BioMed Research International

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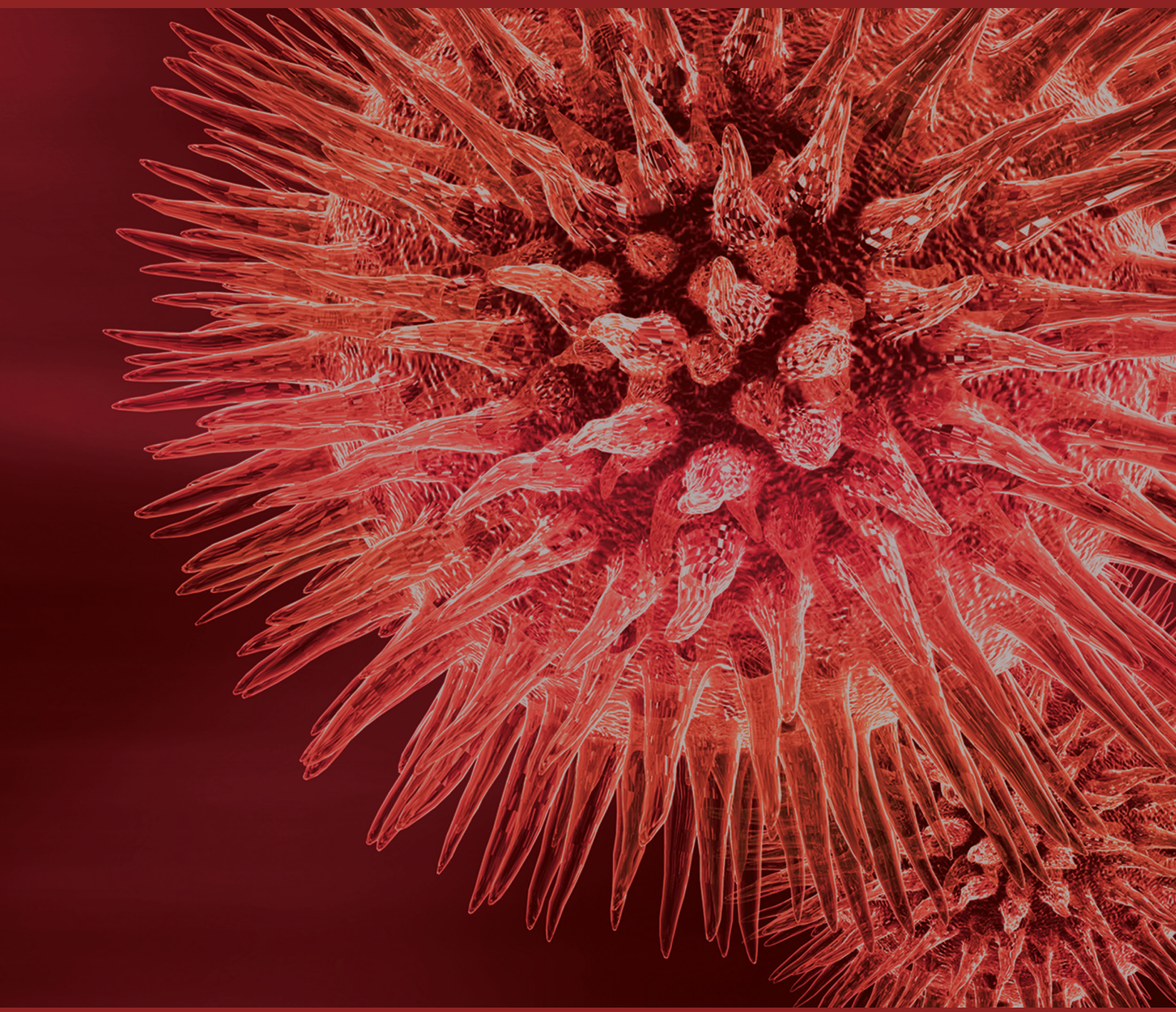
Guest Editors: Giovanni Benelli, Kent M. Daane, Roxina Soler, and Johannes Stökl



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Editorial

Chemical Ecology of Parasitic Hymenoptera

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Over the past one hundred years, the evolutionary chemical ecology of arthropods has been deeply investigated by a wide range of scientists, including chemists, ecologists, neurobiologists, entomologists, and behavioural and evolutionary biologists. Among insects, a substantial number of studies focused on the ecology of parasitic Hymenoptera, particularly wasp species that have evolved to utilize insect herbivores. Parasitoids are therefore considered key organisms in both natural and agricultural systems, for their role in ecosystem services and biological pest control. These fascinating animals rely on a range of communication channels during their life, including visual, auditory, and olfactory channels.

A full understanding of the chemical ecology and evolution of Hymenoptera parasitoids can help develop novel or improve current biological control programs. At an operational level, behavioural knowledge would help to improve mass-rearing techniques, evaluate release rates in a given habitat, and predict parasitoid effectiveness against a variety of hosts. Although extensive research has been carried out on these topics in recent years, there are still significant gaps in our knowledge of the chemical ecology of many parasitic wasps. In this scenario, this special issue presents review and original research papers covering different facets of the chemical ecology of parasitic wasps.

The effects of abiotic factors on biotic interactions mediated by herbivore-induced plant volatiles have received only limited attention. In the review article "Effects of Abiotic Factors on HIPV-Mediated Interactions between Plants and Parasitoids," C. Becker et al. highlighted that HIPV can be influenced by the plant growing conditions, which could have major implications for pest management. Indeed, quantitative and qualitative changes in HIPV blends can improve or impair biocontrol. Enhanced emission of HIPV may attract a larger number of natural enemies. Reduced emission rates or altered compositions, however, may render blends imperceptible to parasitoids and predators. Predicting the outcome of these changes is important for food production and for ecosystems affected by global climate change.

Even though Braconidae wasps are employed as biological control agents against Tephritidae flies, their use is still limited in olive groves against the olive fruit fly, due to low parasitisation efficiency. Besides visual cues, olfactory stimuli can provide key information driving parasitoid host location processes. In "VOCs-Mediated Location of Olive Fly Larvae by the Braconid Parasitoid *Psyttalia concolor*: A Multivariate Comparison among VOC Bouquets from Three Olive Cultivars," G. Giunti et al. investigated the effect of *Bactrocera oleae* infestation on olive fruits of different cultivars. Particular emphasis was given to the impact of infestation on volatile organic compound emissions, in order to evaluate the occurrence of putative HIPV, which may be useful to enhance the parasitoid efficacy in control programs against the olive fruit fly.

In "Electrophysiological and Behavioral Responses of *Theocolax elegans* (Westwood) (Hymenoptera: Pteromalidae) to Cereal Grain Volatiles," G. Germinara et al. studied the olfactory responses of *T. elegans*, a pteromalid wasp that parasitises immature stages of stored-product insect pests,

to volatiles emitted by healthy wheat grains, their hexane extracts, and different doses of three individual compounds previously identified in cereal grain odours. In Y-tube olfactometer bioassays, odours from healthy wheat grains and their hexane extracts were attractive to both sexes of *T. elegans*. Hexane extracts elicited arresting effects in Petri dish arena. The three synthetic compounds valeraldehyde, maltol, and vanillin elicited dose-dependent responses in both male and female adult wasps confirming the capability of the peripheral olfactory systems to perceive cereal volatiles. In behavioural bioassays, different doses of vanillin were significantly attractive to both sexes.

The cues routing the host-searching behaviour of Mymaridae wasps have been scarcely studied. In the research article "*Anagrus breviphragma* Soyka Short Distance Search Stimuli," A. Berzolla et al. pointed out that chemicals soluble in polar solvents are more important than host-borne physical cues, when *Anagrus breviphragma* females search for *Cicadella viridis* eggs. Notably, the stimuli that elicit probing and oviposition are not subjected to learning.

Furthermore, two original research articles present information on the identity and behavioural role of some compounds involved in the courtship and mating behaviour of two parasitoids of economic importance. The study "Sexy Mouth Odour? Male Oral Gland Pheromone in the Grain Beetle Parasitoid *Lariophagus distinguendus* (Förster) (Hymenoptera: Pteromalidae)" by K. König et al. analysed the courtship and mating behaviour of *L. distinguendus* from two different lineages, which are sexually isolated because males fail to elicit receptivity in foreign females. They showed that in *L. distinguendus* a nonvolatile male oral pheromone is essential to release the female receptivity signal. In contrast, male wing fanning and antennal contact play a minor role. Additionally, the composition of the oral pheromone depends on the developmental host and females learn the composition upon emergence from the host substrate. In the study "Species Specificity of the Putative Male Antennal Aphrodisiac Pheromone in *Leptopilina heterotoma*, *Leptopilina boulardi*, and *Leptopilina victoriae,*" I. Weiss et al. focus on male antennal aphrodisiac pheromones eliciting female receptiveness in *Leptopilina* parasitoids. They studied the species specificity of the putative male aphrodisiac pheromone of *L. heterotoma, L. boulardi*, and *L. victoriae*. Males elicited receptiveness only in conspecific females, never in the manipulated heterospecific females. Chemical analyses showed the presence of species-specific unsaturated hydrocarbons on the antennae of males. Only trace amounts of these hydrocarbons were found on the antennae of females. These results are of importance for a full understanding and identification of antennal pheromones in parasitic wasps.

Overall, we are grateful to this journal for hosting this special issue, and we hope that the knowledge presented here will be helpful to researchers in chemoecology, behavioural ecology, and biological control, to boost ecofriendly pest management strategies.

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Research Article

VOCs-Mediated Location of Olive Fly Larvae by the Braconid Parasitoid *Psyttalia concolor*: A Multivariate Comparison among VOC Bouquets from Three Olive Cultivars

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Herbivorous activity induces plant indirect defenses, as the emission of herbivorous-induced plant volatiles (HIPVs), which could be used by parasitoids for host location. *Psyttalia concolor* is a larval pupal endoparasitoid, attacking a number of tephritid flies including *B. oleae*. In this research, we investigated the olfactory cues routing host location behavior of *P. concolor* towards *B. oleae* larvae infesting three different olive cultivars. VOCs from infested and healthy fruits were identified using GC-MS analyses. In two-choice behavioral assays, *P. concolor* females preferred infested olive cues, which also evoked ovipositional probing by female wasps. GC-MS analysis showed qualitative and quantitative differences among volatiles emitted by infested and healthy olives. Volatile emissions were peculiar for each cultivar analyzed. Two putative HIPVs were detected in infested fruits, regardless of the cultivar, the monoterpene (*E*)-β-ocimene, and the sesquiterpene (*E*-*E*)-α-farnesene. Our study adds basic knowledge to the behavioral ecology of *P. concolor*. From an applied point of view, the field application of the above-mentioned VOCs may help to enhance effectiveness of biological control programs and parasitoid mass-rearing techniques.

1. Introduction

The olive tree (*Olea europea*) is an economically important crop in the Mediterranean basin, holding about 98% of world's olive groves [1]. In the last decades, olive crop was also widespread in novel regions, such as China, Brazil, and South Africa, increasing olive production up to 20.4 million tons in 2013, one of the highest production levels ever recorded. On the other hand, the olive crop spread has determined diffusion of the most devastating insect pest of olives, the olive fruit fly, *Bactrocera oleae* (Rossi) (Diptera: Tephritidae). Its diffusion occurred in the Mediterranean regions for over 2000 years, and, more recently, in California olive grows [2]. *B. oleae*, is a monophagous pest, feeding exclusively on *Olea* species. Olive fruit fly females lay an egg under the fruit surface; thus the larvae develop inside olive fruits until they open an exit hole before pupate. On table olive groves the oviposition puncture leads to a serious reduction of crop value, while exit holes and pulp degradation can determine a quality and quantity loss of olive oil production. *B. oleae* infestation can reduce oil yield [3, 4], alter several quality parameters (e.g., acidity, peroxide value, UV absorbance) [1, 5–7], and even negatively impact chemical composition, which determine oil taste and flavor [1, 6–10]. Volatiles profiles are known to be influenced by abiotic factors [11], but also *B. oleae* infestation could induce critical changes of volatile emissions [12].

Herbivorous feeding activity is known to induce a variety of biochemical changes in plants. It is well known that plants respond to herbivores' presence activating their defense system [13], but they can also trigger indirect defenses, as the emission of herbivorous-induced plant volatiles (HIPVs,

hereafter) [14, 15]. The role of kairomones on parasitoid host location has been widely investigated [16–18] and it has been demonstrated that many plants rely on volatile signals induced by phytophagous feeding to attract their natural enemies [14, 19, 20]. Moreover, despite the evidence about the influence of *B. oleae* infestation on the quality and the quantity of volatile compounds emitted in olive oils [7, 8], no information is available to assess the presence of HIPVs produced by infested olive fruits. However, differential emissions have been already proved for Tephritidae-infested and healthy fruits [21–24], highlighting the production of several HIPVs able to evoke electrophysiological and behavioral responses in parasitoid wasps [23, 24].

Psyttalia concolor (Szépligeti) (Hymenoptera: Braconidae) is a koinobiont larval pupal endoparasitoid, able to parasitize at least fourteen tephritids on different wild and/or cultivated plants, including *B. oleae* and *Ceratitis capitata* (Wiedemann), the Mediterranean fruit fly [25]. *P. concolor* females rely on a number of stimuli to successfully locate their host. Indeed, female wasps are able to distinguish between infested and healthy fruit, preferring the first one, even if just olfactory cues are provided [23]. In addition, it was demonstrated that apple and peach fruits infested by *C. capitata* larvae emitted peculiar volatiles, recognized by *P. concolor* wasps and able to attract selectively mated females [23]. HIPVs from apple and peach fruits are also able to attract and prolong the time spent performing searching behavior in *P. concolor* virgin males, probably raising their chances to locate receptive females nearby host microhabitat [26]. Furthermore, even synthetic blends reproducing infested peaches or apples were found able to be attractive for *P. concolor* mated females and virgin males [23, 26].

In this research, we investigated the importance of olfactory cues used by *P. concolor* females to locate their host microhabitat. We hypothesize that the HIPVs from *B. oleae*-infested olive fruits may play a pivotal role in affecting *P. concolor* host location, as described for the same parasitoid on a different tephritid host [23]. Olive fruits from three different cultivars were tested to determine parasitoid attractiveness and volatile organic compounds (VOCs) emissions: cv. Frantoio and cv. Leccino (traditionally cultivated in Italy) and cv. Arbequina (typical of Spanish olive groves). Firstly we evaluated females' preferences among healthy and infested fruits in two-choice bioassay, providing both visual and olfactory cues or olfactory stimuli alone, in order to evaluate the magnitude of volatiles attractiveness. Subsequently, volatiles emitted by healthy and infested olive fruits were SPME-sampled and analyzed by gas chromatography-mass spectrometry (GC-MS) to estimate differentially emissions attributable to herbivores' activity and to indicate possible HIPVs.

2. Material and Methods

2.1. Parasitoid Rearing. *P. concolor* wasps were reared as described by Canale and Benelli [25]. Insects were maintained in Pisa Laboratory under controlled conditions (22°C ± 1, 50–60% relative humidity and natural photoperiod) during their entire life. Adult parasitoids were allowed to emerge in transparent Plexiglas tubes (diameter 40 cm, length 50 cm) into which 1500 adults were introduced (male : female sex ratio 0.3–0.5). To obtain pupae, from which the adult emerged, a nylon mesh bag containing around 700 third instar *C. capitata* larvae was posed into a cage and exposed to *P. concolor* wasps for 20 minutes. Parasitized pupae were placed into smaller Plexiglas cages (diameter 20 cm, height 30 cm) and there *P. concolor* adults were allowed to emerge at a density of 50 specimens per cage (males : females sex ratio 0.3). Insects were stored at laboratory conditions [22 ± 1°C, 50 ± 5% relative humidity and 16 : 8 (L : D) photoperiod] for 7 days after the parasitoids' emergence to allow mating before testing. Adult insects were fed on a semisolid diet (honey mixed with pollen) and with water ad libitum.

2.2. Plant Material. Olive fruits from three different cultivars (Frantoio, Leccino, and Arbequina) were used for behavioral assay and GC-MS analysis. Olives were collected on September 15, 2014, in Tuscan olive groves [Frantoio and Leccino from Torrita di Siena (43°15′49.86″N, 11°78′96.58″E) and Arbequina from Rapolano Terme (43°27′70.97″N, 11°60′70.98″E)] from 5–3-year olive trees. Healthy or infested olives from each cultivar were collected manually, stored into glass jars (diameter 10 cm, length 20 cm) and transferred to laboratory conditions within 3 hours. The fruits were firstly divided according to the maturation index (MI), whereby the skin and flesh colors were scored to a 0 to 7 scale [27], and olives with MI from 2 to 7 were discharged. Among infested olives, we selected the ones attacked second or early third instar larvae, with no exit holes on the olive surface. Healthy fruits were selected avoiding crushed and naturally damaged ones.

Before being tested fruits were stored at laboratory condition for 2–5 days. All olives used for both behavioral and GC-MS tests were subsequently dissected to check the presence or the absence of *B. oleae* larvae inside the fruits.

2.3. Effect of Olfactory Cues from Infested Olives on Parasitoid Attractiveness. Bioassays were conducted using the still air arena described by Benelli et al. [28]. A Plexiglas unit (150 × 150 × 30 mm) was covered on the top with a removable glass panel to create the arena. The unit presents a circular chamber (diameter 40 mm) in the center to release the specimen and two other identical chambers connected with linear paths (length 20 mm; width 10 mm) where the stimuli were allocated.

To assess if infested olives are attractive for *P. concolor*, in a first experiment, mated females were allowed to choose among three healthy or infested olive fruits of each cultivar. In addition, to investigate the role of olfactory stimuli in leading parasitoid host location, a second experiment was designed. As in the first experiment three infested or healthy fruits were placed into the test chambers, but a piece of filter paper was posed ahead of the fruits to avoid visual contact with the parasitoid female released in the central chamber.

A replicate starts when a wasp was gently transferred to the released chamber and observed for 8 min. A wasp was

considered to have to choose a cue when it remains in the same chamber for at least 20 s actively searching for a host and the replicate was considered complete when the wasp left the chamber. Wasps that show no choice after 7 min were not considered. With each new wasp, the arena was rotated 90° and the relative position of cues was randomized. After each assay the arena was cleaned washing firstly with warm water, then rinsed in a water bath with mild soap, subsequently washed with hot water, and eventually cleaned with distilled water [29]. 30 mated females were tested for each treatment. For each bioassay the (i) latency time (time elapsing from the start of the replicate and the effective choice), (ii) female's first choice, (iii) time spent on the chosen chamber, (iv) number of antennal drumming series (performed in close proximity of the stimulus), and (v) number of oviposition attempt (performed on the fruits or on the filter paper surface) were recorded.

For each choice-test, a likelihood chi-square test with Yates correction (with $\alpha = 0.05$) was used to compare the proportion of parasitoids choosing a given cue [30]. The other measured variables were analyzed in JMP 7® by using a general linear model with one fixed factor (i.e., the treatment).

2.4. Effect of B. oleae Infestation on VOCs Production. Supelco (Bellefonte, PA, USA) SPME devices coated with polydimethylsiloxane (PDMS, 100 μm) were used to sample the headspace of three olive fruits (healthy or infested by *B. oleae*) inserted into a 30 mL glass vial and allowed to equilibrate for 30 min. SPME sampling was performed using the same new fibre, preconditioned according to the manufacturer instructions, for all the analyses. Sampling was accomplished in an air-conditioned room (25 ± 1°C) to guarantee a stable temperature. After the equilibration time, the fibre was exposed to the headspace for 30 min. Once sampling was finished, the fibre was withdrawn into the needle and transferred to the injection port of the GC-MS system. All the SPME sampling and desorption conditions were identical for all the samples. Furthermore, blanks were performed before each first SPME extraction and randomly repeated during each series. For each cultivar, three replicates (either containing three olives) for both infested and healthy fruits were provided. Quantitative comparisons of relative peaks areas were performed between the same chemicals in the different samples.

Gas chromatography/electron impact mass spectroscopy (GC-EIMS) analyses were performed with a Varian CP-3800 gas chromatograph equipped with a DB-5 capillary column (30 m × 0.25 mm; coating thickness = 0.25 μm) and a Varian Saturn 2000 ion trap mass detector (emission current: 10 microamps; count threshold: 1 count; multiplier offset: 0 volts; scan time: 1.00 second; prescan ionization time: 100 microseconds; scan mass range: 20–300 m/z; ionization mode: EI). The following analytical conditions were used: injector and transfer line temperature at 250 and 240°C, respectively; oven temperature programmed from 60 to 240°C at 3°C min^{-1}; carrier gas, helium, at 1 mL min^{-1}; splitless injection. Identification of the constituents was based on comparison of the retention times (RT) with those of pure compounds, comparing their linear retention indices (LRI) relative to the series of *n*-hydrocarbons and on computer matching against commercial (NIST 98 and ADAMS) and homemade library mass spectra built from pure substances and components of known oils and MS literature data [31–35].

For each compound and chemical class, the area integration report was transformed into log values, before statistical analysis. The normal distribution of data was checked using Shapiro-Wilk test. To evaluate differences in volatile emissions between infested and healthy fruits of the three cultivars, the variance was analyzed with JMP 7 by using a general linear model with one fixed factor (i.e., fruit health status). In addition a general linear model with two factors, health status and cultivar, was performed: $y_j = \mu + I_j + C_j + (H_j \times C_j) + e_j$, in which y_j is the observation, μ the overall mean, I_j the fruit infestation status (j = 1-2), C_j the cultivar (j = 1–3), $I_j \times C_j$ the interaction infestation status × cultivar, and e_j the residual error.

Principal Component Analysis (PCA) was achieved on normalized values of each VOC to derive different variables (principal components) that summarize the original data. PCA analysis was performed using JMP software. PCA calculated linear combination of the original data extracting eigenvalues and eigenvectors of a correlation matrix of volatiles' areas and highlighted principal components, the orthogonal and linear combination of the original variables. Two-dimensional score plots were created to determine if volatiles from different olive fruit cultivar or with different infestation degree could be clustered into classes. Then, a Multifactorial Analysis (MFA) was performed to assess common factors explaining volatiles' variability using a maximum likelihood estimation procedure and a VARIMAX orthogonal rotation technique by JMP. Scores of common factors were calculated as described by Macciotta et al. [36]. Furthermore, factors scores were analyzed using a general linear model with infestation status and cultivars as fixed factors, to enlighten the relationship between a common factor and the various treatments. Discriminant analysis, also performed using JMP software, used different volatiles, which can be highly correlated to a given fixed variable (i.e., infestation status), as a set of independent variables. A step-wise method was used to select a set of independent variables with $R^2 > 0.1$. The ratio (Wilks's lambda) between the generalized within-category dispersion and the total dispersion was considered [37].

3. Results

3.1. Effect of Olfactory Cues from Infested Olives on Parasitoid Attractiveness. *P. concolor* mated females showed significant preferences for infested fruits over healthy ones when both visual and olfactory cues were provided (Arbequina: $\chi^2 = 10.8333$, df = 1, $P = 0.0010$; Frantoio: $\chi^2 = 8.5667$, df = 1, $P = 0.0034$; Leccino: $\chi^2 = 6.5667$, df = 1, $P = 0.0104$) (Figure 1). No significant differences were recorded for latency times and times spent on the chosen chamber (Table 1), while wasps which had preferred infested fruits of

Table 1: Choice time spent by *Psyttalia concolor* females during searching behavior on healthy and *Bactrocera oleae*-infested olives in two-choice bioassay in still air arena.

Cultivar	Treatment	Infested olives		Healthy olives		F	P value
		Choice time Mean ± SE (s)	Replicates	Choice time Mean ± SE (s)	Replicates		
Arbequina	Visual + olfactory	268 ± 30	25	255 ± 70	5	0,0327	0,8579^{ns}
	Olfactory	336 ± 25	24	208 ± 64	6	4,7747	0,0374*
Frantoio	Visual + olfactory	268 ± 28	23	218 ± 43	7	0,7723	0,3870^{ns}
	Olfactory	158 ± 26	22	125 ± 48	8	0,4192	0,5227^{ns}
Leccino	Visual + olfactory	346 ± 29	22	308 ± 45	8	0,4642	0,5013^{ns}
	Olfactory	222 ± 35	23	166 ± 63	7	0,5883	0,4495^{ns}

Within a row, the asterisk indicates a significant difference ($P < 0.05$).
ns: not significant.
SE: standard error.

Table 2: Number of antennal drumming series performed by *Psyttalia concolor* females during searching behavior on healthy and *Bactrocera oleae*-infested olives in two-choice bioassay in still air arena.

Cultivar	Treatment	Infested olives		Healthy olives		F	P value
		Drumming series Mean ± SE (N)	Replicates	Drumming series Mean ± SE (N)	Replicates		
Arbequina	Visual + olfactory	5,6 ± 1,1	25	1,0 ± 0,6	5	3,4609	0,0734^{ns}
	Olfactory	7,0 ± 0,9	24	2,5 ± 1,3	6	5,3001	0,0209*
Frantoio	Visual + olfactory	7,9 ± 1,3	23	1,6 ± 0,8	7	6,833	0,0142*
	Olfactory	2,3 ± 0,3	22	1,5 ± 0,4	8	1,6977	0,2032^{ns}
Leccino	Visual + olfactory	9,8 ± 1,6	22	1,4 ± 0,5	8	9,6881	0,0042*
	Olfactory	2,1 ± 0,5	23	0,1 ± 0,1	7	5,531	0,0259*

Within a row, the asterisk indicates a significant difference ($P < 0.05$).
ns: not significant.
SE: standard error.

