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ORIGINAL PAPER

Locomotion behavioural responses of *Varroa destructor* exposed to western honey bee (*Apis mellifera*) semiochemicals

Michael Light[1,4] · Dave Shutler[1] · Nicoletta Faraone[2] · G. Christopher Cutler[3] · N. Kirk Hillier[1]

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Abstract

To locate their western honey bee (*Apis mellifera*) hosts, parasitic *Varroa destructor* mites depend on tactile and especially chemosensory cues. Modifying these cues in the honey bee colony environment may show potential for use as a means of managing *Varroa destructor* mite populations. We tested whether chemical compound, previously detected in honey bee colonies or extracted from honey bees and *V. destructor* mites, modified *V. destructor* locomotion behaviour. In experiments quantifying time spent by *V. destructor* mites within areas treated with different chemical compounds, we observed non-significant increasing tendencies in concentration-dependent locomotion behavioural responses. *Varroa destructor* responses towards compounds tested with different emission sources (e.g. stearic acid, sebacic acid, and racemic ocimene) suggest that mites may use multiple cues to orient within a colony environment. Determination of *V. destructor* locomotion behavioural sensitivity to individual compounds and blends provides baseline information for future exploration into managing mite infestations using low-volatility compounds at concentrations relevant to *V. destructor*.

Keywords Apiculture · *Apis mellifera* · Pest management · Semiochemical · *Varroa destructor*

Key message

- Semiochemical cues are important in honey bee–*Varroa destructor* relationships.
- We tested compounds associated with various within-colony sources for effects on *V. destructor* locomotion.
- No concentration–dependent responses were observed among the chemicals tested in locomotion assays.
- *V. destructor* responded strongest towards ethyl oleate, sebacic acid, and stearic acid.
- Compounds eliciting strong responses could be useful in developing lures for *V. destructor*.

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✉ Michael Light
mikelight@acadiau.ca

[1] Department of Biology, Acadia University, Wolfville, NS B4P 2R6, Canada

[2] Department of Chemistry, Acadia University, Wolfville, NS B4P 2R6, Canada

[3] Department of Plant, Food, and Environmental Sciences, Dalhousie University, PO Box 550, Truro, NS B2N 5E3, Canada

[4] Present Address: Department of Forestry, Daniels Faculty of Architecture and Design, University of Toronto, Toronto, ON M5S 3B3, Canada

Introduction

Extensive use of *Apis mellifera* L. (Hymenoptera: Apidae; hereafter, honey bee) in agriculture creates opportunities for rapid spread of parasites among colonies (De Jong et al. 1982; Schmid-Hempel 1995). *Varroa destructor* (Acari: Varroidae) mites have a near-global distribution and are considered the most virulent parasite of honey bees (De Jong et al. 1982; van der Zee et al. 2012), causing death of infested bee colonies unless beekeepers intervene (Fries et al. 2006; Ritter 2008; Dietemann et al. 2012; Seeley and Smith 2015).

Management of *V. destructor* relies heavily on chemical treatments despite a desire to reduce chemical inputs and ongoing risks of evolution of acaricide resistance (Ferland et al. 2017, 2021; Plettner et al. 2017; Bubnič et al. 2021). Alternative pest management approaches can be challenging and inferior to traditional chemical controls, often requiring

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multiple treatments that increase management costs (Milani 1999; Currie et al. 2010; Ferland et al. 2017).

Improved understanding of the chemical ecology of *V. destructor*–host interactions and chemical communication (i.e. semiochemicals) within honey bee colonies may provide insight into solutions that reduce reliance on miticides. An approach would be to interfere with the olfactory or gustatory system of *V. destructor* mites (Plettner et al. 2017). Honey bee semiochemical cues from different life stages are important in eliciting behavioural responses in *V. destructor* (Yoder and Sammataro 2003; Plettner et al. 2017). These cues originate from multiple honey bee colony substrates, suggesting that the context in which they are presented to *V. destructor* may evoke different locomotion behavioural responses (Boot 1994; Rickli et al. 1994; Calderone and Lin 2001; Pernal et al. 2005). Testing *V. destructor* locomotion behavioural responses towards these compounds in a concentration-dependent manner may elucidate which chemicals should be a focus of future investigation in the context of *V. destructor* management.

Behavioural responses of *V. destructor* to cuticle extractions have been studied (Rickli et al. 1994; Nazzi et al. 2004), but mite responses to some individual compounds and other mixtures have not been investigated. The identity and quantity of chemicals obtained from insect cuticle washes in solvent may differ from those of chemicals collected from headspaces of live insects. For example, some honey bee produced fatty acids (e.g. oleic acid) naturally oxidize into other compounds at ambient temperature; and these derivatives may be missed in cuticular extractions. Behavioural testing of honey bee colony-relavent headspace chemicals could improve our understanding of the relevance of these chemicals to *V. destructor*.

