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Chemical footprints as cues to foraging bumblebees and pollination ecologists

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DECLARATION OF SELF-CONTAINED WORK

Herewith, I confirm that I composed the dissertation

“Chemical footprints as cues to foraging bumblebees and pollination ecologists”,

single handed without utilizing illegitimate resources. All experiments conducted comply with the “Guiding principles in the care and use of animals” and with current laws of the Federal Republic of Germany. I used no other than the cited references and facilities. This work has not been previously handed in to another university and was not subject to miscellaneous examinations.

Düsseldorf, 26. Mai 2010 Sebastian Witjes

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ABSTRACT

Many plant species are known to emit species-specific floral scents to attract or guide pollinators, thereby ensuring cross pollination. In combination with visual traits, pollinators use these cues to localize floral resources and to specialize on the most rewarding plant species. To complicate matters, each individual flower is exploited by other visitor individuals/species as well, and visitors are faced with the task to find floral rewards in a heterogeneous and fluctuating market. This thesis investigates the deposition and detection of chemical "footprints" on flowers by bumblebees, which allow them to discriminate against recently visited/depleted flowers. My studies corroborate the view that discrimination between individual flowers is based on the perception of non-polar chemicals left on flowers by previous visitors. In an artificial meadow, individual workers of *Bombus terrestris* were able to locate unvisited "flowers" unless the chemical deposits from previous visits were removed by the experimenter. Given natural reward conditions (small rewards that can be completely depleted during a single visit) the deposits act as repellent "scent-marks", inhibiting repeated visits to depleted flowers. In agreement with studies of other authors, the results of my experiments suggest that the chemical deposits are not evolved communication signals, but simple footprint cues, because the repellent effect was also elicited by footprints deposited on "neutral" (non-feeder) surfaces. Although long-chain hydrocarbons are the major chemical constituents in bumblebee footprints, my experiments indicate that more volatile trace components are the behaviourally active compounds: fresh (directly collected) footprints were rejected significantly more often than old footprints (collected with a 90 min. delay).

While hydrocarbons may not be the perceptually relevant compounds for bumblebees, they could be a cumulative indicator of flower visitation for pollination ecologists. Bumblebee epicuticular lipids consist of alkanes, alkenes, and alkadienes with chain length between 19 and 34 carbon atoms, in a highly species-specific composition. I showed that traces of these cuticular hydrocarbons remain on flowers after bumblebee visitation and are retained in the plants cuticular waxes. In solvent extracts of flowers of foxglove (*Digitalis grandiflora*) and primrose (*Primula veris*) the amount of bumblebee-derived unsaturated hydrocarbons (UHCs) was a close correlate of the number of bumblebee visits. Furthermore, bumblebee-derived nonacosenes were retained on flowers in near unchanged quantities for 24 hours

independent of temperature regime (15°C and 25 °C), suggesting that bee hydrocarbons accumulate over much of an individual flower life time. The results of a 3 year field survey on wild comfrey, *Symphytum officinale*, show that the analysis of hydrocarbon footprints can be used to reconstruct the visitor community and to estimate the seed set of this pollinator-limited plant. We successfully designed and applied a mathematical algorithm, which allowed us to estimate the visitation frequency of different bumblebee species separately from chemical footprint data. Thereby, we were able to derive visitation frequency of the most abundant bumblebee species, and even separately for workers and drones in some species. I conclude that bee footprints on flowers may not only be informative for the bees themselves, but represent a reliable and easy trace cue for pollination biologists.

CONTENTS

CHAPTER I INTRODUCTION

I.I CHEMICAL ECOLOGY OF PLANT-POLLINATOR INTERACTIONS

Flowers attract pollinators through a combination of visual and olfactory stimuli (Robacker et al. 1988, Dobson 2006). Evolutionary studies of floral characteristics have usually focused on visual traits as attractants to pollinators (Majetic et al. 2009), but floral scent has received growing attention in the last decade (Dobson 2006). The knowledge about scent chemistry of flowers has increased vastly, due to the improved sensitivity of analytical methods (gas chromatography/mass spectrometry) used in the analysis of volatiles in the headspace of flowers (Tholl and Röse 2006). The scent-emission of about 1000 plant species (belonging to 100 families) have been analyzed so far and more than 1700 compounds have been identified in their floral headspace (Knudsen et al. 2006). Most of the compounds found are low-molecular-weight volatiles, with high vapour pressure, which promotes the release and dispersal under moderate temperature regimes (Knudsen 2006, Knudsen et al. 2006, Baldwin 2010). The greatest proportion of plant volatiles are lipophilic (Baldwin 2010) and volatile blends are usually dominated by terpenoids, aliphatics, benzenoids, and phenylpropanoids (Knudsen et al. 2006, Baldwin 2010). Floral scent varies considerably in its quantitative and qualitative composition both between and within plant species and is assumed to be a product of phylogenetic constraints and pollinator and florivore mediated selection (Raguso 2001). The primary function of floral scents in flowering plants is the attraction of pollinators, including long distance attraction to patchily distributed resources (Knudsen et al. 1999), but also the guidance of flower visitors to the reward-producing floral organs (Raguso 2004). Studies with honeybees, a model organism of insect learning capacity, reveal that foraging decisions of flower visitors are based on a combination of innate and learned components. Innate preferences for specific floral odours (similar to innate colour preferences) are thought to facilitate flower choice of flower-naive pollinators (Raguso 2008), but are continuously updated with experience (Real 1991). Honeybees are able to distinguish a large number of odours (Hildebrand and Shepherd 1997) and successfully learn to associate both complex mixtures and single compounds with the availability of reward, and thus learn to predict the reward distribution from qualitative as well as quantitative differences in olfactory floral traits (Smith et al. 2006). Floral scent may mediate both, generalized as well as highly specialized plant-pollinator interactions (Raguso 2008). One

intriguing example for the latter is the “euglossine pollination syndrome”. Male orchid bees of the tribus Euglossini (Apidae) use plants as sources for volatiles which they use as pheromone-analogues (Eltz et al. 1999, Zimmermann et al. 2006). One of their major sources of fragrance compounds are orchids, with about 700 species being exclusively pollinated by scent seeking male orchid bees. Orchids produce species-specific fragrance blends of 3 to 10 compounds, which attract only a single or a small set of euglossine species and thus may serve as an important isolation mechanism between sympatric orchid species (Williams and Whitten 1983, Gerlach and Schill 1991). Conversely, plant species that fall under a generalist pollination syndrome, (e.g. Apiaceae, Arecaceae, Ranunculaceae, and Rosacea) are pollinated by many different insects, and pollen and nectar are typically presented in open and radially symmetrical flowers (Proctor et al. 1996). Although the produced floral scents do not show a particular unifying pattern, most of the compounds are common floral volatiles and thus attract many different pollinators, including bees, flies, beetles, and butterflies (Dobson 2006). Restricting the chemical communication between plants and their potential pollinators to plant-produced floral volatiles, would however present an oversimplified view of the chemical ecology of plant-pollinator interactions. Flower visiting insects may themselves leave odoriferous substances on flowers (Goulson 2003), and thus modify the information about reward availability mediated via floral scents. Such scent deposits can be seen as “surrogate floral odours” (Raguso 2008) and their detection and interpretation helps foraging bees to reduce the time spend searching for rewards and thus promotes the effective exploitation of floral pollen and nectar on foraging trips (Schmitt and Bertsch 1990, Stout et al. 1998). This thesis is about substances deposited by bumblebees on flowers, and about the informative content of such “footprints” for the bees themselves and for pollination ecologists.

I.II BIOLOGY OF BUMBLEBEES

Distribution Bumblebees (Apidae: Bombini) are widespread in temperate, alpine, and arctic environments of the northern hemisphere. They are common throughout Europe, North America and Asia with species richness peaks in eastern Tibet and the mountain ranges of central Asia (Williams 1994). There are about 250 known species of bumblebees (Williams 1985, 1994) from which 38 are resident in Germany (Westrich et al. 2008). All bumblebee-species occupy a broadly similar niche, as they exhibit little interspecific morphological variation, are active at largely overlapping times of the year, and exclusively feed on nectar and pollen throughout their lives (Goulson 2003). Although this indicates a high potential for interspecific competition (Heinrich 1976), between 6 to 16 species are commonly found to occur sympatrically in Europe (Goulson et al. 2008). The rather high number of coexisting bumblebee species remains yet to be adequately explained, but resource partitioning is usually attributed to interspecific differences in tongue length (Heinrich 1976, Graham and Jones 1996), preference of different sized flower patches (Goulson et al. 1998b), and heterogeneity in peak worker abundance (Goodwin 1995).

Phylogeny Together with honeybees (*Apini*) stingless bees (*Meliponini*) and orchid bees (*Euglossini*), bumblebees (*Bombini*) constitute the monophyletic clade of corbiculate bees within the family of the Apidae. Since bumblebees are morphologically very similar among species, their taxonomy is rather problematic. Subdivision of the genus *Bombus* was initially based on differences in colour patterns, a highly variable trait in bumblebees both within and between populations, which has led to a division into at least 38 different subgenera (Michener 2007, Williams 2007, Williams et al. 2008). Consequently, Williams (2008) has recently suggested a revision of the phylogenetic relationships based on the combination of morphological, behavioural, ecological and DNA sequencing data, which would lead to a division of the genus into 15 subgenera.

Life cycle In temperate regions bumblebee colonies usually have an annual life cycle, although they seem to be capable to breed continuously in regions with mild winters; e.g. *Bombus terrestris* nests have recently been reported to persist through winter in New Zealand and southern England (Goulson 2003). Impregnated queens emerge from hibernation between late winter and early spring (February until May). The time of emergence varies considerably between species but seems to be synchronized with the

blooming of the first flowers. They build their nests in species-specific sites, preferentially in abandoned cavities or nests of small mammals or birds, below or above the ground. The queens provision their nests with pollen, which they form into a brood clump into which up to 16 eggs are laid. They incubate the brood, maintaining a constant temperature between 30 and 32 °C (Heinrich 1979a) until the larvae hatch after about four days. Until the first workers emerge (after approximately four weeks) the queens have to constantly forage for nectar and pollen to supply the larvae and to provide the energy needed to maintain the incubation temperature of the brood. With the emergence of the first workers, the queens cease foraging as this duty is taken over by worker bumblebees and colony growth accelerates rapidly. The longevity of nests varies considerably between species and may last between 14 weeks in *B. pratorum* and *B. hortorum* to 25 weeks in *B. pascuorum* (Goodwin 1995). At the end of the life cycle bumblebee colonies switch to the rearing of reproductives, with the time of the switching depending on the absolute number of workers compared to the number of larvae in colonies (Alford 1975, Goulson 2003). Queens are able to control the sex of their offspring. Since they are the only mated individuals in colonies female progeny exclusively is produced by the queens, whereas males can develop from unfertilized eggs laid by either queens or workers. Worker reproduction is at first prevented by a queen produced pheromone, which suppresses ovarian development in workers. At a specific point (competition point) in the life cycle of the colony queens cease pheromone production and workers start to rear their own male offspring soon afterwards (Duchateau and Velthuis 1988). Young virgin queens and males leave the nest a few days after hatching in order to choose a compatible mate. After mating the young queens continue feeding on flowers before searching for suitable hibernation sites, preferentially situated in loose soil in species-specific depths. During hibernation queens feed on fat reserves laid down shortly before hibernation. The old nests quickly degenerate and the founding queens and workers perish (Alford 1975, Goulson 2003).

Bumblebees as pollinators Bumblebees are amongst the most abundant and reliable native pollinators in temperate areas (Goulson 2003). In Europe they are responsible for the pollination of at least 25 major crops and are the exclusive pollinators of a large number of wild plants (Corbet et al. 1991, Goulson 2003). The apparent decline of bumblebees could therefore have dramatic ecological and economical consequences (Allen-Wardell et al. 1998) as a loss of pollinator service may result in a decrease of pollen transfer to stigmas (Ashman

et al. 2004) and subsequent reduction in plant reproductive success through decreased fruit and seed set (Bierzychudek 1981, Louda 1982, Rathcke and Jules 1993, Kearns and Inouye 1997).

Pollination of crops Although cross pollination is not essential to all crops grown in the EU, it may promote uniform ripening (Williams et al. 1987) of often higher quality seeds and fruits or the production of more vigorous offspring, even in fully self fertile plants (Stoddard and Bond 1987). Due to their large colonies and the relatively easy management, honeybees were widely accepted as the most important pollinators of crops, but it has become evident that bumblebees are more efficient in pollinating some of them (Goulson 2003). The most prominent bumblebee pollinated crops are probably glasshouse tomatoes, which are now almost exclusively pollinated by commercially reared *B. terrestris* colonies in Europe. Further examples include alfalfa (*Medicago sativa*), clovers (*Trifolium* spp.), cherrys (*Prunus* spp.), cucumber (*Cucumis sativus*) and pumpkins (*Cucurbita* spp.) and a wide variety of other important crops (Corbet et al. 1991, Goulson 2003).

Pollination of wild plants Although many wild plants are reported to rely exclusively on bumblebees as pollinators little is known about the pollination requirements of the majority of naturally occurring plant species. Plant families which are thought to be partly dependent on bee pollination include the Boraginaceae, Ericaceae, Iridaceae, Lamiaceae, Malvaceae, Orchidaceae, Fabaceae, Scrophulariaceae, Solanaceae, and Violaceae (Corbet et al. 1991), but reliable data is missing. Potential pollinators of plants are often deduced from the pollination syndrome of flowers (coevolutionary morphological adaptions between flowers and pollinators), an approach probably reliable on the level of insect orders at most, as pollinations systems are often more generalized as suggested by floral morphology (Waser et al. 1996). Evaluation of physiological characteristics of bumblebees could give further clues to their importance as pollinators of wild plants. By vibrating their flight muscles bumblebees are able to generate internal heat, which allows foraging even under low temperatures (Heinrich 1979a) and makes them unsusceptible to adverse weather conditions (Corbet et al. 1993). Compared to the relatively uniformly sized honeybees, bumblebees vary considerably in size both within and between species and exhibit species-specific differences in tongue length, which allows them to handle a broader range of different sized flowers (Heinrich 1979a, Goulson et al. 2002).

I.III CHEMICAL COMMUNICATION OF BUMBLEBEES

Bumblebees are mostly described as primitively social, because they tend to have a simpler social organisation compared to the highly eusocial honeybees, and communication about availability or distribution of nectar resources, was thought to be rudimentary at most (Dornhaus and Chittka 1999). It is now evident, however, that communication is much more sophisticated than originally assumed.

Patrolling For mating, males of many bumblebee species mark particular parts of the vegetation (e.g. tree-trunks or shrubs) with pheromones secreted in their labial glands and establish flight paths between them, which are regularly patrolled (Schremmer 1972, Lloyd 1981, Bergman and Bergstrom 1997, Hovorka et al. 1998). The composition of the deposited pheromone is highly species-specific (Bergström et al. 1981, Bertsch et al. 2008) and seems to be largely comprised of long chain primary alcohols and hydrocarbons in most species exhibiting patrolling behaviour (Bergström et al. 1981). Although it is generally thought to act as a species-specific sexual attractant, gynes have rarely been observed at pheromone marked objects. Instead, intraspecific male attraction has been recorded far more frequently (Goulson 2003), suggesting that labial gland deposits could serve as aggregation pheromones, allowing virgin queens to choose between different males.

Recruitment Bumblebees do not recruit to specific locations of profitable food sources, and it was thus supposed that the detection of food is not communicated between foragers within colonies (Dornhaus and Chittka 1999). However, a series of recent experiments revealed that communication about food and recruitment does occur. The return of successful *B. terrestris* foragers into nests stimulated nest mates to leave and start to search for food. The overall activity of nest mates increased after successful foragers performed irregular runs through the nest combined with frequent wing fanning. Fanning behaviour seems to be combined with the release of a pheromone, as it could be shown in laboratory experiments that the activity of non-foraging colonies increased significantly when air from a foraging colony was introduced (Dornhaus and Chittka 2001). The major components of the recruitment pheromone in *B. terrestris* seem to be eucalyptol, farnesol and ocimene, which most probably originate in the tergal glands of the last three abdominal tergites. Abundance of the three aforementioned chemicals increased significantly in the airspace of nests with

the number of successfully foraging workers (Granero et al. 2005). Furthermore, foraging behaviour was initiated as soon as colonies were exposed experimentally to synthetic eucalyptol, farnesol and ocimene, both as a mixture and as separate compounds (Granero et al. 2005, Molet et al. 2008). This confirmed the behavioural relevance of the pheromones` major components. The intensity of forager activation depends both on the quality of the provided food (e.g. sugar concentration) and on the nectar reserves in the nests (Dornhaus and Chittka 2004, 2005, Molet et al. 2008), suggesting that bumblebees are able to respond to the recruitment pheromone differently depending on their nests nutritional status. Although bumblebees do not recruit to specific locations they do communicate the scent of nectar sources in the nest. They regularly sample and probe the content of the honey pots in nests and thus were shown to be able to learn the currently most promising food source (Dornhaus and Chittka 1999).