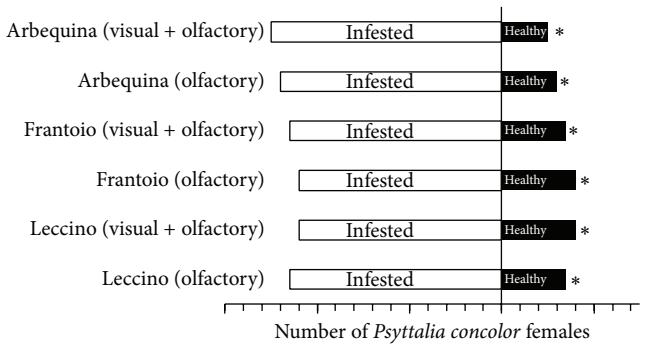

Figure 1: Attractiveness of *Bactrocera oleae*-infested fruits towards *Psyttalia concolor* mated females: effect of visual and olfactory. Two-choice bioassays were conducted in a still air arena with olive fruits, infested or not by olive fruit fly larvae, providing visual and olfactory stimuli associated or only olfactory cues. Thirty wasps were tested in each bioassay. For each test, asterisks indicate significant differences in the number of wasps choosing different cue (χ^2 test with Yates correction, $P < 0.05$).

Frantoio and Leccino varieties performed a greater number of drumming series on infested fruits (Table 2). No oviposition attempts were noted when females visited healthy olives, conversely to oviposition and probing behaviors recorded in infested fruits of all varieties.

Infested olives were positively located and chosen even in absence of visual stimuli, when fruits were hidden by filter paper. Indeed, *P. concolor* females preferred to prospect chambers containing infested fruits over healthy ones (Arbequina: $\chi^2 = 13.3667$, df = 1, $P = 0.0003$; Frantoio: $\chi^2 = 6.5667$, df = 1, $P = 0.0104$; Leccino: $\chi^2 = 8.5667$, df = 1, $P = 0.0034$) (Figure 1). No significant differences for latency times were found, but *P. concolor* females spent longer times in the chamber with Arbequina infested fruits than with healthy Arbequina olives (Table 1). Indeed when Arbequina or Leccino infested fruit odor was preferred by tested wasps, females accomplished a higher number of drumming series (Table 2). No oviposition attempts were recorded for wasps choosing healthy fruit chamber, while, interestingly, some wasps were noted to perform probing behavior in the filter paper or in the glass walls of the chamber containing infested olives.

3.2. Effect of B. oleae Infestation on VOCs Production of Olive Fruits from Different Cultivar. GC-MS analysis identified over 100 different volatile compounds. Differential emissions attributable to herbivore activity were found for all cultivars. In detail, we found 6 compounds significantly increased

in infested olives and 6 volatiles were exclusively produced by infested fruits of Arbequina variety (Supplementary Table S1; see Supplementary Material available online at http://dx.doi.org/10.1155/2016/7827615). In Frantoio, 3 compounds were exclusive and 3 increased and one decreased in infested olives (Supplementary Table S2), while in Leccino we found 4 compounds increasing and 4 exclusively present in infested fruits (Supplementary Table S3). In detail, the three cultivars present 2 common VOCs prevalently produced by infested olive fruits: (*E*)-β-ocimene and (*E-E*)-α-farnesene. Among chemical classes, monoterpenes hydrocarbons increased in all cultivars (Arbequina: $F = 8.0698$, df = 1, $P = 0.0468$; Frantoio: $F = 35.4752$, df = 1, $P = 0.0040$; Leccino $F = 14.3467$, df = 1, $P = 0.0193$). Indeed, Arbequina infested fruit showed different emissions of ketones ($F = 10387.18$, df = 1, $P < 0.0001$) and sesquiterpenes hydrocarbons ($F = 24.1958$, df = 1, $P = 0.0079$), while Frantoio increased monoterpenes oxygenated ($F = 17.5960$, df = 1, $P = 0.0138$) and aromatic hydrocarbons ($F = 50.5679$, df = 1, $P = 0.0021$). From two factors general linear model, 36 compounds, and 6 chemical classes were noted to be significant for at least one factor (Supplementary Table S4).

Furthermore, PCA followed by discriminant analysis allowed a more precise partition of cultivar and infestation effects on volatile emission from fruits. The Kaiser coefficient was around 1.00 since no correlations existed between the majorities of the compounds. Six principal components were analyzed, explaining 66.730% of variation (Table 3). Figure 2 shows PCA results and two-dimensional score plots were created to highlight different clusters relative to different olive fruit cultivar and different infestation status (Figure 3). Eigenvectors of single VOCs are provided in Supplementary Table S5 and rotated factor patterns in Supplementary Table S6. The rotated factors with an eigenvector of at least ±0.5 were marked in bold and considered for the following analysis. A two-way general linear model was provided to understand which sources of variation had a significant effect on the six analyzed factors, as reported by Supplementary Table S7. On this basis, we labeled the six factors as: Factor 1 "Infestation," Factor 2 "cv. Frantoio," Factor 3 "Italian Varieties," Factor 4 "Infestation cv. Leccino," Factor 5 "cv. Arbequina," and Factor 6 "cv. Leccino." Discriminant analysis was provided for one source of the variations (i.e., infestation status). Wilks' Lambda test showed a P value < 0.0001 and no misclassified variables were recorded. Step-wise method emphasized 11 variables highly correlated to infestation status (Table 4). Two VOCs (6-methyl-3-methylene-5-hepten-2-one and 2,6,11-trimethyldodecane) resulted positively correlated with Canonical 1, representing compounds typically associated with healthy olives, while the other 9 compounds were expression of infested status (Figure 3).

Table 3: Principal component identified after Principal Component Analysis (PCA) of volatile emissions from three olive cultivars. Bolded components were analyzed using a General Linear Model to determine source of variation.

Principal component	Eigenvalue	Percentage	Cumulative percentage
1	**195.168**	**20.986**	**20.986**
2	**136.287**	**14.654**	**35.640**
3	**108.109**	**11.625**	**47.265**
4	**65.221**	**7.013**	**54.278**
5	**62.379**	**6.707**	**60.985**
6	**53.423**	**5.744**	**66.730**
7	50.994	5.483	72.213
8	43.666	4.695	76.908
9	39.679	4.267	81.175
10	35.437	3.810	84.985
11	33.083	3.557	88.542
12	25.223	2.712	91.255
13	21.784	2.342	93.597
14	20.431	2.197	95.794
15	17.363	1.867	97.661
16	11.942	1.284	98.945
17	0.9813	1.055	100.000

Table 4: Volatiles identified after discriminant analysis. Positive correlations with Canonical1 indicate volatiles representative of healthy fruits, while negative correlations compounds are expressive of infested olives.

Compound	Correlation with Canonical1
6-Methyl-3-methylene-5-hepten-2-one	0,15583672
Dihydromyrcenol	−0,316570777
Terpinolene	−0,446911371
Methyl carvacrol	−0,759893869
Linalool acetate	−0,445435614
2,6,11-Trimethyldodecane	0,421648972
Cyclosativene	−0,355430074
(*E,E*)-α-Farnesene	−0,535648427
Liguloxide	−0,117965501
1-Hexadecene	−0,21094794
trans-Methyl dihydrojasmonate	−0,279958519

4. Discussion

Olfactory stimuli from host-infested fruits are known to be essential during host location behavior for many braconids, including species attacking larval stages [24, 38–43]. For *P. concolor*, the presence of chemical compounds was demonstrated produced by *C. capitata*-infested apples and peaches able to attract both mated females and virgin males [23, 26]. The evidence that olfactory cues from infested fruits evoke behavioral responses from mated *P. concolor* females and of the presence of compounds that were produced exclusively or in higher amount by infested olives supports our hypothesis that VOCs could act as short-range attractant, playing a key role during host-seeking also in this tritrophic system. *P. concolor* were attracted preferentially by infested olives, both when visual stimuli were provided or not, suggesting that the presence of feeding larvae inside the fruit is crucial for

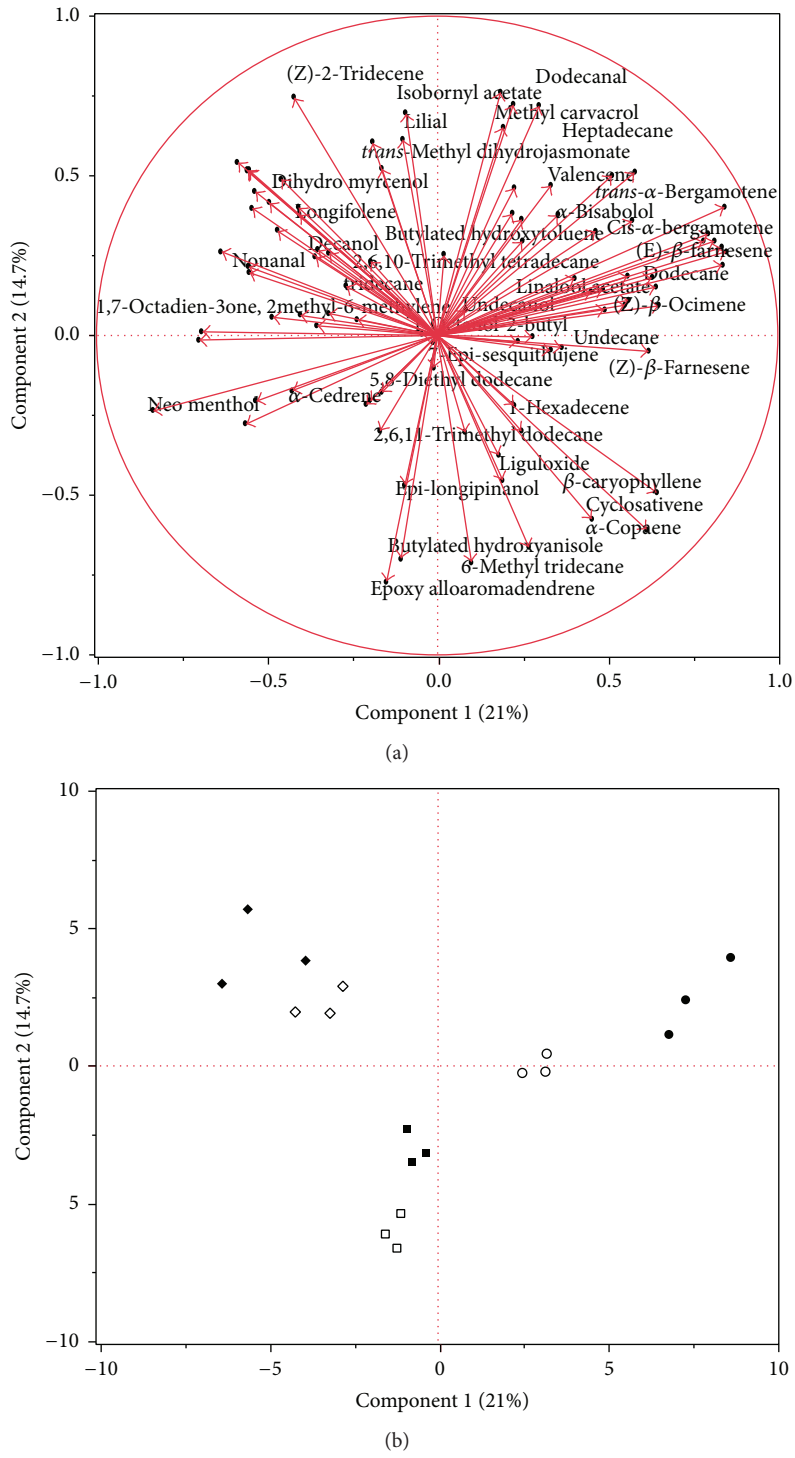

FIGURE 2: Principal Component Analysis (PCA) of volatile profiles from infested and healthy fruits of three different olive cultivars. (a) PCA loading plot, showing volatile correlations with the first and second principal component; (b) PCA score plot, highlighting cluster of volatiles attributable to cultivar or infestation status. ● Arbequina infested fruits; ○ Arbequina healthy fruits; ■ Frantoio infested fruits; □ Frantoio healthy fruits; ◆ Leccino infested fruits; ◊ Leccino healthy fruits.

host location. Indeed, oviposition behavior was performed from females just when they chose infested fruit stimuli. Interestingly ovipositor probing responses were performed also by *P. concolor* females which did not come directly in contact with olive fruits, but only sensing infested olive odors. This behavior, already described for *P. concolor* females attracted by some synthetic HIPVs [23], is uncommon among parasitic wasps, since usually they need an integration of

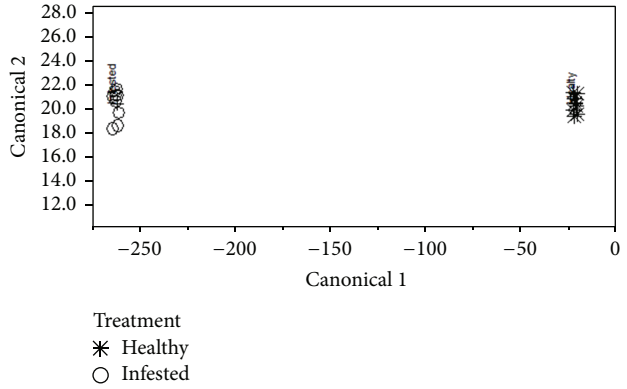

Figure 3: Canonical plot from discriminant analysis showing compounds highly correlated with Canonical 1 variable representing the infestation status in olive cultivars.

visual and olfactory stimuli to perform a complete host location sequence [21]. Moreover, *P. concolor* females showed probing behaviors on the chamber glass surface in presence of volatile emitted by all the three cultivars, inducing also longer active searching activities, with particular reference to antennal drumming.

To determine whatever change in volatile emissions could explain parasitoid behavior, *B. oleae*-infested and healthy olive fruits were analyzed. Among over 100 VOCs identified by SPME and GC-MS techniques, only two volatiles were found to increase in infested olives in all the three cultivars, a monoterpene, (*E*)-β-ocimene, and a sesquiterpene, (*E-E*)-α-farnesene, which are already known as constituent of the odors of olive oils and processed table olives [11, 44–46]. However, since (*E*)-β-ocimene is attractive to several braconid species, with special reference to *Aphidius* species [47–49] and (*E-E*)-α-farnesene which has been demonstrated to attract *Opius dissitus* Muesebeck wasps [50], these two compounds can be considered as putative kairomones for *P. concolor*. Although we observed mainly quantitative changes in volatile emissions among infested and healthy olives, which is not uncommon even in similar tritrophic systems [23, 51, 52], indeed, several plants react to herbivore damages by producing blends of metabolites with changes in number or in their proportions [21, 24].

Moreover, PCA analysis has highlighted that VOCs emissions are peculiar for each cultivar and chemicals, which were differentially emitted after herbivore infestation, changed depending on the olive varieties. Indeed, after multifactorial analysis, we could describe the variability due to baseline healthy cultivar emissions using three different factors (Factor 2, Factor 5, and Factor 6), explaining each one VOC emission of a specific cultivar. In addition, Italian cultivars (cv. Frantoio and cv. Leccino) showed common volatiles explained by Factor 3, which were never produced by the Spanish one (cv. Arbequina). On the other hand, infestation status could be explained for all the three cultivars by a common factor (Factor 1), but we identified also some exclusive compounds which were emitted only by Leccino olives under *B. oleae* infestation (Factor 4). Moreover, Arbequina variety showed to emit differentially the larger number of VOCs. Beside (*E*)-β-ocimene and (*E-E*)-α-farnesene, Arbequina infested olives increased the emission of other 4 compounds (methyl carvacrol, *n*-tridecane, *trans*-α-bergamotene, and *cis*-β-farnesene) and produced specifically 6 compounds [(Z)-β-ocimene, 2-methyl-6-methylene-1,7-octadien-3-one, cyclosativene, 1-undecanol, *cis*-α-bergamotene, and 3,5-di-tert-butylpyrocatechol]. Most of them are known to be common floral compounds, but interestingly some of them are recognized pheromones for several hymenoptera species [53–55], while (Z)-β-ocimene is known to be an attractant for the braconid *Diachasmimorpha longicaudata* (Ashmead) [24]. When attacked by *B. oleae*, Leccino cultivar similar to Arbequina increased the production of the floral compound 2-methyl-6-methylene-1,7-octadien-3-one. In addition, Leccino infested olives increased the emission of limonene and exclusively produced 4 monoterpenes (isocineole, γ terpinene, dihydromyrcenol, and terpinolene), the majority of which are HIPVs produced by mango fruits, positively tested by Carrasco et al. [24] on *D. longicaudata* wasps. Frantoio cultivar seems to be the less odorant varieties, since it produced only 5 compounds when herbivory attack succeeds. As described for Arbequina, Frantoio infested olives emitted more (*E*)-β-ocimene and methyl carvacrol, but they specifically generated [besides (*E-E*)-α-farnesene] dihydrocitronellol and heptylcyclohexane, which to the best of our knowledge were never investigated for their attractiveness toward insects. Interestingly, infested Frantoio olives showed to decrease the production of 2,6,11-trimethyldodecane, a peculiar VOC never identified on olives or olive oils. Conversely, the majority of the other identified VOCs are common volatiles emitted by olive oils [11, 44, 56–59], processed table olives [45, 46], leaves [44, 60], and olive fruits, regardless of their infestation status [61].

Hence, the cultivar seems to be the higher source of variation for VOC emissions in olive trees. For this reason, we cannot exclude that other VOCs produced specifically by one cultivar or increased in not all varieties may act as attractant toward *P. concolor* wasps. Indeed, it was demonstrated that also healthy fruits can produce volatiles attractive for parasitic wasps [24], and generalist parasitoid, as *P. concolor*, could be able to perceive cues from non-infested plant to locate host microhabitat. Thus, short-range volatiles produced by the plants or as excretion of the feeding larvae and/or vibrational stimuli from the hosts could be useful to the successful localization [62].

Overall, since HIPVs are known to act as kairomones for several parasitic wasps [24, 63, 64], further researches are needed to assess the activity of the highlighted compounds on parasitoid behavior. Indeed, knowledge about tritrophic system communications has potential implications also on biological control programs [65]. Synthetic kairomones have been already tested in field conditions for parasitoid attraction [64–66], but beside field applications, HIPVs may also be employed to enhance mass-rearing techniques.

5. Conclusions

Our results support the hypothesis that chemical cues produced by olive fruits under *B. oleae* attack route the host location behavior of *P. concolor* females, acting as short-range kairomones. Olfactory cues seem to have a key role in host-seeking behavior for two olive cultivars traditionally cultivated in Italy (cv. Frantoio and cv. Leccino) and one Spanish variety (cv. Arbequina). Indeed, behavioral assays have shown that *P. concolor* mated females can perceive the presence of host larvae inside a fruit when visual and olfactory stimuli were provided, but also when visual perception was forbidden. In addition, females choosing fruits infested by *B. oleae* performed longer searching activities, with particular references to antennal drumming series completed on the fruit or in close proximity, and olfactory cues from infested olives evoked ovipositor probing behavior even in absence of direct contact between the parasitoid and the fruits. SPME and GC-MS analysis have also supported the presence of volatiles attributable to herbivore activity which can be indicated as putative HIPVs. In detail, we found 12 volatiles increasing or exclusively emitted by infested Arbequina olives, 5 in Frantoio, and 8 in Leccino ones. Interestingly, the three cultivars showed 2 common VOCs produced as response of *B. oleae* infestation: (*E*)-β-ocimene and (*E-E*)-α-farnesene. Moreover, PCA and MFA highlighted that the cultivar is a higher source of variation for VOC emissions in olive trees.

Since HIPVs are recognized as kairomones of a number of parasitic wasps [23, 24, 63, 64], further studies are necessary to ensure the behavioral activity of these investigated volatiles toward *P. concolor* parasitoids. Synthetic kairomones may be useful to improve biological control programs, but even techniques for parasitoid mass-rearing. Moreover, even if the efficacy of synthetic kairomonal molecules has been already proved in field conditions [54–66], the role of HIPVs on the foraging behavior of beneficial arthropods in agroecosystems needs to be investigated deeper to enable their safe commercial applications [19].

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Electrophysiological and Behavioral Responses of *Theocolax elegans* (Westwood) (Hymenoptera: Pteromalidae) to Cereal Grain Volatiles

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Volatiles emitted by the host's food would be the first signals used by parasitoids in the host location process and are thought to play an important role in host habitat location. In this study, the olfactory responses of *Theocolax elegans* (Westwood), a Pteromalid wasp that parasitizes immature stages of stored-product insect pests developing inside cereal or leguminous grains, to volatiles emitted by healthy wheat grains, their hexane extracts, and different doses of three individual compounds previously identified in cereal grain odors were investigated in Y-tube olfactometer and Petri dish arena behavioral bioassays and electroantennogram recordings. In Y-tube olfactometer bioassays, odors from healthy wheat grains and their hexane extracts were attractive to both sexes of *T. elegans*. Moreover, hexane extracts elicited arresting effects in Petri dish arena. The three synthetic compounds valeraldehyde, maltol, and vanillin elicited dose-dependent responses in both male and female adult wasps confirming the capability of the peripheral olfactory systems to perceive cereal volatiles. In behavioral bioassays, different doses of vanillin were significantly attractive to both sexes.

1. Introduction

The use of natural enemies is considered to be an important component of integrated pest management of stored-grain insect pests [1], particularly in storage areas. Because the adult beneficial insects are external to the grain, they can easily be removed using normal grain-cleaning procedures [2].

In their search for hosts, food and mates parasitoids are guided by multisensory information including visual, vibrational, and olfactory signals [3–5] which are frequently used in an interactive manner [6–8].

The success of parasitic wasps in suppressing pest population depends on their ability to locate hosts; therefore, understanding the host location process is critical for the successful implementation of biological control programs. Host-seeking behavior in parasitoids has been divided into four phases, habitat location, host location, host recognition, and host acceptance [3, 9]. Orientation to host plant or host food volatiles in the absence of any host, host damage, and host derived materials have been demonstrated for a number of parasitic wasps [10–14], including some Pteromalidae species parasitizing stored-grain insect pests [15–17]. These volatiles, generally acting as long distance cues, would be the first signals used by parasitoids in the host location process and are thought to play an important role in host habitat location [18–20]. The identification of such synomones has a great practical interest since they could be used as parasitoid behavior modifying compounds to enhance their biological performances.

Theocolax elegans (Westwood) is a solitary ectoparasitoid of immature stages of economically important stored-product insect pests which develop as internal feeders of cereal and legume grains including *Rhyzopertha dominica* (Fabricius), *Sitophilus* spp., *Sitotroga cerealella* (Olivier), and *Callosobruchus* spp. [21]. *T. elegans* has been extensively studied as a biological control agent of some cereal pests. In large-scale experiments, the wasp was effective in reducing *Sitophilus zeamais* Motschulsky populations by up to 50% [22] and those of *R. dominica* by 50–99% depending on the temperature [23–25]. Moreover, augmentative release of *T. elegans* to control *R. dominica* resulted in a 61–92% reduction of the

total number of insect fragments in wheat flour [2] indicating a positive impact on the quality of stored cereal products.

Preliminary behavioral bioassays suggested a preferential orientation of *T. elegans* adults to odors of healthy wheat grains [26] and the sensitivity of the antennal olfactory systems of both sexes towards a wide range of volatiles identified from various cereal grains [27–29] was shown by electroantennogram (EAG) recordings [30].

In the present study, the behavioral responses of *T. elegans* males and females to odors of uninfested wheat grains and their hexane extracts were carefully investigated using Y-tube olfactometer and Petri dish arena bioassays. Moreover, the biological activity of three individual volatiles (valeraldehyde, maltol, and vanillin) previously identified in the aroma of fresh cereal grains [27] and attractive to the two *T. elegans* hosts *Sitophilus oryzae* [31] and *S. granarius* [32] was investigated in a range of doses by electroantennographic and behavioural bioassays.

2. Material and Methods

2.1. Insects. A colony of *R. dominica* parasitized by *T. elegans* was collected from a cereal storehouse and set up in glass jars (15 cm diameter × 25 cm) half filled with uninfested wheat kernels and closed with fine metallic net (0.2 mm mesh). Colonies were maintained at 28 ± 2°C, 60 ± 5% relative humidity, and 12L : 12D photoperiod. During the following four months, wheat kernel samples (5 g) from the rearing jars were periodically transferred to transparent plastic containers (6 cm diameter × 8 cm) and held to check for the emergence of adult wasps. Emerging insects were collected daily and kept individually in glass vials (1.5 cm diameter × 5 cm) without food supply and in the absence of cereal odors for at least 12 h before the EAG and behavioral experiments. The sex of the insects tested was determined by observing their genitalia with a stereomicroscope.

2.2. Extract Preparation. Solvent extracts of wheat kernels (*Triticum durum* var. Simeto) were prepared by immersing wheat grains (40 g) in hexane (20 mL) for 36 hours at room temperature. Extracts from 3 grain samples were combined and concentrated to 10 mg grain equivalent/μL using a gentle stream of nitrogen and stored at −20°C until being needed.

2.3. Chemicals. Valeraldehyde (1-pentanal), maltol (3-methoxy-2-methyl-4-pyrone), vanillin (3-methoxy-4-hydroxybenzaldehyde), and hexanal used as stimuli and hexane, mineral oil, acetone, and chloroform used as solvents were purchased from Sigma-Aldrich (Milan, Italy) and were 97–99% pure.

2.4. Y-Tube Olfactometer Bioassays. A glass Y-tube olfactometer (each arm 23 cm long at 75°C angle, stem 30 cm long × 3.0 cm i.d.) previously described [33] was used to examine the behavioral responses of *T. elegans* adults to odor stimuli. Each arm of the Y-tube was connected to a glass cylinder (9 cm long × 3.0 cm i.d.) as an odor source container. The apparatus was put into an observation chamber (90 × 75 × 40 cm) and illuminated from above by two 36-W cool white fluorescent lamps providing uniform lighting (2500 lux) inside the tube. A purified (activated charcoal) and humidified airflow maintained at 6 mL/min by a flowmeter was pumped through each arm.

In order to test the attractiveness of volatiles emitted by healthy wheat grains and their hexane extracts to *T. elegans* males and females two dual-choice experiments were performed: (1) wheat grains (25 g) *versus* clean air and (2) wheat grain hexane extracts *versus* control hexane. In this latter, the odor chamber contained a filter paper disk (1.0 cm^2) loaded with 50 μL of a 10 mg grain equivalent/μL hexane extract, while the control chamber contained a filter paper disk loaded with 50 μL of hexane. Both disks were suspended in the center of the cross section. In third set of experiments the behavioral responses of the adult wasps to different doses of vanillin were evaluated. The test stimulus was a filter paper disk loaded with 10 μL of 5 μg/μL vanillin hexane solution, while a similar filter paper disk loaded with 10 μL of hexane was used as control.

