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Reconstructing the pollinator community and predicting seed set from hydrocarbon footprints on flowers

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Abstract The measurement of insect visits to flowers is essential in basic and applied pollination ecology studies but often fraught with difficulty. Floral visitation is highly variable, and observational studies are limited in scope due to the considerable time necessary to acquire reliable data. The aim of our study was to investigate whether the analysis of hydrocarbon residues (footprints) deposited by insects during flower visits would allow reconstruction of the visitor community and prediction of seed set for large numbers of plants. In 3 consecutive years, we recorded bumblebee visitation to wild plants of comfrey, *Symphytum officinale*, and later used gas chromatography/mass spectrometry (GC/MS) to quantify bumblebee-derived unsaturated hydrocarbons (UHCs) extracted from flowers. We found that the UHCs washed from corollas were most similar to the tarsal UHC profile of the most abundant bumblebee species, *Bombus pascuorum*, in all 3 years. The species composition of the bumblebee communities estimated from UHCs on flowers were also similar to those actually observed. There was a significant positive correlation between the observed number of visits by each of three bumblebee species (contributing 3–68% of flower visits) and the estimated number of visits based on UHC profiles. Furthermore, significant correlations were obtained separately for workers and drones of two of the study species. Seed set of comfrey plants was positively correlated to overall bumblebee visitation and the total amount of UHCs on flowers, suggesting the potential for pollen limitation. We suggest that quantifying cumulative footprint hydrocarbons provides a novel way to assess floral visitation by insects and can be used to predict seed set in pollen-limited plants.

Keywords *Bombus* · Cuticular hydrocarbons · Pollen limitation · Pollination · Scent-marks

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Introduction

Insects are responsible for the pollination of about 67% of the world's flowering plants (Tepedino 1979) and 84% of the crops cultivated in the European Union (EU) (Williams 1996). The rapid decline in the numbers of insect pollinators (Corbet 1995; Goulson et al. 2008; Kearns et al. 1998; Williams 1996) has therefore raised concerns about possible economic and ecological consequences (Allen-Wardell et al. 1998). A decrease in pollinator service is likely to reduce the quantity of pollen delivered to stigmas (Ashman et al. 2004) and may lead to decreased fruit- and/or seed set (Bierzychudek 1981; Kearns and Inouye 1997; Louda 1982; Rathcke and Jules 1993), a reduction in individual reproductive success (Ågren 1996; Allen-Wardell et al. 1998; Ashman et al. 2004; Rathcke and Jules 1993), a decrease in plant population size (Aizen and Feinsinger 1994) and, ultimately, local extinction (Kearns and Inouye 1997) or crop failure (Allen-Wardell et al. 1998).

Seed set is limited by the amount or quality of pollen deposited on stigmas in 62–73% (Burd 1994) of flowering plant species (Ashman et al. 2004). Ecologists also fear that habitat alteration and fragmentation due to extended

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agricultural land use may further promote the loss of species richness and abundance, initially of pollinators, but subsequently of pollinator-dependent plant populations (Aizen and Feinsinger 1994; Allen-Wardell et al. 1998; Cunningham 2000; Lamont et al. 1993; Rathcke and Jules 1993). Indeed, there is some evidence that plants in fragmented populations produce fewer seeds (Ågren 1996; Aizen and Feinsinger 1994; Bosch and Waser 1999; Jennersten and Nilsson 1993; Kéry et al. 2000; Kunin 1993; Lamont et al. 1993; Steffan-Dewenter and Tscharntke 1999) and fruits (Ågren 1996; Aizen and Feinsinger 1994; Steffan-Dewenter and Tscharntke 1999) than conspecifics in continuous habitats. However, there are relatively few studies that directly link pollinator visitation with the degree of pollen limitation in plant population studies (Baker et al. 2000; Larson and Barrett 1999).

Pollinator visitation frequency is often low and highly variable (Baker et al. 2000; de Jong et al. 2005; Larson and Barrett 1999) and collecting sufficient data requires considerable time and effort, especially if many replicate populations are to be compared (Baker et al. 2000; Waser et al. 1996). There have been attempts to assess pollinator visitation indirectly by recording the "tripped status" of flowers (Parker 1997) or pollinator claw marks and subsequent color changes of the visited flowers due to floral tissue damage (Matsumura and Washitani 2000). However, both methods provide no information on visitation frequency or the composition of the pollinator community. Here, we report our testing of a new method that uses the hydrocarbon deposits (footprints) of insects on flowers to reconstruct the pollinator community and to predict seed set of forage plants.

Hydrocarbons are major constituents of the insects' epicuticular lipid layer (Lockey 1988), which is believed to have originally evolved as a protective barrier against water loss in terrestrial habitats. However, the secondary functions of cuticular hydrocarbons (CHCs) are manifold. For example, lipid droplets on tarsal attachment pads are thought to enhance adhesion on smooth surfaces (Drechsler and Federle 2006; Jiao et al. 2000; Lockey 1988), and CHCs are used as communication signals in many social insects (Bonavitacougourdan et al. 1991; Dani et al. 2005; Howard and Blomquist 2005; Lahav et al. 1999; Liebig et al. 2000; Ruther et al. 2002; Sledge et al. 2001). CHCs from footprints may also have informative value as chemical cues to conspecifics or heterospecifics. At nesting sites, CHCs are used by wasps and bees to recognize their nest entrance at close range (Butler et al. 1969; Hefetz 1992), and on flowers, CHCs enable bees to avoid flowers that have recently been visited by others and are currently depleted of nectar (Gawleta et al. 2005; Gilbert et al. 2001; Goulson et al. 2000, 2001; Stout et al. 1998). Two recent studies indicate that such "scent marks" are not actively released pheromone signals but, rather, mere cues, obligatorily deposited wherever bees walk: bumblebee (*Bombus terrestris)* workers were found to have "left" CHCs of a similar composition and concentration at feeders, nest, and neutral sites (Saleh et al. 2007), and footprints extracted from feeders or neutral sites elicited similar repellent effects when presented in a foraging situation (Wilms and Eltz 2008).

The origin of the involved hydrocarbons is unclear, but in bumblebees at least, several cuticular glands are likely involved. Solvent extracts of different parts of the cuticle (tarsi, antennae) and Duffour's gland were found to be dominated by similar saturated and unsaturated hydrocarbons (Oldham et al. 1994; Schmitt 1990), with a chain length of 21–31 carbon atoms (Goulson et al. 2000; Saleh et al. 2007; Schmitt 1990), indicating that the epicuticular lipid layer consists of a mixture of different glandular secretions (Oldham et al. 1994). During flower visits, traces of these CHCs remain on the visited flower and accumulate within the epicuticular wax of the corolla, which consequently may hold information about past bee visitation (Eltz 2006). In fact, solvent washes of dead-nettle (*Lamium maculatum*) and comfrey (*Symphytum officinale*) flowers visited by bumblebee (*B. pascuorum*) workers in the field, as well as flowers of *Digitalis grandiflora* and *Primula veris* visited by *B. terrestris* workers in the laboratory, contained several odd-numbered alkenes in addition to the plant's own cuticular lipids (Eltz 2006; Witjes and Eltz 2009). Quantification of hydrocarbons in solvent washes of visited flowers showed that deposited alkenes increased almost linearly with the number of visits the flowers had received (Eltz 2006; Witjes and Eltz 2009). Interestingly, the amount of alkenes remained unchanged for up to 24 h and was independent of two tested temperature regimes (15 and 25°C) (Witjes and Eltz 2009). The CHC profiles of bumblebees show species-specific differences (Eltz 2006; Goulson et al. 2000) that could be used to reconstruct the bumblebee visitor community.

