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Corresponding Author	FamilyName	<b>Vega</b>
	Particle	de la
	Given Name	<b>G. J.</b>
	Suffix	
	Division	Laboratorio de Ecología Química, Facultad de Química
	Organization	Universidad de La República
	Address	Montevideo, Uruguay
	Division	Grupo de Ecología de Poblaciones de Insectos
	Organization	IFAB CONICET-INTA EEA Bariloche
	Address	Bariloche, Argentina
	Phone	
	Fax	
	Email	delavega.gerardo@gmail.com

---

Corresponding Author	FamilyName	<b>González</b>
	Particle	
	Given Name	<b>A.</b>
	Suffix	
	Division	Laboratorio de Ecología Química, Facultad de Química
	Organization	Universidad de La República
	Address	Montevideo, Uruguay
	Phone	
	Fax	
	Email	agonzal@fq.edu.uy
	URL	
	ORCID	

---

Author	FamilyName	<b>Triñanes</b>
	Particle	
	Given Name	<b>F.</b>
	Suffix	
	Division	Laboratorio de Ecología Química, Facultad de Química
	Organization	Universidad de La República
	Address	Montevideo, Uruguay
	Phone	
	Fax	
	Email	
	URL	

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Abstract

Biocontrol agents such as parasitic wasps use long-range volatiles and host-associated cues from lower trophic levels to find their hosts. However, this chemical landscape may be altered by the invasion of exotic insect species. The spotted-wing drosophila (SWD), *Drosophila suzukii* (Diptera: Drosophilidae), is a highly polyphagous fruit pest native to eastern Asia and recently arrived in South America. Our study aimed to characterize the effect of SWD attack on the volatile organic compounds (VOCs) of blueberries, a common host fruit, and to correlate these odor changes with the olfactory-mediated behavioral response of resident populations of *Trichopria anastrephae* parasitoids, here reported for the first time in Uruguay. Using fruit VOC chemical characterization followed by multivariate analyses of the odor blends of blueberries attacked by SWD, we showed that the development of SWD immature stages inside the fruit generates a different odor profile to that from control fruits (physically damaged and free of damage). These differences can be explained by the diversity, frequency, and amounts of fruit VOCs. The behavioral response of *T. anastrephae* in Y-tube bioassays showed that female wasps were significantly attracted to volatiles from SWD-attacked blueberries when tested against both clean air and undamaged blueberries. Therefore, *T. anastrephae* females can use chemical cues from SWD-infested fruits, which may lead to a successful location of their insect host. Since resident parasitoids are able to locate this novel potential host, biological control programs using local populations may be plausible as a strategy for control of SWD.

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Keywords (separated by '-') Spotted wing drosophila - Local parasitoids - VOC blends, biocontrol agents

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# 1 Effect of *Drosophila suzukii* on Blueberry VOC's: Chemical Cues 2 for a Pupal Parasitoid, *Trichopria anastrephae*

3 G. J. de la Vega<sup>1,2</sup> · F. Triñanes<sup>1</sup> · A. González<sup>1</sup>

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## 6 Abstract

7 Biocontrol agents such as parasitic wasps use long-range volatiles and host-associated cues from lower trophic levels to  
8 find their hosts. However, this chemical landscape may be altered by the invasion of exotic insect species. The spotted-wing  
9 drosophila (SWD), *Drosophila suzukii* (Diptera: Drosophilidae), is a highly polyphagous fruit pest native to eastern Asia  
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16 and amounts of fruit VOCs. The behavioral response of *T. anastrephae* in Y-tube bioassays showed that female wasps were  
17 significantly attracted to volatiles from SWD-attacked blueberries when tested against both clean air and undamaged blue-  
18 berries. Therefore, *T. anastrephae* females can use chemical cues from SWD-infested fruits, which may lead to a successful  
19 location of their insect host. Since resident parasitoids are able to locate this novel potential host, biological control programs  
20 using local populations may be plausible as a strategy for control of SWD.

21 **Keywords** Spotted wing drosophila · Local parasitoids · VOC blends, biocontrol agents

## 22 Introduction

23 Global trade is one of the main causes of the expansion of  
24 the range of pest species. The flow of agricultural products  
25 between countries facilitates the movement of species to  
26 novel environments (Anderson et al. 2004; Lantschner et al.  
27 2019), threatening not only the production of goods but also  
28 the local biodiversity (Harvey and Fortuna 2012). When  
29 exotic insect species arrive in local natural communities, the  
30 native food webs can be altered, as is the entire ecosystem  
31 by cascading chemical, physiological and ecological changes

across trophic levels (Chabaane et al. 2015). The presence or  
absence of antagonists (i.e., predators or parasitoids) is a key  
component in the success or failure of non-native species  
in a novel range, involving both top-down and bottom-up  
biotic influences among multiple trophic levels (Schultz  
et al. 2019). In this context, understanding how non-native  
pest species establish and interact with the native commu-  
nity is critical to forecast their success and to devise pest  
management strategies.

Chemical cues and signals are essential mediators in  
the ecological interactions of insects. Trophic interactions  
within native communities often rely on these cues and  
are therefore susceptible to chemical landscape alterations  
that may result from the invasion of exotic insect species  
(Chabaane et al. 2015; Mair and Ruther 2019; Rombaut  
et al. 2017). A common third trophic level involved in plant  
defense involves parasitic wasps, hymenopteran insects  
whose immature life stages occur in or on other arthro-  
pods, mostly other insects-(Cusumano et al. 2020; Schultz  
et al. 2019). To find adequate habitats for potential mates

A1 ✉ G. J. de la Vega  
A2 delavega.gerardo@gmail.com

A3 ✉ A. González  
A4 agonzal@fq.edu.uy

A5 <sup>1</sup> Laboratorio de Ecología Química, Facultad de Química,  
A6 Universidad de La República, Montevideo, Uruguay

A7 <sup>2</sup> Grupo de Ecología de Poblaciones de Insectos, IFAB  
A8 CONICET-INTA EEA Bariloche, Bariloche, Argentina

52 or hosts, parasitic wasps use long-range volatiles and host-  
53 associated cues from lower trophic levels (Desurmont et al.  
54 2020; Mumm and Hilker 2005). While searching for hosts,  
55 parasitic wasps need to integrate specific chemical cues  
56 with context background odors, which may be classified  
57 as irrelevant odors (no role in foraging behavior), masking  
58 odors (interfere and neutralize an attractive odor source)  
59 and enhancing odors (increase the attractiveness of an odor  
60 source) (Schröder and Hilker 2008). In this sense, mixtures  
61 of host-associated volatiles and background odors may be  
62 important mediators in tritrophic interactions involved in  
63 parasitoid host location (Desurmont et al. 2020; Schröder  
64 and Hilker 2008). In agricultural systems, these synergistic  
65 effects may be necessary to enhance the behavioral response  
66 of parasitic wasps to find their hosts efficiently (Liu et al.  
67 2019).