In this study, we presented chemicals to *V. destructor* mites at concentrations within ranges that are naturally present in honey bee colonies (Carroll and Duehl 2012) and at concentrations that approach those reported in cuticular extractions (Pankiw and Page 2001; Ziegelmann et al. 2013b) to determine effects on locomotion behaviour of *V. destructor* mites. We used a concentric circle behavioural bioassay design to examine *V. destructor*'s locomotion behavioural responses to compounds previously detected within honey bee colony environments during key stages of reproduction and host–finding in the mite's life cycle (Table 1). Such bioassays evaluate time spent by *V. destructor* mites on a surface treated with honey bee colony chemical compounds (Rickli et al. 1994; Donzé et al. 1998). We also tested exploratory locomotion behaviour of *V. destructor* mites after their first contact with a surface treated with honey bee colony compounds (Donzé et al. 1998).

Our approach in testing compounds on *V. destructor* in a concentration-dependent manner is, to our knowledge, the first attempt to explore locomotion response thresholds of *V. destructor* mites using chemicals that originate from honey bee colony sources. Using a concentric circle assay design may reveal whether previously known *V. destructor* mite chemical attractants also evoke additional locomotion behavioural responses not previously observed with other assay designs. The responses we report here may be used to develop a baseline for comparing thresholds of behavioural

Table 1 Compounds tested in a concentric circle *Varroa destructor* locomotion bioassay (Figure S1, see text for details)

Compound[1]	Source	*V. destructor* response	n	Citation
Ethyl oleate	Brood, *V. destructor*	Copulation	68	Le Conte et al. (1989), Ziegelmann et al. (2013a, b)
Palmitic acid	Brood, *V. destructor*	Attractant	62	Le Conte et al. (1989)
Oleic acid	Brood, *V. destructor*	Copulation	57	Trouiller et al. (1992), Ziegelmann et al. (2013a, b)
Stearic acid	*V. destructor*	Attractant, arrestment	92	Ziegelmann et al. (2013a, b)
Benzoic acid	Larval food	NS	69	Nazzi et al. (2004)
Sebacic acid	Royal jelly	Repellent[2]	86	Lercker et al. (1981), Drijfhout et al. (2005)
*Synthetic brood pheromone	Brood	Attractant	22	Le Conte et al. (1989, 1990)
*Synthetic sex pheromone	*V. destructor*	Attractant, arrestment, copulation	9	Ziegelmann et al. (2013a, 2013b)
E-β-ocimene	Queen, brood	NPT	109	Gilley et al. (2006), Carroll and Duehl (2012)

Compounds with previously cited responses have been tested in multicomponent mixtures in this study. Compounds cited as eliciting copulation attempts were previously tested using sexually mature male *V. destructor*; n = number of total assays performed in this study

NS = no significant response from *V. destructor* in the previous studies; NPT = not previously tested on *V. destructor*; [1]ocimene isomers were tested in this study as a mixture of approximately 50–70% E-β-ocimene, and only E-β-ocimene was previously detected in honey bee colonies; [2]sebacic acid was a suspected repellent by Drijfhout et al. (2005) in a royal jelly extracted fraction and cited as a repellent in Plettner et al. (2017) but was never tested on its own in *V. destructor* repellency assays; and synthetic *V. destructor* sex pheromone mixture was synthesized based on Ziegelmann et al. (2013a, b). Concentrations were derived from 1-min solvent extraction; synthetic brood pheromone mixture was synthesized based on Pankiw and Page (2001), derived from 1-h extraction, omitting ethyl linolenate and methyl stearate; * refers to Supplementary Tables S1 and S2 for full list of compounds and concentrations used in pheromone mixtures.

responses of both *V. destructor* mites and honey bees, which may allow development of novel management approaches for mite infestations.

Methods

From May through August 2018, four Langstroth honey bee colonies provided by a local beekeeper in Berwick, Nova Scotia (NS), Canada, were used to rear drone brood by caging a queen onto a frame with cell sizes specific for the production of drone brood for 12–24 h. Collection and maintenance of honey bees and *V. destructor* followed methods described in Light et al. (2020a). Briefly, drone frames containing brood were transferred from donor colonies to an untreated *V. destructor*-infested colony in Coldbrook (NS). After drone larvae reached the capped life stage, they were transferred to environmentally controlled chambers (32 °C and 65% relative humidity) at the K.C. Irving Environmental Science Centre at Acadia University (Wolfville, NS). Nurse worker honey bees from the *V. destructor* mite-infested colony were also added at a ratio of two workers for every immature drone honey bee. Queen mandibular pheromone was applied on a glass coverslip every 48 h at a concentration of 0.1 queen equivalents to promote honey bee health and longevity (Grozinger et al. 2007).

