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Varroa destructor mite electrophysiological responses to honey bee (*Apis mellifera*) colony volatiles

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Abstract
Detection and interpretation of chemical cues is essential for *Varroa destructor* Anderson and Trueman, an important parasite of honey bees (*Apis mellifera* L.), to complete its life cycle. We collected volatiles from honey bee brood at various developmental stages and screened for *V. destructor* electrophysiological responses to these with gas chromatography-linked electrotarsal detection. Volatile collections contained several methyl-alkanes that evoked electrophysiological responses from *V. destructor*. Moreover, odors in honey bee colonies that regulate honey bee colony structure and function were also detected by *V. destructor*. Collections from mid- to late-stage larvae had detectable levels of low-volatility odors identified as components of the honey bee brood pheromone and branched alkanes likely originating from brood cuticle. Among these, several mid- to heavy-molecular weight compounds elicited high proportional electrophysiological responses by *V. destructor* relative to their abundance but could not be identified using chemical standards of previously documented honey bee brood odors. We suggest further investigation of these unknown volatiles and future behavioral assays to determine attractiveness/repellency (valence) of those identified through chemical standards.

Keywords Bee brood · Electrotarsograms · GCMS · Semiochemicals · Dynamic headspace

Introduction

Varroa destructor Anderson and Trueman (Acari: Varroidae) mites are considered one of the most important and challenging pests of honey bees (*Apis mellifera* L.). *Varroa destructor* is blamed for increased annual winter colony mortalities (Beetsma 1994; Milani 1999; Currie et al. 2010; Rosenkranz et al. 2010) and has rapidly developed resistance to synthetic

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miticides, increasing motivation to develop effective alternatives and integrated approaches. When left untreated, infested honey bee colonies usually succumb to *V. destructor* within 3 years (Rosenkranz et al. 2010).

Varroa destructor has a two-stage life cycle that is repeated up to 3×during an individual female's lifetime: a phoretic stage and a reproductive stage (Martin and Kemp 1997; Nazzi and Le Conte 2016). Detection and interpretation of chemical and physical cues from different honey bee hosts is essential for *V. destructor* to survive and complete its life cycle (Plettner et al. 2017). *Varroa destructor* mites transfer among adult hosts in the phoretic stage, preferring 7- to 12-day-old honey bees of the nurse caste (Le Conte and Arnold 1987; Kraus 1994; Pernal et al. 2005). The reproductive stage occurs when *V. destructor* leaves phoretic hosts and infiltrates a cell containing a honey bee larva host (Le Conte et al. 1989; 1990; Boot et al. 1994).

Host choice and reproductive development are focal areas in *V. destructor* research, given the apparent importance of timing and host cues in their reproduction (Rosenkranz et al. 2010; Nazzi and Le Conte 2016; Plettner et al. 2017). Development of effective semiochemical lures, repellents, or compounds that disrupt host detection (disruptants) and interrupt the *V. destructor* life cycle is an attractive option for control of this widespread apicultural pest (Yoder and Sammataro 2003; Plettner et al. 2017).

Qualitative and quantitative analysis of honey bee semiochemicals through non-destructive sampling techniques has been limited (Gilley et al. 2006; Thom et al. 2007; Carroll and Duehl 2012). To our knowledge, qualitative and quantitative analysis of volatiles that are electrophysiologically active in *V. destructor* has not been previously explored. Volatile collections from honey bee colonies, specific to different developmental stages of honey bee brood, may contain odors important in *V. destructor* host detection (Carroll and Duehl 2012). We refined methods of Carroll and Duehl (2012) for volatile collection from honey bee brood frames. We collected volatiles from single colony frames that had bee larvae of the same, known age. These modified methods allowed collection of replicate samples from a single frame while eliminating possible background odors associated with propolis and plastics or wood on frame peripheries (described in Carroll and Duehl 2012). Furthermore, we modified a method from Endris and Baker (1993) and Dillier et al. (2003) for performing electrotarsography on live *V. destructor* mites. Our methods allowed us to screen volatile compounds to live *V. destructor* through gas chromatography linked to electrotarsal detection (GC-ETD) by prolonging longevity of prepared *V. destructor* mites for electrotarsograms. Following isolation of electrophysiologically active honey bee volatiles, we attempted to identify these honey bee colony volatiles by cross referencing with databases and chemical standards. From these new approaches, we hope to uncover gaps in current knowledge on odors responsible for *V. destructor* attraction to honey bee fifth instar larvae and gain a better understanding of odor cues involved in attraction or repulsion of *V. destructor* towards honey bee brood at different developmental stages. Odorants identified as putatively important in *V. destructor* detection of honey bee brood can then be further tested in laboratory behavioral assays and potentially used for monitoring or managing *V. destructor* infestations.

Methods

Rearing honey bee brood

All honey bee (*Apis mellifera carnica*) brood-rearing and volatile collections were done using a single Langstroth honey bee colony located in Coldbrook, Nova Scotia (NS), Canada (45° 3′ N, 64° 36′ W), from July to August 2018. All brood-rearing and volatile collections were performed using food-grade plastic Langstroth frames (Pierco, Riverside, CA, USA) with wax comb produced within the same year of volatile collections.

Prior to brood-rearing, high-density polyethylene (HDPE-2) 300-mL cups were used to create indentations of cup diameter on drawn-out Langstroth frames, thereby preventing queens from laying on cup peripheries, later avoiding volatiles produced through incidental damage to honey bee brood (injury volatiles) during volatile collection (Fig. 1). Methods for honey bee queen trapping were used to rear honey bee brood of a uniform age for volatile collections (Human et al. 2013). Brood age was then determined by development time in days post egg-laying; volatile collections were categorized accordingly.

Mite collection

Collection and maintenance of *V. destructor* followed protocols in Dietemann et al. (2013). Drone frames containing brood were transferred from donor colonies in Berwick (NS) to an untreated *V. destructor*-infested queenright colony in Coldbrook (NS). Drone frames were reared in the untreated colony until cells were capped and then frames were transferred to environmentally controlled chambers (described above) at Acadia University. Worker honey bees ranging in age from 1 to 10 days old were added to drone frames at a ratio of 2:1 for every drone to maintain colony structure and to keep *V. destructor* alive longer

