonial mating (Hora & al. 2001, 2005, Féneron & al. 2013). This parasitic ant is a sibling species of its host, E. tuberculatum (Olivier, 1792), from which it may have diverged only recently (Féneron & al. 2013). However, its social integration into the host colony seems to be incomplete or unstable. Indeed, host workers specifically antennate or attack some parasites in their own colony (Hora & al. 2009) and treat them differently from conspecific queens and workers during discrimination tests (Féneron & al. 2013). Thus, we investigated the CHC profiles of E. parasiticum, and compared them with the CHC profiles of the host in order to understand the origin of the imperfect social integration of the parasite.

Materials and methods

Study species and rearing conditions: Eleven colonies of Ectatomma tuberculatum were collected from two sites in Apazapan, Veracruz state, Mexico (19° 19’ 38” N, 96° 43’ 21” W) during January 2009. Seven colonies were collected in one site (site 1) and four colonies from another one (site 2), about 500 m apart. Parasitic queens of E. parasiticum were not present as adults during nest collection but they emerged in three out of the seven E. tuberculatum colonies from site 1 two months later. They could be easily differentiated from the host species due to their intermediate body size between E. tuberculatum queens and workers (Hora & al. 2001). The colonies of E. tuberculatum were facultatively polygyrous including from one to seven dealate queens (i.e., four colonies with one queen, three colonies with two queens, one colony with three queens, one colony with five queens and one colony with seven queens) and when parasitized, they also included several E. parasiticum queens (i.e., three, five and 19 queens). No E. parasiticum males emerged during this two-month period, all parasitic queens were virgin and all of them, but five, were alate.

In the laboratory, ants were housed in plaster nests with several chambers kept dark by a cardboard. The nests were connected to an uncovered foraging arena in which water and food (honey-apple mixture, pieces of Acheta domesticus and larvae of Tenebrio molitor) were supplied. All colonies were fed with the same diet. They were maintained at 28 ± 2°C, 60% of relative humidity and in a 12 h:12 h light-dark cycle.

Chemical extraction and analysis: The CHC profiles were analyzed with gas-chromatography (GC) for 32 host queens (5 from parasitized colonies and 27 from non-parasitized colonies), 27 parasitic queens, and a sample of 68 host workers (20 from parasitized colonies and 48 from non-parasitized colonies). Workers were randomly taken either from the nest or from the foraging arena (n = 6 - 8 workers per colony). CHCs were gathered from living individuals using solid-phase microextraction (SPME). We rubbed the first abdominal segment of the ant with a fiber (Supelco Inc., coated with a 7 µm polydimethylsiloxane film) during 10 minutes (Monnin & al. 1998). The fiber was then inserted into the 1177 injection port of a Varian 3900 gas chromatograph equipped with a Factor Four VF-5ms (Varian) non-polar capillary column (30 m × 0.32 mm × 0.25 µm). The injection port was set at 280°C and the GC was equipped with a FID detector set at 340°C. The column temperature was held at 150°C for 5 minutes before increasing to 220°C at 20°C min⁻¹, then to 320°C at 5°C min⁻² and finally held at 320°C for 10 minutes. Helium was used as the carrier gas at 1 ml min⁻¹ and samples were injected during 5 minutes in the splitless mode.

More specifically, the identification of CHCs was done by GC-MS for one host queen, one parasitic queen and a mix of five workers. For this identification, the host queen was extracted with SPME following the above method but due to low amount of CHCs on their cuticle, the parasitic queen and the workers were extracted in a solvent. The parasitic queen was extracted in 200 µl of pentane for 5 minutes, evaporated and suspended in 20 µl of pentane. The five workers were extracted in 300 µl of pentane for 5 minutes and the extract was evaporated and suspended in 20 µl of pentane. Agilent 7890A GC equipped with a HP-5ms (Agilent J&W) non-polar column (30 m × 0.25 mm × 0.25 µm) connected to an Agilent 5975C mass selective detector was used to identify compounds. The program was the same as previously used with an injection port set at 280°C and the transfer line at 280°C. Helium was used as carrier gas at 1 ml min⁻¹ and samples were injected in splitless mode (5 min for SPME and 1 min for liquid extracts). Electron impact mass spectra were measured at 70 eV with a source temperature at 230°C. Compounds were characterized and identified by their mass spectra and retention times in the GC-MS profiles. Then, GC-FID identification of CHCs was based on the corresponding retention time of the compounds in the GC-MS samples. In addition we compared the spectra for Ectatomma tuberculatum queens with previous analyses (Zinck & al. 2009).