Varroa destructor mite collection followed methods developed by Light et al. (2020a). Groups of 20 honey bees of mixed sex were transferred into wooden holding cages (17 × 12 × 13 cm) using a modified vacuum system (Richard Rogers and Geoffrey Williams, pers. comm.). Adult female *V. destructor* were collected in batches of 15–20 individuals during the dispersal phase (formerly called the phoretic stage) from drone and worker bees using both a fine paintbrush and an aspirator. *Varroa destructor* mites were kept in 50-mL plastic falcon tubes (Thermo Fisher Scientific; NY, USA) with a moist piece of filter paper (2 × 4 mm) to maintain humidity. Falcon tubes were kept in an environmental chamber (0.5 × 0.6 × 1.3 m; Biotronette Mark III; Lab-Line Instruments; Melrose Park, IL, USA) at 30 °C and 60–70% relative humidity while assays were performed. All *V. destructor* were assayed on the day of collection.

Compounds were chosen based on previously reported association with honey bee colony environments and *V. destructor* infestations (Table 1). These compounds were tested at 10^1, 10^2, and 10^3 ng μL^{-1} in behavioural assays. This range of concentrations reflects previously tested and detected concentrations of relevant compounds (Pankiw and Page 2001; Martin et al. 2002; McAfee et al. 2017; Ma et al. 2018; Light et al. 2020a, b). All compounds were diluted in 100% ethanol (Sigma-Aldrich; St. Louis, MO, USA).

Synthetic female *V. destructor* sex and brood pheromone mixtures were made using pure commercial compounds (Sigma-Aldrich; St. Louis, MO, USA). Synthetic honey bee brood pheromone mixture was synthesized based on 1-h cuticle extraction for one 4-day-old larval equivalent, omitting ethyl linolenate and methyl stearate because they were not commercially available at the time (Pankiw and Page 2001; Supplementary Table S1). The effect of omitting these two compounds on *V. destructor* locomotion behavioural responses to the synthetic larval blend is unclear, and they are not previously cited as being *V. destructor*-active when considered individually.

Synthetic sex pheromone from freshly moulted mature female *V. destructor* was synthesized based on 1-min cuticle extraction for one *V. destructor* equivalent (Ziegelmann et al. 2013a, b; Supplementary Table S2). A three-component mixture was made using benzoic, sebacic, and oleic acids at 10^2 ng μL^{-1}. We selected these compounds to test whether a multicomponent mixture originating from different colony sources (larval food identified by Nazzi et al. (2004), royal jelly identified by Drijfhout et al. (2005), and *V. destructor* sex pheromone identified by Ziegelmann et al. (2013b), respectively) elicited responses that differed from responses elicited by *V. destructor* to individual components.

Assays were conducted in the evening (16:00–24:00) from June to August 2018 in an environmentally controlled chamber (0.5 × 0.6 × 1.3 m; Biotronette Mark III; Lab-Line Instruments; Melrose Park, IL, USA) kept at 30 °C and 60–70% relative humidity, illuminated with infrared heat bulbs and active air ventilation. Sixty-mm diameter plastic Petri dishes with filter papers (diameter 55 mm; Fisher Scientific; Ottawa, ON, Canada) were used for locomotion bioassays. Three concentric circles with 12-, 24-, and 36-mm diameters were drawn on filter paper (Supplementary Fig. S1). Filter paper discs were washed in 100% laboratory grade ethanol and air-dried. For each trial, 10 µL of solution containing single compounds or mixtures were applied evenly to a filter paper disc between the 12- and 24-mm diameter rings using a micropipette. Care was taken to avoid blotting of a solution outside of the delineated treatment ring, while ensuring complete coverage. Solvent was allowed to evaporate for 10–15 min from treated filter paper discs under a fume hood prior to conducting locomotion behavioural experiments. During locomotion assays, *V. destructor* mites were only counted as coming into contact with the compound or blend if they had completely crossed onto the delineated treated area.

Due to differences in areas of each concentric circle, there may be bias in time a *V. destructor* mite could spend in a treated area compared to an untreated area. Therefore, we repeated the assay using 10 µL of laboratory grade ethanol as a solvent control to test whether time each *V. destructor* mite spent in different areas was significantly different from time spent in treatment assays. For solvent control assays, we assumed that *V. destructor* mite

locomotion behaviour would provide a baseline for typical movements about the assay area, thereby allowing a contrast with mite locomotion behaviours for assays which were treated with test compounds. We analyzed whether *V. destructor* mite responses in control assays were different than in treatment assays, reporting two-tailed post hoc results.