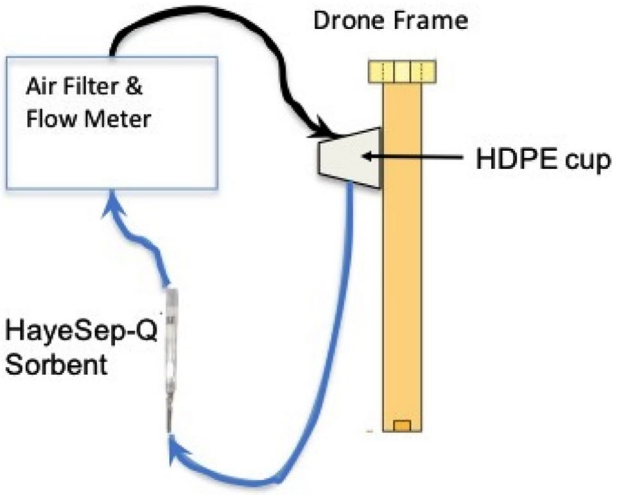

Fig. 1 Example of a closed system volatile collection on a drone frame with a HayeSep-Q volatile collection trap and high-density polyethylene cup (HDPE cup); arrows indicate direction of flow; air flow was regulated by a portable volatile assay system (PVAS22)

within environmental chambers. All frames were placed on wooden racks that had similar dimensions to a honey bee nucleus box (53 × 21 × 24 cm); all bees and frames were kept inside nylon insect-rearing tents (90 × 60 × 60 cm). Queen mandibular pheromone (Intko Supply, Vancouver, British Columbia, Canada) was applied on a glass coverslip every 48 h at a concentration of 0.1 queen equivalents (42.2 ng in 10 µL of 2-propenol) to promote honey bee health and longevity (Grozinger et al. 2007). Adult worker and drone honey bees were transferred in groups of 10–20 into wooden hoarding cages (17 × 12 × 13 cm) using a hand-held vacuum modified as a bee-aspirator (DCV517B; DeWalt, Baltimore, MD, USA; Rogers and Williams, pers. comm.). Honey bees in hoarding cages were then individually examined for phoretic *V. destructor*. Mites were removed from honey bees using both a moistened paintbrush and aspirator and transferred in groups of three to 50-mL falcon tubes (Thermo Fisher Scientific, New York, NY, USA) containing 2 × 4 mm moistened filter paper. All *V. destructor* gathered in falcon tubes were used for electrophysiological experiments on the same day they were collected.

Volatile collection

Volatile collections from honey bee frames were taken in environmentally controlled chambers (1.3 × 1.3 × 1.8 m, Model E-16; Conviron—Controlled Environments, Winnipeg, Manitoba, Canada), at 32 °C and 65% relative humidity. Frames containing early-instar larvae or capped brood and no adults were transported using a colony nucleus box from the donor honey bee colony to environmentally controlled chambers for volatile collections (K.C. Irving Environmental Centre, Acadia University, NS, Canada). Uncapped frames containing larvae were returned to the same colony immediately after volatile collection. Based on visual inspection, brood frames containing honey bee larvae were free of *V. destructor* mites. Capped brood frames were maintained in the incubator along with 100–200 worker nurse bees following volatile collection for honey bee and *V. destructor* rearing. Estimated proportion of adult *V. destructor* reared from capped brood frames used in volatile collections was 20–40% relative to number of honey bee pupae.

Pre-packed volatile collection traps (HayeSep-Q 80/100 mesh, 27+ mg; Volatile Assay Systems, Rensselaer, NY, USA) were cleaned using methods adapted from Kunert et al. (2009) and Molnár et al. (2015). Traps were pre-conditioned before use by flushing sequentially with 1 mL each of methanol (100%; Bebbington Industries, Dartmouth, NS, Canada), acetone (> 98.5% pure; Sigma-Aldrich, St. Louis, MO, USA), and HPLC grade hexane (Fisher Scientific, Fair Lawn, NJ, USA) and dried under compressed ultra-high purity nitrogen following each solvent flush. Volatile collection traps were then kept in an oven at 75 °C for 2 h before use. Prior to volatile collection, traps were flushed with 1 mL HPLC hexane and dried under compressed ultra-high purity nitrogen.

Frames were held vertically in insect-rearing tents using wooden racks. Volatile collections were performed in tandem, with two volatile collections commencing simultaneously on different areas of a brood frame. This method allowed for two replicate volatile collections from brood of a particular age to be collected within the same time span (Raguso and Pellmyr 1998; Kunert et al. 2009; Carroll and Duehl 2012).

Collections were from drone brood of various ages based on previous research indicating a greater attraction of *V. destructor* to drone larvae of the fifth instar (Trouiller et al. 1992; Kuenen and Calderone 1997). Egg and first-instar drone larvae were selected for volatile collections and contrasted to volatile collections from fifth-instar drones. Additional volatiles were collected from honey bee pupae of different ages. Honey bee pupae ages were

quantified based on amount of melanisation of pupal cuticle (Frey et al. 2013; McAfee et al. 2017). A final volatile collection was performed on drone brood killed through cold treatment at 11 °C for 7 h to identify previously unexplored *V. destructor*-responsive volatiles.

Volatile collections were obtained using a portable volatile assay system (PVAS22; Volatile Assay Systems) carbon-filtered continuous airflow pump (0.3 L/min push and 0.1 L/min pull with volatile collection traps in-line) that had Fisherbrand Tygon (0.64 cm I.D. × 15.24 cm length) tubing fitted with Teflon connectors (Fisher Scientific, Pittsburgh, PA, USA). Pre-conditioned volatile collection traps were then connected to the airflow system. HDPE-2 300-mL cups were used to isolate areas of interest in volatile collections. Cups were pushed into initial indentations made in wax comb, forming a seal with the frame. Individual collection cups encompassed approximately 60–80 honey bee brood cells for a particular volatile collection, depending on whether brood were drones or workers, respectively, due to differences in cell size (drones have larger cells; Boot et al. 1995). Elastic bands were used to hold cups to drone frames during volatile collections. Volatile collections were performed for three hours within environmental chambers maintained at above-mentioned climatic conditions comparable to in-colony environments (Carroll and Duehl 2012).

Following collection, volatile traps were sealed with Teflon tape and wrapped in aluminum foil. Solvent elution was performed on the day of volatile collection to reduce sample degradation and contamination. HPLC-grade hexane (Fisher Scientific, Fair Lawn) was used to elute volatile collection traps at 1 mL volume (Raguso and Pellmyr 1998). Extractions were kept in Teflon-sealed 2-mL vials at − 20 °C prior to analysis.

All volatile collection equipment was washed with warm unscented soap (The Unscented Company, Montreal, Canada) and water to remove most contaminants including heavier non-polar compounds (e.g., waxes and fats), and then subsequently flushed with 100% ethanol and air-dried overnight between volatile collections. Ethanol was used to remove possible remaining polar contaminants and cited as an appropriate solvent to clean materials in honey bee research (Torto et al. 2013). Blank volatile collections were performed in the same conditions as above; however, volatile collection cups were wrapped in aluminum foil sealed using elastic bands. Prior to blank collections, aluminum foil was cleaned using 100% laboratory-grade ethanol and air-dried for 1 h.

Electrotarsograms

To reduce their mobility, *V. destructor* were chilled in 55-mm plastic Petri dishes (Fisher Scientific, Ottawa, Ontario, Canada) on ice for 2–3 s, and then mounted on a microscope slide coated in dental wax (Electron Microscopy Sciences, Hatfield, PA, USA). A single mite was placed on its dorsum, without pressing into the dental wax, and held in place with two parallel, horizontally positioned minuten pins (Ento Sphinx, Černá za Bory, Czech Republic) to reduce movement (Light et al. 2020).

