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AKADÉMIAI KIADÓ

Electrophysiological responses of *Varroa* mite to honey bee drone brood volatiles

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RESEARCH ARTICLE

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ABSTRACT

Varroa mite, *Varroa destructor* is the most important ectoparasite of the honey bee, *Apis mellifera* worldwide, contributing to colony collapse. The development of alternative non-toxic methods against this pest is needed, as most of the wide-used acaricides in apiculture are banned in the European Union, which increase the risk of developing resistant mite populations against the remaining few products. In order to reveal biological basis of a new, semiochemical-based method, the aim of this study was to search for olfactory stimuli, used by female *Varroa* mites in orienting to drone brood for egglaying. Volatiles of uncapped drone brood were collected *in situ*, inside bee-hives, using either charcoal, or HayeSep® Q filters. Collections were analyzed by gas chromatograph linked to an electrotarsogram detector (GC-ETD), using the foreleg of female mite. Results showed that most components were present in collections trapped by any of these filters. However, some components appreared only in charcoal-, while others only in HayeSep® collections, respectively. Out of the large number of components, a few elicited electrophysiological responses. Structure elucidation of these active components are underways. Futher behavioral studies should reveal, which components play role in attraction of *Varroa* mites.

KEYWORDS

drone odour, beehive, *Varroa destructor*, kairomone, chemical signal

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INTRODUCTION

The mite, *Varroa destructor* Anderson & Trueman (Acari: Mesostigmata: Varroidae) is an obligate ectoparasite of the European honey bee, *Apis mellifera* L., contributing in direct and indirect ways to colony collapse disorder (CCD). Apart from direct impact, caused by feeding on honey bee fat bodies (Ramsey et al., 2019), *V. destructor* is even more importantly a vector of many bee viruses, such as for example Acute bee paralysis virus (ABPV), Deformed wing virus (DWV) and Sacbrood virus (SBV) (Yañez et al., 2020). Especially, the former two viruses contribute most frequently to CCD (Genersch et al., 2010). In a comprehensive survey it was found that *Varroa* mite contributed predominantly to death of overwintering bee colonies in Canada (Guzmán-Novoa et al., 2010). This applies, in general, to the Northern Hemisphere, where *Varroa* mite is considered to play major role in colony collapse (Dietemann et al., 2012). Similarly, *Varroa* mite is accused to be the most important enemy of bee keeping in Hungary (Tóth, 2022). The application of acaricides againts *Varroa* mite is problematic, because of the risk of polluting honey with residues, and also because of the fear that intense application of acaricides, representing just a few active ingredients which have not been banned yet, would soon evoke resistant *Varroa* populations (Martin, 2004; Lodesani and Costa, 2005).

A traditional practice widely used by beekeepers to reduce *Varroa* mite population in the beehive is the removal of frames with drone-brood (Charrière et al., 2003). This is certainly a useful and chemical-free method, therefore recommendable, however, success is rather limited, especially when the population level of mites in the hive is already high. There are several promising new directions to develop various biological control methods against *Varroa* mites, such as breeding for behaviorally resistant honey bee colonies via suppressed mite reproduction (SMR) (Mondet et al., 2020), applying microbial control agents (James, 2009), or predatory mites (Rondeau et al., 2018), or using essential oils (Ramzi et al., 2017), just to name a few. For recent reviews about the scope and limitations of these methods see Reams and Rangel (2022) and Teski et al. (2022). The search for semiochemicals, which could influence the host location of *Varroa* mites, looks particularly promising (Plettner et al., 2017). Recently, as a fresh idea, it was shown that genes involved in juveline hormone biosynthesis are upregulated in drone larvae, resulting to an enhancement of methyl farnesoate synthesis, in capped cells (Aurori et al., 2021). This compound is a widely known as a semiochemical in other contexts, namely as for example a pheromone components in a Danaid butterfly (Schulz et al., 1993), therefore its involvement, as a signal for *Varroa* mite was postulated by the above authors. In a line of a more traditional semiochemical-based approach, we focused in the present study on volatiles of uncapped drone cells prior capping, as *Varroa* mites oviposit in that period (Calderón et al., 2010; Rosenkranz et al., 2010). The objective of this study was to show whether volatiles of honey bee drone cells elicit olfactory response from *Varroa* mites. This would be the first step towards developing an oviposition attractant for *Varroa* mite, which in turn opened the way to manipulate mite's behavior in the beehive. As for possible future application of these findings in the practice, mites could be attracted to a trap inside the hive, or deter from brood by camouflage signal.