68 Even though invasion ecology is a growing field, studies  
69 seldom focus on the effect of new exotic insects on multi-  
70 trophic interactions in native communities (Carrasco et al.  
71 2017; Chabaane et al. 2015). Our study system involved  
72 three trophic levels: a local population of the parasitoid  
73 wasp *Trichopria anastrephae* Lima (Hymenoptera: Diaprii-  
74 dae), a potential new insect host that has recently arrived in  
75 South America, *Drosophila suzukii* (Matsumura) (Diptera:  
76 Drosophilidae), and one of its soft-skin fruit hosts, *Vac-  
77 cinium corymbosum* (blueberries). *D. suzukii*, known as the  
78 spotted wing drosophila (SWD), is a highly invasive insect  
79 native to eastern Asia (Walsh et al. 2011). In the last decade,  
80 it became a risk for soft-skin fruits affecting a variety of  
81 cherry and berry crops (i.e. strawberries, blueberries) (Bolda  
82 et al. 2010; Walsh et al. 2011). SWD invasions were initially  
83 detected in Europe and North America in 2008 (Cini et al.  
84 2012) and between 2012 and 2015 arrived in South America  
85 spreading from Brazil to the Patagonia region in southern  
86 Argentina (de la Vega and Corley 2019; de la Vega et al.  
87 2019). The fly presents an important difference with most  
88 *Drosophila* species, which oviposit in decaying or overripe  
89 fruit, in that SWD females have a serrated ovipositor to  
90 pierce fruit skin, allowing them to lay eggs inside undam-  
91 aged fresh and ripening fruit (Atallah et al. 2014). Before  
92 *D. suzukii* invaded agroecosystems, wounds on fruits such  
93 as grapes were due to climatic factors (i.e., hail, heat shock,  
94 heavy rain) or physical damages by birds or wasps (Rombaut  
95 et al. 2017). Consequently, the attack of SWD facilitates  
96 *D. melanogaster* infestation. As a consequence, rather than  
97 competing with closely related species, the invader makes  
98 available otherwise non-accessible resources, hence open-  
99 ing a new ecological niche for native, fructivorous insects  
100 (Rombaut et al. 2017).

101 Soft-skin fruits such as blueberries change their profile  
102 of volatile organic compounds (VOCs) as they ripen or  
103 senesce (Farneti et al. 2017). Therefore, fruit VOCs may  
104 potentially be used by *D. melanogaster* and *D. suzukii* to

105 find different fruit stages (Karageorgi et al. 2017; Keesay  
106 et al. 2015). A host preference shift from rotten to fresh  
107 fruit has been proposed for SWD (Keesay et al. 2015), an  
108 ecological shift that may have had an impact on higher  
109 trophic levels as well. In this scenario, understanding mul-  
110 titrophic chemical ecology aspects related to the invasion  
111 of *D. suzukii* to new environments represents an opportu-  
112 nity to understand its effect on established populations of  
113 natural enemies. In turn, this understanding may result in  
114 improvements for integrated pest management programs.

115 There is growing interest in the development of envi-  
116 ronmentally friendly pest management methods to reduce  
117 the application of harmful pesticides (Kruitwagen et al.  
118 2018). Thus, both larval and pupal parasitic wasps have  
119 been tested as biological control agents for SWD. The for-  
120 mer group includes species of the genus *Asobara* (Hyme-  
121 noptera: Braconidae), *Ganaspis* (Hymenoptera: Figitidae),  
122 and *Leptopilina* (Hymenoptera: Figitidae). Pupal parasitoids  
123 include *Pachycrepoides vindemiae* Rondani (Hyme-  
124 noptera: Pteromalidae), *Spalangia erythromera* Förster  
125 (Hymenoptera: Pteromalidae), *Trichopria drosophilae*  
126 (Perkins), and *T. anastrephae* Lima (Hymenoptera:  
127 Diapriidae). These were all able to parasitize *D. suzukii*  
128 under laboratory conditions (Ibouh et al. 2019; Vieira et al.  
129 2019). While potential biological control agents may be  
130 identified in SWD's native range (Lee et al. 2019), com-  
131 plex international regulations and biodiversity risks asso-  
132 ciated with the introduction of exotic natural enemies  
133 underline the need for improving the efficacy of resident  
134 species of natural enemies (Kruitwagen et al. 2018). In  
135 this sense, the presence of *T. anastrephae* populations  
136 in Latin America has been reported since 2001 (summa-  
137 rized in Vieira 2019), although biological control studies  
138 against *D. suzukii* are still only starting in the region (i.e.,  
139 Wollmann et al. 2016; Vieira et al. 2019), with no studies  
140 focusing on the chemical ecology of these interactions. In  
141 this particular system, understanding if parasitoids have an  
142 innate ability to find and exploit fruit infested by SWD by  
143 using volatile cues bears potential implications for the bio-  
144 logical control of this fruit pest. More specifically, if the  
145 parasitoid shows plasticity in exploiting volatile cues from  
146 various infested fruits, it is then capable of switching its  
147 preference patterns toward non-native host fruits or labora-  
148 tory artificial substrates, which becomes also important for  
149 the development of rearing strategies for pest management  
150 programs (Biondi et al. 2017).

151 Using a chemical ecology approach, we here explored  
152 two ecological questions in our tritrophic study system.  
153 First, we investigated the effects of SWD infestation on  
154 the VOCs of ripening blueberries. Second, we addressed  
155 the behavioral response of female parasitoid wasps of a  
156 local population of *T. anastrephae* to fruits infested with  
157 this novel insect host.

## 158 **Methods and Materials**

### 159 **Fruits**

160 Organic grown blueberry fruits (*Vaccinium corymbosum*  
161 var. O'Neil and Blue Jay) were used to analyze the effect  
162 of SWD attack on VOC profiles. The fruits were harvested  
163 weekly from multiple plants between December 2019 and  
164 January 2020 in a local organic farm (La Micaela, Cane-  
165 lones, Uruguay, <http://lamicaelaorganico.com/>). They were  
166 harvested before the fully ripe stage, still on the red stage  
167 as described by Gilbert et al. (2013) and Farneti et al.  
168 (2017) (see Supplementary Data Fig. S1). The fruits were  
169 harvested in the morning (0900–1200 h) and VOC sam-  
170 pling was performed in the laboratory the same afternoon.

