What omnivores don't eat

Nonconsumptive ecological effects of phytophagy by Macrolophus pygmaeus

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Publication date
2018

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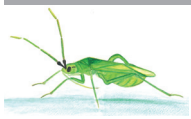

Omnivore induces production of plant volatiles that attract a specialist spider mite predator

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Abstract – Omnivorous predators may induce plant defences that affect the performance and host plant choice of herbivores. Herbivores were found to be less attracted by plants previously exposed to omnivorous predators than clean plants, which suggests that plant volatiles are involved in the host plant choice of the herbivores. These volatiles may also affect the searching behaviour of other predators. Here we show that the predatory mite *Phytoseiulus persimilis* prefer volatiles from plants previously exposed to the omnivore *Macrolophus pygmaeus* to volatiles from clean plants. The predatory mites were equally attracted by plants previously exposed to the omnivore and subsequently infested by spider mites (*Tetranychus urticae*, the prey of the predatory mite) and plants infested with spider mites. In contrast, the predators were more attracted by volatiles from plants infested with prey and subsequently exposed to the omnivore than plants infested with prey but not exposed to the omnivore. The predatory mites were also significantly more attracted to plants on which the omnivores were still present. Experience of the predatory mites with volatiles from plants previously exposed to the omnivore and without prey resulted in a loss of the preference for volatiles emitted by plants exposed to the omnivore. Furthermore, different ratios and quantities of plant volatiles were produced by plants exposed to the omnivore by clean plants. Together, these results suggest that omnivorous predators induce the production of plant volatiles that attract other predators. The volatiles possibly resemble the volatiles induced by the prey of the predators, but subsequent experience with these volatiles in the absence of prey decreases their attractiveness. The consequences of these results for spider mite control remain to be investigated.

Introduction

To protect themselves from herbivory, plants can produce specific compounds that directly reduce the development and oviposition of herbivores, so-called direct plant defences (Karban and Baldwin 1997; Kant et al. 2015). Plants can also reduce damage by herbivores indirectly by involving natural enemies of the herbivores, which is referred to as indirect plant defences (Dicke and Sabelis 1989; Karban and Baldwin 1997; Sabelis et al. 2001). Plants can arrest natural enemies of the herbivores by providing them with food, e.g. extrafloral nectar and pollen (Pemberton and Lee 1996; Heil et al. 2001; Wäckers 2001) and shelter such as domatia (Walter 1996), but herbivory also results in the production of mixtures of volatiles that are attractive to the natural enemies (Turlings et al. 1995; Sabelis et al. 1999; Dicke and van Loon 2000). These plant volatiles do not only emanate from the damaged plant tissue but also systemically from non-damaged tissue (Turlings and Tumlinson 1992; Dicke 1994; Rose et al. 1996; Guerrieri et al. 1999). The volatiles differ qualitatively and quantitatively among plants species (van den Boom et al. 2004), herbivore species feeding on the plants (De Moraes et al. 1998; Birkett et al. 2003), and with the period that herbivores feed on the plants (Takabayashi et al. 1994b; Turlings et al. 1998; Kant et al. 2004), and also vary with other biotic and abiotic factors (Takabayashi et al. 1994a; Dicke and van Loon 2000).

Many carnivorous arthropods use plant volatiles produced by plants attacked by herbivores to locate their prey or hosts from some distance (Turlings et al. 1990; Dicke et al. 1990; Sabelis et al. 1999). Predatory arthropods and parasitoids can discriminate between volatiles from plants attacked by their prey or host and those of plants attacked by non-prey or non-host herbivores (Sabelis and van de Baan 1983; De Moraes et al. 1998), and they can also discriminate between quantitative differences in the composition of major volatile blends from different plants attacked by the same herbivore species (Du et al. 1998; Guerrieri et al. 1999; Birkett et al. 2003). For example, the predatory mite *Phytoseiulus persimilis* preferred volatiles from prey-infested leaves and not those of leaves infested by non-prey (Sabelis and van de Baan 1983). However, it has also been reported that predatory arthropods and parasitoids respond to volatiles from plants attacked by non-prey or non-host herbivores (Shimoda and Dicke 2000; Sabelis et al. 2007).

Although much research has addressed plant volatiles induced by herbivores and their role in plant- arthropod interactions, there is little information on whether omnivores induce the production of plant volatiles. It has been shown that plant feeding by omnivorous predators can induce direct plant defences that affect the performance of herbivores (De Puysseleyr et al. 2011; Pappas et al. 2015; Pérez-Hedo et al. 2015a,b; Naselli et al. 2016; Zhang et al. 2018 [= CHAPTER 2]), and this plant feeding can potentially also induce the production of plant volatiles, and there is indeed some evidence for this

(Moayeri et al. 2007; Pérez-Hedo et al. 2015a; Bouagga et al. 2017, 2018). Here, we investigate the induction of volatile production by the omnivorous predatory bug *Macrolophus pygmaeus* and we assess the response of another predator, the predatory mite *P. persimilis,* to volatiles emanating from plants exposed to the omnivore.

Macrolophus pygmaeus is commercially used for biological control of several pests. Besides feeding on plant tissue (Perdikis and Lykouressis 2000), it attacks a wide range of arthropod pests, such as whiteflies (Montserrat et al. 2000), thrips (Riudavets and Castañé 1998), aphids (Alvarado et al. 1997), spider mites (Hansen et al. 1999), leaf miners (Arnó et al. 2003) and Lepidoptera species, including *Tuta absoluta* (Urbaneja et al. 2009). Earlier, others and we have shown that *M. pygmaeus* affected the performance of the herbivorous pests *Tetranychus urticae* and *F. occidentalis* through induced direct plants defences (Pappas et al. 2015; Zhang et al. 2018 [= CHAPTER 2]). Moayeri et al. (2007a) showed that feeding by *M. pygmaeus* induced the production of 11 additional volatile compounds in bean plants. We also showed that females of *T. urticae* and *F. occidentalis* avoided plants previously exposed to *M. pygmaeus* (CHAPTER 3), which was possibly mediated by volatiles. We therefore sought to confirm the induction of volatile production by performing olfactometer experiments and by analysing the headspace of plants exposed to the omnivore. Although it is possible to test the response of several of the herbivores we studied in an olfactometer (Pallini et al. 1997; de Kogel et al. 1999), these experiments are slow and results are variable (Pallini et al. 1997). We therefore chose to use the predatory mite *P. persimilis*, which relies on olfactory cues to locate plants infested with the two-spotted spider mite, its prey, from a distance (Sabelis and van de Baan 1983; Sabelis et al. 1984; Dicke et al. 1990). Because both the omnivore and the predatory mite are commercially used in biological control, these two species often co-occur in greenhouse crops, but not much is known about their interactions.

To understand the effect of exposure of plants to *M. pygmaeus* on the response of *P. persimilis* to volatiles emanating from these plants, a series of olfactometer experiments was conducted. First, we investigated the response of *P. persimilis* to clean plants and plants previously exposed to *M. pygmaeus*, but without these omnivores being present on the plants. Subsequently, we infested these same plants with spider mites and determined the response of predatory mites again, to check whether previous infestation by omnivores would change the response of predatory mites towards plants with their spider mite prey. We also performed the reverse experiment, infesting plants first with spider mites and then exposing one group to *M. pygmaeus*. We furthermore investigated whether *P. persimilis* responded to volatiles of the mirids themselves by offering them a choice between plants previously exposed to *M. pygmaeus*, of which one group still harboured the mirids and the other group not.

