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Invasive insect herbivores as disrupters of chemically-mediated tritrophic interactions: effects of herbivore density and parasitoid learning

Gaylord A. Desurmont · Antoine Guiguet · Ted C. J. Turlings

Abstract Invasive insect herbivores have the potential to interfere with native multitrophic interactions by affecting the chemical cues emitted by plants and disrupting the attraction of natural enemies mediated by herbivore-induced plant volatiles (HIPVs). In a previous study, we found that the presence of the exotic herbivore *Spodoptera littoralis* on *Brassica rapa* plants infested by the native herbivore *Pieris brassicae* makes these dually-infested plants unattractive to the main parasitoid of *P. brassicae*, the braconid wasp *Cotesia glomerata*. Here we show that this interference by *S. littoralis* is strongly dependent on the relative densities of the two herbivores. Parasitoids were only deterred by dually-infested plants when there were more *S. littoralis* larvae than *P. brassicae* larvae on a plant. Furthermore, the blend of HIPVs emitted by dually-infested plants differed the most from HIPVs emitted by *Pieris*-infested plants when *S. littoralis* density exceeded *P. brassicae* density. We further found that associative learning by the parasitoid affected its preferences: attraction to dually-infested plants increased after parasitoids were presented a *P. brassicae* caterpillar (rewarding experience) in presence of the odor of a dually-infested plant, but not when presented a *S. littoralis* caterpillar (non-rewarding experience). A non-rewarding experience prior to the bioassays resulted in a general decrease in parasitoid motivation to respond to plant odors. We conclude that herbivore density and associative learning may play an important role in the foraging behavior of natural enemies in communities, and such effects should not be overlooked when investigating the ecological impact of exotic species on native food webs.

Keywords Insect–plant interactions · VOCs · Infochemical networks · Range expansion · Invasion ecology

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Introduction

Exotic insect herbivores can impact the interactions among native organisms in multiple ways (Gandhi and Herms 2010; Kenis et al. 2009). By establishing and feeding on native plants, exotic herbivores can affect

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native community members belonging to different trophic levels through plant-mediated effects (Chabaane et al. 2014; Gols and Harvey 2009). For example, herbivory by an exotic herbivore may trigger plant defenses, making it less suitable for subsequent native herbivores (McArt et al. 2013). Similarly, plant-mediated effects may affect pollinators, if herbivory by the exotic herbivore renders the plant less attractive or less rewarding to flower visitors (Schiestl et al. 2014).

The effects of exotic herbivores on the emissions of plant volatile organic compounds (VOCs) and on VOC-mediated insect–plant interactions are of particular interest to understand the indirect impacts of invasive species. Such interactions can take a variety of forms. For example, constitutive or induced plant volatiles can attract or deter insect herbivores in search of host plants (Bruce et al. 2005). Floral odors, on the other hand, are signals aimed at attracting mutualistic pollinators (Schiestl 2010). Finally, herbivore-induced plant volatiles (HIPVs) are known to play a key role in the attraction of natural enemies (predators and parasitoids) to herbivore-damaged plants (Hare 2011; Mumm and Dicke 2010; Turlings and Wäckers 2004).

Exotic herbivores could potentially affect all these interactions by altering the volatile cues emitted by native plants (Desurmont et al. 2014). In a recent study, we found that the generalist herbivore *Spodoptera littoralis*, which is native to Africa and, as a result of global warming, currently expands its range in Europe where it is considered invasive (CABI Invasive Species Compendium: http://www.cabi.org/isc/datasheet/51070), interferes with the attraction of the braconid parasitoid *Cotesia glomerata* to *Brassica rapa* plants infested with caterpillars of *Pieris brassicae*, the main host of the parasitoid. The parasitoid is highly attracted to plants that are only infested by *P. brassicae*, but was found not to be attracted to plants damaged by *S. littoralis*, nor to plants dually-infested by *S. littoralis* and *P. brassicae* (Chabaane et al. 2014). This effect, which corresponds to a change in the blend of volatiles emitted by dually-infested plants, may lead to a reduced foraging efficiency of the parasitoid, which in turn may affect the regulation of *P. brassicae* populations in nature. Here, we further explore how *S. littoralis* may affect the interactions between *B. rapa*, *P. brassicae*, and *C. glomerata* in nature, by evaluating the effects of two factors likely to play a key role in the interference under field conditions: herbivore density and associative learning by the parasitoid. Females of *S. littoralis* are known to lay large egg masses that can contain hundreds of eggs, and *S. littoralis* outbreaks have been documented in agroecosystems in its native and invasive range, indicating that this insect can sometimes occur at very high densities. However, *S. littoralis* is a highly generalist herbivore that may not predictably infest the host plants of *P. brassicae*. Therefore, the frequency of co-occurrences of *S. littoralis* and *P. brassicae* on the same plants in nature and the densities of *S. littoralis* on dually-infested plants are likely to be highly fluctuant in the invasive range of *S. littoralis*.