### 171 **Insect Rearing**

172 *Drosophila suzukii* adults came from a laboratory colony  
173 established from flies collected locally in April 2019. The  
174 rearing was maintained on common cornmeal diet (504 ml  
175 distilled water, 66 g sucrose, 6 g bread yeast, 2.3 g agar,  
176 52 g corn-flour, 1.3 ml propionic acid, 0.8 g nipagin), in  
177 vials (12 cm high, 2.5 cm diam.) placed in an incubator  
178 under controlled conditions ( $21.5 \pm 1$  °C,  $65 \pm 5\%$  relative  
179 humidity, 12:12 h photoperiod).

180 The parasitoid, *T. anastrephae*, also came from a labo-  
181 ratory rearing established at the same time (April 2019)  
182 from field-collected insects. It should be noted that, while  
183 *T. anastrephae* is well known at the regional level, these  
184 field collections represent the first report of the presence of  
185 this parasitoid in Uruguay. To work with wasps naïve with  
186 respect to SWD, the parasitoid rearing was maintained  
187 continuously on *D. melanogaster*, under the same condi-  
188 tions as described above for SWD.

### 189 **Collection of Fruit Volatiles**

190 Blueberries free from external damages or irregularities  
191 (25 g) were placed in glass collecting chambers (20 cm  
192 length, 8 cm diam.) for dynamic headspace volatile col-  
193 lection. VOCs were collected by passing air pushed from  
194 an air compressor at a flow rate of  $0.5 \text{ l min}^{-1}$ . The air  
195 was previously dehumidified with silica gel and filtered  
196 through activated carbon. VOCs were adsorbed in glass  
197 tubes filled with 50 mg of HaySep Q (Alltech, USA) for  
198 24 h. VOC collections were made at a temperature of  
199  $25 \pm 2$  °C,  $70 \pm 5\%$  relative humidity, and a photoperiod  
200 of 12:12 h (L:D). Adsorbed volatiles were eluted with  
201 1 ml hexane, concentrated to 150  $\mu\text{l}$  under a gentle flow of

nitrogen, and stored in 250  $\mu\text{l}$  vial inserts at  $-20$  °C until  
GC–MS analysis. An internal standard solution (100  $\mu\text{l}$ )  
was added prior to concentrating the sample (tridecane in  
hexane, 1  $\mu\text{g/ml}$ ).

### **Effect of Attack by SWD Females on Blueberries**

After the initial 24-h VOC collection the blueberry samples  
were assigned to the different treatments. To obtain SWD-  
attacked blueberries, the fruits were enclosed for 24 h with  
ten SWD couples ranging 2–7 days old. After 24 h the flies  
were anesthetized with CO<sub>2</sub> and removed, and oviposition  
was confirmed under a stereo microscope by the presence  
of egg breathing tubes ( $9 \pm 6$  egg in each blueberry). The  
fruits were stored in clean glass containers covered with a  
fine mesh, under the same conditions as described for the  
insect rearing. Ten days later, a period that correlates with  
egg to pupae development of SWD (Tochen et al. 2014),  
fruit VOCs were collected under the same conditions as  
described above (SWD-attacked samples). Two controls  
were performed to differentiate the effect of SWD attack  
on fruit VOCs from the effect of physical damage and the  
natural ripening of the fruit. Physical damage (Physically  
damaged samples) was mimicked by gently punching all  
fruits in the 25 g cluster with a 5  $\mu\text{m}$  diam. microcapillary  
tube (three holes per fruit). The fruits were punctured ten  
days before VOC collection to match the maturation time of  
SWD-attacked fruit. Natural ripening was allowed under the  
same conditions with no treatment as an additional control  
treatment (undamaged control samples).

### **Chemical Analyses**

Blueberry VOCs were analyzed by gas chromatography cou-  
pled with mass spectrometry (GC–MS) using a Shimadzu  
QP 2010 PLUS (Shimadzu Corp., Tokyo, Japan) equipped  
with a Rtx<sup>®</sup>-5MS column (30 m, 0.25 mm i.d, 0.25  $\mu\text{m}$  film  
thickness; Alltech, USA). Samples (1  $\mu\text{l}$ ) were injected in  
the splitless mode with He as carrier gas at a flow rate of  
1 ml/min (49.7 kPa). The oven temperature was programmed  
from 40 °C for 4 min, then increased to 150 °C at 5 °C/min  
and to 250 °C at 10 °C/min and held for 10 min. Injector  
and MS transfer line temperatures were both set at 250 °C.

Volatile compounds were identified and quantified using  
the GCMS Solution software (Shimadzu GCMS Solution  
V 4.45SP1). The chromatograms were analyzed first by  
comparison with a system blank (without blueberries) for  
background volatiles, then by comparison among the fruit  
VOC samples under the three treatments (SWD-attacked,  
physical damage, undamaged control). VOCs were identi-  
fied from their mass spectra and retention indices, using the  
NIST08 and Adams' MS databases (Adams 2007). Amounts  
of Individual compounds present were calculated relative to

251 the internal standard by peak area comparison and are hence  
252 expressed as  $\mu\text{g}/25\text{ g}/24\text{ h}$ .

## 253 Olfactometer Bioassays

254 The behavioral responses of *T. anastrephae* females to vola-  
255 tiles from blueberries were evaluated using a glass Y-tube  
256 olfactometer consisting of two arms (6 cm long by 0.6 cm  
257 internal diameter) connected to chambers (9 cm long by  
258 5 cm internal diameter) for the volatile stimuli. The cham-  
259 bers were located so that no visual contact was possible from  
260 the Y-tube. Humidified, charcoal-filtered air was pushed  
261 through the stimuli and olfactometer with a pump at a rate  
262 of 0.5 l/min. The olfactometer was laid horizontally on a  
263 glass surface homogeneously trans-illuminated with cold  
264 white LED lights (3600 lumens). To further eliminate visual  
265 cues, the olfactometer was fully enclosed in a box made  
266 from white corrugated plastic with a hole on top to allow  
267 video recording. All bioassays were conducted at  $22 \pm 2\text{ }^\circ\text{C}$ ,  
268  $70 \pm 5\%$  RH, and performed between 09:00 and 18:00 h.  
269 After each trial, the olfactometer arms were rotated to avoid  
270 position bias. At the end of the day the olfactometer was  
271 washed with distilled water and neutral soap, rinsed with  
272 ethanol and acetone, and oven-dried at  $100\text{ }^\circ\text{C}$  for 24 h.

273 *Trichopria anastrephae* females were used 2–5 days  
274 after hatching from *D. melanogaster* pupae. Upon hatch-  
275 ing, females and males were placed in glass containers with  
276 access to honey-water (50:50) absorbed in cotton balls, until  
277 their use in the experiments. To conduct the bioassay, each  
278 female was individually placed at the base of the com-  
279 mon section of the Y-tube, and a 5-min period was video  
280 recorded. From this video, we measured the time spent in  
281 each arm, the first arm selected, and the position at the end  
282 of the recording period. For the three variables, the assign-  
283 ment of the wasp position was defined relative to a perpen-  
284 dicular line at the base of each arm.

