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SHORT COMMUNICATION

Foraging scent marks of bumblebees: footprint cues rather than pheromone signals

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Abstract In their natural habitat foraging bumblebees refuse to land on and probe flowers that have been recently visited (and depleted) by themselves, conspecifics or other bees, which increases their overall rate of nectar intake. This avoidance is often based on recognition of scent marks deposited by previous visitors. While the term 'scent mark' implies active labelling, it is an open question whether the repellent chemicals are pheromones actively and specifically released during flower visits, or mere footprints deposited unspecifically wherever bees walk. To distinguish between the two possibilities, we presented worker bumblebees (*Bombus terrestris*) with three types of feeders in a laboratory experiment: *unvisited* control feeders, *passive* feeders with a corolla that the bee had walked over on its way from the nest (with unspecific footprints), and *active* feeders, which the bee had just visited and depleted, but which were immediately refilled with sugar–water (potentially with specific scent marks). Bumblebees rejected both active and passive feeders more frequently than unvisited controls. The rate of rejection of passive feeders was only slightly lower than that of active feeders, and this difference vanished completely when passive corollas were walked over repeatedly on the way from the nest. Thus, mere footprints were sufficient to emulate the repellent effect of an actual feeder visit. In confirmation, glass slides on which bumblebees had walked on near the nest entrance accumulated hydrocarbons (alkanes and alkenes, C23 to C31), which had previously been shown to elicit repellency in flower choice experiments. We conclude that repellent scent marks are mere footprints, which foraging bees avoid when they encounter them in a foraging context.

Keywords Footprints · Cuticular hydrocarbons · Repellent · Bees · Olfaction · *Bombus terrestris*

Introduction

Bumblebees forage in a dynamic mosaic of renewable resources in which experience is insufficient to predict the reward provided by an individual food item (a flower) at a given point in time. Nectar is secreted rather slowly and in tiny amounts by most bumblebee-visited flowers, which means that the entire standing crop can be harvested during a single visit, rendering the flower unrewarding for minutes or hours to come (Stout and Goulson 2002). Foraging bumblebees can regularly be observed to hover briefly in front of an individual flower, but then leave without landing and probing for reward. These rejected flowers contain on average less nectar than flowers which are probed (Marden 1984). Behavioural experiments have demonstrated that rejection is often not based on direct perception of reward (or lack thereof), but rather on the perception of scent marks deposited by previous visitors (Stout et al. 1998; Goulson et al. 2000). This indirect floral assessment improves the foraging efficiency of individuals by reducing time and energy spent probing depleted flowers (Schmitt and Bertsch 1990; Stout et al. 1998). The origin of the involved marking substances is somewhat unclear. The tarsal glands in the fifth tarsomer of bumblebees were long thought to be the origin of the scent marks (Schmitt et al.

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1991), which now seems uncertain in the light of chemical as well as morphological data (Oldham et al. 1994; Jarau et al. 2005). Whatever the secretory origin, the surface of bumblebee tarsi evidently *carries* behaviourally active substances: solvent extracts of tarsi, containing mostly simple hydrocarbons of uneven chain length (21–31 carbon atoms) (Schmitt et al. 1991), elicited repellency among foraging bumblebees when applied to flowers in the field (Goulson et al. 2000). Furthermore, application of single synthetic alkanes and alkenes present in tarsal extracts also repelled foraging bumblebees (Goulson et al. 2000), suggesting that they were the perceptually and behaviourally active compounds.

The term 'scent marking' implies active labelling, but it is entirely unclear whether bumblebee scent marks are specific pheromones actively released at flowers or unavoidable footprints deposited wherever the bees walk (Stout et al. 1998; Chittka et al. 1999). Notably, the repellent hydrocarbons are near ubiquitous on epicuticles of insects (Lockey 1988) and are known to occur in footprint secretions of beetles and locusts (Kosaki and Yamaoka 1996; Attygalle et al. 2000; Votsch et al. 2002). In the latter, the hydrocarbons are part of the liquid that mediates wet adhesion of tarsal attachment pads to smooth surfaces (Jiao et al. 2000). Tarsal hydrocarbons may serve a similar primary function in foraging bumblebees (see Federle et al. (2002) for the case of wet adhesion in ants).

In the natural habitat, bumblebee scent marks are recognized (and avoided) not only by foraging conspecifics, but also by other bumblebees, honeybees, and even solitary bees (Goulson et al. 1998; Stout et al. 1998; Gawleta et al. 2005). Acknowledging this, and given that bumblebees from several to many colonies/species mix at flower patches (Chapman et al. 2003; Darvill et al. 2004), the information contained in scent marks is practically 'open source' and not likely to predominantly benefit colony members. Thus, the evolution of active pheromone marking through colony level kin selection is difficult to visualize (Thomson and Chittka 2001). Instead, the potential adaptive value of depositing costly chemicals at flowers could be direct: individuals may signal to themselves that they have already depleted a given flower (Thomson and Chittka 2001), a behaviour that would minimize immediate revisits at sites with high floral density (e.g., multi-flower inflorescences).

