J Chem Ecol (2009) 35:1320–1325
DOI 10.1007/s10886-009-9720-7

Hydrocarbon Footprints as a Record of Bumblebee Flower Visitation

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Received: 5 August 2009 / Revised: 16 November 2009 / Accepted: 25 November 2009 / Published online: 15 December 2009

Abstract Bumblebees leave traces of cuticular hydrocarbons on flowers they visit, with the amount deposited being positively related to the number of visits. We asked whether such footprint hydrocarbons are retained on flowers for sufficiently long periods of time so as to reflect bee visitation in pollination studies. In laboratory experiments, flower corollae (*Primula veris*, *Digitalis grandiflora*) visited by *Bombus terrestris* workers retained bee-derived nonacosenes ($C_{29}H_{58}$) in near-unchanged quantities for 24 hours, both at 15 and 25°C. Additionally, synthetic (*Z*)-9-tricosene applied to flower corollae of the deadnettle *Lamium maculatum* was retained for 48 hours in an unchanged quantity. In a field survey, the amount of footprint alkenes on flowers of comfrey (*Symphytum officinale*) plants was positively correlated with the number of bumblebee visits that those plants had received during the day. Together, these data suggest that flowers retain a long-term quantitative record of bumblebee visitation. The analysis of petal extracts by gas chromatography could provide a cheap and reliable way of quantifying bumblebee visits in landscape scale studies of pollination.

Keywords Cuticular hydrocarbons · Cuticular lipids · Footprints · *Bombus* · Scent-marks · Flower visit · Pollination · Pollinator decline

Introduction

The cuticle of insects is covered by a hydrophobic layer of lipids, consisting mostly of long-chain hydrocarbons (Lockey 1988). These epicuticular lipids probably evolved originally as a means for preventing water-loss in terrestrial habitats, but many secondary functions, including tarsal adhesion (Lockey 1988; Jiao et al. 2000; Drechsler and Federle 2006) and communication, are known. Cuticular hydrocarbons play an important role in nest mate recognition in social insects (Lahav et al. 1999; Ruther et al. 2002; Dani et al. 2005), as well as in relating information concerning reproductive status within colonies (Bonavitacougourdan et al. 1991; Liebig et al. 2000; Sledge et al. 2001; Howard and Blomquist 2005).

Cuticular hydrocarbons also provide footprint cues that allow wasps and bees to recognize their nest entrance at close range (Butler et al. 1969; Hefetz 1992). Similarly, footprint hydrocarbons are informative to foraging bees, which use them to discriminate against recently visited (depleted) flowers (Stout et al. 1998; Gilbert et al. 2001; Goulson et al. 2000, 2001; Gawleta et al. 2005). Whereas this discrimination behavior originally was believed to depend on active deposition of lipid "scent-marks" by the bees, two recent studies suggest that chemicals are deposited wherever the bees walk, and are footprint cues rather than pheromonal signals. *Bombus terrestris* workers deposited the same compounds, mostly long chain alkanes and alkenes, in essentially the same concentrations at food, nest, and neutral sites (Saleh et al. 2007). Footprint chemicals collected from neutral surfaces or feeders elicited similar repellent effects, when presented simultaneously in a foraging situation (Wilms and Eltz 2008). These findings strongly suggest that hydrocarbon marks are deposited involuntarily, regardless of the behavioral context (Witjes and Eltz 2007).

