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RESEARCH ARTICLE

Two aggressive neighbours living peacefully: the nesting association between a stingless bee and the bullet ant

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Abstract

Stingless bees often associate with termites, while association with ants is uncommon due to the high costs related to the aggressiveness of ants. Few combative genera of stingless bees can associate with a larger number of hosts, including ants. Here, we describe for the first time the association between a stingless bee (*Partamona testacea*) and the aggressive predator bullet ant (*Paraponera clavata*). In the study area, the colonies of *P. testacea* we spotted were all associated with *P. clavata* or *Atta* sp. ants. COI mitochondrial gene sequences of bees associated with bullet ants and leafcutter ants did not show any divergence, thus revealing no evidence that the two nesting strategies represent a case of cryptic speciation and specialisation on specific hosts. Bees are not unarmed respect to the bullet ants; when ants attempted to penetrate in the colony entrance, they were dragged inside the nest and covered by a resin-like substance. Behavioural experiments focused on ants in arenas and focused on bees at their nest entrance proved that the ants are significantly less aggressive toward associated bees and that guard bees are less alarmed when associated ants are presented. We verified by Gas Chromatography and Mass Spectrometry that *P. testacea* maintains its species-specific cuticular signature in the association with different ant species and that ants and bees possess typical colony signatures. The differential behavioural responses expressed toward associated colony members by both species are likely based on learning these heterospecific cuticular signatures as it occurs in ant parabiotic associations.

Keywords *Paraponera clavata* · *Partamona testacea* · COI · GC/MS · Recognition · Nesting association

Introduction

Nests of social insects and their surroundings can represent shelters for other species establishing peaceful relationships with them or eluding their nest defences. When the nesting association is formed by two species of social insects, two complex and potentially harmful societies are involved in a relationship which can range from a mutualistic to a parasitic symbiosis (Wilson 1971). Stingless bees (Meliponini, Apidae) are eusocial tropical insects establishing perennial colonies composed by up to several thousand workers (Michener 2000). Stingless bees usually nest in cavities but, since their digging abilities are generally scarce, they usually exploit pre-existing hollows including abandoned or inhabited nests of other social insects (Roubik 2006; Siqueira et al. 2012; Carrijo et al. 2012). Several species nest in active colonies of different social insects: these relationships can be obligate or facultative, depending on the stingless bee species (Wille and Michener 1973; Roubik 1983), and they are often species specific (Michener 2000). Other than an

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easy nesting opportunity, the association with other eusocial insects provides stingless bees with the protection benefits of nesting near aggressive colonies (Roubik 1983, 2006). However, establishing and sharing the nesting area with a colony belonging to a potentially aggressive species require adaptations to mitigate or elude the host nest defence. These adaptations depend on the nature of the bee–host interaction (Sakagami et al. 1989; Roubik 2006; Carrijo et al. 2012). Nesting behaviour of stingless bees is extremely various, and little is known about the high diversity of this tribe. Although the relationship can be parasitic or mutualistic, in most of the cases, stingless bees are considered as nesting parasites since they do not show aggressive defensive behaviour and the host does not obtain any evident advantage from the association (Sakagami et al. 1989; Roubik 2006). On the other hand, a few genera such as *Partamona*, *Plebeia*, *Paratrigona* and *Trigona* participate in the defence of the nests and associate with a wider variety of hosts (Roubik 2006). Wille and Michener (1973) reported that, among the studied stingless bee species, the 12% can nest with termites, with the 8% showing obligate associations with termites. Species nesting with ants are fewer, with only the 2% involved in obligate relationships (Sakagami et al. 1989). Host preference does not appear consistent with stingless bee phylogeny, and many sister species (e.g. *Trigona cilipes*, *T. mazucatoi* and members of the genus *Partamona*) show distinct preference for termites or ants (Roubik 2006).

The lower frequency of myrmecophily relative to termitophily can be explained by the generalised higher aggressiveness of ants compared to termites (Sakagami et al. 1989). Stingless bee colonies involved in regular associations with ants are separated by the outer wall of the bee nest, normally represented by a layer of bitumen (Sakagami et al. 1989). These associations seem to rely on an armed peace and the coexistence appears to be maintained by a high tolerance of the ants toward the bees (Sakagami et al. 1989).

This scenario resembles the phenomenon of parabiotic associations, where colonies of different ant species share the same nest. In these cases, ants recognise individuals of the associated colony and tolerate them (Lenoir et al. 2001; Menzel and Schmitt 2012; Emery and Tsutsui 2013). The recognition system of social insects is usually based on the comparison of the cuticular signatures shared among nestmates with the signature occurring on encountered individuals (Breed et al. 1985; Lenoir et al. 2001; van Zweden and d'Ettorre 2010). Since in parabiotic associations, the two species maintain their species-specific chemical profile, a mechanism different from self- vs non-self-recognition is required (Errard et al. 2003; Menzel and Schmitt 2012; Emery and Tsutsui 2013). For this reason, parabiotic associations became models to understand the mechanisms of recognition in insects (Orivel et al. 1997; Lenoir et al. 2001; Menzel et al. 2008; Emery and Tsutsui 2013). Although analyses of cuticular profiles and experiments of recognition between stingless bees and their associated ant colonies have never been carried out, at least to our knowledge, it is possible that similar phenomena can occur also between these organisms.

