

Spitting out information: *Trigona* bees deposit saliva to signal resource locations

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Stingless bees of the species *Trigona spinipes* (Fabricius 1793) use their saliva to lay scent trails communicating the location of profitable food sources. Extracts of the cephalic labial glands of the salivary system (not the mandibular glands, however) contain a large amount (approx. 74%) of octyl octanoate. This ester is also found on the scent-marked substrates at the feeding site. We demonstrate octyl octanoate to be a single compound pheromone which induces full trail following behaviour. The identification of the trail pheromone in this widely distributed bee makes it an ideal organism for studying the mechanism of trail following in a day flying insect.

Keywords: trail pheromone; communication; stingless bees; foraging; salivary glands

1. INTRODUCTION

Pheromone trails play an essential role in the exploitation of food resources in populous and eusocially organized insect species (Hölldobler & Wilson 1990; Pasteels & Bordereau 1998; Wyatt 2003). The communication of highly profitable resources is particularly advantageous when these are limited and several species are competing for them (Johnson & Hubbell 1974; Schaffer *et al.* 1984; Nagamitsu & Inoue 1997; Slaa *et al.* 1997). So far, in-depth studies of pheromones used to mark trails showing the way towards a food source have almost been exclusively carried out in insect species with non-flying foragers. Here, we examine the pheromone used by a stingless bee (*Trigona spinipes*) whose foragers are known to lay scent trails guiding nestmates to a food source (Lindauer & Kerr 1960; Nieh *et al.* 2004).

The genus *Trigona* includes some of the most populous species among the stingless bees (Michener 1974; Wille 1983) and species with highly efficient mechanisms of food source communication (Lindauer & Kerr 1960; Kerr 1963; Jarau *et al.* 2003; Nieh *et al.* 2004), including the known or assumed use of pheromones. However, only little is known about the glandular origin of trail pheromones and their chemical composition in stingless bees.

Foragers of *T. spinipes* do protract their glossae to rub it on the substrate during presumed scent trail marking (Nieh *et al.* 2004). Our aims were to find out whether (i) *T. spinipes* indeed deposits saliva when landing for scent marking and (ii) the saliva contains any attractive

substance or blend of substances representing the pheromone, which induces trail following behaviour. After having found evidence for the presence of such a pheromone we wanted to identify (iii) the active component(s) and (iv) their actual glandular origin. For this purpose, we tested the effectiveness of the synthetic form of the assumed pheromone in behavioural experiments and compared it with that of the natural saliva.

2. MATERIAL AND METHODS

The experiments were carried out on the Ribeirão Preto Campus of the University of São Paulo (Brazil) between November 2004 and March 2006. We studied seven nests of *T. spinipes* (Fabricius 1793; APIDAE, Meliponini). Photographs of field studies are given in the electronic supplementary material.

(a) Trail following bioassays

The artificial scent trails (T1, T2; 10 m long; figure 1) were laid at distances of 60–180 m from the nest, mimicking the natural scent laying pattern (Nieh *et al.* 2004). Feeders contained highly profitable (50% w/w), unscented sugar solution. The amounts of either labial gland extract or octyl octanoate (pentane solution) used for the artificially laid scent trails, increased with the distance (values in metres given in brackets) to the branching point (Bp, figure 1) in the following order (bee equivalents dissolved in pentane): 0.0 (at the Bp, 0 m), 0.05 (1 m), 0.1 (3 m), 0.15 (5 m), 0.2 (7 m), 0.3 (9 m) and 0.9 (at the feeder, 10 m). For control trails, the same amounts of solvent were applied. The distance of the recruitment feeder (RF) from the feeders of trails T1 (open circle) and T2 (filled circle) and the Bp was 10 m. In *T. spinipes*, the scent trails become less effective after 20 min (Nieh *et al.* 2004). We, therefore, renewed the artificial