Gas chromatography electrotarsal detection (GC-ETD) was performed using methods adapted from previous *V. destructor* research (Endris and Baker 1993; Light et al. 2020). Changes in electrical potential were measured from either the left or right foretarsus using tungsten recording electrodes. Small amounts of electrode gel (Signagel; Parker Laboratories, Fairfield, NJ, USA) were placed on prepared *V. destructor* anal plates and all tarsi except the foretarsi to further reduce mechanical responses from mite preparations and improve connectivity (Light et al. 2020). A ground electrode was inserted into the anus at the base of the anal plate of a prepared mite. The recording electrode was inserted just past the apotele of the foretarsus of the mite preparation. Recordings for a given volatile

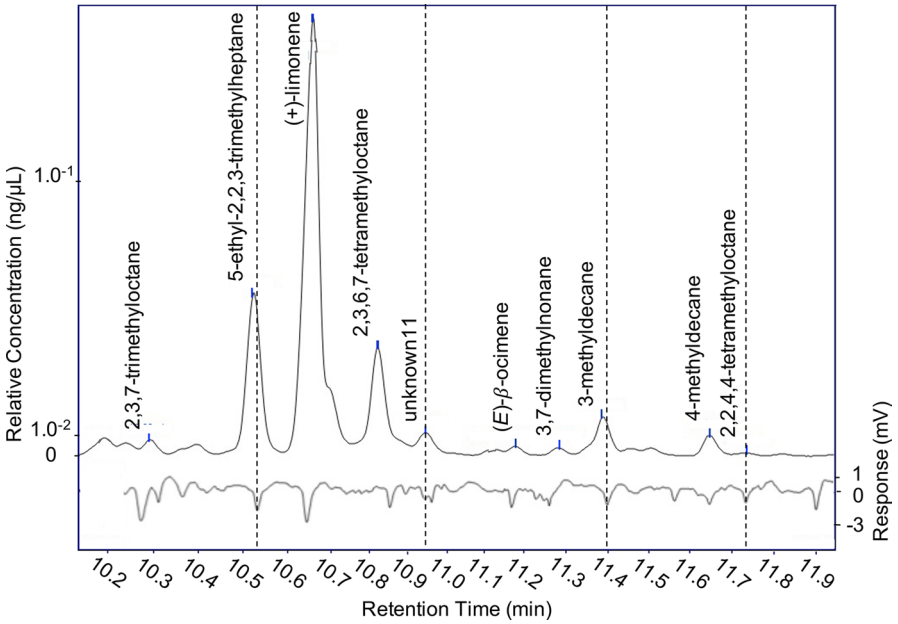

Fig. 2 Typical responses of *Varroa destructor* adult females to volatile compounds from worker larvae of the fourth to fifth instar stage of development. Compounds (+)-limonene (3.1 mV response) and (*E*)-ß-ocimene (1.1 mV response) were identified through chemical standards. Bottom, tarsal recording signal (electrotarsal detection); top, gas chromatography flame ionization detection trace on CIP SIL8-CB column. Relative concentration is represented by proportional peak abundance relative to known amount (3 ng/µL) of internal standard, nonyl acetate. Relative concentration is reported instead of total ion concentration to provide context to amount of analyte versus the strength of *V. destructor* responses

extraction were repeated on at least nine different mite preparations to ensure consistency in electrophysiological responses. GC-ETD signals were collected and amplified by Intelligent Data Acquisition Controller-2 (IDAC-2) (Syntech, Kirchzarten, Germany). Syntech GC-EAD v.1.2.5 software (2010) was used to analyze results (Filter Low Cut-off: 0.05 Hz, Offset: 0, Ext amp: 10). All electrophysiological recordings were manually integrated and peak amplitude (mV) responses to volatile collections were determined from mean responses from mite preparations (Fig. 2). To establish a cut-off of what determines an electrophysiologically active honey bee colony volatile, a minimum of four *V. destructor* mite responses was required out of the nine repeated electrophysiological recordings on a given honey bee colony volatile collection. Volatiles eliciting fewer than four electrophysiological responses from repeated recordings using different mites were not included in the results. Individual varroa may vary in responsiveness to honey bee colony volatiles as they were collected from the phoretic phase and have an unknown age and fertility status, both of which may alter sensitivities to colony volatiles.

Preparation of extractions

Volatile collection samples were divided into 200-µL aliquots and individually concentrated under compressed ultra-high purity nitrogen to 20 µL using 250-µL glass vial inserts. Nonyl acetate was used as an internal standard with a final concentration of 3 ng/µL for

quantification of proportional abundance (Sigma Aldrich, St. Louis) (Carroll and Duehl 2012). Concentrated samples were then immediately used for electrophysiological recording using GC-ETD and subsequent compound identification using gas chromatography mass spectrometry (GC–MS), with remaining solvent extract stored at − 20 °C.

Gas chromatography electrotarsal detection specifications

GC-ETD recordings were performed using Varian 450-GC fitted with a flame ionization detector (FID) in which CIP SIL8-CB (30 m; 0.25 mm diameter; 25 µm) non-polar column was used (Varian, Lake Forest, CA, USA). Oven temperature was held at 50 °C for 5 min, then increasing at 5 °C/min to 200 °C with a final temperature ramp of 25 °C/min to 280 °C, holding this temperature for 5 min. Concentrated volatile extracts were manually injected at a volume of 1 µL, with the inlet held at 250 °C. Helium was used as a carrier gas at a rate of 1.2 L/min. The GC column was split with a sample ratio of 50:50, where half of the sample was delivered to a heated transfer line held at 280 °C (Syntech Temperature Controller TC-02) and introduced into a carbon-filtered humidified airstream at 0.5 L/min over mite preparations.

Chemical identification

Concentrated volatile extractions were analyzed using a Scion 456 gas chromatogram—single quad mass spectrometer (Scion Instruments, Livingston, UK) for identification of compounds eliciting electrophysiological responses with electron impact ionization mode at 70 eV, scanning m/z 40–350 (Restek Rxi-5MS; 30 m; 0.25 mm diameter; 0.25 µm; Restek Corporation, State College, PA, USA). The same oven temperature specifications were used to compare peak retention times with GC-ETD output. Helium was used as a carrier gas at a flow rate of 1.2 mL/min. Concentrated volatile samples were manually injected at 250 °C in splitless mode, with the split closed for 1 min.

Quantitation was performed using the following chromatogram integration parameters: peak width = 4.0 s; slope sensitivity (SN) = 10; tangent = 10%; peak size reject = 2000; using RMS noise calculation, mean three-point smoothing, and a spike threshold factor of 10. Using these integration parameters, peak areas of all odorants eliciting an electrophysiological response from mites were compared to that of 3 ng/µL nonyl acetate internal standard providing abundance and proportional abundance. Relative concentrations compared to the internal standard of each volatile detected were then back-calculated to initial concentration in volatile extracts before concentration under ultra-high purity nitrogen.

