Perspective

Foraging behavior of *Ganaspis brasiliensis* in response to temporal dynamics of volatile release by the fruit–*Drosophila suzukii* complex

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HIGHLIGHTS

- *Ganaspis brasiliensis* G1 is a larval parasitoid highly specific to *Drosophila suzukii*.
- *G. brasiliensis* G1 females attack only young host larvae in ripening fruits.
- VOCs from blueberries infested by young host larvae attract G1 females.
- VOCs from blueberries deteriorated by developing host larvae are repellent to G1 females.
- VOCs emitted by uninfested ripe fruits are not attractive towards G1 females.

GRAPHICAL ABSTRACT
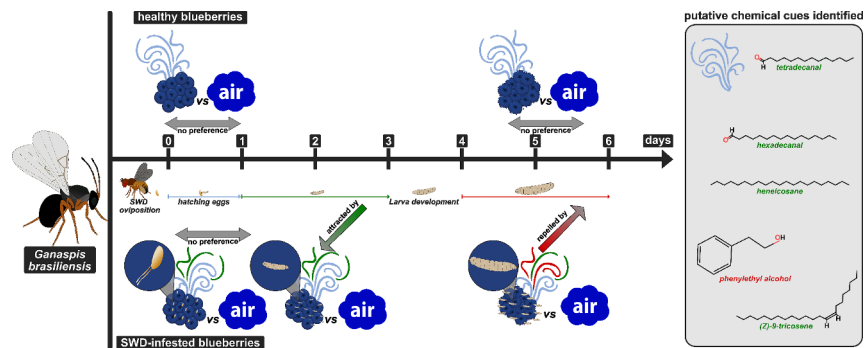
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ABSTRACT

The lineage G1 of *Ganaspis brasiliensis* is a larval parasitoid of the worldwide pest *Drosophila suzukii* and one of its most effective natural enemies in the native area. Because of its high degree of host specificity, *G. brasiliensis* G1 is considered a suitable species for introduction in areas invaded by *D. suzukii* following a classical biological control approach. Indeed, the release of the parasitoid is currently implemented in the USA and Italy. G1 females attack only host larvae developing in ripening fresh fruits on the plant and not larvae that develop in decaying fruits. To date, virtually no information is available on the cues regulating the foraging behavior of G1. In this study, we therefore aimed to find out whether chemical cues are exploited by G1 females to: (i) locate host fruits; (ii) locate suitable host larvae within infested fruit; (iii) discriminate between infested fresh fruits and infested rotting ones. We used a model system composed of blueberries and *D. suzukii* tested in two-choice olfactometer

bioassays (with *D. suzukii*-infested fruits, healthy fruits, and pure air as odor targets), followed by the collection and the characterization of volatile organic compounds (VOCs) released by the tested targets. The results showed a clear time-dependent choice made by G1 females of infested versus healthy fruits related to the concomitant development of *D. suzukii* larvae and fruit degradation. Attraction to infested fruits was recorded during the early stages of infestation, followed by a repellent phase coinciding with fruits largely degraded by larval feeding. We found that the attractiveness of *G. brasiliensis* G1 towards fruits infested by young larvae was associated with the detection of VOCs released by the infested blueberries, and host's cuticular hydrocarbons. Conversely, the repellence of older and deteriorated fruits hosting developed *D. suzukii* larvae was associated with the detection of a fermentation compound produced by microorganisms likely carried inside the fruit by the flies. The response of G1 females to the temporal dynamics of chemical cues emitted by the fruit–host larvae complex was consistent with the high degree of specificity of the parasitoid towards the ripening host fruits and towards *D. suzukii*.

1. Introduction

Drosophila suzukii (Matsumura) (Diptera: Drosophilidae), commonly known as the spotted-wing drosophila (SWD), is native to East Asia and starting from 2008 has become a serious economic threat to the production of berries and stone fruits worldwide (Cini et al., 2012; Asplen et al., 2015; Andreazza et al., 2017; Boughdad et al., 2021). The control of *D. suzukii* populations relies primarily on chemical insecticides, sometimes complemented by other local-area management tactics (Tait et al., 2021). These approaches often fail to prevent crop infestation and yield losses because the wild flora surrounding the cultivated fields offers plenty of fruits where *D. suzukii* can reproduce and overwinter, avoiding the impact of insecticide sprayings (Klick et al., 2016; Tonina et al., 2018; Buck et al., 2023). This situation is exacerbated by the lack of effective indigenous natural enemies in all invaded areas worldwide (Lee et al., 2019; Wang et al., 2020). Hence, the development of effective and sustainable control strategies able to provide an area-wide management of *D. suzukii* has become a critical issue (Haye et al., 2016; Wang et al., 2016). From this perspective, classical biological control, *i. e.*, the introduction of natural enemies from the native area of the pest into an area of new colonization, has been considered a valuable strategy to reduce the density of *D. suzukii* populations in non-crop habitats (Daane et al., 2016). This approach aims to increase the effectiveness of management strategies in crops, reducing the negative side effects of the chemical control. Explorations for natural enemies in the native area of *D. suzukii*, including China, South Korea, and Japan, resulted in the collection of three potential biocontrol agents, namely the larval parasitoids *Asobara japonica* Belokobylskij (Hymenoptera: Braconidae), *Leptopilina japonica* Novkovic and Kimura and *Ganaspis brasiliensis* (Ihering) (Hymenoptera: Figitidae) (Daane et al., 2016; Girod et al., 2018a; Giorgini et al., 2019). While *A. japonica* and *L. japonica* are generalist parasitoids of drosophilid species, *G. brasiliensis* showed a narrow host range. Hence, it was chosen as the best candidate for introduction into Europe and North America hypothesizing limited or null non-target impact (Wang et al., 2020 and references therein).