In the study reported here, we tested to what extent hydrocarbon deposits on comfrey flowers reflect the species composition of the visiting bumblebee community in natural habitats. We asked whether the unsaturated hydrocarbons (UHCs) on *S. officinale* flowers reflect bumblebee flower visitation quantitatively and qualitatively and whether pollination ecologists can use this information to reconstruct the visitor community with reasonable accuracy. We also tested whether seed set of outcrossed *S. officinale* is related to bumblebee visitation and whether it can be predicted by measuring the quantity of bumblebee-derived UHCs on corollas.

Materials and methods

Comfrey (*S. officinale*) is a polycarpous, perennial herb growing in moist habitats. In Germany, it is a common plant on pastures and meadows, especially along rivers (Düll and Kutzelnigg 2005; Hegi 1966). Flowers are open mainly from May to July and are frequently visited by bumblebees. Comfrey has been reported to be self-incompatible (Goulson et al. 1998), but self-pollination may occasionally occur if pollinators are absent or rare (Düll and Kutzelnigg 2005; Hegi 1966).

Surveying the pollinator community of wild comfrey

The field studies took place on 29 July 2007, 3 June 2008, and from 7 May–24 August 2009. Insect flower visitation to plants of comfrey was recorded at 16 different sites in 2007 and 12 sites in 2008, all situated in meadows around Himmelgeist and Urdenbach in southern Düsseldorf. We randomly chose up to four plants per site (a total of 63 in 2007 and 48 in 2008) and recorded the species of flower-visiting insects and the number of visits to flowers per plant. Bumblebees were the predominant visitors and contributed about 99% of visits in 2007 and 98% in 2008. The occasional visits of other insects (unidentified syrphid flies and solitary bees) were therefore excluded from further analysis. It should be noted that workers of the sibling species *B. terrestris* and the much rarer *Bombus lucorum* cannot be reliably distinguished in the field. Thus, counts referred to as *B. terrestris* may occasionally include workers of *B. lucorum*. Flower observation was recorded synchronously by teams of two observers in 10-min intervals distributed evenly over each observation day. Each team observed two sites, regularly switching back and forth between the plants and sites in order to reduce the effect of time per day on counts per plant. In 2009, data were collected by one observer throughout the flowering season of comfrey at ten sites around Urdenbach and Himmelgeist and 17 sites along the river Niers from southern to northern Mönchengladbach. The species of flower-visiting insects and the number of visits to flowers per plant were recorded for three plants per site (a total of 81 plants). As in the preceding years, bumblebees were the predominant visitors and contributed 98% of total flower visits. Consequently, other insects were excluded from further analysis. The observations were carried out as in the two preceding years, but additionally we recorded the sex of the flower-visiting bumblebees, the number of flowers on observed plants, and the number of neighboring conspecific plants within a 25-m diameter.

Surveying seed set of wild comfrey

Symphytum officinale plants set a maximum of four relatively large seeds per flower (Düll and Kutzelnigg 2005; Goulson et al. 1998; Hegi 1966), and the proportion of developed seeds can be assessed accurately for individual flowers. We marked observed flowers with yarn, and at the end of the observation periods, we removed flower corollae that had not already been removed for hydrocarbon extraction (see below) from the flower heads in order to prevent further insect visitation. Bumblebees were nevertheless sometimes seen probing for nectar at the basis of flower heads. As we did not remove the stigmas of flowers, subsequent pollination could have occurred on occasion. After 14 days following the observations, the marked inflorescences were removed, and seeds per flower were counted for 63 of the 81 plants that had been observed in 2009.

Extracting hydrocarbons from flowers of wild comfrey and its pollinators

To assess the quantity and composition of hydrocarbon footprints on flowers, we randomly picked five flowers per observed plant at the end of the observation periods. We removed the flower corollae with clean forceps and extracted them in 1 ml hexane (Merck, Whitehouse Station, NJ) containing 2-undecanone as an internal standard; the extracted fraction was then analyzed by gas chromatography/mass spectrometry (GC/MS). In order to analyze the composition of CHCs of local bumblebee species, individual bumblebees were captured around the campus of the University of Düsseldorf, anaesthetized with CO_2, and their legs cut off at the proximal end of the femur. Individual sets of legs were being extracted for 30 s in 500 µl hexane containing 2-undecanone as internal standard. The bees were then killed by freezing.

Chemical analysis

Gas chromatography/mass spectrometry analyses were performed using an HP 5890 II GC fitted with a 30 m non-polar DB-5 column and an HP 5972 mass selective detector (Hewlett Packard, Palo Alto, CA). A 3-µl volume per sample was injected using an HP 7673 auto-injector. The injection was splitless and the oven heated from 60 to 300°C at a rate of 10°C per minute, with automatic pressure programming. For the quantification of hydrocarbons, peak area (integrated ion current) was compared to that of the internal (2-undecanone) and external (pentacosane) standards. Characterization of the hydrocarbons was based on the comparison of mass spectra and associated retention times with entries in a local library (S. Witjes, unpublished). Alkene isomers were characterized through comparison with authentic reference samples via co-injection.

Analyzing species' specificity of the composition of UHCs

Hydrocarbon analysis was restricted to UHCs (here alkenes and alkadienes), which are common components of the epicuticular lipid layer of bumblebees but absent from the waxes of most unvisited flowers, including those of *S. officinale* (Eltz 2006; Goodwin et al. 2003; Griffiths et al. 1999, 2000; Witjes and Eltz 2009). We performed non-metric multidimensional scaling (MDS) analysis of the composition of UHCs in tarsal extracts of bumblebees and on observed *S. officinale* flowers (2009) with PRIMER (v6.1.6) software (Clarke and Gorley 2006). The absolute quantity of individual UHCs (μg) was standardized to represent their relative contribution to the total amount of UHCs per sample. Pairwise similarities between samples were calculated using the Bray Curtis similarity index and then ordinated into a two-dimensional plot in which the position of individual samples is fitted to reflect best the chemical similarity/dissimilarity between them. Deviations from a perfect fit are expressed in "stress", with values <0.15 representing a good overall fit (Clarke and Gorley 1993). We tested for differences in UHC compositions between different sets of samples (different species and sexes of bumblebees, floral extracts) by using the non-parametric ANOSIM test in PRIMER. To identify the components responsible for similarities among individuals of the same bumblebee species we used the SIMPER function in PRIMER, which calculates the average relative contribution of each component to overall intraspecific clustering.