Data and statistical analyses: The first analyses were performed on the overall chemical profile. The peak area of each compound was determined by the Star Workstation (Varian, Inc.) software. For each individual, we quantified as proportions each compound separately and five classes of compounds, i.e., alkanes, monomethylalkanes, dimethylalkanes, alkenes and esters. For this, the peak area of each compound was divided by the total area of all peaks and the peak area of each compound class was calculated by adding the proportions of each compound belonging to the class. These relative abundances were represented by means (± S.E.) for the three types of individu-
als, i.e., host queens, host workers and parasite queens. In addition, the total amount of CHCs was calculated by adding peak areas of compounds for each type of individual and was weighted with an index of the body size, the thoracic surface. From measurements previously published (HORA & al. 2001), the thoracic surface ($S_T$ in mm$^2$) was estimated by an ellipse using the following formula:

$$S_T = \pi \times \text{pronotum width} \times \text{thorax length} / 4.$$

This index was equal to 9.28 for host queens, 3.60 for host workers and 4.76 for parasite queens, respectively.

Data from the three types of individuals were compared with an ANOVA with general scores and post-hoc permutation tests, using Monte-Carlo simulation. A correction for multiple testing within the same hypothesis was made using Bonferroni sequential method (HOLM 1979, RICE 1989). All tests were performed with the StatXact 8 (Cytel Studio) software.

Secondly, discriminant analyses (DA) were performed on selected dataset, including only compounds supposed to be involved in nestmate recognition (branched alkanes and alkenes) with a mean above 1% of the total abundance calculated from all individuals. DAs were used to determine if the parasites are well-differentiated (1) from the host species, (2) from both castes of the host (i.e., host queens and workers) and (3) from different host colonies according to their localization (i.e., parasitized colonies of site 1, non-parasitized colonies of site 1 and non-parasitized colonies of site 2). For this, we first made a DA with only *Ectatomma tuberculatum* queens and workers and we re-integrated the parasites into the analysis afterwards. This multivariate analysis is particularly suited to our data, because of the sample size (HAIR & al. 1995), the observation /variable ratio (BARTLETT & al. 2001, MITTEROECKER & BOOKSTEIN 2011) and the previous knowledge of the data classification (MARTIN & DRIJFHOUT 2009a). Prior to analyses, data were transformed by an arcsine function (SO- KAL & ROHLF 1981). For these statistical analyses, we used the Statistica 8.0 (Statsoft) software.

**Results**

**Quantitative differences in chemical samples:** Cuticular profiles greatly differed in quantity between the three types of individuals (Fig. 1). When weighted with body size using the $S_T$ index, the total abundance of the 75 identified compounds (Tab. 1) differed significantly among the individuals (ANOVA with general scores, $P < 0.001$, Fig. 2). Parasite queens had one-tenth as much CHCs as host queens (Post-hoc permutation test, $P < 0.001$) and 1.5 times less
than host workers (P = 0.044). Host queens bore much more CHCs than workers (P < 0.001).

**Qualitative differences in chemical samples:** Cuticular profiles, although very similar (Fig. 1), differed in their relative composition. Comparisons between the three types of individuals were different for each compound class, i.e., n-alkanes, monomethylalkanes, dimethyalkanes, alkenes and esters (ANOVA with general scores, P < 0.001). Parastatid parasites possessed less n-alkanes, but more branched alkanes than hosts (Post-hoc permutation tests, P < 0.001).