I.IV BUMBLEBEES FORAGING

Flower choice Bees have specialized on pollen and nectar as a food source and possess the appropriate morphological adaptations (elongated sucking mouthparts, hairs or baskets to trap pollen) for the effective exploitation of plants with different floral morphologies. They face a very heterogeneous environment in which the amount of reward provided by individual flowers is difficult to predict. The composition of forage plants is subject to substantial seasonal change (Zimmerman and Pyke 1986). Furthermore, the reward distribution varies within plant species according to the location of the individual plant (Marden 1984, Zimmerman and Pyke 1986, Real and Rathcke 1988), age and size of the plant (Goulson 2003) as well as with the time of the day (Marden 1984, Zimmerman and Pyke 1986). Within plant individuals the nectar production depends on age, size (Goulson 2003) and the position of the flowers on plants (Zimmerman and Pyke 1986). Effective exploitation of floral rewards is essential for the survival especially of social species, as it could be shown that in bumblebees the number of sexuals produced is a correlate of the collected food (Schmid-Hempel and Schmid-Hempel 1998, Ings et al. 2006) and thus directly influences colony fitness.

Choice of forage plant Bumblebees possess innate preferences for colour purity, dominant wavelength (violet and blue), and colour contrast (Lunau et al. 1996, Raine et al. 2006). The exhibited preferences are thought to facilitate the flower choice of naive bees, since for

example violet and blue coloured flowers have been shown to contain high amounts of nectar in various habitats (Chittka et al. 2004, Raine and Chittka 2007). Such predisposed preferences are far from absolute and may be modified with experience (Gumbert 2000). The recognition of flowers is not restricted to floral colour, but may further include the shape and scent of flowers or a combination of all mentioned traits (Menzel and Erber 1978). During foraging, bumblebees learn to selectively attend to one or several of the aforementioned traits to identify the current forage plant and continue foraging on flowers of the previously most abundant and rewarding plant species (Heinrich 1979b, Waddington et al. 1981). The exhibited fidelity to a plant species (flower constancy) however is not fixed and individual bumblebees have been shown to occasionally probe flowers of different plant species to keep track of changes in the reward distribution (Heinrich 1979b, Waddington et al. 1981, Goulson 2000a).

Choice of forage site Similarly to the choice of the forage plant, the individual choice of the forage site is largely based on the integration of the rewards received on previous flower visits. Bumblebees reliably return to sites which provided a reward on previous occasions (Osborne et al. 1999, Osborne and Williams 2001) and remain longer in flower patches of consistently high rewards (Klinkhamer et al. 1989, Klinkhamer and Dejong 1990, Goulson 2000b). According to Heinrich (1979c) foraging bumblebees exhibit different systematic movement patterns in response to the reward levels obtained. In high rewarding patches individuals had short flight distances and high turning angles between flowers, instead of long flight distances and low turning angles in patches with low levels of reward. This allowed foragers to move rapidly through areas depleted of nectar and concentrate in nectar rich sites. Generally they are thought to prefer large patches with respect to the number of flowers over small ones, although species-specific variation seems to exist. While most authors measured the absolute number of bumblebees in patches and interpreted differences in bumblebee recruitment rates as a generalized response to patch size (Klinkhamer et al. 1989, Dreisig 1995, Grindeland et al. 2005), Goulson et al. (1998b) pointed out that this could be an oversimplification. Whereas workers of *B. terrestris* showed a clear preference to large patches of *S. officinale* (>50 inflorescences per plant), *B. pratorum* workers preferred patches of medium size (<30 inflorescences per plant), and *B. pascuorum* seemed to exhibit no preference at all. This suggests that the choice of patch size could be subject to species-specific variation.

The amount of rewards in flowers does not depend on environmental conditions alone but is markedly influenced by the flower visitors themselves (Zimmerman 1981, Wetherwax 1986). Consequently, systematic movement alone is not sufficient for the effective exploitation of floral nectar and pollen, considering the unpredictability of their distribution. It is evident that bumblebees use several cues for their decision which flowers to probe and thus are able to avoid probing flowers with below average rewards (Heinrich 1979c, Wetherwax 1986). Possible mechanisms include direct visual and olfactory detection of floral rewards in openly structured flowers (Heinrich 1979c, Williams et al. 1981, Zimmerman 1982), but probably in most cases indirect cues, since pollen and nectar is normally hidden within flowers, e.g. in deep corolla tubes.

"Scent-marks" The results of various experiments emphasize that the decision, which individual flower to probe, seems to be mostly based on the recognition of "scent-marks", left on flowers by foraging bees on previous visits. In bumblebees, evidences are largely based on field experiments conducted by the working group of Dave Goulson, although he is not the first to have noticed. Goulson et al. (1998a) reported that worker bumblebees (*B. terrestris* and *B. pascuorum*) foraging on natural populations of comfrey (*Symphytum officinale*) paid significantly fewer visits to flowers already visited by other bumblebees than expected in case of random choice. To identify the underlying mechanism, Goulson et al. (1998a) recorded responses (rejection or acceptance) of bumblebees to inflorescences of *S. officinale* presented to them in choice experiments. Before the choice test, individual inflorescences had either been visited by the test bumblebee itself, a conspecific worker bumblebee, a heterospecific worker bumblebee, or had been randomly chosen (had an unknown history of visits). To exclude direct nectar detection the experiment was repeated with inflorescences which were protected from insect visitation with mesh screens for 1 h preceding the choice tests. Flowers were either unmanipulated (contained high amounts of nectar) or were artificially depleted of nectar. Whereas bumblebees readily accepted previously screened (unvisited) flowers, including those artificially depleted of nectar, they largely avoided flowers that were just recently visited by themselves, a conspecific or a heterospecific. Goulson et al. (1998a) concluded that the most parsimonious explanation was that bumblebees used "scent-marks" to discriminate against recently visited flowers. With variable stringency such effects have been demonstrated in honeybees (Giurfa and Nunez 1992, Giurfa 1993), stingless bees (Hrncir et al. 2004, Jarau et al. 2004, Schmidt et al.

2005) solitary bees (Gilbert et al. 2001, Goulson et al. 2001, Gawleta et al. 2005, Yokoi and Fujisaki 2009), but most often in bumblebees (Cameron 1981, Schmitt and Bertsch 1990, Stout et al. 1998, Goulson 2000b, Goulson et al. 2001, Stout and Goulson 2001). By using "scent-marks" bees are thought to be able to reduce the time spent searching for a reward and thus improve their overall rate of net energy gain (Schmitt and Bertsch 1990, Stout et al. 1998).

Influence on flower choice In agreement with the results obtained by Goulson et al. (1998a) experiments by Stout et al. (1998) revealed that at least in bumblebees "scent-marks" seem to be not exclusively informative to conspecifics but are also detectable by foraging heterospecifics. Workers of *B. terrestris*, *B. hortorum*, *B. pascuorum* and *B. pratorum* equally avoided probing recently visited inflorescences of *S. officinale*, independent of the identity of the previous flower visiting species. Furthermore, solvent extracts of *B. terrestris* legs applied to flowers in bioassays had similar repellent effects in different bumblebee species (Stout et al. 1998, Goulson 2000b), suggesting that the presence of "scent-marks" on flowers provokes an unspecific, repellent response in bumblebees. This seems plausible because kinship analysis between bumblebee workers, which shared the same forage site, revealed, that flowers are commonly visited by members of many different colonies and species. Chapman et al. (2003) and Darvill et al. (2004) both used microsatellite markers to assess the dispersal and degree of resource sharing of two bumblebee species (*B. terrestris* and *B. pascuorum*). According to genetic analysis, on average 20 *B. terrestris* and 55 *B. pascuorum* colonies utilised the same patch of flowers simultaneously in a rural landscape (Darvill et al. 2004). In an urban area resource sharing was even more distinct, with individuals of on average 96 *B. terrestris* and 66 *B. pascuorum* colonies foraging on flowers of the same patch (Chapman et al. 2003). The detection and avoidance of "scent-marks" deposited on flowers by foraging heterospecifics would therefore further promote the effective exploitation of floral resources (Goulson 2009). Correspondingly, the results of recent experiments suggest that bumblebees are able to discriminate against flowers recently visited by honeybees (Stout and Goulson 2001), solitary bees (Gawleta et al. 2005), and even hoverflies (Reader et al. 2005).

Although the use of "scent-marks" is widely accepted as to improve foraging efficiency, there have been discrepancies about the directionality of responses of bumblebee-

individuals to “scent-marked” flowers in bioassays. While in most field experiments “scent-marks” induced repellence (Goulson et al. 1998a, Stout et al. 1998, Goulson 2000b, Gawleta et al. 2005), most laboratory experiments have shown attractant effects (Cameron 1981, Schmitt and Bertsch 1990, Schmitt et al. 1991). Following an argument by Goulson (2003), attractive effects could have been provoked by unnatural reward conditions. Artificial feeders used in laboratory experiments were normally continuously rewarding, encouraging bumblebees to detect and repeatedly visit “scent-marked” (continuously rewarding) feeders. Flowers in natural habitats, however, produce rather low rewards easily extractable during a single flower visit, so that flowers containing a fresh “scent-mark” are most probably empty. The experiments presented in **chapter II.I** had the purpose to resolve this conflict, by implementing near natural reward dynamics in an array of artificial feeders in the laboratory, and to test whether “scent-marks” could indeed be cues, flexibly interpreted under different reward conditions.

“Scent-marks” or footprint cues Communication in animals can roughly be attributed to the recognition of signals or cues. Signals are defined as traits, moulded by natural selection to deliberately transmit information from a signaller to a recipient in order to elicit a specific, often hard-wired response in the recipient. Cues, in contrast, are incidental features in the environment that animals can use to modify their future behaviour (Maynard Smith and Harper 2003).

Considering this the term “scent-marks” is probably misleading, because it implies deliberate labelling of substrates (in this case visited flowers). In a laboratory experiment Saleh et al. (2007) demonstrated that bumblebees leave the same compounds, mostly long chain saturated and unsaturated hydrocarbons, on food, nest, and neutral surfaces. The composition of the detected hydrocarbons closely resembled those found on the tarsi of bumblebees (Oldham et al. 1994, Goulson et al. 2000). “Passive” marks, deposited in a neutral context had an equally strong repellent effect on foraging bumblebees as “active” marks deposited on artificial feeders. This indicates that the involved compounds are most likely involuntary deposits of cuticular lipids remaining on all surfaces bumblebees have walked over (Wilms and Eltz 2008). Furthermore, bumblebees have been shown to respond context-dependent to the same chemical deposits on artificial flowers, intriguingly in a manner most appropriate to efficiently exploit the presented rewards. In agreement with

the results of my laboratory experiments **(chapter II.I)**, Saleh and Chittka (2006) found that bumblebees avoided repeated visits to (presumably "scent-marked") artificial flowers under near natural reward conditions (low rewards, no immediate refills), whereas they showed the opposite behaviour if artificial flowers continuously contained rewards. In another experiment by Saleh et al. (2006) the strength of the repellent effect was shown to depend on flower complexity, with bumblebees being significantly more likely to avoid previously visited flowers when this incurred higher costs, in this case longer handling times. These results clearly indicate that the decision which flowers to probe, at least in bumblebees, is more likely based on recognition of a footprint cue, than on a scent signal, and that the response to the cue is modified by experience.

Chemistry In bumblebees footprint deposits mainly consist of odd numbered saturated and unsaturated hydrocarbons (alkanes, alkenes and alkadienes), which originate in specialized epidermal cells (oenocytes) and then are secreted onto the epicuticle via exocrine glands **(chapter I.V and references therein)**. The hydrocarbons are thought to form a semi-liquid layer of lipids with almost homogenous composition over the entire body of bumblebees (Oldham et al. 1994). Comparisons between solvent extracts of cuticular samples (wing, antenna and leg) and candidate secretory glands (duffour gland and tarsal gland) of bumblebees revealed the same hydrocarbons with chain length from 21 to 31 carbon atoms (Schmitt 1990, Oldham et al. 1994). During foraging traces of these lipids remain on the visited flowers with the quantity depending on the intimacy of contact between the forager and the visited flower. Hydrocarbons are common constituents of the epicuticular lipid layer in a broad range of insects. They presumably originally evolved to reduce water loss in terrestrial habitats, but are now known to have several secondary functions in insects (Lockey 1988, Blomquist et al. 1998) **(chapter I.V)**. Whereas cuticular hydrocarbon composition is rather constant within species, notable interspecific differences seem to exist. Comparisons between five sympatric European bumblebee species revealed significant differences in the qualitative and quantitative composition of cuticular hydrocarbons. While tricosane was a major compound in extracts of all tested *Bombus* species, nonacosenes seemed to be almost exclusive to *B. terrestris* (*B. terrestris terrestris* and *Bombus terrestris audax*) whereas *B. pratorum* extracts were dominated by tricosenes and pentacosenes (Oldham et al. 1994) (see **chapter II.IV** for more details).

Hydrocarbons as footprint cues As major constituents of bumblebee footprint deposits, cuticular hydrocarbons are also thought to be the responsible chemical cues that are perceived by bumblebee flower visitors, allowing them to discriminate against recently visited flowers in natural plant populations (Stout et al. 1998, Goulson et al. 2000). There are however conflicts between the exhibited context-dependent flexibility of bumblebees´ responses to footprint deposits on flowers and the chemical properties (low volatility) of the candidate footprint hydrocarbons. The repellent effect of bumblebee footprints has been shown to decline over time, with the duration of repellence being inversely related to the rate of nectar accumulation of flowers (Williams 1998, Stout and Goulson 2002). It has been hypothesized that the frequency of rejection declines with the concentration of footprint deposits on flowers, presumably because the responsible chemicals evaporate (Stout et al. 1998). Hydrocarbons of the relevant chain length, however, are of very low volatility and are retained on flowers in near unchanged quantities for up to 48 hours **(chapter II.III)**. Eltz (2006) speculated that footprint hydrocarbons could become incorporated into the semi-liquid layer of plant cuticular waxes and thus gradually lose their perceptibility to foragers. Alternatively, footprints could contain small quantities of so far undetected low-molecular-weight volatiles in addition to long-chain hydrocarbons. The experiments in **chapter II.II** tested the two aforementioned hypotheses.

I.V CUTICULAR HYDROCARBONS

Origin and transport The insect cuticle is a membranous outer skin, on the one hand structurally tough enough to protect the insects´ inner organs, yet light and flexible to allow flight. It is comprised of a chitin and protein rich inner procuticle and a non-chitinous epicuticle. The procuticle consists of a rigid exocuticle, in which proteins are sclerotized and an elastic endocuticle. The epicuticle, though relatively thin, is structurally rather complex and consists of an inner and outer epicuticle, the latter being covered by several extracuticular layers. The cuticle as a whole is permeated by lipids, which are classified according to whether they can be extracted with organic solvents as “structural” (insoluble) and “free” (soluble) lipids. “Structural” lipids mainly occur in the inner epicuticle and the exocuticle where they are thought to form a waterproof barrier, protecting insects against desiccation. “Free” lipids are major components of the epicuticle and provide a loose covering (lipid layer) on the outer epicuticles´ surface. Hydrocarbons are major constituents

of the cuticular lipids in insects and, although they are thought to contribute substantially to the cuticles water protective effect, they also play a potential role in insect communication (Lockey 1988, Blomquist et al. 1998, Howard and Blomquist 2005). In insects, cuticular hydrocarbons are synthesized *de novo* from acetate in specialized epidermal cells (oenocytes) (Katase and Chino 1984, Gu et al. 1995). Following synthesis, newly produced hydrocarbons become associated with lipophorin, a lipoprotein carrier in the hemolymph (Gu et al. 1995). It provides a reusable shuttle promoting the distribution of the hydrophobic hydrocarbons through the aqueous hemolymph to specific organs (e.g. secretory glands) (Katase and Chino 1984). The further mechanism of transport is largely unknown, but studies on a Formicine ant (*Cataglyphis niger*) indicate that hydrocarbons accumulate in exocrine glands and then are secreted onto the cuticle surface of insects (Soroker et al. 1994), where they are thought to form a thin layer of epicuticular lipids (Oldham et al. 1994).