The origin of the deposited lipids is somewhat unclear and may involve several glands (Oldham et al. 1994). Solvent extracts of various parts of the cuticle (tarsi, antennae) and Dufours' glands are dominated by saturated

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and unsaturated hydrocarbons (Schmitt 1990; Oldham et al. 1994), mostly the same odd-numbered C21 to C31 ones (Schmitt 1990; Goulson et al. 2000; Saleh et al. 2007), suggesting that lipids from diverse body parts mix on the cuticle surface (Oldham et al. 1994). While probing for nectar, foraging bees touch flower corollae with various body parts, including the tarsi, and, thus, traces of cuticular lipids may pass onto the flower surface. Eltz (2006) speculated that the epicuticular wax of flower corollae could retain a record of past bumblebee visits. In a controlled garden experiment, flowers of the deadnettle, *Lamium maculatum*, with different numbers of visits by worker bumblebees (*Bombus pascuorum*), were analyzed by gas chromatography/mass spectrometry (GC/MS). Several odd-numbered alkenes, in addition to the plants' own cuticular lipids (mostly saturated alkanes), were identified. Pentacosenes (C25:1) were the clearest *B. pascuorum* markers. The quantity of pentacosenes in corolla washes increased positively and linearly with the number of visits that a flower had received. Furthermore, the amount of pentacosenes left on corollae did not change for two hours following the last bumblebee visit (Eltz 2006), thus suggesting that these bee lipids could serve as a long-term information store of bee visits.

In the present study, we further investigated this phenomenon by extending the time scale over which footprint retention is measured and by investigating the extent that retention is affected by environmental variables. We also conducted a field survey that tested the hypothesis that the amount of bumblebee footprint chemicals obtained from corollae of wild comfrey (*Symphytum officinale*) is indicative of the number of bumblebee visits. We show that the alkene amount on flower corollae can be used as a predictor of visit frequency, even in a natural, dynamic foraging environment.

Methods and Materials

All laboratory experiments were conducted in a climate chamber at the Department of Sensory Ecology at the University of Düsseldorf. *Bombus terrestris* colonies (Koppert Biological Systems) were maintained in a nest box (30×30×20 cm), which was connected to a feeding box (40×40×80 cm) via a Plexiglas tunnel (75 cm long). The colonies were fed with 20 ml sugar syrup (ApiInvert®) each day, provided in plastic syringes (5 ml). Pollen was supplied *ad libitum* directly into the nest box. The observation of bumblebee visits on flowers of wild comfrey (*S. officinale*) took place on the 29th July 2007 in pastures and meadows near Himmelgeist and Urdenbach, south of Düsseldorf.

Footprint Accumulation and Retention Under Different Ambient Temperatures Worker *B. terrestris* were allowed to forage on flowers of potted foxglove (*Digitalis grandiflora*; in 2007) or cowslip (*Primula veris;* in 2008) placed in the feeding box. Visits to individual flowers were recorded with the help of a computer and the software clbehave (Compulights 2005). To maintain high attractiveness of the flowers, small amounts of ApiInvert® were pipetted into the corollae at regular intervals.

The number of visits to individual flowers of *D. grandiflora* (2007) was manipulated and varied gradually between 31 and 51, whereas flowers of *P. veris* (2008) were allowed fixed numbers of 0, 20, or 40 visits. In both years, we tested how long the visited flowers retained deposited alkenes by taking corolla samples after 0, 6, and 24 h following the last bumblebee visit. The experiment was replicated under two different ambient temperatures (15 and 25°C) in both years, to test for effect of temperature on alkene retention. Individual flower corollae were removed from the receptacle with clean forceps, and anthers cut off at the base with scissors. Each corolla was extracted for 30 sec. in 500 µl *n*-hexane (p.a., Merck) containing 10 µg of 2-undecanone as an internal standard. The extracts were stored at 2°C until GC/MS analysis (see below). We also analyzed samples of bumblebee cuticular lipids. For this, we randomly sampled workers from the experimental colonies and cut off their tarsi at the proximal end of the femur. All six tarsi of an individual were combined and extracted in the same way as corollae.

We tested for effects of time since the last visit and ambient temperature on the amount of bumblebee-derived nonacosenes (C29:1) in corolla washes. For *D. grandiflora* (2007), in which the flowers had received varying numbers of visits, we performed an analysis of covariance (ANCOVA) in SPSS 15. Time (0, 6, and 24 h) and temperature (15 and 25°C) were specified as factors, and the number of visits received per flower (31–51) was the covariate. For *P. veris* (2008), we used an analysis of variance (ANOVA) and tested for effects of the factors, time (0, 6, and 24 h), temperature (15 and 25°C) and number of visits (0, 20, and 40), on the amount of nonacosenes.