HIPVs are to some extent herbivore density-dependent. More herbivores feeding on a plant means more damage, which typically results in more volatiles released into the atmosphere (Girling et al. 2011; Gouinguené et al. 2003; Shiojiri et al. 2010). Qualitatively, volatiles released in response to high levels of herbivory may also differ from the ones released in response to low herbivory (Horiuchi et al. 2003; Niinemets 2010). In a multi-herbivore context (i.e. more than one herbivore species feeding on a same plant), the respective densities of each herbivore present are likely to play an important role in the blend of volatiles released by the plant, both qualitatively and quantitatively (Holopainen and Gershenzon 2010; Ponzio et al. 2014). For a parasitoid that tracks the volatile cues specifically associated with its host herbivore (e.g. specific compounds or specific ratios of compounds), the presence of a non-host herbivore on the same plant may affect the perception or integration of these cues in a density-dependent manner (De Rijk et al. 2016). Non-host herbivores on a plant may mean "noise" in the HIPV search image targeted by the parasitoid (Desurmont et al. 2015; Dicke et al. 2009), and this noise can be expected to increase with the number of non-host herbivores on the plant. Alternatively, non-host herbivores that manipulate plant defenses systematically (Chung et al. 2013) or trigger highly specific plant defensive pathways may lead to a strong alteration of the HIPVs emitted by a plant even at low densities (Desurmont et al. 2016): in such cases, interference effects may mainly depend on the presence or absence of the non-host herbivore. In general, signal-to-noise ratios are expected to play an important role in parasitoid foraging decisions when navigating complex olfactory landscapes (Beyaert and Hilker 2014).

Many parasitoid species are known for their ability to learn from previous experiences and to use learning as a means to improve their foraging efficiency throughout their lifetime (Geervliet et al. 1998; Meiners et al. 2003). Associative learning typically occurs when a parasitoid encounters a host (i.e. rewarding experience) in presence of odorous or visual cues it may not be innately attracted to, and develops a preference for these cues. This preference may be strengthened by subsequent host encounters in the same olfactory or visual context. A single encounter with a host is sufficient to elicit the formation of long-term memory in several parasitoid species, including *C. glomerata* (Bleeker et al. 2006; Smid et al. 2007). We therefore hypothesized that *C. glomerata* may overcome its innate avoidance of plants dually infested by *P. brassicae* and *S. littoralis* through associative learning. However, foraging on a dually-infested plant means that the parasitoid may encounter the non-host herbivore on the plant, which constitutes a non-rewarding experience. How parasitoids integrate non-rewarding foraging experiences in their olfactory preferences throughout their lifetime is still poorly known, but is a key notion for understanding the evolution of parasitoid foraging strategies in a multi-herbivore context (de Rijk et al. 2013; Takabayashi et al. 2006; Vet et al. 1995). So far it appears that non-rewarding experiences do not necessarily result in negative reinforcement and stronger avoidance of the associated odor (Costa et al. 2010), but encountering a non-host herbivore may lead the parasitoid to leave the plant shortly after the encounter, preventing further herbivore encounters on that plant (Bukovinszky et al. 2012).

Here, we investigated the effects of herbivore densities and associative learning on the interference by *S. littoralis* larvae with the attraction of *C. glomerata* to *Pieris*-infested plants. We first tested the respective attractiveness of dually-infested plants with different ratios of *S. littoralis/P. brassicae* caterpillar numbers, and we compared the volatiles released by these plants to plants damaged only by *P. brassicae* or only by *S. littoralis*. Specifically, we asked: (1) does the number of *S. littoralis* present on a dually-infested plant affect parasitoid preferences, and (2) do volatiles from dually-infested plants differ from volatiles from plants infested by *P. brassicae*, and does this odor divergence accentuate as the number of non-hosts increases on the dually infested plant? We

then tested the effects of associative learning by evaluating the olfactory preferences of *C. glomerata* after a rewarding or non-rewarding experience in presence of the odors of a dually infested *B. rapa* plant. Specifically, we asked the following questions: (3) does the attractiveness of dually infested plants increase once parasitoids have received a rewarding experience in presence of their volatile cues, and (4) do non-rewarding experiences change the olfactory preferences of parasitoids in any way?

Materials and methods

Insect and plant material

Plants used in the study came from a wild accession of *Brassica rapa* grown from seeds that were collected in 2009 and 2012 near Maarssen, the Netherlands. Plants were kept in controlled growth chambers under 16/8 L:D light regime at 25 °C, light intensity 240–260 μmol. Plants were grown in fertilized commercial soil (Ricoter Aussaaterde, Aarberg, Switzerland) in cylindrical plastic pots (4 cm diam, 10 cm high) to fit in the olfactometer. They were watered every other day without supplemental nutrients. Plants used for experiments had three to five fully expanded leaves (approx. 3 weeks old).

Pieris brassicae caterpillars originated from a rearing maintained in our laboratory since 2011. The rearing was initiated with individuals from another laboratory rearing maintained on *Brassica oleracea* for an unknown number of generations at the University of Zürich (Switzerland), and from individuals collected in the field in the Neuchâtel area (Switzerland). Caterpillars were maintained in our rearing with *Brassica rapa* var. *pekinensis* (Chinese cabbage) as a host plant, at a 20–24 °C temperature range and under a 16:8 L:D photoperiod, and had 8–10 generations per year. Every year the rearing was supplemented with field-collected individuals from the Neuchâtel area (Switzerland). *Spodoptera littoralis* caterpillars came from eggs kindly provided weekly by Syngenta AG (Stein, Switzerland). The number of generations *S. littoralis* spent under laboratory conditions in the Syngenta AG rearing is unknown. Newly-hatched *S. littoralis* were fed with an artificial diet at a 20–24 °C temperature range and under a 16:8 L:D photoperiod until they were needed for experimental purposes.