Hydrocarbons in insect communication Communication based on the recognition of cuticular hydrocarbons is common among social insects. The epicuticular lipid layer of most insects consists of a mixture of saturated and unsaturated hydrocarbons, with a highly specific composition. Saturated hydrocarbons (n-alkanes and methyl-branched alkanes) often predominate, whereas unsaturated hydrocarbons (e.g. alkenes and alkadienes) occur in variable proportion. Chain length and methyl-branching pattern are the major distinguishing features of hydrocarbon profiles between species, whereas double bond position in alkenes and alkadienes contribute to specificity with variable stringency (Lockey 1988, Blomquist et al. 1998, Howard and Blomquist 2005). Cuticular lipids have been shown to play an important role in recognition of species, kin and nest mates in many social insects, including ants, termites, wasps, honeybees and bumblebees (Vander Meer et al. 1998, Lahav et al. 1999, Ruther et al. 2002, Breed et al. 2004, Dani et al. 2005, Dronnet et al. 2005, Sramkova and Ayasse 2009), and hydrocarbons are thought to be the most important involved cues (Howard and Blomquist 2005). Species and kin recognition is thought to be especially important to social insects and presumably allows colonies to maintain genetic integrity and facilitates colony defence (Breed et al. 2004). Social insects have been shown to develop a colony specific odour, spread among nest mates via grooming or trophallaxis. Specific variation between colony odours leads to the recognition of non-nest mates and agonistic behaviour against the intruders (Breed and Stiller 1992). Although hydrocarbons

have been shown to be particularly important, the process of discrimination may additionally involve recognition of many other aliphatic compounds (Blomquist et al. 1998, Howard and Blomquist 2005). Furthermore, cuticular hydrocarbons have been shown to relate information about reproductive status within colonies (Bonavitacougourdan et al. 1991, Liebig et al. 2000, Sledge et al. 2001, Howard and Blomquist 2005). Essentially the same cuticular lipids are thought to provide footprint cues, which allow returning foragers of many species of bees, ants and wasps to recognize their nest entrance at close range (Lahav et al. 1999, Ruther et al. 2002, Dani et al. 2005) and could be similarly informative to foraging bees, which are thought to use them to discriminate against recently visited flowers **(chapter I.IV and references therein)**.

Hydrocarbons in chemotaxonomy In addition to the evident importance of cuticular hydrocarbons in the life of insects, there are some characters that render them particularly interesting to biologists. In insects, cuticular hydrocarbons are normally synthesized *de novo*, via genetically controlled pathways (Dallerac et al. 2000). Their composition is therefore an expression of genotype and as such is of use as taxonomic character (Lockey 1988). As semiochemicals, cuticular hydrocarbons are subject to extensive selective pressure for diversification. Consequently, hydrocarbon composition exhibits a high degree of specificity among insects allowing separation of even closely related, sympatrically occurring species (Hefetz 1993). The results of cuticular hydrocarbon-based classification have been found to correspond well with taxonomic grouping and long-chain hydrocarbons are now commonly used in chemotaxonomy, which has been successfully applied to beetles (Symonds and Elgar 2004), cockroaches (Everaerts et al. 1997), Drosophila (Jallon and David 1987), termites (Uva et al. 2004), wasps (Dapporto et al. 2004), grasshoppers (Chapman et al. 1995), butterflies (Dapporto 2007), Formica ants (Martin et al. 2008), and hornets (Martin et al. 2009). Although cuticular hydrocarbon derived chemotaxonomy is mostly used to discriminate between species or sub-specific taxa (Lockey 1988), it has furthermore been successfully applied to separate sibling species and sexes e.g. of insect disease vectors (Carlson and Service 1980). Females of *Anopheles gambiae* and *Anopheles arabiensis* are the principal vectors of malaria in tropical Africa. As members of true sibling species they are morphologically very similar and share the same habitat, which makes them difficult to distinguish. Although they shared the same cuticular hydrocarbons with regard to qualitative

composition, Carlson and Service (1980) found distinct quantitative differences in the relative abundances of components and not only successfully separated the two species, but also the female malaria vectors from males of the same species. Another important feature of hydrocarbons is their extraordinary long term stability. Comparisons between four hornet species by Martin et al. (2009) revealed that species-specific cuticular hydrocarbon profiles remained unchanged on dried specimens for 20 years, allowing the use of dried museum specimens in chemotaxonomy.

Hydrocarbons in mark-recapture studies Ginzel and Hanks (2002) have evaluated the potential use of synthetic hydrocarbons as chemical labels to estimate dispersal of insect populations in mark-recapture studies. Mixtures of synthetic long chain alkanes (C24, C25, C26, C28, and C30) remained qualitatively and quantitatively stable on elytra of milkweed beetle (*Tetraopes tetrophthalmus*) for 2 month, despite exposure to high humidity and temperature. Application of hydrocarbons on live beetles had no effect on individual longevity and mating success and thus seemed not to affect individual reproduction. Due to their durability and low toxicity Ginzel and Hanks (2002) suggested that synthetic hydrocarbons could provide an alternative to the commonly used tags and dyes, which are highly susceptible to adverse weather conditions, and to long lasting but toxic rubidium-isotope markers.

Hydrocarbons as indicator of flower visitation Pollinator service is essential for self-incompatible animal-pollinated plants, because the quantity of pollen transferred to stigmas of female flowers directly influences the reproductive success of plant individuals (Rathcke and Jules 1993, Agren 1996, Allen-Wardell et al. 1998, Ashman et al. 2004). Infrequent pollination therefore may significantly decrease the fertility of allogamous plants, especially in small plant populations, e.g. in fragmented habitats. Although pollen limitation is agreed to be a common phenomenon (Bierzychudek 1981, Burd 1994, Ashman et al. 2004) little is known about the proximate ecological factors involved. Pollen limitation is experimentally demonstrated through an increase in plant fertility after supplemental hand pollination relative to open pollinated controls, but few studies were able to establish a functional link between plant reproduction and flower visitor abundance in natural populations (Agren 1996). Measuring pollinator visitation accurately and with sufficient replication is part of the

problem. Pollinator visitation is highly infrequent and thus the collection of sufficient data by observation is extremely time-consuming (Larson and Barrett 1999, Baker et al. 2000, de Jong et al. 2005). The studies presented in **chapter II. III** and **II.IV** provide evidence that the quantification of pollinator footprints could help pollination ecologists to identify potential pollinators of plants and assess the importance of pollinator visitation for the reproductive success of plant populations. Cuticular hydrocarbons commonly occur on epicuticles of insects in a highly species-specific composition and have recently been shown by Eltz (2006) to be retained on flower corollae after bumblebee visitation. Solvent washes of deadnettle flowers (*Lamium maculatum*) visited by *B. pascuorum* workers in the field contained bumblebee-derived alkenes in addition to the plants own cuticular lipids, as evidenced by gas chromatography/mass spectrometry (GC/MS) analysis. The amount of pentacosenes, which are the major compounds of cuticular lipids in *Bombus pascuorum*, was almost linearly related to the number of visits to flowers, suggesting that the epicuticular wax of flower corollae retains a chemical record of pollinator visitation. **Chapter II.III** and **II.IV** present the results of a series of laboratory experiments and field surveys, conducted to assess the accuracy with which hydrocarbon footprints on flowers could reflect the quantity and composition of bumblebee flower visitation. The experiments presented in **chapter II.III** had the purpose to determine the duration and the stability of bumblebee (*B. terrestris*) footprint retention on natural flowers under different temperatures. Furthermore, it was tested if the amount of hydrocarbon footprints on natural flowers of wild comfrey (*S. officinale*) was reliably related to the overall number of bumblebee visitation. Comfrey is a common perennial herb, preferentially situated at flower rich meadows along rivers. Bumblebees are most likely its only effective pollinators in Germany, because pollen release in comfrey requires high frequent “buzzing” (“buzz-pollination”), a pollination system which is thought to be highly susceptible to pollen limitation (Larson and Barrett 1999). **Chapter II.IV** presents the results of field surveys, in which bumblebee visitation and seed set of wild comfrey plants was recorded and compared to the quantity and composition of potential bumblebee footprints on flowers. A mathematical algorithm was designed to reconstruct bumblebee visitation frequency and species composition. The results are placed in a broader ecological context in order to determine the potential use of cumulative footprint quantification to assess pollinator visitation and seed set of pollen limited plants.

CHAPTER II PUBLICATIONS AND EXPERIMENTS

I. Influence of scent deposits on flower choice: experiments in an artificial flower array with bumblebees

Sebastian Witjes and Thomas Eltz

Apidologie 38 (2007) 12 – 18.

II. Experiment: The perceptual relevance of cuticular hydrocarbons in bumblebee footprints on flower choice

III. Hydrocarbon footprints as a record of bumblebee flower visitation

Sebastian Witjes and Thomas Eltz

Journal of Chemical Ecology 35 (2009) 1320 – 1325.

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

Sebastian Witjes, Kristian Witsch & Thomas Eltz

Manuscript under review in *Oecologia*

CHAPTER II.I

Influence of scent deposits on flower choice: experiments in an artificial flower array with bumblebees

Sebastian Witjes and Thomas Eltz

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Original article

Influence of scent deposits on flower choice: experiments in an artificial flower array with bumblebees*

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Abstract – Foraging bumblebees leave chemical substances when visiting flowers and the detection of these "scent marks" improves their foraging efficiency. Whereas laboratory studies found that scent-marks convey attraction to food sources, all field studies found foragers to be repelled by recently visited flowers. In this study we aim to resolve this conflict by implementing near-natural reward dynamics in a laboratory feeder array. When feeders were filled with small, non-replenished amounts of reward, worker bumblebees (*Bombus terrestris*) avoided revisiting the depleted feeders. As evidenced by a "corolla" replacement experiment, feeder discrimination was based on the perception of chemical cues deposited during previous visits. Pentane extracts of bumblebee tarsi acted as a repellent when applied to glass corollas, whereas pure pentane did not. We suggest that scent-marks are simple cues inherent to footprints and emphasize the importance of context to how these cues are interpreted by foraging bees.

flower discrimination / repellent scent marks / chemical cue / signal / bumble bees / *Bombus*

1. INTRODUCTION

Foraging bumblebees face a heterogeneous environment in which the amount of reward provided by individual flowers is difficult to predict. Within a given plant species the quality and quantity of nectar varies depending on the location of the plant individual, age of the plant, position of the flower on the plant, age of the flower and the time of day (Klinkhamer and van der Lugt, 2004; Leiss and Klinkhamer, 2005). In addition, reward distribution is influenced markedly by the flower visitors themselves, both within and among patches (Wetherwax, 1986; Zimmerman, 1981). The existing variability in nectar standing crops represents a formidable challenge to individual bumblebees, whose foraging behaviour has evolved not only to meet their own energetic requirements but also to provide for their colony (Heinrich, 1979a). In natural flower patches, bumblebees frequently can be observed to hover in front of individual flowers or inflorescences, but then depart without actually probing for nectar. The flowers which bumblebees reject contain on average less nectar than flowers which are being probed (Heinrich, 1979b; Marden, 1984; Wetherwax, 1986). Thus it appears that bumblebees use some means of remote sensing for their decision on what flower to probe. Possible mechanisms include direct detection of the nectar by visual (Kevan, 1976; Thorp et al., 1975, 1976) or olfactory (Raguso, 2004) cues. However, many bumblebee-visited plant species have flowers in which nectar is concealed at the base of deep corolla tubes, which makes direct assessment of nectar volume problematic. In such cases more accurate information about reward levels may be obtained by using indirect cues. Several studies have emphasized that bees use scent-marks deposited on the flower by themselves or by conspecifics to identify the availability of a

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reward. With variable stringency such effects have been demonstrated in honeybees (Giurfa, 1993; Giurfa and Nuñez, 1992), bumblebees (Cameron, 1981; Goulson et al., 1998, 2000; Schmitt et al., 1991), stingless bees (Hrncir et al., 2004; Jarau et al., 2004; Schmidt et al., 2005), and solitary bees (Gawleta et al., 2005; Gilbert et al., 2001; Goulson et al., 2001). By interpreting such scent-marks bees are able to reduce the time required to search for a reward and thus increase their overall foraging efficiency (Giurfa, 1993; Schmitt and Bertsch, 1990; Stout et al., 1998).

In bumblebees the glandular origin of the deposited substances is unclear, and it is an open question whether scent-marking is an active process. Insects are known to leave lipid footprints wherever they walk, and those footprints may be used as cues by subsequent visitors (Federle et al., 2002; Schmidt et al., 2005). Several studies have investigated bumblebee scent-marks and their effect on foraging efficiency. There is one striking difference concerning the results of these experiments. In field experiments foragers have always been found to be repelled by recently visited (scent-marked) flowers (Goulson et al., 1998, 2000; Stout et al., 1998; Gawleta et al., 2005), whereas in laboratory experiments an attractant effect has been found (Cameron, 1981; Schmitt and Bertsch, 1990; Schmitt et al., 1991). The present study aims to resolve this conflict. In accordance with an argument made by Goulson et al. (2000), we hypothesize that the origin of the discrepancy is based on unnatural reward conditions in the laboratory studies. Under most natural conditions flowers contain only minimal amounts of nectar, which are easily extracted by foragers during a single visit. Since nectar secretion rates are low in nature (Stout and Goulson, 2001), a fresh scent-mark of another bee spells "empty". Thus, in the natural context, bees learn to avoid recently visited (scent-marked) flowers. In contrast, when rewarding feeders were presented in laboratory experiments, they carried a continuous reward or were immediately replenished (Cameron, 1981; Schmitt and Bertsch, 1990; Schmitt et al., 1991). In this context foragers were encouraged to detect and revisit scent-marked feeders, while at the same time neglecting unmarked feeders. While these results confirmed traditional views of "attractant scent-marks" in social bees, they were derived from an unnatural situation. In the present laboratory study we modelled the reward dynamics much more closely to the natural situation, with feeders carrying only tiny amounts of nectar that, once exploited, were not replenished during an individual foraging bout. We hypothesized that foragers would now avoid recently visited flowers and that this discrimination enhances their foraging efficiency. Furthermore, we tested whether flower discrimination does indeed depend on recognition of deposited substances, and whether the discrimination ability increases with growing experience.

2. MATERIALS AND METHODS

Colonies of *Bombus terrestris* (Koppert Biological Systems) were fed with sugar-water in permanently rewarding feeders (carrying yellow glass corollas identical to those of the test flowers described below) in a feeding box connected to the main nest box. Individual foragers were marked and later introduced into a test cage measuring $60 \times 65 \times 85$ cm and covered with mosquito mesh. One end of the cage was fitted with a disk made of grey PVC (diameter 60 cm) which could be rotated around its central axis and had fittings for up to 21 artificial flowers, spaced more or less uniformly over the entire disk area. The corolla of an individual feeder (Fig. 1) was a tube of yellow quartz-glass 4 cm in length and 2.1 cm in diameter. It sat on a short Plexiglas cylinder in which a 1.5 mm bore had been drilled for the sugar-water reward. At the rear end of the cylinder the bore widened to 3 mm. Before each trial the narrow front ends of the bores were filled with 2 μL of a 50% sugar-water reward. The rear ends, being well out of the reach of the bees' proboscises, received another droplet of the same solution. This was supposed to maintain unaltered odour and humidity even after the sugar-water reward had been collected by the foraging bumblebee. After a bumblebee had been introduced into the test arena its behaviour was recorded with the help of the software clbehave (Compulights GmbH, Mönchengladbach). We logged the sequence of the approached (numbered) feeders and registered whether an approached feeder was *visited*

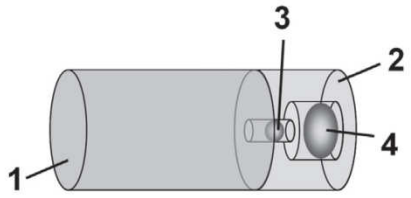

Figure 1. Schematic view of an artificial flower with detachable quartz-glass corolla (1) sitting on a Plexiglas cylinder (2) with a small bore drilled in the front end to receive 2 μL of sugar water reward (3) as well as a larger rear-end bore to receive an additional droplet of sugar water (4), which could not be reached by foraging bumblebees.

or *rejected*. A feeder was defined as being *visited* if a bee crawled completely into the glass corolla, assuming a position that would allow probing (the proboscis extension could not be directly observed from the outside). *Rejection* included all approaches that were not followed by landing or, if a landing took place, it was brief and not followed by in-depth inspection of the flower. During data analysis we were able to establish whether a given visit was actually rewarded or not, depending on whether it was the first visit to that feeder during a particular trial.

