CHEMICAL CUES FROM HONEYDEW AND CUTICULAR EXTRACTS OF *Trialeurodes vaporariorum* SERVE AS KAIROMONES FOR THE PARASITOID *Encarsia formosa*

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ABSTRACT-

Kairomones are semiochemicals that are emitted by an organism of different species to the organism receiver which benefits from these chemical substances. Parasitoids find and recognise their hosts through eavesdropping on the kairomones emitted from the by-products or body of the hosts. Hemipteran insect pests feed on plant sap and excrete the digested plant materials as honeydew. Honeydew serves as a nutritional food source for parasitoids and a medium for micro-organisms whose activity induces the release of volatiles exploited by parasitoids for host location. The parasitoid *Encarsia formosa* preferentially parasitises its host, the greenhouse whitefly, *Trialeurodes vaporariorum*, on tomato *Solanum lycopersicum*, but little is known about the chemicals that mediate these interactions. We investigated the olfactory responses of the parasitoid *E. formosa* to odours from honeydew and nymphs of *T. vaporariorum* in a Y-tube olfactometer. Arrestment behaviour of the parasitoid to honeydew and nymph extracts, as well as to synthetic hydrocarbons, was also observed in Petri-dish bioassays. We found that *T. vaporariorum* honeydew volatiles attracted the parasitoid *E. formosa* but odours from the whitefly nymphs did not. We also found that the parasitoid spent more time searching on areas treated with extracts of honeydew and nymphs than on untreated areas. Gas-chromatography-mass spectrometric analysis revealed that the honeydew volatiles contained compounds such as (*Z*)-3-hexenol, 3-carene, 3-octanone, *α*-phellandrene, methyl salicylate, *β*-ocimene, *β*-myrcene and (*E*)-*β*-caryophyllene which are known to be attractive to *E. formosa*. The cuticular extracts of the nymphs predominantly contained alkanes, alkenes and esters. Among the alkanes, synthetic of nonacosane arrested the parasitoid. Our findings are discussed in relation to how the parasitoid *E. formosa* uses these chemicals to locate its host, *T. vaporariorum*.

INTRODUCTION

Kairomones are chemical signals that are emitted by an organism of a species to mediate interspecific interaction beneficial to only the organism receiver belonging to another species (Dicke and Sabelis 1988; Kost 2008). Eavesdropping by natural enemies on the kairomonal cues from their hosts or prey is a well-documented phenomenon that occurs during the location and recognition of hosts or prey in nature (reviewed in Afsheen et al. 2008). These cues are of different origins including the body of the host at different developmental stages, or their by-products (e.g., frass, honeydew, oral or glandular secretions) (Afsheen et al. 2008; Kaiser et al. 2017). Volatile kairomones serve as long-range signals which are detected through olfactory receptors mainly located on the insect's antenna (Bleeker et al. 2004). Whereas, non-volatile kairomones serve as contact host recognition signals and are detected by olfactory and gustatory receptors during specific behaviours such as antennating, probing, drilling or drumming on the hosts (Bénédet et al. 2002; Iacovone et al. 2016). Knowledge of the specific kairomones that mediate these behaviours can help to develop lures that recruit and retain natural enemies and to improve the efficiency of biological control of crop pests (reviewed in Ayelo et al. 2021a).

Honeydew and insect epicuticle are known to contain some chemical cues that enhance the host finding and parasitism, thereby playing a key role in pest control under field conditions (Tena et al. 2016; Kaiser et al. 2017). Honeydew is a sugar-rich product excreted by sap-sucking insects like aphids and whiteflies (Wool et al. 2006; Roopa et al. 2016), and serves as a growth and nutritional substrate for some microbes (e.g., *Microbacterium testaceum* and *Staphylococcus sciuri*), the activity of which triggers the release of volatiles (Leroy et al. 2011; Fand et al. 2020). Honeydew volatiles are known to act as kairomones for natural enemies, as reported for the parasitoid *Aphidius rophalosiphi* De Stef. (Hymenoptera: Braconidae) which is attracted to indole-3-acetaldehyde, a component of aphid honeydew volatiles (Wickremasinghe and van Emden 1992). Honeydew constituents are involved in host recognition and acceptance by natural enemies, acting as contact host-searching stimulants (Budenberg 1990; Mandour et al. 2005) or oviposition stimulants (Budenberg and Powell 1992). Moreover, honeydew serves as carbohydrate-rich food source for natural enemies (Tena et al. 2016; Kishinevsky et al. 2018), and has been shown to expand lifespan of parasitoids such as the aphid parasitoid *Bracon cephi* (Gahan) (Hymenoptera: Braconidae) that fed on the honeydew excreted by its host, *Rhopalosiphum padi* (L.) (Hemiptera: Aphididae) (Rand and Waters 2020). Apart from honeydew, the body of the different stages of the host is also known

to release volatiles on which their natural enemies eavesdrop to locate them (Morawo and Fadamiro 2016). In addition, extracts of body surface of hosts contain non-volatile chemicals, mainly hydrocarbons, which are known to act as contact kairomones that enable natural enemies to recognise and exploit their hosts (Shonouda 1999; Kaiser et al. 2017).

The endoparasitoid *Encarsia formosa* Gahan (Hymenoptera: Aphelinidae) is known to be efficient in controlling the greenhouse whitefly, *Trialeurodes vaporariorum* (Westwood) (Hemiptera: Aleyrodidae) (Hoddle et al. 1998; De-Vis and van Lenteren 2008; Liu et al. 2015). In the absence of control measures, *T. vaporariorum* can cause between 30 to 100% yield losses to tomato (*Solanum lycopersicum* L. (Solanaceae)) in both open fields and greenhouses in many parts of the world (Hanssen and Lapidot 2012; Gamarra et al. 2016; Perring et al. 2018). Adults of *T. vaporariorum* are known vectors of viruses such as the tomato infectious chlorosis virus (TICV) and the tomato chlorosis viru*s* (ToCV) which are a major cause of the crop damage (Palumbo et al. 2000; Hanssen and Lapidot 2012; Navas-Castillo et al. 2014; Gamarra et al. 2016). *Trialeurodes vaporariorum* is controlled mainly using synthetic chemical insecticides (e.g., neonicotinoids and pyrethroids), and this has led to the development of insect resistance (Kapantaidaki et al. 2018 and references therein), and to detrimental effects on the survival of the whitefly parasitoid, *E. formosa* (Wang et al. 2019). In addition, the use of these insecticides is associated with human and environmental health risks (reviewed by Thompson et al. 2020). The use of biocontrol agents such as the parasitoid *E. formosa* has been reported to play a key role in controlling *T. vaporariorum* on tomato plants grown in greenhouses (Hoddle et al. 1998; Hu et al. 2002; De-Vis and van Lenteren 2008). In the field, this parasitoid has shown parasitism rates of 30-50% on *T. vaporariorum* and a related whitefly species, *Bemisia tabaci* Gennadius (Hemiptera: Aleyrodidae) (Udayagiri and Bigelow 2000; Zhang et al. 2011)*.* Application of kairomones to attract and retain *E. formosa* on tomato field crops could enhance the biological control of *T. vaporariorum* in the field.