Adult female *V. destructor* were placed within the drop zone (centre circle) using a moistened fine-tip paint brush, one individual per Petri dish assay (Supplementary Fig. S1). Assays were replicated for each individual compound at each concentration in groups (N = 6–10 replicates at one time). Different compounds and concentrations were never conducted simultaneously in the same environmental chamber. All assays used open-face Petri dishes to avoid saturation of volatile compounds within enclosed spaces. Digital video camera recorders (Sony Handycam DCR-SR45 and HDR-CX405; Sony of Canada Ltd.; ON, Canada) recorded *V. destructor* mite movement, and videos were analysed to visually assess time spent by *V. destructor* within the treated area. Assays were stopped after 5 min, or if the *V. destructor* mite crossed the outer limits delineated by the 36-mm circle. *Varroa destructor* mites that failed to move from the drop zone (delineated by an "x" in Supplementary Fig. S1) during experiments were discarded from statistical analyses. Locomotion behaviours of *V. destructor* inside the 36-mm circle, but not in the treatment area, were not described, and time spent in these areas was recorded but not statistically analysed. Each *V. destructor* was only used once for a given concentration of a particular compound. Compounds were tested once daily, where day was a random factor to account for possible variability among cohorts of *V. destructor* (Pernal et al. 2005; Dietemann et al. 2013). For each compound, *V. destructor* mite responses at each concentration were statistically compared.

Varroa destructor mites had locomotion behaviours other than moving and stopping in the treated circle during assays. One locomotion behaviour observed repeatedly was *V. destructor* mites crossing in and out of the treated area. A single cross was defined as when a *V. destructor* mite crossed (in and out of) borders delineating the treated circle once. A similar locomotion behavioural response from *V. destructor* mites was previously described as "returning" by Donzé et al. (1998) using a similar assay design. This locomotion behaviour suggests a different response from *V. destructor* mites compared to stopping within a treated area and could be described as resembling host-searching (Rickli et al. 1994). In this study, individual compounds and their mixtures were compared for the frequency of crosses by *V. destructor* in and out of the treatment area.

Data were analysed using R Studio (R Foundation for Statistical Computing 2014; now called Posit). Before modelling compound × concentration interaction effects on *V. destructor* responses, linear models were first used to test whether there were effects of date of experiments. We only report results of parametric tests if non-parametric tests gave similar results; if results were different, we defer to more conservative non-parametric tests, including for some of the following analyses. For pairwise comparisons, Tukey's test was used with the more conservative Tukey's adjustment due to the number of comparisons made for these data (Lee and Lee 2018). For comparing compound responses towards responses of solvent controls, we use Dunnett's test with Dunnett's correction factor for multiple comparisons.

Analysis of time spent by V. destructor within the treated area

The amount of time *V. destructor* mites spent within the treated area was not normally distributed (Shapiro–Wilk $W = 0.83$, $p < 0.001$), and data did not fit other commonly used distributions. We used ordered quantile transformation (Peterson 2019) on the original data to improve model fit (Shapiro–Wilk of transformed data $W = 0.99$, $p < 0.001$). Transformed data were analysed using linear mixed effects regression (LMER) models in the R-package *lme4*, but we also analysed untransformed data non-parametrically with Kruskal–Wallis tests using R-package *coin* (Hothorn et al. 2006) to confirm that transformations did not change the outcome.

After confirming an effect of date for the amount of time *V. destructor* mites spent within the treated area, an LMER model was used with the transformed data, in which date was a random factor (Bates et al. 2015). Post hoc tests using Tukey adjustments were used to compare *V. destructor*'s time spent within the treated area within compounds, among concentrations. A Dunnett's test for multiple comparisons to solvent controls was used to summarize significant differences in mean locomotion responses among individual compounds and control behavioural assays using Dunnett's adjustment factor (Lenth 2020).

Analysis of number of times V. destructor crossed back into the treated circle

The number of times that *V. destructor* crossed back into the treated area was also not normally distributed (Shapiro–Wilk $W = 0.71$, $p < 0.001$). These count data had numerous zeros. To account for this, various models (mixed effects Gaussian, Poisson, negative binomial, and zero-inflated versions) were explored, and results that support the usage of one model over another are reported in Supplementary Table S3 using the R-package *DHARMa* (Hartig 2021).