Individual *T. elegans* adults were released at the open end of the stem. Each experiment lasted for 5 min. A choice was recorded when the insect moved 3 cm up an arm of the Y-tube crossing the decision line (marked on both arms) and remained beyond that line for more than 30 sec. Insects that did not make a decision within 5 min were considered as no response and discarded.

After 5 individuals had been tested, the olfactometer was cleaned with acetone and dried (200°C for 30 min) and treatments between arms were switched to avoid position bias. For each test stimulus at least 20 male and female wasps were tested. A χ^2 test was employed to determine the significance of differences between the number of wasps choosing the treatment or control arm of the olfactometer.

2.5. Electroantennography (EAG). The electrophysiological response of male and female *T. elegans* antennae to increasing concentrations of synthetic valeraldehyde, maltol, and vanillin was measured by the EAG technique used in previous studies [30, 34]. Antennae were excised from 1- to 2-day-old insects. The base of the antenna was put into a glass pipette filled with Kaissling saline [35] which served as the neutral electrode. The tip of the antenna was put in contact with the end of a similar pipette (0.1–0.2 mm diameter) which provided the active electrode.

AgCl-coated silver wires were used to maintain the electrical continuity between the antennal preparation and an AC/DC UN-6 amplifier in DC mode connected to a PC equipped with the EAG 2.0 program (Syntech Laboratories, Hilversum, Netherlands). Stimuli were serial solutions (0.0125, 0.025, 0.05, 0.1, 0.15, and 0.2 mg/μL) of valeraldehyde, maltol, and vanillin, respectively, dissolved in mineral oil, acetone, and chloroform (Sigma-Aldrich) to achieve a satisfactory solution. Just before the experiment, 20 μL of each test solution was adsorbed onto a filter paper strip (1 cm^2, Whatman number 1) placed in a Pasteur pipette (15 cm long), which served as an odor cartridge. After complete evaporation of the corresponding solvent, stimuli were blown by

Table 1: Behavioral response of *Theocolax elegans* adults in a Y-tube olfactometer to different sources of wheat grain odors.

Odor sources	Male				Female			
	N[a]	Treated[b]	χ^2	P value[c]	N[a]	Treated[b]	χ^2	P value
Wheat grains versus control air	20 (20)	18	12.8	<0.001	20 (20)	19	16.2	<0.001
Wheat grain extract versus control hexane	21 (20)	16	7.2	0.007	21 (20)	18	12.8	<0.001

[a]Total sample size (N: number of individuals that made a choice in parentheses).
[b]Number of individuals (of those that made a choice) that chose the treated arm first.
[c]d.f. = 1 for all χ^2 reported.

a disposable syringe into a constant stream of charcoal-filtered humidified air (500 mL/min) flowing in a stainless steel delivery tube (1 cm diameter) with the outlet positioned at approximately 1 cm from the antenna. Over 1 s, 2.5 cm^3 of vapor from an odor cartridge was added. Stimuli were applied in ascending dose [36] and randomly sequenced for each dose. Control (20 μL of mineral oil) and standard (20 μL of 50 μg/μL hexanal mineral oil solution) stimuli were applied at the beginning of the experiment and after each group of 3 test odors. Intervals between stimuli were 30 s. For each compound, EAG responses were recorded from 5 antennae of different insects of each sex.

The amplitude (mV) of the EAG response to each test stimulus was adjusted to compensate for solvent and/or mechanosensory artifacts by subtracting the mean EAG response of the two nearest mineral oil controls [37]. To compensate for the decrease of the antennal responsiveness during the experiment, the resulting EAG amplitude was corrected according to the reduction of the EAG response to the standard stimulus [38]. The activation threshold of dose-response curves was considered to be the lowest dose at which the lower limit of the standard error of the mean response was greater than the upper limit of the standard error for the lowest dilution tested [39]; saturation level was taken as the lowest dose at which the mean response was equal to or less than the previous dose [30]. For each dose tested, the mean EAG responses of each sex to the three compounds were subjected to analysis of variance (ANOVA), Levene's test of homogeneity of variance and ranked according to Tukey's HSD test. Male and female EAG responses to a set test dose of each compound were compared using Student's t-test.

2.6. Petri Dish Arena Bioassays. The behavioral response of *T. elegans* males and females to the hexane wheat extract (10 mg grain equivalent/μL) and valeraldehyde, maltol, and vanillin solutions (0.01, 0.1, 1.0, and 5.0 μg/μL) in hexane [31] was assessed in an arena consisting of a glass Petri dish (15 cm diameter × 2 cm height).

The base of the dish was divided into four sectors. An aliquot of the test stimulus (50 μL of hexane wheat extract or 10 μL of a compound solution) was adsorbed onto a filter paper disk (1 cm^2, Whatman number 1) and placed in one sector after solvent evaporation (2 min). The remaining sectors contained similar filter paper disks treated with equal volumes of hexane used as controls. Treatment and control stimuli were allowed to diffuse into the arena for 3 min before the experiment started. The Petri dish was placed on the bottom of a white box (90 cm × 75 cm × 40 cm) and illuminated from above resulting in 400 lux on the dish.

An insect was released at the center of the Petri dish and its allocation in the four sectors was recorded for 5 min. After 5 individuals were tested, the olfactometer was cleaned with acetone and dried (200°C for 30 min). The position of the odor sample in the Petri dish was changed routinely to avoid position bias. For each treatment, 15 males and females used once were tested. The time spent by insects in the four sectors was analyzed by Friedman two-way ANOVA by ranks and in the case of significance ($P < 0.05$) the Wilcoxon signed ranks test was used for separation of means. Analyses were performed with SPSS (Statistical Package for the Social Sciences) version 10.0.7 for Windows (SPSS Inc., Chicago, IL).

3. Results

3.1. Behavioral Response to Wheat Grain Volatiles. In Y-tube behavioral tests, the percentage of adult wasps responding to stimuli tested was generally high (Table 1). Odors from wheat grains elicited a significant attraction in both male and female wasps. When insects were presented with wheat grain extract *versus* control hexane a significant preference for wheat extract was exhibited by both males and females. The mean allocation time of *T. elegans* males and females in the sector of Petri dish provided with wheat extract was significantly higher (Friedman test, $P < 0.001$; Wilcoxon test, $P < 0.001$) than those in related controls (Figure 1).

3.2. EAG. The sensitivity of male and female *T. elegans* antennae toward increasing concentrations of valeraldehyde, maltol, and vanillin was reported in Figure 2. All compounds elicited EAG dose-dependent responses with action thresholds at the 1 mg dose in both sexes. The amplitude of the EAG response of both sexes to maltol and that of males to vanillin decreased from 3 to 4 mg doses indicating saturation of receptors at the lowest dose.

For each dose tested, the mean EAG response to valeraldehyde was significantly higher than those to vanillin and maltol in both males ($F = 60.10$–593.27, d.f. = 2, $P < 0.001$) ($P < 0.05$, Tukey-HSD test) and females ($F = 27.94$–95.84, d.f. = 2, $P < 0.001$) ($P < 0.05$, Tukey-HSD test). In both sexes, no significant differences were found between the EAG responses to maltol and vanillin at different doses. Male EAG responses to valeraldehyde were significantly higher ($P < 0.05$, t-test) than those of females at the 3 and 4 mg doses. Male and female EAG responses to each dose of maltol

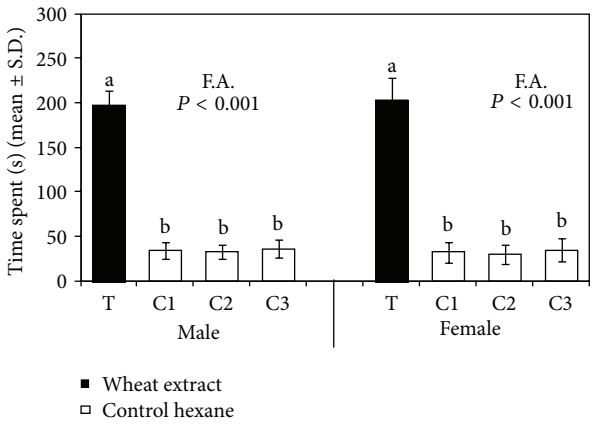

Figure 1: Mean allocation time of *T. elegans* males ($N = 20$) and females ($N = 20$) in the sectors of a Petri dish provided with 50 μL of hexane extract of wheat grains (500 mg grain equivalent) (T) or with 50 μL of hexane (C1, C2, and C3). Means were submitted to Friedman's two-way ANOVA (F.A.). For each sex, values with different letters are significantly different according to Wilcoxon test ($P \leq 0.001$).

and vanillin did not differ significantly ($P > 0.05$, t-test) (Figure 2).

3.3. Behavioral Response to Individual Grain Volatiles. In the range of dose tested, the mean allocation times of *T. elegans* males and females in the sector of Petri dish provided with maltol or valeraldehyde were not significantly different (Friedman test, $P > 0.05$) from those of related controls (Figure 3). A similar result was observed on applying the 100 ng dose of vanillin. Using the 1 μg dose of this compound the mean allocation time in the treatment sector was significantly higher than those in controls in both males (Wilcoxon test, $P = 0.003$) and females (Wilcoxon test, $P = 0.001$) (Figure 3). At the 10 and 50 μg doses, the mean time spent by male and female wasps in the sector with vanillin was significantly higher (Wilcoxon test, $P < 0.001$) than those in controls and higher than those recorded at the 1 μg dose (Figure 3). At the 50 μg dose, vanillin elicited a significant attraction of male ($\chi^2 = 7.20$, d.f. = 1, $P = 0.007$) and female ($\chi^2 = 9.80$, d.f. = 1, $P = 0.002$) wasps in Y-tube olfactometer bioassays.

4. Discussion

T. elegans males and females preferred volatile compounds emitted by healthy wheat grains when offered as an alternative to an empty control in the absence of visual stimuli in a Y-tube olfactometer. Volatile compounds present in hexane extracts of healthy wheat grains were preferred to control hexane in the same apparatus and elicited an arrestant effect in Petri dish arena, thus suggesting the presence of behaviorally active compounds acting as attractants at long distance and arrestants at short range. This implies that both sexes of *T. elegans* are able to detect host substrate on the basis of chemical cues and confirm their capability to perceive cereal grain odors [30]. Plant volatiles are used by female parasitic wasps to locate the habitat of host insects, while male wasps use the same chemical cues to locate the females for mating [40], probably in combination with a sex pheromone.

When, for instance, host population has declined or the host have dispersed from the emergence site, parasitoids may search for a suitable environment with adequate food sources [3, 41]. According to our results, volatiles released by cereal grains more likely drive *T. elegans* adults in the search for a suitable host habitat outside and inside cereal storage.

A possible explanation for the attraction of *T. elegans* adults to cues of healthy grains may be that being hosts concealed inside grains, seed odors probably act as a substitute in situations in which other host related cues, that is, host feces outside the seeds or specific volatiles released by herbivory-damaged grains, are not present to indicate host presence [42]. In this situation, the ability to respond to uninfested grains may serve to prolong parasitoid foraging activity [43, 44] and to exploit a wider range of hosts [42] within the habitat. Alternatively, the blend of volatiles released by healthy seeds may be very similar to that produced by host-damaged seeds causing the parasitoid to respond anyway, at least at long distance. To test this hypothesis, pairwise comparisons of infested and uninfested grains in olfactometer bioassays in combination with chemical analyses of volatile compounds are needed.

The importance of healthy seed volatiles to Pteromalid wasps parasitizing immature stages of internal feeders of cereal or legume seeds was highlighted by previous studies [15–17]. For instance, in pairwise comparisons of attractant stimuli in behavioral bioassays mated females of *Pteromalus cerealellae* (Ashmead) showed preference for the extract of uninfested cowpea seeds compared to larval frass extract [45]. In *Lariophagus distinguendus* (Förster) host feces alone were not attractive to female wasps while healthy grains were attractive even if less than a combination of host feces and herbivore damaged grains [42] indicating positive short range interactions between host and host food cues.

EAG studies showed that *T. elegans* males and females are able to detect the three fresh grain volatiles used in this study. This is in accordance with results of behavioral bioassays and provides further evidences that hosts' food volatiles are used by both male and female parasitic wasps.

For both sexes, different EAG dose-response curves to selected compounds were recorded with valeraldehyde being the most active at all doses tested. This could reflect differences in the number of specific antennal receptors involved in the perception of different cereal volatiles, more than differences in volatility of compounds, and indicate differential selectivity and sensitivity of the peripheral olfactory systems for host habitat odors [40, 46, 47]. Differences in the number of olfactory receptor neurons tuned to different compounds could also explain sexually dimorphic responses observed to high doses of valeraldehyde probably as a result of different roles played by the same plant volatile in the ecology of males and females.

In the dose range from 1 to 50 μg, a significant arresting effect of vanillin to *T. elegans* males and females was observed in Petri dish arena bioassays whereas, in the same

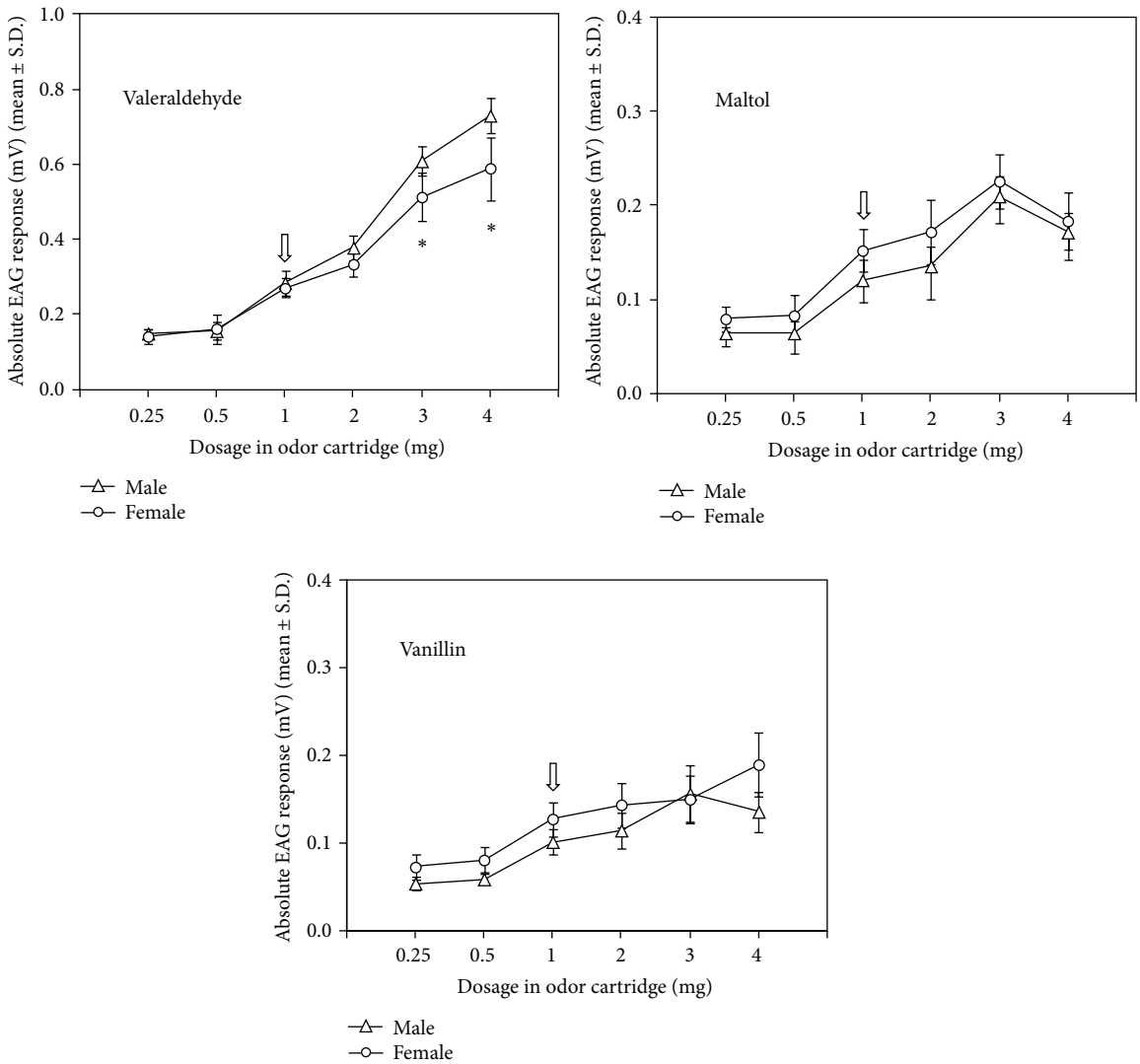

Figure 2: Electroantennogram dose-response profiles of *T. elegans* males and females ($n = 5$) to valeraldehyde, maltol, and vanillin. Arrows indicate the activation thresholds. Asterisks indicate significant differences between male and female EAG responses (Student's t-test, $P = 0.01$).

experimental conditions, maltol and valeraldehyde did not influence the parasitoid behavior. Moreover vanillin was preferred to control hexane in Y-tube olfactometer bioassays. It is worth noting that the behavioral responses elicited by vanillin were comparable to that induced by wheat grain hexane extracts in both apparatuses and even comparable to that of wheat grains in Y-tube olfactometer. Despite the strong attractant activity of vanillin to *T. elegans* adults, the EAG responses evoked by this compound in the range of doses tested showed only moderate amplitudes in both sexes. This is not surprising because a weak correlation between EAG and behavioral activity can occur and it is likely that a few sensilla specifically tuned to an important chemical cue would enhance its detection in the complex blend of plant-based host related volatile compounds [30]. Vanillin was already found to be attractive to *S. oryzae* [31] and *S. granarius* [32]. The sensitivity of a parasitoid and its host to the same volatile compounds emitted by host plants was also observed in other tritrophic systems [40, 48–51].

5. Conclusions

Results of behavioral bioassays demonstrated strong attractant activity of odors emitted by healthy wheat grains to both sexes of *T. elegans*. Electroantennogram recordings in response to three fresh cereal grain volatiles confirmed the capability of the peripheral olfactory systems of male and female wasps to detect chemical cues in a range of doses. Finally, in behavioral bioassays with the same compounds, vanillin was shown to be an effective attractant for both sexes of *T. elegans*. To the best of our knowledge, vanillin is the first synomone of a member of the *Theocolax* genus. This compound could be useful for detecting *T. elegans* and manipulating the

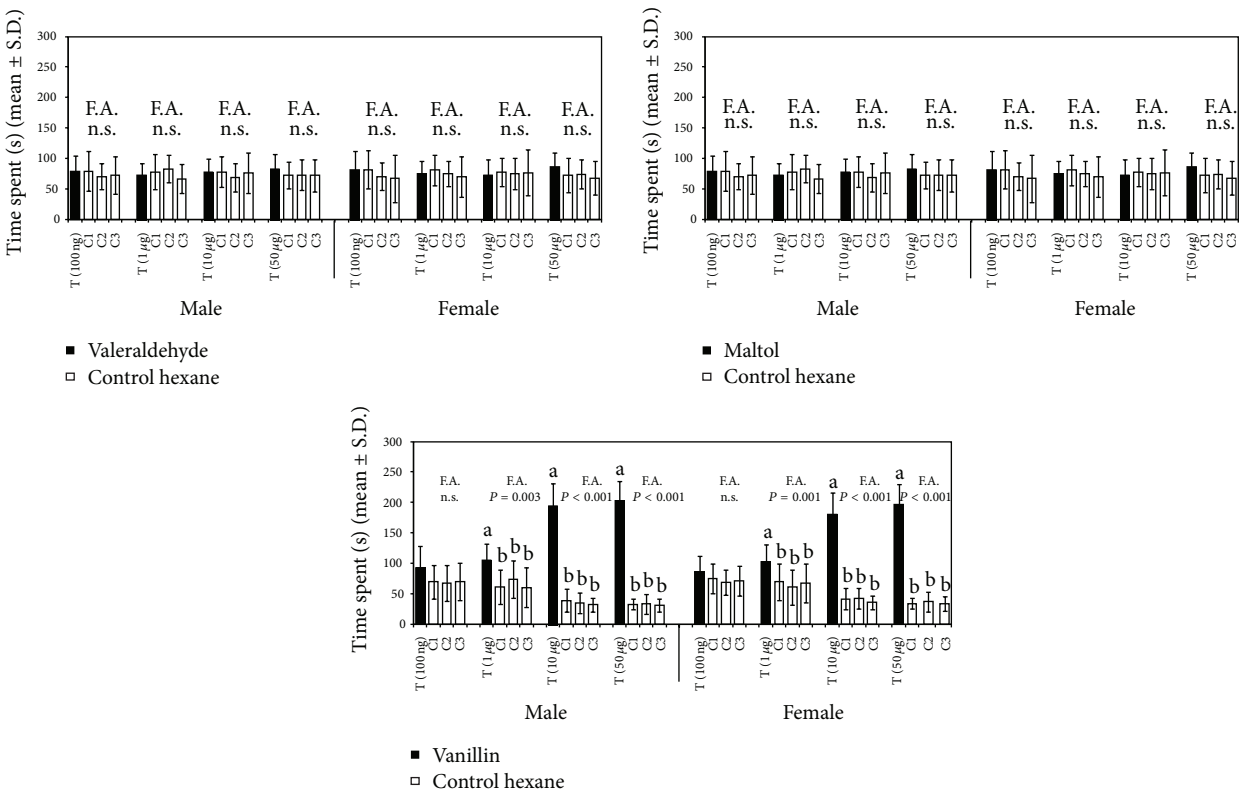

Figure 3: Mean allocation time of *T. elegans* males ($N = 15$) and females ($N = 15$) in the sectors of a Petri dish provided with increasing doses of synthetic compounds (T) or with 10 μL of hexane (C1, C2, and C3). For each dose tested, means were submitted to Friedman's two-way ANOVA (F.A.) followed by Wilcoxon test in case of significance ($P < 0.05$). Values with different letters are significantly different according to Wilcoxon test ($P \leq 0.01$).

parasitoid behavior to ensure suitable population levels in the environments where its hosts are present.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Species Specificity of the Putative Male Antennal Aphrodisiac Pheromone in *Leptopilina heterotoma*, *Leptopilina boulardi*, and *Leptopilina victoriae*

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Male antennal aphrodisiac pheromones have been suggested to elicit female receptiveness in several parasitic Hymenoptera, including *Leptopilina boulardi*. None of the proposed pheromones, however, has been fully identified to date. It is also unknown whether these antennal pheromones are species specific, because the species specificity of mate recognition and courtship elicitation in *Leptopilina* prevented such experiments. In this study we present an experimental design that allows the investigation of the species specificity of the putative male aphrodisiac pheromone of *L. heterotoma, L. boulardi,* and *L. victoriae*. This is achieved by chemical manipulation of the odour profile of heterospecific females, so that males perceive them as conspecifics and show antennal courtship behaviour. Males courted the manipulated heterospecific females and antennal contact between the male and the female was observed. However, males elicited receptiveness only in conspecific females, never in the manipulated heterospecific females. Chemical analysis showed the presence of species specific unsaturated hydrocarbons on the antennae of males. Only trace amounts of these hydrocarbons are found on the antennae of females. Our results are an important step towards the understanding and identification of antennal pheromones of parasitic wasps.

1. Introduction

Chemical senses are widespread in nature and chemical communication was very likely the first mechanism to transfer information between individuals [1]. Chemical compounds that transfer information can be divided into allelochemicals and pheromones [2]. Wyatt [3] defines "pheromones" as "molecules that are evolved signals which elicit a specific reaction, for example, a stereotyped behaviour and/or a developmental process in a conspecific." Sex pheromones are thus signals that are involved in behaviour or processes that relate to mating.

In parasitic Hymenoptera, sex pheromones are important at three different levels of sexual communication [4]:

(1) Mate attraction: one sex attracts the other over some distance with a volatile pheromone.

(2) Mate recognition: less volatile pheromones facilitate reliable recognition of sex and species to identify a specimen as a suitable mate and elicit courtship.

(3) Courtship: during courtship, males release aphrodisiac pheromones to elicit female receptiveness.

Aphrodisiac pheromones are often employed by males to elicit receptiveness in females and their involvement in courtship has been extensively investigated in parasitic Hymenoptera. In several species, including *Leptopilina*, antennal or oral male aphrodisiac pheromones have been proposed [5–10]. Such male aphrodisiac pheromones could allow females to identify a courting male as conspecific, if the pheromone is species specific. Additionally, an aphrodisiac pheromone could also signal male quality [11]. In some species, for example, in the genus *Nasonia*, the male aphrodisiac pheromone lacks species specificity, as heterospecific

courting males may be accepted as a mate by a female. *Nasonia* species also possess very similar sex pheromones, which leads to interspecific courtship [12]. In other genera, such as *Leptopilina*, mate recognition is species specific [13, 14], which usually prevents interspecific courtship.

Isidoro et al. [8] proposed that a male antennal aphrodisiac pheromone also exists in *L. boulardi*. In their work, they demonstrated that antennal contact between males and females during courtship is required to elicit receptiveness in females. Additionally, they described glands and gland openings in the third and fourth male antennomeres. These antennomeres are brought into contact with the distal part of the female antennae during courtship [8]. It is thus assumed that a chemical substance, an aphrodisiac pheromone, is transferred from the male antennae onto the female antennae to elicit female receptiveness. The species specificity of the proposed aphrodisiac pheromone, however, could not be investigated in *L. boulardi* and *L. heterotoma,* as interspecific courtship rarely occurs. Using chemical manipulation of females, we investigated the species specificity of the male courtship signals (putatively pheromones) in *L. heterotoma, L. boulardi,* and *Leptopilina victoriae*. Additionally we analysed the chemical compounds found on the antennae of the males of these three species. Our results are an important step towards the understanding and identification of antennal pheromones on parasitic wasps.