2.1. Experiment 1

We tested whether foragers avoided feeders recently depleted by themselves in order to increase their foraging efficiency, and whether this ability improved with growing experience. Bumblebees foraged on a disk fitted with 21 artificial flowers. Up to 70 approaches were recorded per individual trial and each individual completed four consecutive trials. Following each feeder visit the disk was rotated to exclude the possibility that bees memorized the position of visited feeders. After each trial the bumblebees were released into the colony to deliver the collected sugar-water. The glass corollas were rinsed in acetone, the Plexiglas tubes swabbed with hexane. To analyse the efficiency of exploitation we plotted the number of visits required to receive a given number of rewards (Fig. 2) and compared the observed performance to the performance expected by chance, e.g. in the case that every corolla was selected by random choice. We used the sign-test (STATISTICA 6.0) to test for differences between the observed number of visits necessary to receive

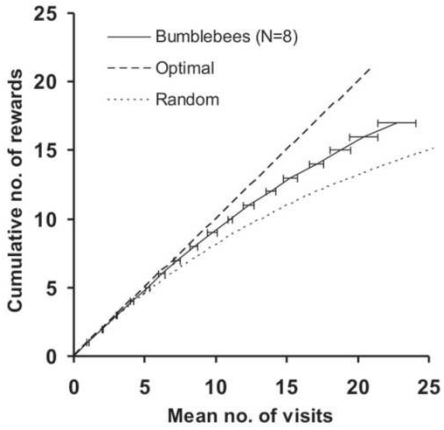

Figure 2. Foraging efficiency of worker bumblebees in a rotating feeder array. The curve shows the mean number (+/– standard deviation) of visits necessary to receive a certain (cumulative) number of rewards. Not that bumblebees were not perfect foragers, but performed better than expected in the case of random choice. Data from all trials pooled for individual bees.

15 rewards and the expected number of visits given that foragers selected flowers randomly. Additionally, we used Repeated-measures ANOVA to test for an effect of experience (No. of trial) on the number of visits individuals required to receive 15 rewards.

2.2. Experiment 2

The second experiment was designed to test whether the discrimination against empty feeders was based on the recognition of a chemical cue deposited on the corolla. Again, the bumblebees foraged on an array of 21 artificial flowers. Each bee had to complete 2 trials. The first trial was identical to the trials of experiment 1 and was used as training. The second trial consisted of two different treatments. In one group of bees the experiment was interrupted after 10 rewarded visits by switching off the light in the test chamber. After the visited corollas had been replaced by clean ones the trial continued (Replacement group). In the other group the visited corollas were not replaced but only lifted briefly and then restored (Control group). We used a t-test for independent samples to test for differences between groups in the number of visits necessary to receive 15 rewards.

2.3. Experiment 3

To investigate the origin of chemical cues on the feeder corolla we simulated bumblebee footprints

by applying tarsal pentane extracts to the corolla surface. The experiment consisted of 2 trials per bumblebee, the first being identical to the first trials in experiment 1 and 2. In the second trial the bumblebees faced 20 rewarding feeders. At ten feeders 5 μL of pentane (p.a.) had been applied to each corolla using a micropipette (0.5–10 μL). The other ten feeders were treated with the same amount of a tarsal extract in pentane. For production of the extract 5 worker *Bombus terrestris* were captured in a clean glass vial and freeze killed. The legs were cut at the end of the tibia and extracted in 1 mL pentane for 30 seconds. We analysed the first 10 rewarded visits and used the Sign-test to test whether individuals visited extract- and pentane-treated flowers with different frequency.

3. RESULTS

In experiment 1, the tested bumblebees quickly learned to avoid previously depleted feeders and thus exploited the array more efficiently than expected by random choice. Figure 2 shows a comparison between the mean foraging performance of the tested bumblebees and the expected performance of a randomly choosing bee. The mean number of visits an individual required to receive 15 rewards was significantly lower than expected by chance when data from all trials were averaged (Sign-test: N = 8; P = 0.013). The same result was obtained for trials 1, 2 and 4, when those were analysed separately, but not for trial 3 in which one individual performed worse than when choosing randomly (Sign-test: N = 8; P = 0.08). Learning of the new context (that feeders could be depleted) appeared to take place during the very first flower visit of the first trial. After having depleted the reward the bees repeatedly returned to the bore as if expecting a reward, sometimes turning around several times in the corolla (a similar behaviour was described by Schmitt and Bertsch, 1990). They rarely did this on subsequent visits or during consecutive trials, but quickly left depleted flowers. Additional experience did not further improve overall foraging efficiency; e.g., there was no effect of trial on the number of visits required by an individual to receive 15 rewards (Repeated-measures ANOVA: N = 8; $F_{3,21}$ = 0.078; P = 0.97).

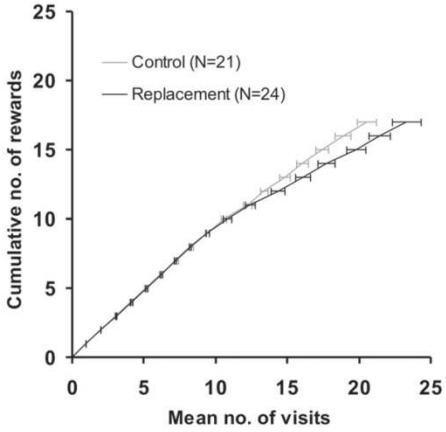

Figure 3. Foraging efficiency of worker bumblebees in a rotating flower array. The curves show the mean number (+/– standard error) of visits necessary to receive a certain (cumulative) number of rewards. In the replacement group visited glass corollas were replaced with clean ones after the first ten successful visits. See text for statistics.

In experiment 2 the intermittent replacement of visited corollas with clean ones (Replacement group) significantly increased the number of visits necessary to receive 15 rewards compared to control bees for which the visited corollas had remained in place (Control group) (t-test: N = 45; t = 2.94; df = 40; P = 0.005). The foraging performance of the replacement group decreased markedly after visit No. 11, shortly after the replacement of the corollas (Fig. 3).

Discrimination against chemical cues deposited on visited corollas was confirmed by the results of the third experiment. Here, the bumblebees made significantly fewer visits to the corollas treated with the tarsal extract than to corollas treated with pure pentane (Sign-Test: N = 10; $P < 0.01$). Of the first ten visits, foragers made on average 7.8 visits to flowers treated with pure pentane.

4. DISCUSSION

Our results demonstrate that foraging bumblebees deposited chemical cues on visited artificial corollas, and that these cues were recognised by the bees when approaching the same feeders on subsequent occasions. Experimental removal of the deposits by

corolla replacement prevented discrimination against depleted feeders and reduced overall foraging efficiency. Our findings are direct evidence for the existence of foraging scent-marks in bumblebees. They suggest that context is of prime importance for shaping individual foraging decisions. Given a reward context that imitates the natural situation (small rewards, no immediate refills) the trace of a previous visit acts as a repellent to inhibit further visits, which is in broad agreement with field studies (Goulson et al., 2000; Stout et al., 1998; Gawleta et al., 2005). The importance of context on the interpretation of scent-marks is also reflected by another recent laboratory study (Saleh et al., 2006). When trained bumblebees were presented with two types of artificial flowers that varied in the time required for handling, the rates of rejection were higher in the type that required longer and therefore offered lower net benefit. This suggests that bumblebees can gradually adjust their choice depending on the expected amount of reward within a certain reward context (Saleh et al., 2006).

Are the deposits actively released by bumblebee individuals with the intention of marking depleted flowers (e.g. for themselves), or are the substances unavoidable footprints that may provide simple cues to any forager? Our data do not allow for a distinction to be made between these alternatives. However, we argue that currently the most parsimonious interpretation is to assume that deposits represent cues rather than evolved signals, and that these cues influence behaviour differentially depending on context. Recent studies on other Hymenoptera confirm that "scent-marking" is frequently passive. For example, stingless bees (*Nannotrigona testaceicornis*) leave chemical traces on Plexiglass on which they have walked, and these substances will later attract other workers when presented in a rewarding context (a permanent feeder) (Schmidt et al., 2005). Similarly, returning yellowjacket foragers follow accumulated footprints of nest mates when these are presented in a "homing context", e.g. within the nest entrance tunnel (Jandt et al., 2005). In bumblebees "scent-marking" probably involves blends of hydrocarbons (mostly uneven-numbered alkanes and alkenes) which are present in large amounts and similar composition all over the bumblebee cuticle (Goulson et al., 2000; Schmitt et al., 1991; Eltz, 2006). On the tarsi, the primary function of these lipids may be the improvement of attachment pad adhesion to smooth surfaces (Federle et al., 2002), with the secondary spin-off of serving as olfactory cues. Both in the field (Goulson et al., 2000) and in the laboratory (this study) the application of tarsal pentane extracts rendered flowers less attractive to approaching bumblebees in comparison to controls, presumably because the extract application imitated the olfactory trace of a previous visit.

All tested bumblebees had experienced continuously rewarding flowers in their foraging life prior to the experiment (in the feeding box). In experiment 1 we had therefore expected that foragers would show an increasing discrimination against previously visited (marked) feeders from trial to trial, reflecting their increasing experience with discontinuous rewards. However, this was not observed, presumably because learning of the new reward situation took place very early in the first trial. When foragers visited their very first experimental feeder they behaved as if expecting a continuous reward and turned around repeatedly inside the corolla, frequently re-probing the empty bore. As the bees did not leave the respective flower while doing so, these attempts were not counted as repeated visits to the same feeder and, thus, did not affect the measure of foraging efficiency for the first trial. On subsequent visits or during consecutive trials foragers normally left depleted flowers without delay. That bumblebees learned to discriminate against previously visited flowers so quickly may reflect an innate aversion to bumblebee odour while foraging. Perhaps this aversion, potentially adaptive in the natural environment, was simply suppressed by encountering continuous rewards in the feeding box, and later revived during the experiment.

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Influence des marques odorantes sur le choix de la fleur par les bourdons : expériences sur fleurs artificielles.

***Bombus* / discrimination / marque odorante / message chimique / signal / butinage**

Zusammenfassung – Die Wirkung von Duftmarken auf die Blütenwahl von Hummeln: Ergebnisse eines Laborexperiments an künstlichen Blüten. Hummelarbeiterinnen sammeln Nektar an Blüten einer Vielzahl von Pflanzenarten und -individuen, wobei der Nektargehalt einzelner Blüten starken Schwankungen unterliegen kann, nicht zuletzt aufgrund der Aktivität von Blütenbesuchern. In früheren Untersuchungen konnte gezeigt werden, dass Hummeln während des Blütenbesuchs Duftmarken hinterlassen, an Hand derer sie in der Lage sind, zwischen belohnenden und weniger belohnenden Blüten zu unterscheiden. Allerdings existieren bis heute unterschiedliche Einschätzungen zur Wirksamkeit der Duftmarken. Während Ergebnisse von Freilanduntersuchungen darauf hindeuten, dass Duftmarken immer abweisende Wirkung auf fouragierende Hummeln haben (Goulson et al., 1998, 2000; Stout et al., 1998; Gawleta et al., 2005), wiesen die Ergebnisse von Laborexperimenten auf einen attraktiven Effekt hin (Cameron, 1981; Schmitt and Bertsch, 1990; Schmitt et al., 1991). Die hier vorliegende Untersuchung soll zur Klärung dieser Diskrepanz beitragen und postuliert, dass die Wirkung der Duftmarken vom Belohnungskontext abhängt: In der Natur regenerieren die meisten Blüten ihren Nektar sehr langsam, weshalb sie normalerweise nur geringe Mengen an Nektar enthalten und von Hummeln komplett erschöpft werden können. Hier wird die Duftmarke als Anzeiger kürzlich geleerter Blüten interpretiert und wirkt als Repellent. In Laboruntersuchungen wurden Hummeln dagegen auf kontinuierlich belohnende oder schnell regenerierende Blütenattrappen dressiert, die von einzelnen Individuen nicht erschöpft werden konnten. In diesem Fall erfolgte eine positive Konditionierung auf die Duftmarken und Attraktion. In unseren Laborexperimenten verwendeten wir deshalb zur Nachahmung der natürlichen Situation Blütenattrappen mit nur einmaliger und äußerst geringer (2 µL) Zuckerwasserbelohnung. In diesem Belohnungskontext vermieden Hummelarbeiterinnen den nochmaligen Besuch bereits zuvor aufgesuchter (geleerter) Blüten, wie dies auch im Freiland der Fall ist. Die Diskrimination erfolgte hierbei durch einen auf der Korolla zurückgelassen chemischen „Hinweis“, was durch ein Korolla-Austausch-Experiment belegt werden konnte. Die Ergebnisse sprechen dafür, dass es sich bei den Duftmarken um einfache Fußabdrücke handelt, die von Hummeln in Abhängigkeit des Belohnungskontexts unterschiedlich bewertet werden können („cues rather than signals“).

Blütendiskriminierung / abweisende Duftmarken / chemischer Reiz / Signal / *Bombus*

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Experiment: The perceptual relevance of cuticular hydrocarbons in bumblebee footprints on flower choice

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Introduction Hydrocarbons as major constituents of footprints are also thought to be the responsible olfactory cues that are used by bumblebees to identify recently visited flowers in their natural habitats. The application of tarsal extracts as well as of synthetic single hydrocarbons on flowers was shown to mimic the repellent effects of recent previous visits by conspecifics (Stout et al. 1998, Goulson et al. 2000). However, Marden (1984) discovered that bumblebees also used unspecific cues associated with the lack or the availability of nectar, including human fingerprints on flowers. Therefore, the repellent effect of synthetic hydrocarbons in bioassays does not necessarily prove their significance in the natural discrimination process. Moreover, the repellent effect of footprint deposits has been shown to wane over time, with the duration of repellence being inversely related to the rate of nectar accumulation of flowers. Whereas bumblebees almost immediately revisited flowers with high nectar secretion rates (after about 2 min. in *Borago officinalis*) they avoided revisiting flowers with low nectar secretion for several hours (24 hours in *Lotus corniculatus*) (Williams 1998, Stout and Goulson 2002). It has been hypothesized that the frequency of rejection declines with the concentration of footprint deposits on flowers presumably because the responsible chemicals evaporate (Stout et al. 1998). Consequently, bumblebees could be able to learn specific footprint concentration thresholds, resulting in appropriate re-visitation intervals to flowers with different nectar secretion rates. Hydrocarbons of the relevant chain length, however, are of very low volatility and are retained on flowers in near unchanged quantities for up to 48 hours, and have been shown to accumulate in almost linear fashion with the number of bumblebee visits (Witjes and Eltz 2009). There are two other plausible explanations for the loss of the repellent effect over time. According to Eltz (2006) footprint hydrocarbons could become incorporated into the semi-liquid layer of plant cuticular lipids and gradually lose their perceptibility to foragers. Alternatively, footprints could contain small quantities of so far undetected low-molecular-weight volatiles in addition to long-chain hydrocarbons. Perception of those could enable bumblebees to adjust their behavioural responses to footprints according to the plants reward dynamics.

Methods and materials For the laboratory experiments a *B. terrestris* colony was kept in a nest box connected to a feeding box via a plexiglas tunnel. Workers were fed on a 50%

sugar-water solution supplied in permanently rewarding feeders. The feeders consisted of a quartz-glass tube as corolla analogue fitted onto a plexiglas cylinder (see Witjes and Eltz (2007**, chapter II.I**) for a more detailed description of the feeding and foraging environment).