Cotesia glomerata parasitoids originated from a laboratory colony started in 2012 with field-collected individuals from the Neuchâtel area and supplemented yearly with new field-collected individuals (8–10 generations per year). *Cotesia glomerata* parasitoids were maintained on *P. brassicae* larvae from our rearing. Parasitoid cocoons were collected after emergence from parasitized caterpillars and were placed in a cage without any host or plant material. Newly-emerged adults were provided water and droplets of honey and were kept in an incubator at 25 °C for 48 h, then in an incubator at 13 °C. Mated naive females that were 2–4 weeks old were used for experiments to test the effects of herbivore density. Parasitoids used for associative learning bioassays were 1–2 weeks old.

Olfactometer tests

To test the effects of herbivore density on *C. glomerata* preferences, naïve females were placed in a 4-arm olfactometer (Turlings et al. 2004). In this setting, four individual air flows (0.8L/min) were connected to glass bottles in which the different treatments were placed, and all air flows converged to a central glass arena where the wasps were released. Here female parasitoids were given the choice between 4 treatments: the odor of (1) a plant infested by *P. brassicae*, (2) a plant dually-infested by *P. brassicae* and *S. littoralis* simultaneously, (3) an undamaged plant, and (4) clean air (=empty bottle). The *P. brassicae* plant was infested with 20 first instar caterpillars and the dually-infested plant was infested with 20 *P. brassicae* caterpillars and 10, 20 or 40 second instar *S. littoralis* larvae. Young *P. brassicae* caterpillars are gregarious, and groups of 20+ caterpillars are very common on their host plants in nature. Densities of 10–40 early instar *S. littoralis* feeding on the same plant are not unrealistic, as females are known to lay eggs in very large batches. Caterpillars were placed on the plants 24 h prior to the first wasp release and were left feeding on the plants during the experiment. After 30 min, the numbers of wasps in the different olfactometer arms were recorded and the wasps were removed from the system. An olfactometer test (=1 replicate) consisted of five consecutive releases of five wasps for this experiment. Each wasp was only used once. A total of five replicates (=25 parasitoids tested per replicate;

125 total parasitoids tested) were conducted for each herbivore density, except for the density with 40 *S. littoralis* ($N = 4$; 100 total parasitoids tested). To test the effects of parasitoid learning, the preferences of *C. glomerata* females were measured in a 6-arm olfactometer setting (Turlings et al. 2004) with the following treatments: plant infested by 20 *P. brassicae*, plant infested by 20 *S. littoralis*, plant dually infested by 40 *S. littoralis* and 20 *P. brassicae*, undamaged plant, and two empty arms. The *S. littoralis* treatment was included to investigate whether or not an exposure to *S. littoralis* (non-rewarding experiment) prior to the tests may result in an increased attraction to plants infested by *S. littoralis*. For each olfactometer test, the dually-infested plant was used to "train" parasitoids. For this we used glass learning chambers ($12 \times 6.5 \times 3.5$ cm) that had two openings. One opening was connected to the glass bottle containing the dually-infested *B. rapa* plant; an air flow (0.8 L / minute) was pushed from the bottle to the learning chamber, ensuring that the odors of the plant was carried inside the chamber. The second opening was used to place and remove insects from the chamber. The chamber was filled with either 50 1st instar *P. brassicae* (rewarding experience) or 50 1st instar *S. littoralis* (non-rewarding experience). Groups of five naïve females were placed in the chambers until each wasp had attacked (i.e. inserted its ovipositor into a caterpillar for more than one second) between two and five caterpillars, which is much lower than the maximum number of hosts females can parasitize before becoming egg-limited (Tagawa 2000; Vos et al. 1998). When parasitoids were placed with *S. littoralis* they frequently did not attack the caterpillars. In such cases, the wasps were considered trained and removed from the chamber after a period of 30 min. All trained wasps were then transferred into glass vials with a drop of honey until the olfactometer tests (approx. 3 h after training). An olfactometer test (=1 replicate) consisted of six releases of five wasps, alternating between releases of wasps that received a rewarding experience and wasps that received a non-rewarding experience. In other words, 15 wasps that received a rewarding experience and 15 wasps that received a non-rewarding experience were used per replicate. In addition, tests were carried out with naïve females and the same treatments: each test (=1 replicate) consisted in six consecutive releases of five naïve wasps. A total of nine replicates were carried out with trained wasps

(9 × 15 = 135 total wasps from each training regimen tested), and six replicates with naïve wasps (6 × 30 = 180 total naïve wasps tested). For each olfactometer test, parasitoid motivation was calculated as the percentage of parasitoids that had made a choice after 30 min in the olfactometer. Herbivore-infested plants used in the tests were prepared the same way they were prepared for the chemical analyses described below. Plants were changed and the glassware was cleaned between two replicates. Damaged leaves were collected from the herbivore-infested plants after the tests and scanned. The damage area was measured using Abode Photoshop CC.