Host-emitted kairomones are known to be specific and highly detectable, and have been shown to be more reliable than plant volatiles for recognition of hosts by parasitoids (Vet et al. 1991; Vet and Dicke 1992; Rodriguez-Saona and Stelinski 2009). It has been reported that whitefly honeydew serves as an arrestment stimulant (i.e., contact signal that enhances host searching behaviour), and a sugar-rich food source for *E. formosa* (Hirose et al. 2009; Roopa et al. 2016). The presence of honeydew on tomato leaves was also reported to increase the host searching of *E. formosa*, thereby increasing the chance of finding and parasitising *T. vaporariorum* nymphs

(van Roermund and van Lenteren 1995; Romeis and Zebitz 1997). However, to the best of our knowledge, the attractiveness of *E. formosa* for the volatiles from honeydew excreted by *T. vaporariorum* has not been studied, and it is not known if the nymphal stages of *T. vaporariorum* produce volatiles that are attractive to the parasitoid. Nymph cuticular compounds which can act as oviposition stimulants for *E. formosa* have also not been investigated so far. In this study, we hypothesised that chemicals emitted by *T. vaporariorum* honeydew and nymphs serve as kairomones for *E. formosa.* We discussed the results with regard to how these kairomones serve the parasitoid in the exploitation of its host, *T. vaporariorum* in tomato crop fields.

MATERIALS AND METHODS

***Plants*.** Tomato (cv. Kilele F1 Hybrid, Syngenta, Kenya) plants were grown in a greenhouse maintained at 28±5°C, 60±10% RH at the International Centre of Insect Physiology and Ecology (*icipe*) (Nairobi, Kenya) with provision of water and fertilizer, but without pesticide treatments, as described in Ayelo et al. (2021b).

***Insects*.** Rearing was done in the laboratory at *icipe*, under 25±2°C temperature range, with 60±10% RH, and a photoperiod regime of 12L: 12D. The rearing procedure is described in details in Ayelo et al. (2021b).

Trialeurodes vaporariorum was reared in Plexiglass cages (40×40×50 cm) on tomato plants of six weeks old, corresponding to the stage 2 (i.e., formation of side shoots for Solanaceae) according to the BBCH (Biologische Bundesantalt, Bundessortenamt und CHemische industrie) scale on the description of plant phenological stages (Moreno et al. 2016). The whitefly adults were allowed to oviposit for three days, after which the infested plants were transferred to a greenhouse (28±5°C, 60±10% RH). Infested leaves with mature nymphs (mainly the fourth instar nymphs) were cut off and their petioles inserted into water-soaked floral foam, then returned to the rearing cages and maintained until emergence of adults.

For rearing of *E. formosa, T. vaporariorum*-infested tomato plants (bearing nymphs of the third and fourth stages) were offered to parasitoid wasps in a Plexiglass cage (40×40×50 cm) for parasitism for three days. Thereafter, the plants with parasitised nymphs were transferred to another Plexiglass cage and kept until emergence of the wasps. Newly emerged parasitoid adults were returned to the original cage. The parasitoid wasps were provided with water and 80% honey solution twice a week. The insects were deprived of host and plant for 48

h to reduce effect of previous experience on the choices of honeydew and nymphs but provided with 80% honey solution ad libitum for survival prior to the bioassays. Naïve three to five days old *E. formosa* females were used in the experiments as the parasitoid fecundity and parasitism rates are known to be higher within this age interval (Qiu et al. 2004).

Distant responses of Encarsia formosa to honeydew and nymph odours. Bioassays were conducted using a Y-tube olfactometer (0.5 cm i.d.; 6 cm stem; two 6 cm side arms at 60° angle) that was oriented vertically inside an observation chamber made of cardboard box (35×35×55 cm) which was illuminated with a 220-240 V cool white fluorescent light providing uniform lightning, as described in Ayelo et al. (2021b). The air was sucked using a vacuum pump (KNF lab LABOPORT Type: N86KT.18, Merck, France), then filtered by passing through a container with charcoal before entering the odour source containers at a constant flow rate of 120 mL min^{-1} that was set using an AALBORG flow meter (Orangeburg, NY, USA). The olfactory responses of *E. formosa* females to volatiles from *T. vaporariorum* honeydew and nymphs were assessed by testing (i) air vs. air (control), (ii) air vs. 50, 100 or 200 *T. vaporariorum* nymphs, and (iii) air vs. 15, 30 or 60 mg of honeydew excreted by *T. vaporariorum* nymphs. Test samples were placed in 250 mL quick-fit glass jars (Sigma Scientific, Gainesville, FL, USA) which were connected between the edges of the olfactometer arms and the air flow meter using Teflon tubes (PTFE, 4 mm i.d. x 6 mm e.d.). Third and fourth instar nymphs of the host were used in the assays as they are known to be preferred by *E. formosa* (Hu et al. 2002). Differentiation of the *T. vaporariorum* third and fourth nymphal instars was based on the size and colour of the nymphs following the description reported by Gamarra et al. (2016). Droplets of honeydew produced by nymphs were collected from leaves of tomato plants heavily infested by *T. vaporariorum* nymphs at densities of about 500 to 700 per leaf. The honeydew was collected on aluminium foil between 8 to 10 AM, using a fine hairbrush, and then kept inside a Petri dish at room temperature and used within the next five hours. An amount of about 6 mg (6 ± 0.63 mg, n = 5) was collected at 21 to 25 days post-infestation from a leaflet with about 100 nymphs (not all nymphs produced honeydew at the collection time), and a ten-fold higher dose (i.e., 60 mg) was tested. This dose found to be attractive to the parasitoid, was thereafter reduced to half and one-fourth which were also tested in the bioassays. A honeydew dose was used once in the bioassays, within a maximum of 1 h 30 min per choice test. Bioassays followed the methods described in Ayelo et al. (2021b). To assess the responsiveness of the insects, a single *E. formosa* female was introduced at the base of the stem of the Y-tube, and the insect observed

for five minutes during which its first choice was recorded. A choice was scored when the insect walked and reached the end of a given arm, and remained there for 30 s. Non-responsive insects (i.e., which made no choice within the five minutes) were not included in the data analysis. Overall, 80 insects were tested per choice test on eight days (i.e., 10 insects tested per day per choice test). On each day, after testing five insects, the Y-tube was cleaned with dichloromethane and turned by 180°, and the edges of the Teflon tubes (PTFE, 4 mm i.d. x 6 mm e.d.) used to connect the quick-fit jars (odour containers) to the Y-tube were switched between the arms (left and right) to prevent contamination and positional bias. Between choice tests (treatments) on the same day, the previous test materials (i.e., honeydew or nymph) were removed from the odour containers, then air flow was allowed to pass through the olfactometer set-up for 60 minutes to remove the odour of the previous treatment. Thereafter, the Y-tube and the quick-fit jars were renewed with clean ones before testing another treatment. At the end of the bioassays on each day, the Y-tubes and the quick-fit jars (odour containers) were cleaned with hot water and Teepol odourless detergent, then rinsed with acetone and distilled water, and thereafter dried in the oven at 100°C overnight, before they were used again on another day.

Collection of honeydew volatiles. *Trialeurodes vaporariorum* honeydew volatiles were sampled using solid phase micro-extraction (SPME) stableflex equipped with a 65-µm absorbent fibre inside (PDMS-DVB, Supelco, Bellefonte, PA, USA) under a static collection system. Prior to volatile collection, the SPME fibre was cleaned by conditioning in gas chromatography (GC) at 250°C for 15 minutes. Droplets of crude honeydew excreted by *T. vaporariorum* nymphs on tomato leaves were collected using a fine camel hairbrush, similar to the method used by Hung et al. (2019). The honeydew was collected on aluminium foil between 8 to 10 AM, and was thereafter immediately used in the volatile collection. A sample of the honeydew (60 mg, i.e., dose found to be attractive to the parasitoid) was placed in a 2 mL vial with a rubber septum lid. The SPME fibre was inserted through the lid and deployed at 1 - 2 cm above the sample for 24 h volatile collection, after which it was drawn into the protecting needle (holder of fibre), then retracted from the collection device. Volatiles were immediately analysed thereafter by gas chromatography-mass spectrometer (GC-MS) in four replicates.