Retention of Synthetic (Z)-9-Tricosene on Flowers Tricosenes are among the most dominant components in the cuticular lipids of bumblebees (e.g., Goulson et al. 2000). The (Z)-9-isomer has, among other hydrocarbons, been detected in footprint deposits of bumblebee workers (Schmitt et al. 1991; S. Witjes, unpublished data). Potted deadnettles were introduced into a climate chamber and habituated to an ambient temperature of 25°C. We applied 0.1 µl of (*Z*)-9-tricosene (Aldrich, Milwaukee, WI, USA), as a model compound, to unvisited flower corollae using a 5 µl Hamilton syringe. The syringe was connected to an assembly micrometer gauge (Holex, Munich, Germany) to facilitate adjustment of the exact volume. Corolla samples

either were taken immediately (0 h treatment), or after 24 or 48 h following application. Individual corollae were extracted for 30 sec. in 1.5 ml *n*-hexane. We performed ANOVA to test for effect of storage time on the amount of (*Z*)-9-tricosene in corolla extracts.

Footprint Accumulation on Flowers of Wild Comfrey Comfrey is a common perennial plant in pastures and meadows along the river Rhine, where it is frequently visited by local bumblebees for nectar and pollen. On the 29th July 2007, we recorded insect visits to flowers of 63 individual plants in 10-min.-intervals distributed evenly over the day from 800 to 1600 h (on average 5.5 intervals per plant, or 55 min of observation). Individual plants were chosen from a total of sixteen patches, and visit data were recorded synchronously by eight teams of two observers, each team switching back and forth regularly between patches and individual plants, so as to reduce the effects of time of day on counts per plant. Bumblebees were the only regular flower visitors (99% of visits), and the occasional visits by other insects (unidentified syrphid flies and solitary bees) were excluded from further analysis. We recorded the species of visiting bumblebee and calculated the average number of bumblebee visits received in 10-min observation intervals per flower and plant for each bumblebee species. At the end of the observation period, we randomly picked 5 flowers from each observed plant with clean forceps and extracted the corollae in 1 ml *n*-hexane (p.a., Merck) containing 10 µg of 2-undecanone as an internal standard for GC/MS analysis. We performed a linear regression analysis to test for an effect of the number of bumblebee visits on the amount of retained alkenes on flower corollae.

Chemical Analysis GC/MS analysis was performed with an HP 5890 II GC, equipped with a 30-m non-polar DB-5 column, connected to a HP 5972 mass selective detector, and an HP 7673 autoinjector (in splitless mode). The column oven was heated from 60 to 300°C at 3°C min^{-1}. Hydrocarbons were characterized by comparison of their mass spectra and retention times with that of authentic reference samples. For the purpose of the present study we did not differentiate between different alkene isomers, but pooled all alkene peaks of a given chain length.

Results

Footprint Accumulation and Retention under Different Ambient Temperatures Tarsal extracts of *B. terrestris* (*N*=10) contained *n*-alkanes and alkenes with chain length from 21 to 31, while corolla extracts of unvisited *D. grandiflora* (*N*=10) and *P. veris* (*N*=10) contained saturated alkanes, but no detectable amounts of unsaturated alkenes (Fig. 1). Nonacosenes (C29:1) were the most abundant class of alkenes on the tarsal cuticle of *B. terrestris* (Fig. 1) and, therefore, these chemicals were quantified as an indicator of bumblebee footprints. There were significant positive effects of the number of bumblebee visits on the amount of nonacosenes in corolla extracts of *D. grandiflora* in 2007 (ANCOVA: $N=77$; $df=1$; $F=9.664$; $P<0.05$) and of *P. veris* in 2008 (ANOVA: $N=180$; $df=2$; $F=69.018$; $P<0.001$) (Fig. 2). In neither case was there an effect of ambient temperature on the amount of nonacosenes in corolla extracts (*D. grandiflora*, $N=77$; $df=1$; $F<0.001$; N.S; *P. veris,* $N=180$; $df=1$; $F=1.074$; N.S) (Fig. 2). The amount of nonacosenes was not significantly affected by the time elapsed since the last visit to *P. veris* ($N=180$; $df=2$; $F=0.69$; N.S) (Fig. 2). In *D. grandiflora*, there was a marginal effect of time ($N=77$: $df=2$; $F=3.126$; $P=0.05$), with the amount of nonacosenes being, on average, reduced by 14% on corollae extracted 24 h, compared to 0 h, after the last visit.