Estimation of visitor communities

The estimation of the bumblebee visitor community is based on a linear model of UHC deposits on *S. officinale* flowers. The amount of a given UHC, for example a certain alkene, on a flower is the product of the amount of this UHC deposited on the flower per visit by a given bumblebee species and the number of visits that members of that species have made to the flower, summed over all bumblebee species. This equation can be calculated for each different UHC. In mathematical terms, this is a system of linear equations, $A.x = b$. Here A is a rectangular matrix. The number of columns is the number of different species and sexes of bumblebees (6 in 2007 and 2008 and 11 in 2009). The number of rows is the number of different UHCs (63 in all years) measured. The entries in a row of A are the mean amounts of the different UHCs deposited by individuals of a given bumblebee species/sex per flower visit, i.e., species-specific absolute deposition profiles. To calculate these values, we calibrated quantities of UHCs in tarsal extracts using deposition data from a controlled flower visitation experiment. For this experiment, we chose workers of *B. pascuorum* as a medium-sized representative species and quantified the total amount of UHCs deposited on *S. officinale* flowers per visit (Witjes, unpublished). We then calculated species-specific deposition profiles, assuming that UHCs are deposited on flowers in the same proportion as they occur in tarsal extracts. The vector x is the number of visits per species, which shall be determined. The vector b is the amount of UHCs found on flowers of a given plant. Since there are more equations than unknowns in $A.x = b$, we applied Gauß's "least squares solution" to solve the system of linear equations, which is the solution x that minimizes the square of the length of the vector $b - A.x$ and is known as the maximum likelihood estimation of x in case of a normal distribution of errors in the measurements (Press et al. 2007). Because x contains the number of visits, it should be a nonnegative integer number. We omitted the requirement "integer", since it is not important in this case. Due to inherent errors, small negative values were occasionally computed instead of small positive ones. We avoided this by applying a modification of the least squares algorithm using the program "lsqnonneg" in MatLab (MathWorks, Natick, MA), which always computes nonnegative solutions (see Lawson and Hanson 1974 for details). To estimate the accuracy with which bumblebee visitation could be derived from UHC deposits on flowers, we compared the mean number of bumblebee visits that plants had received per flower during the day (extrapolated from bumblebee visitation counts in 10-min observation intervals) with the results from the least squares solution using a Spearman rank correlation in Statistica 6.0 (StatSoft, Tulsa, OK).

CHC profiles, seed set, and pollen limitation

We tested for correlations between the total number of visits that plants had received per flower during the day (extrapolated from bumblebee visitation counts in 10-min observation intervals) and the overall quantity of UHCs per flower per plant as well as the average number of seed set per flower per plant, using Spearman rank correlation in Statistica 6.0 (StatSoft). In a second step, we tested for influences of environmental variables (number of plants in the surrounding area and the number of flowers on observed plants) on bumblebee flower visitation.

Results

Analyzing species' specificity of the composition of unsaturated hydrocarbons

The tarsal extracts of the observed bumblebee species contained alkenes and alkadienes with chain lengths of

19–33 C-atoms (Table 1). According to the PRIMER SIMPER algorithm, the average similarity of UHC profiles within groups (individuals of different species and sexes) ranged between 64.34% (in workers of *B. hortorum*) and 92.03% (in drones of *B. pascuorum*) (Table 1). In most groups, the within-group similarity was based on five major components, which contributed approximately 90% to the similarity within the groups, with the exception of *B. hortorum* workers, in which the major components contributed approximately 70% to the inner-group similarity (Table 1). Overall, the UHC composition in tarsal extracts was specific for different bumblebee species and sexes (ANOSIM: $n = 207$; $R = 0.948$; $P < 0.001$), and MDS produced non-overlapping clusters for all species and sexes except *B. pascuorum* (Fig. 1). UHCs of workers and drones of *B. pascuorum* were not significantly different in terms of composition [ANOSIM: $n = 57$; $R = 0.002$; $P =$ not significant (N.S.)], and the two sexes were therefore pooled for further analysis.

Estimation of visitor communities

Comfrey plants had been visited by six different bumblebee species in 2007 and 2008. In 2009, we recorded workers and drones of the same six species (Fig. 1). UHC signatures in floral extracts provided information on visitation frequency, both for the entire guild of bumblebees as well as for some of the more abundant bumblebee species separately. The number of bumblebee visits that plants had received per flower during the day (extrapolated from bumblebee visitation counts in 10-min observation intervals) was significantly correlated to the overall bumblebee visitation frequency derived from chemical signatures with the least squares method in 2007 (Fig. 2a; $n = 378$, $R = 0.48$, $P < 0.0001$), 2008 (Fig. 2b; $n = 288$, $R = 0.46$, $P < 0.0001$), and 2009 (Fig. 2c; $n = 891$, $R = 0.55$, $P < 0.0001$). On the species level, the visitation frequency derived from chemical profiles was significantly correlated to the extrapolated visitation counts in *B. pascuorum* and *B. hortorum* in 2007 ($n = 63$, $R = 0.60$, $P < 0.0001$ for *B. pascuorum*; $n = 63$, $R = 0.43$, $P < 0.001$ for *B. hortorum*) and 2008 ($n = 48$, $R = 0.59$, $P < 0.0001$ for *B. pascuorum*; $n = 48$, $R = 0.56$, $P < 0.0001$ for *B. hortorum*), which were the most abundant flower visitors, contributing 92.05% (2007) and 75.24% (2008) of all visits in these years. In 2009, there was a significant correlation for *B. pascuorum* (Fig. 3a: $n = 81$, $R = 0.62$, $P < 0.0001$), workers and drones of *B. pratorum* (Fig. 3b: $n = 81$, $R = 0.58$, $P < 0.0001$ for *B. pratorum* workers; Fig 3c: $n = 81$, $R = 0.69$, $P < 0.0001$ for *B. pratorum* drones), and workers and drones of *B. hortorum* (Fig. 3d: $n = 81$, $R = 0.49$, $P < 0.0001$ for *B. hortorum* workers; Fig. 3e: $n = 81$, $R = 0.26$, $P < 0.05$ for *B. hortorum* drones), which were the most frequent flower visitors and contributed 92.51% of all observed flower visits in 2009.

CHC profiles, seed set, and pollen limitation

In accordance with the results presented above, the absolute amount of UHCs on visited flowers was a strong correlate of bumblebee visitation frequency in 2009 ($n = 63$, $R = 0.71$, $P < 0.0001$) (Fig. 4b). Flower visitation itself was related to the number of flowering plants in the area surrounding the observed plant ($n = 63$, $R = 0.34$, $P < 0.05$) and to the number of flowers on the observed plant itself ($n = 63$, $R = 0.29$, $P < 0.05$). The average number of seeds set per plant was positively and significantly correlated to the total number of bumblebee visits that the plants had received per flower during the day (extrapolated from bumblebee visitation counts in 10-min observation intervals) ($n = 63$, $R = 0.38$, $P < 0.001$) (Fig. 4a), indicating that seed set was pollen limited under the conditions and circumstances of our study. Importantly, seed set was also correlated to the amount of UHCs on flowers of the respective plant ($n = 63$, $R = 0.43$, $P < 0.001$) (Fig. 4c). Thus, the amount of UHCs on flowers of a given plant, presumably through its relationship with bumblebee visitation, functioned as a predictor of seed set of the respective plant.

Discussion

Our results suggest that hydrocarbon footprints on flowers are a reliable and valuable source of information for pollination ecologists. We confirm our previous finding that the overall amount of footprint hydrocarbons is an indicator of cumulative bumblebee visitation to flowers of wild comfrey (Witjes and Eltz 2009). Furthermore, we show for the first time that UHC profiles also hold information on the composition of the visiting bumblebee community, allowing us to estimate visitation frequencies separately for the most abundant species. Finally, we demonstrate that UHC deposits can be used to predict seed set in plant species/situations where seed set is limited by the number of pollinator visits received.