285 Three olfactometer experiments were performed: one  
286 bioassay tested undamaged blueberries versus clean air, the  
287 second tested SWD-attacked blueberries versus clean air,  
288 and the third compared SWD-attacked blueberries versus  
289 undamaged blueberries. Physically-damaged blueberries  
290 used as a control in VOC chemical analyses were not used  
291 in the behavioral bioassays because the VOC profiles from  
292 undamaged and physically-damaged blueberries were not  
293 different (see “Results”). For the experiments with SWD-  
294 damaged fruit, 25 g of blueberries were exposed to oviposi-  
295 tion by SWD under the same conditions as described for  
296 the VOC collections, and similarly kept for 10 days prior  
297 to the bioassays. Undamaged blueberries were stored under  
298 identical conditions. Blueberries for olfactometer bioassays  
299 were of commercial origin (*Vaccinium corymbosum*, Frusan,  
300 Frutera San Fernando, Chile); they were used fresh upon  
301 purchase and gently washed with distilled water.

## Statistical Analyses

302 All statistical analyses were performed using R (Version  
303 3.6–2) (R Core Development Team 2019).  
304

305 To visualize VOC profiles in the different treatments  
306 a nonmetric multidimensional scaling (NMDS) was per-  
307 formed on the amount of VOCs matrix by using a Bray–Curtis  
308 distance matrix (Vegan package V2.4-6 in R). The data  
309 include many zero values, so it was fourth-root transformed  
310 (Hervé et al. 2018). To test for significant differences in the  
311 chemical composition of VOCs produced among the treat-  
312 ments (SWD-attacked, physical damage, and control) a per-  
313 mutational multivariate analysis of variance (perMANOVA)  
314 was also carried out on the distance matrix based on 9999  
315 permutations. The analysis was performed using the *adonis*  
316 function in R (Vegan package V2.4-6).

317 The behavioral response of parasitoid wasps was analyzed  
318 by a paired *t*-test for the time spent in each arm, and an exact  
319 binomial test both for the first arm chosen and the position at  
320 the end of the bioassay. The exact binomial test performs an  
321 exact test of a simple null hypothesis about the probability  
322 that the number of wasps for first choice of either olfactom-  
323 eter arm had a 50:50 distribution. Also, to test the position  
324 at the end of the bioassay we performed the same test with a  
325 probability of 0.33 for the number of wasps present in either  
326 olfactometer arm or the common section. Females that did  
327 not respond were excluded from the analyses.

## Results

### Effects of Attack by SWD on Blueberry VOC's

329 Analyses of collection of volatiles from blueberries before  
330 initiation of the treatments and visualization by NMDS con-  
331 firmed that the batches assigned to treatments were homog-  
332 enous (Supplementary Data Fig. S2). We then measured  
333 VOCs from 15 samples of SWD-attacked blueberries, 10  
334 samples of physically damaged fruit, and 15 samples of  
335 undamaged control fruits. Twenty-four compounds were  
336 identified in the VOC extracts from blueberries (Table 1,  
337 Fig. 1, Supplementary Data Table S1). Of these, nine com-  
338 pounds were exclusively present in the VOCs from blue-  
339 berries attacked by *D. suzukii* (SWD-attacked) (Table 1).  
340 Taking into account the frequency in which it was found  
341 and the relative amount, isoamyl acetate was one of the main  
342 compounds that separates VOCs of SWD-attacked blueber-  
343 ries from VOCs of the fruit treatments (Table 1). Overall,  
344 volatiles produced in the highest amounts were short-chain  
345 aliphatic esters (Table 2).  
346

347 Multivariate analyses also showed that the VOC profiles  
348 of SWD-attacked blueberries differed from the VOC pro-  
349 files of both control treatments (perMANOVA:  $F_{2,37} = 1.91$

**Table 1** Amounts of volatile organic compounds (VOCs) found in collections from blueberries subjected to the different treatments: attacked by *Drosophila suzukii* (SWD-attacked); physical damage and natural fruit ripening (undamaged control)

