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1 **Electrophysiological and Behavioral Responses of Vine Weevil,**
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3 ***Otiorhynchus sulcatus* (Coleoptera: Curculionidae), Adults to**
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6 **Host Plant Odors**
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19 **Abstract**

20 Vine weevil, *Otiorhynchus sulcatus* F. (Coleoptera: Curculionidae), is an economically
21 important pest species in many soft-fruit and ornamental crops. Economic losses arise from
22 damage to the roots, caused by larvae, and to the leaves, caused by adults. As adults are
23 nocturnal and larvae feed below ground, infestations can initially be missed, and controls
24 may, as a result, be applied too late. In the absence of a vine weevil sex or aggregation
25 pheromone being identified, the development of an effective semiochemical lure for better
26 management of this pest is likely to focus on host-plant volatiles. Here, we investigate the
27 electrophysiological and behavioral responses of adult vine weevils to volatile organic
28 compounds (VOCs) originating from their preferred host plant *Euonymus fortunei*, and
29 synthetic VOCs associated with this host when presented individually or as blends.
30 Consistent electroantennographic responses were observed to a range of generalist VOCs.
31 Behavioral responses to VOCs, when presented individually, were found to be influenced by
32 the concentration of the compound to which the weevils were exposed. Vine weevil adults
33 showed directional movement towards a mixture of seven plant volatiles (methyl salicylate,
34 1-octen-3-ol, (*E*)-2-hexenol, (*Z*)-3-hexenol, 1-hexanol, (*E*)-2-pentenol, and linalool) even
35 though either no response or negative responses were recorded to each of these compounds
36 when presented individually. Similarly, vine weevils showed directional movement towards a
37 1:1 ratio mixture of (*Z*)-2-pentenol and methyl eugenol. Results presented here point to the
38 importance of blends of generalist compounds and concentrations of VOCs in the
39 optimization of a lure.

40
41 **Key Words** – Vine weevil, *Euonymus fortunei*, monitoring, electroantennography, air
42 entrainment, olfactometry.

44 **Introduction**

45 Vine weevil (also known as black vine weevil), *Otiorhynchus sulcatus* F. (Coleoptera:
46 Curculionidae), is one of the most economically important pest species of soft-fruit and
47 ornamental crops globally (van Tol et al. 2012). Vine weevils are female and reproduce via
48 thelytokous parthenogenesis, resulting in very little genetic diversity within the species
49 (Lundmark 2010). The flightless adults lay their eggs at night into cracks in the soil or
50 growing medium (Smith 1932). Upon hatching, the larvae complete four to nine molts before
51 pupating in earthen cells (Masaki and Ohto 1995). Typically, vine weevils are a univoltine
52 species, but as a winter diapause is not required and their development rate is temperature-
53 dependent (Son and Lewis 2005), overlapping generations may occur in protected
54 environments. Crop damage and economic losses are the result of feeding on plant roots,
55 corms and rhizomes by larvae and on leaves by adults (Moorhouse et al. 1992).

56 The conventional method of controlling vine weevil has been with synthetic chemical
57 insecticides incorporated into plant growing media for larval control and foliar applications at
58 dusk to target the adults (van Tol et al. 2012). Despite the widespread use of these
59 insecticides, vine weevil has remained an important pest. Furthermore, repeated applications
60 of insecticides in fruit crops can have negative impacts on beneficial arthropods (Solomon et
61 al. 2001), resulting in outbreaks of secondary pests (van Tol et al. 2012). Over recent years
62 there is has been a move to use entomopathogenic nematodes and fungi to control vine
63 weevil larvae (e.g. Ansari et al. 2008; Georgis et al. 2006; Shah et al. 2006; Willmott et al.
64 2002), but management of adult weevils still typically relies on the use of broad-spectrum
65 insecticides (Moorhouse et al. 1992; van Tol et al. 2012).

66 A key component of an integrated pest management (IPM) program is to base the use
67 of control methods on careful monitoring of pest numbers in relation to action thresholds (e.g.
68 Kogan 1998). For vine weevil, this is made difficult by the nocturnal activity of the adults

69 and the fact that the larvae feed below ground. Consequently, growers often do not observe
70 vine weevils within crops through direct monitoring before significant damage has already
71 occurred (van Tol et al. 2012). During the day adult weevils can be found in leaf litter, under
72 pots or other suitable refuges. This behavior has been exploited using grooved boards placed
73 on the ground (Gordon et al. 2003; Li et al. 1995), corrugated card wrapped around stems of
74 larger bushes (Phillips 1989), and plastic crawling insect ‘traps’ (Pope et al. 2018) that
75 provide adult weevils with artificial refuges and so help to focus monitoring efforts. Indirect
76 monitoring of adult weevils is based on the presence of damage in the form of characteristic
77 ‘leaf-notching’ from adult feeding to determine whether a crop is infested (Moorhouse et al.
78 1992), but this approach also leads to a delay in detecting the presence of adult weevils
79 within crops, allowing oviposition to occur before control measures can be applied (van Tol
80 et al. 2012).

81 Development of an effective semiochemical lure would improve vine weevil
82 monitoring reliability and sensitivity, which may contribute to development of novel control
83 methods such as autodissemination of entomopathogenic fungi (Pope et al. 2018).
84 Identification of semiochemicals suitable for use as a vine weevil lure has proved
85 challenging. As vine weevil reproduce asexually they do not produce a sex pheromone (van
86 Tol et al. 2012). However, adults display strong aggregation behavior (Kakizaki 2001; Pickett
87 et al. 1996) and attraction to conspecifics in a laboratory bioassay has been reported by van
88 Tol et al. (2004b) and Nakamuta et al. (2005). Although male-produced aggregation
89 pheromones have been identified from several weevil species (e.g. Blight and Wadhams
90 1987; Faustini et al. 1982; Gunawardena et al. 1998; Jayaraman et al. 1997; Rochat et al.
91 1991; Tumlinson et al. 1969), an aggregation pheromone for the vine weevil has not yet been
92 identified.

93 Attraction of vine weevil to kairomones from host plants has also been investigated.
94 Attraction to weevil frass has been reported by Pickett et al. (1996) and van Tol et al.
95 (2004b), and it is possible this may explain the attraction of vine weevils to conspecific
96 weevils. Although considered to be polyphagous, vine weevil adults show preferences for
97 plants such as yew, *Taxus baccata* (L.) and spindle tree, *Euonymus fortunei* (Turcz.) Hand.-
98 Maz. (van Tol and Visser 2002; van Tol et al. 2012), and two compounds identified in
99 volatiles from *E. fortunei*, (*Z*)-2-pentenol and methyl eugenol, have been found to increase
100 numbers of weevils in the area around traps (van Tol et al. 2012). These studies suggest that
101 vine weevil adults use olfactory cues to locate host plants and for aggregation. Here we report
102 on the electrophysiological and behavioral responses of adult vine weevils to volatile
103 chemical stimuli originating from host plants and synthetic chemical compounds, presented
104 both individually and as blends, with the aim of informing development of an effective lure
105 for improved monitoring and potentially control of this pest.

107 **Methods and Materials**

108 **Insect Cultures** Adult vine weevils, *Otiorhynchus sulcatus* F., were collected from commercial
109 strawberry crops grown in Shropshire and Staffordshire UK during the summer in 2012, 2017
110 and 2018. Recovered vine weevils were maintained on branches of yew, *Taxus baccata* (L.),
111 and moist paper towels inside insect cages (47.5 x 47.5 x 47.5 cm) (Bugdorm, MegaView,
112 Taiwan) placed in a controlled environment room (20 °C; 60% RH; L:D 16:8 h) (Fitotron,
113 Weiss Technik, Ebbw Vale, Wales).

