

1 **Detection of volatile pheromone candidates from the**
2 **white-spotted longicorn beetle, *Anoplophora malasiaca***
3 **(Coleoptera: Cerambycidae)**

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13

14 **Abstract** *Anoplophora malasiaca* (Thomson) (Coleoptera: Cerambycidae) is a
15 serious pest that affects various crop trees and landscapes in Japan. We collected and
16 analyzed volatiles from male and female *A. malasiaca*. 4-(*n*-Heptyloxy)butan-1-ol and
17 its aldehyde, pheromone components in *A. glabripennis* and *A. chinensis*, were
18 detected in the male volatile extracts and nonanal both in the male and female volatile
19 extracts. Nonanal was absent from the extracts of twigs of the willow host plant. Gas-
20 chromatograph-electroantennographic responses showed that nonanal and 4-(*n*-
21 heptyloxy)butan-1-ol elicited responses from both male and female antennae, but 4-(*n*-
22 heptyloxy)butanal did not. Volatiles of eight artificially reared males, analyzed every 3
23 or 4 days for 60 days from adult emergence showed that they all produced nonanal and
24 4-(*n*-heptyloxy)butan-1-ol. The two compounds produced no short -range female
25 attraction but in males, the short-range attraction to nonanal was dose-dependent and
26 significant in higher dose, but did not depend on 4-(*n*-heptyloxy)butan-1-ol. When
27 wounded willow twigs were added to nonanal and 4-(*n*-heptyloxy)butan-1-ol, the
28 frequency of male responses was higher than in all other treatments, but the same as
29 wounded willow twigs alone. The wounded hostplant willow twigs might thus be
30 enough for male attraction. The identified volatiles from the beetles in the present
31 study might have weaker function for attraction from the distance compare to their
32 hostplant volatiles.

33

34 **Keywords** *Anoplophora* · GC-EAD · Pheromone · Volatiles

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36

37 **Introduction**

38 The white-spotted longicorn beetle, *Anoplophora malasiaca* (Thomson)
39 (Coleoptera: Cerambycidae), is widely distributed in Japan (Muraji et al. 2011;
40 Ohbayashi 1992), where it is a serious pest requiring control. Its larvae destroy
41 horticultural crops, such as citrus, pear, and apple, and landscape trees, such as the
42 oriental plane tree and willow (Kojima and Nakamura 1986; Ohbayashi 1992).
43 *Anoplophora malasiaca* has a wide range of host plants, which includes 108 known
44 tree species (Sjöman et al. 2014). Controlling this species is highly desirable in Japan,
45 as is the case with the Asian longhorned beetle, *A. glabripennis* (Motschulsky), and the
46 citrus longhorned beetle, *A. chinensis* (Forster), in North America and Europe (Haack
47 et al. 2010; Hérard et al. 2006). However, controlling these species with insecticides is
48 difficult because the developing larvae are hidden within the trunk or roots of their
49 hosts.

50 In the laboratory, males have been found to be attracted to volatile chemicals that
51 originate from wounded plants of their host species when they are released close to a
52 female dummy (Fujiwara-Tsujii et al. 2012; Yasui et al. 2007b, 2008, 2011). In a
53 population fed mandarin-oranges, β -caryophyllene and α -humulene emitted from twigs
54 damaged by feeding adults were found to attract mate-seeking males (Yasui 2009;
55 Yasui et al. 2007b, 2008). In a willow-fed population, nerol emitted from wounded
56 willow twigs was found to attract males (Yasui et al. 2011). These volatiles, when
57 emitted from their original host plants, attract males but not females (Fujiwara-Tsujii et
58 al. 2012; Yasui et al. 2007b, 2008, 2011). Therefore, we previously proposed a
59 hypothesis that male beetles use the hostplant volatiles as information for mate location
60 (Yasui 2009; Yasui et al. 2007b, 2008, 2011). Even if there is no volatile pheromone,
61 this species might be able to find mates by wounded hostplant volatiles.

62 Recently, male-produced volatile pheromone components were identified in *A.*
63 *chinensis* (Hansen et al. 2015). The same components had already been identified in *A.*
64 *glabripennis* (Zhang et al. 2002). In *A. chinensis*, and trap catches for the pheromone
65 candidates were significantly higher than those for control in the field bioassays
66 (Hansen et al. 2015). In *A. glabripennis*, pheromone blend showed weak attraction to
67 females in short-range, and adults were not attracted to the pheromone blend in
68 greenhouse bioassays (Nehme et al. 2009). If male-produced volatile pheromone
69 components exist in *A. malasiaca*, it could be available for monitoring or controlling
70 this species, although it is uncertain that this species use these volatiles in mate
71 location.