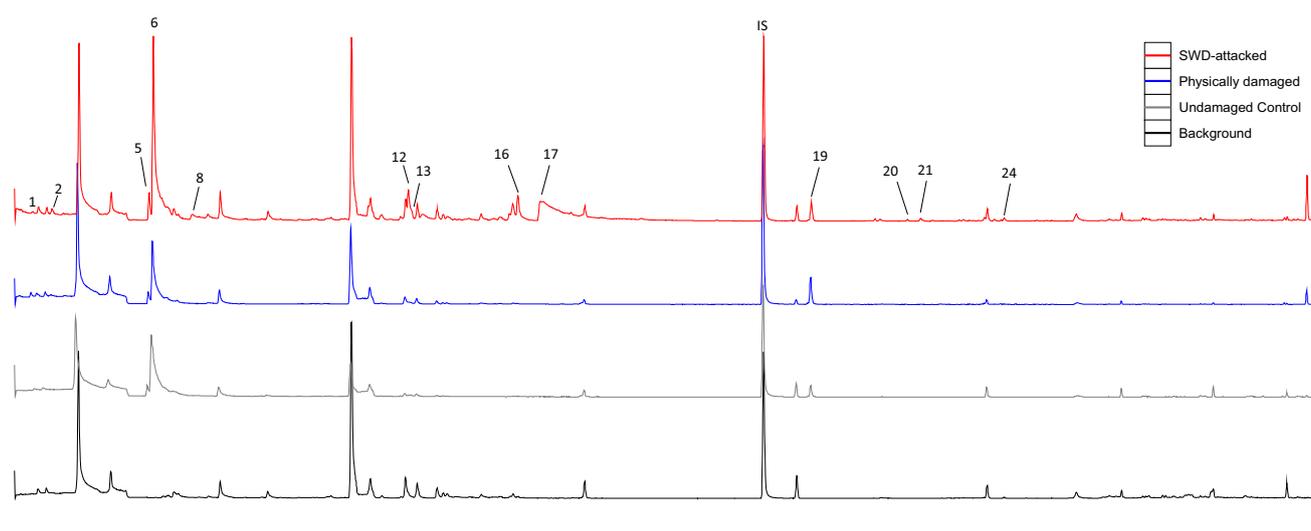
Compound	Amount ( $\mu\text{g}/25 \text{ g}/24 \text{ h}$ ; mean $\pm$ standard deviation)					
	SWD-attacked	<i>N</i>	Physical damage	<i>N</i>	Undamaged control	<i>N</i>
<i>Short-chain aliphatic esters</i>						
1. Ethyl isobutyrate	0.025 $\pm$ 0.03	3	0.021	1	0.005	1
2. Methyl 3-methylbutanoate	0.503 $\pm$ 1.15	8	0.341 $\pm$ 0.09	2	0.125 $\pm$ 0.10	4
3. Ethyl butanoate	0.257	1	0.149	1		
4. Methyl 3-methyl-2-butenate	0.073 $\pm$ 0.03	4				
5. Ethyl 2-methylbutanoate	0.167 $\pm$ 0.15	15	0.097 $\pm$ 0.07	10	0.143 $\pm$ 0.10	15
6. Ethyl 3-methylbutanoate	2.559 $\pm$ 3.45	15	0.973 $\pm$ 1.31	10	1.549 $\pm$ 1.54	15
8. 3-Methylbutyl acetate (isoamyl acetate)	0.144 $\pm$ 0.09	7				
9. isoPropyl 3-methylbutanoate	0.020 $\pm$ 0.01	5	0.034 $\pm$ 0.03	2	0.037 $\pm$ 0.02	2
10. Ethyl 3-methyl-2-butenate	0.112 $\pm$ 0.08	2	0.045	1	0.036 $\pm$ 0.00	2
15. 3-Methylbutyl 2-methylbutanoate	0.03	1				
16. 3-Methylbutyl 3-methylbutanoate	0.293 $\pm$ 0.10	2				
<i>Sesquiterpenes</i>						
19. delta-Elementene	0.182 $\pm$ 0.12	12	0.269 $\pm$ 0.15	9	0.246 $\pm$ 0.21	13
20. $\beta$ -Caryophyllene	0.003 $\pm$ 0.003	4	0.008 $\pm$ 0.01	2	0.014 $\pm$ 0.02	4
21. cis-Thujopsene	0.015 $\pm$ 0.01	6	0.002 $\pm$ 0.001	2	0.006	1
23. 4,5-di-epi-Aristolochene	0.037 $\pm$ 0.02	2				
24. trans-beta-Guaiene	0.007 $\pm$ 0.005	4	0.009 $\pm$ 0.01	4	0.014 $\pm$ 0.01	6
<i>Monoterpenes</i>						
11. Anhydrolinalool oxide	0.090 $\pm$ 0.04	3	0.049	1	0.043 $\pm$ 0.01	3
12. Limonene	0.093 $\pm$ 0.15	11	0.087 $\pm$ 0.08	4	0.133 $\pm$ 0.23	9
13. Cineole	0.032 $\pm$ 0.01	3				
14. Terpinolene	0.023 $\pm$ 0.01	3	0.072	1	0.053 $\pm$ 0.05	4
<i>Aromatics</i>						
17. 2-Phenylethanol	0.178 $\pm$ 0.03	3				
18. Ethyl benzoate	0.059 $\pm$ 0.02	3				
<i>Others</i>						
7. 1-Hexanol	0.055 $\pm$ 0.03	2				
22. 12-Methyl-oxacyclododec-9-en-2-one	0.069 $\pm$ 0.04	2			0.039	1

VOC components are classified in groups. Compounds are numbered as in Fig. 1 and Table S1 *N* indicates the number of samples in which a compound was found. See Table S1 for details of compound identification

350  $P = 0.04$ , permutation = 9999). The NMDS ordination of  
 351 the VOC composition partially separated VOC's of SWD-  
 352 attacked fruits from physically damaged and control VOCs  
 353 (Fig. 2). Further, the multivariate pairwise comparison  
 354 showed significant differences between the VOCs from  
 355 SWD-attacked blueberries and those from physically  
 356 attacked controls (perMANOVA:  $F_{1,23} = 2.63$ ,  $P = 0.03$ ,  
 357 permutation = 9999), as well from SWD-attacked blueber-  
 358 ries and undamaged control fruit VOCs (perMANOVA:  
 359  $F_{1,28} = 2.26$ ,  $P = 0.04$ , permutation = 9999). Finally, the  
 360 NMDS ordination did not graphically-show differences  
 361 between VOCs from physically damaged and undam-  
 362 aged blueberries, nor did the multivariate analyses (per-  
 363 MANOVA:  $F_{1,23} = 0.34$ ,  $P = 0.83$ , permutation = 9999).

### Olfactory Responses of *Trichopria anastrephae* to Blueberry Volatiles

364  
 365  
 366 In the Y-tube bioassay, females of *T. anastrephae* reared  
 367 on *D. melanogaster* responded preferentially to volatiles  
 368 from blueberries attacked by *D. suzukii*, in comparison  
 369 to undamaged blueberries or clean air. In both bioassays  
 370 involving SWD-attacked fruits (vs. air-control and vs.  
 371 undamaged blueberries), about 60% of females made a  
 372 choice during the 5-min test period (72 out of 109 and  
 373 53 out of 92, respectively). In contrast, in the bioassay  
 374 comparing undamaged control blueberries vs. clean air  
 375 only around 30% of the females made a choice (28 out of



**Fig. 1** Typical GC–MS chromatogram (TIC) of VOC collections from blueberries attacked by SWD (red), physically damaged control (blue), undamaged control (grey) and ambient background volatiles (black). Numbers indicate compounds as described in Tables 1 and

S1. Non-numbered peaks correspond to background volatiles. Missing numbers are blueberry VOCs not found in the samples chosen for the Figure (IS internal standard 100 ng tridecane)

376 103). In general, the behavioral responses of *T. anastre-*  
 377 *phae* females showed a tendency to prefer the blueberry-  
 378 treated arm. Parasitoid wasps were significantly attracted  
 379 to SWD-attacked blueberries when compared to clean air  
 380 in all three variables measured (first choice binomial test  
 381  $P = 0.01$ ; final position binomial test  $P = 0.0003$  and time  
 382 in each arm  $t_{52} = 2.52$ ,  $P = 0.01$ ) (Figs. 3, 4). Also, *T. anas-*  
 383 *trephae* females were significantly attracted to the vola-  
 384 tiles from SWD-infested blueberries relative to undamaged  
 385 blueberries when considering their final position (binomial  
 386 test  $P = 0.002$ ), but not in the first arm choice (binomial  
 387 test  $P = 0.1$ ) or the time spent in each olfactometer arm  
 388 ( $t_{71} = 1.28$ ,  $P = 0.20$ ). In the case of undamaged blueberries  
 389 compared to clean air, there were no significant differences  
 390 in any of the three variables measured (first choice bino-  
 391 mial test  $P = 0.28$ ; final position binomial test  $P = 0.33$  and  
 392 time in each arm  $t_{27} = 1.31$ ,  $P = 0.19$ ).

