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Tracing Pollinator Footprints on Natural Flowers

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Abstract Many insects are known to leave lipid footprints while walking on smooth surfaces. Presumably, the deposited substances improve tarsal adhesion. In bumblebees, footprint hydrocarbons also function as scent marks that allow detection and avoidance of recently depleted flowers. I used GC-MS to detect hydrocarbons deposited by bumblebee (*Bombus pascuorum*) on flowers of *Lamium maculatum*. In addition to the plants' own cuticular lipids, extracts of corollas that had been visited by bumblebees contained odd-numbered alkenes. The amount of pentacosenes ($C_{25}H_{50}$) on corollas was linearly related to the number of bumblebee visits, with workers depositing approximately 16 ng per visit (extrapolated to a total of 65 ng of bumblebee cuticular hydrocarbons). Pentacosenes were retained on visited flowers without loss for 2 hr, and probably longer. This and results from flight cage experiments suggest that flower epicuticles retain a chemical record of pollinator visitation, including information on visiting bee species. Continuous footprint accumulation necessitates new explanations concerning the reversibility of “repellent scent marks” of bumblebees.

Keywords Footprints · Cuticular hydrocarbons · Hydrocarbon signature · Scent marks · *Bombus* · *Lamium*

Introduction

Insect cuticular lipids serve a range of functions, including reducing evaporative water loss over the body surface as well as improving adhesion for movement on smooth surfaces (Lockey, 1988; Jiao et al., 2000). Additionally, the same lipids may constitute chemical signals for conspecifics, e.g., by mediating mate recognition or relating information on reproductive status (Blomquist et al., 1998). In foraging

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bees, especially bumblebees (*Bombus* sp.), cuticular lipids are thought to function as scent marks that allow foragers to detect and avoid flowers that have been previously visited and depleted (Goulson et al., 1998, 2000, 2001; Stout et al., 1998; Williams, 1998; Gilbert et al., 2001; Gawleta et al., 2005). When approaching an inflorescence, bumblebee workers briefly hover in front of a depleted flower, but then reject it without actually probing for the concealed nectar reward. Behavioral experiments (Stout et al., 1998; Goulson et al., 2000) have indicated that the remote assessment of flowers is not based on a direct perception of nectar, but rather indirectly on the perception of hydrocarbon marks deposited by previous visitors. Flowers treated with hexane extracts of bumblebee tarsi elicited a repellent effect similar to that of a natural bumblebee visit, with nectar levels remaining unaltered. Similar results were obtained when researchers applied several pure synthetic hydrocarbons (*n*-alkanes, alkenes), the main contents of cuticular lipids of bumblebees (Schmitt, 1990; Oldham et al., 1994; Goulson et al., 2000), to flowers (Goulson et al., 2000). It is unclear whether the deposition of repellent substances is an active process that merits the term “scent marking” or whether the deposits are unavoidable “footprints” (Thomson and Chittka, 2001; Gawleta et al., 2005). A further corollary is that the repellent effect is only temporary. In most cases, repellency of visited flowers is lost after 20–60 min, which has been attributed to a progressive evaporation of the deposited substances (Stout et al., 1998; Stout and Goulson, 2002).

So far, no attempts have been made to directly measure bumblebee chemical traces on natural flowers. Schmitt et al. (1991) analyzed filter paper attached to artificial feeders of *B. terrestris* laboratory colonies and found cuticular hydrocarbon profiles that were similar to those of extracts of *B. terrestris* tarsi. The same substances might be detectable on real flower petals after natural visits of bumblebees. In this case, hydrocarbon signatures of bees would have to be discernable within the hydrocarbon profile of the plant (e.g., Griffiths et al., 1999). In the present study, I compared the cuticular hydrocarbons of the common Deadnettle *Lamium maculatum* (Lamiaceae) with those of its bumblebee visitors. I asked whether bumblebee hydrocarbons are detectable on visited flowers, and explored the temporal dynamics of footprint accumulation.

Methods and Materials

The study was conducted in patches of *L. maculatum* growing in the University of Düsseldorf Botanical Gardens. At the time of the study (August 10 to September 3, 2004), the flowers were mostly visited by workers of *Bombus pascuorum*. A 1 × 1 m area of the patch was covered by a mosquito mesh tent to prevent bees from accessing these flowers.