Estimation of visitor communities

The concept of reconstructing visitor communities from footprints rests on two preconditions. First, it relies on a certain amount of species specificity in terms of the chemical composition of footprint hydrocarbons of the visitors and, second, it assumes those hydrocarbons are well preserved on the visited flowers. The second prerequisite (preservation) has received strong direct and indirect support. In general,

Table 1 Most frequently detected alkenes and alkadiens in tarsal extracts of bumblebees foraging on *Symphytum officinale* in 2009

Retention time	Substance	*Bombus terrestris* ♀ Avg. similarity:68.36 $n = 65$		*B. terrestris* ♂ Avg. similarity:89.12 $n = 10$		*B. hortorum* ♀ Avg. similarity:64.34 $n = 13$		*B. hortorum* ♂ Avg. similarity:81.74 $n = 4$		*B. pascuorum* ♀ Avg. similarity:87.06 $n = 50$		*B. pascuorum* ♂ Avg. similarity:92.03 $n = 7$	
		Abundance	Contribution	Abundance	Contribution	Abundance	Contribution	Abundance	Contribution	Abundance	Contribution	Abundance	Contribution
16.42	Nonadecene 1	0.02	0	–	–	0.02	0	19.86	16.92	–	–	–	–
16.55	Nonadecene 2	–	–	–	–	0	0	–	–	–	–	–	–
18.46	(Z)-9-Heneicosene	–	–	–	–	0.15	0.03	0.82	0.49	–	–	–	–
18.52	(Z)-7-Heneicosene	–	–	–	–	4.51	4.22	–	–	–	–	–	–
18.62	Heneicosene 3	–	–	–	–	0.65	0.61	–	–	–	–	–	–
19.36	Docosadiene	–	–	–	–	–	–	–	–	–	–	–	–
19.43	Docosene 1	–	–	–	–	0.1	0.04	–	–	–	–	–	–
19.49	Docosene 2	–	–	–	–	0.11	0.02	–	–	–	–	–	–
19.59	Docosene 3	–	–	–	–	–	–	–	–	–	–	–	–
20.26	Tricosadiene	–	–	–	–	0.12	0.02	–	–	–	–	–	–
20.35	Tricosene 1	–	–	–	–	14.29	15.17	–	–	–	–	–	–
20.37	(Z)-9-Tricosene	0.3	0.19	0.13	0.06	1.73	0.34	2.55	2.86	1.05	0.89	0.51	0.37
20.42	(Z)-7-Tricosene	0.01	0	–	–	16.09	14.73	0.4	0.08	0.12	0.06	0.19	0.08
20.52	Tricosene 4	0.33	0	–	–	7.78	7.99	–	–	–	–	–	–
21.15	Tetracosadiene	–	–	–	–	–	–	–	–	–	–	–	–
21.21	Tetracosene 1	–	–	–	–	0.49	0.43	–	–	–	–	–	–
21.24	Tetracosene 2	–	–	–	–	–	–	0.16	0.1	0.36	0.21	0.29	0.26
21.30	Tetracosene 3	–	–	–	–	0.38	0.37	–	–	–	–	–	–
21.39	Tetracosene 4	–	–	–	–	0.09	0.05	–	–	–	–	–	–
21.93	Pentacosadiene 1	–	–	–	–	–	–	–	–	–	–	–	–
21.98	Pentacosadiene 2	–	–	–	–	0.7	0.61	–	–	0.05	0.01	–	–
22.07	Pentacosene 1	0.08	0.02	–	–	13.34	15.27	–	–	–	–	–	–
22.10	(Z)-9-Pentacosene	0.73	0.16	0.72	0.33	4.32	2.5	11.77	11.7	48.03	49.54	55.19	58.01
22.17	(Z)-7-Pentacosene	0.15	0.01	–	–	12.02	14.89	0.56	0.58	2.19	2.07	1.62	1.53
22.5	(Z)-5-Pentacosene	–	–	–	–	6.4	6.63	–	–	0.03	0	–	–
22.78	Hexacosadiene	–	–	–	–	–	–	–	–	–	–	–	–
22.87	Hexacosene 1	–	–	–	–	–	–	–	–	–	–	–	–
22.92	Hexscosene 2	–	–	–	–	–	–	0.64	0.7	0.48	0.24	0.66	0.43
22.97	Hexacosene 3	–	–	–	–	0.07	0.03	–	–	–	–	–	–
23.51	Heptacosadiene 1	0.03	0	–	–	–	–	–	–	–	–	–	–
23.55	Heptacosadiene 2	0	0	–	–	–	–	–	–	0.18	0.02	–	–
23.61	Heptacosadiene 3	–	–	–	–	–	–	–	–	–	–	–	–
23.66	(Z)-11-Heptacosene	2.33	1.54	–	–	4.54	4.84	–	–	–	–	–	–
23.72	(Z)-9-Heptacosene	1.9	1.43	19.2	17.18	2.65	1.99	23.01	23.96	20.56	22.22	18.54	18.87
23.77	(Z)-7-Heptacosene	1.08	0.55	1.25	1.1	2.26	2.82	1.65	1.63	0.99	0.83	0.97	0.93
23.85	Heptacosene 4	–	–	–	–	1.2	1	–	–	–	–	–	–
24.42	Octacosene 1	0.11	0.02	–	–	–	–	–	–	–	–	–	–
24.49	Octacosene 2	0.23	0.12	1.47	1.15	–	–	0.78	0.52	0.18	0.06	0.18	0.12