Choice experiments For choice tests individual bumblebees were introduced into a foraging arena containing a PVC-disc (diameter 60 cm), which could be rotated around its central axis and on which 20 of the same feeders were arranged. These artificial flowers contained only 2 µl of sugar-water which was not refilled during trials. At the beginning of the experiments the tested bumblebee performed a training trial, to allow habituating to the low reward conditions in the foraging arena. To test whether flower discrimination is based on the recognition of hydrocarbons or on so far undetected volatiles I recorded the foraging decisions (acceptance=landing and probing; rejection=approaches without probing) of *B. terrestris* workers on three types of artificial flowers. I presented 14 "unvisited" feeders, which had not been touched by bumblebees prior to the trial as well as 3 "delayed" and 3 "immediate" feeders, which had been walked through 10 times by other *B. terrestris* workers on the way from the nest to the feeding box. Corollae of the "delayed" category were kept at room temperature for 1.5 hours before the test trial, whereas corollae of the "immediate" category were introduced into the array of artificial flowers shortly (max. 5 min.) after they had been walked through. Only the response (acceptance or rejection) following the first approach to each flower was used for the analysis.

Chemical analysis of footprints I solvent-washed feeders (i.e. their glass corolla) of the aforementioned types and chemically analyzed the dissolved deposits via gas chromatography/mass spectrometry (GC/MS). Three corollae of each type were combined and extracted in 4 ml hexane containing 2-undecanone as an internal standard. The solvent was evaporated to a volume of 300 µl afterwards. A more detailed description of the methods used for GC/MS analysis is given in Witjes and Eltz (2009) (**chapter II.IV**).

Assumptions Bumblebees are expected to avoid repeated visits to feeders in this experimental setup, because rewards can be completely removed during a single visit, and previously visited feeders are therefore empty. If discrimination between flowers is based on hydrocarbons (null hypothesis), *B. terrestris* workers should avoid "delayed" feeders with the same probability as "immediate" feeders because the long-chain hydrocarbons

quantitatively remain on the quartz glass corolla and are perceptible to the bees (in contrast to natural flowers, where they could become incorporated into the wax layer). If, however, minor volatile components are responsible for the repellent effect (alternative hypothesis), workers are expected to avoid "immediate" feeders with greater probability than "delayed feeders" because in the latter these volatiles have already evaporated completely or partly at the time of the trial.

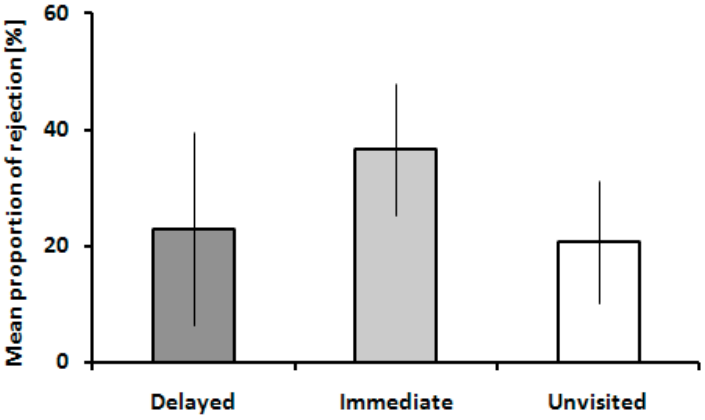

Fig. 1 The responses of foraging bumblebees to „unvisited" artificial flowers compared to flowers nest mates had walked over 10 times, either 1.5 hours before trials ("delayed" treatment) or shortly before trials ("immediate" treatment).

Results *B. terrestris* workers rejected "immediate" feeders significantly more often than "unvisited" feeders (paired t-test: N=10; dF=9; P<0.01) and "delayed" feeders (N=10; dF=9; P<0.01). Artificial flowers that were stored before experiments ("delayed" treatment) were not discriminated against (N=10; dF=9; N.S.) and were equally likely accepted as "unvisited" (**Fig. 1**). No hydrocarbons were found on "unvisited" glass corollae. Walked-over corollae, contained alkanes and alkenes of 21 to 31 C-atoms in similar composition as they are usually found in tarsal extracts of *B. terrestris* (Oldham et al. 1994, Goulson et al. 2000). The overall amount of hydrocarbons (**Fig. 2**) as well as the amount of alkanes and alkenes of each hydrocarbon separately did not differ between "delayed" and "immediate" corollae (N=10; dF=17; P=N.S.).

Discussion The results indicate that the recognition of recently visited flowers is based on the presence of so far undetected, volatile footprint compounds. Bumblebees avoided repeated visits to "immediate" feeders, whereas they did not discriminate between "unvisited" and "delayed" feeders. Furthermore, footprint hydrocarbons remained quantitatively unchanged during 1.5 hours of exposure on glass corollae and thus remained potentially perceptible to the foraging *B. terrestris* workers. Close examination of corolla extracts, however, did not reveal any conspicuous volatiles of low-molecular-weight and their existence in bumblebee footprints remains to be proven. Chemical analysis of future

studies could profit from the use of more polar solvents and a GC-column with a more polar coating, in order to trap possible candidate volatiles. Bioassays with solvent extracts of bumblebee footprint deposits, on natural flowers could give further indications of the relevance of volatile footprint compounds on bumblebee foraging decisions. A possible experiment would include the collection of footprint deposits from neutral surfaces (e.g. glass slides) individual bumblebees had walked over either "immediately" or at different times prior to solvent washes ("delayed treatment") and application of the collected "immediate" and "delayed" footprint deposits on natural flowers in choice tests. Given that volatile footprint components are relevant to bumblebees in natural foraging environments, test bumblebees should, similar to the here presented laboratory experiments, reject flowers treated with "immediate" footprint solvents significantly more often than flowers treated with "delayed" footprint solvents as well as "unvisited" controls. The relevance of volatile footprint compounds seems plausible, considering the multitude of insects bumblebees share natural flowers with. Flowers should become "contaminated" with a growing amount of footprint hydrocarbons of changing composition with the day, rendering cuticular hydrocarbons crude predictors of floral rewards at best.

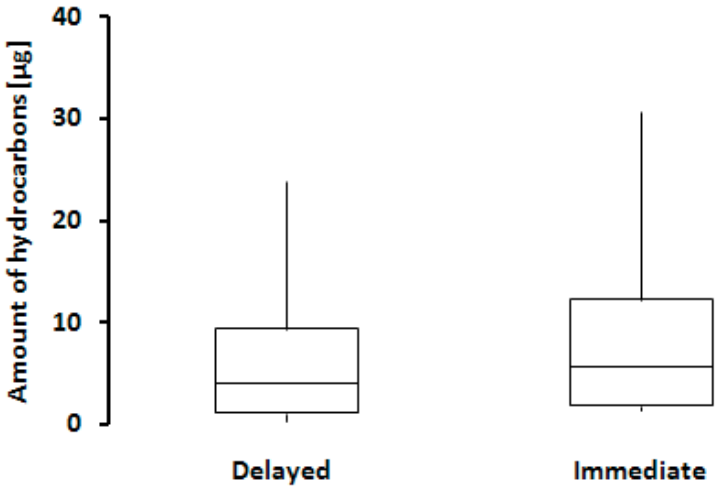

Fig. 2 The mean amount of hydrocarbons on quartz glass corollae bumblebees had walked over 10 times, either 1.5 hours before analysis ("delayed" treatment) or shortly before analysis ("immediate" treatment"). Presented are medians with quartile ranges and non-outlier ranges.

Hydrocarbon footprints as a record of bumblebee flower visitation

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Hydrocarbon Footprints as a Record of Bumblebee Flower Visitation

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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 cIbehave (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).

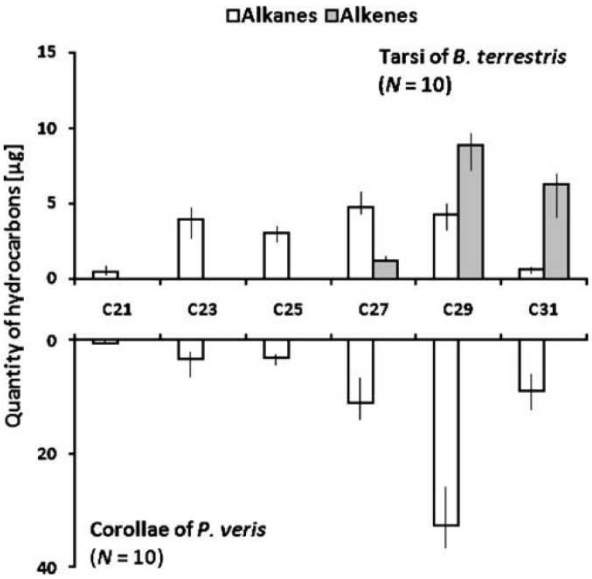

Fig. 1 Quantities of odd-numbered hydrocarbons in extracts of *Bombus terrestris* tarsi (µg/insect; $N=10$) and unvisited corollae (µg/corolla) of *Primula veris* ($N=10$), shown as medians with quartile ranges

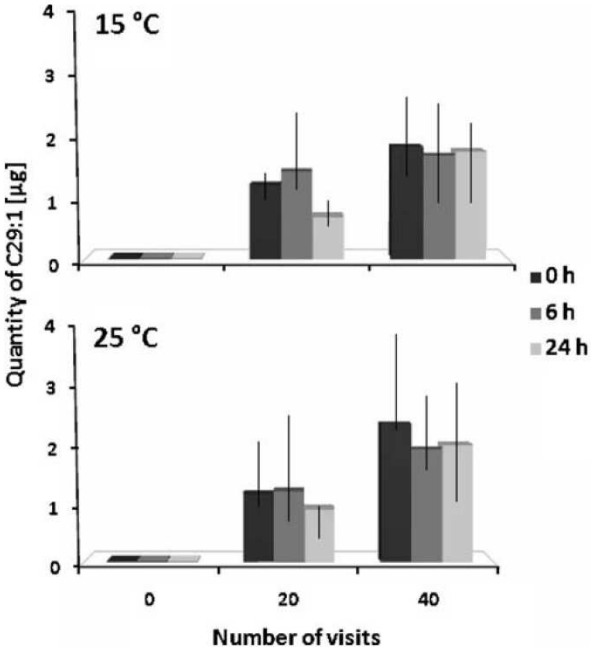

Fig. 2 Quantities (µg/corolla) of nonacosenes from *Primula veris* corollae, extracted 0, 6, or 24 h following the last bumblebee visit. The experiment was carried out at 15°C (*top*) or 25°C (*bottom*) ambient temperature. Corollae received 0, 20, or 40 visits by workers of *Bombus terrestris*. Median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

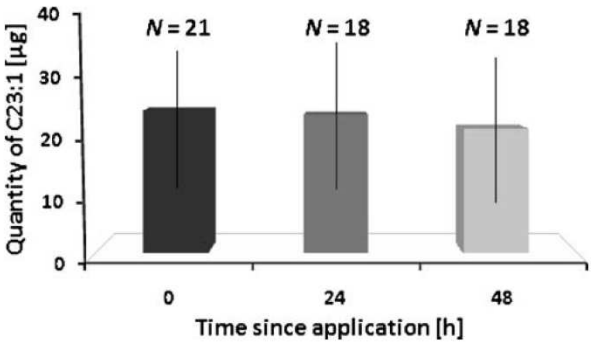

Fig. 3 Quantities (µg/corolla) of (*Z*)-9-tricosene from corollae of *Lamium maculatum* 0, 24, or 48 h after application. Data are shown as mean values with standard deviation

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.

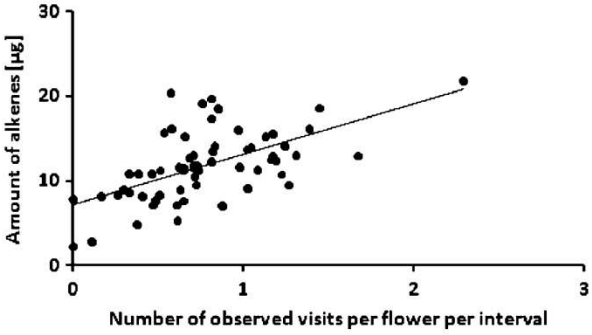

Fig. 4 Quantities of total alkenes on corollae of wild *Symphytum officinale* plants (N=63) in relation to the mean number of bumblebee visits the plants had received per flower in 10-minute observation-intervals during the day

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.

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CHAPTER II.IV

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

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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 flower visitation is essential for basic and applied pollination ecology, but is often fraught with difficulty. Flower visitation is highly variable and observational studies are limited in scope due to the considerable time necessary to acquire reliable data. Our study investigates whether the analysis of hydrocarbon residues (footprints), deposited by insects during flower visits, allows to reconstruct the visitor community and to predict seed set for large numbers of replicate plants. In three 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. The UHCs washed from corollas were most similar to the tarsal UHC profile of the most abundant bumblebee species, *Bombus pascuorum* in all three years. The species composition of the bumblebee communities estimated from UHCs on flowers were similar to those actually observed. We recovered significant positive correlations between observed and estimated visitation frequency for three different bumblebee species, contributing 3 to 68 % of flower visits, and separately for workers and drones of *Bombus pratorum* and *Bombus hortorum*. Seed set of plants was positively correlated to overall bumblebee visitation and the total amount of UHCs on flowers, indicating that reproduction of comfrey was pollen limited under the circumstances of our study. We suggest that quantifying cumulative footprint hydrocarbons provides a potential way to facilitate the assessment of insect flower visitation and could serve as predictor of seed set in pollen limited plants.

Key words Bombus, pollination, pollen limitation, cuticular hydrocarbons, scent marks.

Introduction

The reproductive success of allogamous plants often depends on animal pollen vectors (Burd 1994), especially insects, which are responsible for the pollination of about 67 % of the worlds flowering plants (Tepedino 1979) and 84 % of 264 different crops cultivated in the EU (Williams 1996). The rapid decline of pollinators (Corbet 1995, Williams 1996, Kearns et al. 1998, Goulson et al. 2008) has therefore raised growing concerns about possible economic and ecological consequences (Allen-Wardell et al. 1998). A decrease or loss in pollinator service is thought to reduce the quantity of pollen delivered to stigmas of female flowers (Ashman et al. 2004) which may result in decreased fruit- and/or seed set (Bierzychudek 1981, Louda 1982, Rathcke and Jules 1993, Kearns and Inouye 1997), a reduction in individual reproductive success (Rathcke and Jules 1993, Agren 1996, Allen-Wardell et al. 1998, Ashman et al. 2004), 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). According to metaanalyses the proportion of plant species in which seed set is limited by the amount or quality of pollen deposited on stigmas appears to be high, with an estimated 62% (Burd 1994) to 73% of plant species (Ashman et al. 2004) being pollen limited. Furthermore, ecologists fear that habitat alteration and fragmentation due to extended agricultural land use may further promote the loss of species richness and abundance, initially of pollinators, but subsequently of pollinator-dependent plant populations (Lamont et al. 1993, Rathcke and Jules 1993, Aizen and Feinsinger 1994, Allen-Wardell et al. 1998, Cunningham 2000). Indeed, there is some evidence that plants in fragmented populations produce fewer seeds (Jennersten and Nilsson 1993, Kunin 1993, Lamont et al. 1993, Aizen and Feinsinger 1994, Agren 1996, Bosch and Waser 1999, Steffan-Dewenter and Tscharntke 1999, Kery et al. 2000) and fruits (Aizen and Feinsinger 1994, Agren 1996, Steffan-Dewenter and Tscharntke 1999) than conspecifics in continuous habitats. However, there are relatively few studies that were able to link flower visitor abundance (Burd 1994 and Ashman 2004 exclusively reviewed studies using supplemental hand pollination) to the degree of pollen limitation in plant population studies (Larson and Barrett 1999, Baker et al. 2000). Pollinator visitation frequency is often low and highly variable (Larson and Barrett 1999, Baker et al. 2000, de Jong et al. 2005) and collecting sufficient data requires considerable time and effort, especially if many replicate populations are to be compared (Waser et al. 1996, Baker et al. 2000). There have been attempts to assess pollinator visitation indirectly by observing alterations to flowers following insect visitation, for example recording the “tripped status” in flowers of the invasive plant species *Cytisus scoparius* in North America (Parker 1997) or by recording pollinator claw marks on flowers of *Primula sieboldii* in Japan (Matsumura and Washitani 2000). However, both methods hold no information on the visitation frequency or the composition of the pollinator community. Here we test 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, secondary functions of cuticular

hydrocarbons (CHCs) are manifold. For example, lipid droplets on tarsal attachment pads are thought to enhance adhesion on smooth surfaces (Lockey 1988, Jiao et al. 2000, Drechsler and Federle 2006), and CHCs are used as communication signals in many social insects (Bonavitacougourdan et al. 1991, Lahav et al. 1999, Liebig et al. 2000, Sledge et al. 2001, Ruther et al. 2002, Dani et al. 2005, Howard and Blomquist 2005). CHCs from footprints may also have informative value as chemical cues to conspecifics or heterospecifics. At nesting sites they are used by wasps and bees to recognize their nest entrance at close range (Butler et al. 1969, Hefetz 1992) and on flowers they allow bees to avoid flowers that have recently been visited by others and are currently depleted of its nectar resources (Stout et al. 1998, Goulson et al. 2000, Gilbert et al. 2001, Goulson et al. 2001, Gawleta et al. 2005). Two recent studies indicate that such "scent marks" are not actively released pheromone signals but mere cues, obligatorily deposited wherever bees walk: Bumblebee (*Bombus terrestris)* workers "left" CHCs of 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 at least in bumblebees several cuticular glands are likely involved. Solvent extracts of different parts of the cuticle (tarsi, antennae) and Duffours´ gland were dominated by similar saturated and unsaturated hydrocarbons (Schmitt 1990, Oldham et al. 1994), with chain length of 21 to 31 carbon atoms (Schmitt 1990, Goulson et al. 2000, Saleh et al. 2007), 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 of past bee visitation (Eltz 2006). In fact, solvent washes of deadnettle (*Lamium maculatum*) and comfrey (*S. 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 plants 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 hours 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 (Goulson et al. 2000, Eltz 2006) which could be used to reconstruct the bumblebee visitor community. In the present study 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. Furthermore, we 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 (Hegi 1966, Düll and Kutzelnigg 2005). 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 (Hegi 1966, Düll and Kutzelnigg 2005).

