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Original article

Volatiles of foraging honeybees *Apis mellifera* (Hymenoptera: Apidae) and their potential role as semiochemicals*

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Abstract – Nestmate and kin recognition play a major role in maintaining the integrity of social insect colonies. In *Apis mellifera* it has been suggested that recognition of nestmates is mediated by contact chemoreception. However, there is evidence that volatiles emanating from honeybee workers might transmit recognition cues. These volatiles from the cuticle might also be used as kairomones by females of the European beewolf *Philanthus triangulum* to identify their prey. Here we analysed which compounds occurs on the cuticle of honeybee workers. Additionally, we used a foraging arena that allows the sequestration of volatiles from undisturbed foraging honeybees with a SPME-fibre, followed by GC-MS analyses. We could detect hydrocarbons with a chain length of up to 29 C and some new compounds in the headspace of foraging honeybees. We also found (*Z*)-11-eicosen-1-ol on the cuticle and in the headspace of honeybee workers, thus, it might be used as a kairomone by females of the European beewolf.

***Apis mellifera* / cuticular hydrocarbon / nestmate recognition / volatiles / *Philanthus triangulum* / kairomone / (*Z*)-11-eicosen-1-ol**

1. INTRODUCTION

The cuticle of insects is coated with a mixture of hydrocarbons whose primary role is the prevention of desiccation (Hadley, 1994; Buckner, 1993). However, cuticular hydrocarbons have also been shown to play an important role as semiochemicals in social insects, particularly for species, nestmate, caste, and kin recognition (Howard and Blomquist, 1982; Howard, 1993; Breed, 1998; Singer, 1998; Vander Meer et al., 1998). In honeybees, cuticular hydrocarbons are involved in nestmate recognition (Smith and Breed, 1995, 1998) and potentially in kin recognition (Breed et al., 1994). The majority of compounds on the cuticle of honeybees are long-chain alkanes, branched alkanes, alkenes and esters (Blomquist et al., 1980; Francis et al., 1985, 1989; Carlson, 1988; Ogden et al., 1998).

Nestmate recognition and colony defence of honeybee hives is mainly executed by guard bees (Butler and Free, 1952). They patrol the nest entrance, inspect entering bees, and exclude non-nestmates or other intruders probably using chemical cues for recognition

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(Moore et al., 1987; Moritz et al., 1991; Breed et al., 1992; Beekman et al., 2002). Guard bees antennate approaching bees for identification. Therefore it has been suggested that the relevant compounds have a relatively low volatility and can only be perceived by contact chemoreception (Free, 1987, see p. 102). However, a study by Kalmus and Ribbands (1952) has shown that foraging honeybee workers can distinguish between nestmates and non-nestmates at food sources without contact, suggesting that compounds are involved which has been transmitted through the air. There is even the possibility that volatiles emanating from workers or groups of workers can be used for kin recognition (Getz et al., 1986; Moritz and Southwick, 1987; Breed et al., 1994).

The colony odour on the cuticles of honeybees is a combination of cuticular hydrocarbons and compounds of the wax combs of the nest (Breed et al., 1988, 1998). The compounds from the comb wax include pheromones produced by other workers as well as floral scents brought to the nest via pollen and nectar. Recognition assays have suggested that constituents with functional groups such as fatty acids, esters, hydroxy alkyl esters, and primary alcohols, as well as nonpolar hydrocarbons such as hexadecane, octadecane and heneicosene, are likely to be the key components for nestmate recognition (Breed, 1998; Fröhlich et al., 2001). In this study, we identify compounds of low quantity from the cuticle of honeybee workers and, for the first time, we analyse the composition of emanated substances in the headspace of foraging honeybees in the field under undisturbed conditions.

A second aim of this study was to test particularly for the occurrence of (*Z*)-11-eicosen-1-ol on the cuticle and in the headspace of foraging honeybees. This alcohol is a major component of the honeybee alarm pheromone and has an alerting and attractive effect on nestmates (Free et al., 1982, 1983; Pickett et al., 1982). Females of the European beewolf *Philanthus triangulum* Fabricius (Hymenoptera, Sphecidae) hunt honeybees and provision them as food for their progeny. Beewolf females use (*Z*)-11-eicosen-1-ol as an essential olfactory cue to identify their prey (Herzner et al., 2005). This secondary function as a kairomone requires that honeybee workers constantly smell of (*Z*)-11-eicosen-1-ol during foraging and not only during alarm conditions. Therefore, we focus on the detection of, at least, traces of (*Z*)-11-eicosen-1-ol on the cuticle and in the headspace of foraging worker honeybees.

