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Chemical basis for inter-colonial aggression in the stingless bee *Scaptotrigona bipunctata* (Hymenoptera: Apidae)

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Abstract

Inter-colonial aggression was tested using three colonies of *Scaptotrigona bipunctata* in a natural setting when their nests were moved and by artificial contact between individuals. Examination of the cuticular lipids of individuals from two colonies kept under identical conditions showed clear differences in their cuticular hydrocarbon profiles. The cuticular lipids were a mixture of hydrocarbons (saturated and unsaturated alkanes and alkenes) within the range of C_{23}–C_{29}. The use of multivariate analysis (PCA and discriminant analysis) showed that seven of the identified surface compounds are enough to separate workers from colonies A and B from each other.

Keywords: Stingless bees; Guard bees; Colony aggression; Cuticular hydrocarbons; Insect wings; *Scaptotrigona bipunctata*

1. Introduction

All stingless bees are pantropical social bees, building nests of varied and complex structures, shapes and material (Nogueira-Neto, 1997), with colonies of varied number of individuals. *Scaptotrigona bipunctata* Lepeletier, 1836 from the Brazilian States of Minas Gerais, São Paulo and Mato Grosso do Sul, like related species *S. xanthothrica*, Moure, 1950; *S. postica*, Latrille, 1807; *S. polysticta*, Moure, 1940; *S. tubiba*, Smith, 1863 and *S. depilis*, Moure, 1942, build very large nests with enormous colonies inside tree trunks or logs. The nests have large entrances that communicate with the interior through long tubes. The worker bees collect large amounts of pollen, nectar and resins, which are preserved inside their nest; the food is stored in pots and the resin placed in heaps. At the entrance, a group of guard bees permit the passage only of their own nestmates. The workers protect the resources of the nest, the brood and young bees, making mass attacks on any intruders, nipping the skin (humans), biting hairs (other mammals) or body parts of enemies (insects) with their strong mandibles (Kerr et al., 1967).

Scaptotrigona, like all social bees, have complex social interactions inside their nests as well as with the natural world around them and may be able to employ a large variety of chemicals in their way of life. The ability to recognize nestmates and distinguishing them from individuals of other colonies is a key property of social insects (Wilson, 1971; Hölldobler and Wilson, 1990). After brief antennation, social wasps, bees, and ants typically show an immediate discriminative response towards nestmates or others that are not members of the colony. This suggests that non-volatile surface chemicals on the cuticle play an important role in nestmate recognition. The profiles of cuticular substances, in particular hydrocarbons, are often species- and colony-specific. Whether a bee is regarded as a

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nestmate or a "foreign intruder" is therefore correlated with similarities in their hydrocarbon profiles (Obin, 1986; Bonavita-Cougourdan et al., 1987; Morel et al., 1988; Nowbahari et al., 1990; Smith and Breed, 1995; Singer, 1998).

Among social insects, there are nuances in the aggressive behaviour against nestmates and intruders ranging from acceptance to biting and even killing, as evidenced in ants (Gordon et al., 1993; Gordon and Mehdiabadi, 1999), and wasps (Pfennig et al., 1983; Pfennig, 1990; Starks et al., 1998). For *Apis* species, it is known that the "recognition" capacity of bees is not only based on cuticular hydrocarbon profiles, but also on genetic or environmental factors or a combination of all of them (Breed et al., 1985; Breed, 1998; Downs and Ratnieks, 1999). Similar behaviour patterns have been little investigated in stingless bees.

The capacity for recognition is the first step in aggressive and defensive behaviour. The guard workers of meliponine bees, such as *S. bipunctata*, develop defensive behaviour immediately after an "enemy" or "intruder" is detected. They are also able to communicate to their nestmates whether an intruder is present. Hundreds of workers then leave the colony for its protection and show aggressive behaviour against all outside animals.

It is becoming increasingly clear that the cuticular lipids function as recognition substances between individuals in social insects, but the role of the hydrocarbons in kin recognition in meliponine species has yet to be investigated.

We have examined here the aggression between worker guard bees of three colonies of *S. bipunctata* and analysed the surface lipids on two of them to see if differences in individual cuticular patterns could account for the recognition of "foreign" workers.

