

1 **Association between olfactory sensitivity and behavioral responses of *Drosophila suzukii* to**
2 **naturally-occurring volatile compounds.**

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5 **Corda G¹, Solari P¹, Dettori MA², Fabbri D², Delogu G², Crnjar R¹, Sollai G^{1*}**

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7 ¹Department of Biomedical Sciences, Section of Physiology, University of Cagliari, 09042
8 Monserrato (CA), Italy; ² CNR Institute of Biomolecular Chemistry, 07100 Sassari, Italy

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10 **Corresponding author:** Giorgia Sollai, Department of Biomedical Sciences, Section of Physiology,
11 University of Cagliari, SP 8 Km 0.700, 09042 Monserrato (CA), Italy. E-mail: gsollai@unica.it;
12 Phone: +39 070 6754160; Fax: +39 070 6754191

13

14 **Abstract**

15 *Drosophila suzukii* Matsumura (Diptera: Drosophilidae) is an invasive, destructive crop pest that
16 originated in South-East Asia. *D. suzukii* recently invaded Western countries and is threatening both
17 European and American fruit industries. It is extremely fond of otherwise undamaged, ripening fruits,
18 unlike most other *Drosophila* species which attack only decaying or rotten fruits. Recent studies on
19 different insect species showed that several naturally-occurring compounds of easy market
20 availability showing deterrent action may be used to supplement mass catches with food traps. Based
21 on these considerations, aim of the present work was to test the effects of some natural compounds
22 (alone or in mixture) on the olfactory system of the *D. suzukii* and the behavioral responses evoked.
23 To this end, we measured by EAG recordings, the olfactory sensitivity of antennae to increasing
24 concentrations of eugenol, vanillin, menthol, *cis*-jasmone; eugenol + vanillin, + menthol, + *cis*-
25 jasmone; vanillin + menthol, + *cis*-jasmone. In addition, the behavioral responses to same compounds
26 and mixtures were evaluated. Our electrophysiological results show a dose-response relationship
27 between the EAG amplitudes and the increasing concentrations of the olfactory compound. The
28 behavioral results show that the number of laid eggs is significantly different between the standard
29 diet and the standard diet + natural compound. These results underline a specificity in the olfactory
30 sensitivity and in the ovipositing behavior of *D. suzukii* females; also, they could be valuable for the
31 identification of key chemicals aimed at the future development of strategies in the management and
32 control of this harmful insect for crops.

33

34 **Key Words:** *Drosophila*; olfactory sensitivity; behavior; natural compounds; “push and pull”
35 strategy.

36 1. Introduction

37 In insects, host selection behavior, both in term of identification of suitable sites by ovipositing
38 females and of relevant food sources for the offspring, is strongly influenced by sensory input from
39 their chemical senses (Lebreton et al., 2017; Masala, Solari, Sollai, Crnjar, & Liscia, 2008; Masala,
40 Solari, Sollai, Crnjar, & Liscia, 2009; Merivee et al., 2002; Solari, Masala, Falchi, Sollai, & Liscia,
41 2010; Sollai, Biolchini, Solari, & Crnjar, 2017; Sollai, Biolchini, & Crnjar, 2018a; Sollai & Crnjar,
42 2019; Yarmolinsky, Zuker, & Ryba, 2009; Zhang et al., 2013). Among the chemical senses, the
43 olfactory system detects volatile compounds, such as repellents, attractants, pheromones and food
44 odors, from a potential source by means of olfactory sensilla mainly located in the antennae, but also
45 in the maxillary palps and the ovipositor (Anton et al., 2003; Galizia & Roessler, 2010; Martin, Boto,
46 Gomez-Diaz, & Alcorta, 2013; Sollai et al., 2010). The olfactory sensory neurons (OSNs) housed in
47 the olfactory sensilla transduce the information contained in odor stimuli into action potentials and
48 project their axons to the brain, where they synapse with the local interneurons and the projection
49 neurons in the glomeruli of the antennal lobes (Solari et al., 2016; Strausfeld & Hildebrand, 1999).

50 Odorant compounds are recognized by specific odorant receptors (ORs) in the dendrites of the OSNs
51 and only one or very few of these ORs are expressed in each OSN (Benton, Vannice, Gomez-Diaz,
52 & Vosshall, 2009; Vosshall, Wong, & Axel, 2000). The expression of the specific ORs in each OSN
53 is influenced by the characteristics of the environment in which the insect lives and contributes to
54 individual variation in olfactory behavior and in olfactory sensitivity, not only among different insect
55 species, but also among different populations within the same species (Rollmann et al., 2010). This
56 means that insects differ for the volatile compounds that their olfactory system may detect and for the
57 consequent behavioral response (de Bruyne, Clyne, & Carlson, 1999; de Bruyne, Foster, & Carlson,
58 Rollmann et al., 2010; Yao, Ignell, & Carlson, 2005). In addition, the response profile of OSNs to
59 volatiles may be modified by different factors: for example, in *Manduca sexta* a circadian control has
60 been reported to exist in the olfactory sensitivity to pheromones (Schendzielorz, Schirmer, Stolte, &
61 Stengl, 2015). Likewise, the environmental temperature appears to affect olfactory behavior and

62 modify antennal electrical responses in *Drosophila* spp. (Martin, Riveron, & Alcorta, 2011; Riveron,
63 Boto, & Alcorta, 2009).