After confirming an association between date and the number of times that *V. destructor* crossed in and out of

the treated area, model selection indicated that negative binomial mixed effects model best fit these data (Table S3). The negative binomial model was used to explore compound × concentration interactions in *V. destructor* locomotion responses with date as a random factor. Post hoc tests were then used on model outputs with a Tukey adjustment, and Dunnett's test was used for pairwise comparisons to controls using Dunnett's adjustment (Lenth 2020).

Analysis of Varroa destructor responses to synthetic mixtures

Varroa destructor mite locomotion behaviour towards synthetic mixtures was analysed separately from concentration-dependent locomotion responses and followed the same statistical procedures described above for both time spent by *V. destructor* within the treated area and the number of times *V. destructor* mites crossed in and out of the treated area. *Varroa destructor* mite locomotion responses towards mixtures and some individual components at similar concentrations were compared to identify whether there were compound interactions (i.e. synergisms or antagonisms) with respect to the mixtures.

Analysis of correlation between Varroa destructor response variables

Spearman's rank correlation tests were used to test whether concentration or time spent in a treatment area was related to the frequency of crosses by a mite in and out of the treatment area.

Results

Time spent by V. destructor within the treated area

In total, 742 assays were performed with nine compounds and mixtures (Table 1). Only in bioassays with ethyl oleate at 10^2 ng μL^{-1} did *V. destructor* mite responses vary by date ($F_{1, 25} = 6.2$, $p = 0.02$); for the remaining 21 tests, all $F_{1, 15–67} \leq 3.7$, and all $p \geq 0.07$.

Treatment had a significant effect on the time *V. destructor* mites spent in the treated area among individual compounds and concentrations after removing ethyl oleate 10^2 ng μL^{-1} (Kruskal–Wallis $\chi^2 = 36.3$, df = 20, $p = 0.01$). A LMER model on normalized data was used to identify which compound and concentration contributed to the differences detected while accounting for the effect of date. Results of the LMER model are summarized in Fig. 1, and the model summary output is provided in Supplementary Table S4. *Varroa destructor* mites spent significantly more time within the treated circle relative to solvent controls for most compounds for at least one concentration with the exception of palmitic acid and oleic acid (Supplementary Table S5). Time spent within the circle treated with sebacic acid, stearic acid, and ocimene isomers approached a maximal response at 10^3 ng μL^{-1} (Fig. 1). There was no difference among synthetic mixtures and individual compounds in the time *V. destructor* mites spent in treated circles, suggesting that synergisms or antagonisms did not occur (linear mixed effects model using normalized data with a random effect for date; AIC = 1715, $\chi^2 = 13.1$, $df = 10$, $p = 0.22$).

Number of times V. destructor crossed back into the treated circle

Frequency of *V. destructor* mites crossing back into the treatment area after exiting differed among dates on which locomotion bioassays were performed (Kruskal–Wallis $\chi^2 = 73.5$, $df = 19$, $p < 0.001$; Supplementary Table S6 summarizes compounds and concentrations for which *V. destructor* mite responses varied by date).

After accounting for date, *V. destructor* crosses in and out of treatment areas were seldom significantly higher for test compounds than for solvent controls (Supplementary Table S7). Concentration-dependent responses among individual compounds were not detected (all $Z \leq 2.3$ and all $p \geq 0.07$ using two-tailed Tukey adjustment after accounting for the effect of date).

For compound mixtures, synthetic *V. destructor* sex pheromone elicited the greatest frequency of crossing in and out of the treated area (Fig. 2), and this was significant when compared to solvent control responses (Supplementary Table S8). *Varroa destructor* mite crossing behaviour for compound mixtures was not significantly different from crossing behaviours for some individual components of these mixtures tested at the same concentrations and suggests that compound synergisms or antagonisms did not exist for this locomotion behavioural response (Fig. 2).

The frequency of *V. destructor* mites crossing into the treatment area after exiting it was not related to the time spent within the treated area for any of the test compounds or mixtures (Spearman's rho = 0.04, $p = 0.33$). A positive correlation between the time *V. destructor* spent within the treatment area, and the frequency of crosses in and out of the treated area was detected with solvent control assays (Spearman's rho = 0.4, $p = 0.001$), even though *V. destructor* spent the least amount of time within the assay for solvent control trials.