Materials and methods

Cultures

Sweet pepper plants (*Capsicum annuum* L. Spider F1 Enza Zaden Beheer, The Netherlands) were grown from seeds in pots (14 cm Ø) with soil in a climate room dedicated to growing clean plants (25 ± 1°C, 60-70% RH, 16:8 L:D). Water was supplied twice a week. Plants with 6-10 leaves were used for experiments. Four-week-old plants with 6-8 true leaves (about 20 cm high) were used for experiments. Plants of 5-8 weeks old were used for the rearing of spider mites, thrips and aphids.

A culture of *M. pygmaeus* was established with fifth instar nymphs from a commercial company (Koppert Biological Systems, Berkel en Rodenrijs, The Netherlands). They were reared in insect-proof cages (BugDorm-44545F, 47.5 × 47.5 × 47.5 cm, mesh size: 160 µm) in a separate climate room (conditions as above) with *Ephestia kuehniella* eggs as food and 4- to 5-week-old sweet pepper plants as both food supply and oviposition substrate. New *E. kuehniella* eggs were added twice a week and new plants were added every 2 weeks. Adults from the colony were used for experiments.

The culture of two-spotted spider mites *T. urticae* was started with individuals that were obtained from a cucumber colony in our lab (see Janssen 1999 for details), and was reared on intact sweet pepper plants in a separate climate room (conditions as above). New plants were provided twice a week. The culture was cultivated for 10 months on sweet pepper plants before being used for experiments, hence, was adapted to sweet pepper plants.

The predatory mites *P. persimilis* originating from a culture (Koppert strain) reared with spider mites on bean plants (van Wijk et al. 2008), were transferred to sweet pepper leaves infested with spider mites. These leaves were placed on a plastic platform in a tray filled with water with soap, preventing the mites from escaping. New sweet pepper leaves infested with spider mites were provided 5-6× per week. Predatory mites were reared in this way for more than three generations before being used in the experiments, to allow them to adapt to sweet pepper plants.

Olfactometer experiments

Responses of predatory mites to different plant volatiles were tested using a Y-tube olfactometer (Sabelis and van de Baan 1983). It consisted of a Y-shaped glass tube with a metal wire in the middle on which the predators could walk freely. A transparent hose connected each arm of the Y-tube to a separate glass container (50 × 40 × 40 cm) that contained a volatile source and had an air inlet and outlet covered with fine mesh (80 µm). A tray with three sweet pepper plants that had received one treatment was placed inside one container 30 min prior to the experiment, the other container received a tray with three plants of another treatment. The base of the Y-tube

was connected to a vacuum pump, creating an air flow in the olfactometer (0.45 ± 0.05 m/s). Anemometers (VelociCalc® Air Velocity Meter 9545-A, TSI, USA) were used to measure the wind speed in both arms of the olfactometer, which were calibrated with valves inserted in the transparent hoses. With equal wind speeds in both arms, two separated volatile plumes are formed in the base of the Y-tube, with their interface coinciding with the metal wire (Sabelis and van de Baan 1983; Janssen et al. 1997).

After disconnecting the vacuum pump from the base of the Y tube, an individual starved gravid female of *P. persimilis* was introduced onto the beginning of the metal wire in the Y-tube with a fine brush. Immediately afterwards, the vacuum pump was connected. The predatory mite was observed until it had reached the end of one of the arms or until 5 min had passed. Subsequently, it was removed and the next female was introduced. After five females that made a choice, the volatile sources were switched by connecting the hoses to the other arm or to the other container and the wind speed was measured and calibrated. This was done to correct for any unforeseen asymmetry in the experimental set-up that could influence the choice of the predatory mites (Janssen et al. 1997). Each olfactometer replicate consisted of 20 female predatory mites that made a choice for one volatile. In between replicates, the olfactometer and the hoses were washed with detergent and rinsed with demineralized water, and were left to dry. The containers containing the plants were cleaned with alcohol (60%) and air-dried. All female predatory mites were only used once for an experiment. Individuals that did not make a choice within 5 min were not included in the statistical analyses; on average, 1.92 mites per replicate did not make a choice.

To make sure that females of *P. persimilis* from our culture responded to volatiles from sweet pepper plants, a preliminary olfactometer test was conducted. Three sweet pepper plants (4-5 weeks old) were infested for 2 days with 240 adult spider mites per plant with an average of 30 adults per leaf. Subsequently, an olfactometer experiment was conducted with these plants as one volatile source and another group of plants of the same age and size, but without spider mites as the other volatile source. This test was replicated 2×, and *P. persimilis* significantly preferred treated plants to clean plants in both tests (clean: 17.5 ± 2.5%, plants with spider mites: 82.5 ± 2.5%; GLM: $\chi^2 = 18.35$, d.f. = 1, $P < 0.001$), showing that they can recognize volatiles from infested sweet pepper plants.

Response to plants exposed to *Macrolophus pygmaeus*

Six sweet pepper plants (4 weeks old with 6-8 true leaves) were transferred each into a separate insect-proof cage (as above) in a separate climate room (conditions as above). Five adult females and five adult males of *M. pygmaeus* were released in

three cages. Plants in the other three cages were clean, serving as control. Four days later, all *M. pygmaeus* were removed from the three treated plants, and the treated and control plants served as volatile sources for an olfactometer experiment. The response of *P. persimilis* to these two groups of plants was tested as described above. Five replicates were conducted in total, with five different groups of plants and predatory mites.

Response to plants exposed to *Macrolophus pygmaeus* and infested with *Tetranychus urticae*

After the olfactometer experiment, the groups of plants from four out of the five replicates of the above experiment were transferred back to their cages, and 240 female spider mites were subsequently released on each plant. Each plant was placed in a tray filled with water to prevent mites from escaping. Two days later, another olfactometer experiment was conducted with these three plants previously exposed to *M. pygmaeus* and subsequently infested with spider mites as one volatile source and the three plants only infested with spider mites as the other volatile source. The spider mites were present on the plants during the test. Subsequently, other groups of plants were treated with spider mites and *M. pygmaeus*, but the order of the treatment was changed compared to the experiment above. Six sweet pepper plants were infested with 240 adult spider mites per plant for two days. Subsequently, an olfactometer experiment was conducted as described above with two groups of three spider mite-infested plants to make sure that these two groups of plants had similar attractiveness to the predatory mites before they were exposed to *M. pygmaeus*. Thereafter, all plants were transferred back to their cages, and the group of three plants that attracted a (non-significantly) lower number of predatory mites subsequently received five adult males and five adult female *M. pygmaeus*. The other three plants were kept without mirids but with spider mites. After 4 days, the remaining mirids were removed from the three plants, and their attractiveness was compared with that of the other three plants in the olfactometer. Four replicates were conducted, each with a new group of plants.