64 Within the "melanogaster group", the spotted-wing *Drosophila suzukii* Matsumura (Diptera:
65 Drosophilidae) is a polyphagous invasive pest insect, with a broad climate range tolerance; in fact, it
66 originated from South-East Asia and quickly spread to North and South America, Europe and the
67 Mediterranean basin (Asplen et al., 2015; Tait et al., 2017). The high invasive potential of this species
68 is due to: a) a great ability to adapt to cold climates and seasons (Tait et al., 2018); b) multiple
69 generations with rapid population growth during the summer months (Renkema, Buitenhuis, &
70 Hallett, 2017); c) adult female flies lay eggs on unripe and undamaged fruits by means of a serrated
71 ovipositor (Rota-Stabelli, Blaxter, & Anfora, 2013; Walsh et al., 2011). This difference in ecology
72 between *D. suzukii* and *D. melanogaster* is reflected in neurological and physiological adaptations
73 aimed at finding unripe oviposition substrates (Ometto et al., 2013; Rollmann et al., 2010).

74 On the basis of these considerations, the aim of this study was to evaluate peripheral sensitivity of
75 adult females of the spotted-wing *Drosophila suzukii* towards volatile compounds, which in other
76 insect species and populations of *D. suzukii* have shown a repellent action (Del Fabbro & Nazzi,
77 2013; Kamsuk et al., 2007; Munerato, Sinigaglia, Reguly, & Rodriguez de Andrade, 2005; Park, Jang,
78 Yoon, & Kim, 2016; Renkema, Wright, Buitenhuis, & Hallett, 2016; Renkema et al., 2017; Sobhy et
79 al., 2017; Zeringota et al., 2013), by means of an electrophysiological and behavioral approach. In
80 this respect, we evaluated the antennal olfactory sensitivity towards increasing concentrations of
81 repellent compounds (both alone and as binary mixtures) and the subsequent ovipositing behaviour
82 evoked in adult females of *D. suzukii*.

83

84 **2. Materials & Methods**

85 *2.1. Insects*

86 Four to ten-day adult females of *Drosophila suzukii* (Diptera: Drosophilidae) were obtained from a
87 lab-reared colony at the Dept. of Biomedical Sciences of the University of Cagliari (Italy). In the

88 larval stage, flies were fed on *Drosophila* standard diet (Dalton et al., 2011) under controlled
89 conditions (23°C, 70% of relative humidity, 14L/10D photoperiodic regime) (Biolchini et al., 2017).

90

91 2.2. Stimuli

92 The following compounds were tested as olfactory stimuli: eugenol (Sigma-Aldrich, Italy), menthol
93 (Sigma-Aldrich, Italy), vanillin (Sigma-Aldrich, Italy) and *cis*-jasmone (Alfa-Aesar, Thermo Fisher
94 Scientific, Germany). Excluding *cis*-jasmone with 90% of purity, all compounds were purchased with
95 a purity $\geq 99\%$ (GC) and used without further purification. These compounds were chosen on the
96 basis of data available in the literature on different species of insects: eugenol, a phenolic compound
97 used in perfumes, flavorings and as a local antiseptic and anaesthetic (Jadhav, Khandelwal, Ketkar,
98 & Pisal, 2004; Sell & Carlini, 1976), presents a repellent activity (Del Fabbro & Nazzi, 2013;
99 Zeringota et al., 2013); menthol, the main component of peppermint essential oil (Ibanez & Amparo
100 Blazquez, 2018), shows repellent activity (Park et al., 2016; Renkema et al., 2016; Renkema et al.,
101 2017); vanillin, an aromatic compound and flavouring agent, shows synergic effect with essential oils
102 with repellent activity (Kamsuk et al., 2007); *cis*-jasmone, a volatile compound that enhances the
103 plant defense against herbivores and insects (Sobhy et al., 2017).

104 A 50 μL volume of each compound solution, previously dissolved (100 $\mu\text{g}/\text{ml}$) in dichloromethane
105 (Sigma-Aldrich, Italy; CH_2Cl_2), was pipetted onto a pleated strip of filter paper (80x5 mm), to yield
106 a final dosage of 5 μg . By means of decadic dilutions in CH_2Cl_2 , we obtained the relative lower
107 solutions and 50 μL of each of them was pipetted onto filter paper strips to obtain a $0.5 \div 0.0005 \mu\text{g}$
108 load on strip; compounds were tested in increasing sequence of concentrations (five in all) (Solari et
109 al., 2007).

110 Preliminary electrophysiological experiments showed that when the filter paper was filled with 50
111 μL of CH_2Cl_2 alone, the CH_2Cl_2 evaporated totally and the strip was not chemically stimulating. In
112 order to test the presence of a possible mechanoreceptor response component, before each stimulation

113 sequence (i.e. the five increasing solutions of the same compound) in each experiment, the response
114 to air (blank control) was also tested and, if present, its value was subtracted from those of the
115 chemical stimuli. Stimuli were presented in a randomized sequence, with a blank interstimulus
116 interval long enough to allow for complete repolarization, according to Sollai et al. (2018). Each test
117 was replicated 10 times.

