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ORIGINAL PAPER

Distinguishing signals and cues: bumblebees use general footprints to generate adaptive behaviour at flowers and nest

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Abstract Chemicals used in communication are divided into signals and cues. Signals are moulded by natural selection to carry specific meanings in specific contexts. Cues, on the other hand, have not been moulded by natural selection to carry specific information for intended receivers. Distinguishing between these two modes of information transfer is difficult when animals do not perform obvious secretion behaviours. Although a number of insects have been suspected of leaving cues at food sites and nest entrances, studies have not attempted to experimentally distinguish between cues and signals. Here, we examine the chemical composition of the scent marks left by the bumblebee *Bombus terrestris* at food sites and compare it to those found at a neutral location. If bees are depositing a cue, we expect the same chemicals to be found at both sites, but if they deposit a signal we only expect to find the scent marks at the food site. We were also interested in identifying the chemicals left at the nest entrance to determine if they differed from those used to mark food sites. We find that bees deposit the same chemicals at food, nest and neutral sites. Therefore, bumblebees leave behind general chemical footprints everywhere they walk and we propose that they learn to use these footprints in a manner that ultimately enhances their fitness, for example, to improve their foraging efficiency and locate their nest. Experimentally, distinguishing these two modes of information transfer is crucial for understanding how they interact to shape animal behaviour and what chemical bouquets are under natural selection.

Keywords Chemosensory cue · Cuticular hydrocarbons · Exocrine gland · Foraging · Pheromone · Trail-laying

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Introduction

Sources of information used by animals are generally placed into two broad categories: signals and cues. Signals are defined as traits that evolved for a specific role in communication (Karlson and Luscher 1959) and are often believed to elicit hard-wired responses (Beauchamp et al. 1976). Chemical signals are often referred to as pheromones. Cues, on the other hand, are defined as incidental features present in the environment (Seeley 1995, p. 270). They have not been moulded by natural selection to carry a specific meaning for intended receivers. Therefore, their meaning can vary between individuals of the same species. Animals should rely on both cues and signals to generate adaptive behaviour (Seeley 1998). Distinguishing between these two modes of information transfer is essential for identifying which chemical bouquets are shaped and maintained through natural selection.

Bumblebees use scent marks at flowers to indicate rewarding and unrewarding food sites (Saleh and Chittka 2006), and numerous behavioural studies have investigated the usage of these scent marks by bumblebees (e.g. Cameron 1981; Schmitt and Bertsch 1990; Goulson et al. 2000; Saleh et al. 2006). Several authors have proposed that the scent marks, deposited by bumblebees to improve their foraging efficiency, may be signals (i.e. a foraging phero-

mone), moulded by natural selection to carry specific information in a foraging context (Giurfa and Núñez 1992; Stout et al. 1998), while others have suggested they are incidental cues, left everywhere bees walk (Stout and Goulson 2002; Eltz 2006). The reason for this controversy is the existence of evidence that suggests these scent marks may be cues rather than signals. Firstly, bumblebees do not perform any obvious marking behaviour, to the human observer, as found in other bee species (Schmitt and Bertsch 1990; Nieh 2004). Secondly, bumblebees learn to use these scent marks to indicate both high and low value food sources (Saleh and Chittka 2006), suggesting that the meaning of these scent marks can change with experience. Thirdly, although the tarsal gland has been suggested as the source of the scent marks (Schmitt et al. 1991), no obvious channels through the bee's cuticle have been found (Pouvreau 1991). In addition its contents, linear hydrocarbons, resemble those found on the cuticle (Oldham et al. 1994), which are most likely secreted from cuticular tissue to protect the insect from desiccation (Schal et al. 1998). These cuticular hydrocarbons are liquids that can easily be leaked passively onto a substrate (Oldham et al. 1994). Therefore, it is not clear if these chemical marks are left behind on flowers through active secretion from the glands, or if the cuticular hydrocarbons on the bees' feet are passively left behind. Although the chemicals at the food source have been identified, and chemical bioassays investigating their influence on bumblebees' behaviour have been conducted (Schmitt et al. 1991; Goulson et al. 2000), the presence of these chemicals at the food source does not tell us if these chemicals are foraging pheromones left only at the food source. To examine this question we need to compare the chemicals left at the food source to those left in other areas.