In the present study, we ask whether repellent scent marks are pheromone signals actively released at flowers, or mere unspecific footprints (cues). We address this question by quantifying the degree of repellency that feeders elicited in worker *Bombus terrestris* depending on whether the feeders had been previously subjected to an actual foraging visit (1) or simply walked over by the bee on the way from the nest (2).

Material and methods

B. terrestris colonies (Koppert Biological Systems) were used for laboratory experiments. The colonies were fed with sugar-water supplied in permanently rewarding feeders in a feeding box. This feeding box was connected to the main nest box with a plexiglass tunnel (75 cm). Halfway through that tunnel, an aluminium frame could accept three experimental quartz-glass corollas for accumulation of bumblebee footprints (see below).

Choice experiments Individuals foraging in the feeding box were marked and later introduced into a novel foraging situation in a test cage (60×65×85 cm). In one end of the cage a disk made of grey PVC (Ø 60 cm) was fitted, which could be rotated around its central axis and had fittings for 20 feeders. Each feeder consisted of a cylindrical yellow quartz glass 'corolla' (4 cm length, Ø 2.1 cm) sitting on a Plexiglas cylinder with a 1.5 mm drilled bore for the sugar–water reward (test cage and feeders are described in detail in the work of Witjes and Eltz (2007)). Before each trial the feeder bores were filled with 2-µl sugar–water reward. To test whether scent marking is active or passive three types of feeders were presented to individual bees: *unvisited* feeders with clean corollas, *passive* feeders with a corolla that the test bee had walked through on its way from the nest immediately prior to the trial (see above), and *active* feeders which the bee had actually visited and probed after being introduced into the test cage, but which were immediately (manually) refilled with 2-µl sugar–water (potentially with active "scent mark") To allow refilling and installing the corollas the light was switched off for approximately 3 min, during which the bee sat on the floor of the test cage. Then, when the light was switched on again the bee took flight and faced an array with three active, three passive and 14 unvisited feeders, all carrying a single 2-µl sugar–water reward. Two microliters is only a small fraction of the full crop load of a worker bumblebee, stimulating the forager to visit several to many feeders. The foraging behaviour was recorded and the sequence of approached (numbered) feeders was logged with the help of the software clbehave (Compulights GmbH, Mönchengladbach). It was registered whether an approached feeder (defined as one that the bee had clearly targeted and inspected at a distance of less than 2 cm) was visited or rejected. A feeder was defined as visited if a bee crawled completely into the glass corolla probing for reward. Rejection included all approaches that were not followed by landing or, if a landing took place, it was brief and not followed by crawling into the corolla. After every feeder visit the array was turned randomly to disable the bee to memorize the position of a depleted flower. After 40 approaches the trial was stopped, and only the first

approach to a given feeder was included in the analysis. Before the next trial, all glass corollas were rinsed in acetone and dried in an oven at 50°C. Each individual bee completed several trials (10 to 29, on average 19.2) and the data from all trials of an individual were pooled to test for effects of feeder type on the individual frequency of rejection (using Fisher's exact test). Five individuals were tested for each of two series of the experiment. In the first series passive corollas were walked through once back and forth (similar to an actual feeder visit during which the bee crawls into the corolla and out again) by the test bee, and in the second series five times. *T* tests for paired samples were used to test for effects of feeder type on rejection rates on the population level.

Bumblebee footprints To analyze the chemical composition of bumblebee footprints on glass surfaces we used gas chromatography coupled with mass spectrometry (GC/MS). Microscopic slides with the same length as the glass corollas were positioned in the plexiglass tunnel and crossed 0, 10, 20, 30, 40, 50 times by bumblebee workers on their way to the feeding box. The glass slides were then extracted for 30 s in 400 µl *n*-hexane (p.a., Merck) containing 10-µl 2-undecanone as an internal standard (N=14), for each number of passes, except 0 (N=8) and 30 (N=15). For comparison, individual sets of tarsi of workers were also extracted for 30 s in 400-µl *n*-hexane. GC/MS was performed with a HP 5890 II GC fitted with a 30-m nonpolar DB-5 column and a HP 5972 mass selective detector. Injection was splitless, the oven programmed from 60 to 300°C at 10°C/min. Hydrocarbon contents were quantified based on internal and external (pentacosane) standards.