2. Material and Methods

2.1. Insects. We reared *L. boulardi, L. heterotoma,* and *L. victoriae* using *Drosophila melanogaster* as the host species. *Drosophila melanogaster* was reared on a corn-based diet (504 mL water, 66 g sugar, 6 g baker's yeast, 2.3 g agar, 52 g cornmeal, 1.3 mL propanoic acid, and 0.8 g Nipagin) at 25°C ambient temperature, with roughly 75% humidity, and a 16 : 8 h L : D cycle. About 30 flies (mixed sexes) were placed into a jar for each rearing. The jar contained fresh fly food. The flies were removed from the jar after 48 h, and about 10 wasps (both sexes) were put into the jar. Parasitized fly pupae were removed from the jars before the adult wasps emerged and put singly into 1.5 mL microcentrifuge tubes to obtain naive and virgin wasps of known age.

2.2. Extraction. Virgin 1-day-old females were extracted in batches of 30–50 with 5 μL dichloromethane (DCM) per female for 10 min. Afterwards, the DCM was evaporated under a gentle stream of nitrogen. Then, the residue was redissolved in 1 μL acetone per 5 μL original volume. The final concentration of the extract thus equalled 1 female per 1 μL.

For the analysis of the antennae we killed 1-2-day-old naive males by freezing them. We then cut off the antennae and extracted them in batches of 10 (=5 males) in 25 μL DCM. The body of the males was also extracted in 50 μL DCM. We analysed 10 to 12 samples per species.

2.3. Chemical Analysis. Extracts and fractions were analysed on a GC2010 gas chromatograph (GC, Shimadzu, Duisburg, Germany) connected to a QP2010 plus mass spectrometer (MS, Shimadzu, Duisburg, Germany). The GC was equipped with a nonpolar capillary column (BPX-5, 30 m length, 0.25 mm inner diameter, and 0.25 μm film thickness, SGE Analytical Sciences, Milton Keynes, UK). Helium was used as carrier gas with a constant linear velocity of 50 cm s^{-1}. The temperature of the GC oven started at 80°C and was raised by 5°C min^{-1} to 280°C, where it was kept for 20 min. The MS was run in electron impact (EI) mode at 70 eV and set to a scan range from 35 to 600 mz^{-1}. Sample volumes of 1 μL were injected splitless at an injector temperature of 280°C. The n-alkanes in the extracts of males of all three *Leptopilina* species were identified by comparing mass spectra and retention indices to those of synthetic reference compounds. Methyl-branched hydrocarbons were identified by interpretation of diagnostic ions resulting from the favoured fragmentation at the branching points [15] and comparison of linear retention indices with literature data [16].

2.4. Mating Trials. To investigate whether males from each species could elicit readiness to mate with con- and heterospecific females, mating trials were conducted. For this, naive, virgin males were allowed to court naive, virgin females and we recorded whether females showed readiness to mate. Trials were conducted in a small plexiglass arena (15 mm diameter and 2 mm height) covered with a glass lid and lasted 120 s. Trials were terminated early when the female showed readiness to mate. As males court only conspecific females, heterospecific female odour profiles had to be chemically manipulated, so the females were perceived as conspecifics by the males. The female odour profiles were manipulated by applying 0.1 μL (equalling 0.1 female equivalents) female extract redissolved in acetone from the male's species to the female. Previous studies [17, 18] have revealed that many parasitic wasps tolerate the application of acetone extracts without any visible intoxication. Conspecific females were also treated with extract. The extract was applied using on-column GC syringe (Hamilton, Bonaduz, Switzerland). After applying the extract, females were allowed to recover for 120 s. Females that did not recover after the application were discarded from the experiment (only 2 of all treated females did not recover within 120 s). After recovering, females were carefully placed into the arena and a single male was added. We recorded whether the male courted the female and whether the female showed readiness to mate. For each possible combination of male and female species, we conducted experiments until male courtship including antennal stroking was observed in 10 replicates.

2.5. Statistical Analysis. The number of mating trials conducted until courtship including antennal stroking was observed 10 times in each combination of male and female species and was analysed with the chi-squared test. The relative amounts of the compounds found in the extracts were arc-sinus transformed before statistical analysis. To compare the chemical profiles of the three species we performed a nonparametric multidimensional scaling based on the Bray-Curtis distance between samples. Statistical tests were performed in Past 3 [19] and R version 3.1.1 [20].

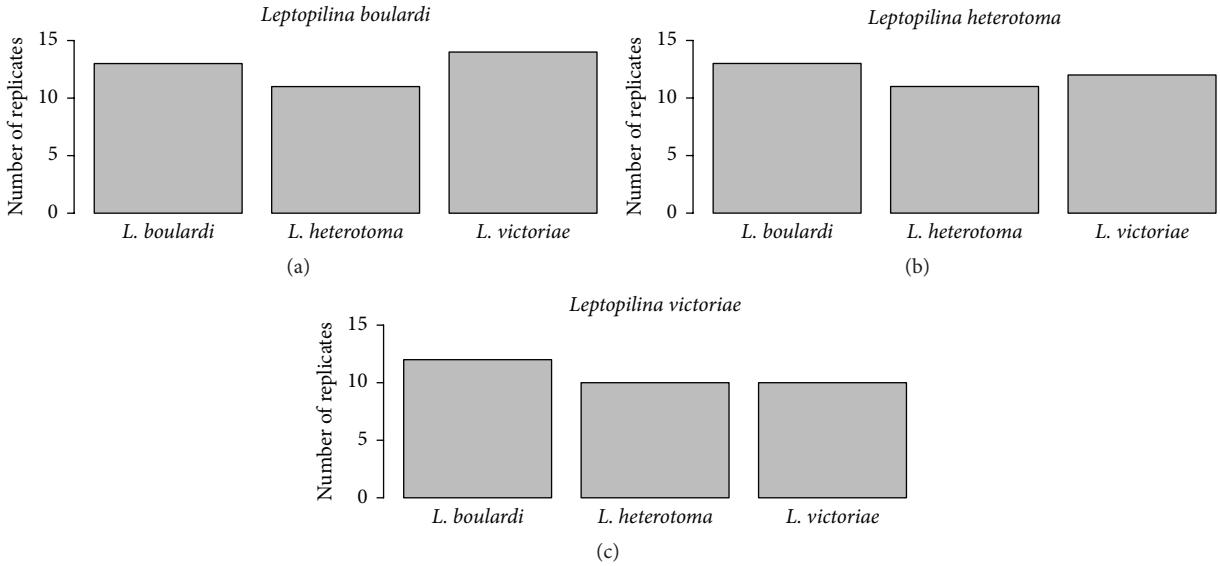

Figure 1: Number of replicates conducted until courtship including antennal stroking was observed 10 times in intraspecific and interspecific mating trials. A statistical analysis of the number of conducted replicates showed no significant differences between the different species combinations (chi-squared test: $\chi^2 = 0.2527$; df = 4; $p = 0.9927$).

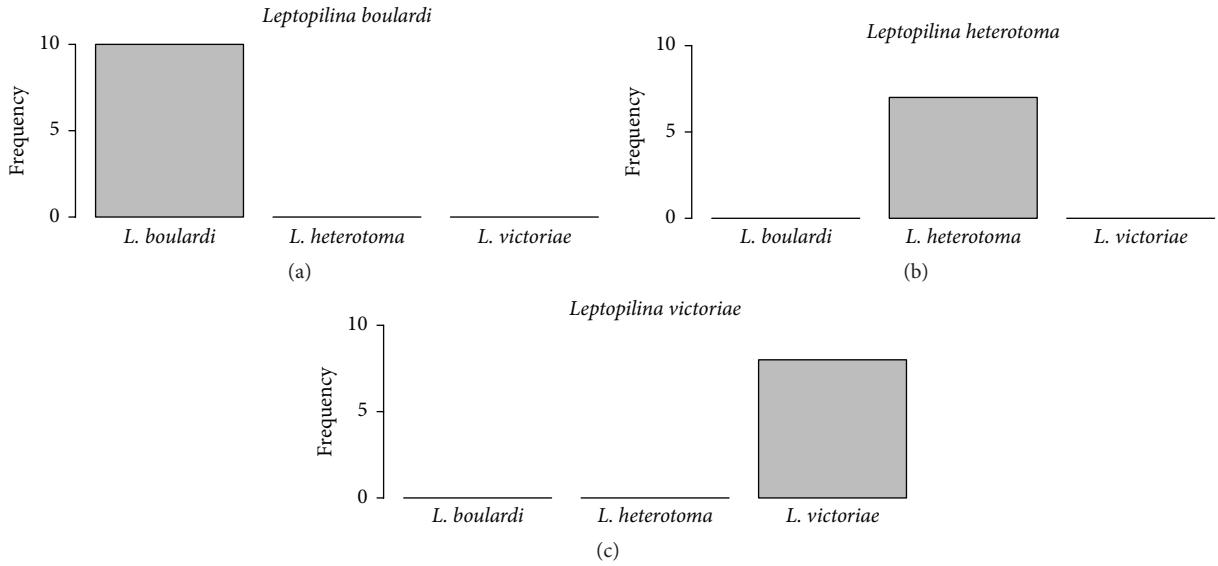

Figure 2: Number of (a) *L. boulardi* males, (b) *L. heterotoma* males, and (c) *L. victoriae* males that elicited readiness to mate in mating trials with conspecific and heterospecific females. For each experiment $n = 10$.

3. Results

Males of all three species readily courted both conspecific females and heterospecific females treated with extract of conspecific females (Figure 1). The statistical analysis of the number of conducted replicates indicated no significant differences between all combinations of male and female species (chi-squared test: $\chi^2 = 0.2527$; df = 4; $p = 0.9927$). The manipulation of the females' odour profiles was an effective means to reliably elicit interspecific courtship. However, males elicited readiness to mate only with conspecific females but never with heterospecific females (Figure 2).

We found 24 compounds in the extracts of the antennae of males (Table 1). The chemical profiles of males of *L. heterotoma* and *L. victoriae* were dominated by pentatriacontadiene (C35), while males of *L. boulardi* produce high amounts of two methyl-branched alkenes with a chain length of 31. Those unsaturated hydrocarbons are only present in trace amounts in the chemical profiles of females [14]. The chemical compounds found on the antennae of males were also found on the other parts of the body of males (not shown). The statistical analysis (NMDS stress = 0.12) showed that the chemical profiles of the antennae of males are species specific (Figure 3).

Table 1: Mean (±SD) relative amounts and linear retention indices of the compounds extracted from the antennae of males of *L. heterotoma, L. boulardi,* and *L. victoriae*.

Compound	Retention index	*L. heterotoma*		*L. boulardi*		*L. victoriae*	
		Mean	SD	Mean	SD	Mean	SD
4-Methyl octacosane	2858	10.95	2.47	21.59	3.40	6.97	1.02
Unidentified	2944	0.53	0.24	0.69	1.05	0.26	0.20
Methyl triacontane[1]	3024	0.08	0.14	3.81	0.99	0.27	0.09
4-Methyl triacontane	3056	16.70	4.93	10.69	1.25	26.79	6.78
Methyl hentriacontene[1]	3106	0.20	0.33	14.04	1.22	0.05	0.03
Methyl hentriacontene[1]	3114	0.16	0.30	11.82	2.22	0.31	0.17
Methyl hentriacontane[1]	3121	1.37	0.92	1.15	0.25	2.30	0.99
Unidentified	3140	4.42	1.59	7.96	1.18	5.09	1.24
Methyl hentriacontane[1]	3157	0.53	0.43	0.15	0.11	0.44	0.15
Methyl hentriacontane[1]	3168	0.59	0.46	0.22	0.10	0.82	0.39
Methyl dotriacontane[1]	3222	0.24	0.20	1.38	0.33	0.61	0.31
Tritriacontadiene[1]	3242	2.83	1.12	15.29	4.93	2.07	0.56
Tritriacontadiene[1]	3249	2.24	0.89	0.03	0.04	3.26	1.26
4-Methyl dotriacontane	3255	3.59	1.03	0.27	0.14	3.92	1.14
Methyl tritriacontene[1]	3305	0.17	0.38	9.05	2.38	0.02	0.02
Methyl tritriacontene[1]	3314	1.73	1.57	0.23	0.08	0.32	0.18
Methyl tritriacontane[1]	3320	1.57	1.63	0.10	0.08	0.78	0.36
Unidentified	3336	0.95	0.37	1.14	1.71	0.00	0.00
Unidentified	3341	1.00	0.58	0.01	0.01	0.00	0.00
Unidentified	3342	0.00	0.00	0.00	0.00	0.96	0.50
Methyl tritriacontane[1]	3349	0.61	0.72	0.10	0.20	0.00	0.01
Methyl tritriacontane[1]	3355	0.98	0.96	0.02	0.05	0.00	0.00
Pentatriacontadiene[1]	3441	48.57	8.27	0.27	0.20	42.04	7.33
Pentatriacontadiene[1]	3448	0.00	0.00	0.00	0.00	2.72	0.85

[1]The position of the methyl branch and/or the double bond could not be determined.

4. Discussion

We have found that the male courtship signal (putatively an antennal aphrodisiac pheromone) in *Leptopilina* is species specific. Males of the species *L. heterotoma, L. boulardi,* and *L. victoriae* can elicit readiness to mate only with conspecific, but not in heterospecific females.

Isidoro et al. [8] demonstrated that antennal contact during courtship is essential in *L. boulardi*. They showed elegantly by amputation of antennae that males cannot elicit readiness to mate with females if antennal contact is prevented. In their experiments, they used individuals that had one of their antennae amputated. When females and males had the antennae amputated on the same side, males were still able to elicit receptiveness in the females, but when females and males had the antennae amputated on different sides, males failed to elicit receptiveness. However, Isidoro et al. [8] could not investigate the species specificity of the assumed male antennal aphrodisiac pheromone, as *L. heterotoma* males did not court *L. boulardi* females—and vice versa—in the bioassays. The absence of cross-specific courtship is no surprise, as mate recognition is species specific in *Leptopilina* [14]. We could overcome this problem by chemically manipulating the odour profile of heterospecific females, so males perceived them as conspecifics. Males of the three investigated species readily courted these manipulated females, which allowed us to investigate the species specificity of the male antennal pheromone.

One possible explanation for the required antennal contact demonstrated by Isidoro et al. [8] is that the signal is a tactile one. Males from different species could show different stroking patterns and, for example, the stroking speed could signal mate quality. Such signalling is known from, for example, cucumber beetles, in which the female decides to reject or accept the male's spermatophore based on antennal stroking speed [21]. In the mating trials conducted in the present study, however, no obvious species specific antennal stroking patterns were observed.

Antennal glands in males, on the other hand, are a common feature in Hymenoptera [10], and they are putatively involved in courtship in a number of parasitic Hymenoptera. Strong evidence for male antennal aphrodisiac pheromones has been found in, for example, *Amitus spiniferus* (Hymenoptera: Platygastridae) [6], *Pimpla turionellae* (Hymenoptera: Ichneumonidae) [7], *Trichopria drosophilae* (Hymenoptera: Diapriidae) [10], and also *L. boulardi* [8]. We thus assume that the species specific signal in *Leptopilina* is indeed a pheromone.

This assumption is supported by the results of the chemical analysis. Males of all three species produce double

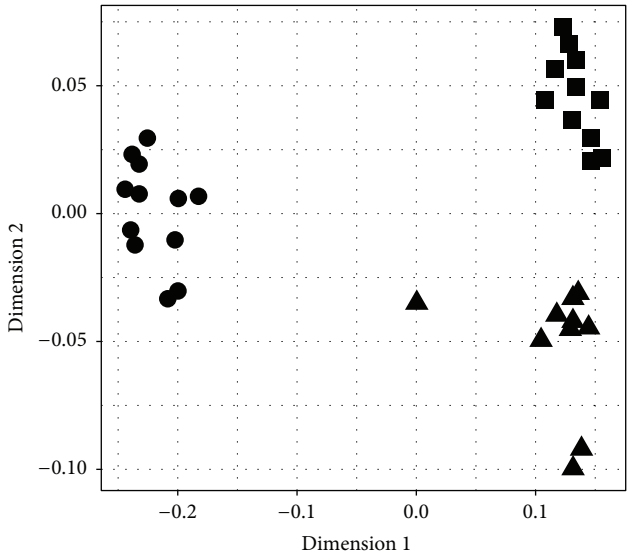

Figure 3: Nonparametric multidimensional scaling (NMDS) analysis of the chemical profiles on the antennae of males of *L. heterotoma* (triangles), *L. boulardi* (circles), and *L. victoriae* (squares).

unsaturated (*L. heterotoma* and *L. victoriae*) or methyl-branched monounsaturated hydrocarbons (*L. boulardi*) with a chain length of 31 to 35. These compounds dominate the cuticular hydrocarbon profiles of males but are only found in trace amounts in females. For example, in *L. heterotoma*, the diene accounts for more than 45% of the total amount of hydrocarbons found on the male antennae (Table 1). The chemical profiles found on the male antennae are also species specific (Figure 3) and are thus ideal compounds to form the putative male aphrodisiac pheromone. The very low volatility of the compounds fits well to the direct contact of the antennae needed to elicit receptiveness in the females. We found the male specific compounds also on the other parts of the males' body, not only on the antennae. The grooming behaviour regularly shown by the males (personal observation) could account for an even spread of the compounds over the whole body of the males.

We found that the male courtship signal (putatively a pheromone) is indeed species specific. This is noteworthy, as a species specific courtship signal establishes a barrier to heterospecific matings, even though mate recognition is already species specific [14]. There are thus two independent recognition mechanisms: the female pheromone eliciting courtship in the male and the male courtship signal eliciting receptiveness in the females. This might reflect a high selective pressure against heterospecific matings. *Leptopilina* females are, like most (solitary) parasitic Hymenoptera, monandrous ([22], personal observation for *L. heterotoma*). In combination with the arrhenotoky found in *Leptopilina* and the *Wolbachia*-mediated cytoplasmic incompatibility [23, 24], this imposes a great fitness loss upon females that accept a heterospecific mate. Only male offspring will be produced from heterospecific matings, which results in a reduced fitness as compared to producing female and male offspring. This is especially true for species that experience local mate competition, which is true for at least *L. heterotoma* [25].

Despite the mentioned range of literature showing the great interest in such male aphrodisiac pheromones in parasitic Hymenoptera, no such putative pheromone has been identified to date. Our experimental setup allows us to have males courting heterospecific females, in which they cannot elicit readiness to mate. In subsequent experiments, the identified pheromone candidate compounds should now be applied to male antennae in heterospecific courtship trials to investigate their ability to elicit female receptiveness.

5. Conclusions

In this study we could show that the male courtship signal of *Leptopilina* males is species specific. We assume that this courtship signal is a male antennal aphrodisiac pheromone and we found species specific long-chained hydrocarbons on the antennae of males which seem well suited to form these putative pheromones. The results are an important step towards the first identification of a male antennal pheromone in a parasitoid wasp.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

Effects of Abiotic Factors on HIPV-Mediated Interactions between Plants and Parasitoids

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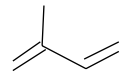
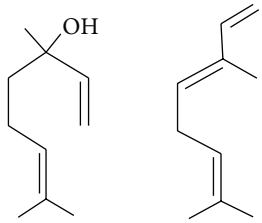
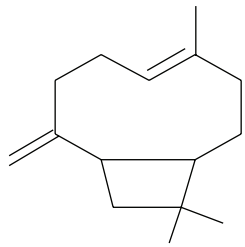
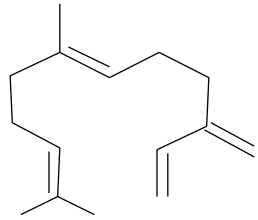
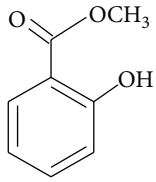
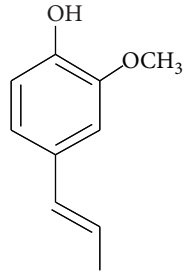
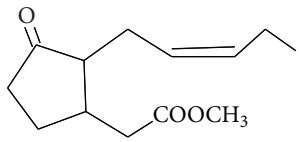
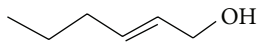
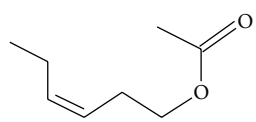
In contrast to constitutively emitted plant volatiles (PV), herbivore-induced plant volatiles (HIPV) are specifically emitted by plants when afflicted with herbivores. HIPV can be perceived by parasitoids and predators which parasitize or prey on the respective herbivores, including parasitic hymenoptera. HIPV act as signals and facilitate host/prey detection. They comprise a blend of compounds: main constituents are terpenoids and "green leaf volatiles." Constitutive emission of PV is well known to be influenced by abiotic factors like temperature, light intensity, water, and nutrient availability. HIPV share biosynthetic pathways with constitutively emitted PV and might therefore likewise be affected by abiotic conditions. However, the effects of abiotic factors on HIPV-mediated biotic interactions have received only limited attention to date. HIPV being influenced by the plant's growing conditions could have major implications for pest management. Quantitative and qualitative changes in HIPV blends may improve or impair biocontrol. Enhanced emission of HIPV may attract a larger number of natural enemies. Reduced emission rates or altered compositions, however, may render blends imperceptible to parasitoides and predators. Predicting the outcome of these changes is highly important for food production and for ecosystems affected by global climate change.

1. Introduction

Plants emit volatile organic compounds in considerable amounts: each day, land plants release up to 10% of the carbon they assimilated from carbon dioxide (CO_2) back into the air [1]. The blend of plant volatiles (PV) emitted by leaves comprises a diverse array of compounds (Table 1), mainly terpenoids as well as fatty acid derivatives, benzenoids, phenylpropanoids, and other amino acid derivatives, methanol and ethylene [2, 3]. While some are emitted constitutively, herbivore feeding and oviposition lead to the release of a special blend of PV called herbivore-induced plant volatiles (HIPV; [4]) and oviposition-induced plant volatiles (OIPV; [5]), respectively. HIPV and OIPV comprise compounds of the same classes that are either not produced by undamaged plants or emitted in different amounts by damaged ones [4]. HIPV have a high information content which is coded by quality and quantity of the HIPV blend [4]. Presence and concentration of, as well as ratio between, compounds can convey highly specific information on the involved herbivore and plant species, possibly even giving away their developmental stages and the plant cultivar [6–9].

As multifunctional infochemicals, HIPV constitute a subcategory of semiochemicals which are mediating interactions between plants and several trophic levels of insects [10, 11]. On the one hand, HIPV can act as allomones in direct defence, having toxic and/or repellent effects on herbivores [8, 12]. On the other hand, they can be perceived by natural enemies (parasitoids/predators) of herbivores who use them to detect their host/prey [13]. This fascinating interaction is also called "cry for help" [4, 14]. Here, they function as synomones—beneficial for both participating parties—providing indirect plant defence by attracting natural enemies of the herbivores and facilitating host/prey detection for the latter [4, 15].

Table 1: Biosynthesis of main compounds classes of herbivore-induced plant volatiles.

Terpenoids	
Terpenoids are basically synthesized in three consecutive steps as described by Dudareva et al. [20]: first, formation of the primary C5 units, the isoprene building blocks. Two or more of these C5 units can, in the second step, be condensed into C10 or C15 units which are, in the third step, conversed into the respective mono- or sesquiterpenes. Step two can be skipped to convert a single C5 unit into a hemiterpene. There are two pathways producing the C5 units in plant cells. The MEP pathway is located in the plastids and produces C5 units for hemi-, mono-, and diterpene synthesis. The MVA pathway is located in the cytosol, producing C5 units for sesquiterpene synthesis. Cross talk between these two pathways is happening. Eventually, enzymatic alterations can improve the volatility and/or change functionality of the hemi-, mono-, sesqui-, and diterpenes. The large enzyme family of terpene synthases is responsible for the last steps in terpene biosynthesis, creating an astounding diversity of terpenoids. Volatility decreases with increasing molecule size: hemi- and monoterpenes are considered volatiles while sesquiterpenes are semivolatiles and diterpenes are nonvolatiles.	Hemiterpene  Isoprene Monoterpenes  Linalool (*E*)-β-Ocimene Sesquiterpenes  (*E*)-β-Caryophyllene  (*E*)-β-Farnesene
Benzenoids and phenylpropanoids	
The shikimate pathway synthesizes the amino acid *L*-phenylalanine which is the common precursor of benzenoids and phenylpropanoids which contribute to the HIPV bouquet [2, 17]. After *L*-phenylalanine is deaminated by the enzyme phenylalanine ammonia-lyase, the resulting trans-cinnamic acid can be transformed into benzoic acid, the precursor of benzenoids, or into phenylpropanol, the precursor of volatile phenylpropenes like eugenol and chavicol. Volatile phenylpropanoids, however, are produced from *L*-phenylalanine directly. Either way, the final biosynthetic steps are dominated by the enzyme superfamilies of acyltransferases and methyltransferases.	Benzenoid  Methyl salicylate Phenylpropanoid  Eugenol
Fatty acid derivatives	
The LOX pathway produces derivatives of C18 fatty acids released from damaged cell membranes [20]. Methyl jasmonate and "green leaf volatiles" (GLV) like hexenol and hexenyl acetate are all breakdown products of C18 unsaturated fatty acids like linoleic and linolenic acid [20]. In the first step of the LOX pathway, the fatty acids are stereospecifically oxygenated into 9- or 13-hydroperoxy intermediates, feeding two separate branches of the pathway: methyl jasmonate and C6 GLVs are produced from the 13-hydroperoxy intermediates while C9 GLV are produced from the 9-hydroperoxy intermediates [146].	 Methyl jasmonate "Green leaf volatiles"  (*E*)-2-Hexenol  (*Z*)-3-Hexenyl acetate

MEP = methylerythritol phosphate pathway, MVA = mevalonate pathway, LOX = lipoxygenase pathway.

Being sessile organisms, plants cannot run away from pestering herbivores. Chemical communication to summon their enemy's enemy is an important element of their armoury. This kind of indirect plant defence appears to be rather common: it has been described for 49 plant species from 25 different families and insects from 5 different orders [16]. Tritrophic interactions between plants, herbivores, and their natural enemies are presumed to have a long history of coevolution [17]. Recently, using genetically modified tobacco (*Nicotiana attenuata*), the ability of HIPV to increase plant fitness has been demonstrated in field assays for the first time [18].