Extraction of cuticular compounds from nymphs of Trialeurodes vaporariorum. Nymph's cuticular compounds were extracted according to the method described in Buckner and Jones (2005). Extracts were obtained from 300 ice-chilled nymphs of third and fourth instars placed in a 2 mL glass vial containing 300 µL of hexane or pentane. The vial with its content was

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gently vortexed for one minute, and the extract supernatant was gently transferred into a glass storage vial, and the volume was concentrated to 150 µL under a gentle stream of nitrogen, then stored at -80 °C until use. Extracts obtained with hexane caused an increase in the time spent by the parasitoid compared to control, whereas extracts obtained with pentane did not (see Results, section on contact responses of *Encarsia formosa* to cuticular extracts from nymphs of *Trialeurodes vaporariorum*). Hence, hexane was used to obtain nymph extracts in subsequent chemical analyses. A 50 µL sample of extract was spiked with 2 µL of octadecane solution (50 ng/µL) used as an internal standard prior to analysis by GC-MS. Four replicates were performed for chemical analyses.

Contact responses of Encarsia formosa to honeydew and nymph extracts. The responses of *E. formosa* females to *T. vaporariorum* nymphs-associated cues were assessed in Petri dish bioassays using crude honeydew and extracts of honeydew and nymphs as sources of contact cues. A filter paper disc (5.5 cm diam., about 24 cm^2 area) was divided into two equal sections and used to cover the bottom section of the Petri dish (5.5 cm diam., 1.3 cm height) which was used as the observation arena. In this way, the parasitoid had free access to a blank area in half-Petri dish (control) and an area treated with extract in the other half (treatment). The honeydew components were extracted by dissolving in a solvent mixture of distilled water: methanol at a ratio of 9:1 according to the method described by Pringle et al. (2014). Doses of 15, 30 or 60 mg (used in the distant response bioassays) of crude honeydew were dissolved in 1 mL solvent in a storage vial of 2 mL, and vortexed vigorously for five minutes. Then the solution was filtered through glass fibres to remove finer particles, and the liquid was used for bioassays. The cuticular components were extracted from a group of 300 *T. vaporariorum* nymphs placed in 300 µL of hexane or pentane for one minute, as described above (see section on extraction of cuticular compounds from nymphs of *Trialeurodes vaporariorum*). First, we assessed any effect of the extracting solvents on the parasitoid searching behaviour by monitoring, through direct visual observation, and recording the time spent for searching and residency by individual wasps between a blank area and an area treated with 50 µL solvent. In preliminary assays, the searching time of the parasitoid did not differ between area treated with solvent (9 water: 1 methanol; pentane or hexane) and blank control area, indicating that the solvents did not affect the parasitoid searching ability. Afterwards, one section of the filter paper disc was treated with 50 µL solvent (control area) and the second section (treatment area) treated either with 15 mg crude honeydew or 50 µL extract of honeydew or nymph. The test aliquot of 50 µL corresponds

to amount extracted from 100 nymphs. A single *E. formosa* female was released on the control area at start, and its movement observed for ten minutes. We recorded times spent by the parasitoid on the control area and on the honeydew droplet or extract-treated area. Only insects which stayed on the filter paper for at least 60 seconds were considered responsive, and 30 responsive insects were used in the data analysis.

Chemical analyses. Analyses of honeydew volatiles and nymph extracts were carried out using a 7890A gas chromatograph (Agilent Technologies) equipped with an HP-5MSI low bleed non-polar capillary column (5% phenyl and 95% methylpolysiloxane, 30 m × 0.25 mm × 0.25 μm film thickness) under a constant helium flow rate of 1.2 mL min^{-1}. The oven temperature was set at 35°C for 5 min and then programmed to increase at 10°C min^{-1} until reaching a final temperature of 280°C, which was held for 10.5 min for the honeydew volatile analysis, and 20.5 min for the nymph extract analysis. The ion source temperature was set at 250°C with an interface temperature of 270°C, and spectra were recorded at 70eV. Compounds were tentatively identified based on their retention time, library mass spectra (NIST11, Adams2 and Chemecol), and Kovats retention indices (RIs) determined using retention times of standards of straight chain alkanes (C_8-C_{33}). The retention index (RI) was calculated using the following formula:

$$(RI) = [RT(\gamma) - RT(\alpha)]/[RT(\alpha + 1) - RT(\alpha)]*100 + (100*\alpha)$$

where RT(γ) is the retention time of the studied compound γ, RT(α) is the retention time of the alkane with α carbons that eluted just before γ, and RT($\alpha + 1$) is the retention time of the alkane with $\alpha + 1$ carbons that eluted just after γ. Thereafter, a blend of available synthetic standards was run to confirm some of the identified compounds, through comparison of their retention times and mass spectra. Compounds in the nymph extracts were quantified relative to the peak area and the concentration of the internal standard (octadecane), as follows:

$$Ccp = \frac{\left(\frac{PAcp}{PAist}\right) \text{x Cist x 2μL}}{V}$$

where Ccp is the concentration (ng/μL) of the compound in the extract; PAcp is the peak area of the identified compound; PAist is the peak area of the internal standard; Cist is the concentration of internal standard (i.e., 50 ng/μL of octadecane) and V is the volume of the extract (i.e., 50 μL) in which the aliquot (2 μL) of internal standard has been applied.

Chemical standards. The synthetic standards: (*Z*)-3-hexen-1-ol, *p*-xylene, 1-octen-3-ol, *α*-pinene, *β*-pinene, 2-carene, *α*-phellandrene, 3-carene, *α*-terpinene, *β*-phellandrene, *β*-ocimene (mixture of *E* and *Z* isomers), linalool, linalool oxide (furanoid), methyl salicylate, *p*-cymene, (*E*)-*β*-caryophyllene, *γ*-terpinene and *α*-humulene with chemical purities between 90-99%, except for *α*-phellandrene (85%), were purchased from Merck, France, and used to confirm some of the identified compounds. Standards of the straight chain hydrocarbons nonacosane and hentriacontane (both 98% purity) used in the bioassays were purchased from Merck, France.

Contact responses of Encarsia formosa to synthetic hydrocarbons. Nonacosane and hentriacontane, the two most abundant straight-chain hydrocarbons, were chosen for bioassays based on the results from the chemical analysis. Solutions of of the synthetic standards of these chemicals were tested using the Petri dish bioassays following the methodology described above (see section on contact responses of *E. formosa* to honeydew and nymph extracts). Each compound was prepared at concentrations corresponding to amount extracted from 1, 2 and 4 nymphs/µL (31, 62 and 124 ng/µL for nonacosane, and 22, 44 and 88 ng/µL for hentriacontane). An aliquot of 50 µL was dispensed on half (about 12 cm^2) of the filter paper disc (5.5 cm diam.); thus, the test doses corresponded to amounts extracted from 50, 100 and 200 nymphs (same densities which were used during the distant response tests). The parasitoid arrestment behaviour was observed, and the time spent on the treated vs. control areas was recorded. Forty responsive insects per choice test were used for the data analysis.