Hydrocarbon Contrast between Bees and Flowers

The following samples were collected for GC-MS: (1) individual sets of tarsi of worker *B. pascuorum* (cut at the distal end of the tibia, combining all six legs per individual sample; $N = 6$), (2) corollas of screened unvisited inflorescences ($N = 8$), and (3) corollas of screened inflorescences that were immediately presented to for-

aging bumblebees, allowing exactly one visit per corolla ($N = 7$). For items (2) and (3), five corollas from the same inflorescence were combined in one sample. Corollas were removed by tearing them from the receptacle with clean forceps; anthers were removed at the base with scissors. Every sample was extracted for 30 sec in 1 ml *n*-hexane (p.a., Merck) containing 10 μg 2-undecanone as internal standard. In the laboratory, the solvent was reduced to 0.15 ml at room temperature.

Footprint Accumulation on Visited Flowers

Unvisited inflorescences were cut, placed individually in water-filled vials, reduced to five open flowers, and presented to bumblebees in the field. Within 15 min, 0, 5, 10, 15, 20, 25, or 30 bee visits were allowed per inflorescence, corresponding to 0 to 6 visits per flower. Larger numbers of visits were increasingly difficult to induce, because the bees strongly rejected these inflorescences. Two series were done synchronously. In one of the series, the corollas were extracted in hexane immediately after the 15-min visitation time window. In the other, the inflorescences were placed in an insectary (not accessible to foraging bees) for 2 hr and then extracted. The same procedure was replicated three times, resulting in $7 \times 2 \times 3 = 42$ samples. For a given sample, all five flowers of the inflorescence were combined and extracted for 30 sec.