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115 **Collection of Volatiles** For collection of volatiles from intact plants during 2012, potted *E.*
116 *fortunei* plants (Homebase, Buckinghamshire, UK) carrying approximately 50 g foliage were
117 enclosed individually in a polyethyleneterephthalate oven bag (37 x 25 cm x 12 µ thick; J

118 Sainsbury plc, London, UK) (Stewart-Jones and Poppy 2006). Charcoal-filtered air (600
119 ml/min) was pumped into the bag to maintain positive pressure while air was drawn out (500
120 ml/min) through a collection filter containing Porapak Q (200 mg, 50-80 mesh; Supelco,
121 Gillingham, Dorset, UK) held between two silanized glass wool plugs in a disposable glass
122 pipette (4 mm i.d.). Six collections were carried out over a period of 24 h in a controlled
123 environment room at 25 °C, 12:12 h L:D and 50% relative humidity.

124 During 2018, volatiles were collected from samples of cut branches of *E. fortunei* as
125 used in the bioassay experiments. Cut stems (approx. 60 g) from one of the authors' garden
126 in Kent, UK, were contained in round-bottomed flasks (3 liter) maintained in a controlled
127 environment room as above. Air was drawn into the flask through an activated charcoal filter
128 (20 cm x 2 cm; 8-10 mesh) and out through a filter containing Porapak Q as above.
129 Collections were made for 6 h and then a further 18 h from four samples.

130 Volatiles were eluted from the Porapak Q filters with dichloromethane (2 x 0.5 ml;
131 Pesticide Residue Grade; Fisher Scientific, Loughborough, UK), concentrated approximately
132 five times by evaporation under a gentle stream of purified nitrogen, and stored at -20 °C
133 before analysis. All collections were analyzed by GC/MS before and after concentration to
134 confirm that no impurities had been introduced and no significant changes in composition
135 had occurred during concentration. In other work, this concentration step caused a 15% loss
136 of limonene but no detectable loss of less volatile compounds such as decyl acetate.

137 For GC/EAG analyses, the collections estimated to contain most material were used.
138 For collections from intact plants in 2012, this was one made from 50 g plant material over
139 18 h, estimated to contain 4.4 ng/g plant material/h of the major component, (*E,E*)- α -
140 farnesene, by comparison with an external standard. For collections from cut plants in 2018,
141 the collection was made from 54 g plant material for 21 h, estimated to contain 5.9 ng/g/h
142 (*E,E*)- α -farnesene.

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2 144 **Analysis by Gas Chromatography coupled to Mass Spectrometry (GC/MS)** Analyses were
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5 145 carried out on a CP3500 GC coupled to a CP2200 Ion Trap Detector (Varian, now Agilent
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7 146 Technologies, Cheadle, UK). The GC was fitted with a fused silica capillary column (30 mm
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10 147 x 0.25 mm x 0.25 µm film thickness) coated with DBWax (Supelco). Manual injections (1
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12 148 µl) were made in splitless mode (220 °C), with oven temperature programmed from 40 °C for
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14 149 2 min then at 10 °C/min to 240 °C. Compounds were identified according to their mass
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17 150 spectrum, retention index relative to retention times of *n*-alkanes, and co-chromatography
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19 151 with authentic compounds.

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22 152 The enantiomeric composition of linalool present in collections of volatiles from *E.*
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24 153 *fortunei* was determined by GC with flame ionization detection (FID) using a fused silica
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26 154 capillary column (25 mm x 0.32 mm i.d. x 0.25 µm film thickness) coated with a cyclodextrin
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29 155 stationary phase (Chirasil-DEX CB; Varian). Injection was splitless (220 °C), detection was
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31 156 by FID (250 °C), and carrier gas was helium (2.4 ml/min). The oven temperature was
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34 157 programmed at 60 °C for 2 min then at 5 °C/min to 200 °C. Retention times of (*R*)-(-)- and
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36 158 (*S*)-(+)-linalool were 11.39 min and 11.48 min, respectively.

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41 160 **Analysis by Gas Chromatography coupled to Electroantennography (GC/EAG)** Analyses were
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44 161 carried out with a HP6890 GC (Agilent Technologies) fitted with flame ionization detector
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46 162 (FID) and fused silica capillary columns (30 m x 0.25 mm x 0.32 µm film thickness) coated
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49 163 with DBWax and DB1 (Supelco). Injections onto the DBWax column were in splitless mode
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51 164 (220 °C), with the oven temperature programmed from 50 °C for 2 min and then at 10
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53 165 °C/min. to 250 °C for analyses in 2012 and from 50 °C for 2 min and then at 20 °C/min. to
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56 166 250 °C for analyses in 2018. The effluents of the two columns were combined with a glass
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58 167 push-fit Y-tube connector (Agilent Technologies) connected to a second Y-tube connector
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168 with deactivated fused silica tubing (10 cm x 0.32 mm i.d.). One arm of this connector was
169 connected with fused silica tubing (50 cm x 0.32 mm i.d.) to the FID (250 °C) and the other
170 to an equal length of deactivated silica tubing passing through a heated transfer line (250°C;
171 Syntech, Hilversum, The Netherlands, now Kirchzarten, Germany) into a glass tube (4 mm
172 i.d.) through which air passed (500 ml/min) over the EAG preparation.

173 Electroantennogram recordings were made with a portable INR-02 device (Syntech)
174 connected as a second detector of the GC for A/D conversion. Glass electrodes containing
175 electrolyte solution (0.1 M potassium chloride with 1 % polyvinylpyrrolidone) were attached
176 to silver wires held in micromanipulators. Vine weevils were anaesthetized using carbon
177 dioxide before excising the head using a scalpel. The reference electrode was inserted into the
178 back of the head and the circuit was completed by bringing the recording electrode into
179 contact with the tip of one antenna. Both the FID and EAG signals were collected and
180 analyzed with EZChrom software (Elite v3.0; Agilent Technologies).

181 This system was used both for analyses of collections of volatiles from *E. fortunei*
182 plants (2 µl injected) and for measurement of EAG responses to synthetic compounds. For
183 the latter, the test compound (10 ng) was injected with 1-hexanol (10 ng) as internal standard
184 eliciting a strong EAG response from vine weevil antennae in our system.

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186 **Chemicals** Unless stated otherwise, chemicals were purchased from Sigma Aldrich
187 (Gillingham, Dorset, UK) and were > 95% pure by GC analysis. 1-Octen-3-ol was from
188 International Flavors and Fragrances (Haverhill, Suffolk, UK). (*E*)-4,8-Dimethyl-1,3,7-
189 nonatriene (DMNT) was synthesized by Wittig reaction of methylphosphonium bromide with
190 geranial prepared by oxidation of geraniol with pyridinium dichromate in dichloromethane.
191 (*E,E*)-4,8,12-Trimethyl-1,3,7,11-tridecatetraene (TMTT) was synthesized analogously from
192 (*E,E*)-farnesal prepared by oxidation of (*E,E*)-farnesol. Pure (*E,E*)- α -farnesene was obtained

193 by washing apples in hexane followed by chromatography on silica gel eluted with hexane.
194 Germacrene-D was a major component (40%) of ylang-ylang essential oil (Holland and
195 Barrett, Nuneaton, Warwks, UK) with (*E,E*)- α -farnesene (45%) and β -caryophyllene (15%).
196 Cadinols were isolated from Hinoki essential oil from *Chamaecyparis obtusa* (Shieh et al.
197 1981) by liquid chromatography on silica gel eluted with a gradient of diethyl ether in
198 hexane. The fraction eluted with 50% diethyl ether in hexane contained 16% tau-cadinol and
199 32% alpha-cadinol according to their mass spectra and GC retention times in GC/MS
200 analyses (El-Sayed 2019).

201
202 **Y-tube Olfactometer Bioassays** The behavioral responses of adult vine weevils to chemical
203 stimuli were tested using a Y-tube olfactometer (Sci-Glass Consultancy, Bere Alston, UK)
204 (Supplementary Information Fig. S1) based upon the design previously used by van Tol et al.
205 (2002). The stem length was 120 mm, arm length 190 mm and internal diameter 18 mm.
206 Airflow in each arm was 600 ml/min and odor sources were held in Drechsel bottles (500
207 ml). *Euonymus fortunei* plant material (20 g) was cut from the plant and the cut stem
208 wrapped in moist cotton wool and aluminum foil. Synthetic compounds were diluted in
209 paraffin oil (Fisher Scientific) to give the required amount in 10 μ l which was applied to
210 Whatman glass microfibre filters (934-AH grade; 47 mm diameter; Sigma Aldrich). Test
211 samples equilibrated in the Drechsel bottles for 1 h prior to tests.