Statistical analysis. In the olfactometer tests, the number of parasitoids that chose (first choice) an odour source or the control was computed and then a chi-square test was applied to compare the frequencies of the choices. In the Petri-dish assays, the recorded times spent by the parasitoids were checked for assumptions of normality and homogeneity of variance by performing a Shapiro-Wilk's test and Bartlett's test, respectively. As the data did not meet these assumptions, a non-parametric Wilcoxon paired signed rank test was used to compare the times spent by the parasitoids between solvent-treated and host kairomone-treated patches. All the statistical analyses were performed in R software, version 3.4.3 (R Core Team 2017).

RESULTS

Distant olfactory responses of Encarsia formosa to host volatiles. Volatiles of *T. vaporariorum* nymph honeydew were attractive to the parasitoid *E. formosa* at 60 mg honeydew dose compared to clean air ($\chi^2 = 9.92$, *df* =1, *P* < 0.01) (Fig. 1). On the other hand, lower honeydew doses of 15 mg ($\chi^2 = 0.01$, *df* =1, *P* > 0.05) and 30 mg ($\chi^2 = 2.17$, *df* =1, *P* > 0.05) did not evoke attraction in *E. formosa*. Likewise, the parasitoid was not attracted to odours from *T. vaporariorum* nymphs at any host density, i.e, 50 ($\chi^2 = 0.32$, *df* =1, *P* > 0.05), 100 ($\chi^2 = 1.87$, *df* =1, *P* > 0.05) or 200 *T. vaporariorum* nymphs ($\chi^2 = 0.12$, *df* =1, *P* > 0.05) compared to clean air (Fig. 1).
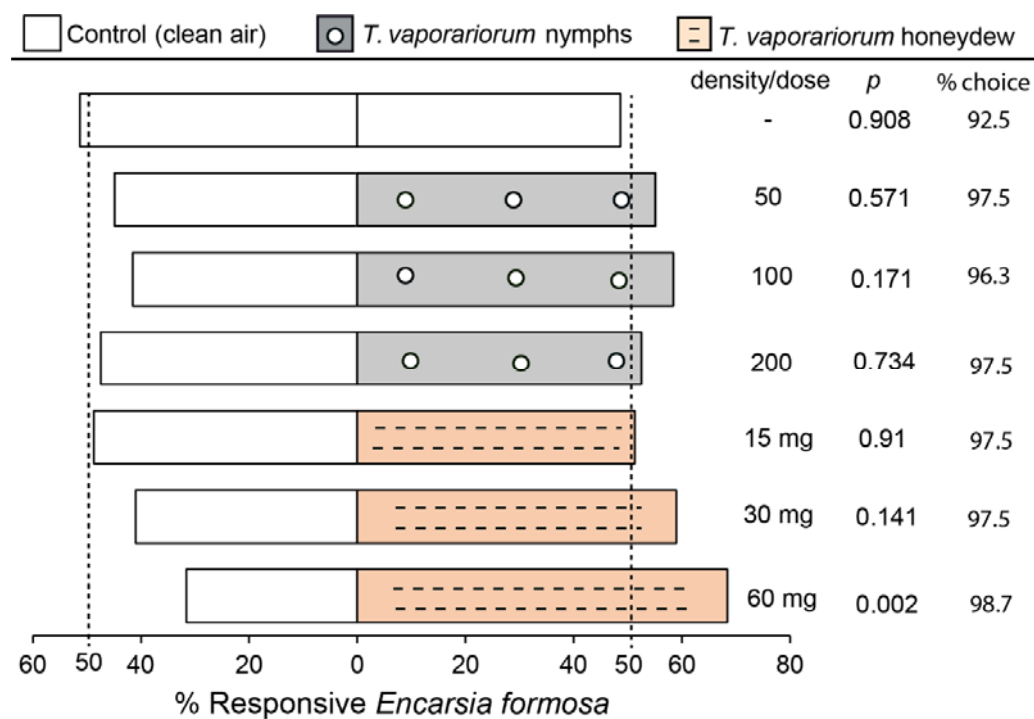

Fig. 1 Behavioural responses (%) of *Encarsia formosa* to odours from nymphs (50, 100 and 200) and honeydew (15, 30 and 60 mg) of *Trialeurodes vaporariorum* in a Y-tube olfactometer. % choice indicates the proportion of responsive insects (i.e., insects that made a choice) out of 80 insects tested per choice test. χ^2 test at $\alpha = 0.05$. Vertical lines are added at 50% to show the nymph density and honeydew dose chosen by at least 50% of the responsive parasitoids

Contact responses of Encarsia formosa to crude and extract of honeydew. The presence of honeydew increased the host searching and residency time by the parasitoid *E. formosa* (Fig. 2). Time spent by the parasitoid was significantly greater on patches treated with crude

honeydew compared to the control (blank area) (Wilcoxon test, $Z = 465$, $P < 0.001$). Likewise, the time spent by *E. formosa* was higher on areas treated with extracts of 15 (Wilcoxon test, $Z = 465$, $P < 0.001$), 30 (Wilcoxon test, $Z = 464$, $P < 0.001$) or 60 mg/mL honeydew solution (Wilcoxon test, $Z = 465$, $P < 0.001$) compared to that spent on the control (area treated with solvent, i.e., 9 water: 1 methanol mixture) (Fig 2).
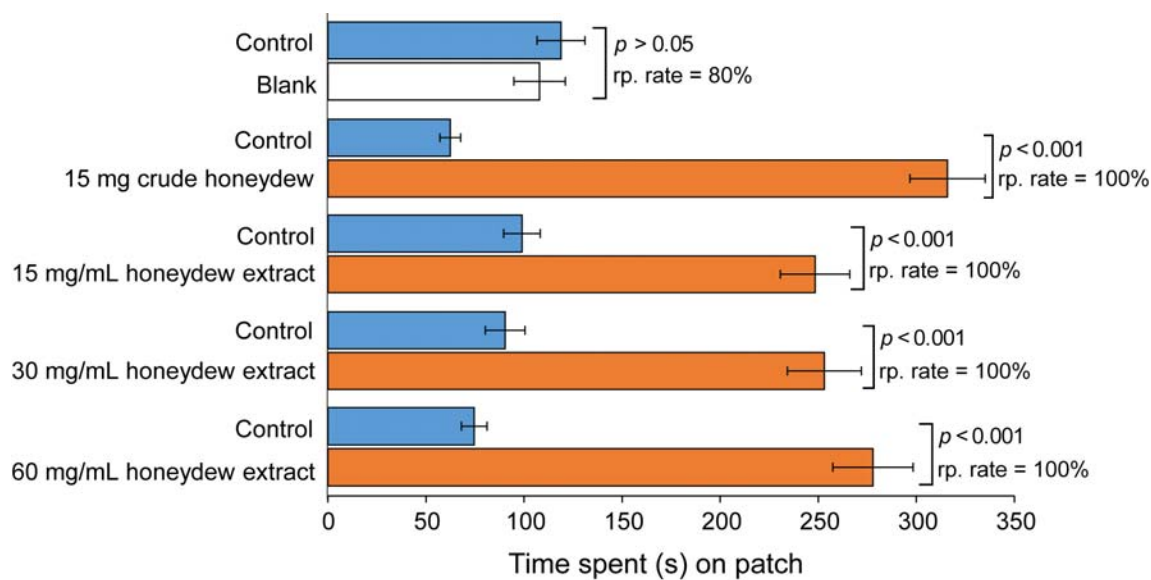