The thorough characterization of *G. brasiliensis* required for its introduction revealed a high genetic variability of the species, differentiated into five main lineages, each characterized by specific bioecological features and host ranges. Therefore, the existence of a complex of cryptic species was hypothesized (Nomano et al., 2017; Giorgini et al., 2019; Seehausen et al., 2020). Among the *G. brasiliensis* lineages, the G1 was found to have the highest degree of host specificity. Ecological observations in the native area suggested that G1 females can attack only *Drosophila* larvae developing in ripening fresh fruits on the plant, thus limiting its parasitic activity towards true carpophagous species belonging to the *D. suzukii* species-group (Nomano et al., 2017; Matsuura et al., 2018; Giorgini et al., 2019). Besides, *G. brasiliensis* G1 was never collected in fruit-baited traps containing decaying fruits hosting drosophilid larvae (Giorgini et al., 2019). Laboratory observations confirmed all these features rendering *G. brasiliensis* G1 a suitable species for introduction within the framework of classical biological control programs. In fact, it was shown that G1 females rarely parasitize *D. suzukii* or other drosophilid larvae within artificial diets or rotting

fruits (Seehausen et al., 2020). Furthermore, although the parasitoid could develop in other *Drosophila* species closely related to *D. suzukii* (*i. e.*, *D. melanogaster* Meigen and *D. simulans* Sturtevant) when forced to attack them in fresh fruits, it always preferred *D. suzukii* when given the choice (Seehausen et al., 2020). This result was even more evident in choice tests under semi-field conditions where G1 females were found to parasitize *D. suzukii* larvae in fresh fruits and almost no *D. melanogaster* larvae in rotting fruits (Seehausen et al., 2022). Taken together, all these results suggest that the specific ecological niche occupied by the *G. brasiliesnsis* G1 in the field, *i.e.*, the larva in fresh ripe fruits on plants, is even narrower than the physiological host range. Consequently, the release of *G. brasiliensis* G1 for classical biological control of *D. suzukii* was approved in Italy (Lisi et al., 2022) and the USA (Beers et al., 2022). A Japanese strain of *G. brasiliensis* G1 (Girod et al., 2018a), provided by the quarantine laboratory at CABI, Delémont, Switzerland, was introduced in both countries since the 2021 (Fellin et al., 2023).

To date, virtually no information is available on the cues regulating the foraging behavior of *G. brasiliensis* G1. While it shows specificity to fresh fruits infested by *D. suzukii* larvae, the parasitoid appears to be quite a generalist regarding fruit species (Girod et al., 2018a; Giorgini et al., 2019). In this study, we consequently aimed to answer the following questions: (i) Is there any chemical cue exploited by G1 females to locate host fruits? (ii) Is there any chemical cue from the *D. suzukii*-infested fruit complex exploited by G1 females to locate suitable host larvae inside the fruit? (iii) Is there any chemical cue involved in parasitoid preference for infested fresh fruits over infested rotting ones? To answer these questions, we used a model system composed of blueberries and *D. suzukii* tested in two-choice olfactometer bioassays against G1 females (with *D. suzukii*-infested fruits, healthy fruits, and pure air as odor targets), followed by the collection and the characterization of volatile organic compounds (VOCs) released by the tested targets.