212 All bioassays were undertaken in complete darkness in a controlled environment
213 room (20 °C; 60 % RH; 16:8 h L:D). Prior to their use in a bioassay, vine weevils were
214 starved for a minimum of 24 h unless stated otherwise. Groups of 40 vine weevils were
215 introduced into the olfactometer via a release chamber (100 mm diameter). Each pair of odor
216 sources was tested six times with fresh individuals for 20 min, and the numbers of weevils
217 reaching the end of each arm during this time were recorded. The positions of the odor

1 218 sources were alternated between replicates to eliminate directional bias. After each pair of
2 219 odor sources had been tested six times, all glassware was thoroughly cleaned by rinsing with
3
4 220 warm water followed by HPLC-grade acetone (Sigma Aldrich) before baking in a glassware
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7 221 oven at 120 °C for 15 min.
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9 222 Nine compounds were tested individually at source loadings of 0.1 mg, 1 mg and 5 mg to
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11 223 provide a wide range of doses that caused significant behavioral effects in previous work by
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13 224 Karley et al. (2012). These were (*Z*)-2-pentenol and methyl eugenol, compounds reported to
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15 225 be attractive to vine weevil by van Tol et al. (2012), and methyl salicylate, 1-hexanol, (*Z*)-3-
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17 226 hexenol, (*E*)-2-hexenol, (*E*)-2-pentenol, (\pm)-linalool, and 1-octen-3-ol, compounds found to
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19 227 elicit strong EAG responses from vine weevil in our own work. Two blends were tested at
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21 228 loadings of 1 mg total: a binary blend of equal amounts of (*Z*)-2-pentenol and methyl eugenol
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23 229 (van Tol et al. 2012) and a blend of equal amounts of the other seven electrophysiologically
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25 230 active compounds: methyl salicylate, 1-hexanol, (*Z*)-3-hexenol, (*E*)-2-hexenol, (*E*)-2-
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27 231 pentenol, linalool and 1-octen-3-ol.
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36 233 **Statistical Analyses** All statistical analyses were performed using R (Version 3.5-3) (R Core
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38 234 Team, 2019). Y-Tube olfactometer bioassay data were analyzed using an exact binomial test
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40 235 against the null hypothesis that the number of vine weevils reaching the end of either
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42 236 olfactometer arm had a 50:50 distribution. Prior to performing statistical analyses, the
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44 237 replicated results from each of the odor pairs tested were pooled with non-responding
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46 238 individuals being excluded from statistical analyses.
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52 53 240 **Results**

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55 241 **Y-Tube Bioassay with Host Plant Material** Vine weevils starved for at least 24 h exhibited a
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57 242 preference for the Y-tube olfactometer arm containing air blown over *E. fortunei* plant
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243 material with 83 % of responding individuals choosing this arm over the clean-air control arm
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2 244 ($P < 0.001$).

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7 246 **GC/EAG and GC/MS Analyses of Volatiles from *Euonymus fortunei*** In analyses of volatiles
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10 247 from *E. fortunei* by GC coupled to EAG recording from a vine weevil antenna, 22
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12 248 reproducible responses were observed in analyses of volatiles from intact plants (Fig. 1) and
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14 249 20 responses in analyses of volatiles from cut branches (Fig. 2). The identities of the
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17 250 compounds responsible are summarized in Table 1 along with their relative proportions
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19 251 present in the collections used for GC/EAG analyses. There were responses to 13 compounds
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22 252 present in both sets of samples: aldehydes hexanal, (*Z*)-3-hexenal, heptanal, (*E*)-2-hexenal,
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24 253 octanal, nonanal, and decanal; alcohols 1-hexanol, (*Z*)-3-hexenol, (*R*)-(-) linalool; aromatic
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26
27 254 1,2-dimethoxybenzene (veratrole), sesquiterpene (*E,E*)- α -farnesene; and the oxylipin *cis*-
28
29 255 jasmone. (*E,E*)- α -Farnesene was the most abundant compound in both sets of samples,
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31 256 although the EAG response to this was always relatively weak (Figs. 2 and 3).

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34 257 In analyses of volatiles from intact plants, responses were also observed to ketones 6-
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36 258 methyl-5-hepten-2-one and geranyl acetone; alcohols 1-octen-3-ol and 2-ethyl-1-hexanol;
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39 259 acetic acid; sesquiterpene α -cadinol; and three unidentified compounds present at very low
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41 260 levels. Acetic acid and 2-ethyl-1-hexanol were also present in collections of volatiles from
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44 261 cut plants, although no EAG response was observed, while the other compounds were not
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46 262 detected in volatiles from cut plants.

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49 263 In analyses of volatiles from cut plants, responses were also observed to alcohols (*E*)-
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51 264 2-hexenol, benzyl alcohol and 2-phenylethanol; acids hexanoic acid and 2-ethylhexanoic
52
53
54 265 acid; methyl salicylate; and indole. Of these compounds, only methyl salicylate was detected
55
56 266 in samples of volatiles from intact plants.

267 EAG responses were not observed to other relatively ubiquitous plant volatiles
268 present in one or both sets of collections, such as (*E*)-ocimene, (*Z*)-3-hexenyl acetate,
269 copaene, α - and β -caryophyllene, and (*E*)-nerolidol, or to the stress-related volatiles DMNT
270 and TMTT.

271 (*E*)-2-Pentenol, (*Z*)-2-pentenol and methyl eugenol, compounds reported from *E.*
272 *fortunei* by van Tol et al. (2012), could not be detected (<0.1%) in any of the collections from
273 intact plants (6 samples) or cut plants (8 samples) by comparison of GC retention times and
274 mass spectra with those of authentic standards (Table 2).

275
276 **GC-EAG Analysis of Synthetic Chemicals** In experiments during 2012, consistent EAG
277 responses were observed to (*E*)-2-pentenol, (*Z*)-2-pentenol, (*E*)-2-hexenol, (*Z*)-3-hexenol, 1-
278 hexanol, 1-octen-3-ol, (\pm)-linalool, and methyl salicylate (Table 2 and Supplementary
279 Material Figs. S2, S3), while (*E,E*)- α -farnesene and methyl eugenol elicited responses
280 approximately 50 % of the time they were tested (Table 2). No response was observed to
281 DMNT or β -caryophyllene, consistent with the absence of responses to these compounds in
282 volatiles from *E. fortunei* (Table 2 and Supplementary Material Figs. S2, S3).

283 Subsequent experiments during 2018 confirmed the consistent response to 1-hexanol
284 and showed a similarly consistent response to *cis*-jasmone (Table 2 and Supplementary
285 Material Fig. S4). Analysis of the cadinol fraction from Hinoki essential oil showed a
286 consistent response to tau-cadinol but not to α -cadinol (Table 2 and Supplementary Material
287 Fig. S5), in contrast to what was observed previously in GC-EAG analyses of volatiles from
288 intact *E. fortunei* plants. No EAG response was observed to indole (Table 2) even though a
289 response was sometimes observed to this compound in volatiles from cut *E. fortunei* plants
290 (Fig. 1 and Table 1).

291

292 **Y-Tube Bioassays** When tested at 0.1 mg at source, significantly more vine weevils moved to
293 the control arm relative to the treatment arm for five of the nine chemical compounds tested :
294 1-hexanol ($P < 0.001$), (*Z*)-3-hexenol ($P = 0.02$), (*E*)-2-hexenol ($P < 0.001$), 1-octen-3-ol (P
295 < 0.001), and (*Z*)-2-pentenol ($P = 0.02$) (Fig. 3). For the other four compounds, methyl
296 salicylate, (*E*)-2-pentenol, linalool and methyl eugenol, there was no significant difference
297 between the numbers in control and treatment arms ($P > 0.05$) (Fig. 3).