Results

All 10 individual workers rejected active and passive feeders significantly more often than unvisited controls during the choice experiments (Fisher's exact tests: $p<0.05$ in all cases). In the first series, when passive corollas were walked over only once, three out of five individuals rejected active feeders significantly more often than passive feeders ($p<0.05$). In the second series, when passive corollas were walked over five times, there was no significant difference in rejection frequency between active and passive feeders in any of the individuals. See the Electronic supplementary material (S 1) for graphs and statistical details of all ten tested individuals. On the population level, rejection rates of active and passive feeders differed significantly in first series (paired *t* test: $t_5=4.20$, $p=0.014$), but not in the second ($t_5=0.71$, $p=0.52$) (Fig. 1).

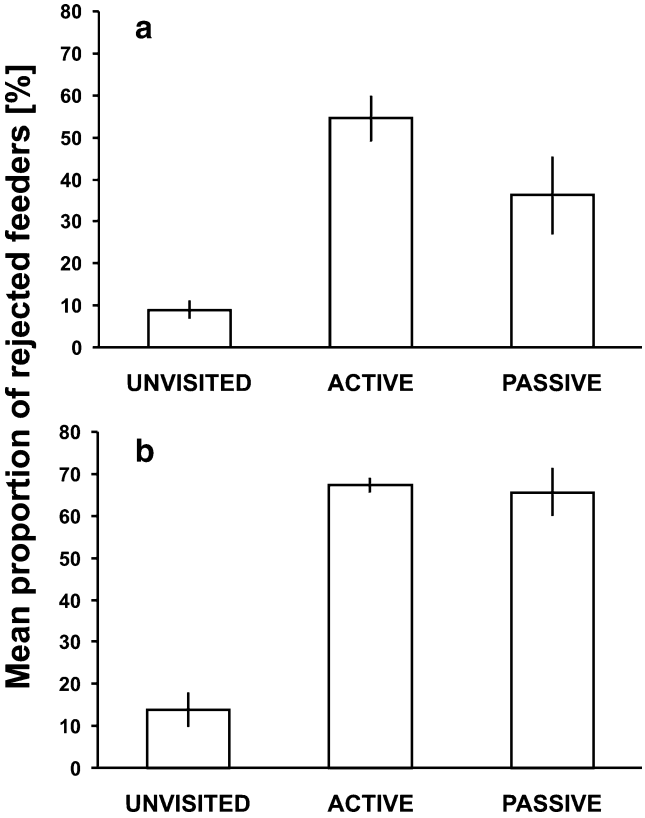

Fig. 1 Rejection of three types of feeders by worker *B. terrestris* during two series of choice experiments (N=5 per series, standard deviation). In the first series passive corollas where walked through once on the way from the nest (**a**) and in the second series five times (**b**)

Hexane washes of microscopic slides contained straight saturated and monounsaturated hydrocarbons of uneven chain length (21 to 31, Fig. 2a), very similar in composition to tarsal washes of *B. terrestris* workers (Fig. 2a; see also Goulson et al. (2000)). The total amount of hydrocarbons deposited on the slides was highly variable between samples. However, there was a significant correlation with the number of bumblebee passes (Spearman rank order correlations: $r_{79}=0.61$, $p<0.001$; Fig. 2b). Individual bees deposited on average 5.6 ng of hydrocarbons per pass (linear regression: $y=5.6x+5.9$).

Discussion

Our results demonstrate that foraging bumblebees are repelled by their own footprints when these are presented to them in a foraging context. This finding is consistent with the view that repellent scent marks of foraging bumblebees are unspecific footprints that represent simple cues to foragers. To further strengthen that conclusion, our data emphasize the importance of quantitative rather than qualitative aspects of chemical deposition. Active feeders were slightly more repellent than passive feeders during the