72 In this study, we focused on the detection of pheromone candidates from *A.*
73 *malasiaca* volatiles. Furthermore, we confirmed the presence of male-produced
74 pheromone components that have previously been reported in other *Anoplophora*
75 beetles. We identified and analyzed chemicals that induced an antennal response and
76 continued collecting volatiles from each individual for 60 days after adult emergence to
77 understand their biological activity throughout adulthood. We also surveyed the beetles'
78 behavioral and attraction responses to the pheromone candidates in the laboratory.
79 Because volatiles from mandarin-orange-fed beetles contain many terpenes derived
80 from the host plant (Yasui et al. 2007b, 2008), which might mask the peaks of insect-
81 produced pheromone candidates, we used willow-fed beetles in this study.

82 Based on the results, we compared *A. malasiaca* with *A. glabripennis* and *A.*
83 *chinensis* and discussed the possibility of using these compounds to control *A.*
84 *malasiaca*.

85

86 **Materials and methods**

87 **Adult insect rearing for egg collection.** *Anoplophora malasiaca* adults
88 were collected by hand from groves of mandarin oranges, *Citrus unshiu* Marc.
89 (Rutaceae), on the Kunisaki Peninsula, Oita Prefecture, Japan in mid-June 2015, 2016,
90 and 2017. The beetles were individually reared in clear plastic cups (~11 cm diam. × 9.5
91 cm height) at 25°C under a 15L:9D photoperiod and illuminated by fluorescent lamps.
92 Each beetle was fed *C. unshiu* twigs collected from the field sites where the beetles
93 were sampled then transported to the Central Region Agricultural Research Center
94 laboratory, NARO. All cut twigs were stored at 5 °C and used within 10 days.

95 **Egg collection and laboratory rearing to adults.** *Anoplophora*
96 *malasiaca* eggs were obtained from 200 females collected from mandarin orange groves
97 in mid-June of 2015, 2016, and 2017. Eggs laid on the citrus twigs were collected then
98 the larvae were reared to adults as described in Fujiwara-Tsujii et al. (2016).
99 Throughout their larval stages, all larvae were reared on an artificial diet (Silkmate 2S,
100 mulberry leaf-based diet, Nihon Nosan Kogyo, Yokohama, Japan). The emerged adults
101 were individually contained in transparent plastic cups (~11 cm diam. × 9.5 cm height).
102 The adults started feeding on willow *Salix schwerinii* E. L. Wolf (Salicaceae) twigs
103 cultivated in NARO (Tsukuba, Japan) one week after emergence. All adults used in the
104 laboratory experiments were reared on willow twigs.

105 **Chemicals.** Nonanal (> 95% purity) was purchased from Wako Pure Chemical
106 Industries, Ltd (Osaka, Japan); 4-(*n*-heptyloxy)butan-1-ol was provided by Prof.
107 Jocelyn G. Millar of University of California Riverside; and 4-(*n*-heptyloxy)butanal was
108 obtained by oxidation of 4-(*n*-heptyloxy)butan-1-ol with pyridinium dichromate (> 98%
109 purity, Sigma-Aldrich, St. Louis, MO, USA). HPLC grade *n*-hexane was used as a
110 solvent, and diethyl ether was distilled just before use.

111 **Insect-produced volatile collection by PorapakQ for coupled**

112 **gas chromatography-electroantennogram detection (GC-**
113 **EAD).** Adult *A. malasiaca* that had been fed on willow twigs (20 – 25 days after
114 adult emergence) were held individually in wire mesh cages. To collect the headspace
115 odors, beetles of the same sex were placed in two separate wire mesh cages inside
116 stoppered 1 L glass flasks. Charcoal-filtered air was pushed (30 – 50 mL/min) through
117 the flasks for 7 h by portable vacuum pumps (MP-2N, Shibata Scientific Technology,
118 Tokyo, Japan). The air outlets were fitted with volatiles traps made of Porapak Q
119 adsorbent (200 mg; Sigma-Aldrich, St Louis, MO, USA) secured in glass tubes by
120 glass wool plugs. The trapped volatiles were eluted with diethyl ether (1 mL) then the
121 extract was concentrated under reduced pressure at room temperature. After being
122 resolved with *n*-hexane, the extract was stored at -30°C before use.