298 When tested at 1 mg at source, significantly more weevils were recorded in the
299 control arm relative to the number in the treatment arm for three chemicals: 1-octen-3-ol ($P <$
300 0.001), linalool ($P < 0.001$), and 1-hexanol ($P = 0.02$) (Fig. 4). Significantly more weevils
301 were recorded in the treatment arm for two of the compounds: (*Z*)-2-pentenol ($P = 0.03$) and
302 methyl eugenol ($P = 0.001$), while there was no significant difference between the numbers in
303 control and treatment arms for (*E*)-2-pentenol, (*Z*)-3-hexenol, (*E*)-2-hexenol, and methyl
304 salicylate ($P > 0.05$) (Fig. 4).

305 When tested at 5 mg at source, seven of the nine chemical compounds tested evoked a
306 behavioral response in the vine weevils. Significantly more weevils were recorded in the
307 control arm relative to the treatment arm for five compounds: methyl salicylate ($P < 0.001$),
308 (*E*)-2-hexenol ($P < 0.001$), linalool ($P < 0.001$), 1-octen-3-ol ($P < 0.01$), and methyl eugenol
309 ($P < 0.001$) (Fig. 5). Significantly more weevils were observed in the treatment arm relative
310 to the number in the control arm for two compounds: 1-hexanol ($P = 0.01$) and (*Z*)-2-
311 pentenol ($P < 0.001$) (Fig. 5). There was no significant difference between the number of
312 weevils in the control and treatment arms of (*Z*)-3-hexenol and (*E*)-2-pentenol ($P > 0.05$).

313 Two blends of chemicals were tested at 1 mg total at source (Fig. 6). With a binary
314 blend of equal amounts of (*Z*)-2-pentenol and methyl eugenol, a significantly greater number
315 of the responding vine weevils demonstrated a preference for the olfactometer arm containing
316 the treatment over the control arm ($P = 0.01$). Similarly, with a seven-component blend

1 317 containing equal amounts of methyl salicylate, 1-hexanol, (Z)-3-hexenol, (E)-2-hexenol, (E)-
2 318 2-pentenol, linalool and 1-octen-3-ol, significantly more of the weevils responding were
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4 319 recorded in the treatment arm ($P < 0.001$) (Fig. 6).
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8 9 10 321 **Discussion**

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12 322 Vine weevil larvae and adults are polyphagous (Masaki et al. 1984; Smith 1932), although
13
14 323 adults show a strong preference for certain plant species in the families Rosaceae, Ericaceae
15
16 324 and Taxaceae (van Tol et al., 2004a). Olfactory cues play an important role in these
17
18 325 behavioral responses, with vine weevil adults preferring the odor of *E. fortunei* over blank
19
20 326 controls and other potential hosts such as *Fragaria* or *Rhododendron* (van Tol et al., 2004a).
21
22 327 The results presented here confirm that adult vine weevils move toward air streams
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24 328 containing VOCs originating from *E. fortunei*. Furthermore, it is apparent that it is the blend
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26 329 of generalist compounds and the concentrations of VOCs that are important in determining
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28 330 whether a positive behavioral response is elicited.
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34 331 In GC-EAG analyses of volatiles from intact and cut *E. fortunei* plants, consistent
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36 332 EAG responses from vine weevil antennae were observed to 13 common plant volatiles
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38 333 present in both sets of collections. These were seven aliphatic aldehydes, three alcohols, one
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40 334 sesquiterpenes, one aromatic and the oxylipin, *cis*-jasmone. In GC-EAG analyses of volatiles
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42 335 from intact plants, 9 additional EAG responses were observed. These additional EAG
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44 336 responses included those to 6-methyl-5-heptenone and geranyl acetone, which are,
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46 337 paradoxically, characteristically emitted by damaged plants (War et al. 2011). In analyses of
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48 338 volatiles from cut plants, as used in the laboratory bioassays, additional EAG responses were
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50 339 observed to three alcohols, two acids, methyl salicylate, and indole. Several of the volatiles
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52 340 eliciting EAG responses in this study, such as (Z)-3-hexenol, (E)-2-hexenol, 1-octen-3-ol, and
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54 341 (*E,E*)- α -farnesene, have previously been detected from GC-EAG analyses of volatiles from
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342 crushed and variously damaged *E. fortunei* plants (van Tol et al. 2012). Other relatively
343 ubiquitous plant volatiles detected in one or both sets of collections, such as (*E*)-ocimene, β -
344 caryophyllene, germacrene D and the stress-related volatiles DMNT and TMTT, did not elicit
345 EAG responses despite being previously reported to do so (van Tol et al. 2012). van Tol et al.
346 (2012) also reported EAG responses to (*Z*)-2-pentenol, methyl benzoate, estragole, 4-*tert*-
347 butylcyclohexanol, 2-phenoxyethanol and methyl eugenol presented in *E. fortunei* air
348 entrainments. These volatiles were never detected in our analyses even though they were
349 specifically looked for by comparison with mass spectra and GC retention times of the
350 authentic compounds. This may in part be due to the poor resolution of the GC peaks in the
351 analyses of van Tol et al. (2012), and questionable structural assignments in this earlier study
352 – e.g. (*E*)-ocimene does not elute after DMNT on the column used.

353 Results of GC-EAG analyses of volatiles from *E. fortunei* plants were supported by
354 similar analyses of a selection of synthetic compounds using vine weevil antennae. In
355 particular, strong and consistent EAG responses were observed to alcohols 1-hexanol, (*E*)-2-
356 hexenol, (*Z*)-3-hexenol, 1-octen-3-ol and linalool. These results are similar to those reported
357 by van Tol and Visser (2002). Strong and consistent EAG responses were also observed in
358 this study to *cis*-jasmone, which has not previously been reported. DMNT and β -
359 caryophyllene did not elicit EAG responses, as observed in GC-EAG analyses of the plant
360 volatiles, but (*Z*)-2-pentenol and methyl eugenol, although not detected in the GC-EAG
361 analysis of the plant volatiles, did elicit EAG responses.

362 Despite their absence in our study samples, both (*Z*)-2-pentenol and methyl eugenol
363 were tested in Y-tube olfactometer bioassays as van Tol et al. (2012) reported that release of
364 (*Z*)-2-pentenol on its own or as 1:1 ratio mixture with methyl eugenol from boll weevil traps
365 in strawberry crops increased numbers of vine weevil adults in the area surrounding the traps.
366 This response could result from orientated movement of vine weevil adults from distance

1 367 towards the release point of these VOCs or be due to the VOCs arresting the weevils after a
2 368 chance arrival in the area close to the traps (Kennedy 1977). The olfactometry results
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4 369 presented here confirm that vine weevil adults do indeed show orientated movement towards
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6 370 both compounds when 1 mg was presented individually. For (*Z*)-2-pentenol, when 5 mg was
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8 371 presented a similar response was recorded but at 0.1 mg weevils were found to move away
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10 372 from this odor source. In addition, when 1 mg of a 1:1 ratio mixture of (*Z*)-2-pentenol and
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12 373 methyl eugenol was presented, vine weevils were again found to orientate towards this odor.
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17 374 The apparent sensitivity to concentration seen for (*Z*)-2-pentenol was also seen for the
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19 375 other individual compounds tested, which elicited either no or negative behavioral responses
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21 376 depending on the concentration presented. The only exceptions were (*E*)-2-pentenol, which
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23 377 did not elicit a behavioral response at any of the loadings tested, and 1-hexanol, to which
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25 378 weevils responded positively only at the 5 mg loading. Few studies have investigated the
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27 379 importance of plant VOC concentration in determining the behavioral responses of
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29 380 phytophagous insects during host plant location or selection (Dicke and Baldwin 2010). For
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31 381 example, a meta-analysis of using plant VOCs to manipulate phytophagous insect pest
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33 382 behavior does not list concentration as a factor influencing insect behavior in any of the 34
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35 383 published studies analyzed (Szendrei and Rodriguez-Saona 2009). Despite this, it has been
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37 384 argued that VOC concentration is important (e.g. Bruce et al. 2005). Often in the case of
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39 385 individual compounds where an insect encounters higher concentrations than would be found
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41 386 in nature a misleading response may be recorded, as demonstrated in electrophysiological
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43 387 experiments (e.g. Anderson et al. 1995; Blight et al. 1995; Hansson et al. 1999; Larsson et al.
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45 388 2005). Nonetheless, in this study negative behavioral responses were recorded to many plant
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47 389 VOCs considered common throughout the plant kingdom at either low or high concentrations
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49 390 (War et al. 2011). The results presented in this study suggest that the concentration of
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51 391 individual VOCs can influence the behavioral response of vine weevil adults under controlled
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5 392 laboratory conditions. Webster et al. (2010) reported similar results for the black bean aphids
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7 393 (*Aphis fabae*) responding to different concentrations of individual VOCs identified from their
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9 394 host plants.