GC-ETD and GC–MS methods for volatile screening and identification were designed to reduce the potential of co-elution of similar molecular weight compounds while at the same time minimizing duration of a single run (39 min) to ensure reliable sustained electrophysiological recordings from mites.

Differences in retention times between GC-ETD and GC–MS were calculated and accounted for through a hydrocarbon standard series (Sigma Aldrich, St. Louis, MO, USA). Compound identification was performed using a combination of National Institute of Standards and Technology (NIST) Database, Kovats retention index calculated using the equation for temperature-programmed chromatography, and chemical standards when available. Compounds were then classified into three categories of confidence (Stein et al. 2011). Compounds identified with a 'high' level of confidence were confirmed through chemical

standards. Compounds labeled as 'medium' confidence had a NIST reverse match ranging from 700 to 900. Compounds with 'low' confidence had a NIST reverse match of < 700. Calculated Kovats retention times were then cross-referenced using online chemical databases when information was available for GC columns with the same active phase. Compounds deemed low confidence and lacking a matching Kovats retention index were categorized as 'unknown' (n = 13) and relegated to Supplementary Materials S1. Peak identities that could not be narrowed to a single possible compound match through both NIST and Kovats (e.g., dimethyl-, trimethyl-, and tetramethyl-alkanes) were deemed low confidence. For this reason, some of these compound identities may be repeated. To improve confidence in compound identity, further chemical analysis would be required using multiple stationary phases.

Nonyl acetate was used as an internal standard in all volatile collections to identify relative abundance of individual volatiles using Eq. 1 (Torto et al. 2013). Moreover, we discovered *V. destructor* had electrophysiological responses towards nonyl acetate, allowing for identification of proportional peak response (in mV; Eq. 2). Proportional peak abundance was then compared to proportional peak response relative to the internal standard (Eq. 3).

$$\text{Proportional peak abundance} = \frac{\text{Peak area of analyte}}{\text{Peak area of internal standard}} \tag{1}$$

$$\text{Proportional response (mV)} = \frac{\text{Response to analyte (mV)}}{\text{Response to internal standard (mV)}} \tag{2}$$

$$\text{Relative response} = \frac{\text{Proportional response (mV)}}{\text{Proportional peak area}} \tag{3}$$

Statistical analysis

Varroa destructor proportional responses towards honey bee colony volatiles were tested against proportional peak abundances to determine whether brood of a particular life stage gave stronger relative responses. Volatile collections were compared by functional group to determine whether a given brood stage has a different chemical signature recognized by *V. destructor*. Statistical analyses were performed using R statistical software v.01.0.136 (R Core Team 2018). A general linear model was used to test for differences among volatile collections in proportional electrophysiological response versus proportional peak abundance using post-hoc pairwise comparisons with Tukey's adjustment (using R software packages lme4, emmeans, dplyr, fitdisterplus, and coin). Initial proportional response over proportional peak abundance data were log-transformed to meet requirements of normally distributed residuals in the model. Another general linear model was used to test for patterns in occurrences of volatiles of a particular functional group among volatile collections using Tukey's post-hoc adjustment factor for pairwise comparisons (software packages lme4, emmeans, and car).

Results

Volatile collections had both new (n = 99) and previously cited (n = 9) compounds from honey bee colony frames, all of which evoked electrophysiological responses from *V. destructor* mites (see Supplementary Tables S2–S7 for compound lists separated by

volatile collection). All uncapped frames containing honey bee larvae were free of adult *V. destructor* mites. We identified 20–40% infestation of capped brood used in honey bee colony odor collections, which means there is a potential for some of the electrophysiologically active odors from capped brood stages to have originated from *V. destructor* mites, mite offspring, and associated frass accumulated during mite development.

Several GC-ETD-active compounds were detected in volatile collections but varied in their proportional abundance among brood developmental stages [e.g., (+)-limonene and 3,7-dimethylnonane]. 3,7-dimethylnonane was detected in all volatile collections, with higher proportional abundance detected in early instar brood and brood killed through cold treatment. The greatest number and relative quantity of GC-ETD-active alkanes were detected in late-stage larvae volatile collections, suggesting that they could be important in host detection.

Table 1 Alcohols, aldehydes, esters, and ketones from honey bee brood that elicited *Varroa destructor* electrophysiological responses through gas chromatography linked electrotarsal detection

Compounds	n	Retention (min)	Kovats	Concentration (ng/μL)	SE	Confid
4-Hexen-3-one	2	4.25	825	0.037	0.045	Med
5-Methyl-3-hexanol	2	4.38	832	0.025	0.033	Med
2-Methyl-3-penten-1-ol (*E* or *Z*)	1	4.48	836	0.001		Med
2,5-Dimethyl-5-hexen-3-ol	1	6.24	920	0.757		Low
2,4-Dimethyl-1-heptanol	2	8.37	1003	0.008	0.009	Low
2-Nonenal (*E* or *Z*)	2	11.97	1130	< 0.001	< 0.001	Med
2-Methyl-3-nonanol	3	12.56	1151	0.442	0.765	Low
Methyl 4,6-dimethyloctanoate	1	12.87	1162	0.005		Med
5-Ethyl-4-nonanone	2	14.84	1232	0.579	0.812	Low
2-Ethyl-2-propyl-1-hexanol	1	15.44	1254	0.002		Low
Ethyl 4-methyloctanoate	2	15.76	1266	0.001	0.001	Low
2-Undecanone	2	16.63	1297	0.002	0.001	Med
Nonyl acetate	6	16.91	1308	IS		High
2-Ethylhexyl-2-ethylhexanoate	1	24.09	1596	0.005		Low
Tetradecanoic acid	1	27.64	1757	< 0.001		LOW
Methyl palmitate	2	31.04	1930	NA	NA	High
Palmitic acid	1	31.68	1971	NA		High
2,5-Heptadecadione	1	32.50	2041	NA		Low
Stearic acid	1	33.43	2161	NA		High

Compounds are ordered by ascending GC retention times (retention on an Rxi-5MS capillary column)

NA indicates peak did not meet requirement for integration. *Concentration* was calculated using proportional peak abundance relative to internal standard and calculated to sample volume before concentration under ultra-high purity nitrogen. Compounds were detected across honey bee brood volatile collections of varying developmental stages

Confid confidence in identification NIST reverse match < 700 and multiple Kovats matches (low), NIST reverse match 700–900 and single Kovats match (med), confirmation using chemical standards and Kovats match (high);

SE standard error for volatiles detected in more than one volatile collection

n number of volatile collections where a particular volatile elicited an electrophysiological response from *V. destructor*

Table 2 Alkanes, cycloalkanes, and alkenes from honey bee brood that elicited *Varroa destructor* electrophysiological responses through gas chromatography linked electrotarsal detection