2. Material and methods

2.1. Insects

The experiments were conducted in the laboratories of the Fondazione Edmund Mach, San Michele all'Adige, Italy (FEM), the National Research Council, Institute for Sustainable Plant Protection, Portici, Italy (IPSP) and the Department of Agricultural, Forest and Food Science, University of Torino, Italy (DISAFA). The colony of *D. suzukii* was initiated from infested fruits collected in Trento province, Italy, during 2021. Fly larvae were reared on a standard cornmeal-based artificial diet according to Rossi-Stacconi et al. (2022). A permanent rearing of the G1 lineage of the larval endoparasitoid *G. brasiliensis* was established at the laboratories of FEM, IPSP and DISAFA within the framework of the Italian national classical biological control program against *D. suzukii* (Lisi et al., 2022; Fellin et al., 2023). Parasitoids were reared on *D. suzukii*-infested blueberries (*Vaccinium corymbosum* L.) (Ericaceae) according to the protocol described by Rossi Stacconi et al. (2022). The starting colony of G1 derived from wild individuals collected in Naganuma Park (Hachioji, Tokyo, Japan) from 2015 to 2017 (Girod et al.,

2018a), and was provided by CABI's Swiss centre (Delémont, Switzerland) to FEM in 2021. Both *D. suzukii* and *G. brasiliensis* G1 cultures were maintained at the following conditions: 24 ± 1 °C temperature, 70 ± 5 % RH and 16L:8D photoperiod.

2.2. Olfactometer bioassays

The foraging behavior of G1 females was investigated in a dynamic Y-tube olfactometer consisting of 1-cm-diameter Y-shaped glass tube with a 9-cm-long base and two 8-cm-long arms as described in detail by Cascone et al. (2019).

Healthy fruits of blueberry variety Brigitta were picked in an organic farm only a few hours before running the olfactometer bioassay. In the field, *D. suzukii* infestation of tested fruits was prevented by covering them in insect-proof net before veraison to avoid any oviposition. Infested fruits were prepared in the laboratory by allowing a hundred mated *D. suzukii* females to oviposit in a fruit sample of 50 g (corresponding to an average of 31 blueberries) for 4 h inside an aerated plastic box (35 × 25 cm and 30 cm high). An infestation of 8.59 ± 4.16 (mean ± s.d.) *D. suzukii* eggs/fruit resulted, leading to an average of 3.68 ± 1.27 (mean ± s.d.) puparia/fruit (some eggs did not hatch and/or some larvae did not complete development).

Parasitoid females were tested against three different targets, healthy (uninfested) blueberry fruits (H-BB), *D. suzukii*-infested fruits (SWD-BB), and pure air (Air) presented in the following combinations: (i) SWD-BB vs H-BB; (ii) SWD-BB vs Air; (iii) H-BB vs Air. The fruit sample tested in the olfactometer consisted of 50 g of infested blueberries (SWD-BB) or heathy blueberries (H-BB). To assess the effect of the temporal combination of host development and fruit degradation level on the behavioral response of G1 females, the same bunch of SWD-BB or H-BB fruits was tested at seven time points: immediately after *D. suzukii* oviposition (T0) and thereafter, every 24 h for six consecutive days (T1-T6). Under the experimental conditions, blueberries were infested with *D. suzukii* eggs at T0, the first larval instars would have hatched at T1, and only *D. suzukii* larvae would have present in the fruits from T2 onward.

Mated G1 females having no prior oviposition experience were used in olfactometer bioassays. Parasitoid adults were collected as soon as they emerged from parasitized puparia and transferred in aerated plastic boxes (13 cm height and 11 cm diameter) in absence of fruits and host flies and provided with honey and water. Each box contained 30 adults (sex ratio 1:1). Males and females of *G. brasiliensis* mate successfully as soon as they emerged (<12 h old) (Girod et al., 2018b,c), and since the egg load reaches the peak in 6–8 days (Wang et al., 2018), we tested 7-day-old females. These conditions also guaranteed the highest chance of testing mated females. Indeed, female wasps were all fertilized (always produced female progeny) in our experimental setting.

Target fruit samples were placed individually inside a 20-L glass jar, which was subsequently closed tightly for 15–20 min prior to the beginning of a choice test to allow a consistent diffusion of the odor plumes and to allow the fruits to acclimatise within the olfactometer system. Each of the two glass jars was independently connected to an arm of the Y-tube by a Teflon tube. A stream of purified (at 99,99 %) air was split into two equal streams, each set at 100 mL min^{-1} by a flow meter and directed to a glass jar. Choice tests were conducted between 12:00 and 16:00 in a laboratory evenly illuminated by fluorescent lights (PPFD of 700 $\mu mol\,m^2s^{-1}$) and at 24 ± 1 °C. The position of the jars with fruit samples was swapped every five parasitoids tested to avoid any position bias.