Here, we describe for the first time, the nesting association between *Partamona testacea* and the bullet ant, *Paraponera clavata* (Fig. 1). *Partamona testacea* is a group of sister/cryptic stingless bee species widespread in South American rainforests (de Camargo 1980). Species of this group were reported to establish facultative associations with termites of the genus *Syntermes* and with *Atta* leafcutter ants (Camargo and Pedro 2003). *Paraponera clavata* is a well-known generalist predator of arthropods, characterised by a high predation performance (Young and Hermann 1980) besides for its pugnacious nest defence and its extremely harmful sting. We aimed to describe and unravel the mechanisms underlying the relationship between these two species by answering four main questions: (Q1) is the association obligate, at least in the study area? (Q2) Is the host choice between different ant species the result of a cryptic divergence in *P. testacea* or does it represent alternative nesting strategies of the same species? (Q3) Do individuals belonging to associated colonies show a reduced aggressiveness respect to individuals belonging to non-associated colonies? (Q4) Are cuticular signatures a potential cue for recognition between individuals of associated colonies?

We answered these questions by integrating (1) field data about occurrence of associated and solitary colonies of ants and bee in the study area (Q1); (2) comparison of

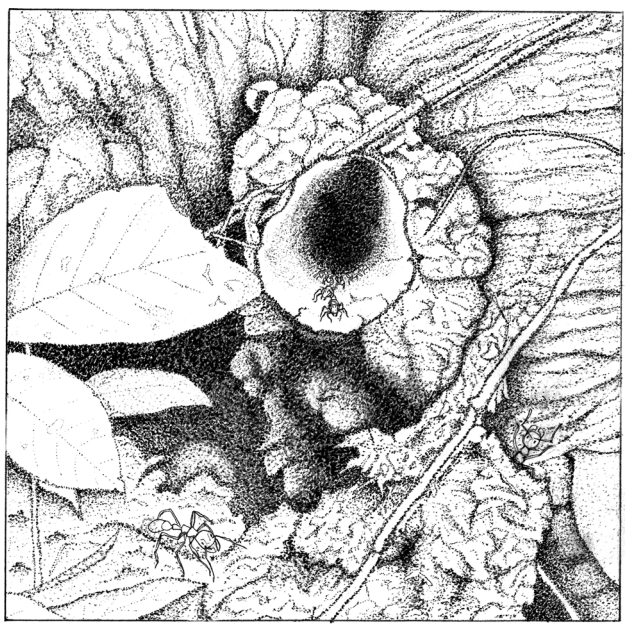

Fig. 1 A representation of the association between *P. testacea* and *P. clavata* (drawing by GM)

COI mitochondrial gene sequences (DNA barcoding, Hebert et al. 2003) of *P. testacea* colonies nesting in *Atta* sp. and *P. clavata* nests (Q2); (3) behavioural experiments, assessing reciprocal aggressiveness between associated and non-associated *P. testacea* and *P. clavata* colonies (Q3); and (4) chemical analyses of cuticular hydrocarbons to verify the existence of typical colony signatures in ants and bees and to verify if *P. testacea* mimics the cuticular hydrocarbon blend of the associated ant species (*P. clavata* and *Atta* sp. in our case) (Q4).

Materials and methods

Study area and experimental colonies

This study has been carried out in the lands of the Urku Estudios Amazónicos centre, located in Tarapoto (San Martin region, Perù). The study area embraced 13 ha of tropical rainforest in the buffer zone of the Área de conservación regional Cordillera Escalera. The colonies of *P. testacea* and *P. clavata* were spotted during a 2-week survey by three operators (AB, MM and GM). Each encountered nest has been geo-referenced and the colonies of *P. testacea* and *P. clavata* were categorised depending on nesting association as: (1) colonies currently involved in the association, when the colonies of both species occurred at the basis of the same tree (associated), (2) colonies not involved in the association (solitary) when only one of the species was present and (3) colonies involved in the association in the past, when one of the colonies was no longer in the association (formerly associated). In fact, the peculiar structures of *P. testacea* (mostly the external structure of the nest entrance, Fig. 1) and *P. clavata* nests were still detectable even if the nests were recently abandoned. In the surrounding of the Urku Estudios Amazónicos centre, we also spotted some colonies of *P. testacea* associated with *Atta* sp. leafcutter ants (Picture_S1).