Bumblebees also leave a chemical trail to their nest, and behavioural studies have shown that they follow this chemical trail to locate their nest entrance (Cederberg 1977; Foster and Gamboa 1989; Pouvreau 1996). Such a trail can also be laid to feeders when bees are forced to forage in darkness (Chittka et al. 1999). Bumblebees nest in underground cavities, which can become covered with grass and shrubs obscuring the entrance of the colony. Reliance on these chemical marks is expected to allow bees to locate their nest faster, and distinguish it from other nests nearby (Foster and Gamboa 1989). In addition, the vegetation surrounding the nest can change, rendering visual cues less reliable. Therefore, olfactory cues may provide a reliable backup system (Jandt et al. 2005). Bumblebees can discriminate between chemical trails laid by their nestmate, conspecific and heterospecific workers, and are more likely to choose entrances marked by their nestmates (Pouvreau 1996). However, we do not know the chemical composition of these chemical trails. They share some characteristics with the chemicals left at the food source in that they both continue to elicit behavioural effects 20 h after they are deposited and dissolve in solvents for lipid type compounds e.g. pentane and hexane (Cameron 1981; Foster and Gamboa 1989). Therefore, it is possible that bumblebees use the same chemicals to mark their food and nest sites.

The purpose of this study was to (1) compare the chemicals deposited at a neutral site to those deposited at the food site to resolve if the scent marks are general footprints or foraging pheromones (2) determine the chemical composition of the scent marks left at the nest entrance.

Materials and methods

If the scent marks are pheromones (signals) we expect to find them only in areas that hold a resource value, such as the food and nest sites, not in a relatively neutral site. However, if they are cues, left everywhere bees walk then we should also find them at relatively neutral sites. In order to test this we needed bees to walk in relatively neutral areas that held no resource value. We did this by having the bees exit the hive into an unrewarding arena connected via a large tunnel to another empty unrewarding arena (see Fig. 1). This section of the setup should hold no resource value to the bees and therefore should have no scent marks, of the kind left at flowers, if the secretion on flowers is a pheromone.

Experimental setup

Colonies of *Bombus terrestris dalmatinus* were obtained from Koppert Ltd. (Netherlands). They were housed in a wooden nest box ($16(w) \times 28(l) \times 11$ cm (h)) and fed approximately 4 g of pollen into the nest every day. The bees foraged on 50% (w/w) sucrose solution from gravity feeders described below.

The experimental apparatus consisted of three arenas connected to each other via tunnels. Nest samples were collected from a clear plastic tunnel ($3.5 \times 30 \times 3.3$ cm) connected to the colony's nest box. Plastic shutters at the entrance and exit of the tunnel could be used to isolate it from incoming bee traffic and a removable top allowed the experimenter access to the tunnel. This tunnel connected the nest to unrewarding arena 1. Unrewarding arenas ($40 \times 60 \times 30$ cm) never contained food and were empty throughout the experiment. Unrewarding arena 1 was connected to unrewarding arena 2 via a large black plastic tunnel ($10 \times 5 \times 195$ cm) where the neutral samples were collected. The top of this tunnel was covered with wire mesh except for the sample collection area. This area was covered