2. Methods and material

2.1. The colonies

Complete colonies of *S. bipunctata* were collected with their nests in the Atlantic Rain Forest (*Mata Atlantica*) at Cunha in the State of São Paulo (Southeastern Brazil). The colonies were carefully brought to the Bee Laboratory at the University of São Paulo, where they were transferred into standard wooden boxes. Two of the colonies (A and B) were settled in the flower garden of the Bee Laboratory where they stayed for some months before the experiments were performed. A third colony (C) was maintained elsewhere in the grounds of the Bee Laboratory.

2.2. Sample preparation for the chemical analysis

Individual guard workers of *S. bipunctata* from colonies A and B were killed by cooling in a refrigerator. Individual wings were taken off the bees under distilled water, dried with tissue paper and placed in thin-walled soft glass tubes (1.8 mm × 20 mm) (Bagnères and Morgan, 1990; Morgan, 1990)

2.3. Gas chromatography

The cuticular substances from the wings of individual bees were analysed by gas chromatography–mass spectrometry (GC–MS) using the solid-sample injection technique (Morgan, 1990; Bagnères and Morgan, 1990). Gas chromatographic (GC) analyses were carried out on a HP5890 GC with split–splitless injector and flame ionization detector, using a 15 m × 0.25 mm fused silica capillary column coated with RX-BP5 stationary phase, with He as carrier gas (1 ml min^{-1}). The temperature program started at 60 °C for 5 min, followed by an increase to 280 °C at 7 °C min^{-1} and a final constant temperature of 280 °C for 18 min. Mass spectrometry was performed using a HP5970 (electron impact, 70 eV). All components were identified using retention indices and mass spectra of reference compounds.

2.4. Double bond determination

An extract from the surface of three worker bees was prepared in hexane (0.5 ml) and from this was taken 40 μl, mixed with dimethyl disulphide (20 μl) and iodine (10 μl of a 5% solution in ether) and heated to 60 °C for 16 h under nitrogen in a small airtight vial. Products were analysed by GC–MS on the same column as in Section 2.3.

Samples of pure (*E*)-9-tricosene, (*E*)-9-pentacosene, (*Z*)-9-heptacosene and (*E*)-9-heptacosene and an extract of cuticle hydrocarbons were injected into the gas chromatograph under identical conditions to determine double bond geometry by comparing their retention times.

2.5. Behavioural experiments

For recognition and aggression behaviour with guard worker bees of *S. bipunctata*, two experiments were performed.

2.5.1. Experiment 1

Two colonies of *S. bipunctata* located in the garden of the Bee Laboratory separated by about 10 m from each other were chosen: no. 456 (A) and no. 96-01 (B). The behaviour of the guard bees was recorded using a Sony CCD-F350 video camera, in three different situa-

tions: (a) colonies A and B were in their original location. The recording lasted 150 min. No disturbance occurred. (b) Colonies A and B changed places. Colony A was settled where B had been and colony B where A was originally. (c) In the third situation, the colonies were returned to their original positions. Each change of place occurred at 8:50 AM. Five hours each of video registration were made for situations (b) and (c). Since the openings of the colonies are very large, it is easy to observe and video the behaviour of the guard bees standing in the inner part of the entrance. The behaviour of the guard bees during the three situations was compared.

2.5.2. Experiment 2

Pair encounters of guard worker bees were arranged using bees of the same colony (A × A and B × B) and workers of different colonies (A × B). A complementary test was done using colony C and the pair encounters with guard bees were: C × C; A × C and B × C. A total of 120 encounters were performed for the three colonies. The guard bees were collected at the entrance of the colonies A, B and C. Each worker bee of a pair was introduced individually in a glass tube (20 mm × 20 cm). The tubes were held together until one of the bees had moved into the next tube. The tube containing the two bees was then closed with a piece of soft sponge and the behaviour registered and later compared. "Touching" followed by "biting" by one of the members of the pair was considered "aggression". The bee that was the first to make the "biting" behaviour was considered to initiate the aggression. "Biting" was very conspicuous, but whenever there was doubt about which bee had started the attack, no assignment was made.

3. Results

3.1. Behavioural results

3.1.1. Experiment 1

3.1.1.1. Situation (a). In a quiet and undisturbed situation, the guard bees stand either at the border of the entrance or in the entrance tube. The heads of those in the tube are directed to the exterior and their antennae upright. They move only to let foragers enter or leave the colony, returning to their previous position afterwards. Occasionally, a guard bee makes a timid, rapid and very short flight (50 cm), around the entrance, returning within seconds to its attentive behaviour. Guard bees remained on post throughout the period of observation.