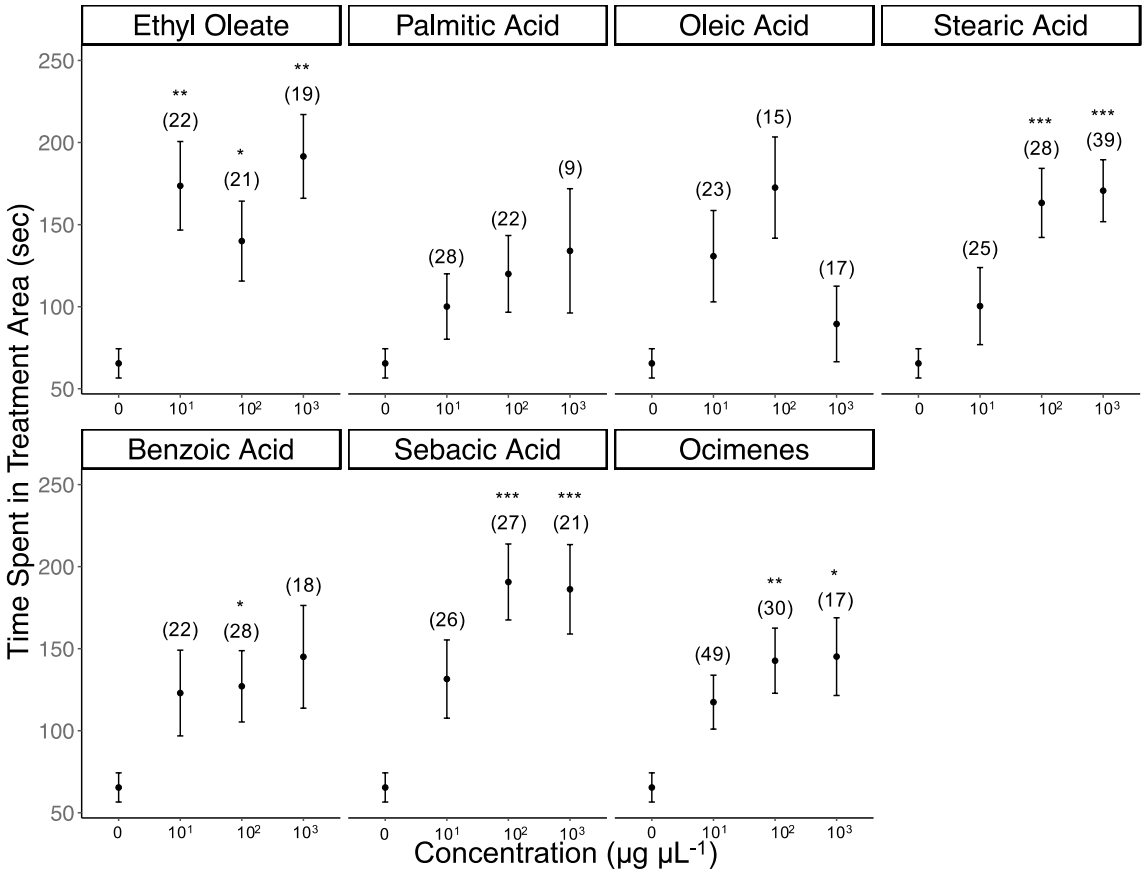

Fig. 1 Mean time (sec ± SE) spent within the treatment area by *Varroa destructor* (seconds) for up to 5 min for compounds from honey bee colonies; numbers within parentheses are numbers of individual *V. destructor* mites tested; significance values are relative to solvent control assays ($N=69$) for each compound; * $p=0.05$, ** $p=0.01$, and *** $p=0.001$; p-values were calculated from pairwise comparisons to solvent controls of compounds by concentration with date as a random factor using Dunnett's correction ($\alpha=0.05$); see Supplementary Table S5 for a complete summary of statistical results

Discussion

We evaluated adult female *V. destructor* locomotion behavioural responses to three concentrations of seven compounds previously identified from honey bee colonies and three synthetic mixtures (Table 1). *Varroa destructor* mites spent more time in treated areas compared to control areas for all compounds we tested in locomotion behaviour assays. Concentration–responses varied across compounds, suggesting different limits of detection by *V. destructor* mites. Stearic acid, sebacic acid, and ocimene isomers elicited the strongest positive concentration–response relationships. The range in volatility among these acids (mean estimated vapour pressure 5.5×10^{-5} mm Hg at 25 °C) relative to ocimene isomers (1.6 mm Hg at 25 °C) suggests that *V. destructor* may behave differently towards putative attractants when presented to mites in different assay designs (Donzé et al. 1998). Putative attractants and repellents could evoke different types of locomotion behavioural responses in *V. destructor*, depending on the concentrations and context (e.g. temperature, humidity, and assay design) in which these compounds are presented. These context-dependent locomotion behaviours should be considered in future research.