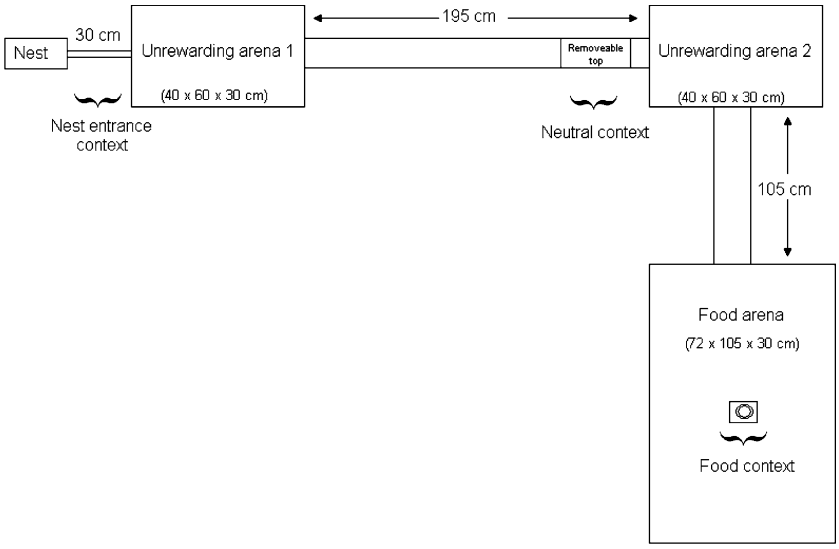

Fig. 1 Experimental setup. Nest samples were collected in tunnel connecting nest box to first unrewarding arena. Neutral samples were collected in large tunnel connecting unrewarding arenas 1 and 2. Food samples were collected at a feeder, indicated by the circular symbol surrounded by a rectangle

with a clear plastic sheet taped to the top of the tunnel, thereby allowing the experimenter easy access to the tunnel. Cardboard shutters were used to control bee traffic. These were inserted before and after the plastic sheet when needed. To encourage bees to walk in this area, we reduced the light level by placing pieces of wood over it. Unrewarding arena 2 was connected to the food arena (72 × 104 × 30 cm) via a second large tunnel (10 × 5 × 105 cm), which was covered entirely with wire mesh. We collected food samples from the food arena. This was the only arena where food was presented to the bees. Bees were fed 50% (w/w) reagent grade sucrose from a gravity feeder (Frisch 1967). It was composed of a glass dish (Ø = 5, *h* = 3 cm) inverted onto a circular Plexiglas plate (Ø = 6, *h* = 0.5 cm). Eighteen equidistant grooves were cut in a radial arrangement on the top surface of the Plexiglas plate. This feeder was elevated from the ground using a platform (8 × 8 × 3.5 cm). Green cardboard was taped onto the arena floors to mimic the green foliage background found in most natural situations of bees foraging.

Data collection

Teflon® disks (Ø = 2 cm; Supelco, Bellefonte, USA), vials and low-volume inserts (QMX, Thaxted, UK) used to collect and treat the samples were sterilised by rinsing them in ethanol, acetone and pentane solutions (HPLC grade, Sigma-Aldrich Co. Ltd, Gillingham, UK) then placing them in the oven for 3 h at 230°C. They were rinsed again with pentane before use. Flame sterilised tweezers were used to handle the vials and Teflon® disks. Glass pipettes, used to transfer the solvents, were new and rinsed at least three times in pentane before use.

We placed aluminium foil along the floor of the nest and neutral tunnels. Aluminium foil also covered the bottom of the gravity feeder and the top of the feeder platform. This minimized contamination from the plastic. We changed the aluminium foil, with tweezers, after each collection. All our samples were compared to an Arena control. To collect the Arena control 12 Teflon® disks were placed onto a sheet of aluminium foil in an 800 ml beaker (Ø = 9.2, *h* = 13.4 cm). The beaker was covered with wire mesh, to prevent bees from walking on the disks, and placed inside one of the arenas.

The nest, neutral and arena control samples were collected together; food samples were collected separately. This prevented contamination by the food foraging pheromone, which alerts nestmates to the presence of food (Granero et al. 2005). Twelve Teflon® disks were placed on the floor of the tunnels to collect the nest and neutral context samples. For the food context, a small part of each of the 12 disks was slipped between the bottom of the feeder and the platform to prevent it from falling on the floor. All Teflon® disks were left for 3 h; meanwhile the bees were allowed to walk on them. The disks were then removed using tweezers and placed into a 4 ml vial containing 1.5 ml of pentane (Schmitt et al. 1991; Jarau et al. 2004). The liquid was swirled for 1.5 min and then transferred, via a glass pipette, to another clean 4 ml vial. Samples not immediately analysed were stored in a –20°C freezer at until analysis. When the samples were analysed, they were concentrated using a gentle stream of dry nitrogen to 200 µl.