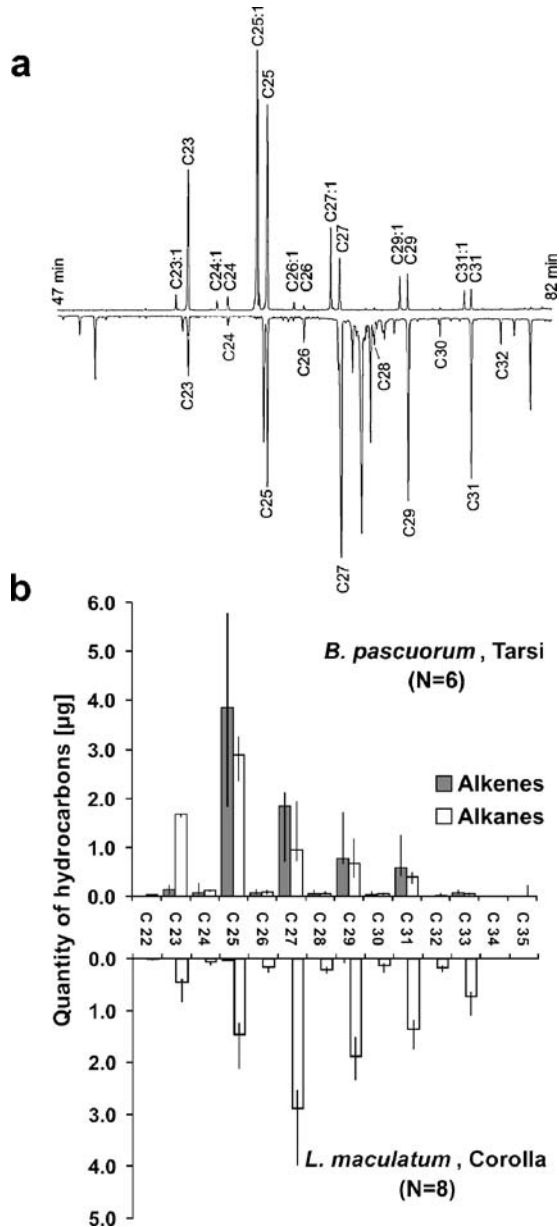
Bee-specific Hydrocarbon Signatures

Worker bumblebees were introduced into a mosquito mesh tent covering approximately 150 unvisited open *Lamium* flowers. Five bees were introduced between 08:30 and 09:30 in the morning and allowed to forage during the day. At 16:00 hr, bees were removed, and three replicate samples of five corollas each were extracted (as above). The experiment was done three times on consecutive occasions, once with *B. pascuorum*, once with *B. hortorum*, and once with *B. terrestris* workers. After an experiment with a given species was completed, all open corollas were removed from stalks with forceps and the next experiment started 2 or 3 d later when new (unvisited) flowers had opened in the tent. Tarsal extracts were taken from individuals of each of the bumblebee species ($N = 3$ per species; tarsi of all six legs pooled per sample). Effects of the (visiting) bee species on alkene composition of tarsal and corolla extracts were analyzed by using nonparametric ANOSIM permutation tests (Clarke, 1999; Clarke and Gorley, 2001). A similarity matrix based on the Bray–Curtis index (Legendre and Legendre, 1998) was calculated based on standardized data. I tested whether the factor (*visiting*) *bee species* had an effect on the rank order of between-sample similarities, separately for tarsal and corolla extracts. I also tested whether *sample type* (bee tarsi, corolla) had an effect.

Chemical Analysis

Gas chromatography-mass spectrometry (GC-MS) was performed with an HP 5890 II GC fitted with a 30-m nonpolar DB-5 column and an HP5972 mass selective detector. Injection was splitless, the oven programmed from 60 to 300°C at 3°C/min with automatic pressure programming. Characterization of hydrocarbons was done by comparison with authentic standards. Quantifications of hydrocarbon contents were based on internal (2-undecanone) and external (pentacosane) standards.

Fig. 1 (a) Total ion chromatograms of hexane extracts of *B. pascuorum* tarsi (on top) and unvisited *L. maculatum* corollas (bottom). *n*-Alkanes and alkenes are labeled and numbered with reference to the number of carbon atoms and double bonds. Unlabelled peaks in *Lamium* extracts are lipids that were not characterized. (b) Amount of hydrocarbons (median and quartile range) in tarsal and corolla extracts. Quantifications are given per individual set of tarsi ($N = 6$) and per corolla ($N = 8$), respectively. On average ($N = 6$), tarsal extract contained $8.02 \pm 2.49 \mu\text{g}$ alkanes and $8.47 \pm 3.51 \mu\text{g}$ alkenes. *n*-Pentacosane (C25) and pentacosenes (C25:1) were consistently the most abundant components, representing 19.4% and 24.0% of total individual hydrocarbons, respectively. Corolla extracts contained on average ($N = 8$) $12.27 \pm 6.44 \mu\text{g}$ alkanes (C:22 to C:33)



Results

Hydrocarbon Contrast between Bees and Flowers

Tarsal extracts of *B. pascuorum* were characterized by a series of *n*-alkanes and corresponding alkenes between C22 and C31, with odd-numbered homologs prevailing, confirming the results of an earlier investigation (Goulson et al., 2000)

(Fig. 1a, b). Corolla extracts of unvisited *L. maculatum* also contained large quantities of *n*-alkanes, but no or only traces of the respective unsaturated alkenes ($0.12 \pm 0.17 \mu\text{g}$; Fig. 1b). They also contained a range of additional lipids that were not further characterized (Fig. 1a). Four odd-numbered alkenes (C25:1 to C31:1) were well-defined markers for bumblebees. In comparison to unvisited controls, these alkenes were more abundant in samples of flowers that had received one visit per flower (Mann–Whitney *U* test: $P < 0.001$ for C25:1 and C31:1, $P < 0.01$ for C27:1, and $P < 0.05$ for C29:1; $N = 15$). As in *B. pascuorum* tarsal extracts, pentacosenes (C25:1) were the dominant alkenes on visited flowers.

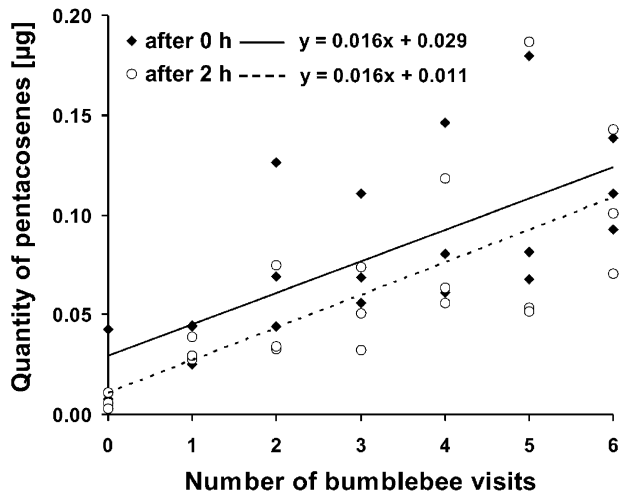
Footprint Accumulation on Visited Flowers