123 **Extract analysis by GC-EAD.** The volatile extracts were analyzed by
124 GC-EAD using an Agilent 6890N GC fitted with an HP-INNOWax column (30 m ×
125 0.25 mm ID × 0.25 µm film thickness; Agilent Technologies, Santa Clara, CA, USA).
126 The injector and flame ionization detector (FID) were set at 220°C, and injections were
127 made in splitless mode. The oven was programmed to 40°C for 1 min, 5°C per min up
128 to 175°C, 15°C per min up to 220°C, and held for 5 min at 220°C. Helium was used as
129 the carrier gas at a constant flow rate of 1.1 mL/min. Nitrogen makeup gas (30
130 mL/min) was added to the column effluent via a stainless-steel T-union, after which the
131 flow was split equally between the FID and EAD with a press-fit Y-splitter. The GC
132 effluent for EAD was directed to a glass transfer tube (15 mm ID) mounted on the GC
133 and was mixed with humidified air (300 mL/min, 20°C) before being passed over the
134 antennal preparation. An antenna (25 – 30 days after adult emergence), including the
135 basal segment, was gently removed from the live beetles using scissors and forceps
136 then mounted on the EAD system's electrodes (Struble and Arn, 1984). Connections

137 were made with electrode gel (Aquasonic Clear®, Parker Lab. Inc., NJ, USA). The
138 EAD and FID signals were recorded simultaneously. Analyses were replicated using
139 antennae from a total of four females and two males, and each antennal preparation
140 was reused for 4 – 5 analyses.

141 **Time course analysis of pheromone candidates in individual** 142 **male volatiles using solid phase microextraction (SPME).**

143 The analyses of the male volatiles started on 6 October 2016. On the day of adult
144 emergence (0 d), eight males were each placed in a 100 mL glass Erlenmeyer flask
145 covered with aluminum foil. Thirty minutes after introduction, an SPME fiber (100 µm
146 polydimethylsiloxane; Supelco, Bellefonte, PA, USA) was inserted into the flask
147 through the aluminum foil cover to collect the headspace volatiles for 90 min. A piece
148 of wire mesh was used to separate the sampling area from the insect. The SPME
149 samples were collected at 25°C in the laboratory.

150 The GC/MS analyses were performed using an Agilent 7890A GC system interfaced
151 to a JMS-T100GC Time-of-Flight Mass Spectrometer (JEOL, Tokyo, Japan) in EI mode
152 with 70 eV at 200°C. Injection was set for splitless mode at 240°C for 1 min. An HP-
153 INNOWax capillary column (30 m × 0.25 mm ID × 0.25 µm film thickness; Agilent
154 Technologies) was used. The carrier gas, helium, was set to a constant flow rate of 1.1
155 mL/min. The GC oven temperature program was held for 1 min at 40°C, increased from
156 40 to 220°C at 5°C min⁻¹, and then held for 5 min at 220°C. Nonanal was eluted at a
157 retention time (t_R) of 12.50 min, 4-(*n*-heptyloxy)butanal at a t_R of 21.05 min, and 4-(*n*-
158 heptyloxy)butan-1-ol at a t_R of 26.15 min.

159 **Nonanal detection in extracts of willow twigs.** Willow is a hostplant
160 of the *A. malasiaca* beetle and was the only plant used in the present study. As nonanal
161 is often detected in plant volatiles, we analyzed the volatiles of wounded willow twigs

162 and an ether extract of willow bark. Willow bark was prepared by peeling bark from
163 the same twigs as used for the adult's food. The head-space volatiles of wounded
164 willow twigs were collected with SPME fibers as described above. The willow
165 volatiles were analyzed on 8 May 2018. Three willow twigs wounded with a knife
166 were placed in a 50 mL glass Erlenmeyer flask covered with aluminum foil. Thirty
167 minutes after introduction, an SPME fiber (100 μ m polydimethylsiloxane; Supelco)
168 was inserted into the flask through the aluminum foil cover to collect headspace
169 volatiles for 90 min. The SPME samples were collected at 25°C in the laboratory. The
170 GC/MS analyses were performed as described above for the male volatile collection in
171 2016, but a different HP-INNOWax column was used. Therefore, a retention time of
172 nonanal was 13.19 min in 2018 experiments.

173 Two grams of peeled willow bark were extracted using 10 mL of diethyl ether for 1 h.
174 The bark was then removed, and the extract concentrated under reduced pressure at
175 room temperature. After being resolved with *n*-hexane, the extract was analyzed with
176 GC/MS under the conditions described above.