HIPV can be emitted from bursting storage organs like resin ducts, glandular trichomes, and vacuoles or can be synthesized *de novo* in the damaged tissue or nearby [3]. Triggers for HIPV emission are of chemical as well as physical nature. Mechanical damage to plant foliage alone, imitating herbivore feeding behavior, can result in PV emissions similar but not identical to HIPV [5, 13, 19]. Additionally, specific molecules in the oral secretions of herbivores and the high pH of the secretions elicit the release of HIPV [2]. The mechanisms of how plants "sense" that they are under attack from herbivorous arthropods have been reviewed in detail by Hilker and Meiners [5]. In general, chewing and piercing/sucking insect herbivores are sensed differently and trigger different defence pathways. Chewing insects cause extensive tissue damage. Fatty acids, like linoleic acid which is originally incorporated in cell membranes, are degraded and transformed into C6 and C9 aldehydes, alcohols, ketones, and their esters which are also called "green leaf volatiles" (GLV) due to their characteristic smell [20]. Together with salivary secretions, this induces a defence pathway in which jasmonic acid plays a major role and leads to the emission of specific HIPV as well as the production of specific defence compounds like proteinase inhibitors [8]. Piercing/sucking insects cause way less mechanical damage and are therefore mainly sensed via elicitor molecules from salivary secretions. They activate a defence pathway in which salicylic acid plays a major role, leading to systemic acquired resistance and HIPV production. Regarding this insect feeding guild, relatively little is known about the involved pathways [8]. Both the jasmonic and the salicylic acid dependent signalling pathways lead to HIPV emission. Several biosynthetic pathways are involved in the production of plant volatiles. The main ones are the mevalonate (MVA) and methylerythritol phosphate (MEP) pathways which are producing terpenoids and carotenoid derivatives, the shikimate and phenylpropanoids pathway producing benzenoids and phenylpropanoids, and the lipoxygenase (LOX) pathway producing GLV, methyl jasmonate, and other fatty acid derivatives [20]. All of them have been previously reviewed and described in detail [16, 20, 21]. Therefore we will only give a brief summary (Table 1) and refer the interested reader to the mentioned publications.

The "cry for help" is a very specific interaction phenomenon. Parasitoids are only attracted by volatile blends which correspond to their host. This information is not necessarily conveyed by major compounds in the blend; minor compounds or compound ratios can be just as important [4]. In the context of current global change, the question arises if HIPV are affected by changing abiotic conditions. Just as plants cannot run away from herbivores, they cannot escape unfavorable changes regarding climate, atmosphere, or soil by going somewhere else. In order to survive, they have to acclimate or adapt. Hence, abiotic factors have a strong impact on plant metabolism. Typically, organic compounds produced by plants are grouped into "primary" and "secondary" metabolism. Primary metabolism includes the very fundamental compounds which mainly consist of fatty acids, amino acids, and sugars. Secondary metabolism comprises compounds which are not imperatively necessary for the plant's survival but can be extremely beneficial and often are of high ecological value, like chlorophyll, flavonoids, alkaloids, terpenoids, and many more.

Abiotic factors affecting primary metabolism are particularly well known.

Climate. Temperature directly affects enzymatic activity and kinetics of chemical reactions. The intensity of photosynthetically active radiation (PAR) strongly influences the photosynthetic rate and stomatal conductance and, thus, the availability of carbohydrates as precursors for a plethora of biosynthetic pathways. Ultraviolet (UV) radiation is not used for photosynthesis but is very energetic and can lead to mutations by causing pyrimidine dimers in the DNA. Air humidity also affects stomatal conductance and thereby the plants evapotranspiration and xylem flow which transport nutrients from the roots to the leaves.

Atmosphere. The CO_2 concentration strongly affects the carbohydrate pool of plants by supplying carbon, influencing photosynthesis, and stomatal conductance. Ozone (O_3) on the other hand is highly reactive and can expose plants to oxidative stress due to enhanced formation of reactive oxygen species (ROS).

Soil. The availability of water is extremely important for plants. Like other factors, it affects photosynthesis and stomatal conductance as well as many more processes in cells. Availability of nutrients in the substrate the plant dug their roots into is another essential factor. Nitrogen, for example, is incorporated in molecules as fundamental as DNA, RNA, proteins, and chlorophyll. Maintaining their mineral homeostasis can be a challenge for plants: they require a certain level for signalling cascades, osmotically regulated processes, and as enzyme cofactors but high levels can be toxic. High salt concentrations can, for example, cause hydric deficit and osmotic shock.

Abiotic changes mostly do not affect one single process but rather a whole range. Primary and secondary metabolism are not two strictly separated pathways but interact and are intertwined in many ways, production and consumption of carbohydrates being only one of these intersections. Because of these interconnections, abiotic factors impacting primary metabolism are likely to have consequences for secondary metabolism. Plant volatiles are classified as secondary metabolites. Considering their involvement in plant defence, the question arises if plants are still able to defend themselves

directly and indirectly in a changing environment. Contrary to constitutive PV emission, the effects of abiotic factors on HIPV-mediated biotic interactions have received only limited attention to date [22]. If the emission of constitutive PV is affected by changing abiotic factors, is this also true for HIPV? Are their quantity and quality consistent although the plant's growing conditions change? Studying the impact of abiotic factors on constitutive plant volatiles provides the opportunity to elucidate their potential effect on HIPV since they share the same biosynthetic pathways.

2. How Do Abiotic Factors Impact Constitutively Emitted Plant Volatiles?

Constitutively emitted plant volatiles have several ecological and metabolic functions. They can attract pollinators and seed dispersers, act as direct defence against herbivores and pathogens, and mediate plant-plant-signalling as well as protecting plants against high temperatures, high light intensity, and oxidative stress [1, 3, 23]. Abiotic factors generally affect the emission of PV which has been discussed and summarized in several reviews [3, 24]. Hence, in the following, we will only provide a brief overview.

Climate. High temperature impact has been studied in short and long term experiments [24]. Temperature immediately affects the vapour pressure of compounds, stomatal aperture, enzymatic activity, and availability of precursor molecules [25]. Consistent with accelerated kinetics of biochemical reactions (Q10 rule), DeLucia et al. [26] found increased concentrations of defensive compounds related to both jasmonic acid and salicylic acid signalling pathways in several plants. In the long run, physiological acclimation and altered gene expression patterns additionally play a role [24]. Although long term studies are scarce, Peñuelas and Staudt [24] refer to rising temperatures as increasing constitutive isoprenoid emission in short and long term experiments. Increased isoprene emissions have been linked to enhanced thermotolerance due to improved lipid membrane stability [27, 28]. However, there are counterexamples where PV emission is not affected by temperature or is decreasing [24, 29]. Niinemets et al. [25] emphasize that the emission of many PV is strongly light-dependent. Among other effects, the intensity of PAR affects stomatal aperture and photosynthesis rates which in turn affects the availability of carbon-based precursor molecules for biosynthesis [25]. In agreement, elevated intensity of PAR has been observed to increase photosynthesis and isoprene emissions in two tropical tree species [30]. Furthermore, terpenoids can function as photoprotectants by dissipating energy and/or scavenging ROS in photosynthetic membranes [31]. Consistently, enhanced exposure to radiation from the UVB spectrum is also reported to increase emission rates of constitutive PV although there appears to be considerable variation depending on the studied species and the applied doses [24]. The "opportunist hypothesis," however, suggests that terpenoid emission is a byproduct of the biosynthesis of essential isoprene-based compounds like carotenoids [32]. The authors propose that emission of terpenoids is high under conditions leading to accumulation of essential isoprenoids, solely because they have common precursors and terpenoids (up to C15) are very volatile. On the other hand, Vickers et al. [33] suggested that isoprenoids generally improve the plant's tolerance to internal oxidative stress regardless which external factor caused it.

Atmosphere. The effect of elevated CO_2 concentrations was unclear in long and short term experiments reviewed by Peñuelas and Staudt [24], although a large number of studies report decreasing PV emissions. DeLucia et al. [26] confirm the considerable variance in the effect of CO_2 concentration on PV emissions, especially when comparing different species. Still, the authors detected the general trend in the literature that elevated CO_2 stimulates the production of phenolics in general but especially tannins and flavonoids while suppressing the production of terpenes. They summarize that the shikimic pathway, regulated by salicylic acid, appears to be enhanced in high CO_2 concentrations while the MEV and MVA pathways, regulated by ethylene and jasmonic acid, appear to be repressed. Peñuelas and Staudt [24] reviewed many indications of increasing emission of constitutive isoprenoids due to enhanced ozone exposure. Ozone poses an oxidative threat and can therefore increase the biosyntheses of antioxidants, like isoprene [33, 34], and, furthermore, have an additional effect on PV by degrading molecules once they have been emitted from the plant [24, 34, 35].

Soil. At first glance, the literature is ambiguous regarding the effect of drought. Looking closer, however, the issue resolves into a dose-dependent response with some variance due to plant species, drought duration, and method used to measure drought: mild drought may increase emissions or have no effect [24] but severe drought generally decreases emissions [36]. Still, approaching the complex situation using a model, the majority of variation in plant isoprene and monoterpene emission could be explained by variation in temperature and light, as well as leaf area index and plant functionality [37, 38]. In Mediterranean ecosystems where drought periods are typical climate events, temperature and PAR are not enough to simulate monoterpene emissions: adding a module on soil water content is necessary to improve simulations [39]. The impact of soil could be strongly species-dependent: terpene emissions of *Rosmarinus officinalis* and *Pinus halepensis* were generally higher on calcareous than on siliceous soil while it was the other way around regarding *Cistus albidus* [40]. The same pattern emerged regarding *Cistus monspeliensis*: terpene emissions were 7 times higher on siliceous than on calcareous substrate [41]. Increasing N-supply has been found to increase isoprenoid emissions [24]. High phosphorous supply, however, coincided with low isoprene emissions in *Phragmites australis* [42]. Salt stress had no effect on isoprene emissions of *Eucalyptus globulus* and *Populus x canescens* [43, 44].

In summary, abiotic factors can affect PV emission at a physiological (e.g., availability of precursors for biosynthesis and enzyme activity) and/or a physicochemical level (e.g., vapour pressure of the compound of interest, the leaf internal

structure, and stomatal aperture) [25]. While a lot of single factors have been studied, there are also countless examples of their interactions [24]. Although rising temperature may increase precursor availability and enhance the compounds' vapour pressure, it can also decrease stomatal aperture [24]. The latter would also be the effect of drought [24]. Deficiencies regarding water and/or nutrient supply as well as salt stress may disturb the osmotic status of plants and therefore likewise affect stomatal aperture.

Chemically, constitutively emitted and induced plant volatiles are not always clearly distinguishable. Terpenoids, fatty acid derivatives, benzenoids, and phenylpropanoids are present in both groups [4]. Some may be emitted in higher concentrations or altered ratios after herbivore attack while other compounds are emitted exclusively then [4]. Both constitutive and induced plant volatiles are derived from the same biosynthetic pathways (Table 1). In order to further illustrate the compounds' resemblance regarding their structure, we assembled examples for both categories of HIPV—involving quantitative or qualitative changes (Table 2). It is highly probable that factors which affect the biosynthesis and emission of constitutive PV also affect HIPV because they depend on the same pool of resources and energy.

3. Do Abiotic Factors Affect the Emission of HIPV?

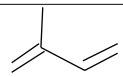
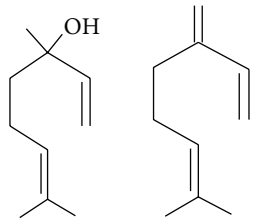
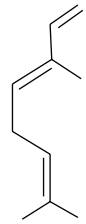
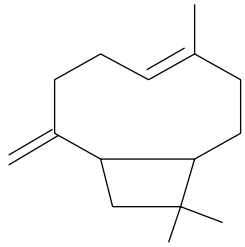
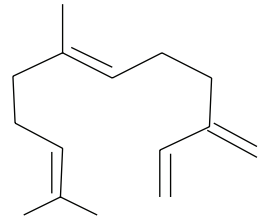
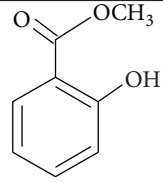
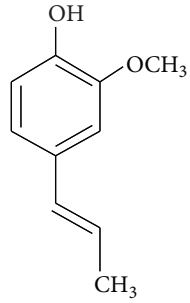
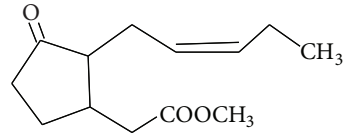
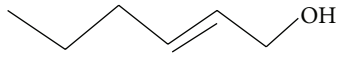
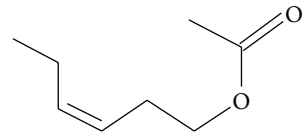
Plants only emit induced volatiles in certain situations. According to the Optimal Defence Hypothesis [45], only producing compounds when they are needed saves resources because plant volatiles come with a metabolic cost. For instance, maize plants that were genetically modified to constitutively emit the HIPV (*E*)-β-caryophyllene and (*E*)-α-humulene showed decreased fitness compared to nonmanipulated plants which only emit these compounds when under attack [46]. However, studies suggest that maintaining signalling pathways may also have considerable metabolic costs for plants [47].

Regarding HIPV emission, there is a large variance caused by several biotic and abiotic factors. Major biotic factors affecting HIPV are the plant species or even the cultivar concerned, the plant organ the damage is afflicted on, the extent of the damage and its duration, and both the feeding guild and species of the herbivore as well as the ontogenetic stage of both plant and herbivore [13, 22, 48]. During plant ontogeny, quantity and quality of HIPV emission change with higher concentrations emitted during their vegetative compared to their reproductive stage [9]. Major abiotic factors affecting HIPV are temperature, light intensity, and ozone, as well as water and nutrient availability [3, 16, 49]. The influence of abiotic factors on HIPV has received much less attention than that on constitutive PV [22]. Generally, all factors that influence stomatal aperture could affect HIPV emission [50]. However, there is heterogeneity in the literature and results are not consistent [3]. The most detailed study on the effect of abiotic factors on HIPV has been published by Gouinguené and Turlings [29] who applied *Spodoptera littoralis* regurgitant on mechanically wounded leaves of young maize plants. Their results are listed in the following paragraphs. Except for air humidity, all tested abiotic factors caused qualitative changes of the HIPV blend. Interestingly, they detected considerable differences among the studied compounds.

Climate. Elevated temperature appears to enhance jasmonic acid, salicylic acid, and ethylene synthesis [26], all of which are involved in plant defence. Yet, the effect of temperature strongly depends on the temperature optimum of the respective plant species [26]. In young maize plants treated with *S. littoralis* regurgitant, HIPV emissions were highest between 22 and 27°C which probably correlates with maximum stomatal aperture [29]. At a relative air humidity of 60%, HIPV emissions were highest—compared to higher or lower humidity—in young maize plants which may also be explained by high stomatal aperture [29]. The authors also report that HIPV emission was generally light-dependent, increasing with radiation intensity, and did not happen in the dark. They suggest that HIPV production is closely connected to photosynthetic activity. Herbivores with strong diurnal feeding rhythms may also add diurnal variation to HIPV emissions: less feeding activity at night is common with many insect herbivores and some HIPV emissions are closely related to herbivore pressure [51]. Still, there is a considerable variation of response and/or emission patterns among HIPV: in cotton plants infested with *Spodoptera exigua*, the induced terpenoids (*E*)-β-ocimene and (*E*)-β-farnesene were emitted in a pronounced diurnal pattern while α-pinene and caryophyllene were not [52]. Additionally, (*E*)-β-ocimene emission continued to follow the diurnal pattern even after the caterpillars had been removed. In contrast, α-pinene emission stopped after insect removal. The authors hypothesize that the emission patterns especially of HIPV released from damaged storage organs depend on the feeding rhythm of the herbivores [52]. Although UVB radiation is known to activate the salicylic acid, jasmonic acid, and ethylene pathways, little is known about its potential to change HIPV [16]. Considering that enhanced UVB radiation can increase isoprene emission in some species [53], it is possible that it also affects HIPV.

Atmosphere. Elevated CO_2 concentrations affect the salicylic and jasmonic acid dependent defence pathways differently [8]. Studies suggest that elevated CO_2 concentration suppresses jasmonic acid while stimulating the production of salicylic acid [26] which may improve the plants ability to sense and signal attacks of piercing/sucking insects but hamper signalling pathway related to chewing insects and the respective production of HIPV. Elevated O_3 levels reduced the total terpenoid emission of nontransgenic and transgenic Bt-*Brassica napus*—oilseed rape which is producing insecticidal *Bacillus thuringiensis* toxin [35]. As mentioned in the previous section, O_3 enhances the level of oxidative stress in plants [16]. Some terpenoid volatiles have antioxidant activity and thus can ameliorate the oxidative damage—they may be synthesized by plants to scavenge ROS [16, 17]. The elucidation of causes and consequences of changed HIPV in elevated O_3 levels is further complicated by its high reactivity which

Table 2: Herbivore-induced plant volatiles emitted constitutively but in increasing concentrations after herbivore attack (quantitative changes) or only after herbivore attack (qualitative changes). Compounds from both categories can have very similar structures and share a biosynthetic pathway (see Table 1). This list is non-exclusive and inter-specific variation can be expected. It is mainly based on results on potato and tobacco (*Solanum tuberosum* and *Nicotiana tabacum*) reported by Dickens [148] and Robert et al. [46] as well as several review articles [16, 20, 147].

Compound class	Constitutively emitted, increasing after herbivore attack	Only emitted after herbivore attack	Reference
Terpenoids Hemiterpenes	 Isoprene		[16]
Monoterpenes	 Linalool β-Myrcene	 (*E*)-β-Ocimene	[16, 46, 83, 147]
Sesquiterpenes	 (*E*)-β-Caryophyllene	 (*E*)-β-Farnesene	[46, 83, 147]
Benzenoids		 Methyl salicylate	[16, 148]
Phenylpropanoids	 Eugenol		[20, 149]
Fatty acid derivatives		 Methyl jasmonate	[20, 150]
"Green leaf volatiles"	 (*E*)-2-Hexenol	 (*Z*)-3-Hexenyl acetate	[46, 148]

can lead to secondary changes of already emitted HIPV [24, 35].

Soil. The salicylic and jasmonic acid dependent defence pathways are also differently affected by drought stress [8]. HIPV emissions of young maize plants were higher in dry compared to wet soil, possibly because water stressed plants invest more in the biosynthesis of defence compounds [29]. In *B. napus* plants grown on nutrient deficient soil, the emission of several HIPV decreased compared to well-nourished ones [54], while HIPV emission of tobacco was not affected by low soil nitrogen [55]. Nitrogen-starved soy plants (*Glycine max*) produced the same range of HIPV like the well-nourished plants, but three compounds were affected in their concentrations [56]. Varying severity of nutrient deficiency may be a crucial factor to explain this heterogeneity of results. Salinity can alter the composition of HIPV blends in maize plants and reduce emissions per plant because it reduces plant growth [57]. Heavy metal stress also has the potential to affect volatile emissions. However, while maize plants exposed to copper stress emitted higher levels of HIPV when damaged by *S. frugiperda*, cadmium-exposure did not result in differential emissions [58].

These examples illustrate the plant side of the interaction, focussing on production and/or emission of induced compounds. In their review, Peñuelas and Staudt [24] pose the question whether the defensive function of HIPV will be retained if their quality or quantity is affected by abiotic changes. Will the parasitoids still be able to decipher the airborne message or will they be confused by the changes? In the following section we will focus on the parasitoid side of the interaction.

4. Do Abiotic Factors Impact Higher Trophic Levels through HIPV?

The emission of HIPV is a well-known characteristic interaction "cry for help" between the first and third trophic levels: the plant and the parasitoid. The chemical composition of a plant, that is, its nutritional value as well as concentrations of chemical defence compounds, shapes the arthropod community that interacts with the plant, notably with respect to the community's size, density, and dynamics [59]. On nitrogen-deficient plants, for instance, herbivore survival can decrease through bottom-up effects [49]. Increased C/N ratios make it harder for herbivores to cover their own nitrogen demand and this can prolong feeding time and slow down their development. This may increase the probability of parasitoids detecting and parasitizing the herbivores and the host may stay in vulnerable stages for a longer time [60]. However, the hosts themselves may be of lower nutritional value for the parasitoids larvae and/or may contain higher concentrations of plant defensive compounds, potentially toxic toward parasitoid larvae [59].

Even if parasitic hymenoptera do not directly feed on plant tissue, they can be affected through bottom-up effects of the plant's nutritious value and/or secondary metabolites which can promote or impede the plants "cry for help" [59]. Abiotic factors which alter the quality or quantity of HIPV may render the chemical "message" incomprehensible to the receiving organisms. Increased HIPV emission due to optimized temperature or air humidity as well as increased PAR intensity may improve signal perception for the parasitoid, also in greater distance of the emitting plant. Ozone, with its high reactivity, could decompose the volatiles and, thus, eliminate the signal. Drought and nutrient deficiency can decrease HIPV concentrations which may make it impossible for parasitoids to locate the plant or to even perceive the signal in the first place, if they are not in the direct vicinity.

One approach to predict if altered HIPV blends will affect parasitoids is to elucidate which compounds they can perceive. Coupled with GC-MS, the electroantennographic detector (EAD) allows for separation and identification of compounds and therefore provide a screening method for compounds which may be behaviorally active [16]. Gouinguené et al. [61] observed via GC-EAD that three species of parasitic hymenoptera, *Cotesia marginiventris*, *Microplitis rufiventris*, and *Campoletis sonorensis*, are able to perceive a variety of HIPV induced by *Spodoptera littoralis* larvae feeding on maize, cowpea, or cotton plants, some of which were only minor compounds. However, whether perception actually leads to a behavioral response and whether it will be positive or negative can only be investigated in observational studies [16]. It is hardly possible to predict the effect a changed blend of HIPV will have on parasitoids. As illustrated by the following examples, changed blends do not necessarily affect the natural enemy's behavior.

Climate. To our knowledge, the effects of temperature and PAR have not been tested yet. Exposure to UVB radiation did reduce oviposition and larval feeding of the moth *Plutella xylostella* on two Brassicaceae species, while increasing parasitization by *Cotesia plutellae* [62, 63]. However, we do not know which are the underlying mechanisms. Caputo et al. [62] report an example of parasitoids discriminating between hosts feeding on UV- and non-UV-exposed plants. While this may be explained by altered HIPV composition, it may just as well be due to other factors like changed host quality. On the other hand, *Cotesia marginiventris* did not discriminate between *Spodoptera frugiperda* larvae feeding on UV- or non-UV-exposed soybean (*Glycine max*) plants [64].

Atmosphere. Elevated CO_2 concentrations affect the feeding guilds differently: while phloem feeders tend to respond positively to the elevated carbohydrate level in plants, foliage feeders tend to respond negatively—possibly due to lower nitrogen concentrations (higher C/N ratio) or due to increased defence compounds [8]. This may in turn affect their parasitization rates. Minor changes of HIPV due to elevated CO_2 concentrations can jeopardize the interaction between parasitic wasps of the genus *Cotesia* and moth-infested (*P. xylostella*) *Brassica* species [65] or not [35]. It is unclear whether this is due to the different species of *Brassica* and *Cotesia* studied. While elevated O_3 levels reduced the total terpenoid emission of nontransgenic and transgenic Bt-*B. napus*, only the latter was negatively affected in its ability to attract *Cotesia vestalis* [35]. In a different study, however, the

communication between *P. xylostella*-infested *Brassica oleracea* and *C. plutellae* was not disrupted, even though elevated O_3 concentrations completely degraded most herbivore-induced terpenes and GLV [66]. The authors suggest that the successful orientation of the natural enemies may have been due to less reactive HIPV like benzyl cyanide and methyl salicylate, respectively. However, as completely clean air is rare in nature, Holopainen et al. [17] suspect that parasitoids may have learned to also associate the breakdown products of HIPV with their host and possibly even use the ratio between originally emitted compounds and their reaction products to estimate the plant's distance. Increased isoprene concentration in the plant periphery of genetically manipulated *A. thaliana* repelled the parasitic wasp *Diadegma semiclausum* but not *Cotesia rubecula* or the lepidopteran herbivores *Pieris rapae* and *Plutella xylostella* [67].

Soil. Nitrogen deficiency has strong bottom-up effects on the leaf miner *Tuta absoluta* feeding on tomato leaves [49], as well as on *S. frugiperda*, feeding on nitrogen-deficient soybean leaves, and its parasitoid *C. marginiventris* [56]. However, the latter authors concluded that indirect plant defence was not compromised because the behavioral response of the parasitoid to the emitted HIPV was unchanged. In maize seedlings treated with the elicitor volicitin, sesquiterpene emissions were higher in nitrogen-deficient compared to nondeficient plants [68], which may improve the attraction of parasitoids.

Additionally, plants may offer shelter or nectar as a food source for parasitoids [59] which may in turn be affected by abiotic conditions: Adler et al. [69] found higher concentrations of alkaloids in nectar of *Nicotiana tabacum* plants that were well fertilized compared to those receiving less nutrients. Such increased concentrations of toxic compounds in nectar may have direct negative effects on survival and/or fitness of parasitoids.

HIPV furthermore have the potential to mitigate various additional interactions among other organisms present in the community. Sensing the presence of beneficial or detrimental organisms, plants can change chemically and/or morphologically with effects on the arthropod communities. Plant defensive compounds can affect the community composition by repelling generalist herbivores but serving as recognition cues to specialists who can detoxify or sequester them for their own defence (e.g., see Desneux et al. [70]) and, thus, indirectly affect the composition of higher trophic levels [59]. HIPV can also have adverse effects on the emitting plants: they may come with negative ecological costs like repelling pollinators [71–73]. Furthermore, communication by volatile compounds is not necessarily a secure connection encrypted to outsiders. Other receivers may be "eavesdropping" on the plant-emitted signals and exploit the intercepted information. Some herbivores use HIPV to find suitable host plants [74] which can turn HIPV into plant kairomones—disadvantageous for the plants themselves. Communication also happens inside the plant community: not-infested neighbouring plants can perceive HIPV and boost their own defence without having suffered from herbivory themselves [15]. Eventually, it seems logical that a system has to be complex to convey information as detailed as observed regarding plant-insect communication. Yoneya and Miki [11] suggest that the multifunctionality of HIPV and variations in plant responses to herbivory are key mechanisms for evolutionary diversification of animal foraging and therefore the structure of ecological networks. Kessler [47] argues in the same direction, suggesting that the dynamics of induced defence compounds, like HIPV, with all the inherent complexity and multifunctionality, should also be seen as an information network.