Table 1 continued

Retention time	Substance	*Bombus terrestris* ♀ Avg. similarity:68.36 *n* = 65		*B. terrestris* ♂ Avg. similarity:89.12 *n* = 10		*B. hortorum* ♀ Avg. similarity:64.34 *n* = 13		*B. hortorum* ♂ Avg. similarity:81.74 *n* = 4		*B. pascuorum* ♀ Avg. similarity:87.06 *n* = 50		*B. pascuorum* ♂ Avg. similarity:92.03 *n* = 7	
		Abundance	Contribution	Abundance	Contribution	Abundance	Contribution	Abundance	Contribution	Abundance	Contribution	Abundance	Contribution
24.55	Octacosene 3	–	–	–	–	–	–	–	–	–	–	–	–
25.07	Nonacosadiene 1	0.32	0.03	–	–	–	–	–	–	–	–	–	–
25.12	Nonacosadiene 2	2.46	2.06	–	–	–	–	–	–	0.06	0	–	–
25.16	Nonacosadiene 3	1.11	0.87	–	–	–	–	–	–	0.06	0	–	–
25.26	(Z)-11-Nonacosene	13.04	12.83	–	–	2.53	2.69	–	–	0.09	0	–	–
25.34	(Z)-9-Nonacosene	27.45	34.88	74.56	78.71	1.53	1.26	20.43	21.73	13.12	13.17	9.87	9.69
25.38	(Z)-7-Nonacosene	1.12	0.29	–	–	1.05	1.29	1.3	1.27	0.43	0.23	0.45	0.42
26.21	Triacontene 1	0.06	0	–	–	–	–	–	–	–	–	–	–
26.25	Triacontene 2	0.18	0.04	–	–	–	–	–	–	0.09	0.01	–	–
26.99	Untriacontadiene 1	2.94	2.87	–	–	–	–	–	–	–	–	–	–
27.05	Untriacontadiene 2	1.98	1.93	–	–	–	–	–	–	–	–	–	–
27.09	Untriacontadiene 3	4.74	4.98	–	–	–	–	–	–	–	–	–	–
27.16	Untriacontadiene 4	4.47	3.68	–	–	–	–	–	–	0.05	0	–	–
27.22	Untriacontene 1	0.43	0	–	–	0.41	0.14	–	–	0.1	0	–	–
27.29	Untriacontene 2	17.62	18.46	–	–	–	–	–	–	–	–	–	–
27.36	Untriacontene 3	12.91	12.41	2.21	1.48	0.23	0.01	15.11	16.91	11.11	10.32	9.63	9.29
27.44	Untriacontene 4	–	–	–	–	0.08	0.01	–	–	–	–	–	–
29.57	Titriacontadiene 1	0.23	0.02	–	–	–	–	–	–	–	–	–	–
29.66	Titriacontadiene 2	0.96	0.36	–	–	–	–	–	–	0.02	0	–	–
29.76	Titriacontadiene 3	0.72	0.23	–	–	–	–	–	–	–	–		–
29.92	Titriacontene 1	0.13	0.01	–	–	–	–	–	–	–	–	–	–
30.01	Titriacontene 2	0.05	0	–	–	–	–	–	–	0.47	0.1	–	–
31.22	Tetratriacontene 1	–	–	–	–	–	–	–	–	–	–	–	–
31.37	Tetratriacontene 2	–	–	–	–	–	–	–	–	–	–	–	–

Retention time	Substance	*B. pratorum* ♀ Avg. similarity:82.37 *n* = 21		*B. pratorum* ♂ Avg. similarity:88.09 *n* = 8		*B. lapidarius* ♀ Avg. similarity:85.21 *n* = 13		*B. lapidarius* ♂ Avg. similarity:80.68 *n* = 5		*B. hypnorum* ♀ Avg. similarity:75.51 *n* = 16		*B. hypnorum* ♂ Avg. similarity:81.58 *n* = 11	
		Abundance	Contribution	Abundance	Contribution	Abundance	Contribution	Abundance	Contribution	Abundance	Contribution	Abundance	Contribution
16.42	Nonadecene 1	–	–	–	–	–	–	–	–	–	–	–	–
16.55	Nonadecene 2	–	–	–	–	–	–	–	–	–	–	–	–
18.46	(Z)-9-Heneicosene	–	–	–	–	–	–	–	–	–	–	–	–
18.52	(Z)-7-Heneicosene	–	–	–	–	–	–	–	–	–	–	–	–
18.62	Heneicosene 3	–	–	–	–	–	–	–	–	–	–	–	–
19.36	Docosadiene	–	–	–	–	–	–	–	–	–	–	–	–
19.43	Docosene 1	–	–	–	–	–	–	–	–	–	–	–	–
19.49	Docosene 2	–	–	–	–	–	–	–	–	–	–	–	–

Table 1 continued

Retention time	Substance	*B. pratorum* ♀ Avg. similarity:82.37 *n* = 21		*B. pratorum* ♂ Avg. similarity:88.09 *n* = 8		*B. lapidarius* ♀ Avg. similarity:85.21 *n* = 13		*B. lapidarius* ♂ Avg. similarity:80.68 *n* = 5		*B. hypnorum* ♀ Avg. similarity:75.51 *n* = 16		*B. hypnorum* ♂ Avg. similarity:81.58 *n* = 11	
		Abundance	Contribution	Abundance	Contribution	Abundance	Contribution	Abundance	Contribution	Abundance	Contribution	Abundance	Contribution
19.59	Docosene 3	–	–	–	–	–	–	–	–	–	–	–	–
20.26	Tricosadiene	–	–	–	–	–	–	–	–	–	–	–	–
20.35	Tricosene 1	4.1	3.54	–	–	–	–	–	–	–	–	–	–
20.37	(Z)-9-Tricosene	2.56	2.11	–	–	2.12	1.8	1.35	0.95	0.52	0.39	0.11	0.07
20.42	(Z)-7-Tricosene	–	–	0.34	0.25	13.21	11.71	5.91	5.73	0.03	0.01	0.05	0.02
20.52	Tricosene 4	0.13	0.01	0.55	0.19	–	–	–	–	–	–	0.01	0
21.15	Tetracosadiene	–	–	0.17	0.14	–	–	–	–	–	–	–	–
21.21	Tetracosene 1	0.49	0.35	–	–	0.1	0.01	–	–	–	–	–	–
21.24	Tetracosene 2	–	–	–	–	–	–	–	–	–	–	–	–
21.30	Tetracosene 3	–	–	0.05	0	1.4	1.26	0.45	0.35	–	–	–	–
21.39	Tetracosene 4	–	–	–	–	–	–	–	–	–	–	–	–
21.93	Pentacosadiene 1	–	–	–	–	–	–	–	–	–	–	–	–
21.98	Pentacosadiene 2	0.1	0.02	55.66	59.75	0.26	0.14	–	–	–	–	0.1	0.03
22.07	Pentacosene 1	29.06	29.98	0.99	0.61	–		–	–	–	–	–	–
22.10	(Z)-9-Pentacosene	7.46	6.44	1.83	1.04	13.73	11.89	11.68	8.77	6.25	5.97	2.4	2.18
22.17	(Z)-7-Pentacosene	–	–	12.95	12.83	54.31	60.86	41.39	48.1	0.77	0.45	1.78	1.77
22.5	(Z)-5-Pentacosene	–	–	0.96	0.88	0.73	0.23	–	–	–	–	–	–
22.78	Hexacosadiene	–	–	0.71	0.62	–	–	–	–	–	–	–	–
22.87	Hexacosene 1	0.95	0.73	–	–	–	–	–	–	–	–	–	–
22.92	Hexscosene 2	–	–	–	–	–	–	–	–	0.1	0.03	0.13	0.02
22.97	Hexacosene 3	–	–	0.15	0.06	0.31	0.22	0.77	0.6	0.4	0.28	0.19	0.08
23.51	Heptacosadiene 1	0.02	0	–	–	–	–	–	–	0.01	0	0.91	0.83
23.55	Heptacosadiene 2	0.64	0.18	8.62	7.6	–	–	–	–	0.05	0.01	2.61	2.58
23.61	Heptacosadiene 3	–	–	1.27	1.07	–	–	–	–	–	–	0.59	0.54
23.66	(Z)-11-Heptacosene	29.6	33.72	2.29	2.16	–	–	–	–	0.01	0	1.79	1.62
23.72	(Z)-9-Heptacosene	7.84	8.15	0.44	0.15	0.83	0.73	2.78	3.01	28.44	31.6	12.85	12.98
23.77	(Z)-7-Heptacosene	–	–	10.02	10.42	5.93	5.43	20.45	19.54	8.93	5.09	22.57	25.3
23.85	Heptacosene 4	–	–	–	–	–	–	–	–	–	–	–	–
24.42	Octacosene 1	0.53	0.45	–	–	–	–	–	–	–	–	–	–
24.49	Octacosene 2	–	–	–	–	–	–	–	–	0.74	0.57	0.39	0.26
24.55	Octacosene 3	–	–	–	–	–	–	–	–	0.54	0.27	0.16	0.05
25.07	Nonacosadiene 1	–	–	–	–	–	–	–	–	0.09	0.02	2.81	2.81
25.12	Nonacosadiene 2	0.44	0.14	2.15	1.8	–	–	–	–	0.13	0.03	6.98	6.72
25.16	Nonacosadiene 3	–	–	–	–	–	–	–	–	0.08	0.01	3.24	3.15
25.26	(Z)-11-Nonacosene	11.29	10.55	0.7	0.41	0.07	0.01	–	–	0.15	0.03	2.87	2.65
25.34	(Z)-9-Nonacosene	3.11	2.81	0.09	0.02	0.45	0.28	1.28	1.3	39.86	44.49	17.7	19.92
25.38	(Z)-7-Nonacosene	–	–	–	–	3.39	3.1	10.06	9.02	6.09	3.48	8.38	8.97