Compounds*	n	Retention (min)	Kovats	Concentration (ng/μL)	SE	Confid
4-Methyloctane	1	4.91	859	0.997		Med
2-Methyloctane	1	5.2	873	< 0.001		Med
2-Methyl-1-octene	1	5.34	881	1.280		Med
2,5,5-Trimethylheptane	1	5.66	897	0.001		Low
Nonane	1	5.7	899	0.373		High
2-Nonene (*E* or *Z*)	1	5.99	910	0.001		Med
2,3,6-Trimethylheptane	2	6.07	914	0.067	0.089	Low
2,5-Dimethyloctane	3	6.29	922	0.107	0.175	Low
2,3,6-Trimethylheptane	3	6.34	924	0.005	0.002	Low
3,5-Dimethyloctane	3	6.53	932	0.005	0.001	Low
2,7-Dimethyloctane	1	6.57	933	0.002		Low
2,6-Dimethyloctane	2	6.66	937	0.011	0.013	Low
2,3-Dimethyloctane	4	7.04	952	0.005	0.002	Med
2,6-Dimethylnonane	1	8.15	995	0.001		Med
Decane	3	8.24	999	0.032	0.023	High
2,3,7-Trimethyloctane	3	8.7	1015	0.027	0.023	Low
5-Ethyl-2-methyloctane	1	8.79	1018	0.007		Low
5-Ethyl-2,2,3-trimethylheptane	1	8.89	1022	0.033		Low
4,5-Dimethylnonane	2	9.43	1041	0.095	0.093	Low
3,7-Dimethylnonane	5	9.6	1047	0.005	0.008	Low
3-Methyldecane	2	9.73	1051	0.015	0.017	Med
5-Ethyl-2,3,3-trimethylheptane	1	9.8	1054	< 0.001		Low
5-Methyldecane	1	9.87	1056	NA		Low
4-Methyldecane	3	10.04	1062	0.006	0.001	Low
2,2,4,4-Tetramethyloctane	1	10.13	1066	NA		Low
4-Methylundecane	1	12.92	1164	< 0.001		Med
3-Methyl-5-undecene (*E* or *Z*)	1	13.11	1170	0.685		Med
2,6-Dimethylundecane	1	14.32	1213	0.002		Low
2,6,11-Trimethyldodecane	2	16.02	1275	0.005	0.006	Low
2,6,10-Trimethyldodecane	1	17.25	1321	NA		Low
Farnesane	2	18.00	1349	0.003	0.004	Low
Tetradecane	3	19.33	1400	0.008	0.011	High
6,9-Dimethyltridecane	1	21.58	1490	< 0.001		Low
Heptadecane	2	26.41	1699	0.365	0.515	High
5-Methylheptadecane	1	27.35	1743	< 0.001		Low
3-Methylheptadecane	1	27.55	1753	< 0.001		Low
2,6,10,15-Tetramethylheptadecane	2	30.77	1912	< 0.001	< 0.001	Low

Compounds are ordered by ascending GC retention times (retention on an Rxi-5MS capillary column)

NA, *concentration*, *confid*, *SE*, *n*: see Table 1 for explanation

*Compound identities with low confidence may be reported more than once due to similarities in ion fragmentation patterns and Kovat retention index values reported in literature and may be incorrect

Table 3 Terpenes and suspected plant secondary metabolites from honey bee brood that elicited *Varroa destructor* electrophysiological responses through gas chromatography linked electrotarsal detection

Compounds	n	Retention (min)	Kovats	Concentration (ng/µL)	SE	Confid
α-Pinene	1	6.55	932	0.096		Med
(+)-Limonene	3	9.08	1028	0.362	0.560	High
β-Ocimene	1	9.54	1045	0.002		High
m-Cymene	1	10.45	1077	0.007		Low
α-Cumyl alcohol	1	10.61	1083	0.001		Med
Citronellal	2	11.99	1131	< 0.001	< 0.001	Low
Menthol	1	13.28	1177	0.001		Med
Methyl salicylate	3	13.67	1190	0.014	0.016	High
Pinanediol	1	15.85	1269	NA		Low
Longifolene	1	19.60	1411	NA		Low
α-Cedrene	1	19.75	1417	NA		Med
Geranyl acetone	1	20.46	1445	NA		High
Lapachol	1	32.53	2045	NA		Low

Compounds are ordered by ascending GC retention times (retention on an Rxi-5MS capillary column)

NA, *concentration*, *confid*, *SE*, *n*: see Table 1 for explanation

Table 4 Aromatic compounds from honey bee brood that elicited *Varroa destructor* electrophysiological responses through gas chromatography linked electrotarsal detection

Compounds	n	Retention (min)	Kovats	Concentration (ng/µL)	SE	Confid
Ethylbenzene	1	4.87	856	0.787		Med
Acetophenone	2	10.06	1063	0.068	0.017	Med
Durene	2	11.50	1114	0.001	< 0.001	Med
4-Ethyl-o-xylene	4	11.61	1118	0.002	0.002	Low
Napthalene	4	13.46	1183	0.089	0.169	Med
m-Di-tert-butyl-benzene	1	15.26	1247	0.003		Low

Compounds are ordered by ascending GC retention times (retention on an Rxi-5MS capillary column)

NA, *concentration*, *confid*, *SE*, *n*: see Table 1 for explanation

Esters and fatty acids were not primary components of volatile collections. These compounds occurred in relatively low abundance and inconsistently among honey bee brood developmental stages because they were, in some cases, likely below detection thresholds (Table 1). ETD-active volatile compounds identified through GC–MS included several methyl-alkanes, possibly originating from wax substrates, with Kovats retention indices between 860 and 1060 (Table 2).

Several suspected plant secondary metabolites (methyl salicylate, α-pinene, citronellal, ß-ocimene, menthol; Table 3) and aromatic compounds (ethylbenzene, naphthalene, and acetophenone; Table 4) were detected across different larvae and pupae developmental stages. (+)-limonene was detected in highest proportional abundance