Surveying the pollinator community of wild comfrey The field studies took place on 29-Jul-2007, on 03-Jun-2008 and from 07-May-2009 until 24-Aug-2009. Insect flower visitation to plants of comfrey was recorded at 16 different sites in 2007 and 12 sites in 2008, 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 the 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 10 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 was 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 25m diameter.

Surveying seed set of wild comfrey S. officinale plants set a maximum of four relatively large seeds per flower (Hegi 1966, Goulson et al. 1998, Düll and Kutzelnigg 2005) 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 removed flower corollae that had not already been removed for hydrocarbon extraction (see below) from flower heads, to prevent further insect visitation. Bumblebees were nevertheless sometimes been seen probing for nectar at the basis of flower heads. We did not remove the stigmas of flowers so that further pollination could have happened occasionally. 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 (p.a., Merck), containing 2-undecanone as an internal standard, for GC/MS analysis. In order to analyze the composition of CHCs of local bumblebee species, individual bumblebees were captured around the campus of the University of Düsseldorf and were anaesthetized in CO_2. Legs were cut off at the proximal end of the femur and individual sets were being extracted for 30 seconds in 500 µl hexane containing 2-undecanone as internal standard.

Chemical analysis GC/MS analyses were performed with an HP 5890 II GC fitted with a 30-m non-polar DB-5 column and an HP 5972 mass selective detector. A volume of 3 µl per sample was injected using an HP 7673 auto injector. 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 internal (2-undecanone) and external (pentacosane) standards. Characterization of hydrocarbons was based on 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 coinjection.

Analyzing species specificity of the composition of unsaturated hydrocarbons Hydrocarbon analysis was restricted to UHCs (here alkenes and alkadienes) which are common components of the epicuticular lipid layer of bumblebees, but absent from waxes of most unvisited flowers, including those of *S. officinale* (Griffiths et al. 1999, Griffiths et al. 2000, Goodwin et al. 2003, Eltz 2006, 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 the software *primer* (v6.1.6) (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 best reflect the chemical similarity/dissimilarity between them. Deviations from a perfect fit are expressed in "stress", with values below 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 (six 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 with 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. This is the solution x which 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). Since 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, sometimes small negative values were computed instead of low positive ones. We avoided this by applying a modification of the least squares algorithm using the program "lsqnonneg" in MatLab (MathWorks Inc. (7.8.0.347)), 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 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 (Stat Soft, Inc.).

CHC profiles, seed set, and pollen limitation We tested for correlations between the total number of visits 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 and the average number of seed set per flower per plant, with Spearman rank correlation in STATISTICA 6.0 (Stat Soft, Inc.). 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 length of 19 to 33 C-atoms (Table 1, supplement). 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 ~90

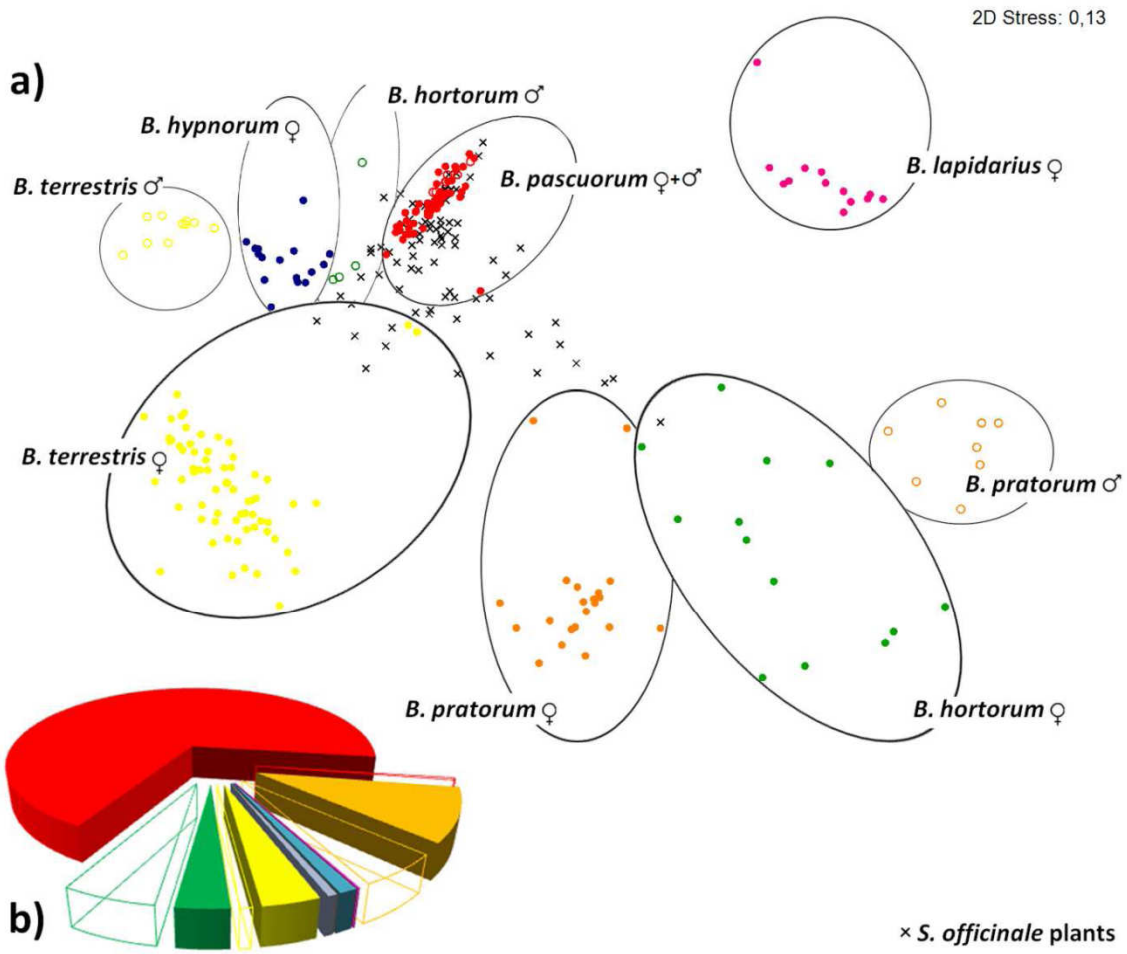

Fig. 1. a) Compositional similarity of UHCs in tarsal extracts of visiting bumblebees and in corolla extracts of visited comfrey (*S. officinale*) plants in (2009) presented in a two-dimensional MDS plot derived from Bray-Curtis similarities based on relative abundances of 63 different alkenes and alkadienes. **Fig. 1. b)** Visitation frequencies of visiting bumblebee species to *S. officinale* flowers in 2009. Colors code for different species of bumblebees. Workers are represented by full symbols, drones by open symbols in both graphs.

% to the similarity within the groups, with the exception of *B. hortorum* workers, in which the major components contributed ~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. 1a). UHCs of workers and drones of *B. pascuorum* were not significantly different in composition (ANOSIM: N=57; R=0.002; P=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. 1a). 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

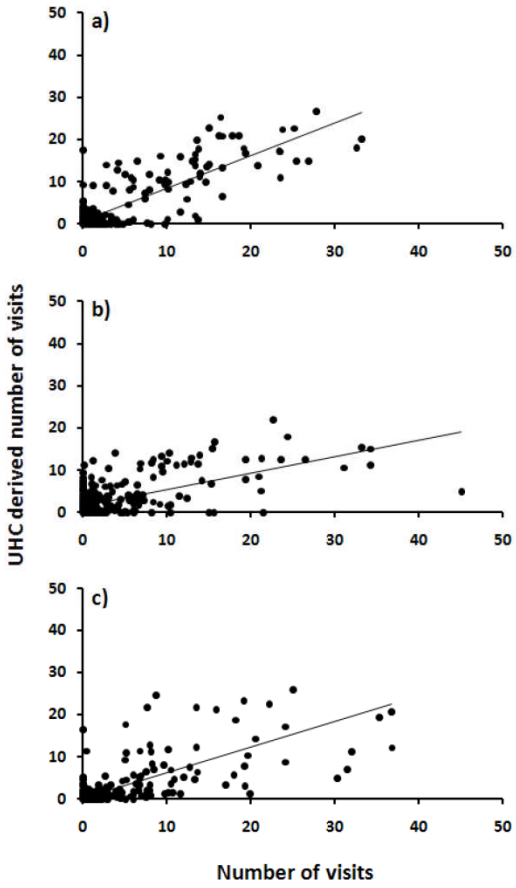

Fig. 2. The mean number of bumblebee visits that *S. officinale* plants had received per flower during the day (extrapolated from bumblebee visitation counts in 10 min observation intervals) in relation to the number of visits derived from UHC-footprints (Least squares approach) in 2007 (a), 2008 (b) and 2009 (c).

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 (Fig. 1b).

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. 4c). 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 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. 4b). 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.

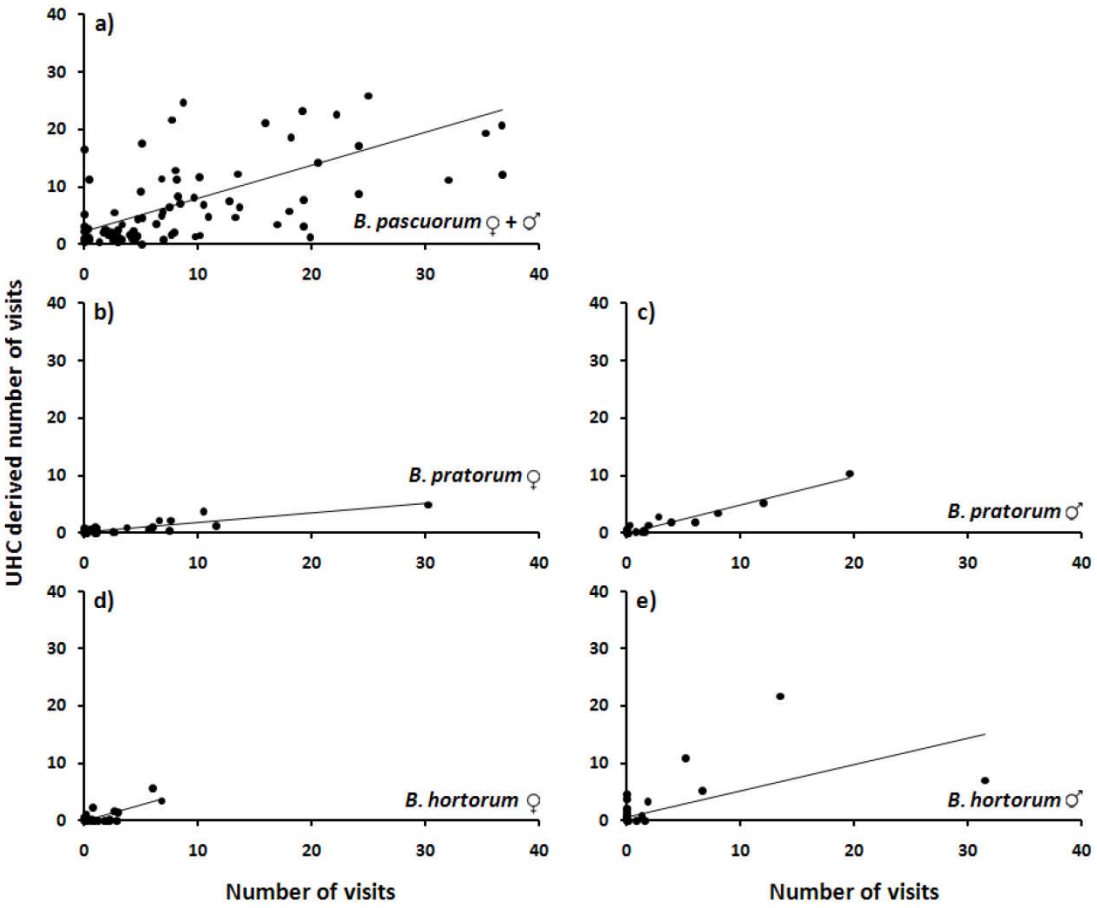

Fig. 3. The mean number of bumblebee visits *S. officinale* plants had received per flower during the day (extrapolated from bumblebee visitation counts in 10 min observation intervals) by the five most common bumblebee groups in 2009 (a – e) in relation to the number of visits derived from UHC- footprints (Least squares approach).

Discussion

Our results suggest that hydrocarbon footprints on flowers are a reliable information source 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 separate visitation frequencies 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 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. Generally, 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 did not measurably evaporate from flowers over 48 hours (Witjes and Eltz 2009), which surpasses the floral life time of many temperate bee-pollinated plant species (Molisch 1929). Finally, Martin (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 predominantly uneven number of C-atoms (Schmitt 1990, Goulson et al. 2000, Saleh et al. 2007). Of those, only the unsaturated alkenes and alkadiens (UHCs) may serve as bumblebee visitation markers, whereas saturated alkanes occur in large quantity on unvisited flowers of most plant species (Griffiths et al. 1999, Griffiths et al. 2000, Goodwin et al. 2003). The overall UHC composition of different species of bumblebees differs significantly (this study, see also (Goulson et al. 2000, Eltz 2006), but there is also substantial between-species overlap in UHC compounds. Of 63 UHC compounds found in the present 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

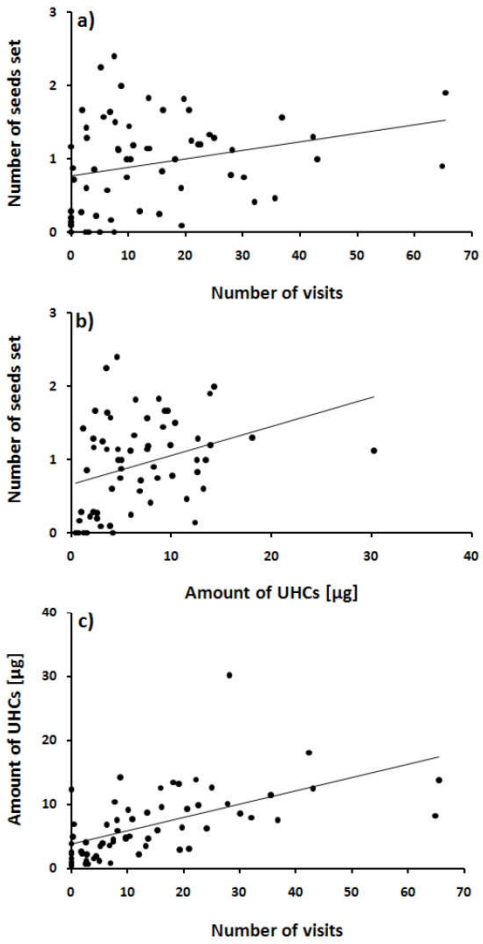

Fig. 4. The total number of bumblebee visits *S. officinale* plants had received per flower during the day (extrapolated from bumblebee visitation counts in 10 min observation intervals) in relation to the mean number of seeds (a) and the total amount of UHCs (c) on a per flower per plant basis. Fig. 4b presents the total amount of UHCs per flower per plant in relation to the mean number of seeds set per flower per plant.