Table 1 continued

Retention time	Substance	*B. pratorum* ♀ Avg. similarity:82.37 *n* = 21		*B. pratorum* ♂ Avg. similarity:88.09 *n* = 8		*B. lapidarius* ♀ Avg. similarity:85.21 *n* = 13		*B. lapidarius* ♂ Avg. similarity:80.68 *n* = 5		*B. hypnorum* ♀ Avg. similarity:75.51 *n* = 16		*B. hypnorum* ♂ Avg. similarity:81.58 *n* = 11	
		Abundance	Contribution	Abundance	Contribution	Abundance	Contribution	Abundance	Contribution	Abundance	Contribution	Abundance	Contribution
26.21	Triacontene 1	–	–	–	–	–	–	–	–	–	–	–	–
26.25	Triacontene 2	–	–	–	–	–	–	–	–	0.19	0.08	–	–
26.99	Untriacontadiene 1	–	–	–	–	–	–	–	–	–	–	0.29	0.06
27.05	Untriacontadiene 2	–	–	–	–	–	–	–	–	–	–	0.3	0.13
27.09	Untriacontadiene 3	–	–	–	–	–	–	–	–	–	–	0.68	0.31
27.16	Untriacontadiene 4	–	–	–	–	–	–	–	–	–	–	0.29	0.11
27.22	Untriacontene 1	1.4	0.81	–	–	–	–	–	–	–	–	–	–
27.29	Untriacontene 2	–	–	–	–	–	–	–	–	–	–	–	–
27.36	Untriacontene 3	0.25	0.02	–	–	0.41	0.25	1.09	0.92	6.38	7.12	2.39	2.09
27.44	Untriacontene 4	–	–	–	–	2.7	2.07	2.59	1.7	0.24	0.05	–	–
29.57	Titriacontadiene 1	–	–	–	–	–	–	–	–	–	–	–	–
29.66	Titriacontadiene 2	–	–	–	–	–	–	–	–	–	–	–	–
29.76	Titriacontadiene 3	–	–	–	–	–	–	–	–	–	–	–	–
29.92	Titriacontene 1	–	–	–	–	–	–	–	–	–	–	–	–
30.01	Titriacontene 2	–	–	–	–	–	–	–	–	–	–	–	–
31.22	Tetratriacontene 1	–	–	–	–	–	–	–	–	–	–	3.16	2.47
31.37	Tetratriacontene 2	–	–	–	–	–	–	–	–	–	–	4.19	2.29

The relative abundance and contribution to intraspecific similarity is given. Unidentified alkene/alkadiene isomers of a given chain length are sorted and numbered with increasing retention time on a DB5-MS non-polar gas chromatography (GC) column (see "Methods" for analytical details)

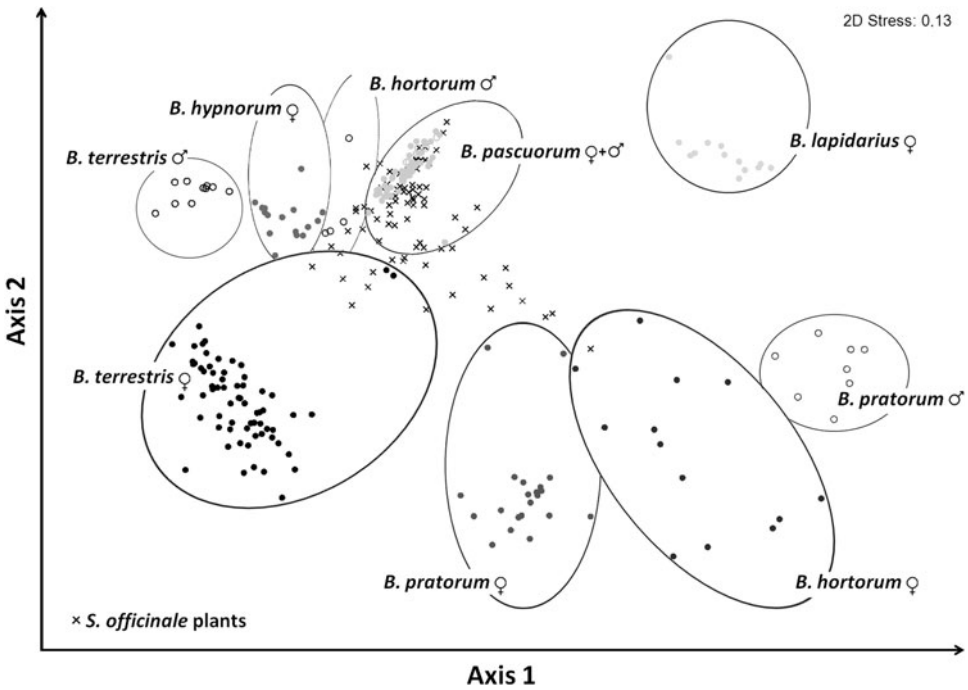

Fig. 1 Two-dimensional multidimensional scaling (MDS) plot showing the compositional similarity of unsaturated hydrocarbons (UHCs) in tarsal extracts of visiting bumblebees and corolla extracts of visited (*Symphytum officinale*). Data are derived from Bray–Curtis similarities based on relative abundances of 63 different alkenes and alkadienes

hydrocarbons of the relevant chain length are of very low volatility under variable temperature regimes (Witjes and Eltz 2009) and remain stable even when exposed to direct sunshine and adverse weather conditions (Ginzel and Hanks 2002). Specifically, bumblebee footprint alkenes do not measurably evaporate from flowers over 48 h (Witjes and Eltz 2009), which surpasses the floral life time of many temperate bee-pollinated plant species (Molisch 1929). Martin et al. (2009) revealed that CHC-profiles of hornets remained almost unchanged after the pinned specimens had been stored for 20 years! Thus, hydrocarbons of relevant chain lengths are highly resilient to evaporation and chemical alteration under a range of conditions and over substantial periods of time.