Response of *Phytoseiulus persimilis* to plants with *Macrolophus pygmaeus*

The following experiment was conducted to check the response of *P. persimilis* to *M. pygmaeus* present on the plants. Two groups of plants, one group treated with *M. pygmaeus*, the other group consisting of clean plants, were prepared as above. All *M. pygmaeus* remaining in the cages with the treated plants were collected after the four days of treatment and kept in 1.5-ml Eppendorf Safe-Lock tubes and the plants were transferred to the container of the olfactometer. The collected *M. pygmaeus*

were subsequently released in this container and their number was supplemented with mirids from the culture to an average of five males and five females per plant. The untreated plants served as the alternative volatile source. The air inlets and outlets of the containers were closed with mesh to prevent mirid escapes. Four replicates were conducted, each with a new set of plants.

We subsequently removed the mirids from the containers after the olfactometer test, all plants were left in the containers overnight with an airflow of about 0.20 m/s to prevent condensation of water on the plants and the container walls, and the response of the predatory mites to the volatiles of these plants and the control plants was measured the next day. This was done with two of the four groups of plants of the previous test

Response of *Phytoseiulus persimilis* with experience with volatiles from plants previously exposed to *Macrolophus pygmaeus*

The previous experiments showed attraction of *P. persimilis* to plants exposed to *M. pygmaeus*. This response was somewhat surprizing because the omnivore can feed on eggs of *P. persimilis*, hence, the predatory mites were expected to avoid plants with the omnivore. We therefore investigated whether experience of *P. persimilis* with the omnivore would change its response towards volatiles of plants exposed to the omnivore. Four plants treated with *M. pygmaeus* and four clean plants were prepared as described above. From one of the treated plants, leaf discs (1.5 cm Ø) were made and placed in plastic cups (2 cm Ø, 3 cm high) filled with water to support the leaf discs and prevent mites from escaping. Leaf discs of such plants have induced direct defences (CHAPTER 2). Subsequently, gravid females of *P. persimilis* collected from the culture were each transferred to a separate leaf disc, which did not contain food for the predatory mites. The cups were closed with lids with a ventilation hole covered with fine mesh (80 µm), thus volatiles could enter and leave the cups. Thereafter, all the cups were placed on top of an upside-down plastic tray (30 × 24 cm) in a cage (same type as above) with one of the *M. pygmaeus*-treated plants. Live *M. pygmaeus* in all three cages were counted and new individuals were added to a total of five adult females and five adult males per cage. Thus, *P. persimilis* were able to perceive cues from the treated leaf disc, volatiles from the treated plant and cues from *M. pygmaeus*. A second group of gravid females of *P. persimilis* were treated similarly, but were incubated on leaf discs from one clean plant and placed in a cage with one clean plant without *M. pygmaeus*. About 24 h later, these predatory mites were used in an olfactometer test. All *M. pygmaeus* were removed from the three plants, and the plants were used as one volatile source; the three clean plants as the other. A third group of *P. persimilis* was collected from the colony and starved for 1 h, and was subsequently also tested in the olfactometer with the same plants. Five replicates

with five different groups of plants were conducted in total; in the last replicate only the first two groups of predatory mites were tested.

Volatile collection and analysis

Three groups of plants with different treatments (as above) were prepared for volatile analysis: 1) clean plants as control (Clean); 2) plants exposed to five females and five males of *M. pygmaeus* for 4 days, after which all *M. pygmaeus* were removed before volatiles were collected (*M. pygmaeus* removed); 3) plants exposed to five females and five males of *M. pygmaeus* for 4 days, with the omnivores present during volatile collection (*M. pygmaeus* present). To ensure that all plants in this last treatment had the same number of omnivores, the omnivores from the culture were added until there were five males and females per plant. Three plants from the same treatment were used as one replicate, in total 21 clean plants, 27 plants from *M. pygmaeus* removed, and 15 plants from *M. pygmaeus* present were used. The three plants were placed in a 40-l desiccator, and volatile sampling was done according to Kant et al. (2004). Briefly, desiccators were ventilated with carbon-filtered pressured air at a flow of 400 ml/min. Air from the desiccator was sampled during 24 h by trapping it on 50 mg of Porapak type Q 80-100 Mesh (Supelco) enclosed in a 5 mm wide glass tube. Volatiles were eluded from the adsorbent using 2 ml pentane-diethyl ether (4:1) spiked with 2.5 ng/ml of benzylacetate (BA) as internal standard. One µl of the eluate was injected (splitless) in the injector port of a Quadrupole Time-of-flight (Q-TOF) and immediately heated to 275°C. Compounds were separated on a HP-5ms column (30 m × 250 µm, 0.25 µm film thickness; Agilent) in an Agilent 7890A gas chromatograph with a temperature program set to 40°C for 5 min, increasing to 250°C at a rate of 15°C per min and an additional 5 min at 250°C. Helium was used as the carrier gas with the transfer flow set to 3 ml/min and a column flow rate of 1 ml/min thereafter. Mass spectra were generated by an Agilent 7200 accurate-mass quadrupole time-of-flight mass spectrometer, operating in electron ionization mode (70 eV) at 230°C and collected with an acquisition rate of 20 scans/s acquiring ions at a 30-500 m/z range. Several volatiles were identified using standard solutions and comparing the retention time (RT) and mass spectra: (*E*)-β-ocimene, linalool, methyl salicylate (MeSA), β-caryophyllene and nerolidol (Sigma-Aldrich). Peaks were detected by chromatogram deconvolution using Agilent MassHunter Qualitative Analysis software using the following settings: signal-to-noise ratio = 2; RT window size factor = 100; minimal peak area = 500; m/z accuracy = 50 ppm. Identified peaks were integrated over the acquired samples using Agilent Masshunter Quantitative Analysis software. Here, the base ion of the peak's mass spectrum was taken for quantification with an accuracy of 50 ppm. Peak areas were corrected with the internal standard. Standards were used for the identification of the peaks when possible. If not, the Kovats Index was calculated using the metabolite's retention time.

Statistical analysis

The preference of the predatory mites for a particular volatile source in each experiment was tested with a log-linear model for contingency tables with a generalized linear model (GLM) with treatment and replicate and their interaction as factors and the numbers of predators choosing for the volatile sources as response variable with a Poisson error distribution (log link) (Crawley 2013). Differences in preference between different experiments were tested by comparing the proportions of predators choosing for a volatile source with a GLM with a binomial error distribution (logit link) with the experimental treatment as factor. All statistical analyses were performed with R (R Development Core Team 2017).

In total, 160 volatiles were found in the volatile analyses. Because we were interested in volatiles associated with the exposure of plants to the omnivore, as a first step, we selected those compounds that were at least a factor 2 higher or lower in exposed plants (either with *M. pygmaeus* present or absent) than in clean plants. This resulted in 20 compounds, of which all the peak areas were scaled relative to the average peak areas of clean plants, and subsequently log(x+0.1)-transformed and analysed with a linear discriminant analysis in R. Three of these compounds were not further identified and were present in significantly lower amounts in exposed plants than in clean plants and were therefore not further analysed. Moreover, the amounts of these volatiles were compared among treatments with an ANOVA. To further confirm the most important volatiles separating the three treatments, we performed a tree analysis (Ripley 2016) and a Partial Least Squares Discriminant analysis (see Supplementary material Ch. 4, Methods).

Results

Response to plants exposed to *Macrolophus pygmaeus*

Phytoseiulus persimilis significantly preferred plants previously exposed to *M. pygmaeus* over clean plants in four out of the five replicates (FIGURE 4.1). No significant preference was found in one replicate, resulting in a significant interaction between treatment and replicate (GLM: $\chi^2 = 20.20$, d.f. = 4, $P < 0.001$). Overall, the predatory mites showed a clear preference for *M. pygmaeus*-treated plants to clean plants.