Chemical analyses

To identify and quantify the volatile organic compounds (VOCs) emitted by undamaged plants or plants damaged by *P. brassica*, *S. littoralis*, or from dually-infested plants, 3-week-old *B. rapa* plants ($n = 5$ plants per treatment) were placed in a VOC collection setup (Ton et al. 2007) for 6 h. Plants infested with one herbivore species were prepared by infesting the plants with 20 1st instar *P. brassicae* larvae or 20 2nd instar *S. littoralis* larvae. For dually-infested plants, three different numbers of *S. littoralis* larvae were used: 10, 20 and 40 2nd instar caterpillars. Caterpillars were not removed from the plants during the volatile collections. VOCs were collected using a trapping filter containing 25 mg of 80–100 mesh SuperQ absorbent. Before use, trapping filters were cleaned with 300 μl of methylene chloride (HPLC grade). After each collection, VOCs were extracted from the filters with 150 μl of methylene chloride. Two internal standards (200 ng of n-octane and nonyl acetate in 10 μl methylene chloride) were added to each sample. VOCs were analyzed with an Agilent 6890 gas chromatograph with a flame ionization detector. A 2 μl aliquot of each sample was injected in the pulsed splitless mode onto a non-polar column (HP-1 ms, 30 m, 0.25 mm ID, 0.25 μm film thickness, Agilent J&W Scientific, USA). Helium was used as carrier gas at constant pressure (15 psi). After injection, temperature was maintained at 40 °C for 3 min, then increased to 100 °C at 8 °C/min, and then to 220 °C at 5 °C/min. The quantities of the major components of the blends were estimated based on the peak areas of the compounds compared to the peak areas of the internal standards. Compounds were identified by

comparing the spectra obtained from the samples with those from a reference database (NIST mass spectral library).
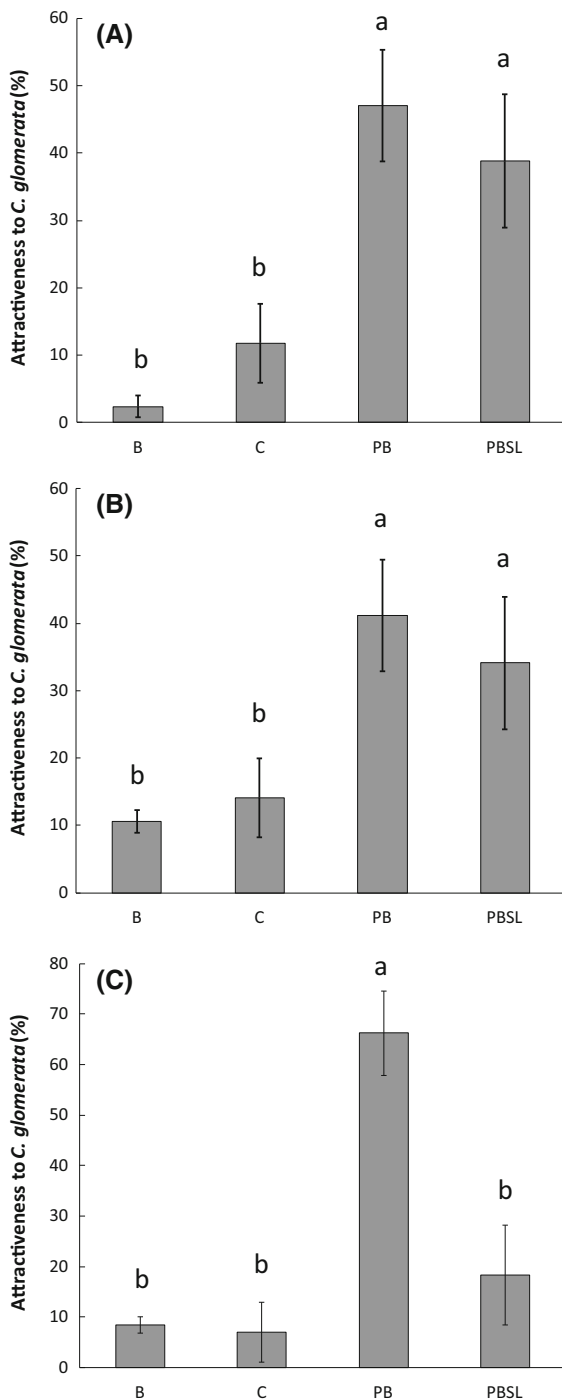
Statistical analysis

Preferences of *C. glomerata* females in olfactometers were analyzed using a generalized linear model with a poisson distribution fitted by maximum quasi-likelihood estimation, using treatment as a fixed effect and counts of parasitoids as the dependent variable. Means were compared using a multiple comparison Wilcoxon rank sum statistical procedure ($\alpha = 0.05$; JMP12, SAS Institute Inc. 2014). The effect of training regimen on wasp motivation was tested with a paired *t*-test comparing motivation of wasps that received a rewarding experience and wasps that received a non-rewarding experience for each replicate (JMP12, SAS Institute Inc. 2014). Results of damage area measures were analyzed using one-way ANOVAs ($\alpha = 0.05$), and treatments were compared using the all-pairwise Tukey-Kramer HSD procedure (JMP12, SAS Institute Inc. 2014).