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. Obviously, it was impossible to obtain separate estimates for entities that are too close in their UHC profiles, as e.g. workers and drones of *B. pascuorum*.

In this study, the pollinator community of *S. officinale* was composed 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 visitor communities composed of, e.g., bees, beetles, flies, and butterflies. It remains to be seen whether more diverse visitor communities 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 activity of certain target species, e.g. the most efficient pollinators. Furthermore, the inclusion of entirely different insect families will also lead to the inclusion of new classes of marker compounds, e.g. branched hydrocarbons and substituted derivatives (Lockey 1988, Ruther et al. 2002, Martin et al. 2009), thereby increasing the power of discrimination of the analysis. At present our experiences with more diverse communities is limited, but we 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 was also related to seed set of flowers, indicating that seed set of comfrey is pollen limited in some circumstances. Our findings are in contrast with 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 (Paige and Whitham 1987, Burd 1994, Agren 1996, Dudash and Fenster 1997, Kunin 1997, Larson and Barrett 1999), 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 summer (June and July) when bumblebee populations were probably near the 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 (Real and Rathcke 1991, Rathcke and Jules 1993). 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.

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 it 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. Especially in small or fragmented plant populations pollinator visits may be infrequent 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 (GC/MS) is relatively cheap and fast, given that a GC system with autosampler is available. The compound calling of peaks in ion chromatograms is likely 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 done in a semi-automated way with the help of spectral deconvolution software, 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.

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Supplement

Table 1 Most frequently detected alkenes and alkadiens in tarsal extracts of bumblebees foraging on S. *officinale* in 2009. 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 GC column (see Methods for analytical details)

	B. terrestris ♀		***B. terrestris*** ♂		***B. hortorum*** ♀		***B. hortorum*** ♂		***B. pascuorum*** ♀		***B. pascuorum*** ♂		***B. pratorum*** ♀		***B. pratorum*** ♂		***B. lapidarius*** ♀		***B. lapidarius*** ♂		***B. hypnorum*** ♀		***B. hypnorum*** ♂	
	av. similarity: 68.36		av. similarity 89.12		av. similarity 64.34		av. similarity 81.74		av. similarity 87.06		av. similarity 92.03		av. similarity 82.37		av. similarity 88.09		av. similarity 85.21		av. similarity 80.68		av. similarity 75.51		av. similarity 81.58	
	N = 65		*N* = 10		*N* = 13		*N* = 4		*N* = 50		*N* = 7		*N* = 21		*N* = 8		*N* = 13		*N* = 5		*N* = 16		*N* = 11	
Ret.time Substance	Abund.	Contr.	Abund.	Contr.	Abund.	Contr.	Abund.	Contr.	Abund.	Contr.	Abund.	Contr.	Abund.	Contr.	Abund.	Contr.	Abund.	Contr.			Abund.	Contr.		
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	-	-	-	-	-	-	4,1	3,54	-	-	-	-	-	-	-	-	-	-
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	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	0,01	0	-	-	16,09	14,73	0,4	0,08	0,12	0,06	0,19	0,08	-	-	0,34	0,25	13,21	11,71	5,91	5,73	0,03	0,01	0,05	0,02
20,52 Tricosene 4	0,03	0	-	-	7,78	7,99	-	-	-	-	-	-	0,13	0,01	0,55	0,19	-	-	-	-	-	-	0,01	0
21,15 Tetracosadiene	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0,17	0,14	-	-	-	-	-	-	-	-
21,21 Tetracosene 1	-	-	-	-	0,49	0,43	-	-	-	-	-	-	0,49	0,35	-	-	0,1	0,01	-	-	-	-	-	-
21,24 Tetracosene 2	-	-	-	-	-	-	0,16	0,1	0,36	0,21	0,29	0,26	-	-	-	-	-	-	-	-	-	-	-	-
21,30 Tetracosene 3	-	-	-	-	0,38	0,37	-	-	-	-	-	-	-	-	0,05	0	1,4	1,26	0,45	0,35	-	-	-	-
21,39 Tetracosene 4	-	-	-	-	0,09	0,05	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
21,93 Pentacosadiene 1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
21,98 Pentacosadiene 2	-	-	-	-	0,7	0,61	-	-	0,05	0,01	-	-	0,1	0,02	55,66	59,75	0,26	0,14	-	-	-	-	0,1	0,03
22,07 Pentacosene 1	0,08	0,02	-	-	13,34	15,27	-	-	-	-	-	-	29,06	29,98	0,99	0,61	-	-	-	-	-	-	-	-
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	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	0,15	0,01	-	-	12,02	14,89	0,56	0,58	2,19	2,07	1,62	1,53	-	-	12,95	12,83	54,31	60,86	41,39	48,1	0,77	0,45	1,78	1,77
22,25 (Z)-5-Pentacosene	-	-	-	-	6,4	6,63	-	-	0,03	0	-	-	-	-	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 Hexacosene 2	-	-	-	-	-	-	0,64	0,7	0,48	0,24	0,66	0,43	-	-	-	-	-	-	-	-	0,1	0,03	0,13	0,02
22,97 Hexacosene 3	-	-	-	-	0,07	0,03	-	-	-	-	-	-	-	-	0,15	0,06	0,31	0,22	0,77	0,6	0,4	0,28	0,19	0,08
23,51 Heptacosadiene 1	0,03	0	-	-	-	-	-	-	-	-	-	-	0,02	0	-	-	-	-	-	-	0,01	0	0,91	0,83
23,55 Heptacosadiene 2	0	0	-	-	-	-	-	-	0,18	0,02	-	-	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	2,33	1,54	-	-	4,54	4,84	-	-	-	-	-	-	29,6	33,72	2,29	2,16	-	-	-	-	0,01	0	1,79	1,62
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	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	1,08	0,55	1,25	1,1	2,26	2,82	1,65	1,63	0,99	0,83	0,97	0,93	-	-	10,02	10,42	5,93	5,43	20,45	19,54	8,93	5,09	22,57	25,3
23,85 Heptacosene 4	-	-	-	-	1,2	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
24,42 Octacosene 1	0,11	0,02	-	-	-	-	-	-	-	-	-	-	0,53	0,45	-	-	-	-	-	-	-	-	-	-
24,49 Octacosene 2	0,23	0,12	1,47	1,15	-	-	0,78	0,52	0,18	0,06	0,18	0,12	-	-	-	-	-	-	-	-	0,74	0,57	0,39	0,26
24,55 Octacosene 3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0,54	0,27	0,16	0,05
25,07 Nonacosadiene 1	0,32	0,03	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0,09	0,02	2,81	2,81
25,12 Nonacosadiene 2	2,46	2,06	-	-	-	-	-	-	0,06	0	-	-	0,44	0,14	2,15	1,8	-	-	-	-	0,13	0,03	6,98	6,72
25,16 Nonacosadiene 3	1,11	0,87	-	-	-	-	-	-	0,06	0	-	-	-	-	-	-	-	-	-	-	0,08	0,01	3,24	3,15
25,26 (Z)-11-Nonacosene	13,04	12,83	-	-	2,53	2,69	-	-	0,09	0	-	-	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	27,45	34,88	74,56	78,71	1,53	1,26	20,43	21,73	13,12	13,17	9,87	9,69	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	1,12	0,29	-	-	1,05	1,29	1,3	1,27	0,43	0,23	0,45	0,42	-	-	-	-	3,39	3,1	10,06	9,02	6,09	3,48	8,38	8,97
26,21 Triacontene 1	0,06	0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
26,25 Triacontene 2	0,18	0,04	-	-	-	-	-	-	0,09	0,01	-	-	-	-	-	-	-	-	-	-	0,19	0,08	-	-
26,99 Untriacontadiene 1	2,94	2,87	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0,29	0,06
27,05 Untriacontadiene 2	1,98	1,93	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0,3	0,13
27,09 Untriacontadiene 3	4,74	4,98	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0,68	0,31
27,16 Untriacontadiene 4	4,47	3,68	-	-	-	-	-	-	0,05	0	-	-	-	-	-	-	-	-	-	-	-	-	0,29	0,11
27,22 Untriacontene 1	0,43	0	-	-	0,41	0,14	-	-	0,1	0	-	-	1,4	0,81	-	-	-	-	-	-	-	-	-	-
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	0,25	0,02	-	-	0,41	0,25	1,09	0,92	6,38	7,12	2,39	2,09
27,44 Untriacontene 4	-	-	-	-	0,08	0,01	-	-	-	-	-	-	-	-	-	-	2,7	2,07	2,59	1,7	0,24	0,05	-	-
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	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	3,16	2,47
31,37 Tetratriacontene 2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	4,19	2,29

CHAPTER III THE AUTHORS´CONTRIBUTION

(II.I) Influence of scent deposits on flower choice: experiments in an artificial flower array with bumblebees

Sebastian Witjes and Thomas Eltz

Apidologie 38 (2007) 12 – 18.

- Design of the experimental setup
- Implementation of the laboratory experiments
- Collection of chemical samples
- Statistical analysis
- Authoring the manuscript

(II.II) Experiment: The perceptual relevance of cuticular hydrocarbons in bumblebee footprints on flower choice

- Design of the experimental setup
- Implementation of the laboratory experiments
- Collection of chemical samples
- Statistical analysis

(II.III) Hydrocarbon footprints as a record of bumblebee flower visitation

Sebastian Witjes and Thomas Eltz

Journal of Chemical Ecology 35 (2009) 1320 – 1325.

- Design of experimental setup
- Implementation of laboratory experiments
- Supervision of field surveys, with T. Eltz
- Collection of chemical samples
- Chemical analysis via gas chromatography / mass spectrometry
- Statistical analysis
- Authoring the manuscript

(II.IV) Reconstructing the pollinator community and predicting seed set from hydrocarbon footprints on flowers

Sebastian Witjes, Kristian Witsch & Thomas Eltz

Manuscript under review in *Oecologia*

- Supervision of field surveys, with T. Eltz (2007 and 2008)
- Implementation of field survey (2009)
- Collection of chemical samples
- Chemical analysis via gas chromatography / mass spectrometry
    - Identification of cuticular hydrocarbons
- Statistical analysis
    - Multivariate similarity analysis of surface chemicals of six endemic *Bombus* species
    - Implementation of least squares approach, with K. Witsch
- Authoring the manuscript

CHAPTER IV SYNTHESIS

Bumblebees face a very heterogeneous environment in which the amount of pollen and nectar in flowers is difficult to predict. The distribution of floral rewards varies substantially within plant species with age and location of the plant as well as between flowers of individual plants due to the age, size and position of the flowers (Marden 1984, Zimmerman and Pyke 1986, Real and Rathcke 1988). Bumblebees depend on pollen and nectar provided by flowers, both for their own sustenance but also to provision their brood. The decisions which flowers to probe therefore directly influences the reproductive success of individual bumblebee colonies. Bumblebees preferentially visit plant species and forage sites that have provided a reward on previous occasions, but the more critical choice is probably between individual flowers within a site, because their respective reward varies drastically due to the influence of the flower visitors themselves. Thus, it is not surprising that bumblebees can discriminate against recently depleted, low rewarding flowers. They do so without actually probing those flowers, hovering briefly at a short distance (~1cm) from the petals. Possible mechanisms include direct visual and olfactory detection of pollen and nectar in dish-like flowers, but probably in most cases the evaluation involves indirect cues.

The results of my experiments corroborate the view that flower discrimination is normally based on the olfactory perception of chemical residues (footprints) left on flowers by previous visitors (Witjes and Eltz 2007, Experiment in **chapter II.II**). Individual workers of *B. terrestris* were able to locate unvisited feeders situated within an artificial meadow unless the chemical deposits from previous visits were removed by corolla replacement. The effect of “scent-marks” on foraging bumblebees seems to be largely dependent on the context in which they are presented. Given natural reward conditions (small rewards, that can be completely depleted during a single visit) “scent-marks” act as repellent, inhibiting repeated visits to depleted flowers. My study strongly suggests that the attractive effects found in earlier laboratory experiments are artefacts of unnaturally high rewards. The importance of context on the effect of “scent-marks” is also reflected by another study, in which the strength of the repellent effect was shown to depend on flower complexity (Saleh et al. 2006). The results of my experiments further indicated that the chemical deposits do not represent evolved communication signals but are simple footprint cues, because the

repellent effect is also elicited by footprints deposited on “neutral” (non-feeder) surfaces **(chapter II.II)**. This view has received further support by another study of our working group (Wilms and Eltz 2008) and by chemical analyses done by Saleh et al. (2007), who demonstrated that bumblebee footprints are essentially chemically identical irrespective of whether they are deposited on food-, nest-, or neutral sites. My own studies (Witjes and Eltz 2007, 2009) agree that footprints are dominated by odd numbered hydrocarbons with 19 to 34 carbon atoms, corresponding closely to the chemicals found on the cuticular surface of bumblebee tarsi. These hydrocarbons are major constituents of the cuticular lipid layer of many insects. They primarily serve to provide waterproofing to the cuticle, but often have secondary functions in insect communication. They may also be the perceptually and behaviourally active compounds in bumblebee “scent marks”, but this is yet debatable. In fact, my own experiments suggest that long-chain hydrocarbons are only the matrix in which the true volatile “scent mark” is contained **(chapter II.II)**. Two lines of evidence argue for this view. First, the cuticular hydrocarbons of bumblebees are all of extremely low volatility and remained quantitatively and qualitatively stable on natural flowers for up to 48 hours (Witjes and Eltz 2009). This is not in agreement with an active function in “scent marks”, because the repellent effect of a bumblebee visit normally wanes after 30 to 60 minutes. Second, when I compared the repellent effect of fresh (directly collected) versus old footprints (collected with a 90 minute delay) on feeder choice in a laboratory experiment, I found that fresh footprints were significantly more frequently avoided by foragers. Intriguingly, the amount of cuticular hydrocarbons was identical in the two types of footprints, again suggesting that hydrocarbons were not responsible for the different behavioural effects. These results suggest that the flower discrimination is based on the recognition of so far undetected low concentration volatile compounds in footprints. Generally, it seems likely that bumblebees will use any available visual or olfactory cue associated with floral rewards, presumably also a combination of cues if necessary.

Cuticular hydrocarbons may not be the behaviourally relevant compounds in bumblebee footprints, but the results of my experiments suggest that they could be a cumulative measure of flower visitation in plant population studies. Pollinator service is essential for reproduction in allogamous plants (Rathcke and Jules 1993, Agren 1996, Allen-Wardell et al. 1998, Ashman et al. 2004). Infrequent pollination may directly decrease plant fertility,

especially in small and fragmented populations. The measurement of pollinator visitation is essential in basic and applied pollination ecology, but is often fraught with difficulty. Pollinator visitation if often low and highly variable and the collection of sufficient data requires considerable time and effort, especially as many replicate samples are needed. I suggest to quantify insect cuticular hydrocarbon residues (footprints) on flowers to reconstruct insect visitation and predict seed set of plants. In bumblebees, epicuticular lipids are largely comprised of alkanes, alkenes and alkadienes with chain length between 19 and 34 carbon atoms in a highly species-specific composition (Witjes et al. 2010, *under review*). In agreement with Eltz (2006) my results show that traces of these hydrocarbons remain on flowers after bumblebee visitation and are retained in the plants´ epicuticular waxes. Solvent extracts of foxglove (*D. grandiflora*) and primrose (*P. veris*) flowers visited by *B. terrestris* workers in the laboratory contained bumblebee-derived unsaturated hydrocarbons (alkenes and alkadienes) in addition to the plants own cuticular lipids, and the amount deposited was a close correlate of the number of bumblebee visits. Furthermore, bumblebee-derived nonacosenes were retained on flowers in near unchanged quantities for 24 hours independent of temperature regime (15°C and 25 °C) (Witjes and Eltz 2009). This indicated that the epicuticular wax of flowers could in fact retain a record of past bumblebee visitation for a period similar to the floral lifetime of many temperate bee pollinated plant species, under a range of environmental conditions. This is further confirmed by the results of recent experiments presented in **chapter II.IV** (Witjes et al. 2010, *under review*). We successfully designed and applied a mathematical algorithm, which allowed us to estimate the visitation frequency of different bumblebee species separately from chemical footprint data. The bumblebee species composition estimated from hydrocarbon deposits on comfrey flowers was similar to those actually observed. Most intriguingly, we were able to predict seed set from absolute amounts of footprint hydrocarbons on flowers, indicating that comfrey reproduction was limited by bumblebee visitation in the context of the study. The quantification of pollinator footprints could facilitate the assessment of flower visitation in studies with multiple replicates, and enable pollination ecologists to obtain pollination-relevant data on large temporal and spatial scales. The analysis of large numbers of floral extracts with gas chromatography/mass spectrometry is relatively cheap and fast compared to the considerable manpower and time needed for direct observations of flowers. Although the reconstruction of the pollinator community was limited to bumblebees in the presented

study it is quite likely that footprints could be used to trace visitation of a larger number of pollinator species. The inclusion of other insect families will also lead to the inclusion of new classes of footprint chemicals, e.g. methyl-branched alkanes, which have been shown to be largely responsible for the chemical disparity between insect species (Lockey 1988, Blomquist et al. 1998, Howard and Blomquist 2005), thereby increasing the power of discrimination of the analysis.