177 **Behavioral responses to synthetic pheromone candidates,**
178 **nonanal and 4-(*n*-heptyloxy)butan-1-ol, and wounded willow**
179 **twigs.** Behavioral assays using nonanal and 4-(*n*-heptyloxy)butan-1-ol were
180 conducted from 18 – 31 May 2016 and 24 – 29 May 2018, and using wounded willow
181 twigs from 25 May – 7 June 2017 and 24 – 29 May 2018. The observation arena was
182 constructed from a sheet of white paper (21 × 30 cm) attached to a plate of the same
183 size then fixed to the bottom of a clear acrylic box (30 × 30 × 30 cm) at a 75° angle.
184 (see Fig. 1 in Yasui et al. 2008). A hole (1.5 cm diam.) was bored through both the
185 plate and paper at point M.

186 The hole was covered with mesh beneath the plate, and a black glass dummy (12

187 mm diam. × 35 mm length) was fixed horizontally in front of the hole to serve as a
188 female model. A plastic cup (5 cm diam. × 3.2 cm height) contained a sheet of filter
189 paper (1 cm × 2 cm), on which a test sample was applied. This was then fixed beneath
190 the observation arena, behind the dummy. Fresh air was supplied to the cup beneath the
191 plate at 18 mL/min through a polytetrafluoroethylene tube (5 mm diam. × 50 cm length)
192 by an air pump (MP-2N, Shibata Scientific Technology, Tokyo, Japan). Air containing
193 volatiles from the sample was pumped through the hole to allow the odor to permeate
194 the observation arena. Males and females were individually introduced to the arena at
195 one of two symmetrically-placed starting points, with the body axis parallel to the
196 vertical. The beetles' walking trails were observed relative to thin grey lines printed on
197 the paper. When the insect veered or curved to walk towards a model before making
198 direct contact, it was considered a positive response. The assay was repeated for 30
199 individuals within 30 min of treating the test material with the filter paper or wounded
200 willow twigs. When the insects failed to adjust their body axis to the vertical or ceased
201 to walk for more than 2 min, the trial was aborted. All behavioral assays were
202 conducted from 10:00 to 15:00, at 25°C (light period: 3:00 to 18:00) in the laboratory.

203 **Statistical analyses.** For behavioral responses to various amounts of nonanal
204 and 4-(*n*-heptyloxy)butan-1-ol, logistic regression analysis was applied to the log-
205 transformed dose (ng). Logistic regression analysis was done using JMP 11.2.1 (2014,
206 SAS Institute Inc, Cary, NC, USA). The assay data with the single chemicals were also
207 analyzed using an $n \times 2$ chi-square test. If this was significant ($p < 0.05$), a paired chi-
208 square test between control and each treatment was then calculated. The assay data
209 with chemical blends were analyzed with an $n \times 2$ chi-square test and subsequent
210 paired chi-square test with Bonferroni's-corrected p values (Sokal and Rohlf, 1995). In
211 Fig. 5, values accompanied by the same letter do not differ significantly at the $p = 0.05$

212 level.

213

214 **Results**

215 **Female and male volatile extract analysis by PorapakQ and**
216 **coupled GC-EAD analysis.** We detected 4-(*n*-heptyloxy)butan-1-ol and its
217 aldehyde in the male volatile extracts (Fig. 1, FID). Nonanal was detected in both the
218 male and female volatile extracts. The mass spectral data and GC retention times of
219 these three compounds matched those of the synthetic compounds. Red arrows indicate
220 the EAG-positive peaks. Extract analysis by GC-EAD showed that male and female
221 antennae both responded to nonanal and 4-(*n*-heptyloxy)butan-1-ol, but not to its
222 aldehyde.

223 **Nonanal detection in willow twigs.** We analyzed both an ether extract of
224 willow bark prepared by peeling willow twigs (Fig. 2a) and the headspace volatiles of
225 wounded willow twigs under the same conditions as for the collection of male volatiles
226 for time course analysis (90 min SPME collection) (Fig. 2b) using GC/MS. Nonanal
227 (t_R 13.19 min in Fig. 2c) was not detected in either extract of willow, although it was
228 detected in the male volatiles (nonanal, t_R 12.52 min in Fig. 2d). Although the retention
229 times shown in Fig. 2c (authentic nonanal) and Fig. 2d were different because the
230 samples were analyzed with different HP-INNOWax columns, we confirmed the 12.52
231 min peak in Fig. 2d as nonanal by t_R and mass spectra as those of authentic nonanal.