To analyze the results of the chemical analyses, the total amounts of the different categories of plant volatiles identified (nitriles, green leaf volatiles, terpenoids, isothiocyanates, and alkanes and aldehydes), the amounts and ratios of specific compounds belonging to each category were compared among treatments using the non-parametric Wilcoxon rank sum statistical procedure ($\alpha = 0.01$; JMP12, SAS Institute Inc. 2014).

Results

Effects of non-host density on parasitoid preferences

In 4-arm olfactometer experiments parasitoids showed significant preferences among treatments for all the non-host herbivore densities tested [full model for density of *S. littoralis* = 10: $\chi^2 = 32.2$, $P < 0.0001$; full model for density of *S. littoralis* = 20: $\chi^2 = 18.3$, $P < 0.001$; full model for density of *S. littoralis* = 40: $\chi^2 = 53.1$, $P < 0.0001$ ($df = 3$ for each model; Fig. 1)]. The effect of dual infestation on the plants' attractiveness to *C. glomerata* females was dependent on relative caterpillar density. There was no difference in

plants infested by *P. brassicae* alone. For all non-host herbivore densities tested, undamaged plants and clean air were not attractive to parasitoids (Fig. 1). Parasitoid motivation ranged from 68% (for SL = 10 and SL = 20) to 71% (for SL = 40), and was not affected by non-host density ($\chi^2 = 0.1$, $P = 0.9$). Leaf area damaged by herbivores differed among treatments ($F_{3,34} = 3.54$, $P = 0.02$): the level of damage was lower on plants infested by *P. brassicae* alone, intermediate on dually-infested plants with 10 or 20 *S. littoralis* larvae, and highest on dually-infested plants with 40 *S. littoralis* larvae (Supplementary material, Fig. S1).

Effects of parasitoid learning on parasitoid preferences

In 6-arm olfactometer experiments, naïve parasitoids were significantly more attracted by plants infested by *P. brassicae* alone than plants dually infested by 40 *S. littoralis* larvae and 20 *P. brassicae* larvae, confirming the results of the 4-arm olfactometer tests (full model $\chi^2 = 124.2$, $P < 0.0001$, $df = 5$; Fig. 2). Experience prior to the olfactometer tests affected parasitoid preferences. Parasitoids that were given an experience with contacting *P. brassicae* larvae (rewarding experience) while perceiving of the odor of a dually-infested plant prior were afterwards equally attracted to dually-infested plants and plants infested by *P. brassicae* alone (full model $\chi^2 = 108.7$, $P < 0.0001$, $df = 5$). In contrast, parasitoids that had experienced *S. littoralis* larvae (non-rewarding experience) in presence of the odor of the dually-infested showed the same patterns of preference as naïve wasps (full model $\chi^2 = 76.5$, $P < 0.0001$, $df = 5$; Fig. 2). Experience also affected parasitoid motivation: parasitoid motivation was 85.1% for naïve wasps and 74.7% for wasps that
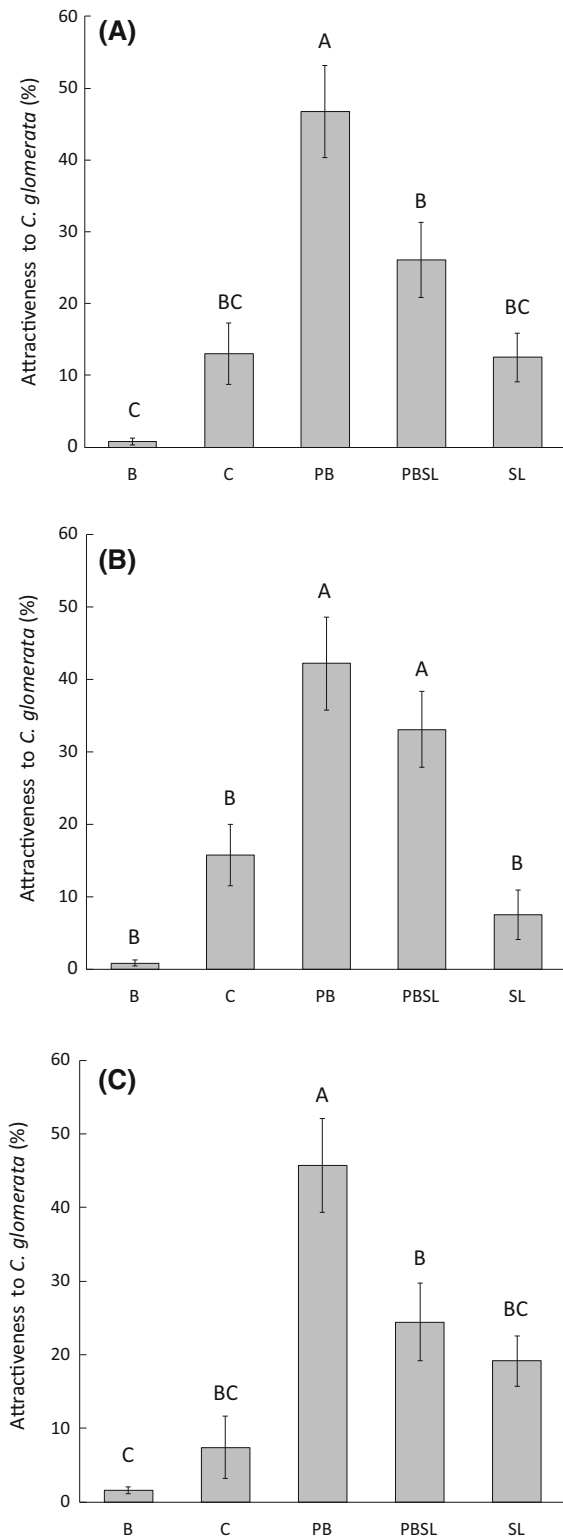
attractiveness between plants dually infested with 20 *P. brassicae* larvae plus 10 or 20 *S. littoralis* larvae and plants infested by *P. brassicae* alone. However, plants infested by 40 *S. littoralis* and 20 *P. brassicae* larvae were significantly less attractive to parasitoids than