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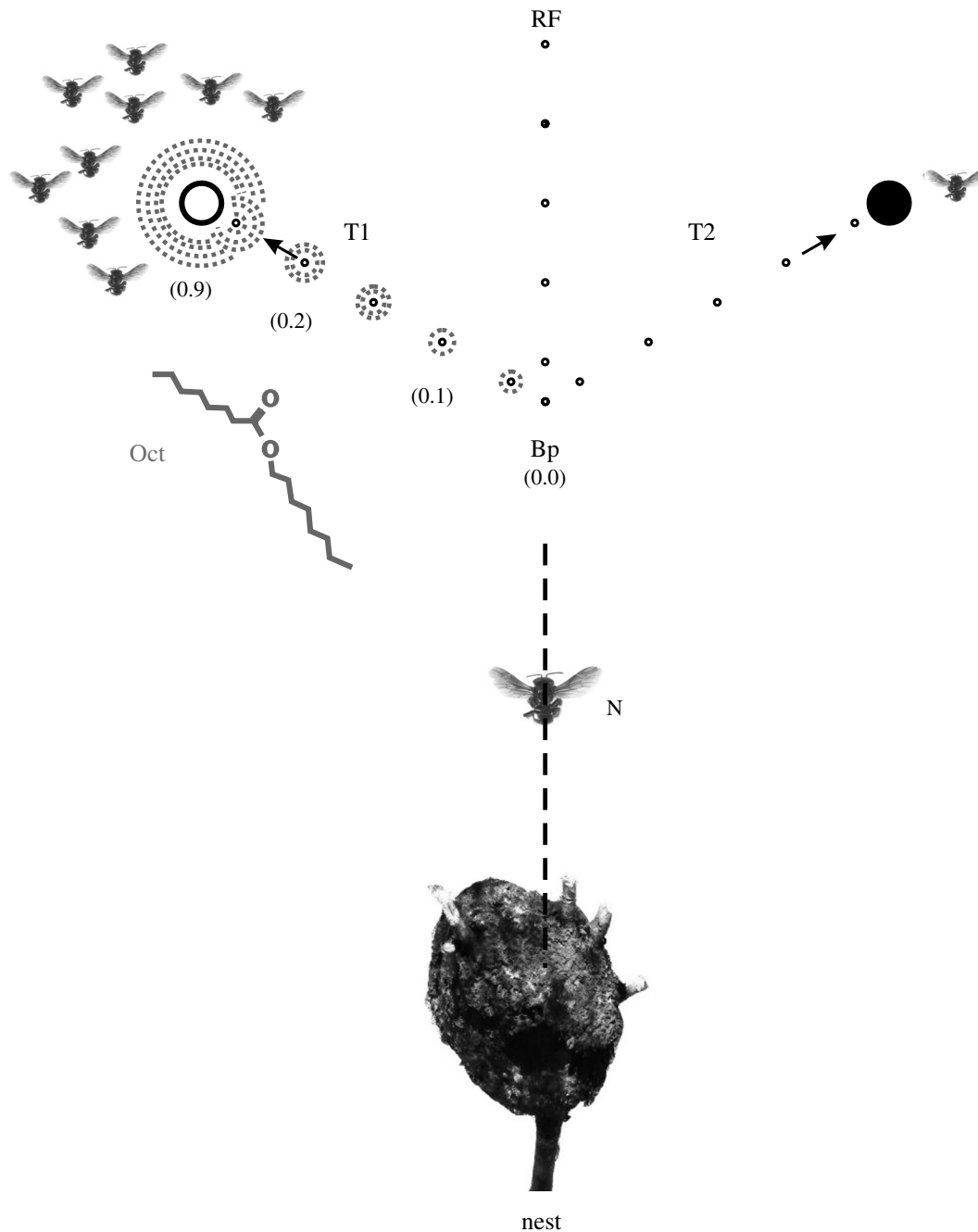


Figure 1. Trail following bioassays: two artificial scent trails (T1, T2) were laid (directions of T1 and T2 were switched in consecutive experiments), beginning at Bp, at some distance from the nest. Scents deposited along T1 and T2 were either the solvent pentane, pentane extracts of labial glands or synthetic octyl octanoate (dissolved in pentane). The amounts of the solutions forming the trails increased towards the respective feeders, where scent concentration was highest (see bee equivalents given in brackets). In the experimental set-up illustrated, T1 was scented with either octyl octanoate (dots; Oct) or labial gland extract (naturally containing Oct) leading to the feeder of T1 (open circle), while T2 was scented with equal amounts of pure pentane (no dots) leading to the feeder of T2 (filled circle). Significantly, more newcomers landed on the feeder at the end of T1 than on that of T2. Fifteen bees feeding at the recruitment feeder (RF) ensured the continued recruitment of newcomers (N) from the nest. A colour version is shown in figure 6 of the electronic supplementary material.