Two colonies were used and at least three sets (set = one nest, one neutral and one food context) of samples were collected from each colony. Volumes of 4 µl of each sample were analysed using a gas chromatograph–mass spectrometer (Agilent Technologies: GC 6890N/MS 5973N) with helium as carrier gas (2 ml/min) on pulsed splitless and constant flow modes. The GC injector temperature was

280°C. The mass spectrometer was in electron impact mode (at 70 eV) and scanned from 50 amu to 550 amu. An HP-1MS column was used (Hewlett Packard: 25.0 m length, 320 μm internal diameter and 0.52 μm film thickness). The temperature program was initially held at 60°C for 1 min, then increased to 300°C at 10°C/min and kept at this temperature for an additional 30 min. The alkanes were identified through retention time comparisons with synthetic compounds (Sigma-Aldrich Biotechnology). A set of samples from one of the colonies was treated with dimethyl disulphide (DMDS) as described in Carlson (1989) to identify the alkenes. Percent peak areas (i.e. relative amounts) of the compounds in each sample were compared via principle components analysis (henceforth PCA) using Brodgar version 2.4.6 (Highland Statistics Ltd).

Results

Samples from all three contexts have very similar chromatograms (see Fig. 2). Detailed identification of the compounds left in each context revealed that 76 out of 77 compounds were present in all three contexts and in similar relative amounts (see Table 1). This similarity was confirmed by the PCA where axis 1 explained 95.19% of the variation. There was no clustering of samples based on the collection context (see Fig. 3), nor were any differences detected between contexts in relative amounts of any compound. Therefore, we conclude that samples collected in the food, nest and neutral contexts are composed of the same chemicals present in similar proportions. The extracts were a mixture of 13 alkanes, 55 alkenes, four alkadienes and five aldehydes. The alkanes were the most abundant compounds, while the majority of the alkenes were present in small amounts (see Table 1).

Discussion

This study provides a simple means for distinguishing between general chemicals and chemicals left only at specific locations. This can be used to categorize chemical marks when the animals do not perform obvious marking behaviour. We did this by identifying the chemicals left at the food and nest sites and comparing them to chemicals found at a relatively neutral site. Other studies generally either draw conclusions based on the presence of correlations between the compounds left on the substrate and a gland or cuticle (e.g. Schmitt et al. 1991; Goulson et al. 2000; Steinmetz et al. 2003; Jandt et al. 2005), or do not control for the possibility that the same *signal* (pheromone) may be left behind in places that hold a resource value (Schmidt et al. 2005). Our results indicate that the same compounds were present in the feeding site, neutral site and nest entrance contexts. Thus bees are leaving behind a general footprint, which they, most likely, learn to associate with different meanings depending upon context and experience.

It is extremely difficult to show experimentally that any area is absolutely neutral. However, we can show that the neutral area in our experimental setup was relatively neutral to the food and nest sites and that if any differences existed between the three locations we would have identified them. Firstly, the neutral area did not hold any obvious resource value, because the bees neither fed nor nested in it. Secondly, we collected the food samples separately to the neutral samples; therefore, it is unlikely that the bees were marking the neutral area with a trail to the food source. Thirdly, it is unlikely we neglected to detect chemicals of high volatility that may be used to distinguish between the different areas because the effect of the chemicals left at the food and nest sites have been reported to last over 20 h (Cederberg 1977; Schmitt and Bertsch 1990; Stout and Goulson 2002), suggesting that the compounds used by the bees are relatively non-volatile. Therefore, we feel that our comparisons of food and nest sites to the neutral site should have revealed differences in deposited chemicals if there were any.