Retention of Synthetic (Z)-9-Tricosene On Flowers No (*Z*)-9-tricosene was detected in extracts of unmanipulated control corollae of *L. maculatum* ($N=24$). In contrast, all treated corollae contained (*Z*)-9-tricosene (Fig. 3). There was no significant effect of the time since application on the amount of (*Z*)-9-tricosene on treated corollae (*ANOVA*: $N=57$; $df=2$; $F=0.358$; N.S.) (Fig. 3).

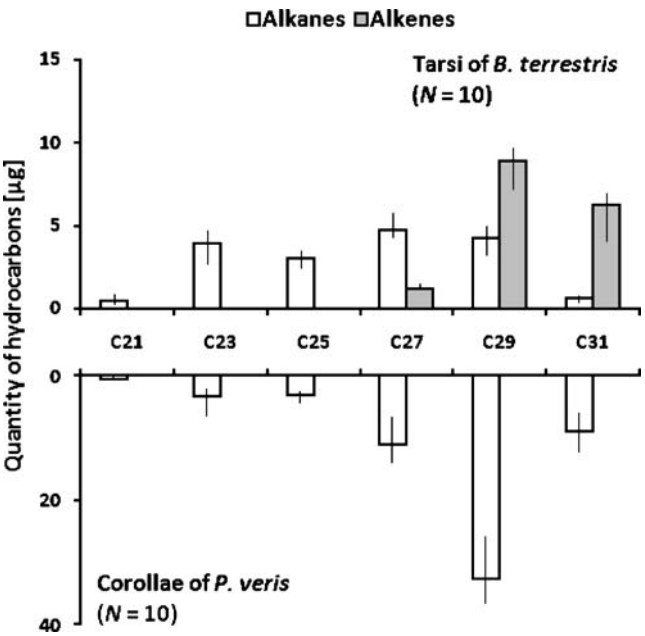

Fig. 1 Quantities of odd-numbered hydrocarbons in extracts of *Bombus terrestris* tarsi (µg/insect; $N=10$) and unvisited corollae (µg/corolla) of *Primula veris* ($N=10$), shown as medians with quartile ranges