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12 395 With green leaf volatiles (GLVs) dominating the list of VOCs detected by vine weevil
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14 396 adults, the EAG results presented here appear to support the hypothesis that this group of
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16 397 plant volatiles play a role in host-plant location by phytophagous arthropods (Bruce et al.
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18 398 2005). With either no or negative behavioral responses to compounds when presented
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20 399 individually, vine weevil adults responded positively to a mixture of equal amounts of seven
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22 400 plant volatiles (methyl salicylate, 1-octen-3-ol, (*E*)-2-hexenol, (*Z*)-3-hexenol, 1-hexanol, (*E*)-
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24 401 2-pentenol, and linalool) when 1 mg of the blend was presented. As such, these olfactometry
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26 402 results with individual and simple blends of VOCs demonstrate that components of a host-
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28 403 plant blend may not be recognized when perceived outside the context of that blend as
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30 404 suggested by Bruce and Pickett (2011). This is again comparable to the study by Webster et
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32 405 al. (2010) who found black bean aphids respond positively to a nine-component blend of host
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34 406 plant VOCs that evoked a negative behavioral response when presented individually.

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36 407 In the absence of a vine weevil sex or aggregation pheromone, the development of an
37
38 408 effective semiochemical lure for vine weevil adults is likely to focus on host-plant volatiles.
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40 409 As the VOC's known to be detected by vine weevil adults are found in many plant families,
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42 410 rather than those that are taxonomically characteristic, it seems likely that optimization of a
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44 411 lure must focus on the blend and the concentration of each component in it. The fact that both
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46 412 blends tested here elicited positive behavioral responses suggests that there is redundancy in
47
48 413 the composition of the blends used by vine weevil adults to recognize host plants with the
49
50 414 potential to substitute some compounds with others (Bruce and Pickett 2011). Further work is
51
52 415 required to identify a blend of VOCs that is effective enough to be deployed within crops as a
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54 416 lure to attract adult vine weevils into traps for monitoring or control purposes.
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548 **Table 1** Compounds identified in GC-EAG analyses of volatiles from intact and cut plants of
 549 *Euonymus fortunei* and relative amounts (EAG responses numbered as in Figs. 1 and 2; RI
 550 is retention index relative to retention times of *n*-alkanes on polar GC column; DMNT is 4,8-
 551 dimethyl-1,3,7-nonatriene; TMTT is 4,8,12-trimethyl-1,3,7,11-tridecatetraene)

Intact plants				Cut plants				Compound
EAG response	RT (min)	RI	Area (%)	EAG response	RT (min)	RI	Area (%)	
1	4.79	1074	1.2	1	3.97	1086	0.48	hexanal
2	5.55	1136	2.0	2	4.47	1147	0.54	(<i>Z</i>)-3-hexenal
3	6.15	1185	1.4	3	4.85	1193	0.31	heptanal
4	6.60	1220	0.7	4	5.11	1226	1.58	(<i>E</i>)-2-hexenal
	7.06	1255	2.2					(<i>E</i>)-ocimene
5	7.57	1293	1.0	5	5.67	1301	0.32	octanal
	7.82	1312	3.7		5.79	1317	3.98	DMNT
	7.97	1323	1.3					(<i>Z</i>)-3-hexenyl acetate
6	8.23	1343	2.1					6-methyl-5-hepten-2-one
7	8.41	1356	0.0	6	6.10	1358	1.07	1-hexanol
8	8.84	1389	4.7	7	6.33	1388	3.63	(<i>Z</i>)-3-hexenol
9	8.98	1399	4.2	8	6.43	1402	1.98	nonanal
				9	6.48	1409	0.54	(<i>E</i>)-2-hexenol
10	9.50	1441	0.0		6.70	1443	0.35	acetic acid?
11	9.66	1454	0.0					1-octen-3-ol
12	10.18	1496	1.6		7.03	1495	1.07	2-ethyl-1-hexanol
13	10.31	1507	4.4	10	7.13	1510	2.43	decanal
					7.17	1516	1.99	copaene
14	10.86	1551	0.8	11	7.39	1550	2.32	(<i>R</i>)-(-)-linalool
	11.46	1600	1.7					hexadecane
	11.58	1610	1.3		7.84	1623	1.79	β -caryophyllene

1						8.28	1700	1.25	α -caryophyllene
2		12.80	1720	2.8					ethyl-benzaldehyde
3									
4						8.40	1721		germacrene D
5									
6									1,2-dimethoxy-
7	15	12.88	1727	0.8	12	8.45	1730	1.28	benzene
8									
9									
10		13.00	1738	0.5		8.50	1739	1.14	α -muurolene
11									
12		13.14	1750	2.3		8.55	1747	1.14	farnesene isomer
13									
14	16	13.19	1754	15.6	13	8.61	1758	25.99	(<i>E,E</i>)- α -farnesene
15									
16		13.54	1786	2.7	14	8.85	1800	0.65	methyl salicylate
17									
18		13.82	1811	0.6					TMTT
19									
20					15	9.12	1852	0.45	hexanoic acid
21									
22	17	14.34	1863	2.1					(<i>E</i>)-geranyl acetone
23									
24					16	9.31	1889	0.92	benzyl alcohol
25									
26					17	9.51	1928	0.70	2-phenylethanol
27									
28						9.61	1948	1.94	benzyl cyanide
29									
30						9.70	1965	0.20	<i>trans</i> -jasmone
31									
32	18	15.28	1956	0.0	18	9.73	1971	1.99	<i>cis</i> -jasmone
33									
34					19	9.89	2002	0.20	2-ethylhexanoic acid
35									
36		16.14	2044	0.8		10.09	2044	6.06	(<i>E</i>)-nerolidol
37									
38		17.44	2183	1.8					tau-cadinol
39									
40	19	17.99	2245	1.4					α -cadinol
41									
42	20	18.92	2353	0.0					unknown
43									
44					20	12.01	2468	3.31	indole
45									
46									
47	21/22	20.94	2588	0.0					unknown

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554 **Table 2** Frequency of EAG responses of vine weevil to 10 ng of synthetic compounds
 555 delivered by GC-EAG (number of times a response was observed out of the total number of
 556 runs); RI is retention index relative to retention times of *n*-alkanes on polar GC column;
 557 DMNT is 4,8-dimethyl-1,3,7-nonatriene; TMTT is 4,8,12-trimethyl-1,3,7,11-tridecatetraene)