Fig. 2 Contact responses of *Encarsia formosa* to components in the crude and extract of honeydew. Thirty responsive insects were analysed per choice test, and rp. rate indicates the responsiveness rate per choice test. The volume (50 μL) of the test extracts was applied on a 12 cm^2, corresponding to 62.5, 125 and 250 ng/cm^2. Wilcoxon paired signed rank test at $\alpha = 0.05$

Contact responses of Encarsia formosa to cuticular extracts from nymphs of Trialeurodes vaporariorum. The time spent by the parasitoid *E. formosa* on the blank area compared to the area treated with hexane (Wilcoxon test, $Z = 227$, $P = 0.918$) or pentane ($Z = 225$, $P = 0.651$, respectively) did not differ (Fig. 3). Nymph cuticular extracts in hexane caused an increase in the host searching and residency time by the parasitoid on the extract-treated area compared to that on the control area (Wilcoxon test, $Z = 0$, $P < 0.001$). The parasitoid shows a tendency to spend more time in the area treated with solutions of the cuticular extracts in pentane (Fig. 3), however, the difference in the time spent was not significant between the control area and area treated with solutions of nymph cuticle extracted in pentane (Wilcoxon test, $Z = 137$, $P = 0.051$) (Fig. 3).
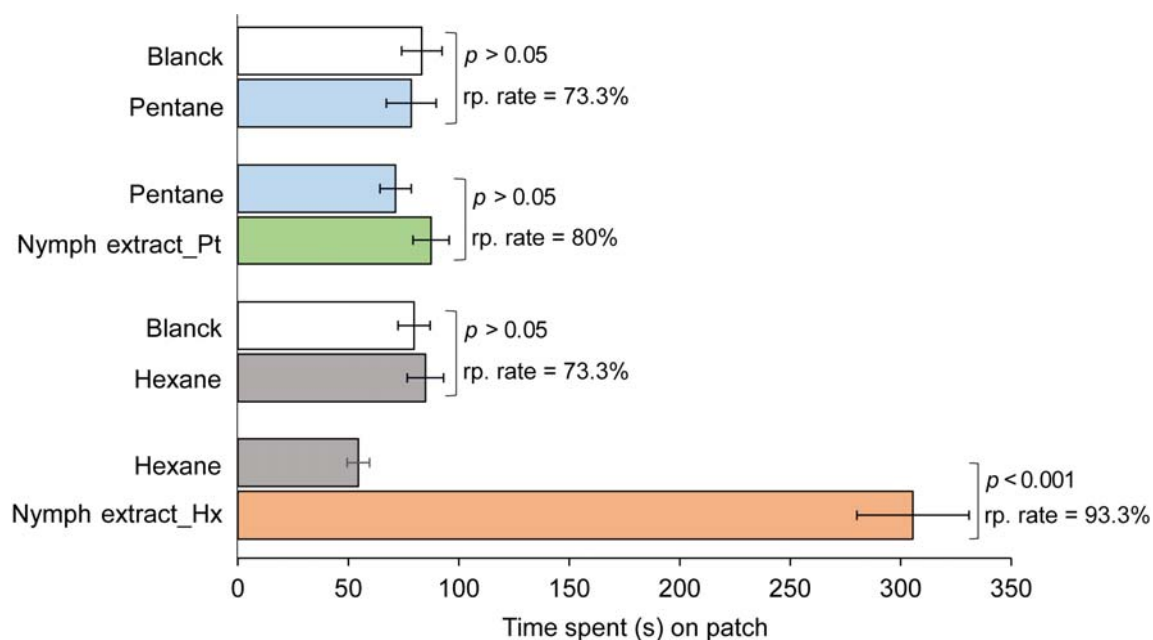
Fig. 3 Contact responses of *Encarsia formosa* to extracts of *Trialeurodes vaporariorum* nymphs. Nymph extract_Pt stands for nymphs extracted in pentane and Nymph extract_Hx stands for nymphs extracted in hexane. The volume (50 μL) of test extract was applied on a 12 cm^2 and corresponded to amount extracted from 100 nymphs. Thirty responsive insects were analysed per choice test, and rp. rate indicates the responsiveness rate per choice test. Wilcoxon paired signed rank test at α = 0.05

Analysis of honeydew volatiles. Twenty-seven volatile organic compounds (VOCs) were detected in the headspace of *T. vaporariorum* honeydew, made up of twelve monoterpenes, five alcohols, four sesquiterpenes, three ketones and three benzenoids (Table 1, Fig. 4).

Table 1: Volatile compounds detected in honeydew excreted by *Trialeurodes vaporariorum* nymphs (n=4)

Peak No.[a]	RT (min)	RI$_{alk}$[b]	RI$_{lit}$[c]	Compound[d]	Chemical class
1	8.08	862	858	(Z)-3-hexenol[£,4]	alcohol
2	8.34	871	865	p-xylene[£,3]	benzenoid
3	8.91	893	892	2-heptanone[4]	ketone
4	9.18	903	902	2-heptanol[4]	alcohol
5	9.83	936	939	α-pinene[£,4]	monoterpene
6	10.73	979	978	β-pinene[£,4]	monoterpene
7	10.79	981	980	1-octen-3-ol[£,4]	alcohol
8	10.93	988	985	3-octanone[2]	ketone
9	11.04	993	992	2-octanone[2]	ketone
10	11.22	1002	1001	2-carene[£,4]	monoterpene
11	11.32	1007	2006	α-phellandrene[£,4]	monoterpene
12	11.41	1012	1011	3-carene[£,3]	monoterpene
13	11.52	1018	1017	α-terpinene[£,3]	monoterpene
14	11.69	1028	1027	p-cymene[£,4]	monoterpene
15	11.80	1034	1032	β-phellandrene[£,4]	monoterpene
16	11.93	1041	1041	(Z)-β-ocimene[£,4]	monoterpene
17	12.11	1051	1051	(E)-β-ocimene[£,4]	monoterpene
18	12.31	1062	1060	γ-terpinene[£,3]	monoterpene
19	12.56	1076	1076	linalool oxide (furanoid)[£,3]	monoterpene
20	12.85	1092	1090	2-methoxy phenol[4]	benzenoid
21	13.01	1100	1097	linalool[£,4]	monoterpene
22	13.32	1118	1117	2-phenylethanol[4]	alcohol
23	14.60	1200	1197	methyl salicylate[£,4]	benzenoid ester
24	16.68	1340	1342	δ-elemene[4]	sesquiterpene
25	17.72	1424	1417	α-cedrene[3]	sesquiterpene
26	17.83	1432	1428	(E)-β-caryophyllene[£,4]	sesquiterpene
27	18.24	1466	1460	α-humulene[£,4]	sesquiterpene

[a]Peak numbers correspond to peaks indicated in Fig. 4

[b]Retention index relative to C$_8$-C$_{23}$ n-alkanes run on an HP-5MSI capillary column

[c]Retention index obtained from the literature

[d]Identification of compounds based on the retention time (RT), retention indices and mass spectral libraries (NIST11, Adams2 and Chemecol), and comparison with published mass spectra and retention indices from online NIST library. Superscript number indicates the number of replicates in which the compound has been detected