MATERIAL AND METHODS

Bee colonies

Five honey bee colonies were installed in the Experimental Station of the Plant Protection Institute, near Budapest, Hungary, in April, 2021, housed in ½ NB („Nagyboconádi") beehives

(a common hive-type, widely used across Hungary). The development of larvae in drone broods was checked at least twice a week. No treatments against *Varroa* mites, or for any other reason was performed in course of the experiments. Also, no smoke was used at all to calm down honey bees when opening the hives for performing volatile collections, in order to avoid unwanted contaminations with alien odours.

Collections of volatiles

Collections were made from 25. May to 23. June, 2021, during daytime (typically between 10:00 A.M.–15:00 P.M., preferably on sunny days, when the ambient temperature was above 20 °C, from the above-mentioned beehives. Sampling of volatiles from the headspace of drone brood cells (*in-situ*, inside the hive) were timed within 24 h of the estimated capping time. A teflon tube was carefully introduced into the beespace, though a tight hole, carved for that reason through the wall of the hive. The tube was placed gently, so that it would cause the possible least disturbance in the normal life of the colony. The filter was inserted near to the opening of the teflon tube at the brood, so most of the sucked air should come from directly above the brood. Duration of collecting volatiles was set to ca. one hour. For trapping volatiles either pre-conditioned charcoal CLSA filter (1.5 mg load, Brechbühler AG, Schlieren, Switzerland), or self-made filter filled by ca. 50 mg of the adsorbent HayeSep® (Q 60–80 mesh and/or 80–100 mesh; SuperCo./Sigma-Aldrich,) were used. The airflow was set to 0.9–1.1 mL min^{-1}, with the help of a DC12/80LC rotary vane pump (Fürgut GmbH, Tannheim, Germany), though an adjustable linear labor power supply (Voltcraft® PS-1152A, Hirschau, Germany). Actual flow was checked by a flowmeter (Kytola Instruments, type BA-4AR). Trapped volatiles were washed of from the filter by either *n*-hexane, or dichloromethane (Merck, Darmstadt, Germany). For wash-off the possible smallest amount of solvent was used (in case of charcoal filter ca 60 µL, while in HayeSep® filters ca 200–300 µL). No further concentration of the samples were performed. Extracts had been placed into cone microvials (Screw neck vial, 1.5 mL with silenized inlets 0.2 mL; Macherey-Nagel, Germany), and stored under minus 30 °C, until analyses. Prior to first usage and after eluting trapped volatiles, filters were rinsed off by a series of pure solvents (methyl alcohol, a 3:1 mixture of methanol/chlorophorm, acetone, dichloromethane and hexane), then gently flashed by nitrogen (purity: 5.0) and heated at 120 °C overnight.

Gas chromatographic screening of collections

Collections were pre-screened for obtaining an overall picture of the pattern of compounds on a 6890N gas chromatograph (GC) (Agilent Technologies Inc., Santa Clara, CA, USA), equipped with either a non-polar HP-5 (J&W, 30 m × 0.32 mm, 0.25 µm film thickness; Agilent Technologies Inc.), or a polar DB-WAX (J&W, 30 m × 0.32 mm × 0.25 µm film thickness) column. Injections were performed to the HP-5 column in splitless mode (injector temperature: 230 °C), while on-column to the DB-WAX column. At analyses ca. 1.5 µL of the samples were injected. The temperature program for the HP-5 runs started from 50 °C with initial time for 1 min, then heating rate was 10 °C min^{-1} to 230 °C. Final temperature was held for 10 min, while for the DB-WAX runs were as follows: oven temperature was held at 60 °C for 1 min, then increased at 10 °C min^{-1} to 220 °C and held for 20 min. In both cases, the carrier gas was helium, with a flow of 4.0 mL min^{-1}. Compounds were detected by a flame ionization detector (FID). The GC was controlled and data acquisition performed by an Agilent Chemstation Program (version Rev. A. 10.02).