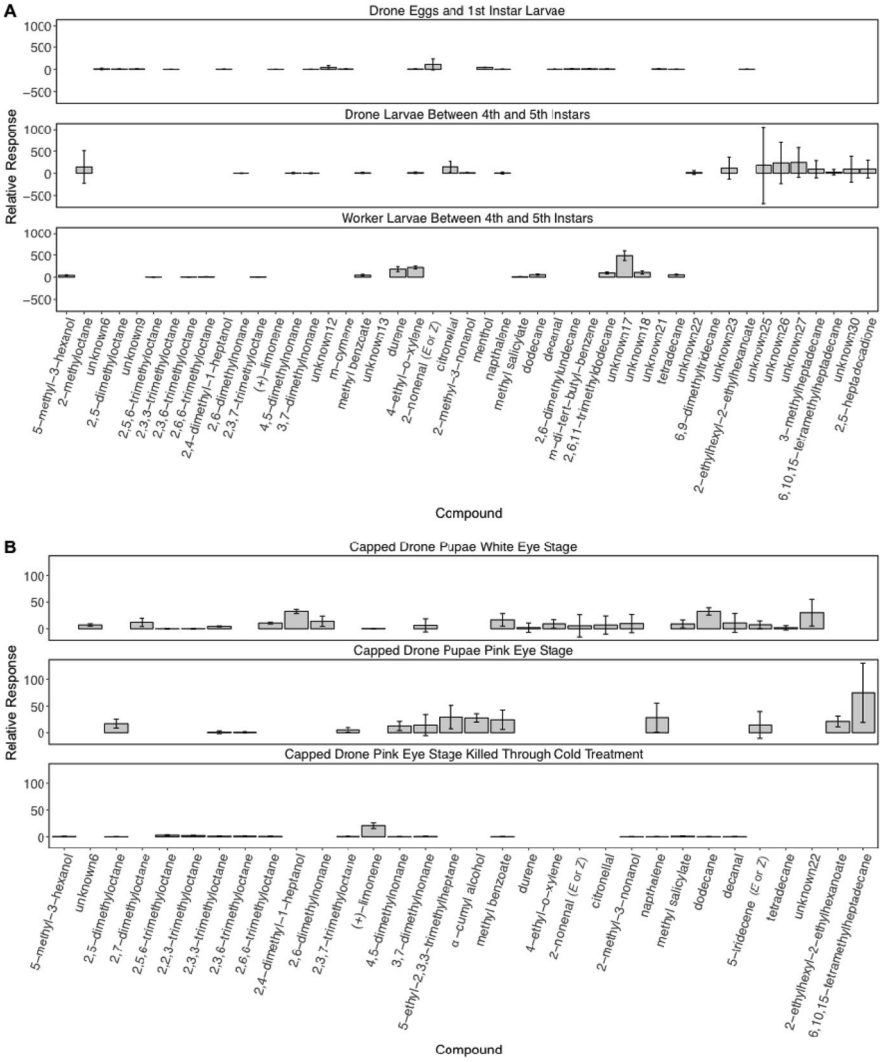

Fig. 3 *Varroa destructor* proportional electrophysiological response (mV) compared to relative compound abundance (peak area) identified as proportions based on nonyl acetate internal standard with error bars indicating standard error of average relative response. Honey bee volatile collections are seperated into panels identifying volatiles collected from larval (**a**) and pupal (**b**) developmental stages. Compounds are arranged according to ascending Kovats retention index, with missing data indicating compounds not detected by *V. destructor* through electrotarsography. Responses to volatiles 6,9-dimethyltridecane and unknown13 were removed from larval volatiles (**a**) to improve visualization because relative responses to these compounds were respectively 14- and threefold greater than most other responses

from volatile collection of early instar brood and had low proportional abundance in capped pupae and brood killed through chilling. Volatiles from early-stage larvae had fewer alkanes and more putative plant secondary metabolites (e.g., menthol, citronellal,

methyl salicylate). In general, plant volatiles were detected in trace quantities in volatile collections of late-stage and chilled brood; however, they still evoked relatively high electrophysiological responses from *V. destructor* suggesting importance in its life cycle (Fig. 3). Although ß-ocimene is included here, it is a honey bee brood and colony-wide odor involved in modulating honey bee behavior (Maisonnasse et al. 2009; Ma et al. 2018).

We identified several dimethyl- and trimethyl-alkanes that may be important in host detection during different stages of honey bee brood development (Fig. 3). Among these, odorants from volatile collections containing mid- to late-stage larvae (WkrL4/5 and DrnL4/5) and pink-eye stage pupae (DrnPiEy) elicited the strongest relative responses from *V. destructor* ($F_{5,200} = 36.2$, $p < 0.0001$; Fig. 3). There was no difference in the number of volatiles of a particular functional group among the six volatile collections ($F_{10,200} = 1.2$, $p = 0.35$).

The relationship of proportional peak abundances to proportional electrophysiological responses relative to a nonyl acetate internal standard revealed volatiles potentially important to *V. destructor* in host detection (Fig. 3). Among the top-10 greatest relative responses from each volatile collection, two components (identified as 6,9-dimethyltridecane; Kovats 1490, and 'unknown13'; Kovats 1095) elicited proportional responses approximately 14- and threefold greater than responses to other odorants, respectively.

Volatile collections yielded some suspected contaminants (xylenes, butylated hydroxytoluene, methyl 2-methylhexyl phthalate); several of these compounds were easily identifiable through their mass spectra. Likely sources for some of these volatiles are plastics used for volatile collections and plastic honey bee frame foundations. We also identified putative colony volatiles associated with honey bee wax in blank (control) volatile collections that elicited electrophysiological responses from mites. Several volatile collections contained tri-methyl alkanes that are not known to be produced by arthropods, and these were regarded as contaminants originating from hive equipment (Table 5).

Discussion

For the first time, we screened a range of honey bee colony volatile components with live *V. destructor* through GC-ETD. Methods for electrophysiological recordings were adapted from previous research (Endris and Baker 1993), and are described in detail elsewhere (Light et al. 2020). GC-ETD recordings contained suspected mechanical responses, typically following responses to odorants (Fig. 3 at 10.4 and 10.9 min retention times); performing repeated GC-ETD recordings allowed confirmation of electrophysiologically-active odorants.

In contrast to previous research on honey bee volatile collections, we identified relatively few suspected plant secondary metabolite compounds (Carroll and Duehl 2012). Additionally, we did not detect previously cited components of the adult honey bee alarm pheromone because adult bees were excluded prior to collections (Table 1; Carroll and Duehl 2012). By restricting volatile collections to the wax comb, this study reduced background volatiles which may be associated with propolis and wax buildup (burr comb) on frame peripheries (Carroll and Duehl 2012; Popova et al. 2014). In addition, we used food-grade plastic frames in our volatile collections and suggest that these may reduce or remove background odors associated with older wood frames and wax foundations (Carroll and Duehl 2012; Torto et al. 2013). Torto et al. (2013) suggested using honey bee equipment < 2 years

Table 5 Compounds attributed to volatile collection background contaminants from honey bee brood that elicited *Varroa destructor* electrophysiological responses through gas chromatography linked electrotarsal detection