118

119 *2.3. Electrophysiological experiments*

120 Recordings were performed on antennae by means of the electroantennogram (EAG) recording
121 technique (Crnjar et al., 1989). A glass micropipette (20 μm tip diameter) filled with saline solution
122 (0.9%NaCl) (Clymans et al., 2019) was inserted into the isolated head through the “foramen
123 magnum” and acted as reference electrode in EAG recordings. The antennae, one per fly, were
124 positioned in such a way as to expose the largest surface to the stimulus-bearing airstream. An air-
125 stimulus control unit (model CS-55, Syntech, Hilversum, the Netherlands) was used for air and odor
126 delivery, according to Sollai et al. (2007). All signals were recorded with a high input impedance
127 ($10^{15} \Omega$) electrometer (WPI, Duo 773), band-pass filtered (DC-1 kHz), digitized by means of an Axon
128 Digidata 1440A A/D acquisition system (sampling rate 10 kHz) and stored on PC for later analysis
129 (Sollai, Solari, Masala, Liscia, & Crnjar, 2008; Sollai, Solari, Corda, Masala, & Crnjar, 2012). The
130 absolute EAG amplitudes during the 2-s stimulation period were calculated by means of Axoscope
131 10.0 software (Solari et al., 2007; Sollai et al., 2007).

132

133 *2.4. Behavioral experiments*

134 Immediately after eclosion, males and females were kept in the same cage to allow mating, which
135 normally occurs on the 4th day (Bretman, Westmancoat, & Chapman, 2013). Adult females ready for
136 oviposition were transferred into vials (5 flies/vial), which served as experimental arenas. Each vial
137 contained an oviposition substrate, consisting of 10 mL standard diet (control; the same diet used to
138 rear the flies) or 10 mL standard diet + 250 μL of a 0.1 mM solution of each single compound or

139 mixture previously tested in the electrophysiological experiments. The females of *D. suzukii* were left
140 free to lay eggs for a period of 72 hours, after which the substrate was removed from the experimental
141 arena and the eggs laid were counted with the aid of a binocular, with the aim of testing oviposition
142 preferences (Sollai, Biolchini, Loy, Solari, & Crnjar, 2017). Ten replicates were carried out for each
143 experiment. The experimental arenas were kept in a climatic chamber, having the same controlled
144 conditions as the rearing one (23°C, 70% of relative humidity, 14L/10D photoperiodic regime).

145

146 *2.5. Statistical analysis*

147 Repeated measures ANOVA was adopted to analyze the effect of increasing concentrations of odor
148 stimuli (eugenol, menthol, vanillin and *cis*-jasmone) on the EAG amplitudes of the antennal sensilla,
149 separately for each stimulus.

150 One-way ANOVA was used to test the effect of rearing substrate (standard diet, standard diet +
151 chemical solution) on the number of eggs laid.

152 Data were checked for the assumptions of normality, homogeneity of variance and sphericity (when
153 applicable). When the sphericity assumption was violated, a Greenhouse-Geisser correction or
154 Huynh-Feldt correction was applied in order to modify the degrees of freedom (Sollai, Tomassini
155 Barbarossa, Masala, Solari, & Crnjar, 2014; Sollai, Tomassini Barbarossa, Solari, & Crnjar, 2015).
156 Post-hoc comparisons were conducted with LSD Fisher's test, unless the assumption of homogeneity
157 of variance was violated, in which case Duncan's test was used (Sollai et al., 2014; Sollai et., 2015).
158 Statistical analyses were performed using STATISTICA for WINDOWS (version 7.0; StatSoft Inc,
159 Tulsa, OK, USA). *P* values < 0.05 were considered significant.

160

161 **3. Results**

162 Examples of EAG recordings in response to 5 µg of the tested stimuli are shown in figure 1.

163 To test for a dose-response relationship, we analysed the EAG amplitudes evoked in the antennal
164 preparations in response to increasing concentrations of each volatile solution, by means of repeated-
165 measures ANOVA. A significant effect of increasing concentrations on EAG values resulted for
166 eugenol ($F_{(2,9,26,4)} = 8.6938$, $p = 0.0004$), menthol ($F_{(4,36)} = 21.386$, $p < 0.0001$) and vanillin ($F_{(4,36)} =$
167 9.1724 , $p < 0.0001$), while no effect was found for *cis*-jasmonone ($F_{(1,6,14,8)} = 0.3207$, $p = 0.6823$) (Fig.
168 2). These results show that the antennal olfactory system is stimulated by eugenol, menthol and
169 vanillin.

170 With the aim of identifying the order of preference of oviposition substrates, we analysed the number
171 of eggs laid on each substrate, by using one-way ANOVA. Results revealed a significant effect of the
172 substrate on the oviposition choice ($F_{(4,45)} = 9.7736$, $p < 0.0001$) and post-hoc comparisons showed
173 that the number of eggs laid on the oviposition substrate containing standard diet + eugenol or +
174 menthol or + vanillin was significantly lower than that on standard diet alone ($p < 0.005$; LSD Fisher's
175 test) or standard diet + *cis*-jasmonone ($p < 0.05$; LSD Fisher's test) (Fig. 3). Instead, no difference in
176 the number of eggs laid was found between the substrate containing standard diet alone and standard
177 diet + *cis*-jasmonone ($p > 0.05$; LSD Fisher's test). These results indicate that eugenol, menthol and
178 vanillin are repellent towards ovipositing females.