Traces displayed on Fig. 1 represent collections from the same drone brood inside the same hive, sampled during the same day (25. May 2021), while traces on Fig. 2 represent collections, sampled from different hives, at 11. June 2021.

Collection of *Varroa* mites for electrotarsogrammic studies

Adult *Varroa* mite females for electrotarsogrammic studies (see next subheading) were freshly collected from the experimental beehives, before use. Exclusively those mites were collected, which either freely walked on brood combs, or were sitting on worker bees while bees were busy on combs. In the latter case, bees with mites were gently placed into a small plastic container with transparent wall, and mites were used for studies when they freely dropped off themself from the hosting bee.

Analyses of selected collection by means of gas chromatography linked to electrotarsogram („electroantennogram") detector (GC-ETG) („GC-EAG")

The GC-ETD system consisted of the above mentioned 6890N GC and HP-5 column, with a split to ETD, connected by an effluent conditioning assembly (SYNTECH, Hilversum, the Netherlands). For mounting the mites, a slightly more robust method was developed, as described by Light et al. (2020a), who used whole body of the mite. Instead, we applied isolated

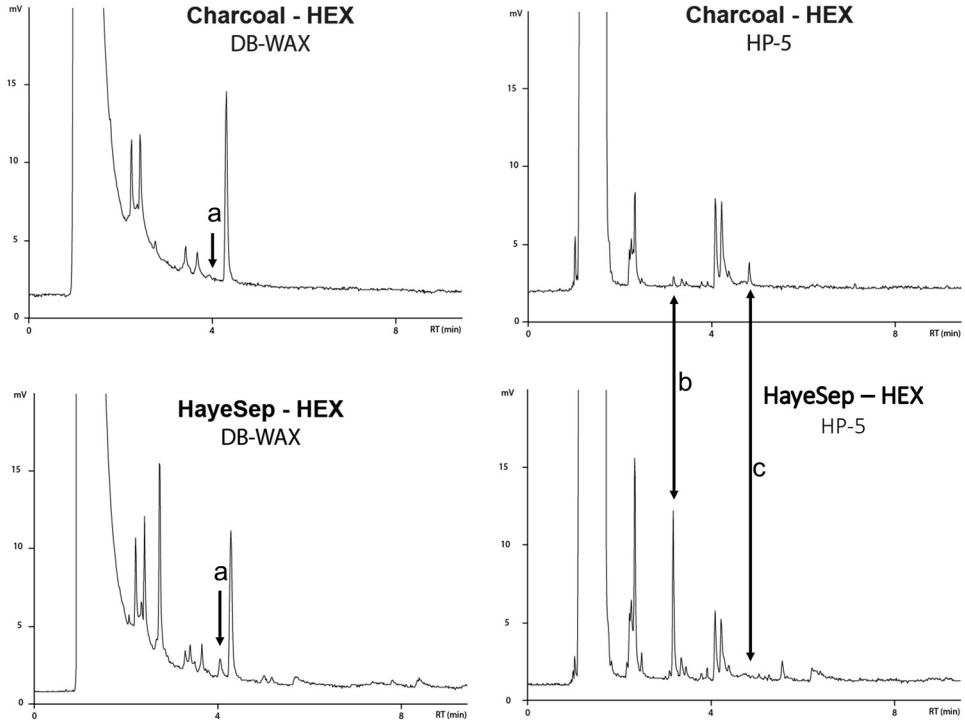

Fig. 1. Gas chromatographic traces of honey bee drone volatiles, collected on either on charcoal, or on HayeSep® Q 60–80 mesh filters, eluted with *n*-hexane (HEX) and separated either on DB-WAX, or on HP-5 capillary column. Arrows, labelled by different letters, point to certain retention time values, where peaks (components) appear predominantly only either in charcoal, or in HayeSep® Q collections