Each olfactometer bioassay included the three two-choice tests (SWD-BB vs H-BB, SWD-BB vs Air and H-BB vs Air) performed using the same fruit samples. Each olfactometer bioassay was replicated 10 times, using a different sample of fruits (SWD-BB and H-BB) in each replicate. Replicates of the olfactometer bioassay were performed one at a time and started on ten different days. Ten or 20 G1 females were tested individually in each replicate of the choice tests. Each female was used only once. Overall, 440 females were tested in each of the three choice tests, divided as follows: 80 (T0), 80 (T1), 80 (T2), 60 (T3), 50 (T4), 40 (T5), 50 (T6). The choice of each parasitoid female was recorded within 5 min from the release in the common arm of the olfactometer. A choice was considered made when the parasitoid arrived inside one of the two trapping bulbs positioned at the end of the Y-tube arms. Wasps not making any choice within 5 min were scored as non-responsive and excluded from the analysis. Overall, only 91 out of 1320 individuals (6.9 %) were non-responsive.

2.3. VOC collection and identification

Fruit samples for gas chromatography-mass spectrometry (GC–MS) analyses were obtained from a single fruit batch of ripe blueberries (cv Brigitta), picked from the field (where fruits were covered by insect-proof net) and cold-stored at 1 °C for one week. Prior to VOC collection, the blueberry batch was divided into three subsamples, corresponding to different treatments: *D. suzukii*-infested blueberries (SWD-BB), artificially pierced blueberries (AP-BB) and healthy blueberries (H-BB). SWD-BB fruits containing 8 to 12 eggs of *D. suzukii* were chosen for this experiment. Artificially pierced blueberries were punctured 10 times in random positions with a fine needle (entomological pin size 000, diameter 0.25 mm) (Austerlitz Insect Pins®, Austerlitz, Czech Republic). Each blueberry sample (50 g) was placed in a polyethylene terephthalate oven bag (25 × 38 cm) (Anfora et al., 2009), and VOCs were collected using the closed-loop-stripping-analysis (CLSA) method (Boland et al., 1984). A 12 V vacuum pump (DC12/16FK type, Fürgut, Tannheim, Germany) circulated air within the bag to an adsorbent trap loaded with 1.5 mg charcoal (CLSA-Filter, Brechbühler AG, Switzerland). The pump was powered by a 6 V rechargeable battery (RS Components Srl, Italy) and the air flow was set at 0.2 L min^{-1}. Volatile collections from the three fruit treatments were performed in parallel for 3 h at 0, 24, 48, 72 and 144 h after *D. suzukii* oviposition or the artificial piercing (time points T0-T3 and T6 of olfactometer bioassays) and replicated 4 times, totaling 60 VOC collections (*i.e.*, 3 treatments × 4 replicates × 5 collection times). Each blueberry sample was subjected to VOCs collection sequentially at the different time points tested and stored in controlled condition (21 °C and 70 % RH) during consecutive collections.

Once collected, each volatile sample was eluted from the charcoal filter into 1 mL-glass vials using 500 μL of dichloromethane (>99.9 % purity; Merck/VWR, Darmstadt, Germany). After each elution, the CLSA filters were cleaned using a set of solvents at different polarity (dichloromethane, methanol, and acetone) to remove any volatile trace and to avoid contamination of subsequent samples. The eluted volatile samples were reduced to 100 μL using a slow stream of nitrogen and stored at − 20 °C until GC–MS analysis. All GC–MS analyses were carried out using a TSQ Quantum XLS (Thermo Scientific, Austin, TX, USA) equipped with a CTC-PAL3 autosampler. The separation was performed using a 30 m × 0.25 mm ID × 0.25 μm Restek Rx Sil MS w/Integra-Guard column (Restek corporation, Bellofonte, PA, USA). A split − splitless injector was set at 270 °C and 1 μL of solution was injected. The GC separation started at 35 °C, was held for 4 min, then was increased with the following intervals: 35 − 80 °C at 20 °C/min, 4 min at 80 °C, 80 − 100 °C at 2 °C/min, 5 min at 100 °C, 100 − 170 °C at 2.5 °C/min, and finally 170 − 270 at 20 °C/min with a 1 min final isotherm at 270 °C. A 1.2 mL/min helium was the carrier gas of choice. The MS signal was obtained by electron ionization at 70 eV, with the transfer line and the ion source both set at 250 °C using a full scan acquisition mode. All the analytical data were processed using XCalibur 4.5 software (Thermo Scientific, Austin, TX, USA). The putative identities of the compounds were characterized by a comparison with synthetic standards considering their GC retention indices and with mass spectra compared via the Wiley mass spectra database.