179 Based on these results, we tested the antennal olfactory sensitivity towards binary mixtures of the
180 compounds previously tested individually. Repeated-measures ANOVA revealed a significant effect
181 on the EAG amplitudes exerted by increasing concentrations of eugenol + vanillin ($F_{(1,9,16,9)} = 35.91$,
182 $p < 0.0001$), eugenol + menthol ($F_{(2,5,22,4)} = 5.3929$, $p = 0.0086$), eugenol + *cis*-jasmonone ($F_{(3,3,29,6)} =$
183 5.9606 , $p = 0.0021$), vanillin + menthol ($F_{(1,7,15,1)} = 5.1752$, $p = 0.024$) and vanillin + *cis*-jasmonone
184 ($F_{(1,3,11,7)} = 12.931$, $p = 0.0024$) (Fig. 4). These results show that the antennal olfactory system is
185 activated by all binary mixtures tested. Accordingly, we found a significant effect of the oviposition
186 substrate on the number of eggs laid when one of the binary mixtures was added to the standard diet
187 ($F_{(5,54)} = 12.904$, $p < 0.0001$) (Fig. 5). In particular, post-hoc comparisons subsequent to one-way
188 ANOVA showed that the number of eggs laid on standard diet containing eugenol + vanillin, eugenol

189 + menthol, vanillin + menthol or vanillin + *cis*-jasmone was lower than that on standard diet alone (p
190 < 0.005 ; LSD Fisher's test) or standard diet containing eugenol + *cis*-jasmone ($p < 0.05$; LSD Fisher's
191 test). In addition, post-hoc comparisons also revealed that the number of eggs laid on the substrate
192 containing eugenol + vanillin was higher than that laid on the substrate containing vanillin + menthol
193 or vanillin + *cis*-jasmone ($p < 0.05$; LSD Fisher's test). These results suggest that, among the
194 oviposition substrates, those containing vanillin + menthol or vanillin + *cis*-jasmone exhibit the
195 highest deterrent effect towards ovipositing females.

196

197 **4. Discussion**

198 Chemoreception plays a key role by regulating essential behaviors such as localization and
199 discrimination of host plants suitable by females for laying eggs and by offspring as food source
200 (Sollai, Biolchini, & Crnjar, 2018b). The goal of this work was to evaluate the olfactory sensitivity
201 of *Drosophila suzukii* females towards naturally-occurring compounds, which showed deterrent or
202 synergic effects both in other species of insects and in different populations of *D. suzukii* (Del Fabbro
203 & Nazzi, 2013; Kamsuk et al., 2007; Munerato et al., 2005; Park et al., 2016; Renkema et al., 2016;
204 Renkema et al., 2017; Sobhy et al., 2017; Zeringota et al., 2013). Firstly, we aimed at studying
205 whether a relationship could be found between olfactory sensitivity and behavioral response to tested
206 compounds. The dose-response relationships we found indicate that female flies present an olfactory
207 sensitivity to eugenol, menthol and vanillin, but not to *cis*-jasmone. Vanillin has been found to elicit
208 EAG responses also in the meadow spittlebug *Philaenus spumarius* (Germinara et al., 2017). In
209 addition, our results indicate that the antennal olfactory system responds in a dose-dependent manner
210 when stimulated with binary mixture of the natural compounds. In particular, we found that the
211 presence of *cis*-jasmone in binary mixture, which is *per se* ineffective in evoking EAG responses,
212 does not influence the stimulating effect of eugenol and vanillin. Besides, no synergic effect of
213 vanillin was found when it was mixed with eugenol or menthol. In fact, the responses obtained with
214 the binary mixtures were not higher than those recorded in response to eugenol or menthol alone.

215 The second aim of the study was to investigate the behavioral effect of the chemical compounds,
216 alone or in mixtures, on ovipositing females of *D. sukuzii*. Our results show that the number of eggs
217 laid on oviposition substrates containing eugenol, menthol or vanillin was significantly lower than
218 that found on substrates containing either the standard diet, or *cis*-jasmone. We also found that, when
219 the oviposition substrate was enriched with one of the binary mixtures, the number of eggs laid was
220 significantly lower than either the control substrate or the one containing the eugenol + *cis*-jasmone
221 mixture. Furthermore, the number of eggs counted on the substrate containing eugenol + vanillin was
222 significantly higher than that found on the substrates containing vanillin + menthol or vanillin + *cis*-
223 jasmone. Analogously, a repellent effect has been suggested for each of the compounds tested in this
224 study. In detail, eugenol has been found to have a toxic effect on the larvae of *D. melanogaster*
225 (Munerato et al., 2005) and a repellent action against different species of ticks, such as *Ixodes ricinus*
226 (Del Fabbro & Nazzi, 2013), *Rhipicephalus microplus* and *Dermacentro nitens* (Zeringota et al.,
227 2013); menthol was found to have a repellent effect on different populations of *D. sukuzii* in the North
228 American (Canada) and Asian (Korean peninsula) continents (Park et al., 2016; Renkema et al.,
229 2017); vanillin increases the repellent effect of DEET (a standard synthetic repellent) against various
230 species of insects belonging to the genus *Aedes* and *Culex* (Kamsuk et al., 2007). Instead, our results
231 show that no effect of *cis*-jasmone was found on both peripheral olfactory sensitivity and the behavior
232 of ovipositing females of *D. sukuzii*, opposite to what reported for the aphid *Macrosiphum euphorbiae*
233 (Sobhy et al., 2017). Taking into account that *cis*-jasmone plays a key role in activating the metabolic
234 pathway of plant defense, in the present study the reduced effects by *cis*-jasmone could be due to the
235 fact that all experimental assays were carried out in the lab excluding plants and their environment
236 (Bingham, Alptekin, Delogu, Gurkan, & Moores, 2014).