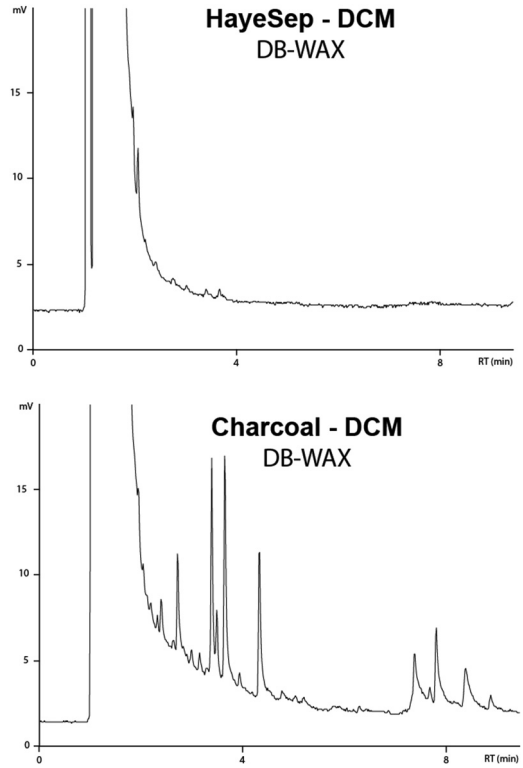

Fig. 2. Gas chromatographic traces of honey bee drone volatiles, collected on either on charcoal, or on HayeSep® Q 60–80 mesh filters, eluted with dichloromethane (DCM) and separated on DB-WAX capillary column

foreleg preparations, as foreleg had been shown a sensilla-rich sensory organ, including odour-receptors (Lei et al., 2019). The probe electrode was connected to the tip of the foreleg, while the ground electrode was inserted into the base. The type of the electrodes was that of glass capillary (1.17 mm i.d.), filled with a Ringer solution (Beadle and Ephrussi, 1936) and equipped with silver-wires for transmitting the electric signal to the pre-amplifier. Connections were established with the help of MP15 micromanipulators. Electric signals were pre-amplified by an IDAC2 amplifier and analyzed by SYNTECH GC-EAD 2014 v. 1.2.5. software (SYNTECH, and its successor, Ockenfels SYNTECH GmbH, Kirchzarten, Germany). At runs, ca. 1.5 µL of samples were injected. Ten repetitions were made per types of extracts, using different specimens.

RESULTS

Altogether 17 volatile collections were made. The specifications of collections by the type of filters and solvents are listed on Table 1. Each collections were analysed by gas chromatography using HP-5, as well as DB-WAX capillary columns. Viewing the runs revealed that the FID patterns of volatiles collected on charcoal, or on HayeSep® Q 60–80 mesh filters and washed thereafter by *n*-hexane had rather similar overall appearance, however, some compounds were

Table 1. Number of drone brood volatile collections, trapped either on charcoal, or on HayeSep® filters, thereafter washed off from the filter either by hexane, or by dichloromethene

Filter	Hexane solvent	Dichloromethane solvent	Total number of collections
Charcoal	3	5	8
HayeSep® (Q 60–80 mesh)	4	3	7
HayeSep® (Q 80–100 mesh)	0	2	2
Total number of collections	7	10	17

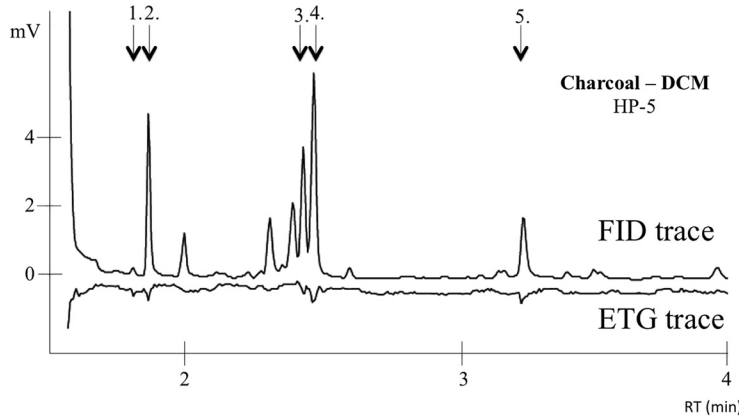

Fig. 3. Gas chromatographic analyses, equipped with synchronized data collection from a flamme ionization (FID) and an electrotarsographic detector (ETG), of a honey bee drone brood volatile collection. Numbered arrows indicate components, which evoked ETG responses from foreleg of *Varroa destructor*