**Parasite categorizations:** Only 17 out of the 75 peaks (bold highlighted in Tab. 1) corresponding to the most abundant peaks were used to categorize the parasites according to the species, the caste (host queens and workers, parasite queens) and the site origin, respectively. The first discriminant analysis (DA) separated *Ectatomma parasiticum* from *E. tuberculatum* well, and also the different castes of the host species (Wilks’ λ = 0.02, F_{26,224} = 52.8, P < 0.001, Fig. 4). The second DA demonstrated that parasites are also related to their own host colony (Wilks’ λ = 0.18, F_{16,180}
Fig. 2: Total abundance of the identified peaks relative to the body size using the thoracic surface ($S_T$) index for the three types of individuals. Different letters show significant post-hoc permutation tests with sequential Bonferroni correction.

Fig. 3: Mean proportions (± S.E.) of the five classes of compounds (i.e., n-alkanes, mono-methylalkanes, dimethylalkanes, alkenes and esters respectively) for host queens in grey, host workers in white and parasites in black. All ANOVAs are statistically significant and different letters show significant post-hoc permutation tests with sequential Bonferroni correction.

Fig. 4: Discriminant analysis applied on the 17 most abundant peaks to characterize the three types of individuals (host queens in grey circles, host workers in white squares and parasites in plus signs). Factor 1 accounts for 66% and Factor 2 for 34% of the total variance, respectively. Confidence ellipses are shown as including 90% of the data.

Fig. 5: Discriminant analysis applied on the 17 most abundant peaks to characterize the localization of the parasites compared to the Ectatomma tuberculatum colonies (queens and workers of the non-parasitized site in open circles, queens and workers of the parasitized site in triangles, non-parasitized colonies in open triangles and parasitized colonies in black ones, and parasites in plus signs). Factor 1 accounts for 78% and Factor 2 for 22% of the total variance, respectively. Confidence ellipses are shown as including 90% of the data.

Discussion

Our results show that the social parasite, Ectatomma parasiticum, is chemically distinct from its host, E. tuberculatum. But the CHC profiles of both species overlap and no specific compound is found exclusively on the parasite’s cuticle. This suggests a chemical mimicry strategy (sensu von Beeren & al. 2012) where the parasite could either acquire or biosynthesize the host odours to achieve the host colony usurpation.

The chemical similarity between both species may have a genetic basis. Indeed, the inquiline ant Ectatomma parasiticum was found to be a sibling species of its host (Fénéron & al. 2013), following the strict version of the Emery's rule (Busching 1990, Bourke & Franks 1991, Bu-
Moreover, the chemical congruency may result from the mixing of individual cues among all nestmates, thus shaping a common colony odor (the Gestalt model, CROZIER & DIX 1979, HEFETZ 2007). It may explain why the *E. tuberculatum* parasitized colonies exhibited a CHC profile matching that of the parasite and well-differentiated from that of the non-parasitized colonies even in the same site. Such changes in the chemical cues of both associated species have been experimentally demonstrated (e.g., D’ETTORRE & ERARD 1998, KREUTER & al. 2012). In addition, parasites can promote odor transfer through social behaviors, such as licking (FRANKS & al. 1990). The fact that *E. parasiticum* queens, but not the host queens, initiated frequent social contacts towards the workers has been previously interpreted as an attempt to homogenize colony odor and favor social acceptance (HORA & al. 2009). Our chemical analysis, however, shows that the total amount of compounds present in the parasite’s cuticle is weak. If a behavioral mechanism to transfer odors exists between both species, it is rather imperfect or at least selective to some compounds.

The major difference between the parasite and its host concerns the reduced amount of chemicals found on the cuticle of the parasite. Chemical abundance is known to differ with body size, age and reproductive status (e.g., LIEBIG & al. 2000, CUVILLIER-HOT & al. 2001, TENTSCHERT & al. 2002, LOMMELEN & al. 2006, HORA & al. 2008). In this study, we verified that difference in chemical abundance cannot be accounted for difference in body size. *Ectatomma parasiticum* queens, although intermediate-sized (HORA & al. 2001, FEITOSA & al. 2008), have a lower level of CHCs than both host queens and host workers. Moreover, chemical samplings were performed several months after parasites had emerged from the brood collected in field colonies of *E. tuberculatum*. So, the parasites were younger than the host queens, but not callow. This precludes any confounding effect with the cuticular chemical insignificance that occurs early in adult life to facilitate the social integration of newly hatched individuals (LENOIR & al. 1999, 2001). Unfortunately, we could not control the reproductive status of the parasites, but we know that the parasites were not inseminated, and some of them were reproductively active and produced male eggs. Even if the weak chemical signature expressed by the parasites could be linked to this temporary reproductive status, it could more likely be a specific chemical strategy named chemical insignificance (but see VON BEEREN & al. 2012).