237 In conclusion, these results indicate that *D. sukuzii* females show a specificity in the olfactory
238 sensitivity profile that conditions their ovipositing behavior and characterizes its diversity compared
239 to other species. The knowledge of the olfactory response specificity and of the consequent behavior
240 of ovipositing females of *D. sukuzii* towards volatiles, in particular naturally-occurring ones, becomes

241 important for the development of control techniques for the populations of this insect harmful for
242 crops. In fact, treatments with insecticides, which are repeated several times during the season
243 (Shawer, Tonina, Tirello, Duso, & Mori, 2018; Van Timmeren & Isaacs, 2013), present several
244 disadvantages: negative effects on natural enemies, development of outbreaks of secondary pests,
245 reduction of pre-harvest intervals and onset of resistance to insecticides (Klick et al., 2019; Van
246 Timmeren & Isaacs, 2013). This means that the development of additional strategies for population
247 control and crop protection are of particular importance. These techniques include “push and pull”
248 strategy (Cook, Khan, & Pickett, 2007), which involves the use of compounds with repellent action
249 to pest insects that damage valuable crops (“push” component) and a “pull” component, represented
250 by bait traps. On the basis of these results, we propose a more environment-friendly alternative by
251 applying naturally-occurring compounds readily available on the market as push component.

252

253 **Acknowledgements**

254 We are grateful to Dr. Marco Melis, Dept. of Biomedical Sciences, University of Cagliari, for his
255 technical assistance.

256

257 **Author Contributions**

258 Conceptualization, R.C., G.D. and G.S.; Methodology, G.C., P.S, M.A.D, D.F., G.D., R.C. and G.S.;;
259 Software, G.C. and G.S.; Validation, G.C., P.S., M.A.D, D.F., G.D., R.C. and G.S.; Analysis and
260 Investigation, G.C. and G.S.; Resources, R.C. and G.S.; Data Curation, G.S.; Writing – Original Draft
261 Preparation, G.S.; Writing – Review & Editing, P.S., G.D., R.C. and G.S.; Supervision, G.D., R.C.
262 and G.S; Funding Acquisition, P.S., G.D., R.C. and G.S.;

263

264

265 **Funding**

266 This research was supported by the Regione Autonoma della Sardegna, Italy (Grant No. SULCIS-
267 821008; delibera CIPE N. 31 del 20.02.2015 e deliberazione N. 52/36 del 28.10.2015 “Progetto
268 Strategico Sulcis” – Progetti di ricerca pubblico-privati).

269

270 **Conflicts of Interest**

271 The authors declare no conflict of interest.

272

273 **Highlights**

274 The olfactory system of *D. suzukii* is responsive to various naturally-occurring volatiles in a dose-
275 response manner.

276 Some of these compounds affect the choice of the oviposition substrate by *D. suzukii* females.

277

278 **Legends of figures**

279 **Fig 1.** Sample EAG recordings from antennal preparations following stimulation with eugenol,
280 menthol, vanillin, *cis*-jasmone, eugenol + vanillin, eugenol + menthol, eugenol + *cis*-jasmone,
281 vanillin + menthol, vanillin + *cis*-jasmone at 5 µg.

282

283 **Fig 2.** Dose-response relationship between EAG amplitudes and increasing concentrations of
284 eugenol, menthol, vanillin and *cis*-jasmone (0.0005÷5 µg). All values are mean ± s.e.m.; N=10
285 antennae (1 antenna/fly). Asterisks indicate significant differences between a concentration and the
286 next lower (* p < 0.05; ** p < 0.01; LSD Fisher's test subsequent to repeated-measures ANOVA).

287

288 **Fig 3.** Mean values ± s.e.m. of the number of eggs laid on different oviposition substrates counted
289 after 72 h from the beginning of the behavioral trials: standard diet alone (SD) or standard diet +
290 eugenol (eug), + menthol (men), + vanillin (van) or + *cis*-jasmone (jas). N = 10 replicates for each
291 compound. Different letters indicate significant differences (p < 0.05; LSD Fisher's test subsequent
292 to one-way ANOVA).

293

294 **Fig 4.** Dose-response relationship between EAG amplitudes and increasing concentrations of eugenol
295 + vanillin, eugenol + menthol, eugenol + *cis*-jasmone, vanillin + menthol, vanillin + *cis*-jasmone
296 (0.0005÷5 µg). All values are mean (± s.e.m.); N=10 antennae (1 antenna/fly). Asterisks indicate
297 significant differences between a concentration and the next lower (* p < 0.05; *** p < 0.001; LSD
298 Fisher's test subsequent to repeated-measures ANOVA).

299

300 **Fig 5.** Mean values ± s.e.m. of the number of eggs laid on different oviposition substrates counted
301 after 72 h from the beginning of the behavioral trials. Standard diet alone (SD) or standard diet + one
302 of the following binary mixtures: eugenol + vanillin (eug-van), eugenol + menthol (eug-men),

303 eugenol + *cis*-jasmone (eug-jas), vanillin + menthol (van-men), vanillin + *cis*-jasmone (van-jas). N =
304 10 replicates for each compound. Different letters indicate significant differences ($p < 0.05$; LSD
305 Fisher's test subsequent to one-way ANOVA).