2. MATERIALS AND METHODS

2.1. Composition of cuticular hydrocarbons of honeybees

Foraging honeybee workers were collected from various colonies maintained by the apiary of the University of Würzburg. Only foraging workers of *Apis mellifera carnica* Pollmann exiting the hive were caught and stored at –20 °C. Five bees were individually soaked in 1 mL distilled hexane for 10 min. These extracts were evaporated to a residue of approximately 100 μL. We used 1 μL for GC-MS analyses. These were carried out on a HP GC System 6890 coupled to a MS 800 (quadrupole type) from Fisons Instruments. The GC was equipped with a DB-5 capillary column (0.25 mm ID $\times$ 30 m; film thickness 0.25 μm, J & W Scientific, Folsom, Ca, USA). Helium was used as a carrier gas with a constant pressure of 90 mbar. A temperature program from 60 °C to 300 °C with 5 °C/min and finally 10 min at 300 °C was employed. A split/splitless injector was used at 240 °C and in the splitless mode for 60 s. The mass spectra were recorded with an ionisation voltage of 70 eV and a source temperature of 220 °C.

The software Xcalibur (ThermoFinnigan, Egelsbach, Germany) for windows was used for data acquisition. Identification of the components was accomplished by comparison with purchased chemicals and the use of a commercial MS database (NIST 4.0).

2.2. Volatiles in the headspace of foraging honeybees

Volatile chemicals from the headspace of foraging honeybee workers were collected using solid phase micro extraction (SPME). Because of the relatively nonpolar compounds on the honeybee cuticle we used a poly dimethylsiloxane

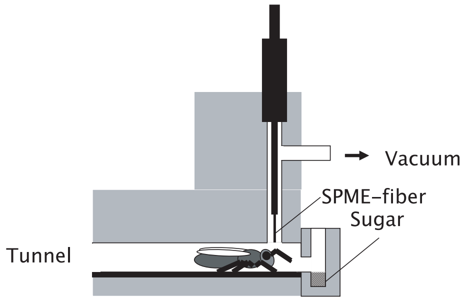

Figure 1. Schematic draft of the arena for the sampling of volatiles emitted from undisturbed foraging honeybee workers.

coated (100 μm) fibre (SUPELCO, Deisenhofen, Germany). Honeybee workers were trained to forage on a sugar solution in an arena made of Plexiglas that consisted of an about 5 cm long tunnel with a sugar solution supply at its end (Fig. 1). A SPME fibre could be inserted in a boring of the arena in such a way that the honeybees could not touch it but the air surrounding the honeybees could be drawn over the fibre using a vacuum pump. An air flow of 0.4 L per minute was chosen to optimize chromatography. A second identical arena, from which workers were excluded by closing the entrance with a valve served as a control and was run simultaneously to analyse the chemical background of the surrounding air and chemicals that were emitted by the arena. This experiment was conducted four times, for two hours each. The arena was established at a distance of around 20 m from 10–15 beehives. One trial lasted for 120 min and the arena was visited by hundreds of bees during one trial.

SPME fibres that were loaded with volatiles from foraging bees and control fibres were immediately analysed. The GC-MS system and temperature program were the same as for the extracts described above.

Pentadecanol, a major component emitted by the Plexiglas arena, was used as standard and the relative amounts of all substances were calculated. There are constituents identified from the headspace of foraging honeybees as from the air of the control arena. Therefore, to distinguish between background chemicals from the surrounding air and those that are emitted by honeybees, we considered all compounds that were less than twice as abundant in the experimental arena than in the control arena to come from the surrounding air. These were not included in the analysis.

3. RESULTS

3.1. Composition of cuticular hydrocarbons of honeybees

Besides the known long-chain saturated and unsaturated aliphatic hydrocarbons and long chain esters (Francis et al., 1985; Salvy et al., 2001), we found traces or minor components, mainly with shorter chain lengths (alkanes, alkenes, one terpene and (Z)-11-eicosen-1-ol) that have not yet been reported as components on the cuticle of honeybees (Tab. I). Several compounds could not be identified.

3.2. Volatiles in the headspace of foraging honeybees

In the headspace of foraging workers we found the major alkanes present on the cuticle up to a chain length of C29. Also minor alkanes as well as alkenes, and in one trial geraniol and farnesol, occurred in the air surrounding bees. However, the proportions differed considerably from the cuticle extracts. Generally, more volatile compounds are overrepresented in the head space compared to the cuticle. Octadecan and some aldehydes from C9 to C17 which were not found on the cuticle occurred in high proportions in the headspace of the foraging bees. Most interestingly, traces (0–0.1%) of (Z)-11-eicosen-1-ol could be unequivocally identified using the "extract ion" mode with specification of the characteristic masses of (Z)-11-eicosen-1-ol (278 (M^+), 250, 109 and 96) and taking into account its retention time (Tab. I).