2.4. Statistical analysis

Statistical analyses of data originated from Y-tube olfactometer bioassays were run in R (R Core Team, 2023). Generalized linear models were built to assess the effect of VOC blends produced by SWD-BB or H-BB samples on attractiveness towards *G. brasiliensis* G1 females at the time points T0-T6. Each choice test (SWD-BB vs H-BB, SWD-BB vs Air, and H-BB vs Air) was analysed individually. Ten replicates for each choice test were included in the analysis. The number of parasitoids that responded to one or the other odor source was included in the model as a dependent variable while the time point (T0-T6, the sequential temporal combination of host development and fruit degradation) was included as an explanatory variable. The data distributions were fitted to a binomial model using the logit link function. Overdispersion was checked by the dispersion test function of the DHARMa package (Hartig, 2022) using non-parametric tests to compare simulated data dispersion with observed data dispersion. Wald Chi-Square statistics were used to assess the overall significance of time on the parasitoid attraction to the volatile blend produced by blueberry samples. Post-hoc tests for multiple comparisons were run using the Šidák method at the confidence level of 95 % to determine statistical differences between the time points T0-T6. Within each two-choice treatment, the two-tailed binomial test was used to assess whether the attraction of G1 females to any odor source in the Y-tube olfactometer differed from a random choice (50:50).

3. Results

3.1. Olfactometer bioassays

The impact of *D. suzukii* infestation of blueberry samples on the attraction of *G. brasiliensis* G1 females over 6 days is reported in Fig. 1. In both choice situations where SWD-BB fruits were offered to G1 females, time had a significant effect on attractiveness to the parasitoid (SWD-BB vs Air, Wald-χ^2 = 46.60, df = 6, p < 0.001; SWD-BB vs H-BB, Wald-χ^2 = 32.38, df = 6, p < 0.001). Parasitoid females were attracted to SWD-BB fruits during the first three days after fly oviposition, with significant differences at T1 and T2 compared to the air treatment (binomial test, p = 0.037 and p = 0.022, for T1 and T2, respectively), and at T3 compared to the H-BB treatment (binomial test, p = 0.005). Conversely, no preference for SWD-BB fruits was observed at T4 to T6. In fact, G1 females

showed a significant repellence to SWD-BB fruits tested against the air at time T4, T5 and T6 (binomial test, p = 0.008, p < 0.001 and p = 0.008 for T4, T5, and T6, respectively) and compared to healthy fruits at time T5 and T6 (binomial test, p = 0.002 for T5 and p = 0.019 for T6). When testing H-BB vs air, G1 females did not show any preference and no effect of the age of the fruits was recorded (Wald-χ^2 = 6.66, df = 6, p = 0.353).

3.2. VOC collection and identification

Analysis of the CLSA extracts revealed 19 VOCs (Table 1). Four compounds, tetradecanal, hexadecanal, heneicosane and (Z)-9-tricosene were detected in consistent quantities in the infested blueberries (SWD-BB) at all time points tested (T0-T3, and T6) while no detection or trace amounts (tetradecanal) were recorded from both AP-BB and H-BB samples (Table 1, Figs. S1-S4). Phenylethyl alcohol was not detected or detected in traces in all samples from T0 to T3 (Table 1, Fig. S4). At 144 h after exposure to *D. suzukii* (T6), phenylethyl alcohol was detected in much higher proportion in the SWD-BB samples compared to the H-BB and AP-BB samples (Table 1, Fig. S5). The remaining 15 compounds were detected, at different rates, in all treatments and at all times (Table 1).

4. Discussion

The success of oviposition in or on its host by a parasitoid female is a complex phenomenon largely relying on chemical communication becoming increasingly reliable as the foraging parasitoid gets closer to the host. Parasitoids of generalist herbivorous insects can locate their hosts on many plant species and in different environments. Chemical cues from uninfested plants are usually unreliable signals for host location, while herbivore-induced plant volatiles (HIPVs), produced after the insect host starts feeding or laying eggs, represent abundant and reliable cues exploited by parasitoids over long distances (Dicke and Baldwin, 2010; Turlings and Erb, 2018). Additionally, some parasitoids of generalist hosts can also exploit long-range volatiles produced by uninfested plants through associative learning (*i.e.*, after experiencing a cue from the plant and associating it to the host presence) (Geervliet et al., 1998; Segura et al., 2016). At short distances, host-derived chemical cues, including volatile compounds and non-volatile contact
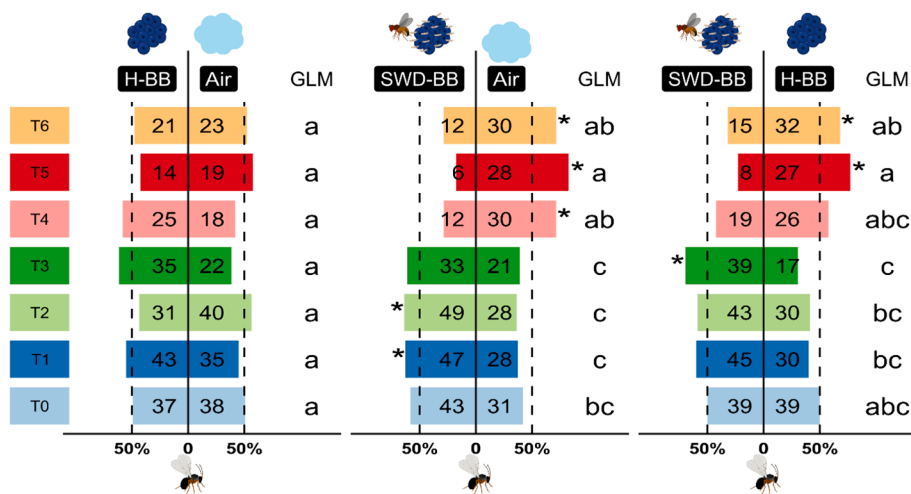