306

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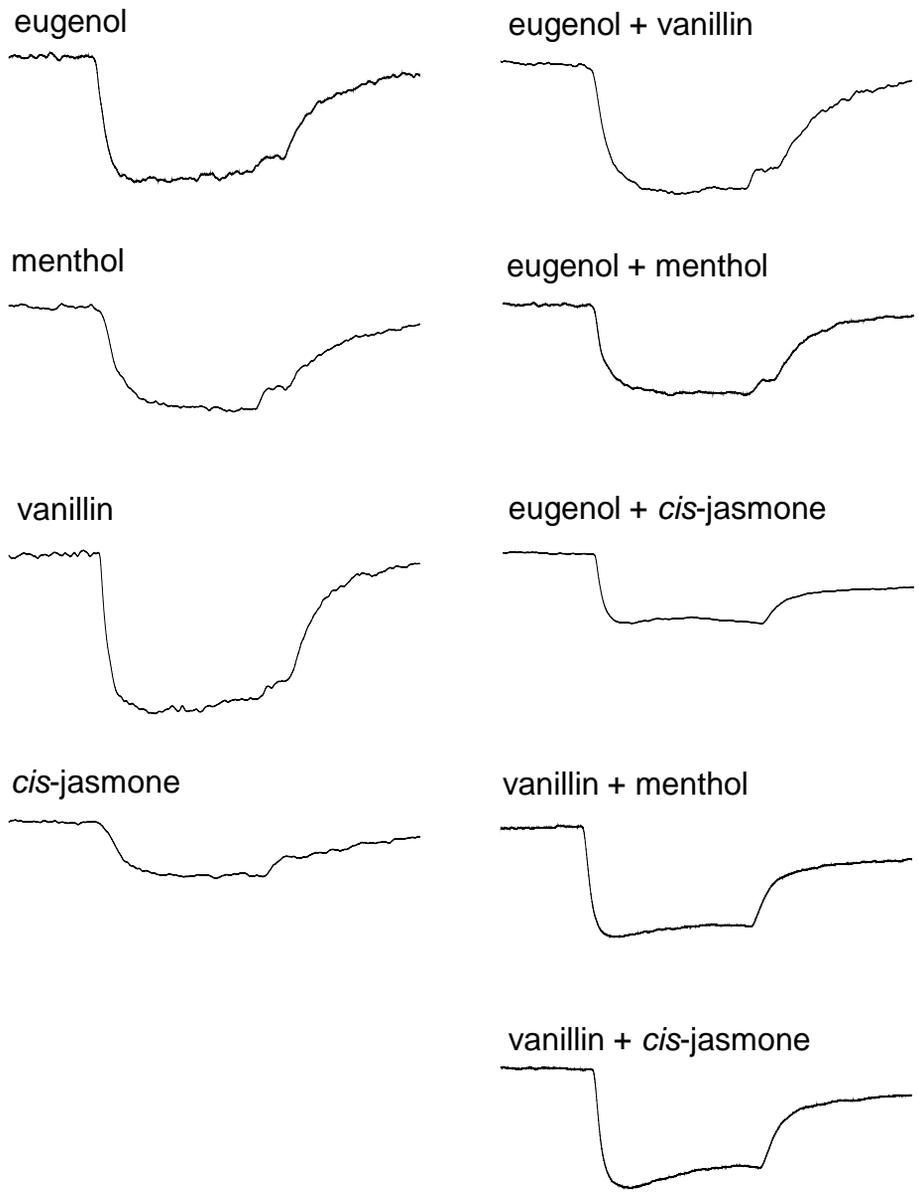