Mitochondrial DNA characterization

We obtained tissue samples (legs) for *P. testacea* associated with *P. clavata* or *Atta* sp. and preserved them in ethanol at 4 °C. The genomic DNA was isolated from single legs using the protocol of "Smarter Nucleic Acid Preparation" (Stratec). We amplified the DNA barcoding region of cytochrome oxidase subunit 1 (Folmer et al. 1994) using the LC01490 (5′-GGT CAA CAA ATC ATA AAG ATA TTG G-3′)/HC02198 (5′-TAA ACT TCA GGG TGA CCA AAA AAT CA-3′) primer pair. PCR amplifications were carried out using the GoTaq DNA polymerase kit (Promega) following the manufacturer's protocol. Thermal cycle program included: initial denaturation for 5 min at 95 °C; 33 cycles of 30 s at 95 °C, 45 s at 55 °C, 30 s. at 72 °C; final extension for 7 min at 72 °C. The PCR products were then purified using ExoSAP-IT PCR Product Cleanup Reagent (Thermofisher) and sequenced with the Sanger method at Macrogen Inc. Europe Lab. Sequence chromatograms were checked with SeqTrace 0.9.0 (Stucky 2012).

Behavioural experiments

We carried out behavioural experiments to verify whether individuals belonging to associated colonies of *P. testacea* and *P. clavata* show different relationships compared to individuals belonging to non-associated (alien) colonies. In this aim, we performed two distinct experiments focusing on the reaction of ants towards stingless bees and vice versa.

To compare the reaction of bee colonies toward ants belonging to associated and alien colonies, we carried out experiments in the field. For each trial, we collected a single ant from its colony entrance from 10 to 30 min before the experiment and kept it in a glass drug container (4 × 2.5 cm). The ant was softly sedated for 3 min in a field refrigerator at 4 °C. Then, we placed the ant on the nest entrance using forceps (movie S1) and we video recorded the reaction of bees and the interactions between the individuals until the ant left the nest entrance. The focus of the video recording was on the guard bees. The general reaction of the guard bees was consistent and represented by a retreating, then guard bees hid in the nest entrance while the ant occurred on the structure. Once the ant left the entrance, the guard bees repositioned in the nest entrance to the initial position (movie S2). Coherently with this generalised response, we collected the following parameters: (1) number of guard bees visible at the nest entrance before the beginning of the trial, (2) number of guard bees visible in the presence of the ant, (3) number of guard bees repositioned when the ant left the nest entrance, (4) repositioning time, corresponding to the time interval spent in repositioning by the bee guards after the ant left the entrance. We performed all the possible combinations between bee and *P. clavata* ant colonies involved in an association, following a timetable organised to minimise repetitive stress on the bee colonies (combinations can be inspected in the supplementary dataset, Appendix S2).

To test the reaction of ants toward bees belonging to associated or non-associated colonies, we designed a different experiment. Indeed, the peculiar shape of the entrance of the bullet ant colony appears as a 1–2 cm gap running around the basis of the tree trunk where this species usually nests. It was thus impossible to identify a localised nest entrance, and generally guard ants are not visible from outside. For this reason, experiments focusing on the ant reaction were performed in artificial arenas represented by plastic Petri dishes (9 cm diameter). Bees and ants were collected directly from their colony entrances and individually kept in glass

containers. In each trial, after a soft sedation (see above), one ant and one stingless bee were placed in the arena. We video recorded their interactions for 3 min after the first active contact. In this experiment, we focused on ant reactions toward bees and we scored the following events in each trial: (1) physical contacts between individuals, (2) mandible openings following contacts, (3) bites and stings (normally coupled) (4) crouching behaviour and (5) suction of abdominal secretions. Opening of mandibles is a well-known aggressive display in ants, widely used as a measure of the aggression level (Guerrieri and d'Ettorre 2008). Ant workers in some cases faced the bees in the arena by crouching, a behaviour described by Dejean (2011) in *Platythyrea conradti*, an arboreal ponerine ant, and expressed when facing termites defending their nests and toward alien ant species. In crouching posture, ants crouch with the antennae folded backward, likely to avoid that opponents grab the appendixes and usually move forward very slowly. But, differently to the behaviour described by Dejean (2011) for *P. conradti* workers, crouching *P. clavata* workers did not widely open with their mandibles in our experiments (movie S4, S5). We performed all available combinations between bee and ant colonies involved in an association.

Bee reaction toward intrusion into the nest

While performing the presentation on the bee nests, we occasionally observed that some anesthetised ants which were not able to run away and moved too deep in the nest entrance were attacked by bees. We thus designed another experiment to describe this behaviour. We performed one trial per bee colony with an ant collected from the same nesting association. We deeply sedated the ant by refrigerating it and we tied, in the junction between the head and the thorax, the extremity of a 50 cm long nylon cord (0.5 mm diameter). After an eventual further sedation, we introduced the ant in the deepest visible portion of the bee entrance using forceps and we video recorded bee reactions. We then rescued the ant using the nylon cord.