There were significant positive effects of the number of visits on the amount of pentacosenes extracted from corollas, both when extraction was immediate or delayed (Fig. 2). Although samples with delayed extraction contained slightly lower amounts of pentacosenes, this effect was not significant (one-way ANOVA: $N = 42$; $F = 1.40$; $P = 0.24$). Inclusion of the number of visits as a covariate did not change this result ($N = 42$; $F = 2.84$; $P = 0.10$). Judging from the slopes of the linear regressions in Fig. 2, individual bumblebees deposited on average 16 ng pentacosenes per flower visit.

Bee-specific Hydrocarbon Signatures

Extracted tarsal alkenes differed qualitatively and quantitatively between different species of bumblebees (ANOSIM: $N = 9$; $R = 1$, $P < 0.01$), as did alkenes extracted from corollas visited by the different species of bumblebees ($N = 9$; $R = 1$, $P < 0.01$). There was no overall difference of alkene composition between tarsal extracts and visited corolla extracts ($N = 18$; $R = 0.043$; $P = 0.58$), with alkene profiles extracted from corollas closely corresponding to those of the visiting species of bumblebee (Fig. 3). The chain length of the dominant alkene in deposited footprints increased

Fig. 2 Relationship between the number of bumblebee visits per flower and the amount of pentacosenes extracted from corolla samples per flower. Black diamonds represent corolla samples that were extracted immediately after bumblebee visitation (linear regression: $N = 21$; $R^2 = 0.50$; $t = 4.39$, $P < 0.001$). Circles are corolla samples that were extracted after a delay of 2 hr ($N = 21$; $R^2 = 0.53$; $t = 4.66$, $P < 0.001$)



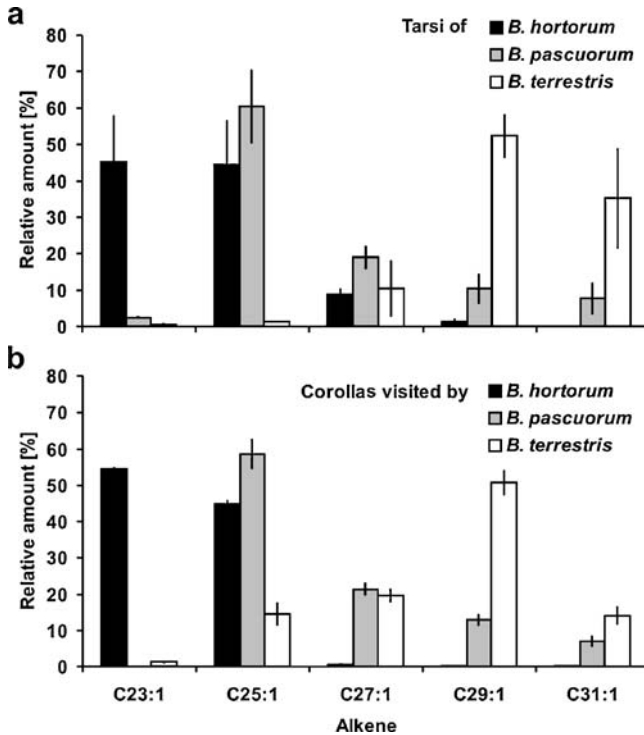


Fig. 3 (a) Alkene profiles of tarsal extracts of three species of bumblebees ($N = 3$ per species) and (b) of corolla extracts of *Lamium maculatum* visited by the three bumblebee species ($N = 3$ per visiting bee species). The mean and standard deviation are given

from tricosenes in *B. hortorum*, to pentacosenes in *B. pascuorum* and nonacosenes in *B. terrestris*.