3.1.1.2. Situation (b). The change of positions of the nests between colonies A and B caused great disturbance and aggressive behaviour of the bees. The guard bees at the border of the entrance let some of the arriving foragers enter the "wrong" colony inadvertently. Within a few minutes, the guard bees standing in the inner part of the tube, after touching the intruders with their antennae, became very disturbed and these were the ones to start attacking the intruders. Soon, many bees flew from the nest to attack whatever was nearby including the researcher. Such situations lasted at least 5 h. The following aggressive behaviour was observed: biting off the appendages of the intruders; pulling the intruders to the exterior; deposition of resin on the body of the intruder; and often even killing them.

3.1.1.3. Situation (c). After the return of the colonies A and B to their previous position, the guard bees were still disturbed, flying around the entrance and attacking aggressively the incoming foragers. This situation lasted for about 1 h, quieting down afterwards.

3.2. Experiment 2

It was observed that in confrontations, the bees first touch each other with their antennae. In the case that the guard bee workers came from the same colony (A × A; B × B; C × C), only once or twice was aggressive behaviour observed. During confrontations between guard bees from different colonies, one of the bees of the pair was the first to touch and attack. The results for 20 encounters of A × B; B × C and A × C are given in Table 1. Kruskal–Wallis analysis ($H = 4.324$, gl = 2, $p = 0.115$) showed that there were no differences among the colonies for the initiation of aggression.

3.3. Chemical analysis

We have found that bumblebees (Oldham et al., 1994) and honeybees (unpublished) can pick up many contaminants from plants and their nests, especially on their legs, so that analysis of the surface of individuals can give variable results. The part least subject to contaminants are the wings, which contain the same mixture of cuticular hydrocarbons as on the rest of the

Table 1
Aggressive encounters between individual guard bees of *Scaptotrigona bipunctata* from three laboratory colonies A, B and C. Twenty encounters were observed for each situation. Encounters between A × A, B × B, and C × C were statistically without aggression

Colony	Colony	Number of aggressive attacks	Attack initiated by
A	B	16	A: 6, B: 10
A	C	17	A: 10, C: 7
B	C	17	B: 11, C: 6

body. Therefore, the wings only of each bee have been analysed in this work.

Cuticular hydrocarbon profiles obtained by gas chromatography of wings of individual guard bee workers differed considerably between the two investigated colonies. Both colonies contained C_{22}–C_{29} hydrocarbons. Trace components, representing less than 0.5% of the total, were docosane and octacosane and still smaller amounts of methyl-branched C_{24} and C_{26} alkanes. These were excluded from the calculations, but all other compounds, representing 0.5% or more were included. The position of double bonds in the alkenes was determined from the fragmentation patterns in the mass spectra of dimethyl disulphide addition products. The geometry of the double bonds in the C_{23}, C_{25}, and C_{27} alkenes was shown, by comparison with synthetic samples to be (*Z*)- or *cis* in each case. The major compounds of both colonies were therefore (*Z*)-9-pentacosene, (*Z*)-7-pentacosene, pentacosane, (*Z*)-9-heptacosene, and tricosane. A few individuals were contaminated with ketones from the mandibular glands, including 2-tridecanone, 2-pentadecanone, 2-heptadecanone and 2-undecanone (in decreasing order of amount). There were also in a few samples small quantities of tetradecanal and hexadecanal. These have been excluded from the percentage calculations for the surface.