Cuticular hydrocarbon characterization

We extracted cuticular hydrocarbons (CHCs) from *P. testacea* individuals belonging to the six colonies involved in the experiment (five associated with *P. clavata* and one formerly associated). Furthermore, we collected bees from two colonies associated with colonies of *Atta* sp. leafcutter ants, found nearby the study area. Four individuals per *P. clavata* and *P. testacea* colony and four *Atta* sp. individuals belonging to one of the colonies associated with *P. testacea* were sacrificed by freezing and were then washed in pentane (500 µl for ants, 250 µl for bees). We dried the extracts in the field station and re-eluted them in the Florence University lab with heptane (100 µl for ants, 40 µl for bees). In the Florence lab, we injected 1 µl of the solution in an Agilent technology instrument 7820 gas chromatograph (GC) coupled to a 5977B mass selective detector (using 70 eV electronic ionisation source). An Agilent silica capillary column (30 mm × 0.25 mm × 0.25 µm) was installed in the GC. The injector port and transfer line temperatures were set at 280 °C and the carrier gas was helium (at 13 psi head pressure). The temperature protocol was from 70 to 150 °C at a rate of 15 °C/min held for 3 min and from 150 to 310 °C at 5 °C/min held for 16.7 min. Injections were performed in split mode. Data acquisition and recognition of substances based on their mass spectra and equivalent chain length have been done using the Agilent MassHunter Qualitative Analysis Version B07.00 ©Agilent technologies.

Statistical analyses

COI sequences were aligned as amino acids using MAFFT v. 7 with the auto-parameter setting (Katoh and Standley 2013) and subsequently retro-translated to nucleotides. The number of mutations and their segregation between *P. testacea* associated with *P. clavata* and *Atta* sp. colonies were inspected through MEGA v.7.

An author (AB) unaware of the colony membership of bees and ants involved in each video recording, scored the selected behaviours using BORIS, the open source software for video/audio coding and live observations created by the University of Turin, Italy. Since the counts and time measurements of the behavioural responses showed a Poisson distribution, we used the "glmmadmb" function in the "glmmADMB" package (http://glmmadmb.r-forge.r-project.org/) to fit Generalised Linear Mixed Models (GLMMs). We included the different count measures (repositioning guards and repositioning time of bees; physical contacts, mandible openings, crouching and suction of secretions of ants) as response variables, the relationship of association between colonies as a factorial binomial (associated versus non-associated) predictor and colony membership of bees and ants as two random factors (see R scripts). For the petri dish experiments (reaction of ants), the physical contacts between individuals were also entered as an additional predictor in the GLMMs to account for a possible increase in responses after an increased number of encounters. In these cases, we computed estimated marginal means to plot variables in graphs using the "emmeans" function of the "emmeans" R package. Since the number of cases (associated versus non-associated) were not balanced among groups, we used type III sum of squares as returned by the "Anova" function of the "car" R package.

To reduce the bias due to the use of compositional data in multivariate analyses of cuticular substances, we

transformed the area of each chromatographic peak by the following formula (Aitchison 1982):

$$Z_{ij} = \ln(Y_{ij}/g(Y_j)),$$

where Y_{ij} is the area of peak i for individual j, $g(Y_j)$ is the geometric mean of the areas of all peaks for individual j, and Z_{ij} is the transformed area of peak i for individual j. To visualise the general pattern of similarity among individuals and colonies, we first performed Partial Least Square Discriminant Analyses (PLSDA) using species or colony membership as a priori grouping variable. Among conspecifics, we also verified if nestmates were chemically more similar to each other than non-nestmates. In this aim, we scaled the frequencies of each compound (mean = 0, standard deviation = 1) to avoid an unbalanced contribution in Euclidean dissimilarity and compared the obtained dissimilarity matrix among individuals with the corresponding membership matrix indicating whether each pair belong to the same colony. Due to the non-independence of data, we used a Mann–Whitney *U* test and assessed the *P* value by comparing the observed U value with the values obtained after re-sampling 10,000 times the original membership matrix. A similar analysis has been done to verify whether bees and ants belonging to the same association show a higher inter-specific similarity. For this analysis, we selected the compounds shared among *P. testacea* and *P. clavata* and calculated a dissimilarity matrix as described above. Then, we extracted the rectangular section of the dissimilarity matrix containing the chemical distances among ants and bees. We verified if the pairs of associated ants and bees show a lower chemical distance compared to non-associated ones using a GLMM (Gaussian family, glmmPQL function of the MASS R package) using bee and ant colonies as random factors.

Results

Frequency of colony association

Within the Urku reserve, we spotted 19 colonies of *P. clavata* and 6 colonies of *P. testacea*. All the colonies of *P. testacea* were associated with active bullet ant colonies except one which was in association with *P. clavata* until 2016, but then the ants disappeared from the colony (MM personal observation). In the surrounding area of the reserve, we identified six colonies of *P. testacea* associated with *Atta* sp. leafcutter ants (Fig. S1 in Appendix S1).

COI characterization

The 28 COI sequences we obtained were 678 bp long, and correctly translate for 225 amino acids. Overall, no nucleotide variation was scored and, therefore, we recovered a single haplotype (Genbank acc. no. MN542421) out of 28 individuals from 6 colonies in association with *P. clavata* and 3 in association with *Atta* sp.