Response to plants exposed to *Macrolophus pygmaeus* and infested with *Tetranychus urticae*

When both groups of plants of the previous experiment were subsequently infested with spider mites, the preference for *M. pygmaeus*-treated plants disappeared (FIGURE 4.2a). *Phytoseiulus persimilis* showed no significant preference in three out of four replicates, and showed a preference for plants that had not been exposed to *M. pygmaeus* in the fourth replicate, resulting in a significant interaction between treat-

ment and replicate (GLM: χ^2 = 14.83, d.f. = 3, P = 0.002). The difference in preference between the previous experiment and the current one was highly significant (cf. FIGURES 4.1 and 4.2a, GLM: χ^2 = 16.9, d.f. =1, P < 0.0001), showing that the preference for plants exposed to *M. pygmaeus* disappeared when these plants plus the clean plants were subsequently attacked by spider mites.

When plants were first infested with spider mites and not exposed to *M. pygmaeus*, none of the two groups of plants attracted significantly more *P. persimilis* (FIGURE 4.2b). However, because we assigned the groups of plants that were slightly more attractive to one treatment (FIGURE 4.2b, left-hand bars) and the other groups to the other treatment (FIGURE 4.2b, right-hand bars), there was an overall significant preference for the groups of plants that were more attractive (GLM: χ^2 = 7.32, d.f. = 1, P = 0.007). When the least attractive groups of plants were subsequently exposed to *M. pygmaeus,* they became equally attractive as the groups of plants not exposed to *M. pygmaeus* (FIGURE 4.2c, GLM: χ^2 = 0.45, d.f. = 1, P = 0.50). The change in preference due to exposure to *M. pygmaeus* was significant (cf. FIGURE 4.2b,c, GLM: χ^2 = 5.77, d.f. = 1, P = 0.016).

Response of *Phytoseiulus persimilis* to plants with *Macrolophus pygmaeus*

When adults of *M. pygmaeus* were present on the plants, *P. persimilis* again preferred plants exposed to *M. pygmaeus* (FIGURE 4.3a, GLM: χ^2 = 8.61, d.f. = 1, P = 0.0034). When subsequently, the omnivores were removed from the plants and their attraction was tested again the following day (FIGURE 4.3b), the preference for mirid-exposed plants was even somewhat more pronounced (cf. panels 4.3a and b), but

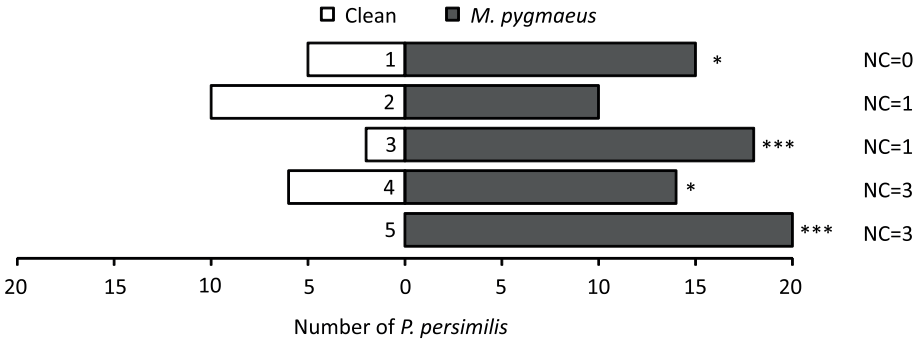

FIGURE 4.1 Numbers of *Phytoseiulus persimilis* responding to the volatiles emanating from two groups of plants: clean plants (white bars) vs. plants previously exposed to *M. pygmaeus* (grey bars). Each bar represents the result of a different replicate, each with new groups of plants and predatory mites. NC indicates the number of predatory mites that did not respond. Significant preference for one of the two volatiles is indicated by asterisks (*: 0.01 < P < 0.05; ***: P < 0.001)

this difference was not significant (FIGURE 4.3, GLM: $\chi^2 = 1.66$, d.f. = 1, $P = 0.20$). The results presented in FIGURE 4.3b further confirmed earlier results showing the attraction of *P. persimilis* to plants previously exposed to *M. pygmaeus* without these mirids being present on the plants (FIGURE 4.3b, GLM: $\chi^2 = 12.8$, d.f. = 1, $P = 0.0003$, cf. FIGURE 4.1).

Effect of experience of *Phytoseiulus persimilis*

Phytoseiulus persimilis with and without experience with the volatiles from plants exposed to *M. pygmaeus* showed different attractiveness to plants previously exposed to the omnivore and clean plants (FIGURE 4.4, GLM: $\chi^2 = 17.40$, d.f. = 2, $P =$

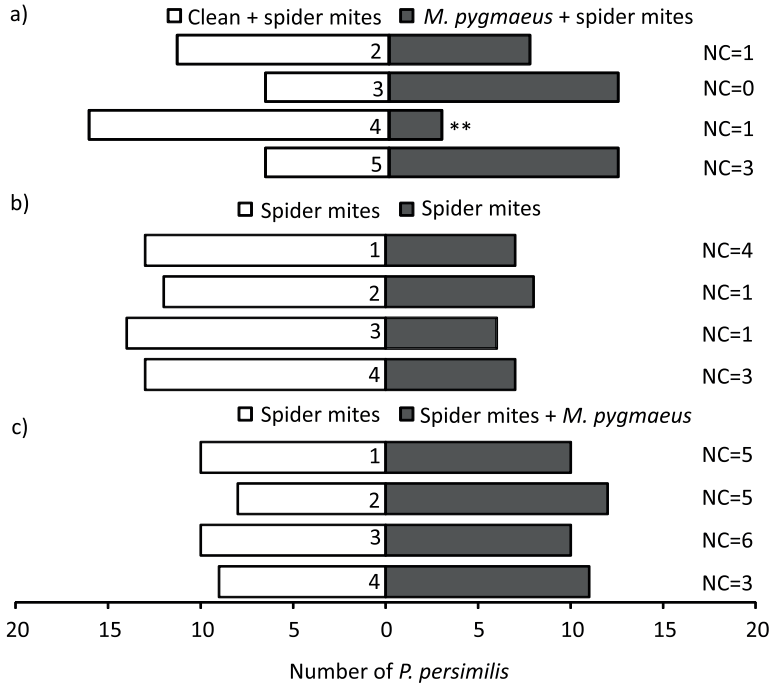

FIGURE 4.2 Numbers of *Phytoseiulus persimilis* responding to the volatiles emanating from two groups of plants: (a) clean plants subsequently infested with spider mites for 2 days (white bars) vs. plants previously exposed to *Macrolophus pygmaeus* for 4 days and subsequently infested with spider mites for 2 days (grey bars); (b) plants infested with spider mites for 2 days (white bars) vs. plants infested by spider mites for two days (grey bars); (c) plants infested with spider mites for 6 days (white bars) vs. plants infested with spider mites for 6 days and exposed to *M. pygmaeus* for the last 4 days (grey bars). Within each panel, each bar represents the result of a different replicate, each with new groups of plants and predatory mites. Bars with the same numbers in (a) and FIGURE 4.1 share the same groups of plants, and bars with the same numbers in (b) and (c) share the same groups of plants. NC indicates the number of predatory mites that did not respond. Significant preference for one of the two volatiles is indicated by asterisks (**: $0.001 < P < 0.01$)