Consistent with previous studies on the scent marks left by bumblebees at food sources, we have found that the footprint is a mixture of alkanes and alkenes with a minor occurrence of alkadienes and aldehydes, which resemble those found in the tarsal gland and cuticle extracts (Schmitt et al. 1991; Oldham et al. 1994). There are some variations in the compounds present and their quantities in our study compared to those of Schmitt et al. (1991) and Goulson et al. (2000). These differences are probably due to natural variation within the species, as similar differences were observed in honeybee cuticular extracts (Dani et al. 2005). Although all the three studies used *B. terrestris*, it is possible that the subspecies used may influence the ratio of hydrocarbons present. We used the South-Eastern European variety *dalmatinus*, but subspecies information was not provided in the previous studies.

It is unlikely that we overlooked differences in minor compounds in the samples. This is because each sample was compared to the arena control with which it was collected and only compounds that were not present in the control or present in quantities above those of the control were included in the analysis. Therefore, any consistent differences, even if minor, would have been noticed by the experimenter and should have been detected by the PCA analysis. In addition, it is unlikely that bees are responding to only one compound, rather a mixture of different compounds (Schmitt et al. 1991; Goulson et al. 2000; Schiestl and Ayasse 2000).

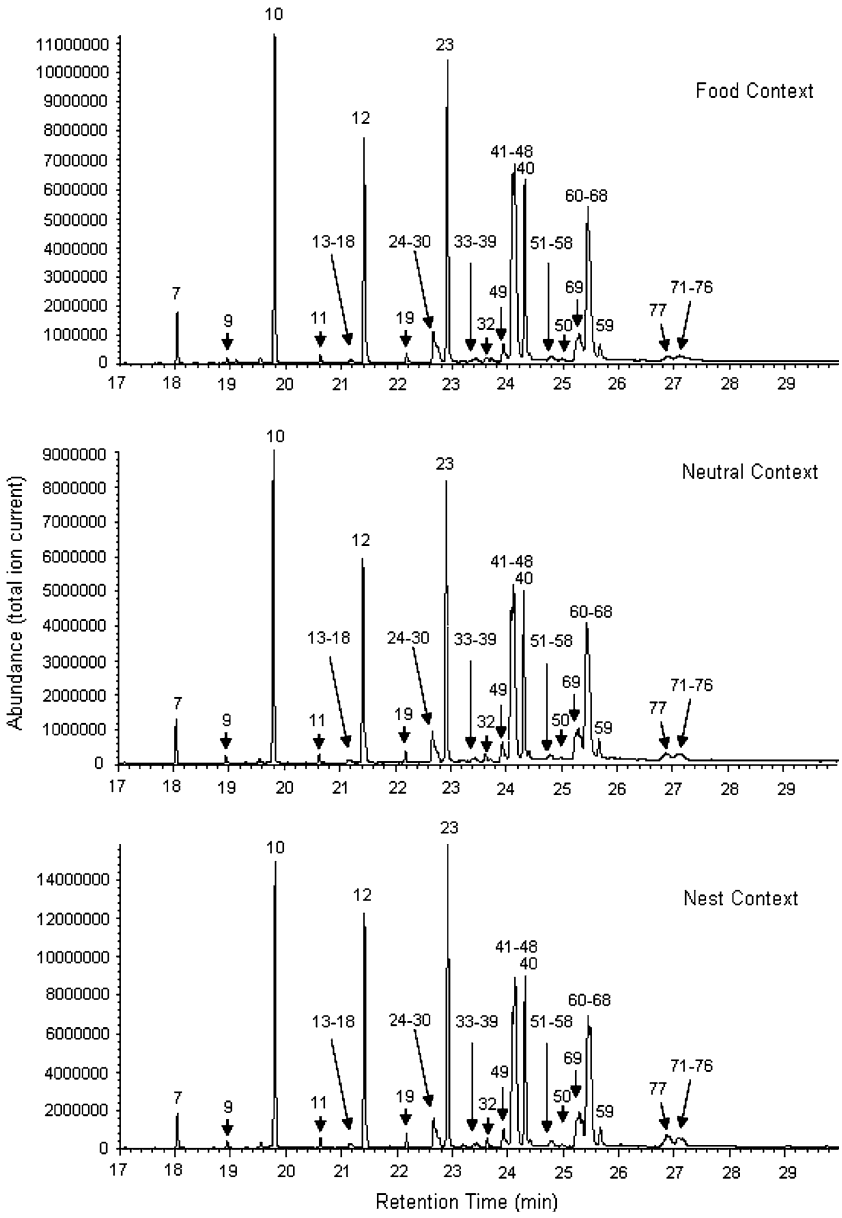

Fig. 2 Chromatograms of samples collected in food, nest and neutral contexts. The three chromatograms are very similar indicating the presence of similar compounds in each context. Results shown here are for samples collected from colony A. Numbers correspond to compounds identified in Table 1

It is known from previous studies that experience greatly influences how a bee interprets these chemical footprints. We know that during foraging these scent marks can be attractive or repellent (Saleh and Chittka 2006), and they are relied on to different degrees depending on the handling time of flowers (Saleh et al. 2006) and its replenishment rate (Stout and Goulson 2002). Now we know that the same scent marks are used to locate the bees' nest entrance. Therefore, bees learn to associate these scent marks with multiple meanings that may, at times, be unique to each individual. Although we have shown that bees deposit general footprints where they walk, in theory, it is possible that bumblebees actively mark all areas they have walked on. Therefore, the next step is to determine if these scent marks are actively or passively left behind. This would involve identifying the source of the compounds to determine if they are glandular or cuticular. This will also shed some light on whether these compounds are the same as the cuticular hydrocarbons used in nestmate recognition (Dronnet et al. 2005).