Behavioural experiments

Bee guard reaction

At the end of the experiment, we carried out 47 trials (18 among associated bee and ant colonies and 29 between non-associated bee and ant colonies). When the ant was presented on the nest entrance, the guard bees invariably retreated; after the ant left the structure of colony entrance, they simultaneously advanced in a line and repositioned (movie S1, S2). The ratio between guard bees immediately repositioned after the ant left the nest entrance and the number of guard bees occurring before the arrival of the ant showed a normal distribution with a mean value of 0.576 ± 0.238 s.d. This value revealed that almost half of guard bees did not promptly reposition after the occurrence of a bullet ant on the nest entrance. This ratio did not differ between ant belonging to the colony associated to the focus bee colony and to a non-associated ant colony (Table 1, Fig. 2a). Repositioning time was rather variable among trials (mean 4.4 s ± 4.1 s.d.) and in a Poisson GLMM, bees revealed to have repositioned faster when an ant belonging to a non-associated colony was presented (Table 1, Fig. 2b).

Table 1 Results for the GLMMs comparing six examined behaviours of bees and ants when individuals of the counterpart belonging to the associated and non-associated colonies were presented

Focus	Behaviour	Variable	Chi square	*P*
Bees	Repositioning guards	Colony membership	0.055	0.814
	Repositioning time	Colony membership	7.790	0.005
Ants	Physical contacts	Colony membership	0.094	0.759
	Mandible openings	Physical contacts	89.150	< 0.001
		Colony membership	11.580	< 0.001
	Crouching	Physical contacts	23.702	< 0.001
		Colony membership	4.562	0.033
	Suction of secretions	Physical contacts	8.269	0.004
		Colony membership	4.784	0.029

For ants also number of physical contacts in the arena have been included in the models

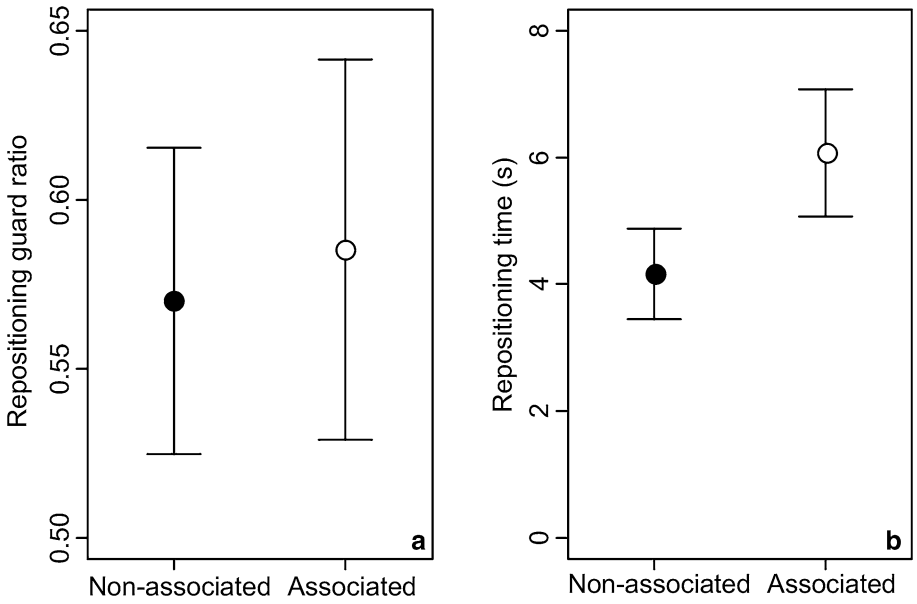

Fig. 2 Mean and standard deviation for the ratio of guard bees repositioned after an associated and a non-associated bullet ant left the nest entrance (**a**) and of the repositioning time showed by guard bees (**b**)

Bee reaction toward intrusion into the nest

All ants introduced in the deepest visible portion of the bee entrance were grabbed by bees hidden behind the nest entrance and dragged inside the vestibular chamber of the bee nest (Camargo and Pedro 2003). Once rescued, after about 30 s following their disappearance inside the nest, ants were covered by a highly gluey substance, presumably composed by resins, (picture_S2) carried by bees on hind legs, using the corbiculae, manipulated and applied by mouth parts (movie_S3). This substance impeded the ant from escaping by slowing them and sticking them on any contacted surface.

Ant reaction toward bees

At the end of the experiment, we carried out 39 trials (21 among associated bees and ants and 18 between non-associated bees and ants). A Poisson family generalised linear mixed model using ant colony as a random factor showed that associated and non-associated ants and bees did not differ in the number of physical contacts in the arenas (Table 1, Fig. 3a); number of mandible openings highly depended on number of physical contacts but was also higher when non-associated bees and ants were examined (Table 1, Fig. 3b). A similar effect was found in crouching behaviour (Table 1, Fig. 3c), but the frequency was lower when associated bees