present, or appeared with sharply pronounced amplitude only in case of one of these filters (Fig. 1). Volatiles collected on HayeSep® Q 80–100 mesh filters, resulted in fewer components and peaks were generally smaller, than in case of Q 60–80 mesh filters (not shown on the figures). It was also evident that much better separations were achieved on HP-5 column, under the given chromatographic conditions (Fig. 1). An example is provided on Fig. 2, that dichloromethane rinse of the filter yielded a similarly wide arrays of components, as using *n*-hexane, however, interestingly only in case of charcoal filter. Therefore, volatiles prepared with the help of charcoal filter and dichloromethane were chosen for GC-ETG measurements, where components were separated on HP-5 column.

Analyses of collections by GC-ETG revealed several repeatable ETG peaks, corresponding to FID peaks. Five ETG peaks were displayed on Fig. 3, where the ETG trace was composed as an average of two runs.

DISCUSSION

It was demonstrated in this study that airborne volatiles of honey bee drone brood can be collected *in-situ*, from inside the hive, using either charcoal or HayeSep® Q 60–80 mesh filters. Samples collected on HayeSep® Q 80–100 filter generally resulted in a smaller number of peaks,

moreover those peaks which appeared on the gas chromatographic traces had, in general, smaller amplitudes (quantity), which could result, at least in part, because larger volume of solvent was needed to rinse off this type of filter. Regarding what the samples represent, although air was sampled directly above drone brood, nevertheless, it should be taken into consideration that overall background odour of the inside area of the hive was certainly collected, too. Also, this collecting technique did not allow to separate background odour from the volatile of the drone brood. Nevertheless, it can be supposed that the ETG signals recorded from the foreleg of *Varroa* mites refer mostly to components, exploited by the mites in their search for uncapped drone brood. Five components were revealed to elicit ETG response, which means that these components are perceived by the mites. To our knowledge this is the first case that components of drone brood volatiles were pinpointed, as possible olfactory signals for *Varroa* mite.

Reviewing related studies in the literature, a set of volatile compounds, related to honey bee larvae, were collected from hives and chemically isolated by means of gas chromatography, linked to mass spectrometry (Carrol and Duehl, 2012). However, the approach of Carrol and Duehl (2012) was different from that of the present study, as they did not relate the components to *Varroa* mites. Therefore, the components identified by them could unfortunately not be matched to the ETG-active peaks found in the present study. On the other hand, synthetic compounds with previously supposed, putative effects on *Varroa* mites were screened via ETG by Light et al. (2020a). Here again the approach was different from ours, as Light et al. (2020a) directed their studies towards putative attractants and repellents, regardless the source of compounds (drone brood). This again did not make possible to relate synthetic compounds, studied by them, to ETG-active components in brood volatiles, found in the present study. However, the present study fits well into the scope of another, very comprehensive study of the above authors (Light et al., 2020b). They identified more than 100 compounds from hive volatiles, out of which they relate ca. 70 compounds to brood, and the rest to hive background odour. Variations in methods unfortunately exclude direct comparisons to electrophysiologically active peaks found in the present study, therefore future studies should reveal similarities in the sets of components.

Qin et al. (2019) followed the titer of four components, methyl palmitate, methyl oleate, methyl linoleate, and methyl linolenate, known as capping pheromone components, released by bee larvae and eliciting capping behavior of attendant bees. They presented titers of these components prior and after capping, in worker and drone larvae, respectively, and also postulated biosynthetic pathways and related RNA expressions. It would be of great interest to relate these synthetics to respective eletrotarsogram responses, found in the present study.

Results obtained in the present study by ETG do not provide any evidence on the nature of possible behavioral responses. The ETG-active components found in the present study may serve as attractants for *Varroa* mite, nevertheless other types of behavior, including even avoidance, as an extreme, could theoretically not be excluded. For clarifying this, chemical identification of the presently found ETG-active compound should be completed, followed by behavioural tests. Our present results may represent the first step in that direction.

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