0.00017). Naïve *P. persimilis,* without experience with *M. pygmaeus*-treated plants, starved for 1 h prior to the test, showed a significant preference to plants exposed to *M. pygmaeus* (FIGURE 4.4a, GLM: $\chi^2 = 42.2$, d.f. = 1, $P < 0.0001$). This is consistent with previous results (FIGURE 4.1, 4.3b), again confirming that *P. persimilis* pre-

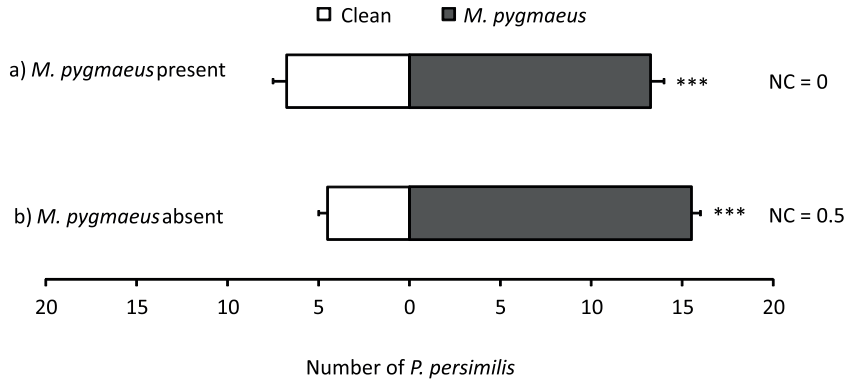

FIGURE 4.3 Average numbers (+ SE) of *Phytoseiulus persimilis* responding to the volatiles emanating from clean plants (white bars) and plants exposed to *Macrolophus pygmaeus* (grey bars), with (a) and without (b) *M. pygmaeus* present on the plants. NC indicates the average number of predatory mites that did not respond. Each bar represents the average of 4 replicates (*M. pygmaeus* present) or 2 replicates (*M. pygmaeus* absent), each with new groups of plants and predators. Significant preferences for one of the two volatiles are indicated by asterisks (***: $P < 0.001$)

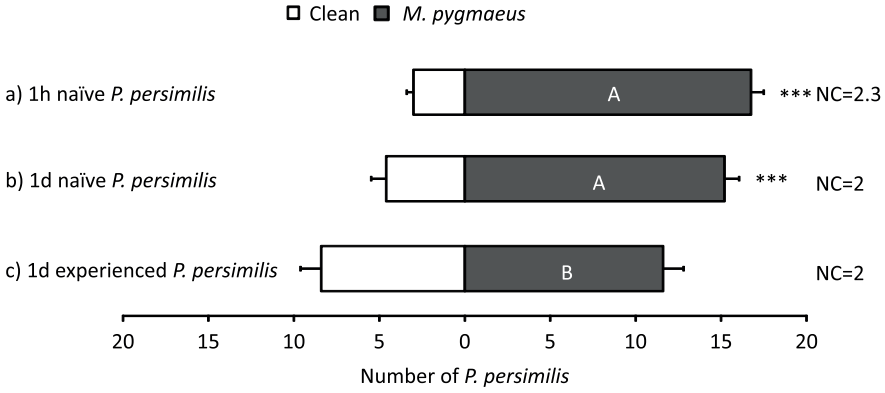

FIGURE 4.4 Response of *Phytoseiulus persimilis* (mean + SE) to volatiles of clean plants (white bars) and of plants exposed to *Macrolophus pygmaeus* (grey bars). Shown are results of three groups of predatory mites with different experience with volatile cues from plants exposed to *M. pygmaeus*: (a) *P. persimilis* without experience, starved for 1h; (b) *P. persimilis* without experience, starved for 1 day; (c) *P. persimilis* experienced with the volatiles from plants with *M. pygmaeus* in the absence of food for 1 day. Each bar represents the average of 4 (1 h naïve) or 5 replicates (1d naïve and 1d experienced), each with new groups of plants and predators. NC indicates the average number of predatory mites that did not respond. Different capital letters indicate significant differences among the three groups of *P. persimilis* (contrasts after GLM: $P < 0.05$). Significant preferences for one of the two volatiles are indicated by asterisks (***: $P < 0.001$)

TABLE 4.1 Mean (± SE) levels of volatiles from plants exposed to the omnivore (*Macrolophus pygmaeus*) with either the omnivore absent or present during volatile collection relative to clean, unexposed plants

Compound	RT (min)	Kovats Index	*M. pygmaeus* removed	*M. pygmaeus* present
Monoterpene 1	8.5	1023.5	2.6 ± 0.9	1 ± 0.7
(*E*)-β-ocimene † ***	8.6	977.7	11.6 ± 4.4 (b)	40.6 ± 15.9 (c)
Linalool † **	9.2	1007.2	3.2 ± 0.6 (b)	9.5 ± 3.9 (b)
Monoterpene 2 **	9.7	1128.3	3.3 ± 1.2 (ab)	9.5 ± 3.1 (b)
MeSA †	10.3	1200.4	2.7 ± 1	4.8 ± 0.9
E-Jasmone ***	12.2	1403.2	80.1 ± 17.9 (b)	323.1 ± 161.2 (b)
β-Caryophyllene †	12.4	1418.8	0.4 ± 0.2	1.1 ± 0.6
Phenylpropene 1	12.7	1432.6	1 ± 0.3	0.3 ± 0.1
Sesquiterpene 1 *	13.0	1503.3	0.5 ± 0.2 (ab)	0.4 ± 0.3 (b)
Sesquiterpene 2 *	13.1	1507.7	18.9 ± 11.5 (ab)	60.2 ± 30.3 (b)
Monoterpene 3	13.5	1534.3	0.4 ± 0.1 (a)	1.3 ± 0.5 (b)
Nerolidol † **	13.5	1535.9	1.6 ± 0.4 (a)	5.5 ± 1.7 (b)
Alkane 1 *	14.0	1611.7	0.4 ± 0.1 (b)	0.4 ± 0.1 (ab)
Alkane 2	14.0	1617.0	1.1 ± 0.5	8.9 ± 6.8
Phenylpropene 2	14.8	1720.8	1.4 ± 0.5	0.3 ± 0.2
Terpene	15.6	1824.7	1.1 ± 0.5	0.2 ± 0.1

†: Compounds identified with standards

Asterisks indicate significant effects of treatment on the relative levels of volatiles from three groups of plants (*: $0.01 < P < 0.05$; **: $0.001 < P < 0.01$; ***: $P < 0.001$).

Letters in parentheses indicate significant differences between treatments of a compound: (a) no difference from clean; (b), (c): different from clean.

ferred plants exposed to *M. pygmaeus* over clean plants. When naïve *P. persimilis* were starved for 24 h, they were also attracted to plants exposed to *M. pygmaeus* (FIGURE 4.4b, GLM: $\chi^2 = 29.9$, d.f. = 1, $P < 0.0001$). The two groups of naïve mites showed similar attractiveness to plants exposed to *M. pygmaeus* (FIGURE 4.4a,b, contrasts with glht function of package lsmeans). When given 1day of experience with the volatiles from plants exposed to *M. pygmaeus* in the absence of food, the preference of *P. persimilis* for these plants disappeared (FIGURE 4.4c, GLM: $\chi^2 = 2.57$, d.f. = 1, $P = 0.11$). The response of the experienced predatory mites differed significantly from the two groups naïve mites (FIGURE 4.4, contrasts as above).