Compounds*	n	Retention (min)	Kovats	Concentration (ng/μL)	SE	Confid
p-Xylene	2	5.05	866	0.005	0.004	Med
Styrene	1	5.50	889	0.344		Med
o-Xylene	1	5.65	894	0.001		Med
3-Ethyl-4-methylheptane	1	6.83	943	0.001		Low
2,4,6-Trimethyloctane	2	7.19	958	0.042	0.049	Low
2,2,6-Trimethyloctane	3	7.54	971	0.022	0.005	Low
2,2,3-Trimethyloctane	2	7.60	974	0.026	0.007	Low
2,5,6-Trimethyloctane	3	7.78	981	0.086	0.112	Low
2,3,3-Trimethyloctane	4	8.00	990	0.042	0.039	Low
2,3,6-Trimethyloctane	3	8.08	992	0.020	0.007	Low
2,2,4,6,6-Pentamethylheptane	2	8.55	1009	0.006	0.001	Med
2-Ethyl-1-hexanol	1	9.01	1026	0.011		Low
2,3,6,7-Tetramethyloctane	2	9.19	1032	0.023	< 0.001	Low
Methyl benzoate	5	10.87	1092	0.092	0.203	High
Undecane	1	11.09	1100	0.002		High
Nonanal	2	11.18	1103	0.031	0.041	High
3-Decyn-2-ol	1	12.40	1146	NA		Low
Dodecane	3	13.95	1200	0.175	0.300	High
(1,2-Dimethylpropyl)-cyclohexane	1	14.03	1203	0.001		Low
Decanal	3	14.07	1204	0.039	0.047	High
Benzothiazole	1	14.57	1222	< 0.001		Med
Tridecane	2	16.70	1300	0.017	0.022	High
1-Cyclohexyloctane	1	20.54	1449	0.001		Low
2,6-Di-tert-butylbenzoquinone	1	20.81	1459	NA		Low
Butylated hydroxytoluene	3	21.85	1501	0.030	0.006	Med
2,4-Bis(1,1-dimethylethyl)-phenol	4	21.92	1504	0.048	0.058	Med
Dodecyl acrylate	1	26.22	1691	0.004		Med
2-(Octadecyloxy)-ethanol	1	32.56	2049	< 0.001		Low
Methyl 2-methylhexyl phthalate	1	33.17	2123	0.070		Low

Compounds are ordered by ascending GC retention times (retention on an Rxi-5MS capillary column)

NA, *concentration*, *confid*, *SE*, *n*: see Table 1 for explanation

*Compound identities with low confidence may be reported more than once due to similarities in ion fragmentation patterns and Kovat retention index values reported in literature and may be incorrect

old to avoid saturation of volatile collections with frame background odors associated with consecutive brood-rearing and propolis buildup. Although reducing background odors associated with wax and propolis can help in narrowing down odors putatively important to *V. destructor* reproductive cycles, these substances may be important to *V. destructor* orientation within a honey bee colony. In this study, honey bees wax may have adsorbed and released some background odors associated with colony resources (e.g., propolis, pollen, honey) and could contribute towards the detection of some compounds in volatile

Table 6 Ranked five most produced honey bee brood volatiles at various developmental stages in ascending order (A–E) for each volatile collection; each run corresponds to one volatile collection analyzed

Run and rank	Compound	Kovats	Prop area	Relative response
Drone eggs and 1st instar larvae				
A	4-Methyloctane	859	68.1	0.02
B	5-Ethyl-4-nonanone	1232	78.8	0.02
C	2-Methyl-1-octene	881	87.5	0.02
D	2-Methyl-3-nonanol	1151	90.6	0.01
E	Unknown14	1127	376.0	0.00
Worker larvae between 4 and 5th instars, 50% cell occupancy[a]				
A	Unknown11	1035	0.6	8.35
B	Unknown10	963	0.9	3.07
C	2,3,6-Trimethyloctane	992	1.2	4.04
D	2,2,6-Trimethyloctane	971	1.7	2.53
E	2,5,6-Trimethyloctane	981	1.7	2.36
Drone larvae between 4 and 5th instars, 70% cell occupancy				
A	2,3,6-Trimethylheptane	924	0.2	3.58
B	2,6-Dimethylnonane	1014	0.3	2.01
C	2,3-Dimethyloctane	952	0.3	1.52
D	4-Methyldecane	1062	0.5	0.97
E	3,7-Dimethylnonane	1051	0.8	0.63
Capped drone pupae white eye stage				
A	2,2,6-Trimethyloctane	971	1.7	1.23
B	2,3,3-Trimethyloctane	990	1.7	4.52
C	Butylatedhydroxytoluene	1500	1.9	2.41
D	5-Ethyl-2,2,3-trimethylheptane	1020	2.3	0.59
E	(+)-Limonene	1030	5.1	0.53
Capped drone pupae pink eye stage				
A	2,3,3-Trimethyloctane	990	0.5	1.00
B	2,4-Bis(1,1-dimethylethyl)-phenol	1504	0.7	1.66
C	2,3,6-Trimethyloctane	992	1.0	0.86
D	2,2,6-Trimethyloctane	971	1.1	1.08
E	2,3,6,7-Tetramethyloctane	1032	1.5	0.85
Capped drone pupae pink eye stage killed through cold treatment (7 h at 11 °C)				
A	Ethylbenzene	856	53.8	0.02
B	4-Methyloctane	859	68.1	0.02
C	5-Ethyl-4-nonanone	1232	78.8	0.02
D	2-Methyl-1-octene	881	87.5	0.02
E	2-Methyl-3-nonanol	1151	90.6	0.01

Kovats retention index determined from hydrocarbon standards, *Prop area* proportional peak abundance relative to internal standard nonyl acetate, *Relative response* proportional response over proportional peak abundance relative to internal standard nonyl acetate

[a]Percent occupancy was estimated based on number of larvae within areas in which volatiles were collected. Stages of pupal development were identified by uncapping 10 brood cells in areas of volatile collection and referring to the literature for determining pupal developmental stage based on cuticle melanisation (Frey et al. 2013; McAfee et al. 2017). Each volatile collection was composed of two replicates collected simultaneously containing brood of the same age from the same frame; replicates were combined and concentrated under ultra-high purity nitrogen for gas chromatography electrotarsal detection and gas chromatography mass spectrometry analysis

collections from later stages which were not detected in earlier stages of honey bee larvae (Table 6; Torto et al. 2013).

Many honey bee larval volatiles of high molecular weight, which have been previously identified as important in *V. destructor* host detection (Le Conte et al. 1989; Trouiller et al. 1992), were infrequently detected in our volatile collections, although some evoked electrophysiological responses from mites. Potential compounds related to infested and dead pupae could be important in eliciting honey bee hygienic behavior (cell uncapping and removal of infected or dead pupae; Martin et al. 2002; Frey et al. 2013; McAfee et al. 2017). Similarly, *V. destructor* may use these semiochemicals emitted from late-stage pupae to ensure successful completion of the reproductive cycle. Odors from honey bee pupae in the natural context were tested for potential importance in *V. destructor*'s life cycle for the first time in our study.

Volatiles specific to late-stage honey bee larvae (DrnL4/5 and WkrL4/5) evoked greater electrophysiological responses from mites relative to the nonyl acetate internal standard. These volatile collections contained odors in higher relative quantities compared to volatile collections from younger brood stages, potentially attributable to the larger larval size and therefore increased surface area for odorants to be emitted. This suggests that in addition to brood methyl- and ethyl-esters, other compounds (predominantly methyl-alkanes) may be important for host or conspecific detection (Martin et al. 2002; Fig. 3). Late-stage drone larvae contained a number of electrophysiologically active, heavy molecular weight compounds in trace quantities. Several of these trace compounds were not identifiable or were identified with low confidence.