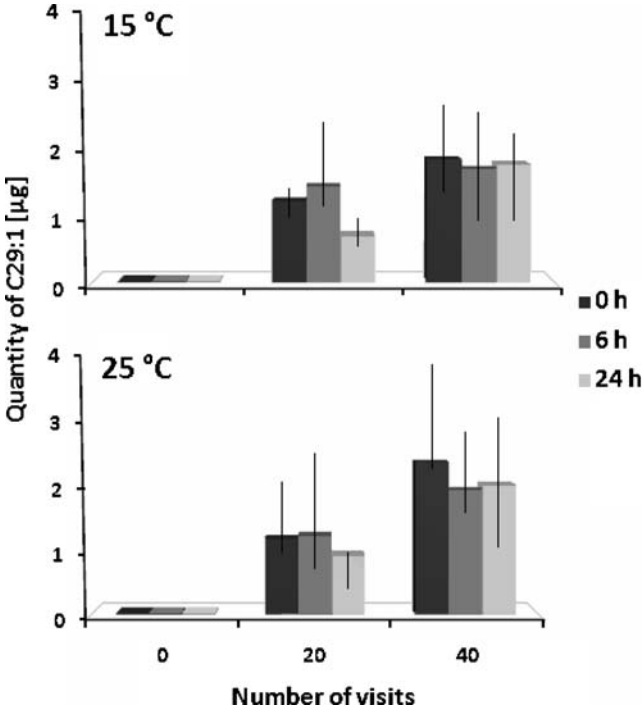

Fig. 2 Quantities (µg/corolla) of nonacosenes from *Primula veris* corollae, extracted 0, 6, or 24 h following the last bumblebee visit. The experiment was carried out at 15°C (*top*) or 25°C (*bottom*) ambient temperature. Corollae received 0, 20, or 40 visits by workers of *Bombus terrestris*. Median amounts with quartile ranges are shown

Footprint Accumulation on Flowers of Wild Comfrey The corollae of unvisited flowers of comfrey contained no detectable quantity of alkenes (S. Witjes, unpublished data). Workers of *B. pascuorum* were the most abundant visitors of comfrey at the time and contributed roughly 80% of all observed flower visits. The remaining 20% of visits were by *B. hortorum* (13 %), *B. terrestris* (4 %) and *B. pratorum* (3 %). The extracts of visited comfrey corollae contained alkenes of chain lengths from 21 to 31, corresponding well with that found in tarsal extracts of the visiting species of bumblebees (Goulson et al. 2000, Eltz 2006, S. Witjes, unpublished data). There was a significant positive relationship between the number of bumblebee visits observed per flower for a given plant during the observation intervals and the overall amount of alkenes on corollae of those plants (Fig. 4; Linear Regression: N=63; dF=62; F=32.83; P<0.001).

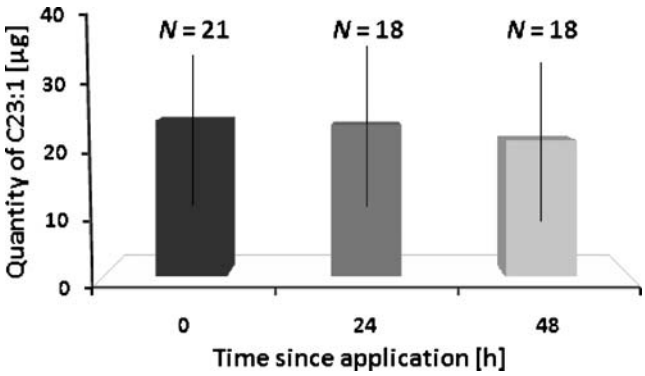

Fig. 3 Quantities (µg/corolla) of (*Z*)-9-tricosene from corollae of *Lamium maculatum* 0, 24, or 48 h after application. Data are shown as mean values with standard deviation

Discussion

This study provides further evidence that flowers retain a long-term chemical record of bumblebee visits. First, the amount of *B. terrestris*-derived nonacosenes washed from corollae was closely related to the number of bumblebee visits to the respective corollae in laboratory experiments. Second, the amount of marker alkenes remained unchanged over periods of 24 (footprint nonacosenes) to 48 (synthetic (*Z*)-9-tricosene) hours after the visits/manipulations, indicating that flower petals retain a quantitative record of bumblebee visits for a period similar to the lifetime of individual flowers of many temperate bee-pollinated plant species (Molisch 1929; Stead 1992). Third, the laboratory results were confirmed by our survey of wild comfrey plants, in which the amount of alkenes on flower corollae was closely related to the number of visits flowers of those plants received during the previous eight hours. Overall, our results indicate that alkene footprints on flower corollae can serve as an information source of bumblebee visits in natural populations of plants, especially since unsaturated alkenes seem to be absent or rare in epicuticular waxes of unvisited flowers (Griffiths et al. 1999, 2000; Goodwin et al. 2003; Eltz 2006). The alkene footprint, due to its cumulative nature, effectively integrates visitation dynamics over the entire exposure time of a flower, possibly providing a more accurate measure of bumblebee visits than an observational method, especially in studies with multiple replicates and limited observers.