These studies illustrate how plants are influenced by their environment and how these changes can be propagated through the higher trophic levels as bottom-up effects. Climate, soil, and atmosphere have a large potential to impact parasitoids directly or indirectly through plants. As they are often employed as biological pest control agents, a dramatic question arises: will global change jeopardize integrated pest management? Can we confront this looming threat with detailed knowledge on the elements involved? Moreover, could we go one step further and even use this knowledge to our advantage and improve the efficacy of parasitoids by manipulating the plants' growing conditions?

5. Significance of HIPV for Integrated Pest Management and Future Prospects

5.1. Could Optimized Abiotic Factors Improve Integrated Pest Management? The effects of abiotic conditions on HIPV and/or on natural enemies have mostly been studied focussing on factors relevant in a changing global environment [16, 24, 75]. However, some of these factors are also relevant in horticultural and agricultural context where parasitoids are often employed as pest control agents. Temperature, water supply, and humidity, for instance, are likewise affected by climate change and managed in horticulture and, to some extent, in agriculture. Plant nutrition, irrigation, temperature, and radiation intensity as well as CO_2 concentration are closely controlled in many modern horticultural production systems. In agricultural production systems, fertilization and irrigation are often manipulated. This may offer opportunities to adapt cultivation practice during biological pest control application to maximize the natural enemies' performances.

Climate. Crop producers using greenhouses may be well advised to increase heating and decrease cooling, respectively, decrease the application of shading screens or add lamps, and adjust a relative humidity of 60% to increase HIPV emission. However, we have to bear in mind that, so far, there is a lack of studies regarding the response of the parasitoids. Furthermore, one can assume that the optimal values for temperature, radiation intensity, and relative air humidity are immensely dependent on both the involved plant and insect species. It is well possible that optimal climatic conditions for high crop yield are not the same as for good parasitoid performance. High temperature, for example, might have positive effects on HIPV emission but is prone to have

negative effects on yield. Regarding many tritrophic systems, finding compromises might be challenging.

Atmosphere. CO_2 enrichment is a common practice in greenhouse crop production. A meta-analysis found that elevated CO_2 concentrations decrease herbivore abundance but increased foliage consumption [60]. The "high carb diet" slowed down herbivore development and, hence, may lead to increased attack rates by natural enemies because of higher exposure time [60]. This may be beneficial when parasitoids are employed to control foliage feeders. However, each tritrophic system has to be evaluated carefully as the response of parasitoids to bottom-up changes due to elevated CO_2 concentration has been observed to vary substantially (see Section 4).

Soil. In hydroponic cultivation systems, nutrient and water supply can be easily manipulated. Reducing the amount of nutrient solution or the frequency of its supply may increase HIPV emission in some plants by establishing mild drought and nutrient deficiency. However, greenhouse crops have hardly been studied in this respect. As we illustrated in the previous sections, existing results are promising but also highlight the interspecific variability.

While existing results definitely show tendencies, clearly more research is needed [24]. Attention has furthermore to be paid to temporal changes in HIPV blends which can affect herbivore and parasitoid preferences [76]. Additionally, most studies investigated the effect(s) of one altered factor while in a changing global context several factors will interact which calls for studies on various factors simultaneously [17].

5.2. Application of Synthetic Blends of HIPV. Instead of manipulating the plant's HIPV emission and to overcome variability due to a variable environment, compounds can be artificially applied to crop production systems to attract natural enemies [77]. Kaplan [78] has recently published a thorough review on HIPV application in biological control, explaining methods and mechanisms, listing which compounds attract which species (target and nontarget effects), describing opportunities and limitations, and we would like to refer the interested reader to his article for further details.

The potential of single synthetic HIPV or of blends in horticulture and agriculture has been the subject of several studies. Simpson et al. [15] list a number of chemicals that were successfully attracting parasitoids in field trials. Namely, these are methyl salicylate, *cis*-3-hexenyl acetate, geraniol, methyl anthranilate, methyl jasmonate, *cis*-jasmone, *cis*-3-hexen-1-ol, 3,7-dimethyl-1,3,6-octatriene, farnesene, octyl aldehyde, and indole. Topic application of plant hormones like jasmonic or salicylic acid can lead to the release of PV, but the quality and quantity mostly differ from actual HIPV blends [79]. In a large study, comparing the effect of methyl salicylate, *cis*-3-hexen-1-ol, and phenylethyl alcohol in maize and soybean fields, 4 and 16, respectively, out of 119 arthropod taxa showed significant responses [80]. The authors summarize that, all in all, repellent effects of HIPV were as frequent as attractive effects and the crop studied has a strong influence. Gols et al. [81] made the appeal that, in order to gain a more realistic understanding of these interactions in an ecological and evolutionary framework, studies should not simply focus on crops but involve wild plants. They found *C. rubecula* to be more attracted to wild than to cultivated cabbage infested by *P. rapae*.

Synergistic effects of blends of HIPV compared to single compounds have been observed a number of times regarding attraction of parasitic wasps [80]. While 13 synthetic HIPV showed activity in EAGs of *Cotesia sesamiae*, only 3 of them elicited behavioral responses when tested at a natural dose and two more at a higher dose [82]. Still, the authors had to combine 9 compounds to create a synthetic HIPV blend that was as attractive as the natural blend emitted by maize plants infested with female stemborers (*Chilo partellus*). Consistently, a study on genetically altered *A. thaliana* found a blend of HIPV and constitutively emitted PV to be more attractive to *C. marginiventris* than the HIPV alone [83]. Studies on natural enemies different from parasitoids point in a similar direction [84, 85]. An explanation of these synergistic effects might be that more compounds can convey more, thus more specific, information than single compounds. Fontana et al. [83] suggested that a successful host finding strategy might involve both constitutive and herbivore-induced volatiles. Specific information about the involved plant and herbivore species may be especially important for specialist parasitoids (see next section).

The use of synthetic HIPV for pest control in agroecosystems is not without risk. A field study showed that application of one single HIPV common in soybean managed to repel and/or attract several arthropod species in a range of up to 8 m from the source [86]. However, the authors observed that braconids were lured from surrounding fields, resulting in a depletion of braconid communities in neighbouring fields—possibly increasing the risk of herbivore outbreaks there. Removing parasitoids from surrounding areas may furthermore disrupt their population dynamics [87]. Meiners and Peri [87] caution that parasitoids which were artificially attracted to a field with low host density might decrease their foraging rates because the cue does not deliver a reward, that is, available hosts, and that higher parasitoid densities may not necessarily lead to higher parasitization rates. Using synthetic HIPV in the field may furthermore have unwanted effects like attracting additional herbivores and disrupting trophic cascades [88].

5.3. Is the Parasitoid's Host Range Relevant to Their Future Employment as Biological Pest Control Agents in a Changing Environment? Considering the plethora of HIPV compounds, blends, and their variability, the ability of predators and parasitoids to discriminate between the chemical cues is immense [16]. Yet, not all parasitoids necessarily use the same molecules for orientation.

As illustrated in the previous section, there is a considerable heterogeneity among the observed responses of parasitic hymenoptera to plant volatiles. With two studies, Ngumbi et al. provided some more detailed insights about the complexity of parasitoid responses, suggesting that the sex of the insect has an influence, as well as its degree of host

specialization. In Y-olfactometer essays, females of *Cotesia marginiventris* and *Microplitis croceipes*, a generalist and specialist parasitic hymenoptera species, responded stronger to HIPV than their respective males [89]. Additionally, at low dosages, the authors observed the generalist to respond strongly to GLV which convey the general information that herbivory is taking place while specialists responded stronger to more specific, host-related HIPV. These behavioral essays correspond well to earlier GC-EAD studies [90, 91]. The authors suggested that specialists use differences regarding compound ratios to determine if the feeding insect is their host or not. Specialists are considered to be rather "narrowly tuned" on host-related volatiles while it is sufficient for generalists to register broad-spectrum herbivory cues like GLV [10, 90–93]. Again, however, we must be alert to exceptions from this rule. A study comparing the generalist *Diadegma fenestrale* and the specialist *Diadegma semiclausum* did not find behavioral differences [71]. Yet, the authors emphasize the importance of ontogeny: both species only differentiated host and nonhost HIPV produced by the plant species they were reared on.

Associative learning describes a process where responses to certain stimuli are newly acquired or existing responses are enhanced by linking them to a reinforcing stimulus [10]. It has often been suggested to be the mechanism responsible for olfactory learning in adult parasitic wasps, increasing the phenotypic plasticity of displayed responses [10, 94–96]. For example, both *Cotesia glomerata* and *C. rubecula* (parasitoids of first-instar *Pieris brassicae* and/or *P. rapae* larvae) showed increasing interest in a previously unattractive host plant after finding suitable caterpillars there [94]. The author emphasizes that the two wasp species showed substantial differences although they are closely related: *C. glomerata* changed its innate preferences from cabbage odours towards odours of another plant already after one single experience and remembered it for at least five days. *C. rubecula* kept preferring cabbage odour and completely quit responding to the new odour after one day. The authors related the differences in learning to the wasps' social and oviposition behavior as well as the oviposition behavior of their hosts. While both *Cotesia* species are considered specialists, *C. glomerata* is described as more of a generalist than *C. rubecula* [94]. This is mirrored by inconsistent reports in the literature, describing *C. glomerata* either as specialist regarding its insect hosts [97] or as generalist [98]. Lately, another study on associative learning in parasitoids found a greater effect regarding the species with a wider compared to a more specialized host range [99]. While both generalists and specialists use infochemicals to find hosts and both have innate odour preferences, learning capacity is more pronounced regarding generalist natural enemies than specialists [100]. The generalist parasitic wasp *Psyttalia concolor* (Hymenoptera: Braconidae), for example, has been trained to associate the previously unattractive volatiles geranyl acetone, nonanoic acid, and decanoic acid with food rewards and the authors suggested the possibility to train mass-reared wasps before using them as biological control agents [101]. Apparently, innate positive responses to HIPV can also be nullified or even reversed: *P. concolor* trained to associate HIPV with electric shocks, grew indifferent to low concentrations, and avoided high concentrations of the actually attractive HIPV ethyl octanoate and decanal [102].

Parasitoids can be specialists on herbivore and plant level, specialists at plant, and generalist at herbivore level and vice versa, as well as generalists on both levels [10]. In Table 3 we listed several parasitic hymenoptera and their host breadth. The list points out the generalists who, on the one hand, may offer reliable efficacy as pest control agents under variable conditions. Tritrophic systems involving parasitoid species from this category may therefore be less vulnerable to altered HIPV blends induced by varying abiotic conditions. On the other hand, generalists on herbivore but not plant level show the highest potential to be trained in order to increase their efficacy as biological control agents [10]. They could possibly be trained to respond to volatiles which are not subject to changes. Table 3 also points out the specialists. Tritrophic systems involving parasitoids with a narrow host range may, on the one hand, be very vulnerable to changes. This could have major ecologic and economic implications against the background of global change and may be a key concern given how many known parasitoids can be classified as specialist to their host and/or to associated plants. On the other hand, these systems might be optimized by adding crucial compounds or enhancing their biosynthesis in plants. So, to answer the question posed in the subsection's title: yes, there are indications that the parasitoids' response to changing abiotic factors is strongly influenced by their degree of specialization.

Based on the observation that generalists rather tend to respond more to unspecific GLV and specialists to specific, host-related HIPV [89, 100], it would be interesting to know if these groups respond differently to abiotic factors. If one of the compound groups was less susceptible to changes, the respective parasitoid-herbivore-plant system should be more resilient and favorable in unstable environments. Unfortunately, existing data so far do not suffice to draw conclusions. Gouinguené and Turlings [29] do report (*E*)-β-farnesene to be emitted in more stable proportions than (*E*)-nerolidol. However, they are sesquiterpenes which are considered rather specific, host-related volatiles. This suggests that the emission is more finely regulated than just based on compound class. Still, this is only one study and in the big picture things might look different. There is a great need to decipher the language used by plants to communicate with insects. Knowing which compounds and/or compound ratios are pivotal for specialists used in biological pest control and how these HIPV are subject to changes due to biotic or abiotic impact factors may be essential for their future employment in food production.

6. Conclusion

Abiotic conditions have the capacity to alter the interaction between parasitic hymenoptera and plants. Changes regarding climate, atmosphere, or soil can increase or decrease the emission of constitutive and herbivore-induced plant volatiles. They can have bottom-up effects on parasitoids by affecting their herbivore hosts or influence orientation of

Table 3: Degree of dietary specialization of several hymenoptera species regarding their insect host and their plant host as well as examples of insect and plant hosts.

Hymenoptera species	Specialist		Generalist		Plant species	Host species	References
	Regarding host plant	Regarding insect host	Regarding host plant	Regarding insect host			
Aphelinus abdominalis				X	Greenhouse crops, wheat	Cereal aphids (e.g., *Sitobion avenae*), greenhouse aphids (e.g., *Myzus persicae* and *Macrosiphum euphorbiae*)	[103, 104]
Aphidius colemani				X	Wheat, cabbage, ascleipas, and others	Over 19 aphid species (e.g., *Aphis* spp., *Myzus persicae,* and *Rhopalosiphum padi*)	[105, 106]
Aphidius funebris				X		*Uroleucon* spp.	[107]
Campoletis chlorideae				X		Several noctuid caterpillar species (e.g., *Helicoverpa armigera*)	[108]
Campoletis sonorensis				X		Several noctuids caterpillar species	[61]
Cotesia marginiventris				X	Several, as the caterpillar species are polyphagous	Several caterpillar genera including *Heliothis* spp. and *Spodoptera* spp.	[89, 99]
Cotesia sesamiae				X		Wide range of stem borer species, mainly noctuid moth larvae (*Busseola fusca, Sesamia calamistis, Chilo orichalcocilielus, Chilo partellus*)	[109]
Dacnusa sibirica				X		*Liriomyza* spp. (e.g., *Liriomyza huidobrensis, L. bryoniae,* and *L. trifolii*)	[110]
Diachasmimorpha longicaudata				X		Tephritid fruit fly species (*Anastrepha, Ceratitis*,and *Bactrocera*)	[111, 112]
Diadegma fenestrale				X			[71]
Lariophagus distinguendus				X	Poaceae and Fabaceae	Over 11 different beetle species (e.g., *Rhyzopertha dominica* and *Sitophilus granarius*)	[113]
Lysiphlebus testaceipes				X	Bean, wheat, cabbage, and others	Over 9 aphid species (e.g., *Aphis* spp., *Rhopalosiphum maidis* and *Toxoptera aurantii*)	[105, 114]
Psyttalia concolor				X			[101]
Telenomus podisi				X		Parasitizing the eggs of various pentatomids in agroecosystems (e.g., *Euchistus* spp., *Nezara viridula*, *Piezodorus guildinii*,and *P. maculiventris*)	[115, 116]
Anaphes iole			X	X	A variety of crop plants	*Lygus* spp.	[117]
Aphidius ervi			X	X	Wheat, cabbage, bean, and others	Over 14 aphid species (e.g., *Acyrthosiphon pisum, Sitobion avenae,* and *Macrosiphum euphorbiae*)	[105, 118]
Cotesia flavipes			X	X	A variety of crops in the "New World"	Stem borers (e.g., *Diatraea saccharalis*, *D. grandiosella*, *Ostrinia nubilalis*, and *Chilo* spp.)	[119]
Diaeretiella rapae			X	X	Wheat, cabbage, bean, asclepias, and other crop plants	Over 23 aphid species (e.g., *Myzus persicae, Brevicoryne brassicae,* and *Sitobion avenae*)	[105, 120]
Diglyphus isaea			X	X	Mainly associated with herbaceous plants	18 different agromyzid species (*L. sativae*, e.g.)	[121]
Opius dissitus			X	X	Pea, celery (*L. huidobrensis*), vegetable leaf (*L. sativae*), and bean (*L. trifolii*)	*Liriomyza huidobrensis, L. sativae, L. trifolii*, and other leafminers	[122]

Table 3: Continued.

Hymenoptera species	Specialist		Generalist		Plant species	Host species	References
	Regarding host plant	Regarding insect host	Regarding host plant	Regarding insect host			
Cotesia glomerata		X		X		Pieridae spp. (e.g., *P. brassicae, P. napi*,and *P. rapae*)	[97, 98, 123, 124]
Apoanagyrus lopezi (= *Epidinocarsis lopezi*)		X			*Manihot esculenta*	*Phenacoccus manihoti*	[125]
Hyssopus pallidus		X			Apple	*Cydia molesta* and *Cydia pomonella*	[124]
Encarsia formosa		X	X		Greenhouse crops like tomato and cucumber	*Trialeurodes vaporariorum* and *Bemisia tabaci* *Alternative host: aleurodes*	[126, 127]
Trissolcus basalis		X	X		Several	*Nezara viridula*	[128]
Roptrocerus mirus	X			X	Conifers	Bark beetle species	[129]
Roptrocerus xylophagorum	X			X	Conifers	Bark beetle species	[129, 130]
Anagrus nilaparvatae		X		X		*Laodelphax striatellus, Nilaparvata bakeri, Nilaparvata muiri, Nilaparvata lugens, Sogatella furcifera, Sogatella panicicola, Toya propinqua,* and *Toya tuberculosa*	[123, 131, 132]
Aphidius rhopalosiphi		X			Poaceae, for example, wheat	Aphids on Poaceae (e.g., *Metopolophium dirhodum, Rhopalosiphum padi,* and *Sitobion avenae*)	[133]
Cardiochiles nigriceps		X			Cotton, tobacco, and others	*Heliothis virescens*	[134]
Chrysonotomyia ruforum		X			*Pinus* spp.	Diprionidae of pine (e.g., *Diprion pini*)	[135]
Cotesia kariyai		X			Tobacco	*Pseudaletia separata*	[136]
Cotesia plutellae		X			Brassicaceae	*Plutella xylostella*	[137]
Cotesia rubecula		X				*Pieris rapae*	[98, 123]
Diadegma semiclausum		X			Brassicaceae	*Plutella xylostella*	[138, 139]
Glyptapanteles flavicoxis		X				*Lymantria dispar, L. obfuscata*	[140]
Microplitis croceipes		X			Over 100 species (*Heliothis* spp. is a generalist)	*Heliothis* spp.	[89, 99]
Trybliographa rapae		X			Cabbage	*Delia radicum*	[141]
Oomyzus gallerucae	X	X			*Ulmus* spp.	*Xanthogaleruca luteola*	[142]
Orgilus lepidus	X	X			Potato	*Phthorimaea operculella*	[143]
Pauesia picta	X	X			Scots pine (*Pinus sylvestris*)	*Cinara pinea*	[144, 145]

parasitoids directly. Some tritrophic interactions are threatened by climate change; others seem more resilient. Active manipulation of abiotic factors in food production systems offers the chance to improve the efficacy of pest control through parasitoids. However, the large variability between the different tritrophic systems and the organisms involved requires thorough investigations and careful application of the gained knowledge.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Sexy Mouth Odour? Male Oral Gland Pheromone in the Grain Beetle Parasitoid *Lariophagus distinguendus* (Förster) (Hymenoptera: Pteromalidae)

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Throughout the animal kingdom, sexual pheromones are used for the attraction of mates and as courtship signals but also enable sexual isolation between species. In the parasitic wasp *Lariophagus distinguendus*, male courtship behaviour consisting of wing fanning, antennal stroking of the female antenna, and head nodding stimulates female receptivity leading to copulation. Recently *L. distinguendus* was reported to consist of two different lineages, which are sexually isolated because males fail to elicit receptivity in foreign females. It is unclear, however, which part of the courtship behaviour triggers female receptivity and therefore could be a mechanism causing sexual isolation. Here we show that in *L. distinguendus* a nonvolatile male oral pheromone is essential to release the female receptivity signal. In contrast, male wing fanning and antennal contact play a minor role. Additionally, the composition of the oral pheromone depends on the developmental host and females learn the composition upon emergence from the host substrate. These results will enable more detailed work on oral sexual pheromones to answer the question of how they are involved in the speciation process of *L. distinguendus* and other parasitoid species, for a better understanding of the huge biodiversity in this group.

1. Introduction

In many animals, sexual pheromones are involved in mate finding and courtship but also enable sexual isolation between species [1] mediated by pheromone divergence [2, 3]. This is also true for the hyperdiverse group of hymenopterous parasitoids [4, 5]. Here pheromones are used for long-range and short-range attraction of mating partners (e.g., [6–10]) as well as during courtship. Thereby, female derived compounds stimulate wing fanning behaviour, mounting, and specific courtship behaviours by males, which in turn induce receptivity by the females (e.g., [11–16]). Male courtship behaviours often consist in head nodding and/or antennal stroking movements of the female's antenna and it has been suggested that male pheromones are applied during this process [5]. The role of sexual pheromones in parasitoid speciation has been studied in detail with the pteromalid wasp *Nasonia vitripennis* Walker (Pteromalidae) [17, 18]. The divergence of pheromones between closely related species was addressed for *Drosophila* parasitoids [19–21].

Because many parasitoid wasps are used for biological pest control, knowledge on their biology is potentially relevant for their application. For example, the parasitoid wasp *Lariophagus distinguendus* (Förster) (Pteromalidae) is used for the biological control of stored product pests as the granary weevil *Sitophilus granarius* (L.) (Dryophthoridae: Curculionoidea), for many years [22, 23]. Its establishment as biocontrol agent was based on detailed studies on its biology (e.g., [24–28]). Recently, we discovered that *L. distinguendus* consists of two genetically distinct lineages, which most likely represent two species. One is specialised on drugstore beetles (*Stegobium paniceum* (L.), Anobiidae) in pantries and has a low fecundity on granary weevils, whereas the other attacks granary weevils and is mostly found in grain stores [29]. Hence it is not advisable to use the first species for biocontrol of granary weevils. A strong indication for the

species status of the two lineages consists in the fact that they are sexually isolated and females do not accept males from the other lineage as mating partners (König et al., in prep.). This suggests a communication breakdown between sexes during courtship. The courtship behaviour of *L. distinguendus* consists of male wing fanning, stimulated by the female cuticular hydrocarbon profile (CHC profile), followed by mounting and antennal stroking of the female antennae by the males. Subsequently, females signal receptivity by lowering their head and open their genital orifice leading to copulation [12, 30–33]. It is unclear if this receptivity signal is stimulated by vibrations due to the wing fanning behaviour of the males [16], by pheromones transferred via the male antennae as suggested in other hymenopterous parasitoids (e.g., [34]), or by a male oral gland pheromone as in the related species *N. vitripennis* [35]. Therefore, the reason for the communication breakdown causing sexual isolation between the two *L. distinguendus* lineages remains to be clarified.

To answer this question, we studied the origin (antennae or mouthparts) and the nature (tactile or chemical) of the male courtship signal of *L. distinguendus*, which induces receptivity in females. We analysed the role of antennae and mouthparts of the males during mating behaviour via video recordings, performed an experiment on the role of antennal contact during courtship, examined courtship success of males with sealed mouthparts, and studied the volatility of a putative pheromone. Finally, because it is known that the composition of sexual pheromones can be influenced by the feeding substrate of an insect [36–38], we addressed the question if development on the two different hosts of the *L. distinguendus* lineages, drugstore beetles and granary weevils, might have caused sexual isolation.

2. Material and Methods

2.1. Insects. For all experiments we used *L. distinguendus* wasps from the SLOgw strain [29]. Wasps were reared in Petri dishes (9 cm diameter) on 40 g wheat grain (cultivar: Batis; Saaten-Union GmbH, Hannover, Germany) infested by either drugstore beetles or granary weevils. Insect cultures were kept under constant conditions of 26°C and 45% r.h. and 16 L : 8 D photoperiod. For host rearing, 1 g of adult unsexed drugstore beetles or 2.7 g of adult unsexed granary weevils was placed on 40 g wheat grains moistened with 1 mL H_2O. After six weeks wasps were placed on the grains infested by drugstore beetle larvae or after three weeks on the grains infested by granary weevil larvae. Developmental time of *L. distinguendus* was 17–21 days. After wasps emerged out of the grain and before having contact to possible mating partners, males and females were kept separately in small Petri dishes (diameter 5.5 mm). For all experiments 2-day-old wasps were used.

2.2. Position of Antennae and Male's Mouthparts during Courtship. To analyse the role of antennae and mouthparts of the males during mating, we observed and videotaped 20 matings. Virgin males and females were placed in an arena consisting of a glass Petri dish (diameter 30 mm) closed with a glass plate (30 mm × 30 mm) and the mating behaviour was video recorded using a Digital Handheld Microscope (Bresser Meade Instruments Europe) fixed on a metal lab support stand. Videos were recorded with 7.5 fps and 1280 × 1024 pixels for a maximum time of 20 minutes or up to copulation behaviour. The camera operates with integrated software for video recording. Magnification was adjusted between 20x and 200x. During the subsequent analysis of the videos we focused on the position and movement of male and female antennae and on male's mouthparts.

2.3. Role of Antennal Contact. To examine if antennal contact is required for releasing the female's receptivity signal, we studied mating success of 20 couples with cross-ablated antennae, that is, after removing the right antenna of the male and the left antenna of the female ($n = 10$) and vice versa ($n = 10$) using a scalpel. Wasps were anaesthetised before the ablation by cold temperature (−23°C for 1.5 min). This procedure has no effect on wasp behaviour (data not shown). After removing the antenna, wasps were allowed to recover for 30 minutes. Experiments were conducted in the same arena as described above and the behaviours (wing fanning, antennal stroking, receptivity signal, and copulation) were registered by direct observation for a maximum of 20 min using a stereomicroscope (Zeiss Stemi SV11).