## Discussion

393  
 394 The aim of our study was to characterize the effect of  
 395 SWD attack on the VOCs of ripening blueberries, and to  
 396 correlate these odor changes with the olfactory-mediated  
 397 behavioral responses of local populations of *T. anastre-*  
 398 *phae* female parasitoids. Our study is the first to use a  
 399 chemo-ecological approach to test the capacity of *T. anas-*  
 400 *trephae* to find *D. suzukii*-infested fruit.

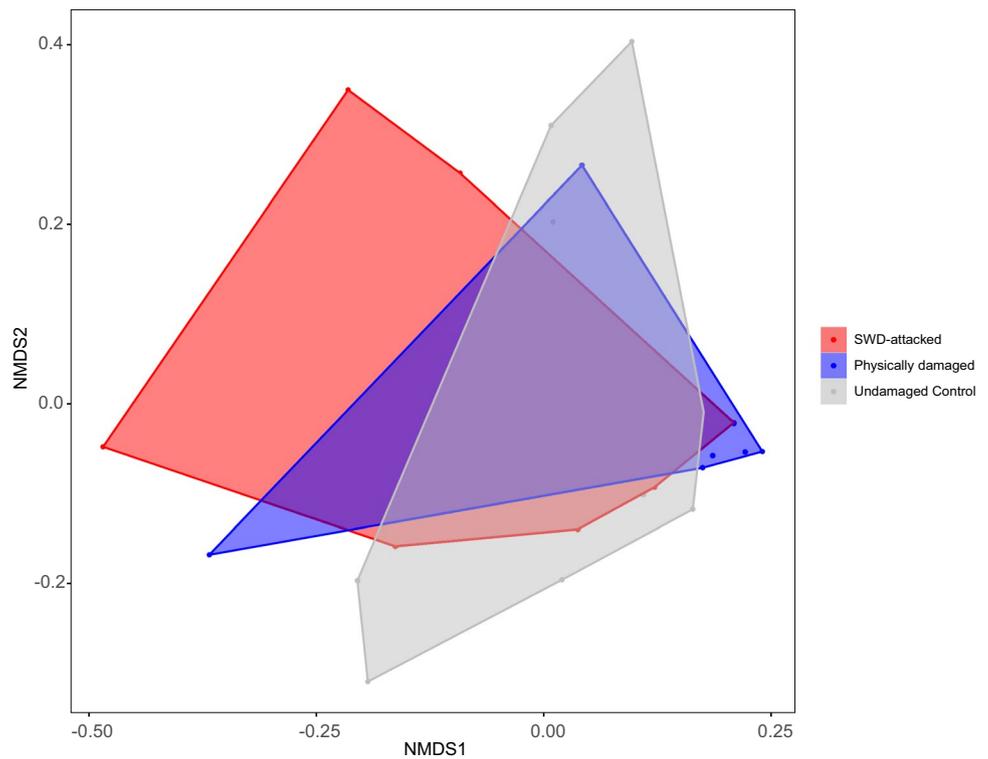
401 Multivariate analyses of the odor blends produced by  
 402 SWD-attacked blueberries showed that the development of  
 403 SWD immature stages inside the fruit generates a different  
 404 odor profile in comparison with physically damaged and  
 405 undamaged control fruits. Ten days after SWD infesta-  
 406 tion, a period that correlates with SWD larval development  
 407 (Tochen et al. 2014), the odor differences among SWD-  
 408 attacked and control blueberries (physically damaged and

**Table 2** Amounts of blueberry volatile organic compounds (VOC's) clustered by chemical group, according to fruit treatments: attacked by *Drosophila suzukii* (SWD-attacked); physical damage control and

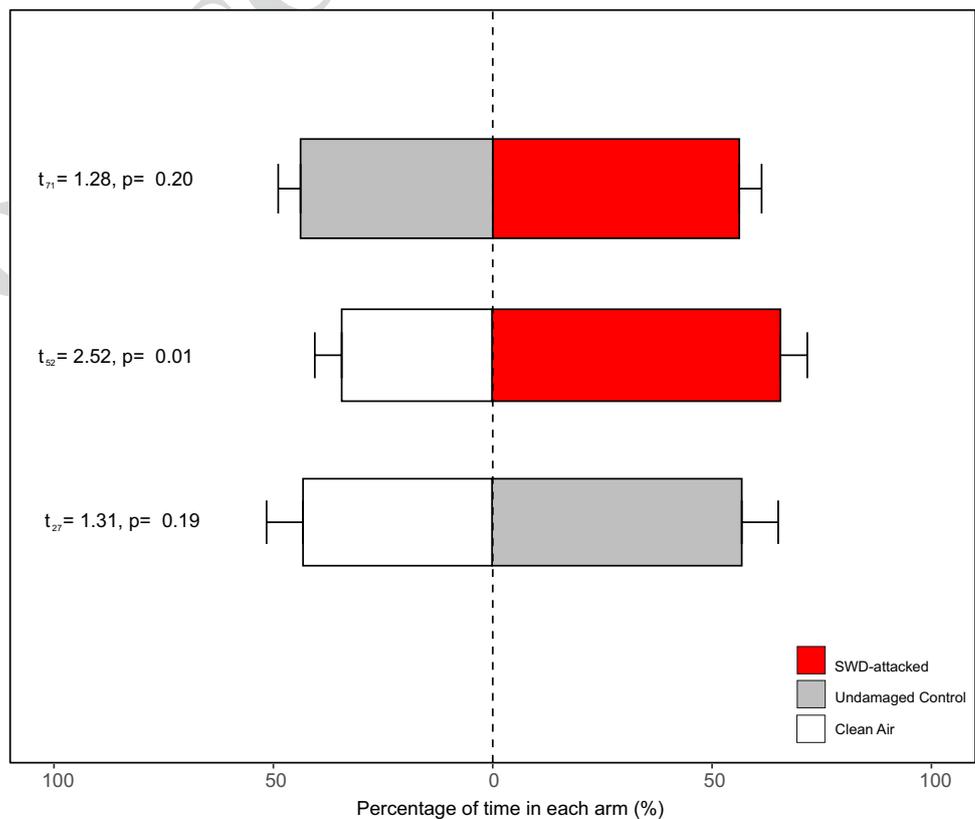
natural fruit ripening (undamaged control). *N* indicates the number of samples in which compounds of the chemical group were found