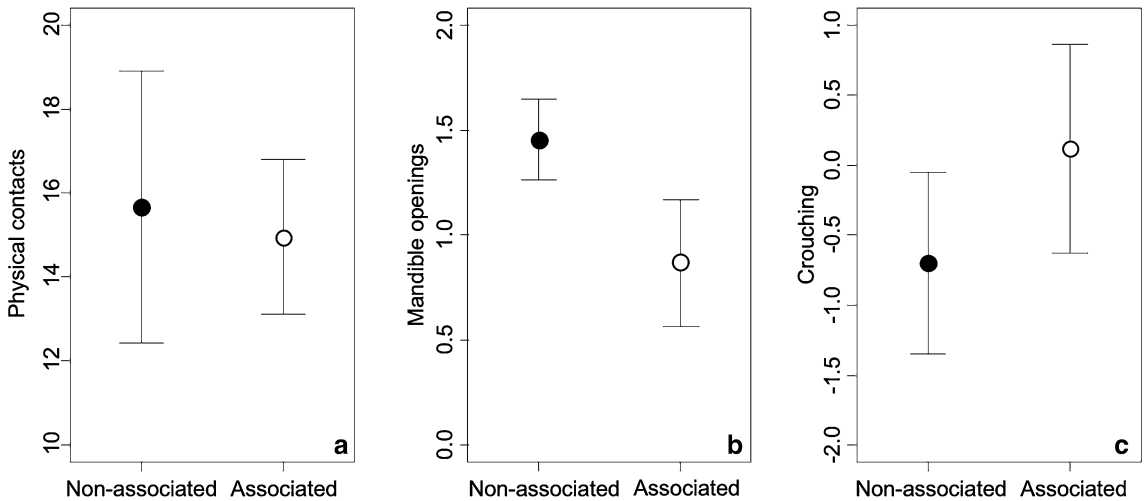

Fig. 3 Comparisons of frequencies of physical contacts (**a**), mandible openings (**b**) and crouching behaviour (**c**) performed by *P. clavata* toward associated and non-associated individuals of *P. testacea.* The values of mandible openings and crouching represent estimated marginal means from GLMMs also including physical contacts as a predictor

were placed in the arenas. Suction of abdominal secretion by ants has been performed in six trials and in five cases, it occurred when a non-associated bee occurred in the arena. Accordingly, in a binomial GLMM, this behaviour showed a significant relationship with number of physical contacts and were significantly more frequent when a non-associated bee occurred in the arena (Table 1). Bites and stinging by the ants were highly uncommon, and they were recorded in three cases only.

Cuticular hydrocarbon characterization

We detected a total of 135 peaks in the 3 species analysed (*P. testacea*, *P. clavata*, *Atta* sp.) corresponding to 1 or more chemical compounds (Table S1). As expected in an inter-specific comparison, many peaks revealed qualitative differences (Fig. 4a, Table S1). A partial least square discriminant analysis using species membership as response variable (also separating *P. testacea* living in association with *P. clavata* and *Atta* sp.) confirmed that the three species have different profile and also that *P. testacea* living with bullet ants and leafcutter ants show highly similar profiles (Fig. 4a,b).

As it occurs in most social species also, *P. testacea* and *P. clavata* showed evidence for the existence of colony-specific signatures as showed by PLSDA (Fig. S1 in Appendix S1) and the chemical similarity among nestmates was significantly higher compared to aliens (bees, Mann–Whitney test, $W = 9382$, permutation $P < 0.001$, Fig. 4e; ants Mann–Whitney test, $W = 10{,}354$, permutation $P < 0.001$, Fig. 4e). A comparison of chemical distances between pairs of ants and bees belonging to associated and non-associated colonies