scent trails every 20 min. Any bee landing on the feeder of T1 or T2 was captured. When the newcomer (unmarked) bees landed on the feeder, there were no other individuals present. In this way, we avoided potential effects of local enhancement (Slaa *et al.* 2003). All the newcomers were marked with colour and included in the statistical analysis. Thus, every bee included in the statistics was only used once, avoiding pseudoreplication. Fifteen colour marked foragers were allowed to forage at RF to ensure the recruitment of newcomer bees. Any other bee landing on RF was removed from the experiment.

(b) *Statistics*

For normally distributed data of equal variance, we used the one-way ANOVA to test for significant differences in the percentages of bees that had landed on either of the two tested feeders. Tukey tests were applied for the pairwise multiple comparisons.

(c) *Chemical analysis*

Approximately 90% of the supposed scent trail markings in *T. spinipes* (Nieh *et al.* 2004) are deposited within 1 m from the food source (in most cases more than 50% at the feeder

itself). We therefore analysed the scent marks left by foragers in the immediate vicinity of the feeder, which was a clean Petri dish (diameter, 14–18 cm) supporting the actual feeding dish. Scent-marking bees always had stopped feeding before leaving a scent mark on the Petri dish (extending their glossae again). Therefore, we were sure that the cause of saliva-discharge on the Petri dish was scent marking and not food uptake. After the experiment, during which 15 recruiting foragers had scent marked ad libitum for 40 min, the feeder was removed and the Petri dish rinsed with 10 ml of the solvent (Pentane, HPLC-grade). The resulting solution was treated in the same way as the gland extracts. For the extraction (24 h at room temperature) of the glands, which were carefully cleaned from other tissues, we used pentane (HPLC-grade) as well. The extracts were reduced to 60 μ l. Internal standard substances (tetradecane and nonadecane) were used to quantify the amount of the detected substances. The percentage of octyl octanoate was calculated by comparing its peak area with the sum of all peaks (except peak areas less than 0.3% relative to the internal standard). Gas chromatographs (HP-5890, HP-GC6890A, Shimadzu GC-2010; carrier gas: hydrogen) with flame ionization detectors were used for quantitative analysis. For qualitative analyses, we used gas chromatography combined with mass spectrometry (Shimadzu GC-2010/GCMS-QP2010; Fisons Instruments GC 8000 series/MD 800; carrier gas: helium). The column was an Agilent DB-5MS column (30 m \times 0.25 mm, 0.25 μ m thickness), the temperature programme started at 50°C (5 min) and increased temperature by 10 °C min^{-1} up to 310°C (15 min). Compounds were identified by the comparison of mass spectra with literature data (Francke *et al.* 2000) and authentic reference substances. Octyl octanoate was obtained from Sigma-Aldrich (3050 Spruce Street, Saint Louis, MO 63103 US; Product Number W281107).

3. RESULTS

Pentane extracts of both the scent-marked substrate and the saliva contained octyl octanoate (saliva: 2.63 $\mu\text{g} \pm 0.45$ s.e.m.; $n=21$ foragers from $N=6$ nests), an ester, which turned out to be highly attractive for foragers searching for food (figure 2). We found octyl octanoate only in the labial gland parts of the salivary system, not however in either the mandibular or hypopharyngeal glands. In bioassays, synthetic octyl octanoate was used to lay scent trails whose attractiveness was simultaneously tested against another artificial trail consisting of a pentane extract of labial glands or of pure pentane (figure 1). To provoke enough newcomer bees (i.e. bees who had never fed at an artificial feeder before) to visit the artificial feeding site in search for food, we installed RF (figure 1). Clearly, newcomers chose to follow the trails scented with octyl octanoate (89.5% ± 2.7 s.e.m. out of a total of $n=1164$ foragers) and labial gland extract (90.3% ± 2.3 s.e.m. out of a total of $n=913$ foragers), respectively, and neglected the trail made of an equivalent amount of solvent (ANOVA, octyl octanoate trail versus solvent trail: $F_{1,10}$, $p < 0.001$; ANOVA, labial gland extract trail versus solvent trail: $F_{1,12}$, $p < 0.001$). As expected from these results, there was no preference when the bees had to choose between following an octyl octanoate trail and a labial gland extract trail (ANOVA: $F_{1,10}$, $p > 0.81$; $n=300$). Likewise, the synthetic compound and the natural extract proved to