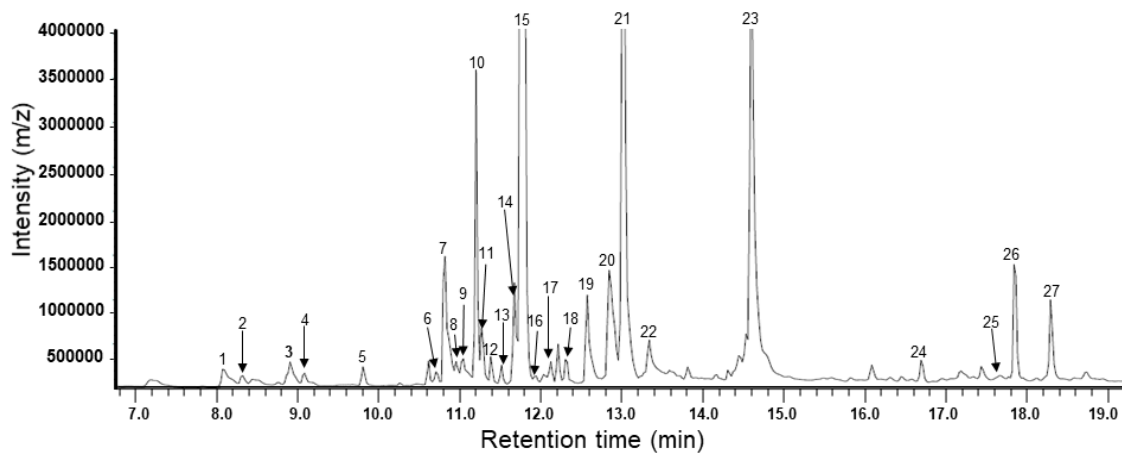£ indicates compounds confirmed with authentic standards

Fig. 4 Profile of GC-MS analysis of volatiles from honeydew excreted by *Trialeurodes vaporariorum* nymphs. Numbers correspond to the compound names in Table 1

***Analysis of components from nymph extracts*.** A total of 19 compounds were detected in the extracts of *T. vaporariorum* nymphs. These include nine alkanes, three esters, one aldehyde, one alcohol and five unidentified alkenes (Table 2; Fig. 5). The alkanes, nonacosane and hentriacontane were the most abundant, with their combined percentage accounting for 37% of the total amount of the chemicals detected in the extracts (Table 2). In addition, two of the unidentified alkenes (detected at retention times of 31.19 and 33.59 minutes) accounted for 22.4% of the total amount of the chemical compounds (Table 2).

Table 2 Amount and percentage composition (Mean ± SE) of compounds detected in *T. vaporariorum* nymph cuticular extracts (n=4)

Peak no.[a]	RT (min)	RI[b]$_{alk}$	RI[c]$_{lit}$	Compound[d]	Mean (ng/nymph)	Mean (ng/µL)	Proportion (%)
1	28.39	2502	2500	pentacosane[£]	0.29±0.03	0.58±0.06	0.21±0.02
2	29.15	2597	2600	hexacosane[£]	0.17±0.03	0.33±0.05	0.12±0.02
3	29.44	2633	2635	tretracosanal	6.64±0.07	1.29±0.14	0.45±0.06
4	29.91	2706	2700	heptacosane[£]	3.27±0.17	6.54±0.35	2.30±0.15
5	30.77	2795	2800	octacosane[£]	0.5±0.17	1.00±0.05	0.35±0.02
6	31.19	2840	-	unidentified alkene1	15.75±0.5	31.49±0.99	11.08±0.44
7	31.74	2905	2900	nonacosane[£]	30.73±1.74	61.47±3.48	21.62±1.36
8	32.94	2992	3000	triacontane[£]	0.57±0.01	1.15±0.03	0.40±0.01
9	33.16	3005	3013	hexacosyl acetate	8.87±1.13	17.75±2.26	6.24±0.80
10	33.59	3032	-	unidentified alkene2	16.10±0.36	32.20±0.71	11.32±0.38
11	34.42	3094	3100	hentriacontane[£]	22.08±0.4	44.16±0.81	15.53±0.19
12	36.05	3195	3200	dotriacontane[£]	0.45±0.04	0.91±0.08	0.32±0.02
13	36.43	3208	3213	octacosyl acetate	11.57±1.0	23.14±1.99	8.14±0.59
14	37.08	3240	-	1,30-triacontanediol	11.13±1.07	22.26±2.14	7.83±0.62
15	38.22	3300	3300	tritriacontane[£]	0.96±0.11	1.91±0.23	0.67±0.08
16	38.40	3309	-	unidentified alkene3	6.91±0.61	13.82±1.21	4.86±0.33
17	41.00	3446	3408	triacontyl acetate	9.75±1.16	19.53±2.32	6.86±0.66
18	41.90	3493	-	unidentified alkene4	2.41±0.49	4.81±0.98	1.69±0.29
19	47.34	-	-	unidentified alkene5	1.32±0.17	2.64±0.35	0.93±0.10

[a]Peak numbers correspond to peaks indicated in Fig. 5

[b]Retention index relative to C_{18}-C_{33} n-alkanes run on an HP-5MSI capillary column

[c]Retention index obtained from literature

[d]Identification of compounds based on the retention time (RT), retention indices and mass spectral libraries (NIST11, Adams2 and Chemecol), and comparison with published mass spectra from online NIST library; all compounds were detected in the four replicates

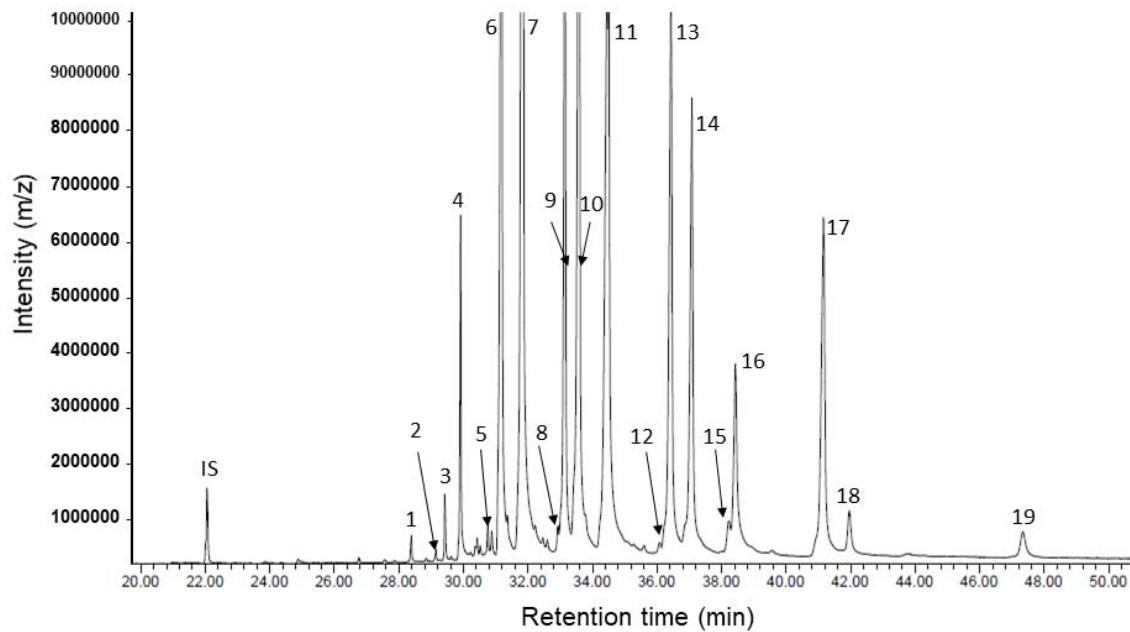[£]Compounds confirmed using authentic standards