2.4. Mouthparts as Source of a Putative Pheromone. To test if a pheromone is released from the male mouthparts, mating experiments were performed with males with sealed mouthparts. Males were collected and mated with virgin females in order to check their ability to release the female receptivity signal. Subsequently, males were anaesthetised by cold temperature (−23°C for 1.5 min) and mouthparts were sealed with solvent-free superglue (UHU easy geruchsfrei, UHU GmbH & Co. KG, Bühl, Germany). To ensure that sealing of mouthparts with glue did not affect the activity of the males, they were kept in a Petri dish for 3 h before being used in the experiments. Males which were inactive during this period were discarded. In the experiments, single males were placed into a mating arena as described above together with one virgin female. Mating behaviour [12] consisting of "wing fanning," "antennal stroking/head nodding," "receptivity signal," and "copulation" was registered for a maximum of 20 minutes using a stereomicroscope. After the first test, each male was retested with a second virgin female for another 20 minutes. When no copulation occurred, females were paired with a second, untreated male. The experiment was performed with 20 males with sealed mouthparts (test) and 20 untreated males (control). To exclude that the presence of glue on the males did affect the experiments, males were treated with a drop of glue on their thorax. Mating experiments were conducted with these males as described above with the exception that only one female per treated male was tested.

2.5. Volatility of the Putative Pheromone. To study the volatility of the putative male pheromone, an experiment from van den Assem et al. [39] with *N. vitripennis* was repeated with some modifications. In a first experiment, two couples

of *L. distinguendus* were placed in one small glass vial each (Supelco, 2 mL Clear Vial, Screw Top). In one couple, the male's mouthparts were sealed as described above; in the other couple the male was untreated. By using a gastight syringe, air was collected from the headspace close to the antennae of the couple with the untreated male during antennal stroking. This air was injected next into the antennae of the couple with the treated male, again during antennal stroking. Then, the occurrence of copulation was registered using a stereomicroscope. The experiment was repeated 20 times.

In a second experiment, the two couples were placed in direct neighbourhood to each other during mating behaviour. Each female was fixed with superglue (s.a.) at the tip of one dissecting needle (Supplementary Figure S1 in Supplementary Material available online at http://dx.doi.org/10.1155/2015/216952). A wheat grain was placed in the middle under the females to enable males mounting the females. To enable that needles are moveable in all directions, they were pinned in a block of polystyrene fixed in a metal sphere, which was placed on a metal ring. Males were released onto the females. As soon as males started antennal stroking, females were brought into close distance from each other (<2 mm) but without having direct contact by moving the needles. Again, the occurrence of copulation was registered using a stereomicroscope. The experiment was repeated 15 times.

2.6. Effect of Developmental Host and Experience on Sexual Isolation. To study if development on the two different hosts might cause sexual isolation, mating experiments were performed with males and females which were reared on granary weevils or for one generation on drugstore beetles. Single couples of virgin males and females from the same or from different hosts were placed in an arena as described above and the behaviours (wing fanning, antennal stroking, receptivity signal, and copulation) were registered using the software package THE OBSERVER 9.0 (Noldus) for a maximum of 20 min or until copulation occurred. To examine the role of experience, the experiment was performed twice. First, we tested naïve wasps without emergence experience, which were dissected out of the grain as pupa and kept in an Eppendorf tube until emergence ($n = 50$). Second we tested experienced wasps, which were collected directly after the emergence out of the grain ($n = 50$). The contact period of these wasps with grain after emergence was <1 min. The occurrence of the different behaviours was compared and statistically analysed separately for naïve and experienced wasps using the 4×2 χ^2-test for an overall comparison, followed by Bonferroni corrected 2×2 χ^2-test for single comparisons.

3. Results

3.1. Position of Antennae and Male's Mouthparts during Courtship. The analysis of videos from 20 matings showed that during courtship the female antennae were stretched forward in a V-shape. Males were sitting on the female with their front legs placed on the female's head, performed wing fanning, and moved their antennae in skewed circles starting from above their heads downwards. The right antenna was moving clockwise and the left one counterclockwise (Supplementary Video 1). During circling, the male antennae often touched the middle of the female's antennae, stroking forward to their tip and rising up again in the air, starting a new circle. Thereby, the female's antennae were bended out of the V-shape into a rather parallel position (Supplementary Video 2). This brought them into the vicinity of the male's mouthparts, which were moved forward and backward along the female's antennae by head nodding. The mandibles were always in close vicinity (Supplementary Video 2), but not always in direct contact with the female's antennae, while contact of the mandibular or labial palps cannot be excluded (Supplementary Video 3).

3.2. Role of Antennal Contact. In these experiments one antenna was ablated in each male and each female in opposite positions to prevent direct contact of antennae during courtship behaviour. Nevertheless, all males showed normal wing fanning and antennal stroking behaviour. Likewise, all females gave the receptivity signal and successful copulation was observed in all 20 couples tested.

3.3. Mouthparts as Source of a Putative Pheromone. When mouthparts of males were sealed with superglue, males performed wing fanning and antennal stroking behaviour, but females did not show the receptivity signal and therefore no copulation occurred (Figure 1). However, when tested females were paired afterwards with untreated males, they all mated readily (Figure 1). Likewise, copulation occurred in all 20 control experiments, in which males were treated with a drop of glue on their thorax.

3.4. Volatility of Putative Pheromone. When the headspace above couples with an untreated male was transferred using a gastight syringe to couples with males with sealed mouthparts, no copulation could be observed ($n = 20$). Likewise, when two couples were brought into close vicinity, the couple with the unsealed male mated normally, while the couple with the sealed male showed no copulation ($n = 15$).

3.5. Effect of Developmental Host and Experience on Sexual Isolation. When wasps were naïve, that is, dissected out of the grains in which they developed, no significant differences were found between the experimental groups for all behaviours, including receptivity signal and copulation (Figure 2). When wasps hatched normally out of the grain, no overall differences were found for wing fanning but for antennal stroking, receptivity signal, and copulation. Single comparisons followed by Bonferroni correction revealed no differences between experimental groups for antennal stroking, but for receptivity signal and copulation. In couples consisting of females that had developed in drugstore beetles and males from granary weevils significantly less receptivity signals and copulations were observed (Figure 3).

4. Discussion

Despite the fact that mating behaviour in *L. distinguendus* has been studied by several authors before [12, 32, 33, 40]

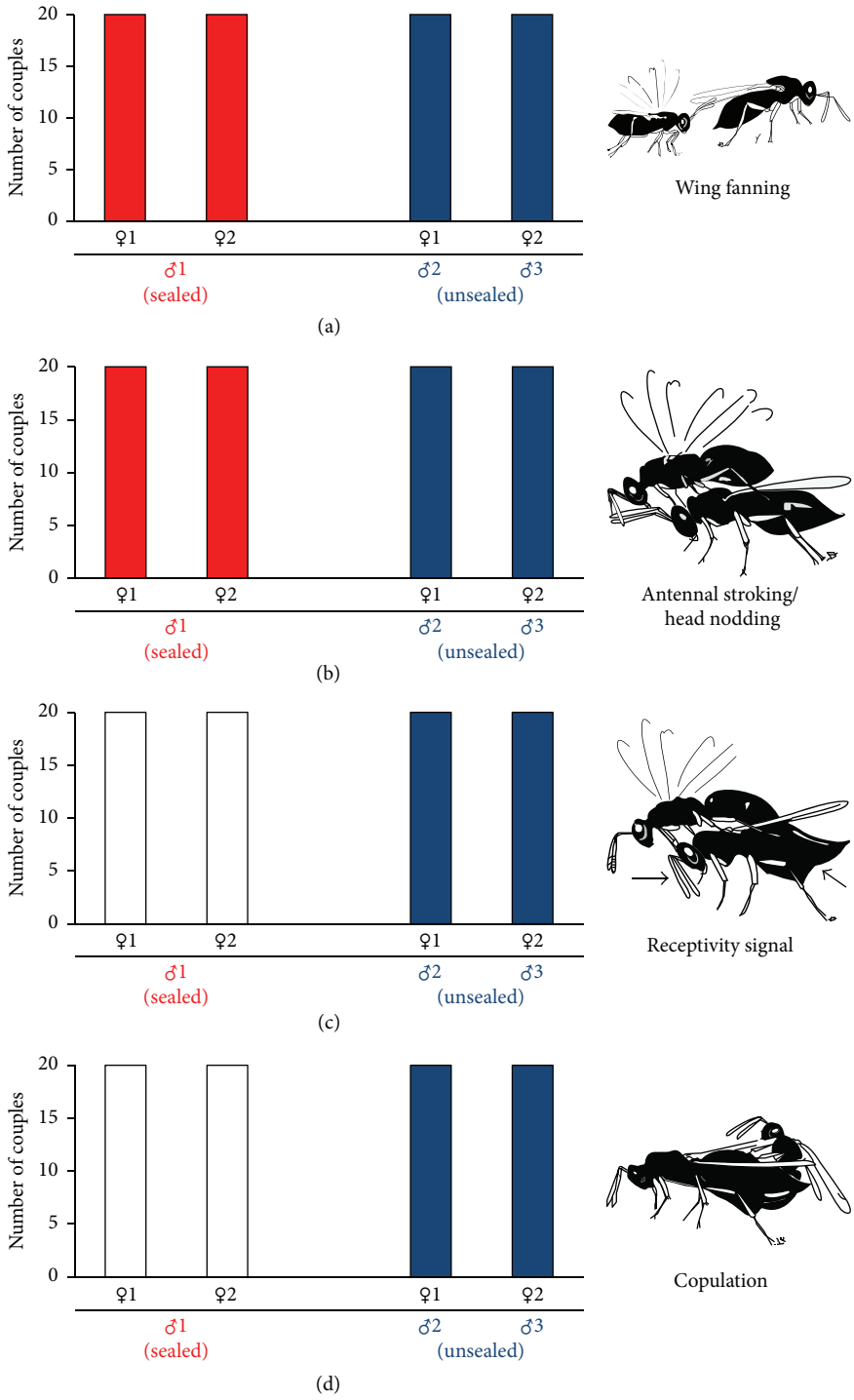

Figure 1: Occurrence of the different mating behaviours ((a) wing fanning, (b) antennal stroking/head nodding, (c) receptivity signal of female, and (d) copulation) in couples with a male in which the mouthparts had been sealed with superglue (red) and in couples with an unsealed male (blue). White bars indicate that the specific behaviour did not occur.

it was still unclear what triggers receptivity in the female. Our analysis of video recordings from matings revealed that during courtship males perform wing fanning and touch the antennae of females with their own antennae and with their mouthparts during head nodding. This is in line with the observations of the former studies [12, 32, 33, 40]. Therefore, based on these earlier studies and our video recordings, receptivity in females could be induced by males through mechanical stimuli due to wing fanning (hypothesis 1) or antennal contact (2), volatile pheromones from antennae (3) or mouthparts (4), or nonvolatile pheromones from antennae (5) or mouthparts (6).

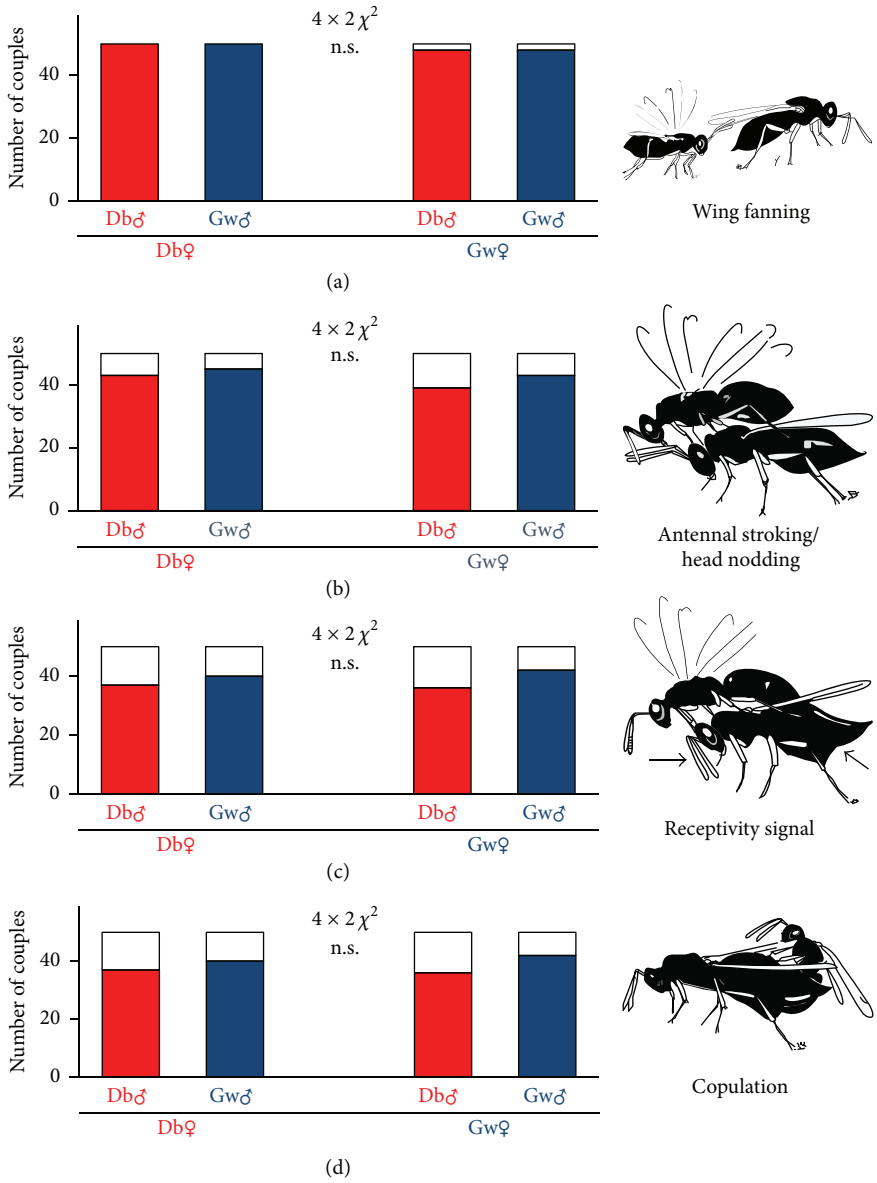

Figure 2: Occurrence of the different mating behaviours ((a) wing fanning, (b) antennal stroking/head nodding, (c) receptivity signal of female, and (d) copulation) in naïve wasps. Red bars are couples with a male that developed on drugstore beetles (Db); blue bars refer to couples with males that developed on granary weevils (Gw). White parts of bars indicate couples, which did not show the specific behaviour. n.s.: not significant (overall comparisons using 4×2 χ^2-test).

Our experiments with wasps, in which antennal contact between males and females was prevented by cross-ablation of antennae, revealed normal matings in all couples tested. Thus antennal contact between mating partners is not necessary to induce copulation. This excludes the hypotheses that the female's receptivity signal is stimulated by mechanical cues via antennal contact or by a male contact pheromone, which is transferred via the male antennae onto the antennae of the females (hypotheses 2 and 5).

In mating experiments with males having mouthparts sealed with superglue no copulations could be observed, despite the fact that males performed normal wing fanning and antennal stroking. This strongly supports the hypothesis that the male's mouthparts are essential for stimulating the female's receptivity by releasing a volatile or nonvolatile pheromone (hypothesis 4 or 6) and falsifies the idea that pheromones from the antennae are involved (hypotheses 3 and 5). Because the closely related *N. vitripennis* uses a male oral pheromone as well [35], this seems to be a general trait for Pteromalidae. Although the role of wing fanning has not been explicitly studied, our results also demonstrate that wing fanning alone is not sufficient (hypothesis 1). In agreement with Benelli et al. [16] we assume that it might play a role as additional signal indicating male quality to enable mate choice decisions by the females.

To analyse the volatility of the male pheromone, we tried to stimulate mating behaviour in a couple with a sealed male by exposing it to putative volatile compounds transferred

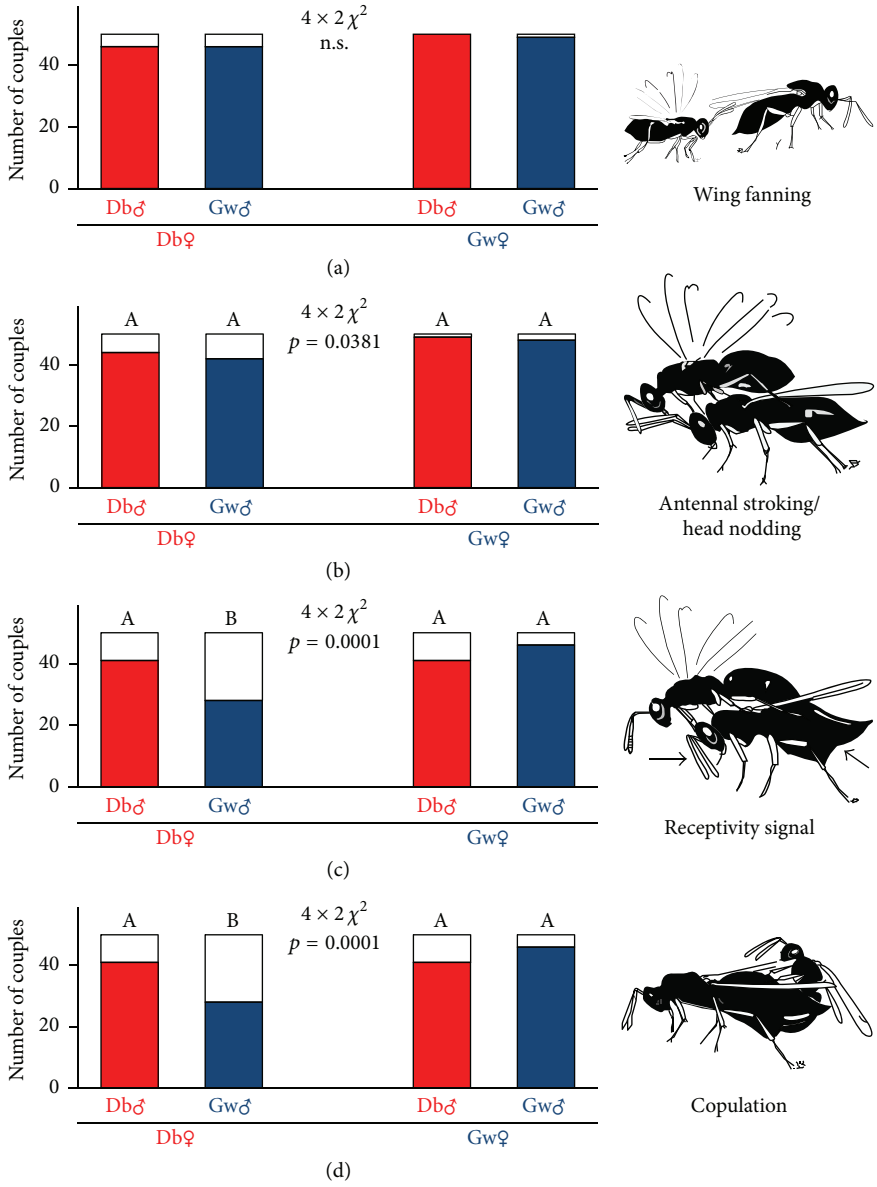

Figure 3: Occurrence of the different mating behaviours of wasps with emergence experience from the host substrate ((a) wing fanning, (b) antennal stroking/head nodding, (c) receptivity signal of female, and (d) copulation). Red bars are couples with a male that developed on drugstore beetles (Db); blue bars refer to couples with males that developed on granary weevils (Gw). White parts of bars indicate couples, which did not show the specific behaviour. p values refer to overall comparisons using 4×2 χ^2-test. n.s.: not significant. Bars with different lowercase letters are significantly different at $p < 0.05$ (Bonferroni corrected 2×2 χ^2-test).

from a normal couple via a syringe and to a mating couple within 2 mm distance. In both cases, no receptivity signal by the female could be stimulated. These results contradict hypotheses 3 and 4 from above and let us assume that the mandibular male pheromone is nonvolatile, acting only at contact or at very close distance (hypothesis 6). Again, this agrees with *N. vitripennis*, where the male oral pheromone has been demonstrated to be nonvolatile and is transferred via contact directly onto the female's antennae [5, 35].

The experiments on the effect of the developmental host on the male pheromone showed that females which developed on drugstore beetles accepted males from granary weevils significantly less often as mating partners than males from their own developmental host. Due to the low relevant p values, ranging from $p < 0.000$ to $p < 0.0049$, and the use of the Bonferroni correction we strongly assume that this result is not based on an alpha-error. It points to the fact that the developmental host influences the composition of the pheromone from the male's mouthparts. This agrees with findings from the CHCs profile of *L. distinguendus* [41] but also with other insects, where pheromone composition has been reported to depend on the feeding substrate (e.g., [36–38, 42]).

Interestingly, the reduced acceptance of males from the other developmental host was only observed in experienced females, which emerged normally out of their grains, but

not with naïve females, which were dissected from their grains. Thus, experience gained during emergence out of the grain must have affected the female's behaviour. This learning process could consist in direct learning of chemical host cues by imprinting upon emergence, which has been demonstrated recently in *L. distinguendus* [29].

The rejection of males was only observed in encounters of females from drugstore beetles and males from granary weevils, but not vice versa. We hypothesise that pheromones from males developing in granary weevils contain all compounds present in pheromones of males from drugstore beetles, whereas the latter contain additional compounds. Therefore, females developing in drugstore beetles reject males from granary weevils because they miss important compounds in the male pheromones, which are synthetised only when wasps develop in drugstore beetles. Alternatively, females might be able to learn at emergence only cues from drugstore beetle, but not from granary weevils. Thus, only the former influenced acceptance or rejection of males.

5. Conclusions

Our study strongly supports the hypothesis that the release of a nonvolatile oral pheromone by the males is essential to induce female receptivity in *L. distinguendus*. We found no support for alternative hypotheses, as mechanical stimuli due to wing fanning or antennal contact, volatile or nonvolatile pheromones from the antennae, and volatile pheromones from the mouthparts. Based on our video recordings we assume that the primary function of the antennal stroking is to move the female's antennae into the vicinity of the male mouthparts where the pheromone is applied. A putative source for this oral pheromone could be the mandibular glands, which are described by several authors for *L. distinguendus* [40, 43] and *N. vitripennis* [44].

Interestingly, the composition of this pheromone seems to be host dependent and is learned by the female during development, possibly during emergence from the host. This enables sexual isolation by development on different hosts within one generation, a phenomenon that has been also described for other insects (e.g., [36–38]). It remains to be studied if this mechanism has played a significant role for the sexual isolation of the two lineages of *L. distinguendus*. Further studies need to address the chemical identification of the pheromone. This will considerably help to answer the question of how pheromones were involved in this speciation process. In addition, our study enables more detailed work on oral sexual pheromones and their role in speciation in parasitoids, for a better understanding of the huge biodiversity in this group.

Conflict of Interests

The authors declare no conflict of interests.

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Research Article

Anagrus breviphragma Soyka Short Distance Search Stimuli

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Anagrus breviphragma Soyka (Hymenoptera: Mymaridae) successfully parasitises eggs of *Cicadella viridis* (L.) (Homoptera: Cicadellidae), embedded in vegetal tissues, suggesting the idea of possible chemical and physical cues, revealing the eggs presence. In this research, three treatments were considered in order to establish which types of cue are involved: eggs extracted from leaf, used as a control, eggs extracted from leaf and cleaned in water and ethanol, used to evaluate the presence of chemicals soluble in polar solvents, and eggs extracted from leaf and covered with Parafilm (M), used to avoid physical stimuli due to the bump on the leaf surface. The results show that eggs covered with Parafilm present a higher number of parasitised eggs and a lower probing starting time with respect to eggs washed with polar solvents or eggs extracted and untreated, both when the treatments were singly tested or when offered in sequence, independently of the treatment position. These results suggest that the exploited stimuli are not physical due to the bump but chemicals that can spread in the Parafilm, circulating the signal on the whole surface, and that the stimuli that elicit probing and oviposition are not subjected to learning.

1. Introduction

Anagrus breviphragma Soyka (Hymenoptera: Mymaridae) is a generalist, tiny egg parasitoid that develops in leafhopper and planthopper eggs inserted into vegetable tissues (leaves, stems, twigs, and shoots) of different plants, depending on the season [1] and on the different hosts: *Agalliana ensigera* Oman, *Dalbulus maidis* (DeLong and Wolcott), *Chlorotettix fraterculus* (Berg), *Cicadella viridis* (L.), *Ciminius platensis* (Berg), *Dechacona missionum* (Berg), *Exitianus obscurinervis* (Stål), *Hortensia similis* (Walker), and *Xerophloea viridis* (Fabricius) (Hemiptera: Cicadellidae) and *Conomelus anceps* (Germar), *Delphacodes kuscheli* Fennah, *Dicranotropis hamata* (Boheman), *Muellerianella fairmaire* (Perris), and *Peregrinus maidis* (Ashmead) (Hemiptera: Delphacidae) [2–6].

The majority of these hosts are indicated as harmful to a variety of agricultural (food, ornamental, and medicinal) and forestall crops. Their damage is due to the eggs oviposition wounds and to their nutrition punctures or to the transmission of viruses and phytoplasmas.

Anagrus breviphragma development (at 20°C) lasts 21–24 days: 3 for the egg stage, 3 for the motionless first instar larva, 6 for the very active second instar larva, 1 for the prepupal stage, and 6-7 for the pupa. Reproduction is anphigonic or parthenogenetic (arrenotokous). Oviposition can take place in host eggs at a different embryonic development, because the active second instar larva can easily disrupt the embryo tissues [7].

Anagrus is one of the most important mymarid genera for biological control, both when it is used in classical biological control programs, introducing laboratory bred parasitoid specimens, and when it keeps pests below damaging levels, in landscape management programs or in natural ecosystems.