232 **Time course analysis of pheromone candidates in the**
233 **individual male volatiles.** Nonanal, 4-(*n*-heptyloxy)butanal, and 4-(*n*-
234 heptyloxy)butan-1-ol in the volatiles of the eight males were tracked individually every
235 3 to 4 days for 60 days after adult emergence (Fig. 3). We used the SPME method,
236 which is convenient for detecting trends in the emission of each compound from the

237 individual male beetles. The males began to emit these compounds on approximately
238 the 10th day after emergence. They emitted nonanal intermittently before the 10th day
239 and until the 60th day. Many males had one substantial nonanal emission between the
240 10th and 35th days. The patterns of 4-(*n*-heptyloxy)butanal and 4-(*n*-heptyloxy)butan-
241 1-ol emission were similar for all the individuals, with two peaks: a large one at around
242 the 20th day and a smaller one at around the 40th day, after which the amount of the
243 emissions decreased.

244 **Behavioral responses to synthetic pheromone candidates,**
245 **nonanal, 4-(*n*-heptyloxy)butan-1-ol, and wounded willow**
246 **twigs.** In the laboratory bioassay, the frequency of the orientation response to
247 nonanal was dose-dependent in males but not in females [logistic regression (1 – 1,000
248 ng), males: *d.f.* = 1, $\chi^2 = 6.956$, *p* = 0.0084; females: *d.f.* = 1, $\chi^2 = 1.014$, *p* = 0.319] (Fig.
249 4a). The frequency of the male response to over 10 ng of nonanal was significantly
250 higher than that to the control (paired chi-square test with a Bonferroni-corrected *p*
251 value). Alternatively, the frequency of female orientation response to 4-(*n*-
252 heptyloxy)butan-1-ol was dose-dependent [logistic regression (1 – 1,000 ng), males:
253 *d.f.* = 1, $\chi^2 = 1.546$, *p* = 0.214; females: *d.f.* = 1, $\chi^2 = 8.910$, *p* = 0.003] (Fig. 4b).
254 However, the frequency of female responses to 4-(*n*-heptyloxy)butan-1-ol were as low
255 as their response to the control (paired chi-square test with a Bonferroni-corrected *p*
256 value). The frequency of male responses to 4-(*n*-heptyloxy)butan-1-ol were also as low
257 as their response to the control.

258 There were no significant differences in the frequencies of the male response to
259 various amounts of 4-(*n*-heptyloxy)butan-1-ol + 10 ng of nonanal. The frequency of
260 female responses to 4-(*n*-heptyloxy)butan-1-ol + nonanal was increased from 0% at 50
261 ng to 23% at 100 ng, but decreased to 6.7% at 500 ng (Fig. 5a). When wounded willow

262 twigs were added to a mixture of 10 ng nonanal and 100 ng 4-(*n*-heptyloxy)butan-1-ol,
263 the frequency of the male responses was significantly higher than that for the two-
264 compound mixture, but was still the same as for the wounded willow twigs alone (Fig.
265 5b). For the females, adding wounded willow twigs to the two compounds led to no
266 significant changes in the responses.

267

268 **Discussion**

269 A male-produced attractant pheromone has previously been identified in *A.*
270 *glabripennis* (Crook et al. 2014; Nehme et al. 2009; Zhang et al. 2002) and more
271 recently in *A. chinensis* (Hansen et al. 2015). We investigated the presence of these
272 types of male-produced pheromone in *A. malasiaca*. Combinations of these attractant
273 pheromones and host-plant-derived attractant chemicals with efficient flight traps could
274 produce to effective methods for monitoring these pests. In the present study, we
275 detected 4-(*n*-heptyloxy)butan-1-ol and 4-(*n*-heptyloxy)butanal in volatile extracts from
276 male *A. malasiaca*. These two compounds have also been detected in the male volatiles
277 of *A. glabripennis* (Zhang et al. 2002) and *A. chinensis* (Hansen et al. 2015). GC-EAD
278 analysis revealed that the male and female *A. malasiaca* antennae only responded to 4-
279 (*n*-heptyloxy)butan-1-ol. Nonanal was also confirmed as a common EAG-active
280 substance in the male and female volatiles. As pheromone candidates, establishing a
281 time course analysis of the emission of these three compounds during the beetles'
282 adulthood is essential for controlling this species. All eight tested males emitted the
283 three compounds from approximately the 10th day after adult emergence to the 60th day,
284 although only a small amount by that time (Fig. 3). *A. malasiaca* might continuously
285 emit these compounds through adulthood in the field, because the adults are active for 2
286 to 3 months. The identified volatiles from the beetles, however, revealed to have weaker

287 function of attraction from the distance compare to their wounded hostplant volatiles.