Fig. 1. *Ganaspis brasiliensis* G1 behavioral response in a two-choice olfactometer bioassay. Percentage of parasitoid females attracted to the two odor sources are reported on the x-axis. SWD-BB: blueberries infested by *D. suzukii*; H-BB: uninfested blueberries. Each two-choice bioassay was carried out over seven consecutive times starting from 0 h (T0) to one-six days (T1-T6) after *D. suzukii* oviposition. Numbers in the bars indicate parasitoids choosing. Asterisks indicate a significant response of *G. brasiliensis* to one of the two odor sources (two-sided binomial test, p < 0.05). Significant differences (p < 0.05) between times (T0-T6) are indicated with different letters (Šidák post hoc test at the 95 % confidence level after GLM analysis with data distribution fitted to a binomial model using the logit link function).

Table 1

Volatile organic compounds (VOCs) identified from healthy (H-BB), artificially pierced (AP-BB) or *Drosophila suzukii*-infested (SWD-BB) blueberries. VOCs were collected at 0 h (T0), one to three days (T1-T3), and six days (T6) after *D. suzukii* oviposition. VOCs specifically produced by SWD-BB blueberries are reported in bold.

#	Name	Formula	RT	RI	H-BB					AP-BB					SWD-BB				
					T0	T1	T2	T3	T6	T0	T1	T2	T3	T6	T0	T1	T2	T3	T6
	Esters:																		
1	Ethyl isovalerate	C7H14O2	4.15	729	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
	Aldehydes:																		
2	(E)-2-Pentenal	C5H8O	4.15	729	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
3	Hexanal	C6H12O	4.41	773	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
4	Nonanal	C9H18O	10.62	1101	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
5	(E)-2-Nonenal	C9H16O	12.17	1148	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
6	Decanal	C10H20O	13.93	1202	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
7	**Tetradecanal**	C14H28O	26.59	1607											x	x	x	x	x
8	**Hexadecanal**	C16H32O	32.14	1811											x	x	x	x	x
	Alkanes:																		
9	Dodecane	C12H26	13.77	1197	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
10	Tridecane	C13H28	17.05	1296	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
11	Eicosane < n->	C20H42	29.11	1697	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
12	**Heneicosane**	C21H44	38.94	2096											x	x	x	x	x
	Alkenes:																		
13	1-Octadecene	C18H36	26.11	1590	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
14	**(Z)-9-Tricosene**	C23H46	41.59	2277											x	x	x	x	x
	Monoterpenes:																		
15	1,8-Cineole	C10H18O	4.83	849	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
16	(E)-β-Ocimene	C10H16	5.82	929	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
17	Linalool	C10H18O	10.48	1096	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
	Alcohols:																		
18	**Phenylethyl alcohol**	C8H10O	10.84	1107															x
	Aliphatic acids:																		
19	Hexadecanoic acid	C16H32O2	35.77	1955	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x

compounds, are responsible for orienting parasitoids toward their host. Some highly volatile host-derived compounds (*e.g.*, some sex pheromones) are also exploited to locate hosts over long distances (Vet and Dicke, 1992; Steidle and van Loon, 2003).

In this paper, we studied the long-range behavioral response of a specialist parasitoid, the lineage G1 of *G. brasiliensis*, towards the chemical cues produced by its generalist host, *D. suzukii*, developing in blueberries. The results we obtained in olfactometer bioassay confirmed the general theory, showing how host-induced VOCs elicited a foraging response by parasitoid females, while volatiles released by uninfested fruits did not. Additionally, we showed that not only chemical cues are involved in host location but also that the time gap from *D. suzukii* oviposition (and thus the development stage of the host larva) is a key factor for success of host location by G1 females. Taken together, the choice tests with infested fruits (SWD-BB vs H-BB, and SWD-BB vs Air) showed that G1 females were attracted to infested blueberries from the first to the third day after *D. suzukii* oviposition, whilst they were repelled by infested fruits after 4–6 days from host oviposition. Conversely, when G1 females were offered a choice between healthy blueberries and pure air, they did not show any preference, regardless of the age of the fruits tested. The timely response of G1 females to infested fruits reflected the parasitoid requirements for successful oviposition. *Ganaspis brasiliensis* females preferentially parasitize very young host larvae (1–2 days old) (Wang et al., 2018) developing in fresh ripe but not rotting fruits (Seehausen et al., 2020, 2022). In our experimental setting, after 4 to 6 days from *D. suzukii* oviposition, infested fruits hosted late instar larvae of *D. suzukii* and were deteriorated by the feeding activity of the fly larvae and their associated microorganisms. Hence, they were recognized as unsuitable by the parasitoid.