The first precondition, species-specific chemical composition of CHCs, is critical for the power of discriminating different visitor species. In bumblebees, the CHCs consist of linear alkanes, alkenes, and alkadienes with a predominantly uneven number of C-atoms (Goulson et al. 2000; Saleh et al. 2007; Schmitt 1990). Of those, only the unsaturated alkenes and alkadiens (UHCs) may serve as bumblebee visitation markers, whereas saturated alkanes occur in large quantities on unvisited flowers of most plant species (Goodwin et al. 2003; Griffiths et al. 1999, 2000). The overall UHC composition of different species of bumblebees differs significantly (this study, but see also Eltz 2006; Goulson et al. 2000), but there is also substantial between-species overlap in UHC compounds. Of the 63 UHC compounds identified in our study, only 18 were not shared by at least two different species, and nine were actually shared by all six. However, shared UHC compounds often occurred in predictably different relative proportions on different species, allowing us to estimate visitor communities quantitatively and with reasonable accuracy. It should be emphasized that the general scarcity of species-exclusive compounds compromises the ability to calculate reliable estimates for very rare species because their signatures are usually obscured by those of more abundant species. Thus, the overall likelihood that we recovered correlations between observed and estimated visitation frequency in different species was related to the abundance of those species at comfrey flowers. Remarkably, we were also able to recover separate visitation frequencies for workers and drones of *B. pratorum* and *B. hortorum*, which were sufficiently abundant and had UHC profiles sufficiently different from each other to allow differentiation by the algorithm. Workers of *B. hortorum* were the least abundant species for which we were able to estimate visitation frequency, contributing roughly 3% of all flower visits in 2009. It was clearly impossible to obtain separate estimates for entities that are too close in their UHC profiles, such as workers and drones of *B. pascuorum*.

In this study, the pollinator community of *S. officinale* consisted almost exclusively of bumblebees, thereby restricting the candidate visitors to a single bee genus of rather similar size and foraging behavior. This avoided the problem of having to deal with excessive variation in the amount of hydrocarbons deposited per visit, as would be the case in more diverse pollinator communities. It remains to be seen whether other pollinator groups will be as amenable to reconstruction as pure bumblebee communities. However, it is quite likely that even in more diverse communities, the UHC-based method can help to trace the

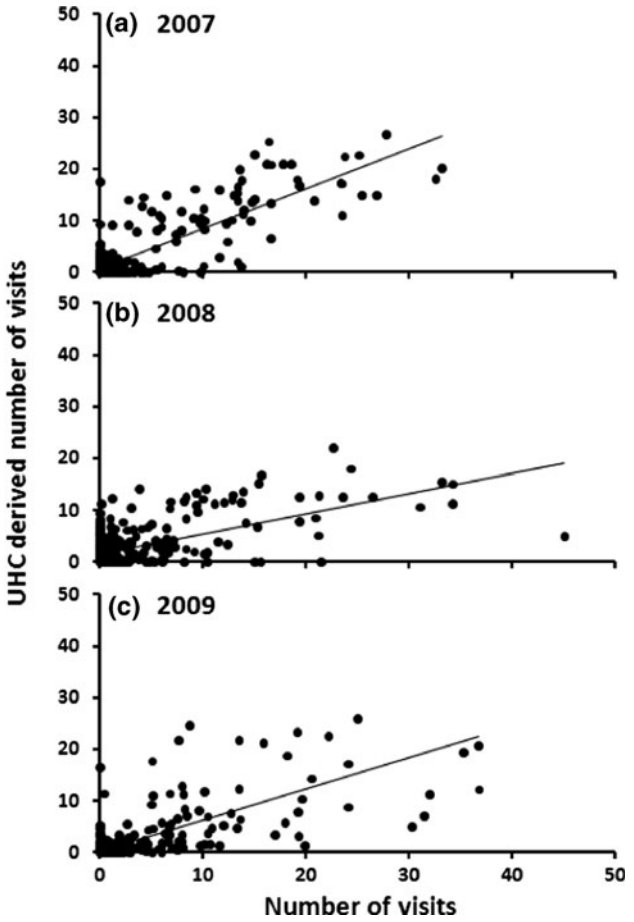

Fig. 2 Mean number of bumblebee visits per flower and day for *S. officinale* (extrapolated from bumblebee visitation counts in 10-min observation intervals) in relation to the UHC-derived number of visits in 2007 (**a**), 2008 (**b**) and 2009 (**c**)

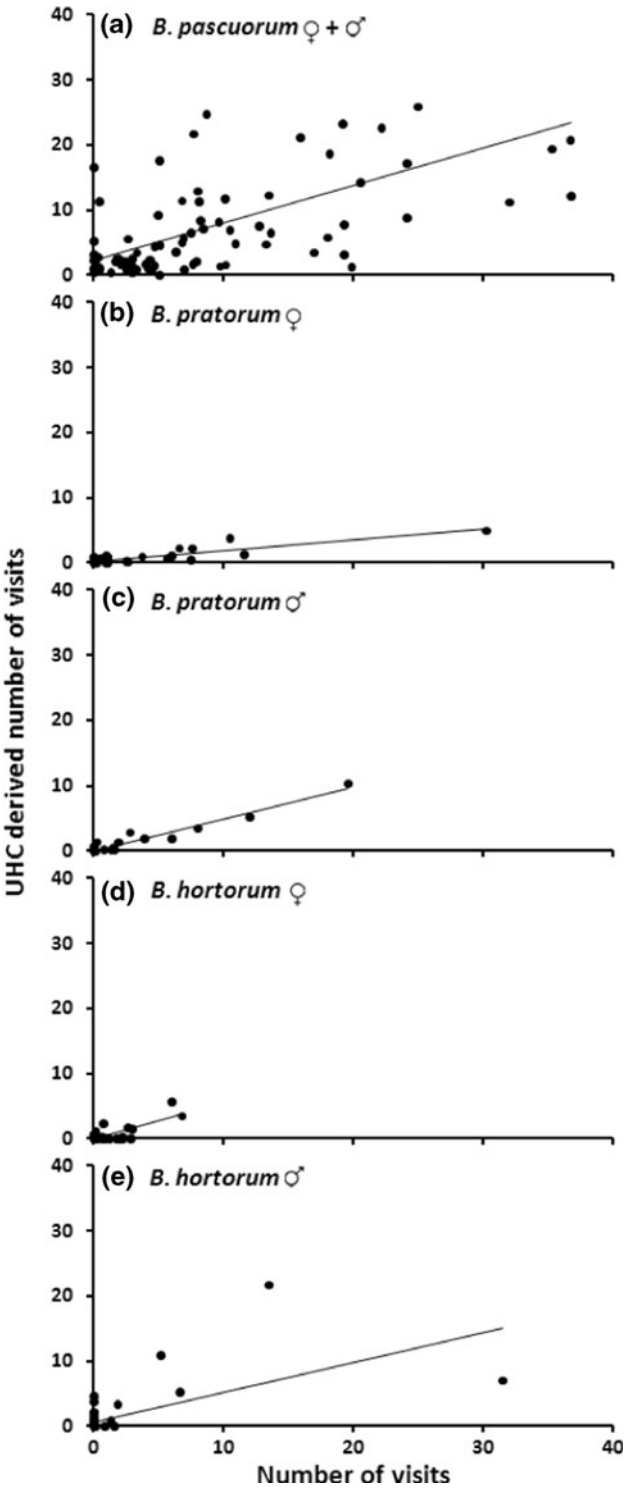

Fig. 3 Mean number of bumblebee visits per flower and day for *S. officinale* (extrapolated from bumblebee visitation counts in 10-min observation intervals) by the five most common bumblebee groups (**a**–**e**) in 2009 in relation to the UHC-derived number of visits

activity of certain target species, such as the most efficient pollinators. Furthermore, the inclusion of entirely different insect families will also lead to the inclusion of new classes of marker compounds, including branched hydrocarbons and substituted derivatives (Lockey 1988; Martin et al. 2009; Ruther et al. 2002), thereby increasing the power of discrimination of the analysis. At present, our experiences with more diverse communities is limited, but we have successfully distinguished the UHC deposits of a solitary bee (*Anthophora plumipes*) from those of bumblebees (Witjes and Eltz, unpublished data). Generally, the diversity of hydrocarbon profiles of insects is both substantial and predictable (i.e. species specific) (Howard 1993; Martin and Drijfhout 2009), encouraging future attempts to use them as visitation tracers.