288 Nonanal has reportedly been detected in female *A. glabripennis* cuticular extracts
289 exposed to ozone or UV and visible light, and male antennae were found to respond to
290 nonanal (Wickham et al. 2012). The laboratory bioassays, using a Y-tube olfactometer,
291 found that *A. glabripennis* males were preferentially attracted to a mixture of nonanal,
292 heptanal, and hexadecanal. In the present study, we detected nonanal in the male and
293 female *A. malasiaca* volatiles, and found that the antennae of both sexes responded to
294 nonanal. Nonanal is often found in plant volatiles but in this study, we detected none,
295 neither in the volatiles nor in the extract of their food plant willow twigs (Fig. 2). The
296 extract of willow bark analyzed was prepared by peeling it from twigs collected from
297 the same willow tree used as food by *A. malasiaca* adults. Therefore, in *A. malasiaca*
298 nonanal was revealed to have been produced by the beetles themselves.

299 We used the laboratory bioassay method described by Yasui et al. (2008) to evaluate
300 the short-range attractiveness of samples to mate locations, because volatiles from
301 wounded host plant twigs had been found to attract *A. malasiaca* males. This method
302 also revealed that *A. malasiaca* males, like *A. glabripennis* are attracted to nonanal in a
303 dose-dependent manner (Fig. 4a). In the case of EAG-positive 4-(*n*-heptyloxy)butan-1-
304 ol, although female beetles responded in a dose-dependent manner, even up to 1000 ng,
305 their response was as low as that to the control. Therefore, neither male nor female
306 beetles were significantly attracted using levels of 4-(*n*-heptyloxy)butan-1-ol between 1
307 and 1000 ng (Fig. 4b).

308 It was not clear whether adding 4-(*n*-heptyloxy)butan-1-ol to nonanal had a
309 synergistic effect on the female orientation responses, but there was clearly no
310 synergistic effect on male responses (Fig. 5a). One possible reason was a low nonanal
311 concentration, however, based on the daily time course experiments, we suspect that the

312 beetles do not emit a large amount of nonanal (Fig. 3).

313 When wounded host willow twigs (Yasui et al. 2011) were added to a blend of 4-(*n*-
314 heptyloxy)butan-1-ol and nonanal, the male response increased but to the same level as
315 for the wounded willow twigs alone. This means that the blend of two compounds had
316 no synergistic effect on the male response to wounded willow twigs. In contrast,
317 females were not attracted to this blend with wounded willow twigs. Because the
318 attraction levels were low, the females may use other signals, such as visual cues, for
319 locating a mate.

320 (3*E*,6*E*)- α -Farnesene has been identified as the third component of male-produced
321 aggregation pheromones in *A. glabripennis* (Crook et al. 2014). This compound is a
322 sesquiterpene, but has not been detected in females nor in the twigs of the striped maple
323 host plant. This compound was EAG-positive for both male and female antennae, which
324 confirmed its attractiveness to the beetles in the laboratory. The same compound, α -
325 farnesene derived from volatiles of the wounded twig of the mandarin orange hostplant,
326 was found to be an attractant for mandarin orange-fed *A. malasiaca* males (Yasui et al.
327 2008). Despite their different origins, it is interesting that both *Anoplophora* species
328 were attracted to the same compound.

329 Male *A. chinensis* volatiles have been found to contain 4-(*n*-heptyloxy)butan-1-ol
330 and its aldehyde, and elicited EAG-active responses in male and female antennae
331 (Hansen et al. 2015). In the field bioassays, trap catches for the pheromone candidates
332 were significantly higher than those for the control; however, the number of beetles
333 captured was very low. In greenhouse bioassays, *A. glabripennis* adults were not
334 attracted to the pheromone blend (Nehme et al. 2009). These compounds were
335 undoubtedly emitted by three different *Anoplophora* species. Although we have not
336 conducted field bioassays with those candidates at willow cultivation sites, our short-

337 range attraction bioassay results showed that those pheromone candidates had a weak or
338 no effect, which might be the same for long-distance attraction.