Compound group	Amounts ( $\mu\text{g}/25 \text{ g}/24 \text{ h}$ ; mean $\pm$ standard deviation)					
	SWD-attacked	<i>N</i>	Physical damage	<i>N</i>	Undamaged control	<i>N</i>
Short-chain aliphatic esters	$3.17 \pm 4.63$	15	$1.17 \pm 1.52$	10	$1.74 \pm 1.61$	15
Sesquiterpenes	$0.17 \pm 0.13$	14	$0.28 \pm 0.16$	9	$0.24 \pm 0.22$	14
Monoterpenes	$0.12 \pm 0.15$	12	$0.12 \pm 0.14$	4	$0.17 \pm 0.29$	9
Aromatics	$0.14 \pm 0.09$	5				
Others	$0.06 \pm 0.03$	4			0.04	1

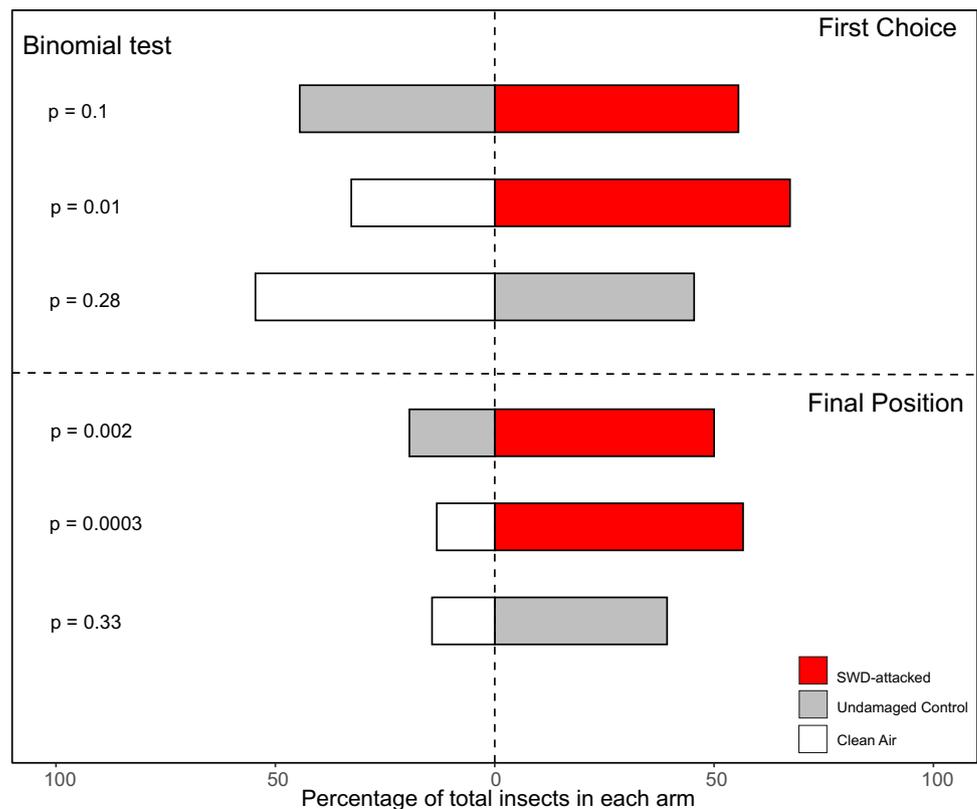
**Fig. 2** Non-metric multidimensional scaling (NMDS) ordination based on Bray–Curtis dissimilarities of the volatile organic compounds (VOC's) from blueberries attacked by *Drosophila suzukii* (SWD-attacked,  $N=15$ ); VOC's from physically-damaged blueberries ( $N=10$ ) and from undamaged fruit (Control,  $N=15$ ). Stress value = 0.1615



**Fig. 3** Percent time spent by *Trichopria anastrephae* females in each arm of an olfactometer tube in choice bioassays testing volatile cues produced by blueberries attacked by *Drosophila suzukii* (SWD-attacked), control blueberries (undamaged) or clean air. Paired  $t$ -test are reported and error bars show Standard Error



**Fig. 4** Percent *Trichopria anastrephae* females in each arm of an olfactometer tube in choice bioassays with volatile cues produced by blueberries attacked by *Drosophila suzukii* (SWD-attacked), undamaged control or clean air. The upper and lower panels show the first arm choice and the final position at the end of a 5-min test period, respectively. *P* values for the binomial test are reported



409 undamaged) were significant and observable in the diver- 436  
 410 sity, frequency, and amounts of volatile compounds. These 437  
 411 differences in the blueberry VOC profiles may be the result 438  
 412 of various factors associated with SWD infestation, such 439  
 413 as fruit tissue collapse due to larval feeding, larval meta- 440  
 414 bolic wastes and their associated microorganisms, and 441  
 415 opportunistic microorganisms associated with oviposition 442  
 416 wounds (Hamby and Becher 2016; Rombaut et al. 2017). 443

417 Short-chain aliphatic esters, particularly ethyl 3-methylb- 444  
 418 utanoate, constituted the most abundant group of chemicals 445  
 419 in all three treatments. Along with the esters, the blueberries 446  
 420 emitted sesqui- and monoterpenes as relatively minor com- 447  
 421 ponents. Among the sesquiterpenes, delta-elemene was the 448  
 422 most abundant and frequent, while limonene was the most 449  
 423 abundant monoterpene in the control treatments. In SWD- 450  
 424 attacked blueberries, however, the amount of limonene was 451  
 425 similar to that of anhydrolinalool oxide, a probable fungal 452  
 426 biotransformation metabolite. VOCs from SWD-attacked 453  
 427 fruits also contained 2-phenylethanol and ethyl benzoate, 454  
 428 volatiles commonly found in flowers and fruits and also typi- 455  
 429 cal from fermentation processes in the case of the former. 456  
 430 These aromatic volatiles were not found in either of the con- 457  
 431 trol treatments. Finally, SWD-attacked blueberries emitted 458  
 432 seven additional compounds that were not found in the VOC 459  
 433 collections from both control treatments. Among these, the 460  
 434 most abundant were 3-methylbutyl 3-methylbutanoate (isoa- 461  
 435 myl isovalerate) and isoamyl acetate. The latter was also 462

the most consistently found among compounds exclusive to

436 SWD-attacked fruits. 437  
 438 Compounds such as hexanol, (*Z*)-linalool oxide and lin- 439  
 440 linalool are probably produced throughout ripening and pre- 440  
 441 served during the last maturation phases, reaching high 441  
 442 amounts in the overripe stage (Farneti et al. 2017; Horvat 442  
 443 et al. 1996). Other compounds seem to be emitted by unripe 443  
 444 fruits and drastically reduced during ripening (e.g. caryo- 444  
 445 phyllene), while compounds such as  $\delta$ -elemene are stable 445  
 446 during all ripening phases (Farneti et al. 2017). In contrast, 446  
 447 esters such as ethyl 3-methylbutanoate (ethyl isovalerate), 447  
 448 ethyl acetate, and methyl 2-methylbutanoate are exclusively 448  
 449 produced in the last phase of ripening, increasing as the 449  
 450 blueberries overripe (Farneti et al. 2017). In this scenario, 450  
 451 our results suggest that SWD attack results in volatile emis- 451  
 452 sions that resemble overripe fruit. 452