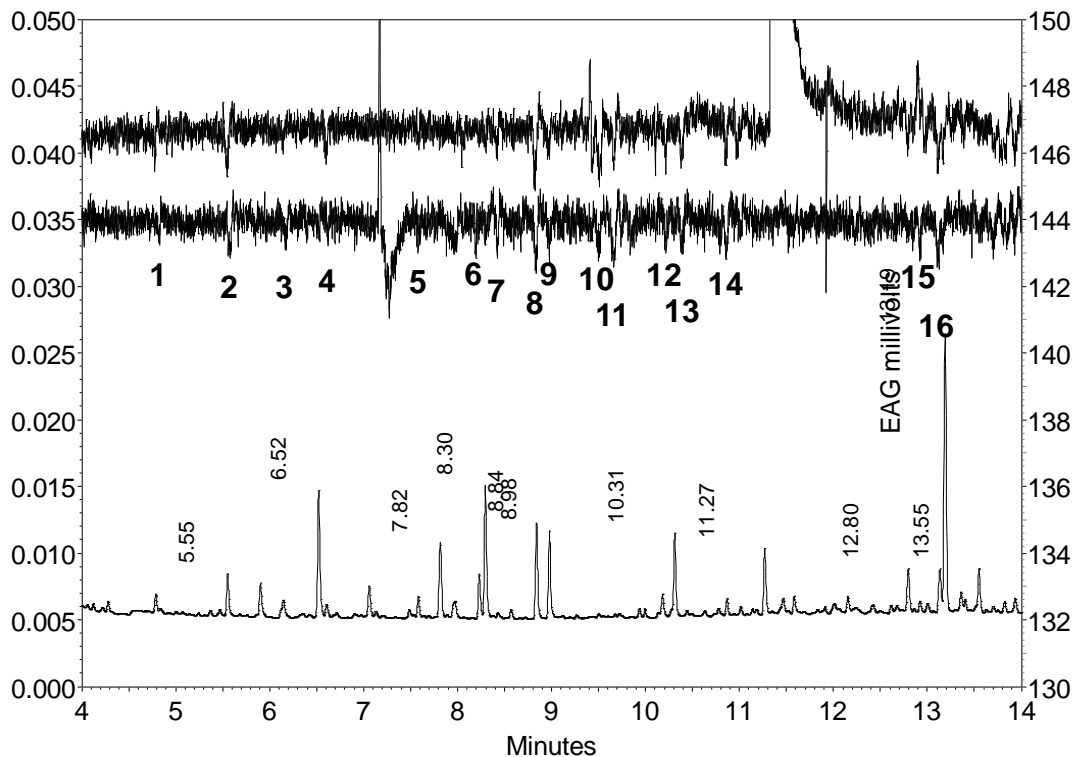
Compound	RT (mins)	RI	Frequency of EAG response
2012 Experiments			
DMNT	6.82	1310	0/2
(<i>E</i>)-2-pentenol	6.88	1315	3/4
(<i>Z</i>)-2-pentenol	6.98	1323	4/4
1-hexanol	7.41	1356	16/16
(<i>Z</i>)-3-hexenol	7.85	1389	4/4
(<i>E</i>)-2-hexenol	8.11	1410	18/18
1-octen-3-ol	8.67	1455	2/2
linalool ^a	9.87	1552	4/4
β-caryophyllene	10.59	1612	0/16
germacrene-D	11.88	1727	0/16
(<i>E,E</i>)-α-farnesene	12.22	1757	5/16
methyl salicylate	12.56	1788	4/4
methyl eugenol	14.91	2018	1/2
2018 Experiments			
1-hexanol	6.09	1358	15/15
<i>cis</i> -jasmone	9.72	1973	14/14
tau-cadinol	10.79	2196	5/5
α-cadinol	11.08	2261	0/5
indole	12.00	2468	0/2

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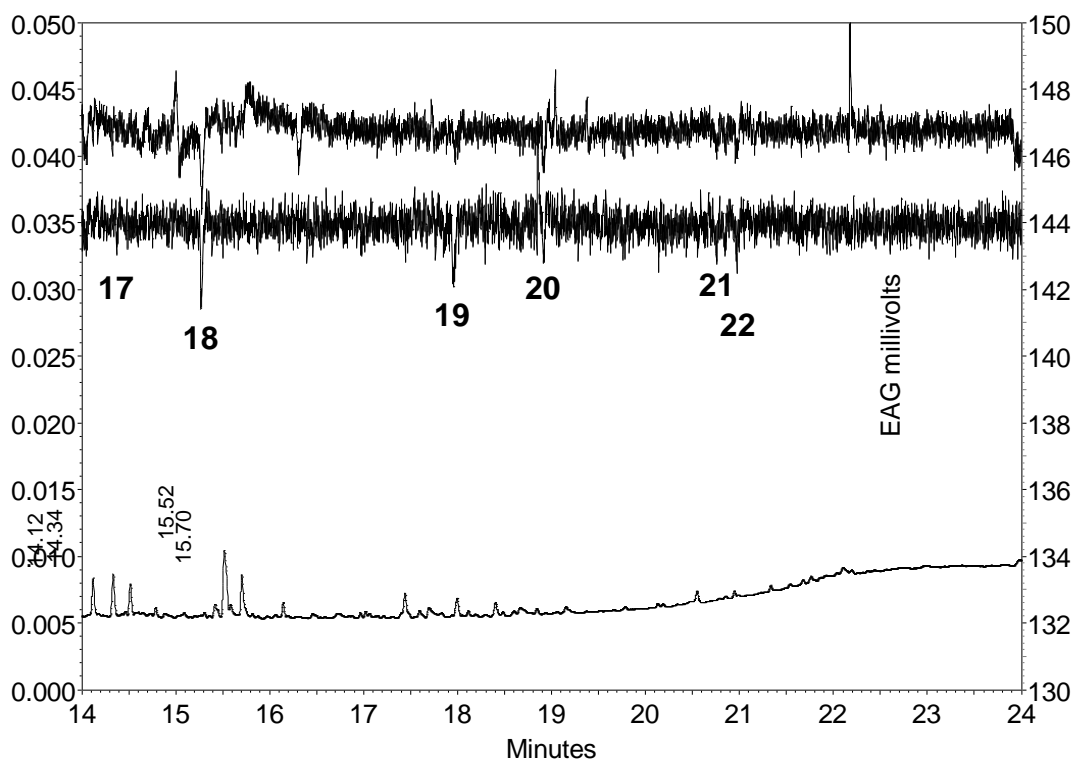
559 ^a Present as a minor component in ylang ylang essential oil