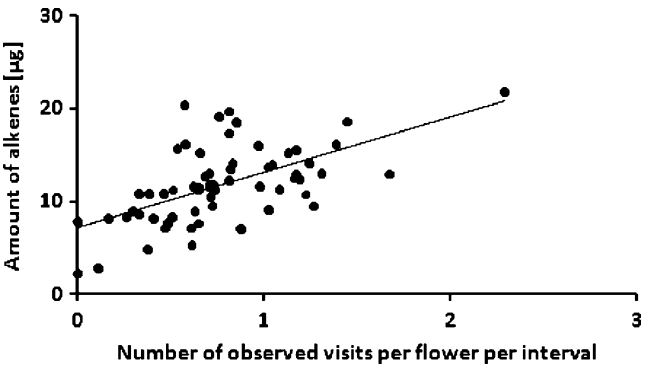

Fig. 4 Quantities of total alkenes on corollae of wild *Symphytum officinale* plants (N=63) in relation to the mean number of bumblebee visits the plants had received per flower in 10-minute observation-intervals during the day

Cuticular hydrocarbons are typically of low volatility, a point illustrated by a study that used combinations of synthetic alkanes (C24 to C31) to mark the elytra of milkweed beetles; these alkane profiles remained unchanged in quality and quantity over weeks despite exposure to direct sun and rain (Ginzel and Hanks 2002). The long-term retention of bee footprints on flowers may be promoted by the physicochemical characteristics of plant surfaces. Following deposition, bee hydrocarbons probably are integrated into the semi-liquid layer of plant cuticular waxes (Jetter et al. 2000; Eltz 2006), reducing their susceptibility to evaporation.

In agreement with Ginzel and Hanks (2002), bumblebee alkene retention was not influenced by changes in ambient temperature, at least over the temperatures (15 or 25°C) used in the experiments. The effects of more extreme temperature regimes or variation in exposure to direct sun were not investigated in detail in the present study. However, preliminary tests in an incubator oven suggest that evaporative losses of (*Z*)-9-tricosene droplets from filter paper are small even at 60°C (7.2 % over 24 h; S. Witjes, unpublished data). This suggests that variability of hydrocarbon retention should be low across a broad range of climatic conditions, thus allowing for comparisons among samples taken at different dates within the same general season/region.

It should be emphasized that the amount of hydrocarbons deposited on corollae of different plant species may vary substantially due to differences in flower morphology and in the way visitors contact corolla surfaces. Thus, each species of plant is likely to require calibration for determination of the number of visits. We currently are testing the applicability of footprint quantification as a tool to retrace the composition of the flower-visiting bumblebee community in wild populations of comfrey. Bumblebees show species-specific differences in hydrocarbon profiles (Goulson et al. 2000; Eltz 2006), and preliminary data indicate that those differences can be used to reconstruct the visiting bumblebee community (Witjes and Eltz, unpublished).

Quantification of hydrocarbon footprints on flowers may represent a cheap and reliable tool to quantify bumblebee visits in pollination studies, thus helping to reduce the problem of insufficient temporal and spatial replication in studies of pollinator decline.

Acknowledgements We thank Klaus Lunau and the members of the Sensory Ecology Group for discussions and comments on the manuscript, and the participants of the 2007 Sensory Ecology course for their help and endurance during the wild comfrey survey. A special thanks to Andreas Fischbach and Wilhelm Rogmann of the Botanical Garden of the University of Düsseldorf for maintaining the plants, as well as Waldemar Seidel of the Feinmechanikerwerkstatt of the University of Düsseldorf for the production of the bumblebees´ nest- and feeding boxes. This study was funded by the DFG grant EL249/3 and the University of Düsseldorf.

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