Anagrus breviphragma is a facultative gregarious parasitoid; in so far as in larger Cicadellidae eggs, five to eight adults per host egg can emerge, while in smaller Delphacid eggs, a single individual per egg develops [7]. This behaviour seems to be determined by the competition between the larvae that hatch from the eggs, often supernumerary within a single host, and not by a decision of the ovipositing female

[8]. This superparasitisation has been observed both in the case of the same female and in the case of different females.

Anagrus breviphragma is attracted to the infested plant thanks to induced plant VOCs (synomones) and host egg kairomones [9]. Once the female is on the leaf, the typical searching and selection behaviours ("standing still," "walking while tapering," "brushing the club," "drilling," and "vibrating the abdomen") have been described [10, 11] even if the specific stimuli that generate them have not been identified.

Cicadella viridis eggs are inserted in the attacked plant tissues in bunches of 5–10 or more, causing a reaction of the vegetal tissues surrounding the eggs or a simple swelling, depending on the attacked organ (leaf, stem, or shoot) and species. Therefore, the scar directly made by the leafhopper ovipositor, usually smeared with a transparent substance [1, 7], is widen, revealing the eggs just below, or it is sealed and situated far from the eggs, that is, along the leaf margin. In such a variegated situation, both physical and chemical stimuli could reveal the presence of the host to the searching parasitoid female. Physical cues could be coupled with the swelling due to the eggs' presence or the lump due to the proliferation of the vegetal cells and could be perceived by the parasitoid with mechanical or visual receptors. Other physical stimuli could be due to the scar and to the exposed eggs. In fact, *C. viridis* eggs are equally parasitized whether partially embedded in *Ranunculus acer* L. (Ranunculaceae) stems and *Alnus glutinosa* (L.) (Betulaceae), *Fraxinus excelsior* L., *Ligustrum vulgare* L. (Oleaceae), *Rosa* spp. (Rosaceae), and shoots [1] or completely hidden in between *Carex riparia* Curtis (Cyperaceae) leaf epidermis [7].

Though the stimuli utilized by ovipositing females in hosts searching behaviour are well known for exposed eggs parasitoids [12–18], both long- and short-range cues are poorly studied for what concerns parasitoids of embedded eggs [12], such as *A. breviphragma*. On the basis of previous, occasional observations, such as the fact that females oviposit also from the flat surface of *Carex* leaves (unpubl. data), and analysing the different oviposition sites exploited by *A. breviphragma*, we hypothesized that physical stimuli are less likely to be used by *A. breviphragma* ovipositing females.

Therefore, in the present study, we investigated the nature of the cues (physical or chemical) that make the female locally search for and recognise the host, probe, and eventually oviposit. In addition, considering the ability of parasitoids to adapt their response to cues based on previous experiences associated with the host presence, particularly significant in generalist parasitoids [19], we examined if the oviposition behaviour can be influenced by the female's learning.

2. Methods

2.1. Biological Material Origin. All *C. viridis* eggs used in this study were obtained from field-collected material. Leaves of *C. riparia*, cut at their base and bearing overwintering eggs of *C. viridis* in uncultivated areas along the Po river in Piacenza, Italy, were collected periodically during the winter months, from November 2013 to February 2014.

Bundles of about 20–30 leaves were wrapped with wet paper towels, placed in a closed plastic bag, and stored in a refrigerator at 1–3°C. The towelling was changed biweekly to avoid the development of mould.

Parasitised *C. viridis* overwintering eggs were the source of *A. breviphragma*.

2.2. Parasitoid Breeding. As overwintering *C. viridis* eggs could be already parasitised by *Oligosita* spp. or *Anagrus* spp. [8] and as, at an early phase, parasitisation is not detectable when the eggs are embedded in the leaf tissues, in our experiments, we used eggs extracted from leaves. In this way, it was possible to check them under a light stereomicroscope to ensure that they were healthy eggs and to confirm that they were all at the same stage of development, namely, embryos without developed eyes.

The extracted eggs, as well as parasitoid adults, were conserved in Petri dishes on wet tissue paper discs, in a conditioned chamber at 20°C, and a long day photoperiod of LD 16 : 8.

Females that emerged from eggs collected in the field were observed under a light stereomicroscope for identification [7]. Those belonging to *A. breviphragma* were put in a Petri dish with conspecific males, white sugar very fine crystals, and healthy *C. viridis* eggs placed on wet tissue paper discs and removed after 24 hours.

Five days later (at second larval instar [20]) parasitised eggs were isolated on wet tissue paper discs in Petri dishes.

After 13/14 days, males and females of the same age emerged and these were used for the tests.

2.3. Oviposition Tests. Preliminary video recording of oviposition behaviour of *A. breviphragma* females on host eggs demonstrated that this parasitoid is too small for automatic image analysis. Therefore, as black dots become visible on the egg shell within about 15 minutes from the ovipositor puncture [7], we decided to rely upon this evidence to verify probing.

Considering that both physical and chemical stimuli could reveal the presence of the host to the searching parasitoid female, we considered treatments that verified each stimulus separately. Parafilm was used to eliminate physical stimuli while solvents were used to remove chemicals from eggs surface. At first, nonpolar solvents were preliminary tested but as in this case the washed eggs became grey and degenerated, polar ones were used instead.

The following treatments were then considered:

Eggs extracted from leaf were used as control since these are frequently utilized for laboratory parasitoid breeding [9] and can mimic those partially exposed (untreated-U).

Eggs extracted from leaf, cleaned with a synthetic brush in distilled water for 3 minutes, washed in ethanol for 2 minutes, and rinsed again in distilled water were used to evaluate the presence of polar chemicals (washed-W).

Eggs extracted from leaf and completely covered with Parafilm (M) (Pechiney Plastic Packaging Inc., Chicago, Illinois) in such a way that it was impossible

for the parasitoid female to reach the eggs were used to avoid physical stimuli due to the bump on the leaf surface, in correspondence with the eggs, the presence of a scar or a lump, and the possibility to "see" the eggs directly or by transparency (Parafilm-P). As Guerra et al. [21] reported that Parafilm alone induced probing (but not oviposition) in *Catolaccus grandis* (Burks) (Hymenoptera: Pteromalidae), notwithstanding the fact that *Anagrus* is a completely different parasitoid, we previously checked, under a light stereomicroscope for two hours, 10 *Anagrus* females on a Parafilm surface without any host egg beneath, but probing was never observed.

Small Petri dishes (⌀ = 1 cm) were prepared twelve hours prior to the experiment starting with wet tissue paper discs and six healthy *C. viridis* eggs just extracted from leaf tissue for each replicate. The Parafilm treatment was prepared cutting a circle of Parafilm just the same size of the Petri dish, placing the wet tissue paper disc on it, positioning the six eggs on the wet paper, covering them with stretched Parafilm and sealing it to the layer underneath. In this way the eggs were closed in a sort of Parafilm pocket with the upper Parafilm surface (on which the wasp was positioned) completely smooth (without any bump). At the beginning of the experiment, one naïve, one-day-old female, left with a male, water, and sugar for the previous 24 hours, was introduced in the Petri dish with eggs.

Two different experiments were performed.

Experiment 1 (one female was offered one treatment). This experiment was performed in order to evaluate the influence of the different stimuli on probing and oviposition behaviours.

Each female was checked every 15 minutes under a light stereomicroscope to detect the eggs with dots in order to obtain the probing start time. After the first dot appeared on the egg chorion, the following checks were performed 5 and 24 hours from the beginning of the test to record the number of eggs with dots per each female. If no dots were observed within 5 hours, the female was left with the eggs until 24 hours from the beginning of the test and checked for dots afterwards. Twenty-four hours after the beginning of the test, the female was removed. In order to confirm parasitisation, we kept the Petri dishes with the eggs on wet tissue paper discs, in a conditioned chamber at 20°C, with a long day photoperiod of LD 16 : 8, until second instar larvae were visible. Fifty replicates were performed per treatment.

Experiment 2 (one female was offered all of the treatments in sequence). This experiment was performed in order to evaluate the influence of a possible learning activity on probing and oviposition behaviours.

Each female was checked every 15 minutes under a light stereomicroscope to record the number of eggs with dots. After two hours on each treatment it was moved to the following one. Six different sequences were possible: U-W-P, U-P-W, W-U-P, W-P-U, P-U-W, and P-W-U. Each of them lasted 6 hours; after this period, the female was removed. In order to confirm parasitisation, the Petri dishes with the eggs were kept in a conditioned chamber at 20°C (photoperiod of LD 16 : 8) until second instar larvae were visible. Ten replicates were performed per sequence.

2.4. Statistical Analysis. Data are presented as N (%) for categorical data with 95% confidence intervals and as mean (SD) for continuous data (eggs number). Chi-square was used to evaluate the differences between categorical variables using Fisher's exact test where appropriate, while the independent t-test and one-way analysis of variance (ANOVA) were applied, where appropriate, to investigate the differences between continuous variables. Paired sample t-test and McNemar test were used to analyse differences within groups in continuous and categorical variables, respectively. Cochran's Q was used for repeated measures when more than two comparisons were made.

The logistic regression model was used to determine which treatment (between subjects) was associated with a greater percentage of oviposition. In order to check for a possible sequence effect that may cause differences in the number of parasitised eggs in experiment 2, repeated measures ANOVA using one between factor (first treatment presented) in the model was performed assessing possible differences within subject.

Kaplan-Meier survival estimates were used to analyse the hazard of probing in *Anagrus* allocated to the three different treatments: untreated, washed, and Parafilm. The time period considered in the survival analyses was 5 hours in order to analyse the effectiveness of treatment; therefore, data were censored at 5 hours.

All of the post hoc tests were adjusted for multiple comparisons using Bonferroni correction.

The alpha level was set at 0.05. Analyses were carried out using SPSS, version 20.

3. Results

Second instar larvae were recognised in all of the eggs with black dots. This result indicates that there was always oviposition after probing, thus confirming that it was correct to rely on black dots to assess probing.

Experiment 1 (one female was offered one treatment). The percentage distribution was not homogeneous at either 5 hours (chi-square$_2$ = 20.66; $P < 0.001$) or 24 hours (chi-square$_2$ = 11.19; $P = 0.004$) (Figure 1). At 5 hours, the percentage in "Parafilm" was higher than those in "washed" (chi-square$_1$ = 20.54; $P < 0.001$) and in "untreated" (chi-square$_1$ = 9.33; $P = 0.002$), while there was no difference between "washed" and "untreated" (chi-square$_1$ = 2.56; $P = 0.11$). At 24 hours, post hoc analysis showed similar relationships between treatments. The percentage in "Parafilm" was higher than those in "washed" (chi-square$_1$ = 9.76; $P = 0.002$) and in "untreated" (chi-square$_1$ = 9.76; $P = 0.002$), while there was no difference between "washed" and "untreated" as the percentage in both was exactly the same (Figure 1).

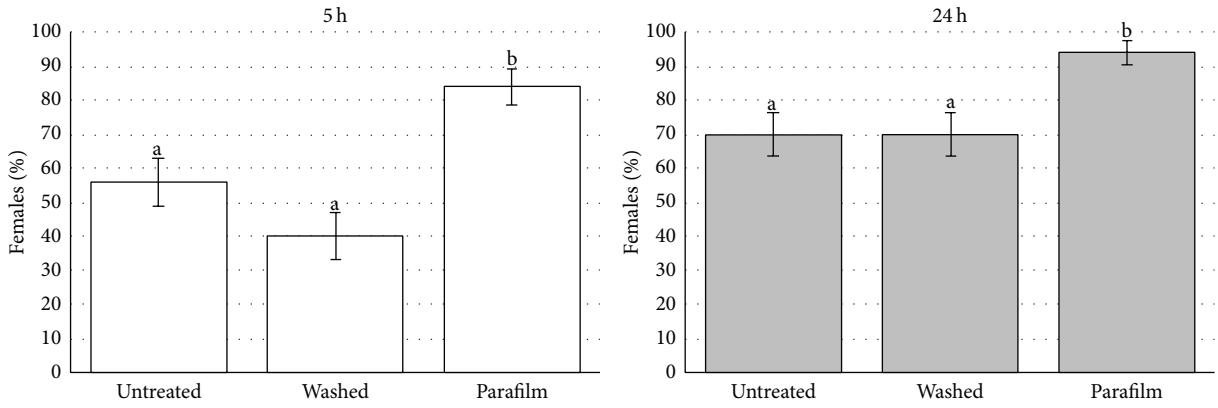

Figure 1: Percentage of females which probed at least one egg in the three considered treatments in 5 or 24 hours' time from the beginning of the test. Vertical lines indicate 95% CIs. In both considered times (5 and 24 h). Bars not sharing the same letter differ significantly ($P < 0.05$).

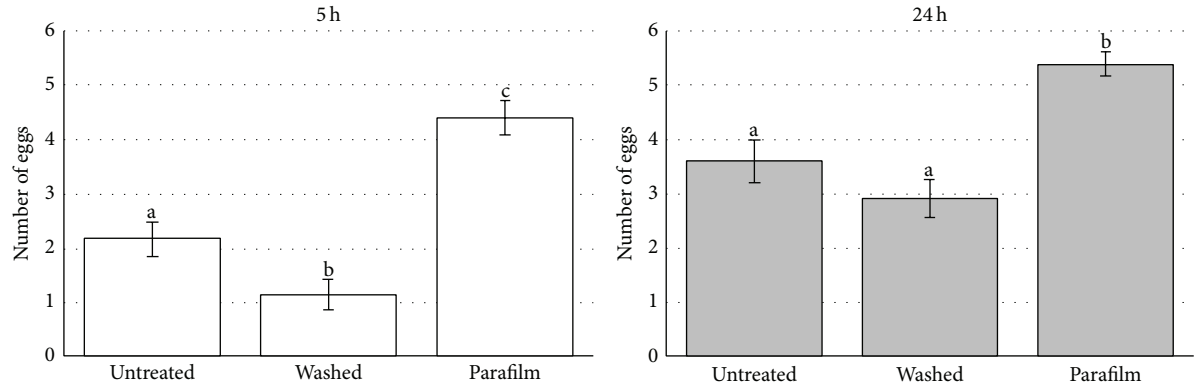

Figure 2: Average number of parasitised eggs per female in the three considered treatments in 5 or 24 hours' time from the beginning of the test. Vertical lines indicate standard deviation. In both considered times (5 and 24 h). Bars not sharing the same letter differ significantly ($P < 0.05$).

In all treatments, the percentage of females which probed at least one egg increased from 5 to 24 hours, but this increase was not significant in "Parafilm" (McNemar; $P = 0.063$). However, we had to consider a "ceiling effect" as, in this treatment, the percentage reached almost 100%. On the other hand, there was a significant difference both in "washed" (McNemar; $P < 0.01$) and "untreated" (McNemar; $P = 0.016$).

To determine the relationship between treatments and the likelihood of oviposition, two logistic regression models were used. In the first model, we used oviposition within 5 hours as the outcome measure and found a fourfold higher oviposition likelihood in "Parafilm" compared to "untreated" (OR = 4.13; 95% CI: 1.16–10.55). In the second model, we used oviposition within 24 hours as the outcome measure and found a sixfold higher oviposition likelihood in "Parafilm" compared to "untreated" (OR = 6.71; 95% CI: 1.80–24.99).

The average distribution of parasitised eggs per female in the three considered treatments is not homogeneous at 5 hours (one-way ANOVA; $F_{2,147} = 31.31$; $P < 0.001$) or 24 hours (one-way ANOVA; $F_{2,147} = 15.62$; $P < 0.001$).

At 5 hours, the average number of parasitised eggs per female in "Parafilm" was higher than those in "washed" ($t_{49} = 3.26$; $P < 0.001$) and in "untreated" ($t_{49} = 3.24$; $P < 0.001$), and there was also a significant difference between "washed" and "untreated" ($t_{49} = 2.47$; $P < 0.015$). At 24 hours, the percentage in "Parafilm" was again higher than those in "washed" ($t_{49} = 2.50$; $P < 0.001$) and in "untreated" ($t_{49} = 1.82$; $P < 0.001$), while there was no difference between "washed" and "untreated" ($t_{49} = 1.30$; $P = 0.196$) (Figure 2).

The average number of parasitised eggs per female at 5 and 24 hours significantly increases in all treatments: "Parafilm" ($t_{49} = 4.25$; $P < 0.001$), "washed" ($t_{49} = 6.10$; $P < 0.001$), and "untreated" ($t_{49} = 5.98$; $P < 0.001$).

The probing start time was very variable among females in all of the treatments (in "Parafilm" it varies from $5'$ to $300'$, in "washed" from $30'$ to $300'$, and in "untreated" from $15'$ to $300'$). Nevertheless, the Kaplan-Meier analysis on probing start time in the three considered treatments shows that the "Parafilm" curve was significantly different from the other two (median time = $238'$ for "washed," $208'$ for "untreated," and $130'$ for "Parafilm") (Figure 3).

Experiment 2 (one female was offered all of the treatments in sequence). The percentages distribution of females which probed at least one egg was not homogeneous (Cochran's

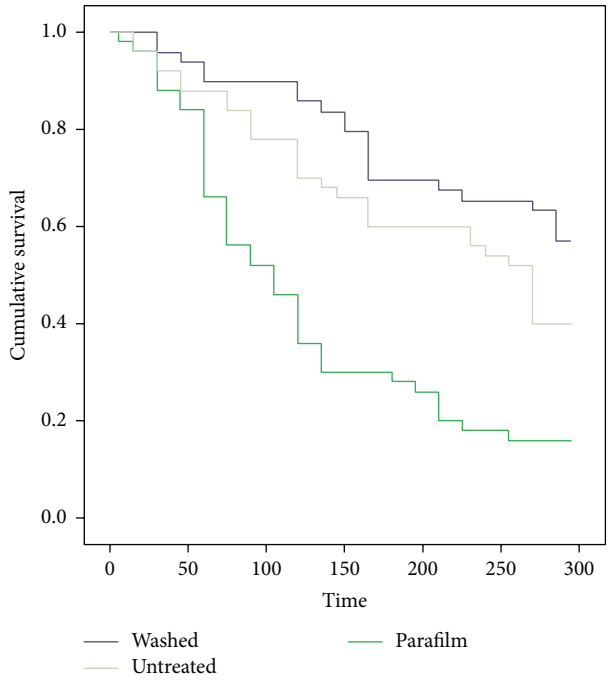

Figure 3: Kaplan-Meier probing starting time (minutes) curves for the three considered treatments at 5 hours.

$Q = 9.88$; $P = 0.005$). The value in "Parafilm" was significantly higher than those in "washed" (McNemar; $P = 0.008$) and in "untreated" (McNemar; $P = 0.041$), while there was no difference between "washed" and "untreated" treatments (McNemar; $P = 0.286$) (Figure 4).

The distribution of the average number of parasitised eggs per female was significantly different ($F_{2,118} = 7.33$; $P = 0.001$) in the three considered treatments. The number of parasitised eggs per female in "Parafilm" was higher than those in "washed" ($t_{59} = 3.37$; $P = 0.001$) and in "untreated" ($t_{59} = 2.49$; $P = 0.015$), while there was no difference between "washed" and "untreated" ($t_{59} = 1.47$; $P = 0.146$) (Figure 5).

The results of the repeated measures analysis of variance show that there was no significant difference due to the first treatment proposed (interaction effect $F_{4,114} = 0.420$; $P = 0.794$).

Indeed, the ovipositing female always preferred the "Parafilm" treatment, independently from the sequence of treatment presentation ($F_{2,118} = 7.33$; $P = 0.001$).

4. Discussion

Results show that *A. breviphragma* females oviposit in untreated eggs extracted from leaf tissues as well as in eggs washed with water and ethanol or covered with Parafilm. Nevertheless, "Parafilm" eggs are always preferred to "untreated" or "washed" eggs with respect to probing starting time and average number of parasitised eggs. As the Parafilm flat surface eliminates all physical cues coupled with the swelling present on the attacked plant surface, or the lump due to cell proliferation, or the scar, or the eggs themselves, these results clearly indicate that *A. breviphragma* females

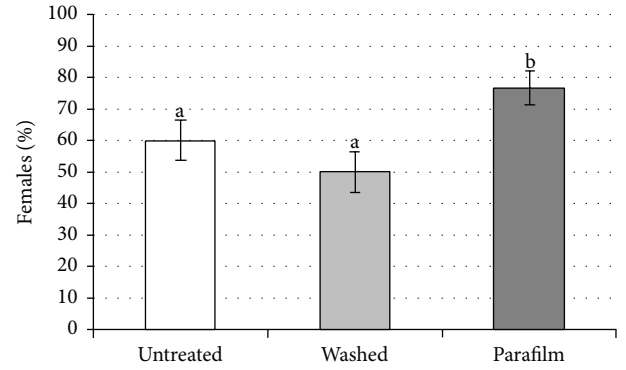

Figure 4: Percentage of females ($n = 60$) which probed at least one egg in the three considered treatments, independently from the sequence in which they had been presented to the females (U-W-P, U-P-W, W-U-P, W-P-U, P-U-W, and P-W-U). Vertical lines indicate 95% CIs. Bars not sharing the same letter differ significantly ($P < 0.05$).

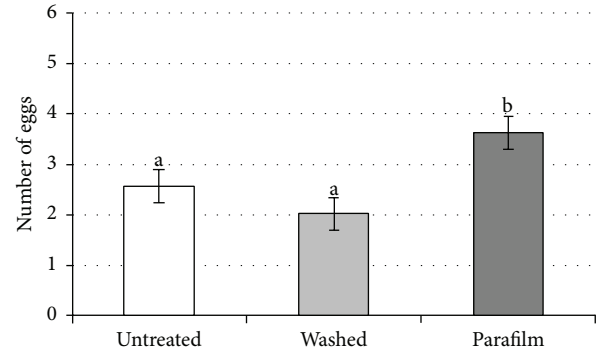

Figure 5: Average number of parasitised eggs per female ($n = 60$ females per treatment) in the three considered treatments, independently from the sequence in which they had been presented to the females (U-W-P + U-P-W + W-U-P + W-P-U + P-U-W + P-W-U). Vertical lines indicate standard deviation. Bars not sharing the same letter differ significantly ($P < 0.05$).

short distance search is determined by chemicals present on the eggs surface and somehow able to cross the Parafilm.

Comparing "untreated" with "washed" eggs, no significant difference relatively to both probing starting time and parasitized eggs is present, except for the number of eggs at 5 hours. This result indicates that these chemicals are not eliminated by washing with polar solvents and should therefore be compounds nonpolar or of intermediate polarity [22]. The fact that there is a significant difference with respect to the number of eggs at 5 hours, besides being canceled at 24 hours, can be explained hypothesizing a partial mechanical removal of oviposition stimulating chemicals due to the brush cleaning. This could also justify the reduced, albeit not significant, attractiveness of the washed eggs.

At the same time, the fact that there is a significantly lower probing start time and higher number of eggs parasitised in "Parafilm" treatment indicates that the presence of a "Parafilm" layer covering the eggs enhances parasitisation. Therefore, these chemicals should be able to form weak bonds with the Parafilm and spread in it, circulating the signal on the whole surface. If so, they could behave in a similar way

in the waxy epicuticular layer of the leaf surface as hypothesized for *Trichogramma brassicae* Bezdeko (Hymenoptera: Trichogrammatidae) parasitizing *Pieris brassicae* L. (Lepidoptera: Pieridae) eggs on Brussels sprouts plants [23].

Long-range search, which recruits the parasitoid to the attacked plant, providing it with precise information that lets it find the leaf where the host eggs are present, is determined by "a synergistic effect of induced plant VOCs and host egg kairomones" [8]. Once the female has landed on the leaf surface, a short distance search starts with "walking while tapering" and "brushing the club" behaviours [11]. Gustatory sensilla, represented by setae (trichodea) and corresponding to those described for *Anagrus atomus* [24], are disposed on the ventral surface and on the tip of the club of *A. breviphragma* female [9]. These sensilla allow the female to perceive the chemicals which are present on the surface beneath her. Therefore, our results are consistent with the behaviours performed by the female before probing its sensilla type and position. The chemicals that elicit such behaviour could be egg kairomones as those exploited by parasitoids of nonembedded eggs [25] and/or plant synomones locally produced as a reaction to oviposition [19]; in this case, especially, learning should be considered [26].

This hypothesis is confirmed by the fact that the curve of probing starting times is lower in "Parafilm." In fact, if the chemicals are "absorbed" and spread in this matrix, the probability that a female tapering on the Parafilm surface perceives the chemicals is higher than that in the other treatments.

After probing, the female can decide whether to oviposit or not. It does so when it perceives something (probably host yolk or haemolymph) [8] that is suitable for larval development; in fact, all probed eggs were parasitised.

In experiment 2, both the percentage of females which probed at least one egg and the number of parasitised eggs are significantly higher when the host eggs are covered with Parafilm, even when the naïve female had experienced uncovered eggs first ("untreated" and "washed"). Thus, this behaviour is not subjective to learning, as eggs under Parafilm were always more probed and parasitised, regardless of which treatment was proposed first. These results appear to be in contrast with the idea that generalist parasitoids should get advantage by exploiting conditioned stimuli [26]. Nevertheless, when "Parafilm" is offered first, the females parasitize more eggs in both "untreated" and "washed" treatments, even if not significantly. This could indicate that the females which had experienced the "Parafilm" treatment acquired a useful knowledge in relation to host finding ability. Therefore, after characterizing the chemicals involved, further research should focus on this aspect, also considering whether a specific sequence, not only in relation to the first treatment proposed, may cause an alteration of probing and oviposition behaviour.

5. Conclusions

This research allowed us to verify that the short-range cues exploited by *A. breviphragma* females to locate the host are not physical but chemical. Therefore, after characterization of these compounds, a manipulation of *A. breviphragma* oviposition behaviour could be feasible under laboratory conditions. This is very important especially in view of artificial breeding of the parasitoid for research or large number production and commercialization for biological control. In the past, we achieved promising results on *in vitro* rearing of *A. breviphragma* on diets devoid of insect components [20, 24]. The possibility to obtain oviposition through the Parafilm surface treated with chemicals that induce search behaviour and probing should consistently increase the realistic opportunity to rear parasitoids on artificial media.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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