There is strong suggestive evidence that honeybees also use general footprints left behind at their nest entrance and food sites, in addition to signalling use of Nasonov gland pheromone (Frisch 1967). Butler et al. (1969) have shown that scent marks collected at the nest entrance increase the frequency of honeybees landing on rewarding food sources. This behaviour was also elicited from the scent marks left on the hive floor, suggesting that the hive floor, nest entrance and food source are marked by the same chemicals. The scent marks are most likely a general footprint

Table 1 Compounds identified in nest, neutral and food contexts

Number	Compound	Mol. wt.	Diagnostic EI ions	Food	Nest	Neutral
1	Nonanal	142		+	+	+
2	Decanal	156		+	+	+
3	undecanal	170		+	+	+
4	dodecanal	184		+	+	+
5	Tridecanal	198		+	+	+
6	*n*-Nonadecane ($C_{16}H_{40}$)	268		+	+	+
7	*n*-Heneicosane ($C_{21}H_{44}$)	296		++	++	++
8	9-heneicosene	388	173/215	+	+	+
9	*n*-Docosane ($C_{22}H_{46}$)	310		+	+	+
10	*n*-Tricosane ($C_{23}H_{48}$)	324		+++	+++	+++
11	*n*-Tetracosane ($C_{24}H_{50}$)	338		+	+	+
12	*n*-Pentacosane ($C_{25}H_{52}$)	352		++	++	++
13	7-pentacosene	444	145/299	+	+	+
14	8-pentacosene	444	159/285	+	+	+
15	9-pentacosene	444	173/271	+	+	+
16	10-pentacosene	444	187/257	+	+	+
17	11-pentacosene	444	201/243	+	+	+
18	12-pentacosene	444	215/229	+	+	+
19	*n*-Hexacosane ($C_{26}H_{54}$)	366		+	+	+
20	9-hexacosene	458	173/285	+	+	+
21	11-hexacosene	458	201/257	+	+	+
22	13-hexacosene	458	229/229	+	+	+
23	*n*-Heptacosane ($C_{27}H_{56}$)	380		+++	++	++
24	7-heptacosene	472	145/327	+	+	+
25	8-heptacosene	472	159/313	+	+	+
26	9-heptacosene	472	173/299	+	+	+
27	10-heptacosene	472	187/285	+	+	+
28	11-heptacosene	472	201/271	+	+	+
29	12-heptacosene	472	215/257	+	+	+
30	13-heptacosene	472	229/243	+	+	+
31	Heptacosadiene	376	376	–	+	+
32	*n*-Octacosane ($C_{28}H_{58}$)	394		+	+	+
33	8-octacosene	486	159/327	+	+	+
34	9-octacosene	486	173/313	+	+	+
35	10-octacosene	486	187/299	+	+	+
36	11-octacosene	486	201/285	+	+	+
37	12-octacosene	486	215/271	+	+	+
38	13-octacosene	486	229/257	+	+	+
39	14-octacosene	486	243/243	+	+	+
40	*n*-Nonacosane ($C_{29}H_{60}$)	408		++	++	++
41	7-nonacosene	500	145/355	+	+	+
42	8-nonacosene	500	159/341	+	+	+
43	9-nonacosene	500	173/327	++	++	++
44	10-nonacosene	500	187/313	++	++	++
45	11-nonacosene	500	201/299	++	++	+
46	12-nonacosene	500	215/285	+	+	+
47	13-nonacosene	500	229/271	++	++	++
48	14-nonacosene	500	243/257	+	+	+