339 Like other *Anoplophora* species, the males were more attracted than the females to
340 plant volatiles (Nehme et al. 2009). Females of all three *Anoplophora* species were
341 barely attracted to any volatile lures, which indicates that olfactory information is not
342 important in long- or short-range orientation for females. We have previously proposed
343 a hypothesis of male mate searching in *A. malasiaca* (Yasui 2009; Yasui et al. 2007b,
344 2008, 2011), but this needs to be explained in more details, e.g. in the case of willow
345 individuals. We hypothesized that the same host plant volatile components will be
346 emitted when either conspecific males or females bite the twigs of the host plant, but
347 only *A. malasiaca* male beetles use the hostplant volatiles as information for mate
348 location. Nerol is emitted from bitten willow twigs and the males will be attracted to
349 this volatile chemical. However, the male beetles do not know who has bitten the host
350 plant until they approach the odor source. When the males approach the volatile source,
351 they might use visual cues to determine whether the source of the bite is conspecific
352 individuals (Fukaya et al. 2004, 2005) then touch those individuals with their antennae
353 or tarsi to recognize conspecific females through detecting cuticular contact sex
354 pheromone components. The contact sex pheromone, specific in *A. malasiaca*, consists
355 of complex mixture of chemicals which elicit males grasping, mounting, and bending
356 their abdomen toward females (Fukaya et al. 2000; Yasui et al. 2003; Yasui et al. 2007a).
357 Therefore, based on this hypothesis, *A. malasiaca* males can meet and mate with
358 conspecific females.

359 Because male beetles produce and emit the three analyzed pheromone candidates
360 throughout their adulthood, which involves a significant cost, they might not emit them
361 aimlessly. One possible function of male-produce chemicals {4-(*n*-heptyloxy)butan-1-ol

362 and its aldehyde} is to avoid encountering males in short-range. When one male
363 approaches to a conspecific beetle, if he detects these volatile chemicals, he recognizes
364 the target will be a male. Nonanal was found to be emitted from both male and female *A.*
365 *malasiaca*, and stimulated short-range attraction to the males. The biological function of
366 nonanal could be an attractant for male mate location when bitten willow twigs are not
367 around the beetle or bitten long time ago, because host plant attractant nerol is only
368 emitted for short period after wounding the twigs (Yasui et al. 2011). Further research
369 will be needed to reveal the alternative function of these volatiles. Other unknown
370 factors may help individuals to find mates. Other factors might be worth analyzing in
371 the *Anoplophora* species, and many factors may synergistically affect their system of
372 mate location.

373

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388

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470

471 **Figure legends**

472 **Fig. 1 Coupled gas chromatography-electroantennogram detection of headspace**
473 **volatile extracts produced by *A. malasiaca*.** a) The top trace shows the response from
474 a male *A. malasiaca* antenna, the middle trace shows the same from a female, and the
475 bottom trace shows the GC detector response of male volatile extracts. b) The top trace
476 shows the response from a male *A. malasiaca* antenna, the middle trace shows the same
477 from a female, and the bottom trace shows the GC detector response of female volatile
478 extracts. Red arrows indicate positive EAG peaks.

479

480 **Fig. 2 Gas chromatography-mass spectrometry analysis of willow twig extracts.** a)
481 An ether extract of willow bark, b) an SPME extract of head space volatiles of wounded
482 willow twigs, c) authentic nonanal, and d) an SPME extract of headspace volatiles of a
483 willow-fed male beetle. a), b), and c) were analyzed by the same HP-INNOWax column,
484 but d) was analyzed by a different HP-INNOWax column. SPME of both b) and d) were
485 collected for 90 min.

486

487 **Fig. 3 Time course analysis of pheromone candidates in the individual male**
488 **volatiles.** From the day of adult emergence (0 d), volatiles of eight males were
489 individually collected with SPME fibers and analyzed by GC/MS. A to H shows each
490 male beetle, with each of his pheromone candidates' emissions [nonanal, 4-(*n*-
491 heptyloxy)butanal and 4-(*n*-heptyloxy)butan-1-ol] displayed vertically. The Y-axis
492 shows the peak area of each compound, and the X-axis shows the time course.

493

494 **Fig. 4 Behavioral responses of *A. malasiaca* adults to synthetic pheromone**
495 **candidates.** a) Nonanal (1 – 1,000 ng) and b) 4-(*n*-heptyloxy)butan-1-ol (1 – 1,000 ng).

496 con: negative control. $N = 30$. Black bars represent males and white bars represent
497 females. Response values of each sex accompanied by asterisk in the same category
498 differ significantly at the $p = 0.05$ level compared with the control ($n \times 2$ chi-square
499 test and subsequent paired chi-square test between control and each treatment with
500 Bonferroni's-corrected p -values).