4. DISCUSSION

Different alkanes, methyl-branched alkanes, alkenes and alkadienes with chain lengths ranging from C19 to C35 had already been identified on the cuticle of honeybees (McDaniel et al., 1984; Francis et al., 1985, 1989; Martin et al., 2001). However, we found traces and minor constituents with shorter chain lengths and characterized them as alkanes, alkenes, alcohols and terpenes. We also

Table I. Identified constituents and relative proportions of extractable cuticular hydrocarbons and volatiles in the headspace of foraging worker honeybees. (* 0–0.1%; ** 0.1–1.0%; *** 1.0–5.0%; **** 5–100%). [1] These compounds occurred only in one of the five analysed bees and in one of the four trials.

	Cuticular hydrocarbons	Headspace
Nonanal		**
Geraniol	**[1]	*[1]
Undecanal		*
Dodecanal		**
Pentadecane (C15)	*	***
Tridecanal		**
Hexadecane (C16)	*	**
Tetradecanal		***
Heptadecene		**
Heptadecane (C17)	*	***
Pentadecanal		***
Farnesol	*[1]	*[1]
Octadecane (C18)		*
Hexadecanal		***
Nonadecene	*	
Nonadecane (C19)	**	***
Heptadecanal		**
Eicosane (C20)	*	**
Heneicosene	*	
Heneicosane (C21)	**	***
Docosene	*	
Docosane (C22)	**	***
11-Eicosen-1-ol	**	*
Tricosene	***	***
Tricosane (C23)	****	****
Tetracosene	**	
Tetracosane (C24)	**	***
Pentacosene	***	**
Pentacosane (C25)	****	***
Hexacosane (C26)	**	
Heptacosene	***	
Heptacosane (C27)	****	**
Me-Heptacosane	**	
Octacosane (C28)	**	
Me-Octacosane	**	
Nonacosene	**	
Nonacosane (C29)	****	**
Me-Nonacosane	**	
Triacontane (C30)	**	
Hentriacontene	***	
Hentriacontane (C31)	***	
Me-Hentriacontane	**	
Tritriacontene	****	
Tritriacontane (C33)	***	

found some substances on the cuticle that have not been identified yet. As should be expected, the more volatile compounds generally increased in their proportion in the headspace of foraging honeybees compared to the cuticle. Similar results have been shown studying the emission of volatiles of honey bee queens (Moritz and Crewe, 1991). Surprisingly however, also long chain alkanes and alkenes up to C29 emanate from the cuticle of honeybee workers in detectable amounts although they have been assumed to be nearly nonvolatile. Arnold et al. (1996, 2000) have shown that 14 long-chain hydrocarbons from C23 to C33 might function as cues for kin recognition in honeybees. Hexadecane, octadecane, Z-9-heneicosene and Z-9-tricosene have already been tested for recognition activity in honeybees and yielded positive results (Breed, 1998; Breed and Stiller, 1992; Breed et al., 1992). Since these components could be detected in the headspace of honeybees, it is possible that they are able to recognize nestmates without direct contact.

The principal constituents of hydrocarbons with functional groups in the headspace of honeybee workers are aldehydes from chain length C9 to C17. The source of these aldehydes might be either the cuticular hydrocarbons of the bees or the comb wax (Blum et al., 1988). However, the large amounts of these aldehydes that we found in the air surrounding honeybee workers suggest that they do not originate from the traces of these aldehydes on the cuticle. A third possible source might be the degradation of alkenes and alkadienes caused by oxygen, heat and sunlight, a process that has been extensively studied with regard to rancidity of food oils (Frankel, 1998). The oxidation of unsaturated hydrocarbons is also known from the cuticle of other Hymenoptera. In these cases the released volatiles are saturated and unsaturated aldehydes (Bartelt and Jones, 1983, Bartelt et al., 1983, 2002; Swedenborg and Jones, 1992).

Other hydrocarbon components such as geraniol and farnesol that were found in only one trial of headspace analyses, are constituents of the Nasonov gland (Free, 1987). Their occurrence in the headspace of foraging bees is possibly the result of the exposure of the Nasonov gland during foraging on the sugar solution (Fernandez and Farina, 2001).