Volatile analysis

The 16 volatiles with the largest differences between the clean plants and the exposed plants are presented in TABLE 4.1. The discriminant analysis based on these 16 compounds resulted in a perfect separation of the three groups (FIGURE 4.5), with the first linear discriminant explaining 97% of the variance. Exposed plants produced significantly higher amounts of (*E*)-β-ocimene, linalool, E-jasmone and nerolidol than clean plants (TABLE 4.1). Plants on which the omnivores were present produced higher amounts of these volatiles than plants with the omnivores removed, but this difference was not always significant (TABLE 4.1). Exposed plants also produced higher

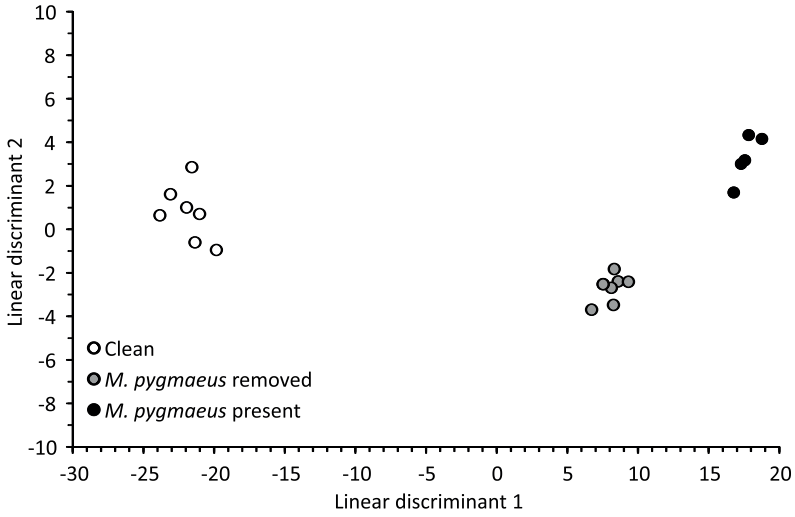

FIGURE 4.5 Grouping of the three treatments as a function of two linear discriminants, based on the 16 selected volatile compounds (see text)

amounts of other compounds than clean plants, but the differences were not significant. In contrast, the exposed plants produced less sesquiterpene 1 and alkane 2 than did clean plants (TABLE 4.1, averages < 1).

Discussion

We show that volatiles of sweet pepper plants exposed to *M. pygmaeus* are attractive to the predatory mite *P. persimilis,* although we observed differences among replicates in the attractiveness (FIGURE 4.1a). This might have been caused by the different survival rate of the *M. pygmaeus* during the plant treatments in different replicates. Lower numbers of *M. pygmaeus* feeding on the plants may induce lower amounts of volatiles, which might be less attractive to predatory mites. However, overall, predatory mites preferred the *M. pygmaeus*-infested plants over clean plants, and this preference was consistently confirmed in subsequent experiments (FIGURE 4.3b, 4.4a). Thus, we conclude that *P. persimilis* was more attracted by the volatiles emanating from plants previously exposed to *M. pygmaeus* than to volatiles from clean plants. This suggests that the presence of *M. pygmaeus* could potentially interfere with the long-distance searching behaviour of the predatory mite, which will be attracted to plants without its prey.

Plants exposed to the omnivore produced different amounts of volatiles than clean plants. The headspace of plants exposed to the omnivore contained higher amounts of (*E*)-β-ocimene, linalool, and nerolidol than that of clean plants. These compounds are known to be produced by plants under attack by herbivores, including sweet pepper plants (Dicke et al. 1990; Turlings and Tumlinson 1991; van den Boom et al. 2004). Moreover, linalool and (*E*)-β-ocimene are known to be attractive to *P. persimilis* (Dicke et al. 1990). Another omnivorous predator, *Orius laevigatus*, was found to induce the production of linalool, MeSA, and nerolidol in sweet pepper plants (Bouagga et al. 2018). The exposed plants produced several volatiles in lower amounts than clean plants, suggesting that plant feeding by the omnivore changed ratios of the volatile blends. When the omnivores were present on the plants during volatile collection, both plant volatiles and volatiles from the omnivore were collected. A study by Moayeri et al. (2007) detected 12 volatile compounds from *M. pygmaeus*; the presence of these compounds in the volatile blends collected here awaits further analysis, involving standards of these compounds.

When plants that had been exposed to the omnivore were subsequently attacked by spider mites, the prey of the predatory mites, the preference for plants exposed to the omnivore disappeared. When plants were first attacked by spider mites and were subsequently exposed to *M. pygmaeus*, they became somewhat more attractive than before exposure. All in all, these results show that suggest that *P. persimilis* remains attracted to plants with spider mite prey, independent of the omnivore. We observed differences among replicates in the attractiveness of volatiles from plants exposed to *M. pygmaeus* and subsequently attacked by spider mites (FIGURE 4.2a). This might have been caused by the different numbers of spider mites during the treatment in different replicates, and lower densities of spider mites resulting in lower amounts of volatiles (Maeda and Takabayashi 2001), making the plants less attractive for predatory mites. We indeed observed lower numbers of spider mites on plants exposed to *M. pygmaeus* than on unexposed plants (126 ± 12.9 vs. 171 ± 7.4, respectively), which was consistent with earlier results showing that spider mites performed less well on plants previously exposed to *M. pygmaeus* than on clean plants (Zhang et al. 2018 [= CHAPTER 2]). Overall, these results show that *P. persimilis* was still attracted to plants with its spider mite prey, irrespective of the presence of the omnivore.

When the omnivores were present on the plants, predatory mites could also perceive cues from the omnivores themselves present on the plants. Plants with the omnivores were still significantly attractive to the predatory mites, but this attractiveness was slightly less pronounced than that to predator induced plants without the omnivore (FIGURE 4.3), however, this difference was not significant. We therefore conclude that cues from the omnivores themselves did not interfere significantly with the choice of the predatory mites.

Because of natural variation in volatile blends, depending on host plant species, herbivore species, plant age and other factors factors (Takabayashi et al. 1994a; De Moraes et al. 1998; van den Boom et al. 2004), natural enemies face the difficult task to discriminate among many different blends (Sabelis et al. 1999; Takabayashi et al. 2006). It is often suggested that the ability to learn to respond to volatile cues associated with their prey or host increases their searching efficiency, which is very important for many predatory arthropods and parasitoids (Papaj and Lewis 1993; Drukker et al. 2000a,b; Takabayashi et al. 2006; Glinwood et al. 2011; Janssen et al. 2014). Given 24 h of experience with the volatiles from plants with *M. pygmaeus* in the absence of prey, *P. persimilis* lost the preference for the volatiles of these plants, whereas the non-experienced predatory mites were still attracted by these plants after 1 and 24 h starvation. This shows that the predatory mites probably associated the volatiles with the absence of food. This suggests that predatory mites that would initially be naïve and attracted to plants with the omnivore, but without food, would subsequently no longer be attracted to such plants.