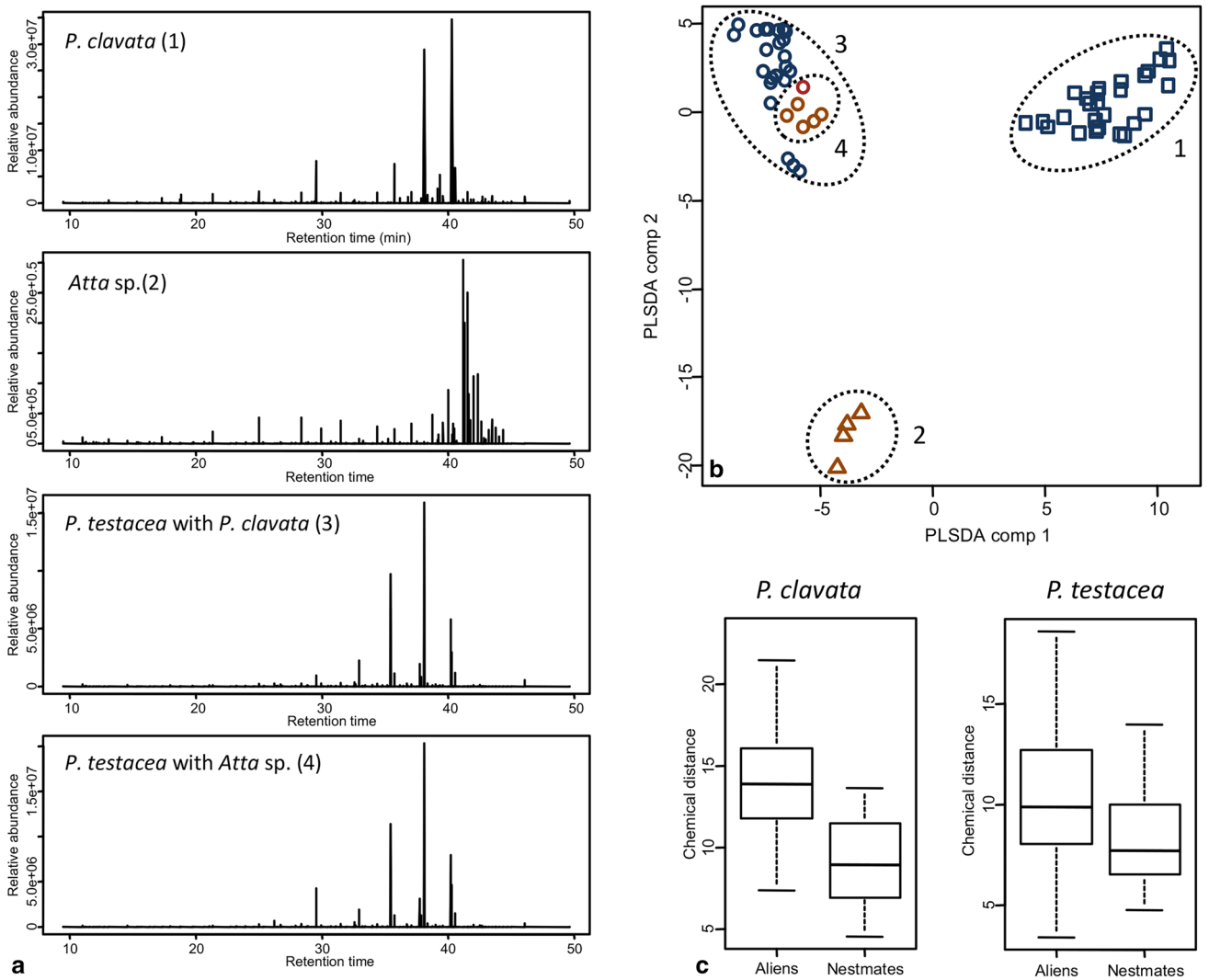

Fig. 4 The average chemical profiles of *P. clavata*, Atta sp., and of *P. testacea* associated to *P. clavata* and *Atta* sp. (**a**). A scatterplot obtained by PLSDA of these four groups of individuals (**b**) (reference numbers as in **a**). Comparisons of chemical distances between nestmate and alien individuals in bees and ants (**c**)

revealed that associated pairs were not characterised by a lower chemical distance (GLMM, $df = 1$, $X^2 = 2.098$; $P = 0.147$).

Discussion

In the study area in the Peruvian Amazon, we described for the first time the association between a stingless bee species, *P. testacea*, and the bullet ant *P. clavata*. All the bee colonies we spotted were found in association with *P. clavata* or *Atta* sp. ants (Q1). Differences in bee host choice were neither mirrored by differences in mitochondrial COI sequences (Q2) nor in cuticular hydrocarbon composition (Q4). During behavioural experiments, both *P. testacea* and *P. clavata* showed a low aggressiveness toward each other, despite in specific circumstances, they were both able to effectively fight. Furthermore, aggressiveness and alertness among individuals belonging to pairs of associated colonies were reduced (Q3). Like in most social insect species, the relative composition of cuticular hydrocarbons of both *P. clavata* and *P. testacea* showed differences among colonies. Such colony-specific profiles likely represent the cue allowing the heterospecific recognition of members belonging to the associate colony (Q4).

All observed *P. testacea* colonies were associated with an ant colony (*P. clavata* or *Atta* sp.), presumably depending on the local availability of the different hosts, and they were found inside cavities produced by active ant colonies. The disappearance of the ant colony from one association did not determine the disappearance of the bee colony, supporting the hypothesis that the association is probably necessary for the foundation but not for the survival of the bees. In our study area, the host choice is not mirrored by differences in COI sequences. This mitochondrial gene is highly variable, not only among species, but also among conspecific lineages, making it the most used marker for the identification of animal species and to detect incipient phenomena of genetic divergence (Hebert et al. 2003; Ratnasingham and Hebert 2013; Dincă et al. 2015). Although the inspection of other markers and genome-wide analyses could show a different pattern, the occurrence of a single haplotype among the 28 individuals over the 9 colonies does not allow us to conclude that nesting habits with different ant species of *P. testacea* is related to genetic divergence among cryptic species, lineages, or even matrilines and supports literature data indicating that this bee can nest with different hosts (Camargo and Pedro 2003).