Figure 1



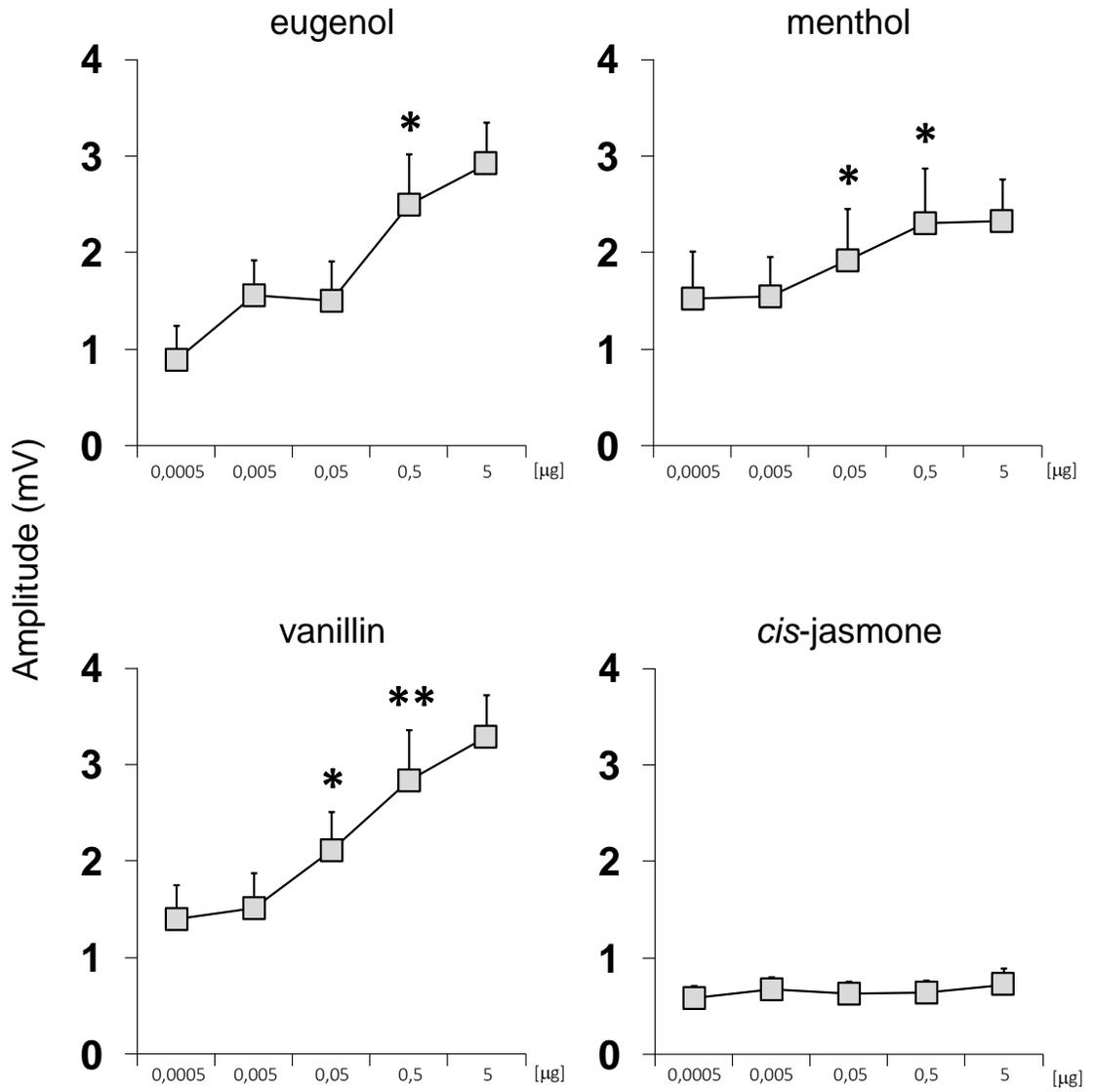


Figure 2

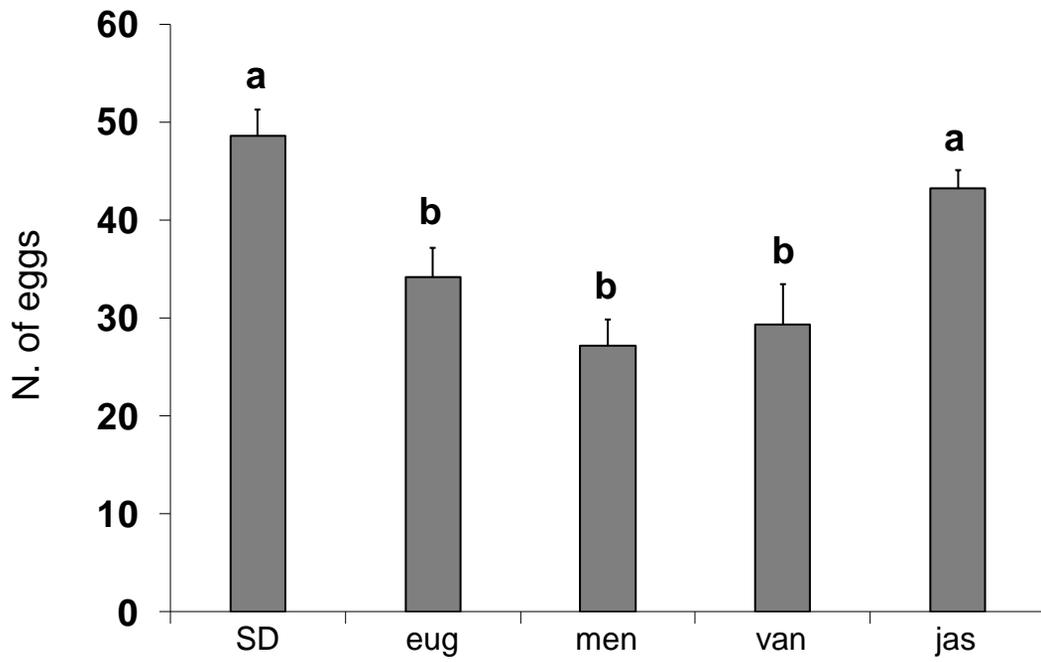