CHC profiles, seed set, and pollen limitation

The overall quantity of UHCs on *S. officinale* flowers was not only a strong correlate of bumblebee visitation, but it was also related to the seed set of flowers, suggesting potential pollen limitation of comfrey plants under the conditions of our study. Our findings are in contrast with

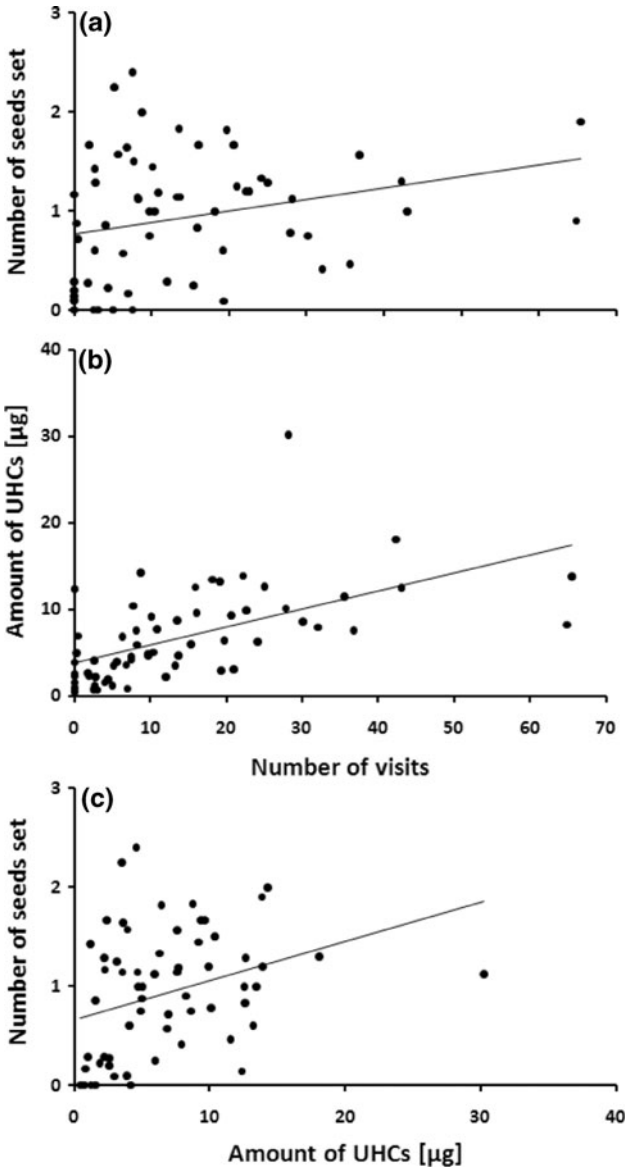

Fig. 4 Relationship between total number of bumblebee visits per flower and day for *S. officinale* (extrapolated from bumblebee visitation counts in 10-min observation intervals) and mean number of seeds (**a**) and total amount of UHCs per flower per plant (**b**). **c** Total amount of UHCs per flower per plant in relation to the mean number of seeds set per flower per plant

those of Goulson et al. (1998), who found no relationship between bumblebee visitation and seed set of comfrey in England. In both studies, the average number of seeds set per flower was far below the maximum of four, indicating that seed production is also limited by factors other than pollination. The degree to which plants suffer from pollen limitation varies substantially among localities and seasons (Ågren 1996; Burd 1994; Dudash and Fenster 1997; Kunin 1997; Larson and Barrett 1999; Paige and Whitham 1987) and might be particularly high very early in the season (Campbell 1985; Campbell and Halama 1993). Our own study included measurements taken very early in the flowering season of comfrey (May 2009), when bumblebee populations were still relatively low. In contrast, the study of Goulson et al. (1998) was conducted in the summer (June and July) when bumblebee populations were probably near their maximum. Thus, seasonal effects and differences in pollinator abundance might explain the differences in pollen limitation between the two studies. This discrepancy further illustrates the need for long-term and multi-replicate studies to gain a more general understanding of the relationship between pollinator visitation and plant fecundity (Rathcke and Jules 1993; Real and Rathcke 1991). In any case, our results suggest that the quantity of CHC deposits on flowers can serve as a crude predictor of seed set of plants in pollen-limited conditions. Within this context, it should be emphasized that the number of pollinator visits may not only affect the quantity of pollen deposited on a flower, but also its quality. For example, an increase in the number of pollinator visits may increase the number of pollen donors (Karron et al. 2006), and thereby offspring genetic diversity.

CHC profiling as a tool for pollination ecologists

Estimating visitation frequency from insect footprints may substantially supplement the toolbox available to pollination biologists and plant reproductive ecologists. In plant population studies, the availability of such a tool could relax the trade-off between the local intensity of a study, which is often high, and the number of replicate populations investigated, which is often quite low. Pollinator visits may be especially infrequent in small or fragmented plant populations, and thus observational studies need considerable time and effort to measure visitation frequency at all (Baker et al. 2000). Consequently, studies often focus on very few plant individuals at a specific time and place, which bears the risk of obtaining a biased sample of the actual visitation status (Waser et al. 1996). Extracting UHC footprints from many flowers may help to assess pollinator visitation on increased temporal and spatial scales, allowing broader generalization of the conclusions reached.

The analysis of large numbers of floral extracts with gas chromatography/mass spectrometry is relatively cheap and fast, given that a GC system with autosampler is available. The compound calling of peaks in ion chromatograms is likely to be the most time-consuming part of the analysis, which also requires some basic skills and experience. However, a few days are normally sufficient for students to learn how to build mass spectral user libraries and how to reliably assign peaks to entries based on their spectra and retention times. Generally, peak calling can be performed in a semi-automated way with the help of spectral

deconvolution software, thereby reducing the amount of time necessary for this task. Obviously, structural characterization (identification) of entries is a more demanding task and may not be possible based on mass spectra alone. Complete structural assignment, however, is not absolutely required in most of the envisioned ecological applications.

In conclusion, tracing pollinator footprints by solvent extraction and GC/MS analysis offers exciting new perspectives for pollination ecologists in many areas, but especially for those interested in population processes on a regional or landscape scale. This approach may also allow a more robust approach to measuring pollinator decline.

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