The time spent moving in and out of the treated area could to be related to *V. destructor* host-searching behaviour (Donzé et al. 1998). Locomotion behaviours recorded using concentric circle assay designs may reflect efforts by *V. destructor* to avoid detection when entering reproductive phases of their life cycles (Rickli et al. 1994). Locomotion behavioural responses we observed are consistent with behaviours identified in previous *V. destructor* research that used similar assay designs (Rickli et al. 1994; Donzé et al. 1998). Collectively, these results can guide future concentration-dependent investigations.

Interestingly, *V. destructor* spent nearly equal amounts of time within the treatment circle of assays treated with ethyl oleate for three concentrations, and responses were significantly different from solvent controls. These findings align with those of Trouiller et al. (1994) and Frey et al. (2013) about the importance of ethyl oleate in *V. destructor*

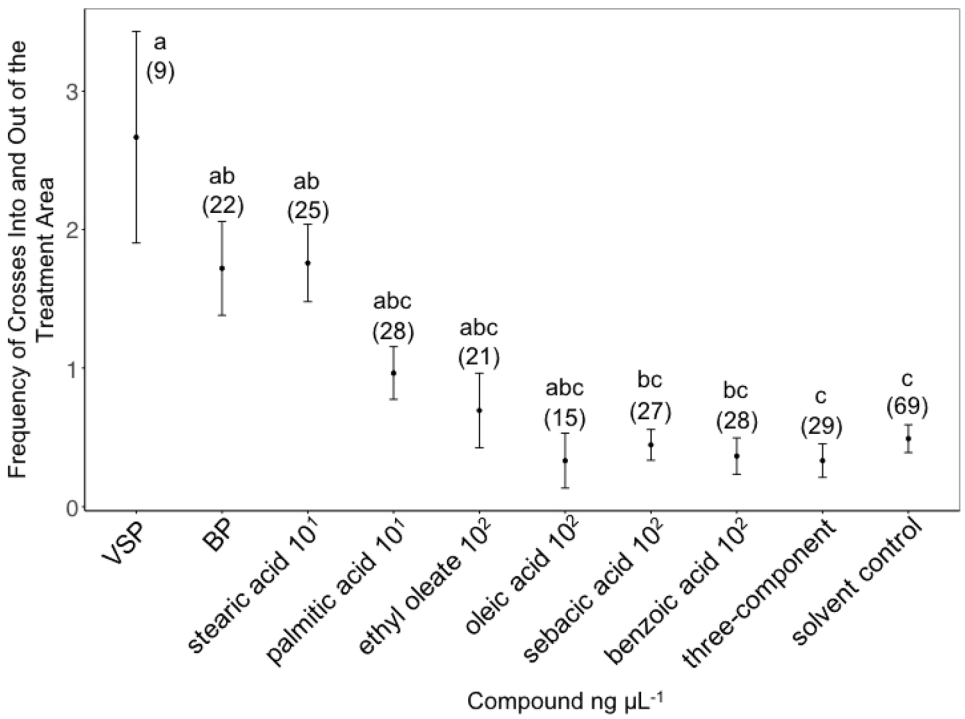

Fig. 2 Pairwise comparisons of *Varroa destructor* mean number of crosses in and out of the treated area in locomotion behavioural assays using Tukey's method for multiple comparisons after correcting for the effect of date on *V. destructor* mite responses; compounds sharing letters were not significantly different; numbers within parentheses denote the number of *V. destructor* mites tested; BP = brood pheromone mixture; VSP = *V. destructor* sex pheromone mixture; three components = mixture of benzoic, sebacic, and oleic acids at 10^2 ng μL^{-1}; points represent means, with standard errors represented by capped whiskers

mite locomotion behaviour and suggest that attraction and time spent within a treated circle may be related locomotion behaviours. Ethyl oleate may also be important in timing of *V. destructor* egg development and serve as an indicator of larval age following cell–capping (Frey et al. 2013). Our results suggest that, at low concentrations, ethyl oleate may be an important semiochemical for *V. destructor*. Further, concentration-dependent assays should be performed to confirm a lack of concentration-dependent responses, and these tests should occur in a variety of assay designs.