Discussion

This is the first study to trace bumblebee “footprints” on natural flowers. Detection in corolla extracts was possible because several straight-chain alkenes served as chemical “bumblebee markers,” as they occurred abundantly on bee tarsi but not on unvisited *Lamium* flowers. Extrapolating from these markers to the entire set of tarsal hydrocarbons (see Fig. 1, assuming that deposition occurs in equal proportions), individual workers deposited approximately 65 ng of hydrocarbons per visited flower.

The origin of the deposited substances is as yet unknown. Schmitt et al. (1991) as well as the present study found that deposited substances were similar in composition to those in tarsal extracts, suggesting that tarsal glands situated in the fifth tarsomer are the secretory source (Schmitt, 1990; Schmitt et al., 1991). However, the tarsal gland reservoir has no opening to the outside in any of the several investigated bee species, which has cast doubt on their involvement in scent marking (Jarau et al., 2005). Furthermore, the hydrocarbons found on bumblebee tarsi can

also be found in similar composition on the cuticle of other body parts (Oldham et al., 1994; Goulson et al., 2000), suggesting that they are part of the hydrocarbon layer covering the entire body surface (Oldham et al., 1994). While probing *Lamium* flowers, bumblebees touch the corolla not only with their feet but also with the head, thorax, and abdomen, thus exposing the plant to a broad surface area of cuticle from which hydrocarbons may be acquired. This may be responsible for the rather large amounts of bumblebee hydrocarbons found on *Lamium* corollas in the present study.

The linear relationship between the amount of deposited pentacosenes and the cumulative number of visits per flower suggests that deposition is not reward-dependent and, therefore, probably passive (see Thomson and Chittka, 2001). Nonetheless, the depositions may be used as cues by foragers that enable them to detect recently depleted flowers (cues rather than signals; see Schmidt et al., 2005 for an example with an attractive effect). An unresolved problem exists concerning the temporal dynamics of the repellent response. In field studies with bumblebees, depleted flowers regain their original attractiveness after relatively short periods of time, in most cases between 20 and 60 min following a visit, depending on plant species and situation (Stout et al., 1998; Williams, 1998; Stout and Goulson, 2002; personal observation). It has been hypothesized that loss of repellency is observed because scent marks evaporate. In conflict with this idea, the amount of pentacosenes on visited flowers was not reduced by a 2-hr time delay before extraction, although longer delays might produce such effects. Generally, long-chain hydrocarbons such as those on bumblebee tarsi are of low volatility. This point is well illustrated by a study of Ginzler and Hanks (2002) that used combinations of synthetic alkanes (C24 to C31) to mark elytra of milkweed beetles. The profiles remained unchanged in quality and quantity over weeks, despite exposure to direct sunshine (Ginzler and Hanks, 2002). Thus, it seems unlikely that evaporation or molecular break down leads to a rapid vanishing of bumblebee footprints from flower corollas.

Why then is repellency reversed? Two alternative explanations seem plausible. First, bumblebee hydrocarbons accumulate, but are only slowly incorporated into the semiliquid layer of plant epicuticular lipids (see Jetter et al., 2000), rendering them increasingly imperceptible to foraging bees. Alternatively, bumblebee footprints may contain small quantities of low-molecular-weight volatiles that have not been detected in hexane extracts. Perception of such compounds could modify the behavioral response elicited by the hydrocarbons alone. Future studies will have to address this issue.

The present investigation shows that detection and quantification of bumblebee footprints is relatively simple and straightforward by using solvent extraction of flowers and GC. Probably the approach is also applicable to other plant–bumblebee systems. Cuticular hydrocarbons of bumblebees typically contain odd-numbered alkenes, with species-specific differences in chain length distribution (Goulson et al., 2000; this study). On the other hand, corolla extracts of bee-pollinated plants lacked these alkenes (Griffiths et al., 1999, 2000; Goodwin et al., 2003; Eltz, unpublished data for *Symphytum officinale* and *Linaria vulgaris*), thus providing an unobscured background for extractive footprint detection. The finding that footprint hydrocarbons accumulate on petals without loss suggests that flowers carry a complete chemical record of bumblebee visitation, including information on visitation frequency and composition of the pollinator community. Reading this record by means of chemical analysis may constitute a powerful tool for pollination ecologists, re-

ducing time and manpower necessary for multireplicate field studies. Pilot studies will have to evaluate the feasibility and scientific potential of such an approach.

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