An open question is why predatory mites were attracted by volatiles emanating from plants exposed to the omnivores. The volatile analysis showed that plants exposed to *M. pygmaeus* produced several volatile compounds for example (*E*)-β-ocimene, linalool, MeSA that are similar to those produced by plants infested with spider mites, and are known to attract *P. persimilis* (Sabelis and van de Baan 1983; Dicke et al. 1990; van den Boom et al. 2004; van Wijk 2007). However, plants produce volatile blends that differ in composition and in quantity upon attack by herbivores of different species feeding on the same plant species (Sabelis and van de Baan 1983; Takabayashi et al. 1991) and by the same herbivore species feeding on different plant species (Takabayashi et al. 1994a; van den Boom et al. 2004). So possibly plants will likely also respond differently to omnivore damage and herbivore damage, and indeed different blends of volatiles were detected in this study. An explanation for the attraction of the predators to plants exposed to the omnivore is that the predators cannot discriminate among the many different volatile blends produced by plants. The prey of the predator tested here, *T. urticae*, is highly polyphagous and induces the production of quantitatively and qualitatively different plant volatiles in different host plants (van den Boom et al. 2004). It might be impossible for the predatory mites to response innately to all these volatile blends induced by spider mites, and it has been suggested that learning the association of the volatiles with the presence of prey helps the predators finding plants with suitable prey (Krips et al. 1999; Drukker et al. 2000a,b; de Boer et al. 2005). Negative experience with volatiles from non-prey plants without the presence of prey may result in aversion to non-prey host plants (Zanen and Cardé 1991; Drukker et al. 2000ab). This may be the case in our study: after experience with volatiles from plants with *M. pyg-*

maeus, the preference for the volatiles produced by these plants disappeared. Besides experiencing the association of *M. pygmaeus*-induced plant volatiles with the absence of food, *P. persimilis* may also have experienced an association of the volatiles with cues left by *M. pygmaeus* on the leaves, such as faeces.

This is not the first study showing that omnivores can induce the production of plant volatiles. Pérez-Hedo et al. (2015a) found indications that the parasitoid *Encarsia formosa* was attracted by tomato plants exposed by each of three species of mirid bugs. Another study by Moayeri et al. (2007) showed that, compared to clean plants, 11 additional compounds were produced when *M. pygmaeus* was feeding on bean plants. More recently, Bouagga et al. (2018) found that another omnivorous predator *Orius laevigatus* induced the production of volatiles by plants. Together with this study, these results confirm that volatile production was induced by plant feeding of the omnivorous predators. Earlier, we also found that the spider mites and thrips preferred clean plants over plants previously exposed to *M. pygmaeus* (CHAPTER 3), and these responses may have been partly or entirely based on the volatile produced by these plants. We conclude that plant volatiles induced by *M. pygmaeus* play a role in plant-herbivore-predator interactions. In this study, much lower numbers of omnivores were used than in the study by Pérez-Hedo et al. (five females and five males here vs. 100), which still resulted in strong effects on the behaviour of other predators. This is remarkable, because these low densities of omnivores hardly cause any damage to the plants (Castañé et al. 2011), and much higher numbers of herbivores are often used to induce direct plant defences or the induction of volatile production (Maeda and Takabayashi 2001).

Cues from prey and plants with prey are important information for prey patch choice, however, cues associated with the presence of competitors also affect patch selection. Avoidance of patches occupied by competitors occurs in parasitoids (Janssen et al. 1995a,b; Tamò et al. 2006) and predatory mites (Janssen et al. 1997), and prey patches with cues associated with spider mites eggs killed by competitors were less attractive for *P. persimilis* (Choh et al. 2017). In our study, spider mites-infested plants were subsequently exposed to *M. pygmaeus,* thus cues associated with killed spider mite eggs were present on these plants. Moreover, other cues associated with the presence of the mirids, e.g., volatiles, chemical marks and faeces from *M. pygmaeus* (Kats and Dill 1998; Moayeri et al. 2007), may also serve as cues for predatory mites. We observed that *M. pygmaeus* can prey on all stages of *P. persimilis*, also in the presence of spider mites. We therefore expected that the predatory mites would avoid plants with the omnivores, but instead, plants with the omnivores were more attractive for *P. persimilis* than clean plants. However, we observed that adult predatory mites could escape from the omnivores (NX. Zhang & J. Brouwer pers. obs.). Thus, *M. pygmaeus* is perhaps not a serious threat for adult female *P. per-*

similis. Furthermore, the predatory mites oviposit inside the web produced by spider mites and use it as a refuge to prevent predation from other predators (Sabelis 1985; Sabelis and Bakker 1992; Cloutier and Johnson 1993; Roda et al. 2000; Lemos et al. 2015), hence, even other stages of the predatory mite may be able to escape from predation by the omnivore. We showed that the presence of *M. pygmaeus* can interfere with the searching behaviour of *P. persimilis*, because the adult female predatory mites are attracted to plants with the omnivore but without prey. However, the adult female predatory mites then gain experience with the volatiles associated with the lack of food, and are subsequently no longer attracted to such plants. Depending on the capacity of the predatory mites to learn to discriminate between plants induced by spider mites and plants induced by *M. pygmaeus*, this may interfere with the biological control of spider mites with *P. persimilis*. This deserves further confirmation.

It is also not clear why plants would produce volatiles when omnivorous predators are feeding on them. One suggested function of herbivore-induced plant volatiles is to attract natural enemies, and one may argue that the plants guarded by the omnivorous predators do not need to produce volatiles to attract more natural enemies. Another suggested function is signalling between different plant parts (Frost et al. 2007), through which unattacked and undefended parts can become primed against future attacks. It is also unclear why these distant parts should be primed when omnivores are present on the plant. Possibly, plants do not produce volatiles to attract natural enemies, but to signal to herbivores that the plant is defended. This was confirmed by our earlier study that spider mites and thrips preferred clean plants over plants previously exposed to *M. pygmaeus* (CHAPTER 3). Pérez-Hedo et al. (2015b) showed that tomato plants damaged by the omnivorous predator *Nesidiocoris tenuis* were less attractive to the whitefly *Bemisia tabaci.* The repellence of herbivores would not only reduce herbivore damage to the plant, but also prevent the transmission of plant pathogens by herbivores. However, without experience, some predators may take this information as indication of the presence of herbivores, which might be the case for *P. persimilis*.

Overall, we conclude plants exposed to the omnivorous predator *M. pygmaeus* are attractive to *P. persimilis* and this might disrupt control of spider mites by this predatory mite. Our earlier study showed that omnivore feeding on the plant interfered with the performance of herbivores through induced direct defences, and the results of the current study suggest that they also induce the production of plant volatiles.

ACKNOWLEDGEMENTS – We thank Enza Zaden Beheer B.V. for the supply of sweet pepper seeds, Koppert Biological Systems for the supply of *Macrolophus* and *Ephestia* eggs, Alexandra Revynthi for spider mites and predatory mites, Robert Schuurink for

providing the GC-MS machine, Peter de Ruiter for comments on the manuscript, and the population biology group for discussions. NXZ received a scholarship from the China Scholarship Council (CSC)

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Supplementary material Ch. 4

Methods

Tree analysis

The separation of the three treatments based on the volatiles collected was further confirmed with a tree analysis (Ripley 2016). In short, the data were successively divided into groups using the explanatory variables, in this case the volatiles, and the value of these variables that maximally separate the response variable, in this case the three treatments. In contrast to the linear discriminant analysis presented in the main text, this analysis thus results in those single volatiles that separate the three treatments best.