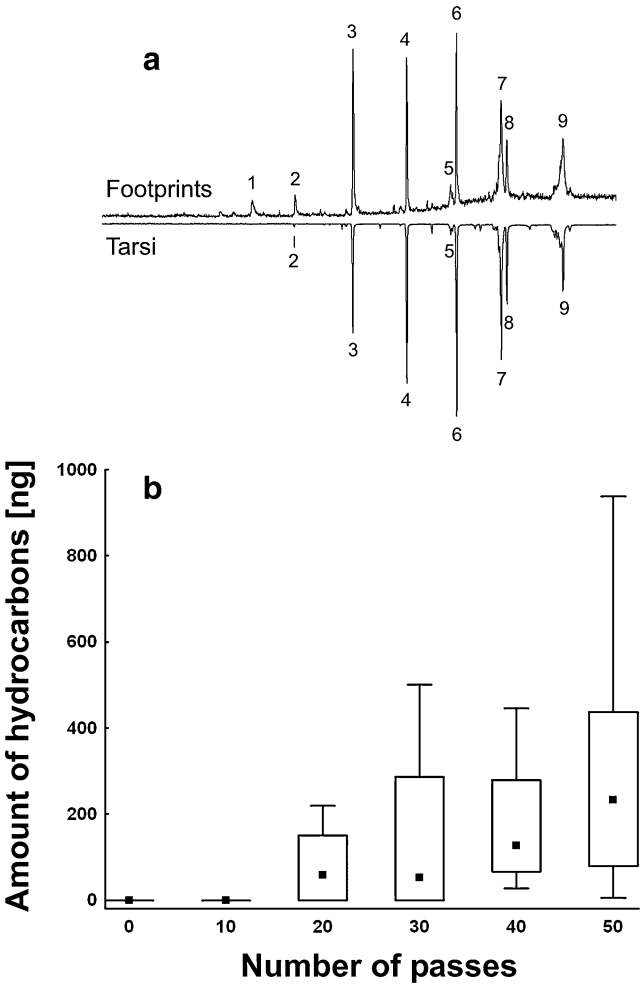

Fig. 2 **a** Ion chromatograms of hexane wash of microscopic slide that was walked over 50 times by *Bombus terrestris* workers in the nest entrance tunnel (*top*) and hexane extract of the tarsi of an individual worker (*bottom*). Peak assignment: *1* contamination, *2* heneicosane, *3* tricosane, *4* pentacosane, *5* heptacosene, *6* heptacosane, *7* nonacosene, *8* nonacosane, *9* untriacontene (ISTD internal standard). **b** Amount of total hydrocarbons in microscopic slide washes in relation to the number of times the slides were passed by bees in the nest entrance tunnel (median, quartile range, and non-outlier range)

first series of choice experiments, which could be because of greater deposition of footprint chemicals during an actual probing visit as compared to a walk-through. This again could be because of the following behaviours. First, when bees landed on a corolla before actively probing a feeder they frequently grappled on the edge of the corolla, struggling to get a hold on the glass surface. Second, after probing the feeder bumblebees had to turn around *inside* the narrow corolla. Both movements likely caused extensive contact of tarsal attachment pads with the corolla and presumably extensive deposition of footprint substances. In contrast, a walk-through of passive feeder corollas was always quick, no grappling was necessary, and bees turned around *outside* of the corolla (for the backwards walk-through). Thus, passive corollas may have carried smaller amounts of bee-derived substances than active corollas during the first series of choice experiments. In the second series, passive corollas were walked through five times instead of once and this caused rejection rates to become equivalent to those of active feeders. It is tempting to conclude that this was because of an equalization of the amount of footprint substances on the glass surface.

Chemical analysis of solvent washes showed that long chain hydrocarbons similar to those found in tarsal washes had accumulated on walked-over glass surfaces, which explains the observed repellency of passive feeders. Previous behavioural experiments in the field have demonstrated that certain hydrocarbons (heneicosane, tricosane, pentacosane, heptacosane, and Z9-tricosene), when applied to flowers, were perceived and avoided by foraging *Bombus lapidarius* (Goulson et al. 2000). In another field study we found that tarsal hydrocarbons of *Bombus pascuorum* workers were present on visited deadnettle flowers, accumulating linearly with increasing numbers of visits (Eltz 2006). Thus, a chain of direct and indirect evidence points to passively deposited footprints as the source of scent cues for foraging bees. It should be noted that our findings do not generally preclude the possibility of active release of repellent pheromones in some situations, but for this there exists no evidence. Also, it is possible that traces of pheromonal compounds retained on the body surface from previous (active) exposures in other situations (e.g., the recruitment pheromone exposed in the nest, see Dornhaus et al. 2003) contribute to the olfactory effect of footprints. Such synergism would be in agreement with a generally passive deposition of foraging scent marks.

Facultative interpretation of unspecific footprint cues can explain reversed ‘scent mark’ effects. In laboratory experiments scent marks were found to be either repellent (Witjes and Eltz 2007) or attractive (Cameron 1981; Schmitt and Bertsch 1990), depending on whether the reward in feeders could be depleted during a single visit or not. These behavioural differences could certainly be because of flower marking with chemically different ‘attractant’ or ‘repellent’ pheromones. However, a much more parsimonious explanation is that the same chemical cue (footprint) can adopt opposite meanings for foragers because of negative or positive conditioning (Saleh and Chittka 2006; Witjes and Eltz 2007). Chemical cues inherent to footprints are used in variable contexts by other Hymenoptera. For example, stingless bees leave chemicals on Plexiglass on which they have walked, and these substances attract other workers when presented in a rewarding context (at a permanent feeder; Schmidt et al. 2005). Similarly, returning yellowjacket and honeybee foragers follow accumulated footprints of nest mates when these are presented in a “homing context”, e.g., within the nest entrance tunnel (Butler et al. 1969; Jandt et al. 2005). We suspect that

responses to simple chemical cues (such as cuticular hydrocarbons) are frequently, and incorrectly, taken as evidence for evolved pheromonal communication in insects.

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