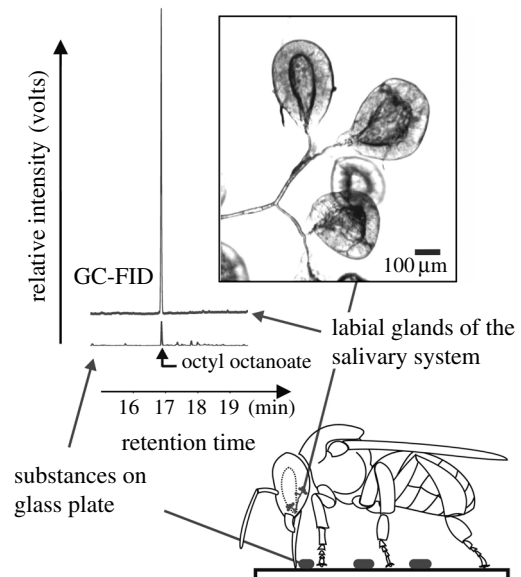


Figure 2. Substances inducing trail following: the recruiting forager deposits saliva from its labial glands on substrates outside the nest. Gas chromatography revealed that extracts of both the labial glands and the substances on the glass plate contain a substantial proportion of octyl octanoate (labial glands: 73.8% ± 2.6 s.e.m, $n=30$, $N=7$; substances on glass plate: 24.1% ± 6.8 s.e.m, $n=6$, $N=3$). A colour version of part of this figure is given in figure 4 of the electronic supplementary material.

be equally attractive when presented subsequently. We conclude that octyl octanoate represents the trail pheromone in *T. spinipes*.

4. DISCUSSION

More than 25 years ago, Kerr *et al.* (1981) pioneered meliponine scent trail communication biology by testing the effect of 2-heptanol (found in cephalic extracts of worker bees) when laid out to form an artificial scent trail in *Trigona* bees. Unfortunately, the results of the only one experiment done can easily be interpreted as providing evidence against 2-heptanol being a food source trail pheromone. The main reasons for this were already discussed earlier (Jarau *et al.* 2004). Our findings together with those of Jarau *et al.* (2004, 2006) demonstrate that it is the labial glands which produce trail pheromones in scent path laying stingless bees. Extracts of the labial glands are also highly attractive to newcomer bees in *Trigona recursa*, who follow artificial scent trails made of labial gland extracts as well (Jarau *et al.* 2004). While we present evidence for a single compound pheromone in *T. spinipes*, in *T. recursa* a blend of compounds not yet fully identified is necessary to induce the full intensity of trail following behaviour (Jarau *et al.* 2006). A particularly important component of this blend is hexyl decanoate, another ester of similar volatility as octyl octanoate. Obviously, different species of stingless bees use different pheromone compounds for communicating food source locations even when closely related to each other phylogenetically. This makes sense in situations when several species of stingless bees compete for the same resource and use similar foraging strategies and ways of communicating. Both species, *T. recursa* and *T. spinipes*, indeed occur sympatrically and both feed on nectar. By

showing that octyl octanoate represents the trail pheromone or at least its most significant component in *T. spinipes*, a widely distributed species of South America, we not only found a very promising model organism to experimentally study the significance of scent trails for the exploitation of food sources in stingless bees, but also a species to investigate the behavioural and neurobiological mechanisms of three-dimensional trail following in a day-flying insect.