560

561 **Figures**



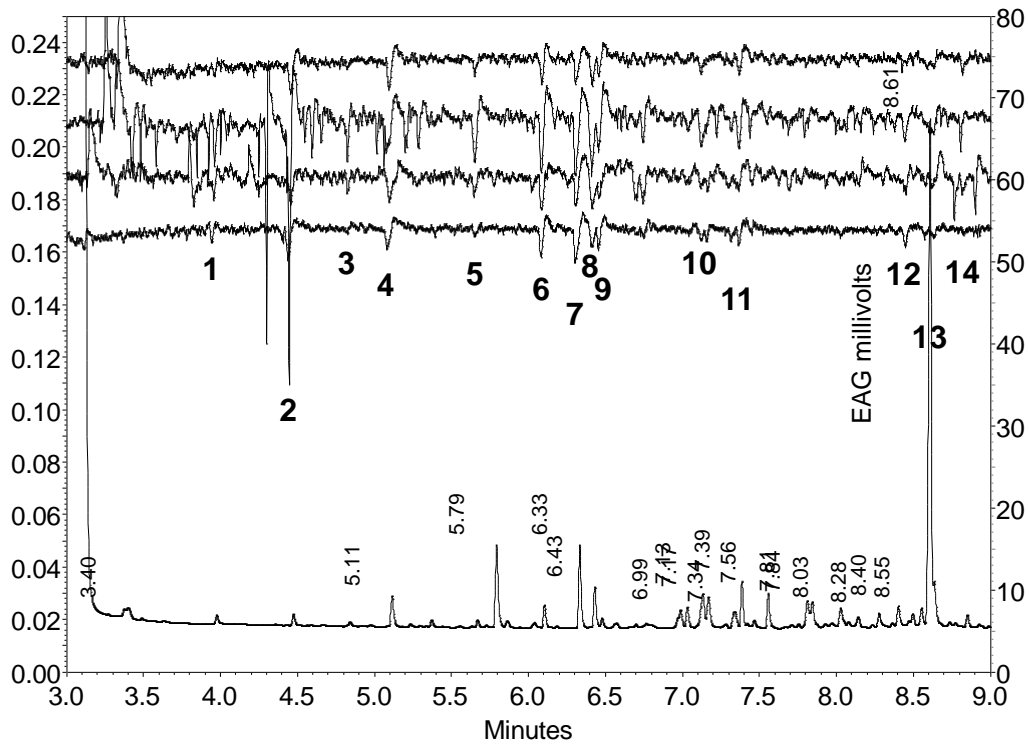
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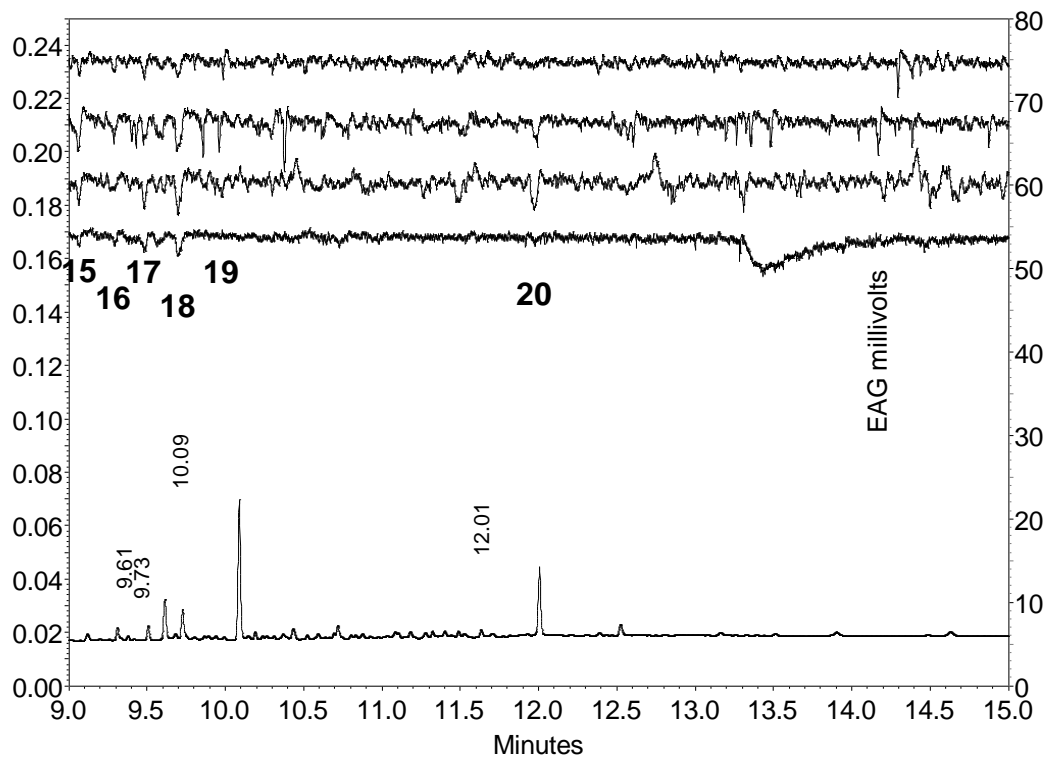
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564 **Fig. 1** GC-EAG analysis of volatiles from intact *Euonymus fortunei* plants on polar GC
 565 column (responses numbered as in Table 1

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Fig. 2 GC-EAG analysis of volatiles from cut *Euonymus fortunei* plants on polar GC column (responses numbered as in Table 1)

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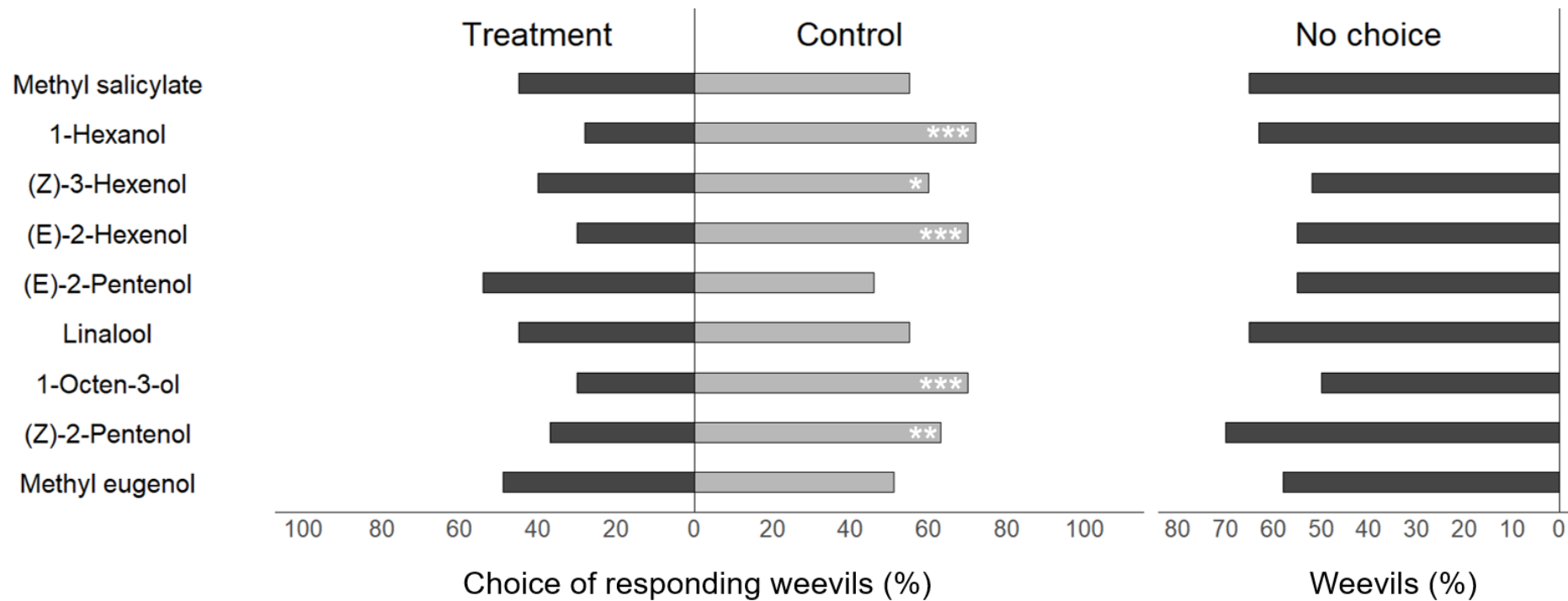
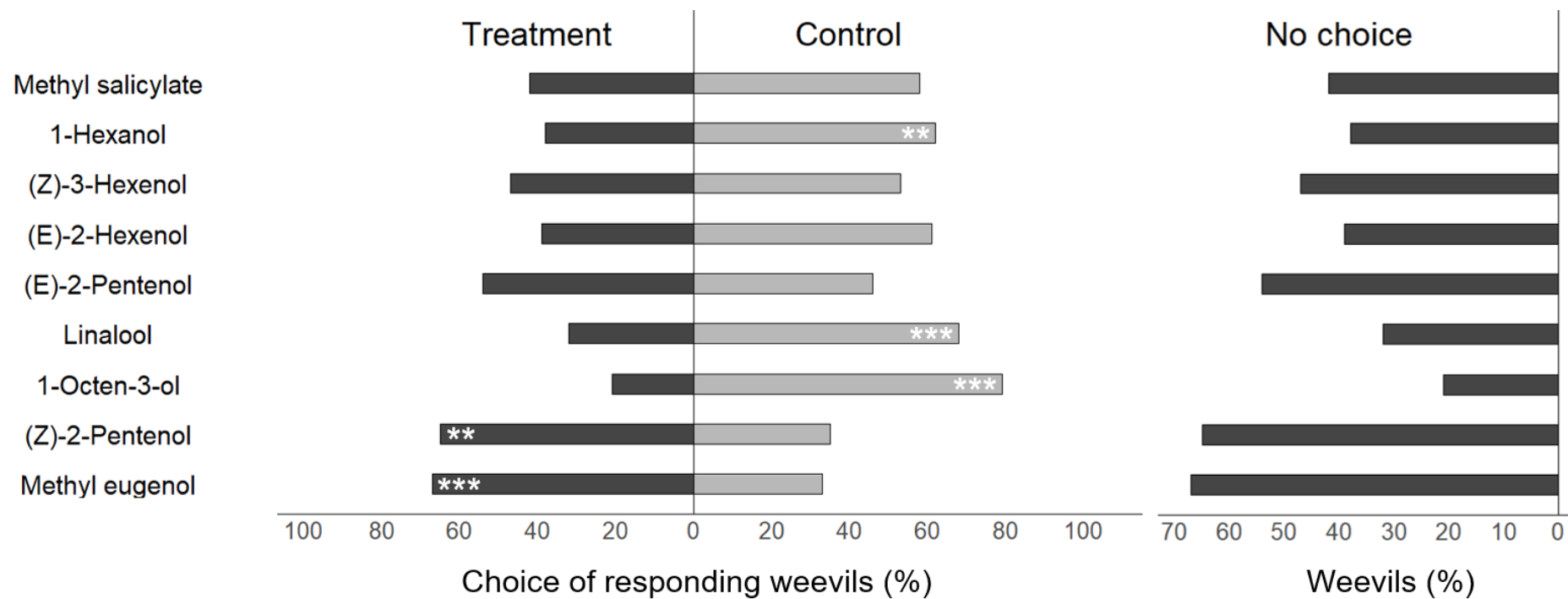