The above strategy commonly occurs during colony infiltration in inquiline ants (FRANKS & al. 1990, LAMBARDI & al. 2007), wasps (LORENTZI & al. 1999, TURILLAZZI & al. 2000, UBONI & al. 2012) and bumblebees (DRONNET & al. 2007), wasps (LORENZI & al. 1999, TURILLAZZI & al. 2000, UBONI & al. 2012) and bumblebees (DRONNET & al. 2007), and *Myrmoxenus* spp. (BRANDT & al. 2005). It has also been selected in other parasitic life-history traits. For example, in the closely related species, *Ectatomma rudivum* (ROGER, 1860), workers which rob food in neighboring conspecific nests (clietobiosis) have low extractable cuticular compounds (JERAL & al. 1997). In slavemaker ants as *Polyergus* spp. (D’ETTORRE & ERARD 1998, LENOIR & al. 2001) and *Myrmoxenus* spp. (BRANDT & al. 2005) queens lack fighting adaptations against host workers but rely on their chemical invisibility to integrate into host colonies. However, after colony infiltration, true inquilines (i.e., queen tolerant inquilines) like *E. parasiticum* have to live in the host colony over an extended period of time. As they gain to be identified as nestmates, they should shift to an alternative chemical strategy to acquire at least some of the host’s chemicals (LENOIR & al. 2001, DRONNET & al. 2005, UBONI & al. 2012). Our results would suggest that inside their native colony and prior to invading another colony *E. parasiticum* queens either do not yet produce a full CHC profile or restrain CHC biosynthesis to a low level. But due to the lack of parasite dispersal in the laboratory and the prolonged association with hosts of the natal colony, the shift in the subsequent strategy, an acquisition of the host odors, could have already occurred. Further investigations with chemical samplings of the parasite before and after host colony usurpation are really needed, but hard to be performed due to the difficulties of rearing parasites in the laboratory.

Another major difference we observed between the parasite and host was the proportion of four cuticular lipid categories. *Ectatomma parasiticum* individuals are characterized by less n-alkanes and more branched methyl- and dimethyl-alkanes than their hosts, and have alkenes in intermediate proportion between host queens and workers. Cuticular hydrocarbons prevent from desiccation but branched alkanes and alkenes also serve to convey information for recognition in different species (AKINO & al. 2004, HOWARD & BLOMQUST 2005, MARTIN & al. 2008, MARTIN & DRIJFHOUT 2009b, BLOMQUST & BÄGNERES 2010). We then expected that parasitic ants should have low levels of branched alkanes and alkenes to avoid host detection, but this is not the case in *E. parasiticum*. However this contradictory result should be nuanced with regards to the reduced abundance of CHCs in this species. Parasites have also a lower amount of esters compared to host queens. These molecules are commonly found on the insect cuticle but their function is still largely unknown in social insects, except in honeybees where they are used as a fertility signal (BREED & al. 1992).

The weak chemical signature and the differentiation in CHCs between the parasite and its host could explain why host workers well-discriminate *Ectatomma parasiticum* individuals from their conspecifics and sometimes attack them (HORA & al. 2009, FÉNERON & al. 2013). Such discrimination suggests a probable failure in the social integration of the parasites which can be due to imperfect chemical mimicry. This suggests a recent divergence of both species in congruency with other traits previously analyzed, i.e., the morphological and genetic similarity between the parasite and its host (FEITOSA & al. 2008, HORA & al. 2008, FÉNERON & al. 2013) and the potential virulence of the parasite through frequent egg cannibalism (HORA & al. 2009). Further experiments, such as the chemical dynamics during the life cycle of the parasite, might help to clarify the underlying mechanisms. Nevertheless, this study highlights the usefulness of chemical approaches in contributing to our understanding of the evolution of interspecific associations.

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