Partial Least Square Discriminant Analysis

Another discriminant analysis was performed with the full data set. Peak areas that were higher than 1000 counts were selected, resulting in 99 compounds. The areas were corrected for the internal standard and log transformed prior to the analysis. Peak areas of the 99 compounds were analysed with a Partial Least Square Discriminant Analysis (PLSDA) in MetaboAnalyst 4.0 (http://www.metaboanalyst.ca). Compounds were selected according to their importance in the linear discriminants (components 1 and 2) that separated the treatments.

Results

The volatile that separated the three treatments best was (*E*)-β-ocimene, which was produced in low amounts by the clean plants and in higher amounts by the two groups of exposed plants. Two cases of the treatment with the omnivore removed were misclassified as being cases with the omnivore present (FIGURE S4.1). To further investigate other volatiles, we constructed a classification tree without (*E*)-β-ocimene. This showed that *E*-jasmone also separated the clean plants from the exposed plants and nerolidol subsequently separated the two exposed groups, also with two misclassifications. Removal of *E*-jasmone resulted in poor separation of the clean plants from the exposed plants. Further removal of volatiles showed that linalool, monoterpene 2 and phenylpropene 1 all separated the two exposed groups with two misclassifications. In conclusion, the main compounds that separated clean plants from exposed plants were (*E*)-β-ocimene and *E*-jasmone. Most of the volatiles with significantly higher peaks in exposed plants (TABLE 4.1) were also important in separating the clean plants from the exposed plants.

The PLSDA analysis separated the three treatments, and the two first components explained 40% of the variance (FIGURE S4.2). Component 1 separated the three treat-

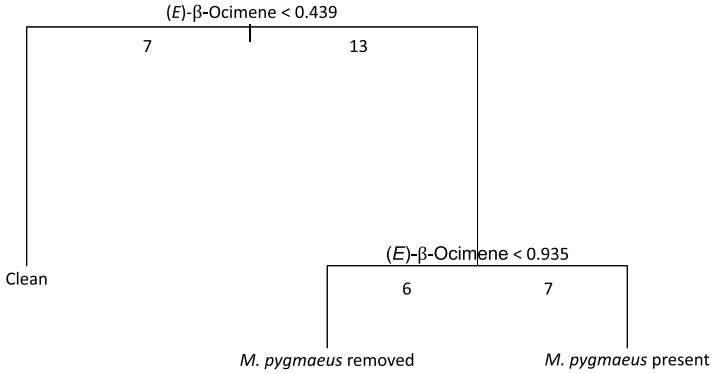

FIGURE S4.1 A tree analysis of the separation of clean plants and plants exposed to the omnivore, either with the omnivore present or removed, based on the volatiles produced by the plants. For the analysis, log-transformed peak areas relative to the clean plants were used. The first separation is between the 7 clean plants and the 13 exposed plants, which produced more (*E*)-β-ocimene. Numbers going into each group are given under the bifurcation. Six of the eight plants without the omnivore (*Macrolophus pygmaeus* removed) produced less (*E*)-β-ocimene than plants with the omnivore present, and were classified correctly. The five plants with the omnivore present plus two plants from which the omnivores were removed were classified as plants on which the omnivore was present

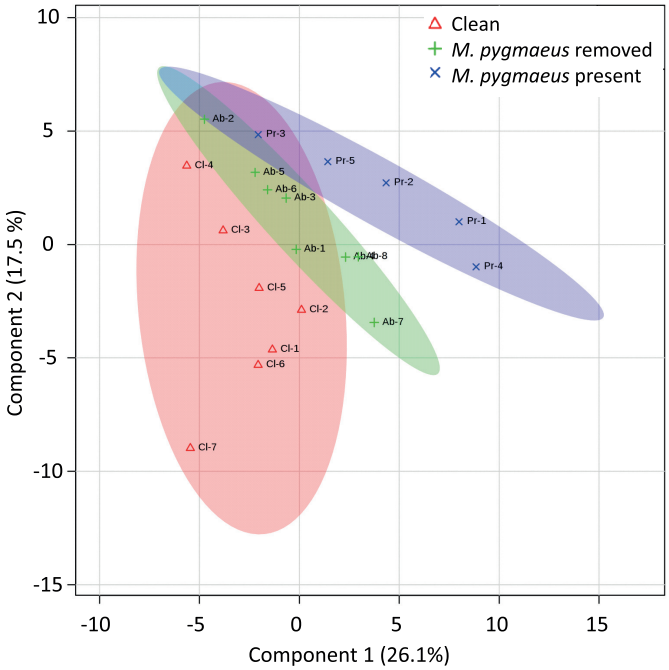

FIGURE S4.2 Grouping of the three treatments based on component 1 and component 2 from the PLSDA analysis. Triangles correspond to clean plants, plusses to exposed plants from which the omnivores were removed before volatile collection and crosses to plants on which the omnivores were present during volatile collection

TABLE S4.1 Compounds in component 1 and component 2 for separation of the three treatments in the PLSDA analysis

Compound	RT (min)	Kovats Index	Comp. 1	Comp. 2
E-β-Ocimene	8.6	977.7	3.12	2.63
E-Jasmone	12.2	1403.2	3.08	2.67
Linalool	9.2	1007.2	2.42	1.95
Sesquiterpene-1	13.0	1503.3	2.20	1.71
Nerolidol	13.5	1535.9	2.11	1.76
Monoterpene-2	9.7	1128.3	2.10	1.69
Sesquiterpene-2	13.1	1507.7	1.89	1.46
Phenylpropene-1	12.7	1432.6	1.80	1.39
Alkane-1	14.0	1611.7	1.75	1.37
Phenylpropene-2	14.8	1720.8	1.59	1.23
C153	15.6	1827.5	1.49	1.18
C129	14.1	1624.8	1.47	1.15
C104	12.7	1435.0	1.46	1.18
C154	15.8	1839.1	1.35	1.12
C22	7.9	951.6	1.32	1.15
Methyl heptenone	7.8	947.6	1.25	1.06
C77	10.8	1231.6	1.22	1.07
Alkane	11.5	1319.2	1.22	0.98
MeSA	10.3	1200.4	1.21	0.96
Alkane	15.3	1750.6	1.16	0.94
C24	7.9	953.6	1.10	1.18
C63	10.1	1144.7	1.08	1.23
Bezyl alcohol	8.4	1019.7	1.02	1.91
C96	12.0	1347.1	0.97	1.20
Alkane-2	14.0	1617.0	0.89	0.85
Sesquiterpene	9.3	1108.5	0.85	1.59
C58	9.7	1127.4	0.47	1.15

Compounds in **bold** are also presented in TABLE 4.1 in the main text.

ments (FIGURE S4.2). Most of the compounds that were important for separating the three groups were also found in the analysis in the main text (TABLES 4.1 and S4.1). (*E*)-β-ocimene was again one of the main compounds that differentiated between treatments (TABLE S4.1).

Reference

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