Table 1 continued

Number	Compound	Mol. wt.	Diagnostic EI ions	Food	Nest	Neutral
49	Nonacosadiene	404	404	+	+	++
50	*n*-Triacontane ($C_{30}H_{62}$)	422		+	+	+
51	8-triacontene	514	159/355	+	+	+
52	9-triacontene	514	173/341	+	+	+
53	10-triacontene	514	187/327	+	+	+
54	11-triacontene	514	201/313	+	+	+
55	12-triacontene	514	215/299	+	+	+
56	13-triacontene	514	229/285	+	+	+
57	14-triacontene	514	243/271	+	+	+
58	15-triacontene	514	257/257	+	+	+
59	*n*-Hentriacontane ($C_{31}H_{64}$)	436		++	++	++
60	7-Hentriacontene	528	145/383	+	+	-
61	8-Hentriacontene	528	159/369	+	+	+
62	9-Hentriacontene	528	173/355	++	++	++
63	10-Hentriacontene	528	187/341	+	+	+
64	11-Hentriacontene	528	201/327	++	++	++
65	12-Hentriacontene	528	215/313	+	+	+
66	13-Hentriacontene	528	229/299	+	+	+
67	14-Hentriacontene	528	243/285	+	+	+
68	15-Hentriacontene	528	257/271	+	+	+
69	Hentriacontadienes	432	432	++	++	++
70	9-tritriacontene ($C_{33}H_{66}$)	556	173/383	+	+	+
71	10-tritriacontene	556	187/369	+	+	+
72	11-tritriacontene	556	201/355	+	+	+
73	12-tritriacontene	556	215/341	+	+	+
74	13-tritriacontene	556	229/327	+	+	+
75	14-tritriacontene	556	243/313	+	+	+
76	15-tritriacontene	556	257/299	+	+	+
77	Tritriacontadiene(s)	460	460	+	+	+

76/77 compounds are present in all three contexts, indicating the scent mark is a general chemical footprint. +, <1%; ++, 1–10%; +++, >10% and –, not present. Alkenes were identified using DMDS treated samples and their DMDS adduct molecular weights are reported. Identification of compounds was done for one set of samples from colony B

because non-foraging bees left scent marks at unrewarding feeders (Ferguson and Free 1979) and the attractiveness of an entrance tube was a factor of the number of bees walking on it (Butler et al. 1969). However, empirical tests need to eliminate the possibility that these sites are marked with the same pheromone by comparing compounds left at a site that does not hold a resource value to those at food and nest sites.