In this study, the behavioral response to infested fruits by the

specialist *G. brasiliensis* G1 was similar to that previously observed for some generalist parasitoids of drosophilids attacking *D. suzukii* (Biondi et al., 2021; de la Vega et al., 2021). These parasitoid species showed a preference for infested fruits in respect to healthy fruits or pure air. Conversely, significant behavioral differences emerged between *G. brasiliensis* G1 and the generalist parasitoids towards decaying fruits. In our study, G1 females were repelled by decaying fruits infested by *D. suzukii* (*i.e.*, blueberries after 4–6 days from *D. suzukii* oviposition) when offered as an alternative to pure air or healthy fruits. In the study by Biondi et al. (2021), the generalist parasitoids *L. japonica* and *A. japonica* preferred decaying fruits to pure air and did not show any preference when the choice was between decaying fruits and infested fruits. In fact, these authors also showed that the olfactometer response by *G. brasiliensis* lineage G3 was similar to that of the generalist parasitoids *L. japonica* and *A. japonica*. The different behavior of *G. brasiliensis* G1, here examined, further confirm the very narrow host range of this lineage with respect to the lineage (sister species) G3 (Seehausen et al., 2022).

The results of olfactometer bioassays with G1 females found substantial support in the characterization of VOCs released by the blueberries tested. *Drosophila suzukii*-infested blueberries differed from healthy ones and from the artificially pierced ones by the release of five specific VOCs of which four (tetradecanal, hexadecanal, heneicosane and (Z)-9-tricosene) were detected at all tested times (T0-T6). The fifth compound, phenylethyl alcohol, was detected in consistently high proportion only six days after oviposition (T6). Fourteen VOCs detected in healthy blueberries were also detected in similar proportions in infested and artificially pierced blueberries; they are known to be components of blueberry aroma and flavor (Gilbert et al., 2015; Farneti et al., 2017; Sater et al., 2020) and blueberry cuticular wax (Trivedi et al., 2019;

Klavins et al., 2020). The results of the chemical analysis of VOCs clearly showed that infested blueberries release compounds associated with the activity of *D. suzukii* and are not derived from mechanical damage to the fruits. Tetradecanal, hexadecanal, heneicosane and (Z)-9-tricosene might be some of the chemical cues that G1 females exploit to locate fruits infested by a suitable host stage. Tetradecanal and hexadecanal are components of the pheromone blend that mediate long-range communication of *D. melanogaster* (Lebreton et al., 2017), but they have not been reported for *D. suzukii* (Dekker et al., 2015; Snellings et al., 2018; Lima et al., 2023). Heneicosane and (Z)-9-tricosene are compounds found in the cuticular hydrocarbon profile of some drosophilids including *D. suzukii*; in particular, (Z)-9-tricosene is released by adults of both sexes and has been suggested to play a role in intraspecific communication by acting as a sexual or aggregation pheromone (Dekker et al., 2015; Snellings et al., 2018; Lima et al., 2023).

The compound 1,8-cineole, which was detected in healthy, infested and artificially pierced blueberries, was exclusively found in infested blueberries in a previous study (de la Vega et al., 2021). Also, these authors found a VOC profile of *D. suzukii*-infested (26 volatile compounds) and healthy blueberries (13 volatile compounds) very different from our results with only three compounds in common (ethyl isovalerate, 1,8-cineole and, exclusively in infested fruits, phenylethyl alcohol). These differences may be partially explained by the different blueberry variety used in the two studies highlighting an important issue about the successful behavior of *G. brasiliensis* G1 when foraging on different blueberry varieties attacked by *D. suzukii*.