received a rewarding experience, but was only 58.0% for wasps that received a non-rewarding experience. This decrease in motivation between rewarded and non-rewarded wasps was significant (t = 2.38, P = 0.044).

Chemical analyses

There were significant differences in the emissions of plant volatiles (VOCs) between undamaged *B. rapa* plants (control) and plants infested by different densities of herbivores. Fourteen compounds belonging to five broad categories [nitriles, isothiocyanates, green leaf volatiles (GLVs), alkanes and aldehydes, and terpenoids] were identified in the samples (Table S2). Control plants produced the lowest total amounts of VOCs, plants with the highest herbivore infestation (PB + 40SL) produced the highest total amounts of VOCs, and plants infested with other herbivore densities produced intermediate amounts of VOCs (χ^2 = 19.6, P = 0.002, df = 5). Production of isothiocyanates (χ^2 = 19.1, P = 0.002, df = 5) and green leaf volatiles (χ^2 = 18.9, P = 0.002, df = 5) followed similar patterns: for these two categories of compounds, production was the highest for plants with the highest herbivore infestation (PB + 40SL), was the lowest for control plants, and was intermediate in all other treatments. Nitriles were consistently produced by all *Pieris*-infested plants (i.e. treatments PB, PB + 10 SL, PB + 20 SL, PB + 40 SL), but were almost absent in *Spodoptera*-infested plants and control plants (χ^2 = 20.1, P = 0.001, df = 5). There were no significant differences in the production of terpenoids and alkanes and aldehydes, among the treatments (Ps > 0.01; Fig. 3). The ratio of nitriles in the VOCs blend was the highest in plants infested by *P. brassicae* only,
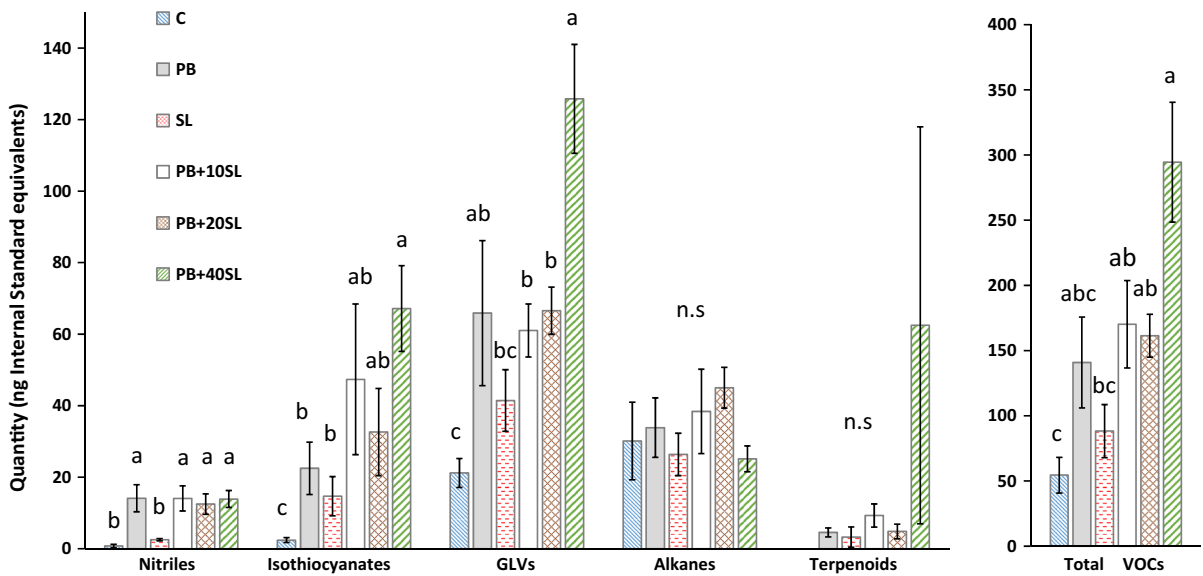
Fig. 3 Effects on non-host herbivore density on plant volatiles. Volatile organic compounds (VOCs, ng internal standard equivalents, mean ± SE) emitted by *B. rapa* plants undamaged (control) or infested by 20 *P. brassicae* larvae (PB), 20 *S. littoralis* larvae (SL), or dually infested by 20 *P. brassicae* caterpillars and 10, 20, or 40 *S. littoralis* larvae (PB + 10 SL, PB +20 SL, PB + 40SL, respectively). Means followed by a different letter are significantly different (Wilcoxon rank test, α = 0.01, JMP12)
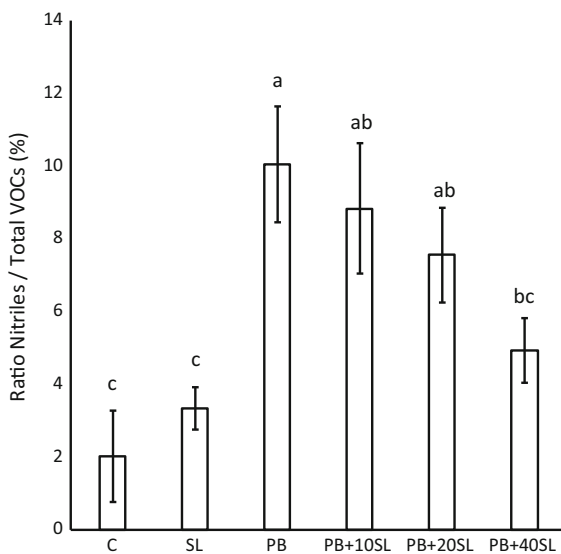