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REFERENCES

- Francke, W. G. *et al.* 2000 Identification of oxygen containing volatiles in cephalic secretions of workers of Brazilian stingless bees. *J. Braz. Chem. Soc.* **11**, 562–571.
- Hölldobler, B. & Wilson, E. O. 1990 *The ants*. Berlin, Germany: Springer.
- Jarau, S., Hrncir, M., Zucchi, R. & Barth, F. G. 2003 Effectiveness of recruitment behavior in stingless bees (Apidae, Meliponini). *Insect. Soc.* **50**, 365–374. (doi:10.1007/s00040-003-0684-2)
- Jarau, S., Hrncir, M., Zucchi, R. & Barth, F. G. 2004 A stingless bee uses labial gland secretions for scent trail communication (*Trigona recursa* Smith 1863). *J. Comp. Physiol. A* **190**, 233–239. (doi:10.1007/s00359-003-0489-9)
- Jarau, S., Schulz, M., Hrncir, M., Francke, W., Zucchi, R., Barth, F. G. & Ayasse, M. 2006 Hexyl decanoate, the first trail pheromone compound identified in a stingless bee, *Trigona recursa*. *J. Chem. Ecol.* **32**, 1555–1564. (doi:10.1007/s10886-006-9069-0)
- Johnson, L. K. & Hubbell, S. P. 1974 Aggression and competition among stingless bees: field studies. *Ecology* **55**, 120–127. (doi:10.2307/1934624)
- Kerr, W. E. 1963 Communication among stingless bees—additional data (Hymenoptera: Apidae). *J. N. Y. Entomol. Soc.* **71**, 80–90.
- Kerr, W. E., Blum, M. & Fales, H. M. 1981 Communication of food source between workers of *Trigona (Trigona) spinipes*. *Rev. Bras. Biol.* **41**, 619–623.
- Lindauer, M. & Kerr, W. E. 1960 Communication between the workers of stingless bees. *Bee World* **41**, 29–41, see also 65–71.
- Michener, C. 1974 *The social behavior of the bees*. Cambridge, MA: Harvard University Press.
- Nagamitsu, T. & Inoue, T. 1997 Aggressive foraging of social bees as a mechanism of floral resource partitioning in an Asian tropical rainforest. *Oecologia* **110**, 432–439. (doi:10.1007/s004420050178)
- Nieh, J. C., Contrera, F. A. L., Yoon, R. R., Barreto, L. S. & Imperatriz-Fonseca, V. L. 2004 Polarized short odor-trail recruitment communication by a stingless bee, *Trigona spinipes*. *Behav. Ecol. Sociobiol.* **56**, 435–448. (doi:10.1007/s00265-004-0804-7)
- Pasteels, J. M. & Bordereau, C. 1998 Releaser pheromones in termites. In *Pheromone communication in social insects* (eds R. K. Vander Meer, M. D. Breed, M. L. Winston & K. Espelie), pp. 193–215. Boulder, CO: Westview Press.
- Schaffer, W. M., Zeh, D. W., Buchmann, S. L., Kleinhans, S., Schaffer, M. V. & Antrim, J. 1984 Competition for nectar between introduced honey bees and native North American bees and ants. *Ecology* **64**, 564–577. (doi:10.2307/1939976)
- Slaa, E. J., van Nieuwstadt, M. G. L., Pisa, L. W. & Sommeijer, M. J. 1997 Foraging strategies of stingless bees (Apidae, Meliponinae): the relation between precision of recruitment, competition and communication. *Acta Hort. (ISHS)* **437**, 193–198.
- Slaa, E. J., Wassenberg, J. & Biesmeijer, J. C. 2003 The use of field-based social information in eusocial foragers: local enhancement among nestmates and heterospecifics in stingless bees. *Ecol. Entomol.* **28**, 369–379. (doi:10.1046/j.1365-2311.2003.00512.x)
- Wille, A. 1983 Biology of the stingless bees. *Annu. Rev. Entomol.* **28**, 41–64. (doi:10.1146/annurev.en.28.010183.000353)
- Wyatt, T. D. 2003 *Pheromones and animal behaviour*. Cambridge, UK: Cambridge University Press.