Figure 3

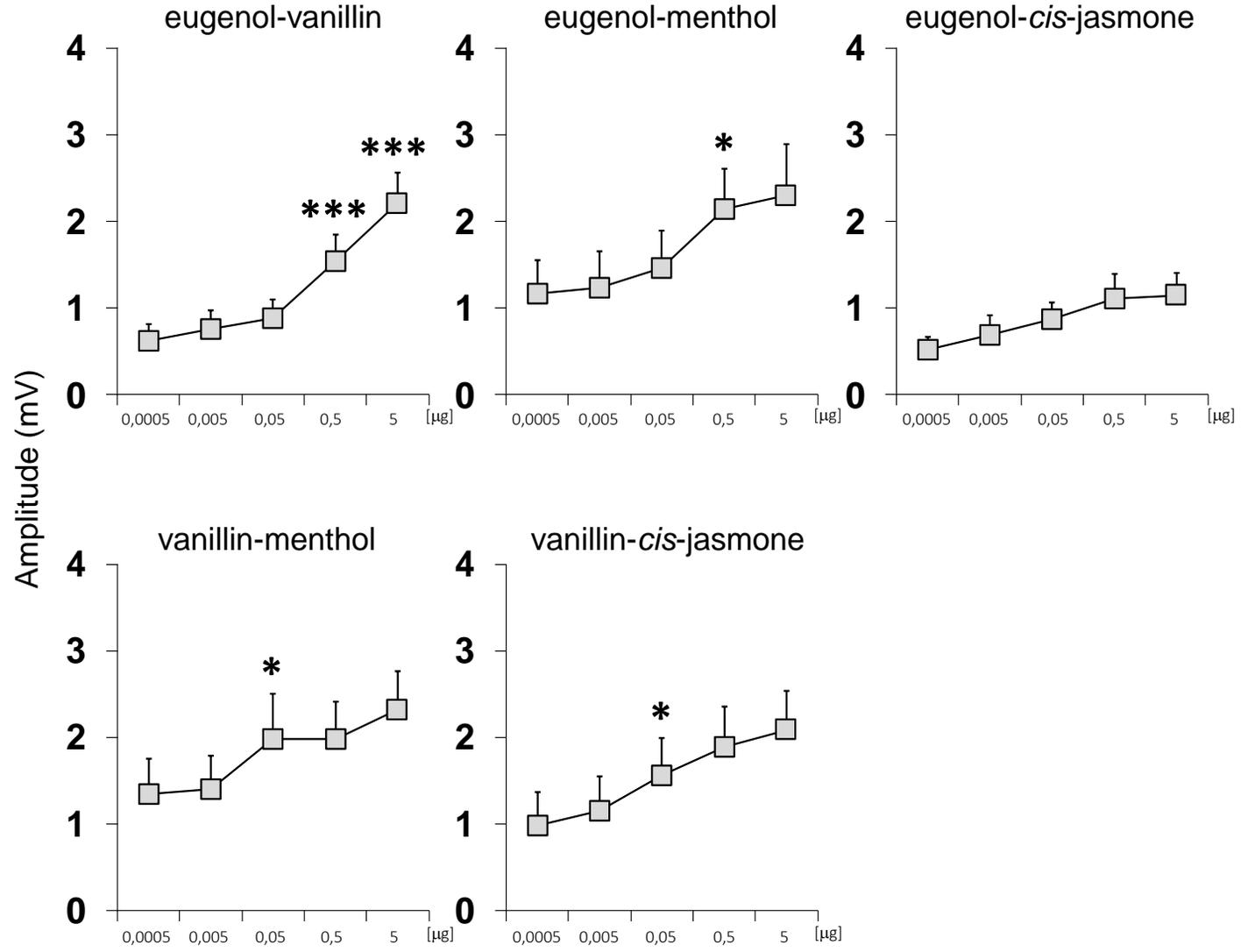


Figure 4

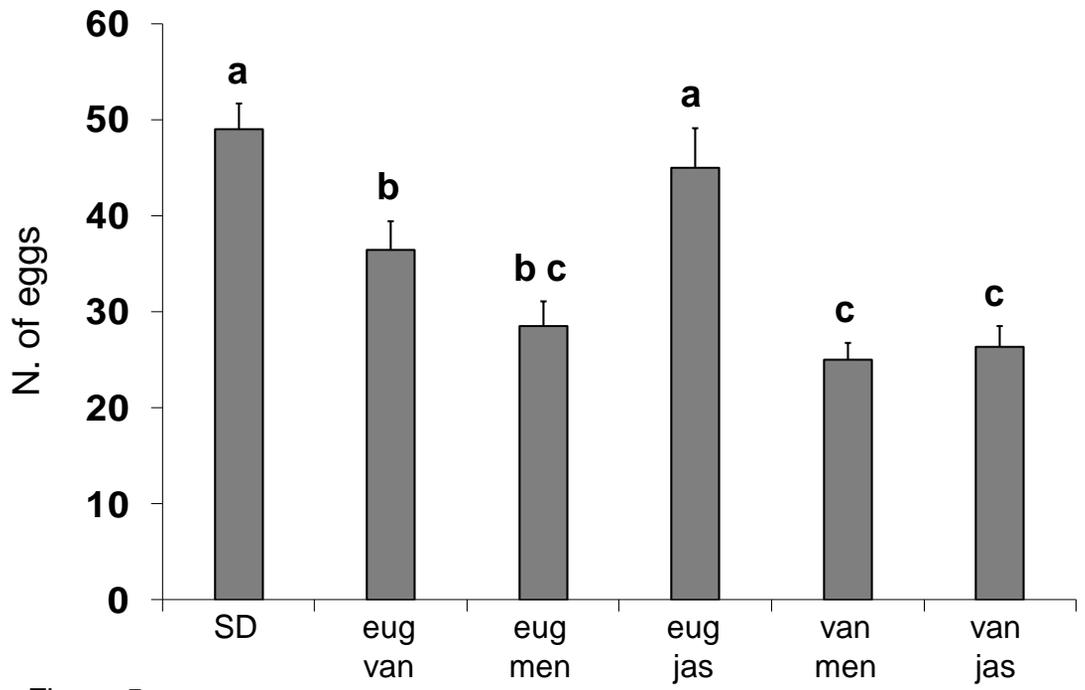


Figure 5