In addition to qualitative differences, some noteworthy quantitative distinctions deserve attention. For instance, in infested fruits tetradecanal was detected in substantial quantities throughout the entire tested period (from 0 to 6 days after oviposition, therefore in the absence of *D. suzukii* adults). Conversely, both healthy and artificially pierced fruits released no amounts or traces of tetradecanal. The significant increase in tetradecanal production by infested blueberries appears to be a direct consequence of their infestation by *D. suzukii*. Therefore, tetradecanal might be considered a candidate HIPV, potentially exploited by G1 females to locate *D. suzukii*-infested blueberries. However, we cannot exclude that tetradecanal and other chemical cues exploited by the parasitoid may originate from its larval host. The cuticular hydrocarbons of *D. suzukii* detected from infested fruits are known to be a product of adult flies. Since *D. suzukii* females were allowed to oviposit for only 4 h and the VOCs were collected from infested fruits always in the absence of adult flies, it is likely that heneicosane and (Z)-9-tricosene are deposited on the fruits during oviposition, as occurs for the aggregation pheromones of several *Drosophila* species (Bartelt et al., 1985; Wertheim et al., 2006). Alternatively, they might be produced by the larvae. In fact, the infested blueberries were not attractive towards G1 females at the end of the oviposition time (T0), hence the cuticular hydrocarbons left by the adult flies on the fruit surface would appear to play a minor role in the foraging behavior of the parasitoid. In the generalist parasitoid of drosophilids *Leptopilina heterotoma* (Thomson), the aggregation pheromone of the host is a highly reliable kairomonal cue exploited for substrate selection over a long distance, while other host cues (*e.g.*, cuticular hydrocarbons, feces) are exploited for host location at a closer spatial scale (Wertheim et al., 2003; Quicray et al., 2023). Considering all the features of the volatile blend released by infested fruits, we might hypothesize that highly volatile compounds (*e.g.*, tetradecanal) represent the primary cues exploited for host location over a long distance, while fly cuticular hydrocarbons (*i.e.*, heneicosane and (Z)-9-tricosene), characterized by a lower volatility (Dekker et al., 2015; Snellings et al., 2018), come into play at a short distance. To confirm this hypothesis, it is pivotal to test the behavioral response of G1 females towards the purified compounds identified in this paper and to trace for each one a specific dose–response curve.

The VOC profile detected from blueberries deteriorated by larval feeding 6 days after *D. suzukii* oviposition supports the repellence of infested fruits towards G1 females 4–6 days from *D. suzukii* oviposition.

In detail, at time T6, the peak proportion of phenylethyl alcohol dramatically increased, resulting in much higher levels in infested blueberries compared to healthy and mechanically damaged fruits. Similarly, de la Vega et al. (2021) collected phenylethyl alcohol from *D. suzukii*-infested blueberries and not from healthy fruits and artificially pierced fruits. Phenylethyl alcohol is a volatile that mediates the attraction of *D. suzukii* and other drosophilids to fermented sweet materials and is a fermentation product of yeast and lactic acid bacteria (Cha et al., 2012; Đurovic et al., 2021). Although phenylethyl alcohol can be a small component of the volatile profile of healthy blueberries (Farneti et al., 2017), our results suggest that the high specific production of phenylethyl alcohol from deteriorated infested fruits may be associated with fermentation microorganisms carried by *D. suzukii*. *Ganaspis brasiliensis* G1 might exploit the huge emission of phenylethyl alcohol (and other fermentation volatiles) to discriminate those fruits harbouring late larval instars of *D. suzukii* that are not suitable for parasitization or that harbour larvae of non-host drosophilid species.

The results of this research pave the way to set up tools to be exploited for enhancing the foraging success of *G. brasiliensis* G1 and in turn the efficacy of the biological control of *D. suzukii*. Specific compounds could be profitably used to attract the parasitoid where its activity is most needed, or to bait traps to check its distribution as, for example, after its release in biological control programs. Nonetheless, it now appears fundamental to assess whether the same attractive compounds are released by different host fruits attacked by *D. suzukii* or if specific VOC blends associate with different infested fruits. If this is the case, it then will be interesting to assess the ability of G1 females to locate its host in different host-fruit complexes.

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CRediT authorship contribution statement

Massimo Giorgini: Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Funding acquisition, Conceptualization. **Marco Valerio Rossi Stacconi:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Roberta Pace:** Writing – review & editing, Investigation, Data curation. **Francesco Tortorici:** Writing – review & editing, Investigation. **Pasquale Cascone:** Writing – review & editing, Visualization, Investigation, Formal analysis, Data curation. **Giorgio Formisano:** Writing – review & editing, Investigation. **Giuseppe Spiezia:** Writing – review & editing, Investigation. **Lorenzo Fellin:** Writing – review & editing, Investigation, Data curation. **Silvia Carlin:** Writing – review & editing, Data curation. **Luciana Tavella:** Writing – review & editing, Project administration, Funding acquisition, Conceptualization. **Gianfranco Anfora:** Writing – review & editing, Project administration, Funding acquisition, Conceptualization. **Emilio Guerrieri:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Project

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biocontrol.2024.105562.

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