Although many stingless bee species perform behaviours that indicate active marking of highly rewarding food sources (Nieh 2004), there are two reports that suggest a general footprint mechanism may be acting in some stingless bees. *Melipona seminigra* leaves compounds on food sites very similar to those left behind by bumblebees (Jarau et al. 2004). The claw retractor tendon glands are believed to be the source of these chemical marks, but the secretion mechanism is thought to be passive. Schmidt et al. (2005) found that *Nannotrigona testaceicornis* leaves the same scent marks at the nest entrance and food sites. Although the authors suggest that the scent marks are general footprints, they do not control for the possibility that both sites are marked with the same signal. This can be easily achieved through comparison with a neutral site. There are also reports on *Vespula vulgaris* (Steinmetz et al. 2003) and *V. germanica* (Jandt et al. 2005) that suggest a footprint mechanism may operate in some wasps. It is, therefore, possible that the use of conspecific chemical cues for adaptive behaviour is common among social insects. This remains to be shown.

When active marking cannot be demonstrated through behavioural observations, very few studies, investigating the mechanisms involved in conspecific communication of

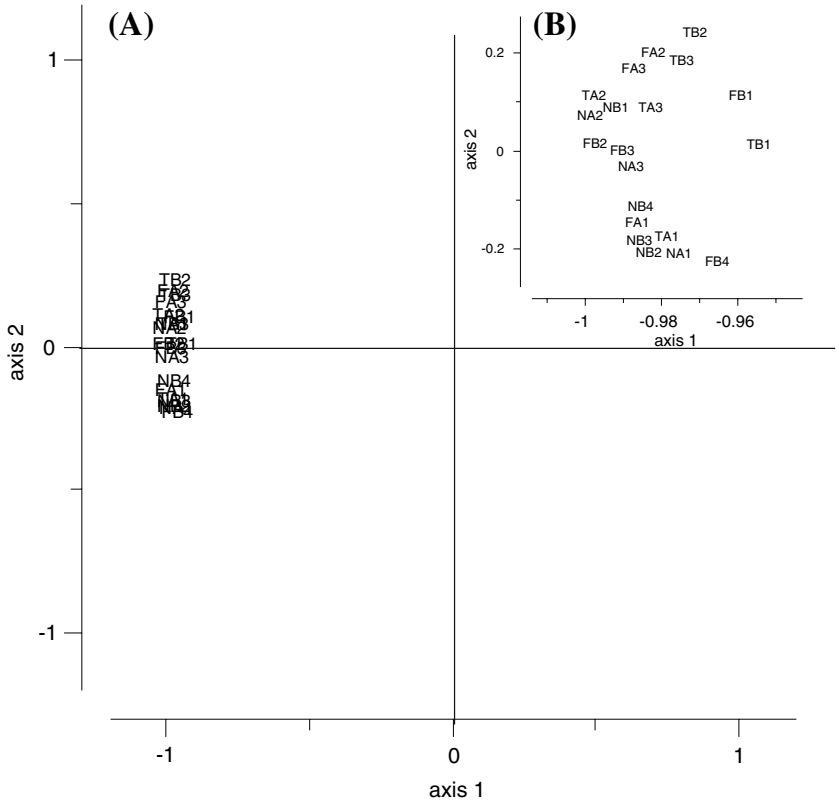

Fig. 3 Results from PCA analysis. (**A**) Samples were highly correlated and grouped together. Axis 1 explains 95.19% of the variation. (**B**) Magnified view of *x*-axis: there was no clustering of samples depending on context, indicating that the scent mark is a footprint. F, sample from food context; N, sample from nest context; T, sample from neutral context; A, sample from colony A; B, sample from colony B

resources, have shown that the compounds left in a specific context are indeed pheromones (left behind only at specific locations), rather than general chemicals left in multiple contexts. Conspecific cues left and used by insects have received little attention in the context of generating adaptive behaviour. However, these types of cues can contain biologically important information such as reproductive status (Ayasse et al. 1995), and can be used to influence biologically important behaviour such as foraging (Giurfa and Núñez 1992), locating nesting sites (Cederberg 1977; Foster and Gamboa 1989; Pouvreau 1996) and the detection of intruders (Dronnet et al. 2005). In order for us to understand the roles certain chemicals play in communication, more studies will need to experimentally determine if the chemical bouquet is a cue or signal. This will increase our understanding of animal communication and provide us with greater insight on how signals and cues interact to shape animal behaviour.

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