Fig. 3 Behavioral responses of adult vine weevils toward synthetic chemical compounds when offered against paraffin oil in a Y-tube olfactometer at a concentration of 10 mg/ml (0.1 mg at source). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ by binomial exact test

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Fig. 4 Behavioral responses of adult vine weevils toward synthetic chemical compounds when offered against paraffin oil in a Y-tube olfactometer at a concentration of 100 mg/ml (1 mg at source). ** $P < 0.01$, *** $P < 0.001$ by binomial exact test

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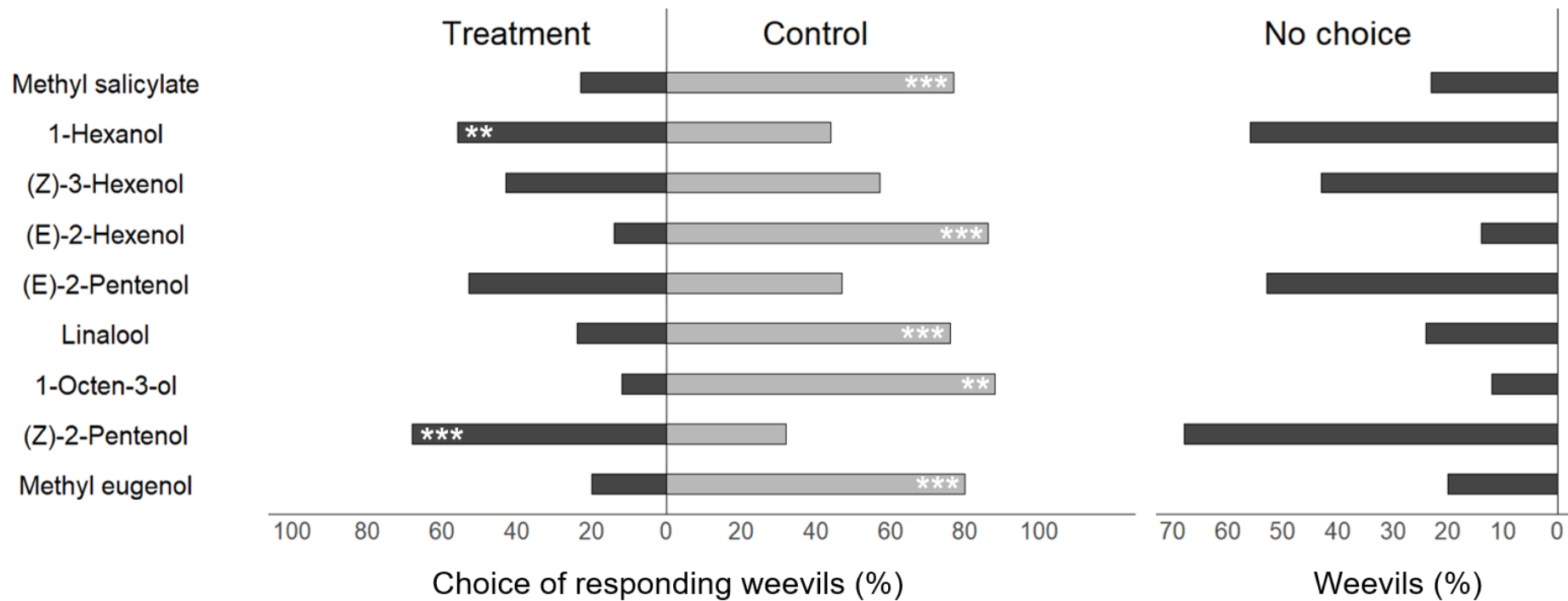
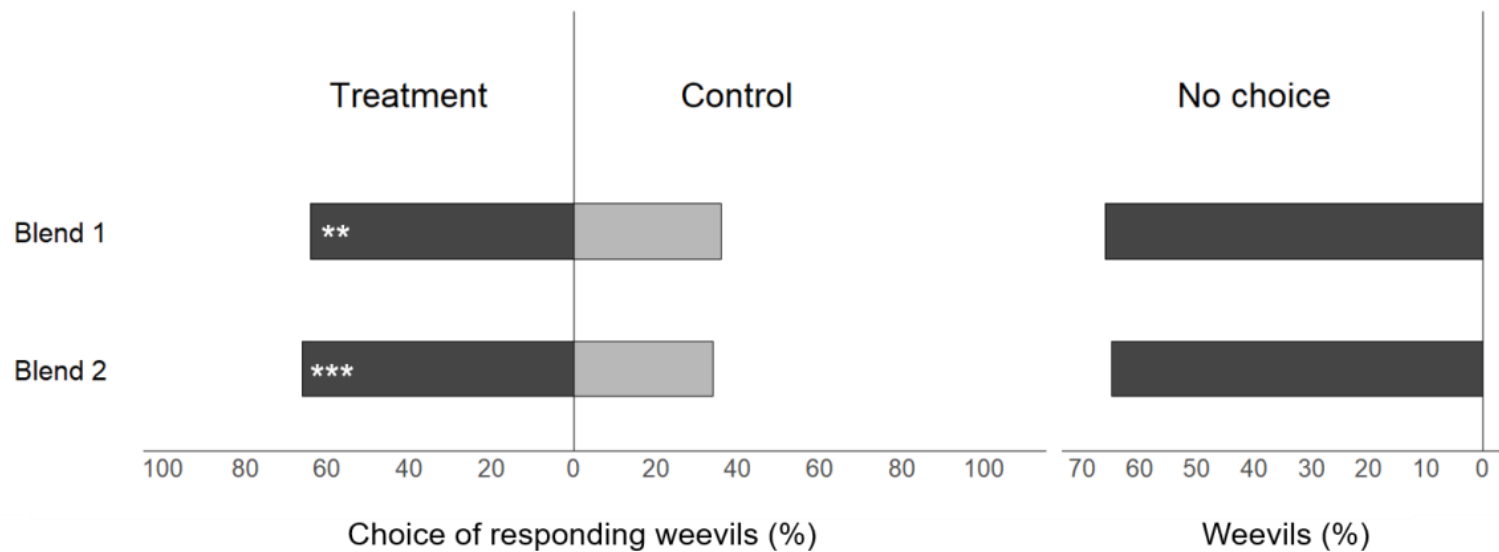


Fig. 5 Behavioral responses of adult vine weevils toward synthetic chemical compounds when offered against paraffin oil in a Y-tube olfactometer at a concentration of 500 mg/ml (5 mg at source). ** $P < 0.01$, *** $P < 0.001$ by binomial exact test



583

584 **Fig. 6** Behavioral responses of adult vine weevils toward synthetic chemical blends when offered against paraffin oil in a Y-tube olfactometer at
 585 a concentration of 100 mg/ml (10 mg at source). Blend 1 contains equal amounts of (*Z*)-2-pentenol + methyl eugenol; Blend 2 contains equal
 586 amounts of methyl salicylate + 1-octen-3-ol + (*E*)-2-hexenol + (*Z*)-3-hexenol + 1-hexanol + (*E*)-2-pentenol + (\pm)-linalool. ** $P < 0.01$, *** $P <$
 587 0.001 by binomial exact test

588



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