Fig. 4 Ratio Nitriles/Total VOCs (%, mean ± SE) for *B. rapa* plants under herbivore treatments: undamaged (control), infested by 20 *P. brassicae* larvae (PB), 20 *S. littoralis* larvae (SL), or dually infested by 20 *P. brassicae* caterpillars and 10, 20, or 40 *S. littoralis* larvae (PB + 10 SL, PB +20 SL, PB + 40SL, respectively). Means followed by a different letter are significantly different (Wilcoxon rank test, α = 0.01, JMP12)

gradually decreased in dually infested plants with increasing densities of non-host herbivores, and was the lowest in plants infested by *S. littoralis* and control plants ($\chi^2 = 19.6$, $P = 0.002$, $df = 5$; Fig. 4). The ratio of alkanes and aldehydes was the highest in control plants, the lowest in plants infested with the highest density of herbivores (PB+40SL), and intermediate in all other treatments ($\chi^2 = 18.4$, $P = 0.002$, $df = 5$). Ratios of isothiocyanates, green leaf volatiles, and terpenoids did not differ among treatments (Ps > 0.01).

Discussion

To evaluate the consequences of invasions by exotic herbivores for native communities, it is important to understand how their effects on native plants affect multitrophic interactions among native organisms. Here we show that the interference of the invasive pest *S. littoralis* with the foraging behavior of a native specialist natural enemy, the parasitoid *Cotesia glomerata*, is highly context-sensitive. Herbivore density, as well as associative learning were found to affect the

olfactory preferences of the parasitoid. In the field, both these factors are likely to play an important role on the realized impact of invasive herbivores on native parasitoids because herbivore densities can highly fluctuate from plant to plant, and because associative learning can shape the preferences of parasitoids throughout their lifetime (Giunti et al. 2015; Turlings et al. 1993). Although our results do not constitute a prediction of the outcome of *C. glomerata*/*S. littoralis* interactions in *S. littoralis* invasive range because many untested variables may affect the frequency and extent of these interactions in the field and because the *S. littoralis* larvae we used could have potentially been affected by artificial selection, they illustrate several ecological mechanisms that could play a role in many scenarios of biological invasions.

Our behavioral bioassays showed that parasitoids are considerably less attracted to dually-infested plants compared to plants only infested by hosts when the number of non-hosts exceeds the number of hosts and are consistent with our previous study (Chabaane et al. 2014), which documented a reduced attractiveness of dually-infested plants with 30 *S. littoralis* larvae and 20 *P. brassicae* larvae, and with a recent study by de Rijk et al. (2016), who found a negative impact of non-host herbivores on the foraging behavior of *C. glomerata*, but only at high non-host herbivore densities. Chemical analyses of the HIPVs emitted by study plants offer a proximal mechanism for this behavioral effect. Indeed, the composition of the volatile blends emitted by plants with the highest numbers of non-host herbivores strongly diverged from all the other herbivore treatments, with a significant increased production of green leaf volatiles and isothiocyanates (Fig. 3). This suggests that high numbers of *S. littoralis* on dually-infested plants affect HIPVs to such an extent that they become unattractive to naïve parasitoids. From the parasitoid perspective, this unattractiveness can be explained either by the fact that (1) parasitoids no longer associate the volatile cues from these plants with the presence of *P. brassicae*, or that (2) parasitoids still detect key *Pieris*-associated cues, but consider these plants of lesser foraging value compared to plants infested only by *P. brassicae*. Among the key compounds that parasitoids may use to detect the presence of *P. brassicae*, nitriles are known to be produced specifically as a result of the detoxification process of glucosinolates by *Pieris* caterpillars (Wittstock et al.