The analysis of the cuticular compounds showed that 13 compounds could be identified in all 12 guard bee samples (see Table 2). These 13 compounds were taken for the statistical analysis. For each guard bee chromatogram, the areas of these 13 peaks were taken as 100% and the percentage of each peak calculated to standardize the data set. To show whether both guard bee sample sets are different, a normal principal component analysis in combination with a discriminant analysis was made using the 13 identified compounds. To judge whether all 13 compounds are really necessary for the size discrimination, compounds scoring less than 0.295 in the component correlation matrix of the principal component analysis were omitted. A normal principal component analysis with successive discriminant analysis was then conducted using the remaining seven compounds (9-tricosene, 7-tricosene, 3-methyltricosane, pentacosane, hexacosane, 7-heptacosene and nonacosane). Factor 1 of the principal component analysis with these seven compounds described 41.27% of the observed variance, factor 1 + factor 2 described 61.21% and factor 1 + factor 2 + factor 3 described 78.21% of the total variance (see Fig. 1). The discriminant analysis with the resulting three factors from the principal component analysis of seven compounds resulted in no group mismatch for all 12 data sets. All six data sets from guard bee samples of colony A were correctly distributed to the "guard bee colony A" group, all six data sets from guard bee samples of colony B were correctly assigned to the "guard bee colony B" group. The results show that the two groups could be separated successfully using only seven of the 13 compounds identified in all 12 guard bee samples (see Fig. 2). Omitting the next least scoring compound resulted in one mismatch in the discriminant analysis, suggesting that these six compounds are really necessary to distinguish between guard bees of the two colonies. The results suggest that there is an underlying structure within these six compounds, which can be recognized for discrimination. That may result in a complex multi-compound recognition receptor, capable of not only recognizing these six compounds but also be able to

Table 2
Mean composition as percentage of the total, with standard deviations, of cuticular lipids for colonies A (456) and B (96-01)

Compound	Colony A		Colony B	
	% of total	±sd	% of total	±sd
(*Z*)-9-Tricosene	4.59	2.56	1.49	0.94
(*Z*)-7-Tricosene	1.89	1.66	0.73	0.44
Tricosane	16.97	5.69	11.28	5.51
3-Methyltricosane	0.85	0.39	2.29	1.18
Tetracosane	2.39	2.32	3.18	3.14
(*Z*)-9-Pentacosene	26.59	7.74	16.68	10.52
(*Z*)-7-Pentacosene	8.27	2.79	10.99	13.62
Pentacosane	13.30	3.20	27.42	13.47
Hexacosane	2.19	2.61	3.23	1.03
(*Z*)-9-Heptacosene	11.28	10.15	9.63	11.55
(*Z*)-7-Heptacosene	1.70	1.58	5.73	2.89
Heptacosane	7.36	5.34	6.23	5.44
Nonacosane	2.62	1.23	1.12	0.66

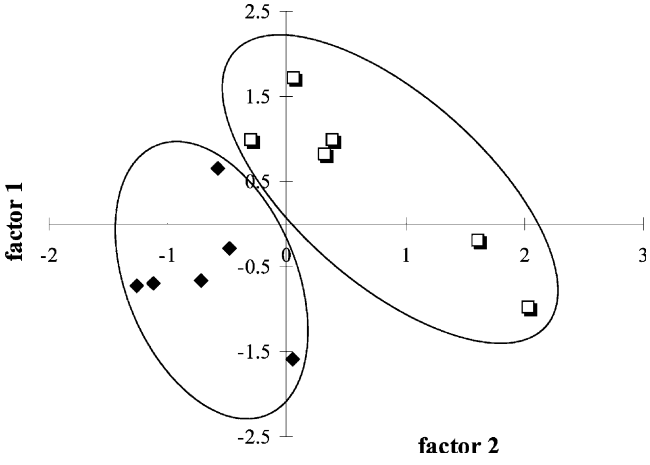

Fig. 1. Principal component analysis results (Equamax with Kaizer normalization) with seven cuticular compounds to distinguish between guard bees of colonies A and B of *Scaptotrigona bipunctata*. The circles suggest no overlapping regions for both sample sets. The *x*-axis shows the factor scores for factor 1 of the PCA, the *y*-axis shows the factor scores for factor 2 of the PCA. Open squares, colony A, closed lozenges, colony B.