453 Fruit volatiles are important in the chemical ecology of 453  
 454 drosophilids and may play a role in niche differentiation 454  
 455 among sympatric species. Even though cosmopolitan *Dros-* 455  
 456 *ophila* species are host generalists, different species may 456  
 457 separate along resource-based niche dimensions such as fruit 457  
 458 maturation time (Nunney 1996). SWD may be attracted to 458  
 459 leaf volatile cues to mate-finding and also fresh unripe fruits 459  
 460 odors to locate areas for oviposition (Cloonan et al. 2018). 460  
 461 As fruits ripen, other drosophilids such as *Drosophila simu-* 461  
 462 *lans*, *D. melanogaster*, and *Drosophila immigrans* may fur- 462  
 463 ther colonize this rotten fruit, following a preference order

for increasing maturation stages (Atallah et al. 2014; Nunney 1996; Rombaut et al. 2017). Fruit volatile esters may provide cues for ripening stages and may encode enough information to enable drosophilid flies to detect and discriminate their niches (Scheidler et al. 2015). For instance, isoamyl acetate, a “fruity” odor often present in ripening, ripe, and early fermenting fruits, is known to attract many drosophilids (Stökl et al. 2010). This compound, along with isobutyl acetate and ethyl hexanoate, were present in headspace VOC samples of fruit-associated yeasts and caused antennal responses in *D. melanogaster* and *D. suzukii* (Scheidler et al. 2015). Moreover, isoamyl acetate was one of the EAD-active compounds from wild blueberries, attractive to *D. suzukii* both individually and as part of a blend (Urbaneja-Bernat et al. 2021). However, tested in formulated blends for trapping SWD, isoamyl acetate showed no attraction and even a decrease in SWD adult captures (Cha et al. 2012). In this case, it is possible that concentration modulates SWD response to volatiles such as isoamyl acetate, since high concentrations may signal an overripe fruit that is not a preferred oviposition site for SWD females (Revadi et al. 2015).

The dynamics of fruit volatile blends associated with the temporal separation of *Drosophila* species may provide host-finding cues to the next trophic level (Vet and Dicke 1992). Using behavioral bioassays, we demonstrated that resident populations of *T. anastrephae*, a pupal parasitoid reared in the laboratory on *D. melanogaster*, responded differentially to volatiles emitted by blueberries infested by SWD. We measured three variables to characterize female wasp responses to the volatiles of blueberries: the first choice, the time spent in each arm, and the final position after five minutes of bioassay. Females of *T. anastrephae* showed a preference in all three variables when SWD-attacked fruits were tested against clean air. When the bioassay compared SWD-attacked and undamaged blueberries, a preference was only found in the position of the wasp at the end of the tested period, a variable that may correlate with active seeking behavior of the preferred source. These results are in line with those obtained from a related parasitoid, *T. drosophilae*. Using an olfactometer arena with four chambers and testing seven wild non-crop fruits as stimuli, Wolf et al. (2020) showed that female *T. drosophilae* spent more time walking over chambers with SWD-infested fruits compared to clean air. When comparing SWD-infested fruits with non-infested fruits, significant preferences were not so consistent, indicating that fruit odors alone may not be sufficient for host location in these fruits (Wolf et al. 2020). The preference showed by *T. anastrephae* females towards volatiles of SWD-infested blueberries appear to be innate, since they have had no access to SWD or SWD-infested fruit prior to the tests. SWD infestation of blueberries resulted in an increase of typical volatile compounds that other *Drosophila* species

use for locating food, mating, and oviposition sites. It is then likely that *T. anastrephae* females use these same general odorant cues to locate their established drosophilid hosts. The foraging females may switch hosts if other alternatives are more abundant (Jaworski et al. 2013) or if they find earlier infested fruits that are more likely to contain pupae. In support of this, the generalist parasitoid *T. drosophilae* showed no differential preference for *D. melanogaster* or *D. suzukii* pupae (Wang et al. 2016). Similarly, a recent study by Biondi et al. (2021) showed that the larval parasitoids, *Asobara japonica* Belokobylskij (Hymenoptera: Braconidae), *Leptopilina japonica* Novković & Kimura, and *Ganaspis brasiliensis* (Ihering) (Hymenoptera: Figitidae), respond to fruit volatile cues associated with the presence of either *D. suzukii* or *D. melanogaster* (Biondi et al. 2021).

From an applied perspective, our results represent a relevant contribution to the development of a biological control program for SWD, since it deepens our understanding of how SWD impact blueberry VOCs, and how a parasitoid responds to the presence of its potential pest host (Biondi et al. 2017). Our study highlights the potential use of established populations of *T. anastrephae* since they are naturally able to cue on VOCs from SWD-attacked fruit and use SWD as a viable host (Vieria et al. 2020). Although most SWD pupae are found in the soil (Wolz et al. 2017), by using fruit VOCs as a long-range cue, parasitoids could target the right ecological niche to then refine their search. Understanding the behavior of this parasitoid in challenging environments is important to obtain higher efficiency in biological control programs against SWD (Krüger et al. 2019), stressing the need for more studies on the biology of *T. anastrephae* in the region. In line with this, the absence of management in adjacent crops or wild fruits could be reservoir of SWD where resident populations of parasitoids could have a greater importance. Our finding of local parasitoid populations suggests that the wasp is well adapted to local environmental conditions, which further underlines its potential as a biological control agent also in unmanaged adjacent crops or nearby wild fruits, which could serve as reservoirs for SWD (Krüger et al. 2019). In a broader sense, studies on these local parasitoid populations at the regional level may offer opportunities to manage SWD in Latin America without the new introduction of exotic species. Hence, a non-native organism such as SWD in Latin America may become controlled by a regulating mechanism in the introduced range, which limits its density and expansion (Schulz et al. 2019).

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572 GD, AG, and FT collected the data; AG identified the chemical com-  
573 pounds; FT led the parasitoid experiments; GD and AG led the analysis  
574 and writing of the manuscript. All authors contributed to the drafts and  
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579 **Availability of Data and Material** Data is available from the correspond-  
580 ing authors on request.

581

582 **Code Availability** Code is available from the corresponding authors  
583 on request.

## 584 Declarations

585 **Conflict of interest** All authors declare that they have no conflict of  
586 interest.

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