Volatile collections from early-stage drone larvae contained the most ETD-active compounds. Compounds unique to this stage were frequently plant secondary metabolites, potentially originating from larval food and/or royal jelly [e.g., menthol, (+)-limonene; Drijfhout et al. 2005; Nazzi et al. 2004], which may be derived from nectar or pollen resources by honey bees (Dillier et al. 2003; Torto et al. 2007; Carroll and Duehl 2012). (*E*)-ß-ocimene was detected in mid- to late-stage larvae volatile collections only, and was found in low proportional abundance, consistent with previous research on brood volatiles (Carroll and Duehl 2012). We observed *V. destructor* electrophysiological responses to (*E*)-ß-ocimene. Further examination demonstrated a logarithmic increase in time spent arrested to increasing concentrations of (*E*)-ß-ocimene (Light unpubl. data). Together, these findings suggest that (*E*)-ß-ocimene is important to *V. destructor* for host detection.

Among plant secondary metabolites (Table 3), geranyl acetone and menthol were present in volatile collections from early- and mid-stage development drone larvae. These plant secondary metabolites activate *V. destructor* noxious stimulus receptor TRPA1, suggesting that they are potentially repellent or aversive compounds (Peng et al. 2015). Butylated hydroxytoluene (BHT), a suspected contaminant, also elicited ETD responses from *V. destructor*. Toluene is a noxious stimulus for vertebrates (Nilius et al. 2012). It is possible that BHT has properties (toxicity, respiratory irritation) similar to toluene. However, it is unknown if either of these compounds are repellent to *V. destructor* (Nilius et al. 2012). It is possible that BHT is not responsible for eliciting electrophysiological responses from *V. destructor* because it has a retention time similar to heptadecane, a compound that elicits a response in *V. destructor* (Pernal et al. 2005). These two compounds could be co-eluting during GC-ETD runs, although confirmation through electrophysiological responses from *V. destructor* exposure to BHT and heptadecane exclusively was not possible. Furthermore, 2,6-di-tert-butylbenzoquinone, found exclusively in volatile collections from honey bee brood killed with cold treatment, may possess insecticidal properties or similarly involve the TRPA1 channel due to similarities with other molecules in structure and properties

(Miller et al. 2007; Nilius et al. 2012). Benzoquinones are produced by some arthropods for protection against predation (Blum 1996; Lis et al. 2011), and can be repellent to other arthropods (Weldon et al. 2003; Bissinger and Roe 2010). Furthermore, it is possible that 2,6-di-tert-butylbenzoquinone is a metabolite of BHT through oxidation reactions (Nieva-Echevarría et al. 2015). Both compounds may be contaminants in honey bee volatile collections but may share potential repellent properties towards *V. destructor* as well.

Methyl salicylate was identified in several volatile collections as ETD-active, but it does not activate the TRPA1 receptor (Peng et al. 2015). A dosage of 5 mg of methyl salicylate per cage of honey bees infested with *V. destructor* resulted in an average mortality of 22% for honey bees and 100% mortality for *V. destructor* (Lindberg et al. 2000; Rosenkranz et al. 2010). Single sensillum recordings within *V. destructor* tarsal pit organs also found that methyl salicylate activated olfactory sensilla (Dillier et al. 2003). Methyl salicylate may be behaviorally relevant to *V. destructor* because it is a component of aggregation pheromones in other species of ticks and predatory mites (de Bruyne et al. 1991; Carr and Roe 2016). In the context of a honey bee colony, methyl salicylate was not identified previously from volatile collections, but is a common floral component (Dillier et al. 2003; Clavijo McCormick et al. 2014). Further research is needed to examine whether methyl salicylate is behaviorally relevant to *V. destructor*. Of those evaluated, we identified the top ten compounds eliciting the strongest relative electrophysiological responses compared to relative abundance (Fig. 3). Alkanes with di- and tri-methyl groups, in the range of C-10–C-15 carbon atoms, elicited strong relative responses. These alkanes were more commonly detected from volatile collections of capped brood stages. Several of these compounds may be important for host or conspecific detection in *V. destructor* (Martin et al. 2002).

Compounds eliciting the strongest relative responses from early stage honey bee drone larvae and eggs were identified as (*E* or *Z*)-2-nonenal and menthol. Early stage larvae between 1 and 3 days old are typically provisioned with royal jelly. Volatile collections from this brood stage contained plant secondary metabolites in higher abundance relative to some alkanes. These compounds may be important in eliciting *V. destructor* repellence towards royal jelly, although they were not detected in previous research (Drijfhout et al. 2005; Nazzi et al. 2009). Moreover, volatile collections from pupae at the pink eye stage and chilled brood potentially offer additional insight into volatiles involved in interrupting *V. destructor* reproductive cycles (Frey et al. 2013). Volatile collections from larvae of mid- to late-stage development (DrnL4/5 and WkrL4/5) stimulated a greater number of high proportional electrophysiological responses relative to volatiles collected from capped pupal stages. Among these, several unknown compounds (Fig. 3, Supplementary Material Table S1) of moderate to heavy molecular weight appear to be important to *V. destructor* based on electrophysiological responses. Kovats retention indices of these compounds ranged from 1390 to 2029 and did not match retention times of previously identified brood or *V. destructor* pheromone components (Le Conte et al. 1989; Pankiw and Page 2001; Ziegelmann et al. 2013). Further investigation in these unknown volatiles may lead to new discoveries for integrated management of *V. destructor*.

Among previously identified fatty acid and respective methyl and ethyl esters from cuticle extractions of late-stage worker and drone larvae (Le Conte et al. 1989, 1990), we detected palmitic and stearic acid in volatile collections from late instar uncapped brood. Identification of these volatiles was possible only by using chemical standards, due to the relatively low abundance of these brood pheromone components. Several components produced by honey bee larvae throughout development are also produced by *V. destructor* during its reproductive stages; among them, stearic acid is a major component (Martin et al.

2002; Ziegelmann et al. 2013). Identification of stearic acid from honey bee larvae volatile collections and attendant ETD responses suggests a possible influence of this fatty acid outside the context of *V. destructor* reproduction.

The absence of several putative attractive brood pheromone components (e.g., methyl palmitate, methyl linoleate, methyl linolenate, ethyl palmitate) from volatile collections of late-stage brood brings into question the relative importance of these components given a wider variety and abundance of additional alkanes found in this study (Le Conte et al. 1989; Trouiller et al. 1992), some of which were determined previously to be behaviorally relevant to *V. destructor* (Donzé et al. 1998). Development of a generic screening method and exploration of relative electrophysiological responses to volatile components has provided a baseline for further research to investigate behavioral relevance of these honey bee brood volatiles. Compounds eliciting a strong relative electrophysiological response from *V. destructor* can further narrow research foci towards new, potentially behaviorally relevant, semiochemicals. Broad scale screening techniques used here could be similarly applied to other acarine pests. This may also lead to better understanding of whether particular sensitivities to compounds (e.g., methyl salicylate) are conserved across species.

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