Partamona testacea belongs to one of the few genera for which the association is not considered as a parasitic relationship (Roubik 2006). In our behavioural experiments, although both *P. clavata* and *P. testacea* appeared to be able to fight against their counterpart, a generalised tolerance between them also emerged. In the arena experiments, physical attacks by ants were rare and when ants were presented to bee colonies, guard bees invariably retreated and hid when facing the ants. Conversely, when we purposely forced the ants to penetrate in the deepest visible portion of the bee nest entrance, guard bees reacted by grabbing and drugging ants inside the nest and applying on them a sticky substance (picture_S2). Nests of species belonging to the genera *Partamona* and *Plebeia* possess a peculiar chamber between nest entrance and the proper nest (vestibule or false nest, Roubik 2006) hosting a reticular structure made of soil and resins (Camargo and Pedro 2003, AB, GM and MM personal observation). The sticky substance applied on the intruder likely traps it inside the reticular structure. Stingless bees also normally apply on the nest entrance resin and other ant repellent materials (Schwarz 1948), and this invariably occurred in *P. testacea* after the intrusion experiments (movie S3).

In the experiments where the reaction toward associated and non-associated individuals was compared, both ants and bees showed a lower frequency of agonistic behaviour and higher levels of avoidance. Ants showed a lower frequency of mandibular openings (a well-known agonistic behaviour, Guerrieri and d'Ettorre 2008). Crouching behaviour was higher when an associated bee occurred in the arena; in the literature, a similar behaviour has been described as an aggressive behaviour expressed by *P. conradti* in heterospecific relationships (Dejean, 2011). However, while in *P. conradti*, the behaviour was expressed with open mandibles and was often followed by attacks, in our experiments, *P. clavata* kept their mandibles almost closed while crouching and this behaviour was never followed by attacks (see movie S6 for an example). In this optic, crouching appeared as a non-aggressive behaviour whose function still needs to be investigated. The experiments on bees conducted in the field on their nest entrance, showed that guard bees exhibit a shorter repositioning time when a non-associated ant left the nest entrance which can be interpreted as a higher level of alertness toward individuals belonging to an unknown colony.

In social insects, the differential behaviour showed toward nestmates and individuals of associated colonies is generally based on the composition of cuticular hydrocarbons (Lenoir et al. 2001; Dani et al. 2005; van Zweden and d'Ettorre 2010; Emery and Tsutsui 2013). Typically, in social insect colonies, the common nest odour (gestalt odour) is maintained through social interactions homogenising chemical profiles among individuals (Breed et al. 1985; Lenoir et al. 2001; van Zweden and d'Ettorre 2010). Nothing was known about the composition and the dynamics of chemical phenotypes in stingless bees associated with other social insect species. Ant species involved in heterospecific nesting association (parabiosis) do not show a convergence of their

species-specific cuticular profiles, as generally occurs in parasitic symbioses, in which parasitic species mimic the cuticular hydrocarbon signatures of their specific hosts (Orivel et al. 1997; Lenoir et al. 2001; Errard et al. 2003; Emery and Tsutsui 2013). Many inquiline and parasitic species show chemically insignificant profiles composed by chemicals which are not involved in recognition processes (e.g. linear alkanes as opposed to alkenes and methyl-alkanes) (Lenoir et al. 2001). This is not the case of *P. testacea*, because the hydrocarbon blend was composed by several chemical compounds, most of which are recognised as having a high significance as semiochemicals like the alkenes (Dani et al. 2005). In this perspective, the reduced aggressiveness and alertness showed in *P. clavata–P. testacea* associations is likely based on a mechanism similar to that existing in ant parabiotic associations, where a heterospecific colonial profile is learned by members of each species allowing a peaceful coexistence in the same nest (Emery and Tsutsui 2013). These rare and particular associations were used as a model to demonstrate that the recognition process in social insects can be based on learned signatures and not necessarily on a self-referent comparison (Lenoir et al. 2001; Menzel and Schmitt 2012; Emery and Tsutsui 2013). Ant species living in parabiotic association show a chemical profile characterised by a high frequency of alkenes with a longer carbon chains compared to congeneric species (Emery and Tsutsui 2013). The repeated evolution of these unusually long-chain compounds suggests that they are a key trait facilitating heterospecific tolerance (Menzel and Schmitt 2012; Emery and Tsutsui 2013). The comparison among the bee chromatograms obtained in this study with those available in the literature reveals that *P. testacea* shows a cuticular profile dominated by longer chain alkenes (C29:1, C31:1, C33:2) with respect to the stingless bee species not involved in association, C25 and C27 and C27:1 in *Melipona scutellaris* (Kerr et al. 2004), C25 and C27 in *Austroplebeia australis* (Leonhardt et al. 2011) and C27 and C29 in *Frieseomelitta varia* (Nunes et al. 2009).

In conclusion, we showed that the association of *P. testacea* and *P. clavata* is far to be a mere case of facultative nesting in close proximity. In fact, ants and bees belonging to the same association showed to recognise each other's similarly to the ant colonies forming complex parabiotic associations. The host generalism showed by *P. testacea* indicates that this stingless bee species evolved plastic strategies to engage in relationships with different ants and termite species. The study of this phenomenon can enlarge our understanding about social insect recognition systems and about mutualistic vs parasitic nesting strategies.

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