CHAPTER V SUMMARY

Hydrocarbons are major constituents of the epicuticular lipid layer of most insects, which most probably originally evolved as a protective barrier against water-loss in terrestrial habitats. Secondary functions, however, are manifold, and cuticular hydrocarbons have been shown to play an important role as communication signals in many social insects. They may also provide footprint cues, informative to conspecifics and heterospecifics in various contexts. This dissertation examines the informative value of bumblebee (Bombus) hydrocarbons in two different contexts. In the first it investigates the use of hydrocarbon-based footprint cues by the foraging bumblebees themselves. This part is comprised of one publication concerning the effect of chemical footprints on flowers on the foraging behaviour of bumblebees, and one supplemental experiment, investigating whether cuticular hydrocarbons are the behaviourally active chemicals in bumblebee footprints. Within the second context this thesis addresses the potential use of footprint hydrocarbons on flowers for pollination ecologists. This part is comprised of one publication, dealing with the durability of hydrocarbon footprint retention on flowers under different temperatures in the laboratory, and one submitted manuscript in which hydrocarbon footprints are used for the reconstruction of the bumblebee visitor community of natural flowers in a three year field survey.

(CHAPTER II.I)

Influence of scent deposits on flower choice: experiments in an artificial flower array with bumblebees

Bumblebees have been shown to use chemical footprints, deposited on flowers by themselves or by conspecifics to evaluate the availability of floral rewards. However, there have been discrepancies concerning the directionality of the effect, because foragers were usually repelled by recently visited flowers in field surveys, but were attracted to visited feeders in the laboratory. Our results demonstrate that attractive effects found in laboratory experiments are artefacts of unnaturally high rewards and emphasize the importance of context on the effects of scent deposits. Under near natural reward conditions (small rewards, easily extractable during a single visit) *Bombus terrestris* workers avoided repeated visits in the laboratory, and were thus able to exploit an array of feeders more efficiently as

expected by random choice. Flower discrimination depended on the availability of a chemical cue, because the removal of scent deposits by corolla replacement significantly reduced the overall foraging efficiency. Furthermore our results suggest that the responsible chemical deposits most probably represent mere footprint cues instead of evolved communication signals.

(CHAPTER II.II)

Experiment: The perceptual relevance of cuticular hydrocarbons in bumblebee footprints on flower choice

Hydrocarbons have been shown to play an important role in insect communication indicating their potential to act as the perceptually and behaviourally active chemicals in bumblebee footprints. The duration of the repellent effect of bumblebee footprints on flowers has been shown to vary substantially between plant species, in a way closely resembling the rate of nectar secretion. It has been suggested that the footprint effect could wane with time as cuticular hydrocarbons either evaporate or become incorporated into the semi liquid lipid layer of flowers and thus gradually lose their perceptibility to foragers. Neither was the case in my experiment. Footprint hydrocarbons did not measurably evaporate on artificial flowers during 1.5 hours of exposure, as evidenced by chemical analysis via gas chromatography/mass spectrometry. Nevertheless, the repellent effect of footprint deposits decreased with exposure time. This indicates that flower discrimination is based on the recognition of so far undetected volatile footprint compounds. This conclusion requires further support, because no volatile chemical of low molecular weight has been detected in bumblebee footprints so far.

(CHAPTER II.III)

Hydrocarbon footprints as a record of bumblebee flower visitation

Bumblebees have been shown to leave traces of cuticular hydrocarbons on flowers they visit and we asked whether these hydrocarbon residues are retained on flowers for sufficient time to reflect bumblebee visitation in pollination studies. Solvent extracts of foxglove (*Digitalis grandiflora*) and primrose (*Primula veris*) flowers visited by *B. terrestris* workers in the laboratory contained clear bumblebee derived unsaturated hydrocarbons (alkenes and alkadienes) in addition to the plants own cuticular lipids. The amount of *B. terrestris*-derived

nonacosenes washed from corollae was a close correlate of the number of visits received, and marker alkenes remained quantitatively unchanged over periods of 24 (footprint nonacosenes) to 48 hours (synthetic (*Z*)-9-tricosene) irrespective of two tested temperature regimes (15 and 25 °C). This suggests that flower petals could in fact retain a chemical record of bumblebee flower visitation for a period similar to the floral lifetime of many temperate bee-pollinated plant species. These results were confirmed by a field survey of wild comfrey plants, in which the overall amount of unsaturated hydrocarbons on individual flowers was closely related to bumblebee visitation. This indicates that analysis of hydrocarbon footprints on flowers could be used to quantify insect visitation frequency, even in natural, dynamic foraging environments.

(CHAPTER II.IV)

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

This manuscript is based on the idea presented in the previous publication **(chapter II.III)** and addresses the question whether analysis of hydrocarbon residues on flowers could be used to reconstruct the visitor community and predict seed set of natural plants. We recorded bumblebee visitation to wild plants of comfrey (*Symphytum officinale*) in three consecutive years and later used gas chromatography/mass spectrometry (GC/MS) to analyze unsaturated hydrocarbon footprints extracted from flowers. We successfully developed and applied a mathematical algorithm which allowed us to estimate the visitation frequency of each bumblebee species separately from the chemical data. The species composition estimated from unsaturated hydrocarbons on comfrey flowers was similar to those actually observed. We were furthermore able to derive visitation frequency of the most abundant bumblebee species, contributing at least 3% of all flower visits and even separately for workers and drones of 2 out of 6 observed species. Seed set was positively correlated to overall bumblebee visitation and the absolute amount of unsaturated hydrocarbons on flowers, indicating that comfrey plants were pollen limited under the circumstances of our study. We suggest that quantifying cumulative footprint hydrocarbons provides a potential way to facilitate the assessment of flower visitation and could serve as predictor of seed set in pollen limited plants.

CHAPTER VI ZUSAMMENFASSUNG

Die kutikuläre Wachsschicht von Insekten setzt sich größtenteils aus Kohlenwasserstoffen zusammen und evolvierte wahrscheinlich ursprünglich, um den Verdunstungsschutz von Insekten in terrestrischen Habitaten zu gewährleisten. Allerdings konnte gezeigt werden, dass kutikuläre Kohlenwasserstoffe vielfältige Funktionen haben können. Unter anderem spielen sie eine wichtige Rolle als chemische Signale in der Kommunikation vieler sozialer Insekten, werden aber auch als indirekte Hinweisstoffe in vielen verschiedenen Kontexten genutzt. Wespen und Bienen können z.B. an Hand arteigener Kohlenwasserstoffsignaturen ihren Nesteingang auffinden und auf Blüten wurde angenommen, dass sie Bienen die effektive Ausbeutung der pflanzlichen Ressourcen erleichtern. Diese Dissertation widmet sich zwei Themenkomplexen. Der erste Komplex beschäftigt sich mit der chemischen Ökologie von Hummeln. In einer Publikation und einem ergänzenden Experiment wird darin der Einfluss von chemischen Fußabdrücken, aber im Besonderen der kutikulären Kohlenwasserstoffe, auf das Fouragierverhalten von Hummeln untersucht. Im zweiten Themenkomplex, werden die chemischen Eigenschaften der auf der Insektenkutikula vorkommenden Kohlenwasserstoffe aufgezeigt und im Zuge dessen ihre mögliche Bedeutung für Bestäubungsökologen untersucht. Er besteht aus einer Publikation, in welcher größtenteils die Langlebigkeit der kutikulären Kohlenwasserstoffe auf Blüten unter verschiedenen Temperaturen getestet wurde, und einem eingereichten Manuskript. Im letzteren werden die Ergebnisse eines 3 jährigen Feldversuchs präsentiert, in welchem die Zusammensetzung der Hummelbesuchergemeinschaft an Hand von Kohlenwasserstoff-signaturen auf natürlichen Blüten rekonstruiert wurde.

(KAPITEL II.I)

Duftmarkenabhängige Blütenwahl von Hummeln im Laborexperiment

Hummeln können an Hand von Duftabdrücken, welche von ihnen selbst oder von Artgenossen während des Blütenbesuchs hinterlassen wurden, Rückschlüsse auf die in Blüten enthaltene Belohnung ziehen. Allerdings gab es Unstimmigkeiten über die Wirkung dieser Fußabdrücke, da fouragierende Hummeln wiederholte Besuche von natürlichen Blüten in Feldversuchen üblicherweise vermieden, während sie Kunstblüten im Labor gezielt mehrfach besuchten. Unsere Experimente zeigen eindeutig, dass die in bisherigen

Laborexperimenten gefundenen attraktiven Effekte von Hummelfußabdrücken Artefakte von unnatürlich hohen Belohnungsmengen waren und heben die Bedeutung des Kontextes auf die Wirkung von Duftabdrücken hervor. In unseren Laborexperimenten enthielten künstliche Blüten niedrige Belohnungsmengen, die denen in natürlichen Blüten ähnelten. Unter diesen Umständen vermieden Erdhummelarbeiterinnen (*Bombus terrestris*) wiederholte Blütenbesuche und waren so in der Lage, eine aus 21 Attrappen bestehende Kunstblütenwiese effizient auszubeuten. Die Diskriminierung zwischen schon besuchten (geleerten) und noch unbesuchten (belohnenden) Blüten fand an Hand von chemischen Abdrücken statt, da der Austausch von bereits besuchten Kunstblüten durch unbesuchte die Fouragiereffizienz signifikant minderte. Die Ergebnisse deuten außerdem darauf hin, dass es sich bei den untersuchten Duftmarken eher um unvermeidbare Fußabdrücke als um evolvierte Kommunikationssignale handelt.

(Kapitel II.II)

Versuch: Die Bedeutung der kutikulären Kohlenwasserstoffe in Fußabdrücken auf die Blütenwahl von Hummeln

Kohlenwasserstoffe sind wichtige chemische Signale für die Kommunikation vieler Insekten und als solche könnten sie auch die verhaltenswirksamen Komponenten in Hummelfußabdrücken sein. Die Dauer der repellenten Wirkung von Fußabdrücken auf Blüten variiert stark zwischen Pflanzenarten und korreliert mit der Nektarsekretionsrate von Blüten. Es wurde vermutet, dass die Intensität der abweisenden Wirkung von der Konzentration der Fußabdrucksubstanzen auf Blüten abhängt und dass mit zunehmender Verdunstung oder Versickerung relevanter Fußabdruckkomponenten in der Pflanzenkutikula deren Wahrnehmbarkeit abnehmen und Blüten wieder attraktiv werden könnten. Tatsächlich ließ die repellente Wirkung von Hummelfußabdrücken in meinem Laborexperiment innerhalb von 1,5 Stunden nach, allerdings schien dieser Effekt unabhängig von der Wahrnehmbarkeit der relevanten Kohlenwasserstoffe zu sein. Der Gebrauch von künstlichen Quarzglasblüten verhinderte die Versickerung von Fußabdrücken, wie sie auf natürlichen Blüten hätte stattfinden können. Des Weiteren konnte durch chemische Analysen von Lösungsmittelextrakten im Gaschromatographen gezeigt werden, dass die in den Hummelfußabdrücken enthaltenen Kohlenwasserstoffe in der relevanten Zeit nicht nachweisbar von Kunstblüten verdunsteten. Dies ist ein Hinweis darauf, dass die

Blütendiskriminierung an Hand einer bis jetzt noch unentdeckten volatilen Fußabdruck-komponente stattfinden könnte, deren Existenz in Hummelfußabdrücken noch nachgewiesen werden muss.

(KAPITEL II.III)

Blüten enthalten ein Kohlenwasserstoffarchiv des Hummelbesuchs

Hummeln hinterlassen während des Blütenbesuchs geringe Mengen von kutikulären Kohlenwasserstoffen und mein Ziel war es zu untersuchen, ob die Retentionszeit dieser Kohlenwasserstoffsignaturen auf Blüten eine Analyse der Hummelbesuchsfrequenz in Bestäubungsstudien ermöglichen könnte. Sowohl Fingerhut-, (*Digitalis grandiflora*) als auch Schlüsselblumenblüten (*Primula veris*), die im Labor von Erdhummelarbeiterinnen (*B. terrestris*) besucht worden waren wiesen neben den blütenständigen Lipiden auch ungesättigte Kohlenwasserstoffe (Alkene und Alkadiene) auf. Die Konzentration von n-Nonacosen auf besuchten Blüten war positiv mit der Anzahl der Besuche von *B. terrestris* Arbeiterinnen korreliert. Die Substanzmenge relevanter ungesättigter Kohlenwasserstoffe blieb dabei über mindestens 24 Stunden (n-Nonacosen) bis 48 Stunden (synthetisches (*Z*)-9-Tricosen) konstant, unabhängig von den zwei getesteten Umgebungstemperaturen. Diese Ergebnisse konnten in Freilandexperimenten bestätigt werden, in denen wir zeigen konnten, dass die Konzentration von ungesättigten Kohlenwasserstoffen auf Beinwellblüten (*Symphytum officinale*) positiv mit der Anzahl der Hummelbesuche korrelierte. Die Ergebnisse dieser Studie legen nah, dass die quantitative Analyse von Kohlenwasserstoffsignaturen genutzt werden könnte um die Besuchshäufigkeit von Blüten zu bestimmen.

(KAPITEL II.IV)

Die Rekonstruktion der Bestäubergemeinschaft und die Vorhersage des Samenansatzes von Blüten an Hand von Kohlenwasserstoffsignaturen

Dieses Manuskript baut auf der vorherigen Publikation auf und beschäftigt sich mit der Fragestellung, ob Kohlenwasserstoffsignaturen auf Blüten genutzt werden könnten, um die Bestäubergemeinschaft von natürlichen Pflanzen zu rekonstruieren und deren Samenansatz

vorherzusagen. In drei aufeinander folgenden Jahren haben wir die Besuchsfrequenz von Hummeln an Beinwellpflanzen gemessen und gleichzeitig die Kohlenwasserstoffsignaturen auf den beobachteten Blüten per Gaschromatographie/Massenspektrometrie (GC/MS) analysiert. Die Auswertung dieser chemischen Daten mit Hilfe eines mathematischen Algorithmus erlaubte uns die Besuchsfrequenz der einzelnen Hummelarten zu berechnen. Die berechneten Besuchsfrequenzen zeigten eine hohe Übereinstimmung mit den tatsächlich beobachteten Blütenbesuchen. An Hand der Kohlenwasserstoffprofile der Blüten konnten wir die Besuchsfrequenz von allen Hummelarten berechnen, die mindestens zu 3% der insgesamt beobachteten Besuche beigetragen hatten. Des Weiteren konnten wir die Besuchsanzahl von Arbeiterinnen und Drohnen von 2 der 6 beobachteten Hummelarten getrennt voneinander bestimmen. Der Samenansatz der Blüten war sowohl positiv mit der Anzahl der Hummelbesuche als auch mit der gemessenen Menge der Kohlenwasserstoffe korreliert. Dies deutet darauf hin, dass die Beinwellpflanzen unter den vorherrschenden Bedingungen Pollen-limitiert waren. Die Ergebnisse unserer Studie zeigen, dass die Analyse von ungesättigten Kohlenwasserstoffsignaturen auf Blüten eine kostengünstige und effektive Alternative zu Blütenbeobachtungen in Bestäubungsstudien darstellt und des Weiteren genutzt werden könnten, um den Samenansatz von Pollen limitierten Pflanzen vorherzusagen.

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