Fig. 5 Profile of GC-MS analysis of *Trialeurodes vaporariorum* nymph cuticular extract. Numbers correspond to names of compounds listed in Table 2. IS = internal standard (octadecane)

Contact responses of Encarsia formosa to synthetics of cuticular hydrocarbons. The parasitoid *E. formosa* spent more time on areas treated with nonacosane at 3.1 µg (Wilcoxon test, $Z = 220$, $P = 0.011$) or 6.2 µg (Wilcoxon test, $Z = 144.5$, $P = 0.001$) than on control areas (Fig. 6). Time spent by the parasitoid did not differ between control areas and areas treated with 1.55 µg nonacosane (Wilcoxon test, $Z = 270.5$, $P = 0.062$) or various concentrations of hentriacontane (Wilcoxon test, $Z = 478$, $P = 0.222$; $Z = 235.5$, $P = 0.32$ and $Z = 270.5$, $P = 0.101$ for 1.1, 2.2 and 4.4 µg, respectively) (Fig. 6).
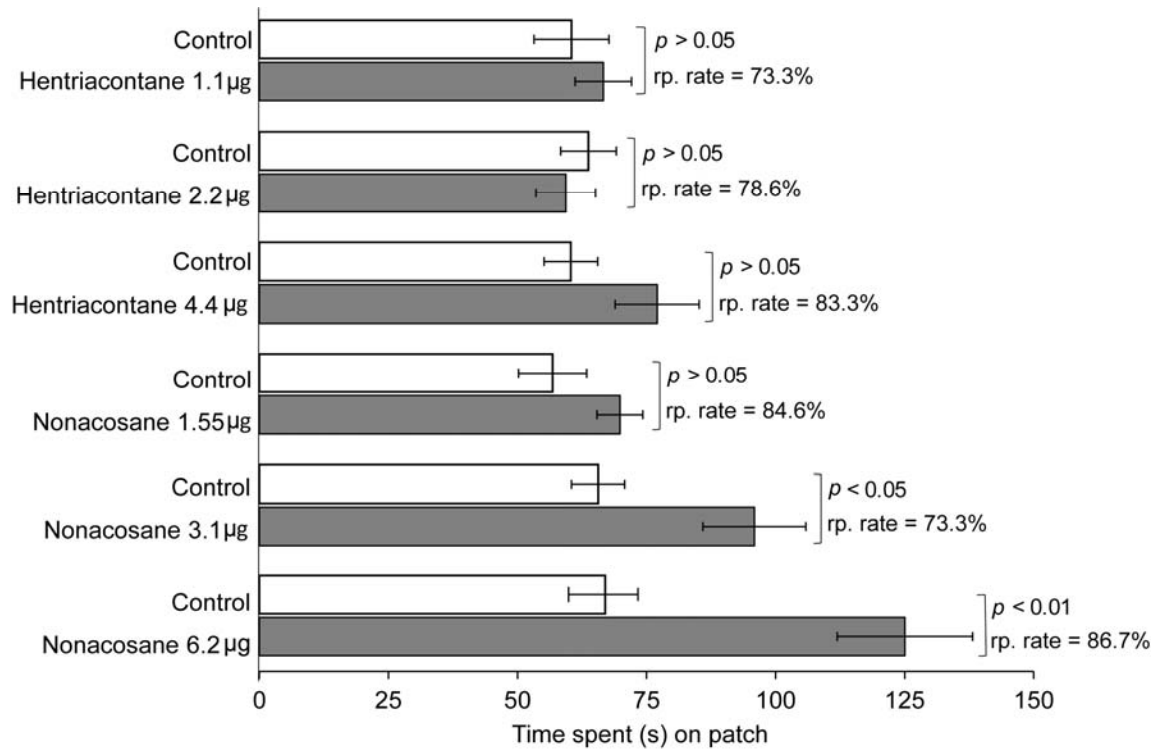
Fig. 6 Contact responses of *Encarsia formosa* to synthetic standards of hentriacontane and nonacosane. The test doses correspond to amounts extracted from 50, 100 and 200 nymphs for one minute in hexane (control). The doses were applied on a 12 cm^2 filter paper, making about 91.6, 183.3 and 366.6 ng/cm^2 for hentriacontane, and 129.2, 258.3 and 516.6 ng/cm^2 for nonacosane. Forty responsive insects were analysed per choice test, and rp. rate indicates the responsiveness rate per choice test. Wilcoxon paired signed rank test at $\alpha = 0.05$

DISCUSSION

Natural enemies locate for their hosts or prey through a three-sequence foraging process involving habitat location, host location and host selection using mainly a combination of long-range, short-range and contact chemical cues (Fatouros et al. 2008). It is known that kairomones emitted by hosts or prey are generally distinct from plant background odours, and thus constitute the most reliable sources of chemical information for natural enemies in the detection of hosts and prey in nature (Vet et al. 1991; Vet and Dicke 1992; Rodriguez-Saona and Stelinski 2009). In this study, we investigated the kairomones that mediate interactions between the parasitoid *E. formosa* and its host *T. vaporariorum*. We found that, at 60 mg honeydew dose, *E. formosa* was attracted to volatiles of the honeydew excreted by *T. vaporariorum* nymphs that fed on tomato plants, while lower honeydew doses did not elicit attraction in the parasitoid. The findings imply that a certain amount of honeydew is required for much emission of volatiles to elicit a long-range detection by the parasitoid in the location of its whitefly host, and low

honeydew doses could only be detected upon contact. The attractive honeydew dose of 60 mg is biologically relevant, and it was obtained from leaves infested with about 1000 nymphs. Similar infestation levels were recorded on tomato plants in the field (Gusmão et al. 2006), suggesting that a single whitefly-infested plant can produce a honeydew dose of 60 mg under field conditions. Parasitoids and predators of phloem-feeding herbivores are known to eavesdrop on kairomones from honeydew excreted by their hosts or prey. For example, the parasitoid *Psyllaephagus pistaciae* Ferrière (Hymenoptera: Encyrtidae) was reported to be attracted to volatiles from honeydew excreted by its host, the common pistachio psyllid, *Agonoscena pistaciae* Burckhardt & Lauterer (Hemiptera: Psylloidae) (Mehrnejad and Copland 2006). Most compounds that we identified in the headspace of the collected honeydew have been documented as volatiles of tomato plants infested with *T. vaporariorum* (López et al. 2012; Darshanee et al. 2017; Ayelo et al. 2021b). The honeydew volatiles were likely a combination of odours from fresh and fermented honeydew, and leaf trichomes because the honeydew droplets excreted by *T. vaporariorum* nymphs were on the leaf surfaces bearing trichomes of which constituents could have dissolved in the honeydew through hydrolysis. The feeding behaviour (i.e., sap sucking) of the whitefly *T. vaporariorum* could also have enriched the honeydew aroma with tomato components, as previously reported for the whitefly *B. tabaci* whose intake of salicylic acid from tomato leaf mesophyll resulted in the secretion of the derivative glycosylate salicylic acid in the insect's crude honeydew (Vandoorn et al. 2015). Host plant-derived volatiles have also been reported from honeydew of the sweet potato whitefly, *Bemisia tabaci* Gennadius (Hemiptera: Aleyrodidae) feeding on cotton, *Gossipium hirsutum* L. (Malvaceae) (Leroy et al. 2012), and that of the aphid *Megoura viciae Buckton (*Hemiptera: Aphididae) feeding on the broad bean, *Vicia faba* L. (Fabaceae) plants (Roopa et al. 2016).