We quantified concentration-dependent locomotion behavioural responses to three fatty acids and one ester (i.e. stearic, palmitic, and oleic acids, and ethyl oleate) which have been identified as part of *V. destructor* sex pheromone, and are present in honey bee brood (Rickli et al. 1992; Ziegelmann et al. 2013a). However, *V. destructor* locomotion behavioural responses are variable depending on the bioassay design and perhaps other physiological factors associated with age of mites and timing of mite collection (Donzé et al. 1998; Pernal et al. 2005). Variation in locomotion behavioural responses of *V. destructor* to palmitic acid across concentrations among studies suggests that a relevant biological range is present; we propose that this range of maximal time spent within the treated areas of concentric circle assays is between 10^3 and 10^4 ng μL^{-1} (Rickli et al. 1992; Donzé et al. 1998). Although palmitic acid is considered a strong attractant of *V. destructor*, assays we performed generated variable locomotion behaviour at similar concentrations tested by Rickli et al. (1992) and Donze et al. (1998). Variability in *V. destructor* mite locomotion behavioural responses in our study may be an artefact of outliers within a small sample or could be related to differences in the mating status of each individual *V. destructor*.

Varroa destructor time spent within areas treated with oleic acid increased from 10^1 to 10^2 ng μL^{-1} and sharply decreased at 10^3 ng μL^{-1} (Fig. 2). Oleic acid is an important component of *V. destructor* sex pheromone (Ziegelmann et al. 2013a). When a concentration of approximately 2×10^3 ng μL^{-1} (two times the maximum concentration used in our study) was applied to honey bee colony frames, mated female daughter *V. destructor* stored fewer spermatozoa (Ziegelmann and Rosenkranz 2014). Oleic acid is not known to trigger hygienic behaviour (e.g. removal of dead or diseased brood) in honey bees (McAfee et al. 2017). These findings collectively suggest promise for using oleic acid in interrupting *V. destructor*'s life cycle. Future studies should test female *V. destructor* mite responses towards oleic acid across a greater range of concentrations than those used in our study to determine if a locomotion behavioural threshold exists.

Varroa destructor mites exposed to synthetic *V. destructor* sex pheromone mixture, and the honey bee brood pheromone mixture crossed in and out of the treated area more frequently than mites exposed to the three-component blend or the solvent control. Differences in *V. destructor* crossing locomotion behaviour among the two synthetic pheromone mixtures and individual components tested at similar concentrations were not significant and suggest that synergism

or antagonism did not occur (Fig. 2). The relative importance of mixtures compared to individual compounds in governing *V. destructor* locomotion behaviour is understudied. The number of crosses made by *V. destructor* in and out of the treatment area may provide additional information on the importance of particular compounds and mixtures (Rickli et al. 1994; Donzé et al. 1998). Testing for this behaviour using different assay designs could elucidate importance of individual compounds and mixtures in *V. destructor* locomotion behaviour.

The concentrations we used for synthetic brood pheromone mixture were 100-fold weaker than previously used concentrations from 1-h cuticle extractions (Pankiw and Page 2001), although these concentrations were within range of those tested with *V. destructor* sex pheromone mixture in our study. It is possible that higher concentrations of these weakly volatile methyl and ethyl esters may elicit stronger locomotion behavioural responses from *V. destructor*, although they may not be representative of concentrations typically encountered in a colony context (Light et al. 2020a). In situ volatile collections are needed to further quantify concentrations of these synthetic brood pheromone components and to provide a better representation of their ratios likely encountered by *V. destructor*.

The three-component mixture tested in this study containing compounds originating from various honey bee colony sources did not elicit locomotion behavioural responses from *V. destructor* mites. Some individual components, such as sebacic acid, did change the amount of time *V. destructor* mites spent in the treated area. Sebacic acid has not previously been tested on its own, but it was found in the *V. destructor*-repellent fraction of royal jelly (Drijfhout et al. 2005) and was subsequently suggested to be a *V. destructor* repellent (Plettner et al. 2017).

Although none of our mixtures synergized or antagonized *V. destructor* mite locomotion behaviour, we should not discount the possibility of other mixtures as being important to *V. destructor* in the honey bee colony given the complexity of the honey bee semiochemical environment and the narrow requirements for *V. destructor* mite survival and reproduction.

Identification of components and mixtures putatively important in eliciting *V. destructor* locomotion behaviour described in our study could lead to the development of lures for mite aggregation (Grenacher et al. 2001; Gries et al. 2015). Worker honey bees respond to primary components within mixtures to a greater extent than towards a complete mixture profile (Joerges et al. 1997; Reinhard et al. 2010), suggesting that synthetic mixtures or individual compounds could be developed to influence *V. destructor* locomotion behaviour, providing that components are not behaviourally relevant to honey bee colony function. By evaluating mite locomotion behavioural responses to individual components and mixtures, we seek to gain an improved understanding of *V. destructor* semiochemical repertoires. Identification of key compounds and mixtures may then provide a basis for novel, environmentally sustainable *V. destructor* management.

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Data availability The datasets generated and analysed during the current study are available from the corresponding author on request.

Declarations

Conflict of interest The authors have no relevant financial or non-financial interests to disclose.

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