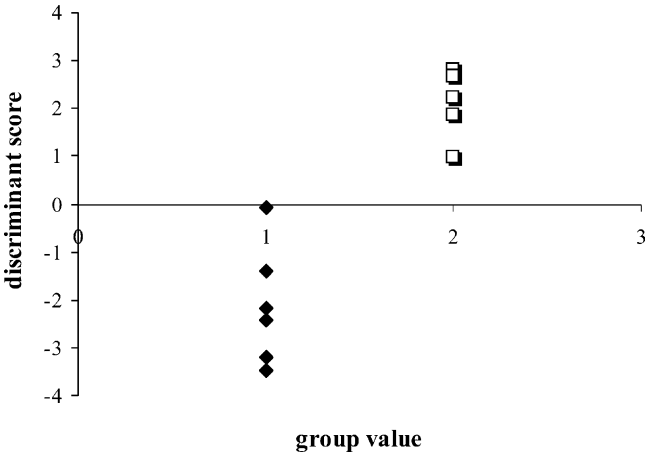

Fig. 2. Plot of the values of the discriminant scores (discriminant analysis) of 12 guard bee cuticular profiles of *Scaptotrigona bipunctata* from colonies A and B, showing that the guard bees of both colonies can be distinguished from each other using seven of the 13 identified cuticular compounds, which could be identified in all 12 guard bee samples. All 12 single data points (samples) were assigned correctly to either colony A group (open squares) or colony B group (closed lozenges).

distinguish among single bees by comparing their intrinsic chemical pattern.

4. Discussion

In the first experiment, after the change of position of the nest between colonies A and B, some of the arriving foragers were allowed to enter the "wrong" colony, unchallenged by the column of guard bees at the entrance. These border guard bees were not able to discriminate the "wrong" foragers immediately. Only after the inner guard bees perceived that they were "intruding" foragers were they attacked and the whole colony became excited. The ecological success of a colony of social insects depends on the organization of the division of labour and on the ability of workers to carry out determined tasks (Bonabeau and Theraulaz, 1999). Perhaps the "landing" of these foragers was too abrupt and the "smell" of the nectar, resin or pollen carried by them mixed with their own chemicals made "recognition" difficult for the border guards. It is also possible that the "tube" guard bees are a little older than the "border" ones and more capable of distinguishing different odours or are more able to produce and spread alarm pheromones to the other members of the colony. Engels et al. (1987) indicate that older bees of *S. postica* contain more of the volatile secretions. When colonies A and B were restored to their original places, it took more than an hour for the colonies to re-establish their quiescent condition, indicating that a kind of "memory" of the previous situation may prevail and a "lag" period intervenes before the original situation can be restored. Roubik (1989) has observed that the presence of intruders near a colony of meliponine bees determines an increase in the number of guard bees. We can suppose that it takes some time before the colony readjusts to the lower number of guard bees under normal undisturbed conditions. Biesmeijer et al. (1998) have observed in workers of *Melipona* species, that the decision process to perform certain tasks such as foraging, depends on the integration of external and internal factors. This may be what causes the observed "lag" period.

In experiment 2, the number of encounters of guard bee workers of different colonies, A × B, B × C and A × C that resulted in aggressive attacks did not vary significantly among those colonies (Table 1). Although colony C had been maintained under somewhat different conditions, this did not affect its interactions with colonies A and B. Suka et al. (1994) in *Scaptotrigona barrocoloradensis* and Inoue and Roubik (1990) in *Melipona fasciata*, have observed that young worker bees of a different colony are accepted easily by the older ones, without inducing aggression. Yet, in *Melipona panamica* under stress conditions, even younger sisters bees were killed in aggressive encounters (Inoue et al., 1999). In *S. bipunctata*, young worker bees are often more aggressive towards their older sisters than the opposite (da Costa, unpublished). In our experiments, the bees that are the first to attack are likely to be the younger ones.

We have found in various experiments that the legs and bodies of bees can be quite heavily contaminated. McDaniel et al. (1984) in analysis of honeybee cuticular hydrocarbons found that they were very susceptible to contamination from beeswax and pollen. We have found that the wings contain the same hydrocarbon surface as the rest of the body, but are much less contaminated, so therefore more reproducible and accurate results are obtained by analysing the wings alone. There is sufficient amount of the material on the wings of a single bee to carry out this analysis.

Although there were large variations in the composition of hydrocarbons on individual bees, as indicate by the large standard deviations given in Table 2, the discriminant analysis showed that worker bees of colony A could be separated from worker bees of colony B (Fig. 1). The results suggest that there is an underlying structure within these seven compounds, which can be recognized for discrimination. That may result in a complex multi-compound recognition receptor, capable of not only recognizing these seven compounds, but also be able to distinguish amongst single bees by comparing their intrinsic chemical pattern under normal undisturbed or unstressed conditions. Chemical analysis of wings of guard bee workers of colony C was not done because this colony had been held under different conditions.

We conclude that the recognition of nestmate or "foreign" individuals in this stingless bee is consistent with current ideas about antennal recognition through surface lipids under normal conditions but may be influenced or disturbed under stressed conditions.

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