We identified (*Z*)-11-eicosen-1-ol as a new component on the cuticle of almost all foraging honeybees and as traces in the surrounding air. It is known as a major component of the honeybee alarm pheromone and it has been shown to have an alerting and attractive effect on nestmates (Free et al., 1982, 1983; Pickett et al., 1982). The occurrence of these compounds in the air surrounding foraging honeybees shows that also pheromone components might be transmitted as volatiles from the body of the bees in very low concentrations and outside the context of the known use, e.g. as alarm pheromone. It is not yet known why (*Z*)-11-eicosen-1-ol is present on the honeybees' cuticles. Possibly, this compound also occurs in the honeybees' Dufour's gland which seems to be slightly leaking (A. Hefetz, unpubl. data.). Alternatively it might be a residue from the last exposure to (*Z*)-11-eicosen-1-ol or the use of the compound. We do not know yet whether the (*Z*)-11-eicosen-1-ol on the honeybees' cuticles has also a meaning for honeybees themselves in non alarm situations. Possibly it provides a cue to discriminate between conspecifics and other species. Since (*Z*)-11-eicosen-1-ol is otherwise rare in nature it would represent a reliable cue for the identification of honeybees by a specialized predator or parasite. In fact, it has been shown that beewolf females use (*Z*)-11-eicosen-1-ol as an essential kairomone for the identification of honeybees (Herzner et al., 2005). That this substance serves as a kairomone for beewolf females despite its very small amounts on the honeybee and its low volatility underlines the extraordinary sensitivity of insect olfaction and the potential meaning of trace components for insect communication purposes.

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Les substances volatiles des abeilles (*Apis mellifera*) butineuses et leur rôle potentiel comme composés sémiochimiques.

***Apis mellifera* / hydrocarbure cuticulaire / substance volatile / reconnaissance des membres de la colonie / kairomone / *Philanthus triangulum* / Hymenoptera / Sphecidae / (*Z*)-11-Eicosen-1-ol**

Zusammenfassung – Flüchtige Verbindungen von fouragierenden Honigbienen *Apis mellifera* (Hymenoptera: Apidae) und ihre potentielle Rolle als Signalstoffe. Die Nestgenossinnen- und Verwandtenerkennung spielt eine bedeutende Rolle innerhalb von Kolonien bei sozialen Insekten. Bei der Honigbiene *Apis mellifera* wurde angenommen, dass die Nestgenossinnenerkennung durch Wächterbienen über den Kontakt der Antenne der Wächterin mit dem Körper einer anderen Biene vermittelt wird. Einige Beobachtungen lassen jedoch vermuten, dass Wächter andere Bienen auch über den Geruch erkennen können, z. B. über das Wahrnehmen von Stoffen, die von der Oberfläche dieser Bienen abgegeben werden. Der Geruch von Bienenarbeiterinnen wird auch vom Weibchen des Europäischen Bienenwolfes *Philanthus triangulum* als Signal bei der Beuteerkennung verwendet. Bienenwolfweibchen nutzen speziell einen Stoff zur Beuteidentifizierung, der ein Bestandteil des Honigbienenalarmpheromons ist. Zunächst haben wir untersucht, ob diese Komponente auf der Kutikula der Honigbiene vorkommt, danach, welche der flüchtigen Stoffe überhaupt von der Oberfläche ungestört fouragierender Honigbienen abgegeben werden. Wir extrahierten Honigbienen in einem Lösungsmittel und analysierten die gelösten Stoffe mittels Gaschromatographie-Massenspektroskopie. Tatsächlich fanden wir kleine Mengen (*Z*)-11-Eicosen-1-ol auf der Oberfläche von fouragierenden Honigbienen (Tab. I). Zusätzlich verwendeten wir eine Fouragierarena, die uns erlaubt, volatile Substanzen aus der Umgebungsluft von fouragierenden Honigbienen auf speziellen Fasern zu sammeln (Abb. 1). Wie erwartet war das Verhältnis von leicht flüchtigen zu schwerer flüchtigen Stoffen der Umgebungsluft höher als auf der Oberfläche. Weiterhin konnten wir auch Kohlenwasserstoffe bis zu einer Kettenlänge von 29 Kohlenstoffatomen in der Umgebungsluft von Honigbienen wiederfinden, obwohl allgemein angenommen wurde, dass diese eine sehr geringe Volatilität besitzen (Tab. I). Folglich sind die Stoffe, welche auf der Oberfläche der Honigbienen vorkommen, gut in ihrem Duft repräsentiert und könnten als Signale zur Nestgenossinnenerkennung verwendet werden. Wir konnten auch (*Z*)-11-Eicosen-1-ol in der Umgebungsluft der Honigbienenarbeiterinnen nachweisen (Tab. I). Dies unterstützt die Ergebnisse der Verhaltenstests, dass diese Substanz vom Bienenwolfweibchen genutzt

wird, um Honigbienen zu identifizieren. Unsere Ergebnisse werfen ein neues Licht auf die Frage nach der Nestgenossinnenerkennung bei Honigbienen und lassen vermuten, dass Individuen sich nicht mit der Antenne berühren müssen, um eine fremde Honigbiene zu erkennen.

***Apis mellifera* / Nestgenossinnenerkennung / Kohlenwasserstoffe der Kutikula / flüchtige Stoffe / *Philanthus triangulum* / Kairomon / (*Z*)-11-Eicosen-1-ol**

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