Some compounds identified in *T. vaporariorum* honeydew volatiles such as (*Z*)-3-hexenol, 3-carene, 3-octanone, *α*-phellandrene, methyl salicylate, *β*-ocimene, *β*-myrcene and (*E*)-*β*-caryophyllene were known as attractants for the parasitoid *E. formosa* (Birkett et al. 2003; Silva et al. 2020; Ayelo et al. 2021b; Chen et al. 2021)*.* The parasitoid was reported to be strongly attracted to the blend of 3-carene, *α*-phellandrene, *β*-ocimene, and *β*-myrcene in Y-tube olfactometer, using between 60 to 800 ng dose (Ayelo et al. 2021b), and to *β*-myrcene and (*E*)-*β*-caryophyllene under tomato greenhouses, using a dose of 375 µg with an average release rate of 169 and 193 ng/h (Chen et al. 2021). The compounds 1-octen-3-ol, 3-octanone, 2-octanone, linalool, linalool oxide (furanoid), 2-methoxy phenol and 2-phenylethanol were detected in the

honeydew volatiles (Table 1), but they have not been reported as volatiles of tomato plants infested with *T. vaporariorum* (López et al. 2012; Darshanee et al. 2017; Ayelo et al. 2021b). Some of these honeydew compounds are also known to serve as kairomones for natural enemies. Linalool was identified from the headspace of fermenting sugars (El-Sayed et al. 2005) and it has been reported to be attractive to the parasitoid *Aphidius ervi* Haliday (Hymenoptera: Braconidae) (Du et al. 1998). The compounds 2-methoxyphenol and 2-phenylethanol were sampled in the headspace volatiles of honey (Machado et al. 2020), and could have resulted from microbial activity in the honeydew we collected, as observed in the volatiles of aphid honeydew inoculated with micro-organisms (Leroy et al. 2011). These compounds serve as kairomones for natural enemies, as reported with regard to the attraction of the parasitoid *Microplitis demolitor* Wilkinson (Hymenoptera: Braconidae) to 2-methoxyphenol (Ramachandran et al. 1991) and the predator *Chrysoperla carnea* (Stephens) (Neuroptera: Chrysopidae) to 2-phenylethanol (Zhu et al. 1999). Attractive volatile compounds could be used to formulate an effective kairomone lure to be deployed in tomato crops to recruit *E. formosa* for enhancing the biological control of the whitefly *T. vaporariorum* (Chen et al. 2021), as reported for the control of other insect pests through the recruitment of their natural enemies under field conditions (Ayelo et al. 2021a).

We also found that honeydew excreted by *T. vaporariorum* nymphs and its aqueous extracts of the act as an arrestant, even at low honeydew dose, and this causes an increase in the searching activity of *E. formosa* on honeydew-contaminated compared to honeydew-free patches. This finding is in agreement with previous reports that *E. formosa* searched more intensively on tomato leaves when honeydew was present, which increased the parasitoid's chance of finding and parasitising *T. vaporariorum* nymphs (van Roermund and van Lenteren 1995; Romeis and Zebitz 1997). Such behaviour has also been noticed in the whitefly parasitoids *Encarsia bimaculate* Heraty & Polaszek (Hymenoptera: Aphelinidae) and *Eretmocerus* sp. on extracts of the honeydew excreted by *B. tabaci* nymphs that fed on poinsettia plants (Mandour et al. 2005, 2007). Honeydew of whiteflies is rich in sugars (Henneberry et al. 2003; Liu et al. 2007; Liu et al. 2007), and the parasitoid *E. formosa* undoubtedly feeds on whitefly honeydew in the field (Burger et al. 2004; Hirose et al. 2009).

The parasitoid *E. formosa* was not attracted to odours from *T. vaporariorum* nymphs. This finding corroborates with those by Chen et al. (2021) who reported that odours of nymphs of the whitefly *B. tabaci* were not attractive to *E. formosa*. However, the parasitoid spent more

time on tomato leaves infested with *B. tabaci* nymphs than on leaves without nymphs (Chen et al. 2021), thus suggesting that the parasitoid only detects the presence of the whitefly nymphs upon contact using the chemical cues found on the epicuticles. Furthermore, our chemical analyses revealed that the cuticular extracts consisted of alkanes, esters, aldehydes, alcohols and alkenes. Also, Buckner et al. (1994) reported the presence of these chemical compounds except for alkenes in the extracts of *T. vaporariorum* adults reared on tomato plants. Among the straight-chain hydrocarbons identified in our study, nonacosane, triacontane, hentriacontane, dotriacontane and tritriacontane have also been detected in the extracts from nymphs of the whitefly *Bemisia argentifolii* Bellows & Perring (Hemiptera: Aleyrodidae) reared on cantaloupe plants (Buckner and Jones 2005). We noted the aldehyde tetracosanal and esters with between 28-32 carbons from cuticular extracts of *T. vaporariorum* nymphs fed on tomato plants, whereas Buckner et al. (1999) identified the aldehydes octacosanal, triacontanal, dotriacontanal and tetratriacontanal, and esters with between 40-60 carbons from the extracts of *B. argentifolii* nymphs fed on cantaloupe plants. The differences in the numbers of carbons for the esters noted in our study and those reported by Buckner et al. (1999) could partly be explained by the difference in the extraction methods used. While we used hexane alone, Buckner et al. (1999) used a double extraction procedure with an initial extraction with hexane which was followed by chloroform. Bioassays using synthetics revealed that the parasitoid *E. formosa* spent more time on areas treated with nonacosane than on control areas, indicating that nonacosane acted as an arrestant for the parasitoid. Alkanes are known to mediate location and acceptance of host by parasitoids. For instance, tricosane identified in scale extracts of the corn earnworm, *Helicoverpa zea* (Boddie) (Lepidoptera: Noctuidae) (formerly known as *Heliothis zea*) induced an increase in the efficiency of host location and parasitism by the parasitoids *Microplitis croceipes* (Cresson) (Hymenoptera: Braconidae) and *Trichogramma achaeae* Nagaraja & Nagarkatti (Hymenoptera: Trichogrammatidae) (Gross et al. 1975; Lewis et al. 1975). It is likely that nonacosane could play a role in the parasitisation of the whitefly *T. vaporariorum* by *E. formosa*, and this warrants further investigations.

Our study reveals that the parasitoid *E. formosa* is attracted to volatiles from honeydew excreted by its whitefly host, *T. vaporariorum*, while being arrested by extracts of honeydew and nymphs. Chemical analyses revealed that the honeydew volatiles contained compounds that are known to be attractive to *E. formosa*. These findings suggest that honeydew volatiles contribute to the long-range signals exploited by the parasitoid *E. formosa* to find its host *T. vaporariorum* on tomato plants. Upon landing on the plants, the parasitoid eavesdrops on contact chemicals

emanating from nymphs and honeydew of the host. . The cuticular extracts of the whitefly nymphs contained straight chain hydrocarbons like nonacosane which acted as an arrestant for the parasitoid. Our findings show that honeydew and nymph cuticular components are important kairomones for *E. formosa*, and these could be exploited to retain the parasitoid on tomato crops for the biological control of the whitefly *T. vaporariorum.*

Authors' contributions: All authors contributed to the conception and design of the research work. P.M.A. conducted the experiments, analysed the data, and wrote the first draft of the manuscript. All authors proofread the manuscript and approved the final version for submission.

Data Availability: Data generated in the study are available upon request via the corresponding author.

Declarations

Conflict of interest statement: The authors state that they have no conflict of interests to declare.

Ethical approval: Not applicable

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