2004), and have been suggested to play an important role in the attraction of *Pieris*-specific parasitoids (Mumm et al. 2008). In our study, nitrile production by *B. rapa* remained stable for *Pieris*-infested plants at all the non-host herbivore densities tested, but the ratio of nitriles in the complete VOCs blend gradually decreased with increasing densities on non-host herbivores; thus, dually-infested plants with the largest number of non-host herbivores had a nitrile ratio comparable to control plants and *S. littoralis*-infested plants (Fig. 4). This could imply that the relative amount of nitriles plays a key role in how *C. glomerata* parasitoids innately evaluate the foraging value of a plant. Further studies directly manipulating the quantity of nitriles present in otherwise identical VOCs blends would be needed to test this hypothesis.

Plants responded very differently to the highest density of herbivores compared to other treatments (Fig. 3), which suggests a non-linear effect of insect density on HIPVs. This is consistent with other studies that investigated the effects of several herbivore densities on plant volatile emissions (Miresmailli et al. 2012; Rioja et al. 2016). Because plants with the highest numbers of herbivores were also the most damaged (Fig. S1), it is possible that plant response to herbivory depends on a damage threshold rather than being linearly density-dependent. Threshold-dependent plant responses have been documented for a range of abiotic stresses such as drought, heat or ozone pollution, with plant responses being highly different in response to mild or severe stresses (Niinemets 2010). For maize plants infested with *Spodoptera littoralis* caterpillars Gouinguené et al. (2003) found an almost linear increase in the quantity of released volatiles with increased amount of damage, but at 60% or more leaf tissue loss the released quantities of volatiles decreased with increasing damage. As yet, there is very little information on how different levels of herbivory are perceived by plants in terms of stress severity, and this topic remains a challenge in the field of insect–plant interactions (Strauss and Agrawal 1999).

Parasitoids that use HIPVs as foraging cues have evolved under strong selective pressure to be able to cope with the great plasticity and diversity of plant responses to herbivory to successfully locate their hosts (Holopainen and Gershenzon 2010). In addition to finely-tuned innate preferences to certain herbivore-induced compounds or ratios of compounds,

associative learning is believed to be a key strategy to improve host finding efficiency in multi-herbivore communities (Giunti et al. 2015). We found that a rewarding experience (parasitoid encountering a host herbivore) in presence of a non-preferred odor indeed increases parasitoid attraction toward this odor; parasitoids changed their innate preference and were also attracted to dually-infested plants with high numbers of *S. littoralis* after a rewarding experience with the odors of such plants. This is in line with a study that investigated parasitoid attraction to plants with simultaneous infestation with an above- and a belowground herbivore (Rasmann and Turlings 2007). Much less is known about the effects of non-rewarding experiences (parasitoid encountering no hosts or non-host organisms) on foraging preferences (Costa et al. 2010; Papaj et al. 1994), in part due to the difficulty of assessing whether or not parasitoids "acknowledge" the presence of non-host herbivores. In our training setting, most *C. glomerata* females put in presence of *S. littoralis* in training chambers simply ignored the caterpillars and did not display behaviors associated with *C. glomerata* in-plant foraging behavior, such as antennal tapping, during the duration of the training period. Parasitoids that received a non-rewarding experience did not show a change in olfactory preferences compared to naïve parasitoids, but they made significantly fewer choices in olfactometers compared to naïve and rewarded parasitoids (58% vs. 85% and 75% motivation, respectively), which indicates that a non-rewarding experience decreased the overall motivation of parasitoids to respond to the odors of *B. rapa*. This result is consistent with findings by Papaj et al. (1994), who found that parasitoids can quickly lose their motivation to forage in certain microhabitats after a non-rewarding experience. It remains to be explored whether this decrease in motivation can be reversed or accentuated with subsequent experiences and to study the consequences of learning for *C. glomerata* fitness under more realistic conditions (Bukovinszky et al. 2012; Rijk et al. 2016).

One of the main challenges faced by invasion ecologists is to understand how the arrival of exotic invaders may realistically affect native communities. The impact of invaders as bio-disrupters of information networks that are mediated by plant chemical cues is particularly difficult to estimate due to the volatile nature of plant responses to herbivory (pun intended) and the behavioral plasticity of organisms using plant signals in their foraging decisions. Our study illustrates that the putative impact of the invasive herbivore *S. littoralis* is strongly affected by relative herbivore density and the ability of the parasitoid to learn rewarding odors. These two factors are likely to interact in nature. For instance, parasitoids certainly become more likely to encounter a non-host on a dually-infested plant as the number of non-host herbivores increases (De Rijk et al. 2016). Therefore, the negative effects of non-host herbivore density on plant detection from a distance and within-plant foraging may be additive or synergistic.

Taken as a whole, the findings of our study suggest a scenario where parasitoids may cope with the presence of exotic non-host herbivores at low or medium non-host densities due to the reliability of HIPVs and the use of associative learning. They may not be able to locate their specific hosts anymore when the non-host density surpasses a certain threshold. At these high densities, there will be higher variability and/or lower signal to noise ratio in HIPVs and there will be a lower probability of having rewarding encounters on dually-infested plants. Such density thresholds are very likely to be plant- and herbivore-specific, implying that each exotic invader has the potential to impact infochemical networks in a different way at different densities. Understanding how plant physiological responses, biotic and abiotic environmental factors, and insect behavior interact under realistic field conditions is the key to constructing realistic models predicting the impact of invasive insect herbivores on multitrophic interactions.

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