501

502 **Fig. 5 Behavioral responses of *A. malasiaca* adults to volatile blends of synthetic**
503 **pheromone candidates, nonanal (9al) and 4-(*n*-heptyloxy)butan-1-ol (Hbol), and**
504 **wounded willow twigs.** a) Nonanal and 4-(*n*-heptyloxy)butan-1-ol, and b) nonanal, 4-
505 (*n*-heptyloxy)butan-1-ol, and wounded willow twigs. con: negative control. $N = 30$.
506 Response values of each sex accompanied by the same letter did not significantly
507 differ at the $p = 0.05$ level ($n \times 2$ chi-square test and subsequent paired chi-squared test
508 with Bonferroni's-corrected p -values). n.s.: not significant.

509

510

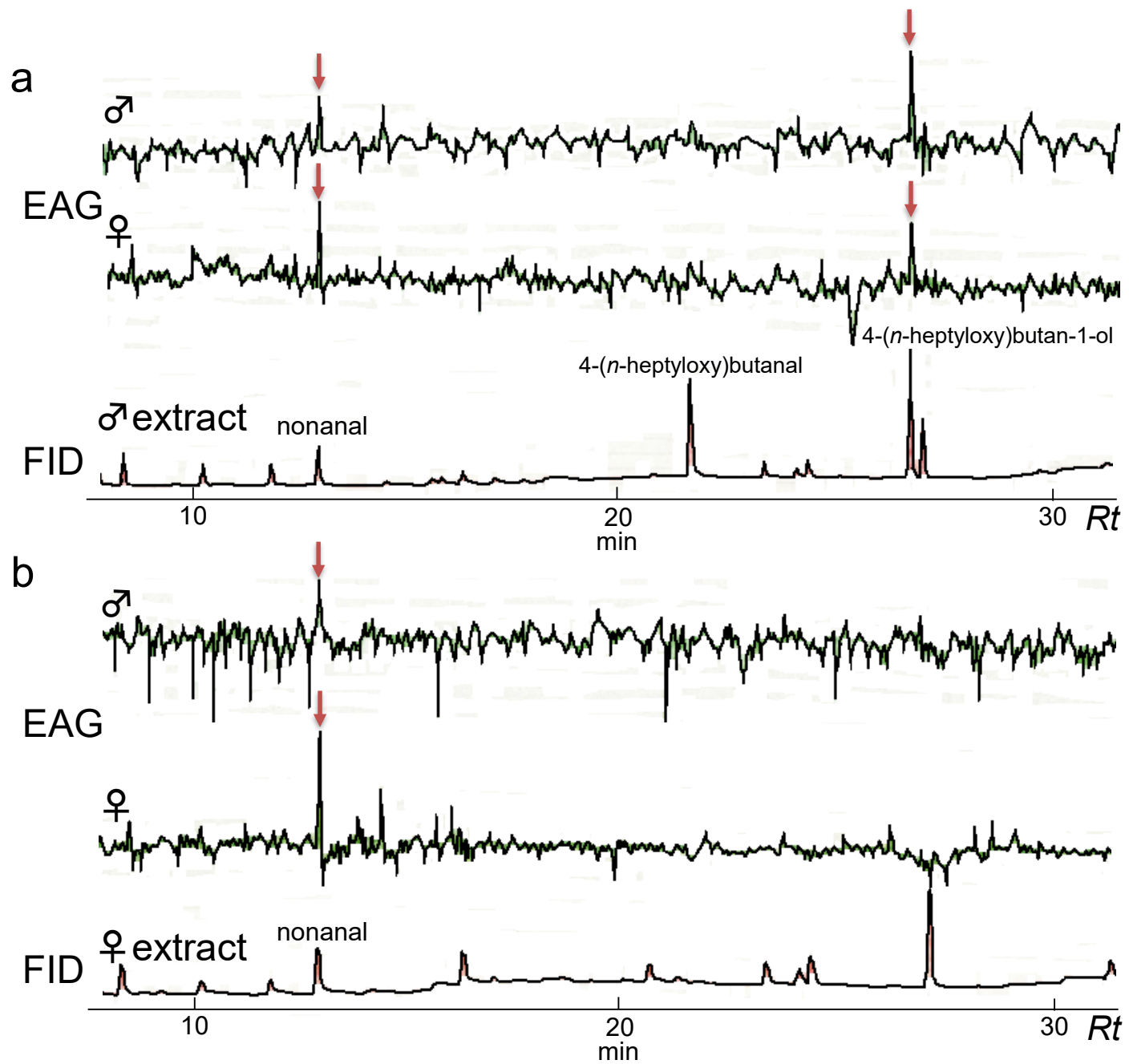


Fig. 1 Coupled gas chromatography-electroantennogram detection of headspace volatile extracts produced by *A. malasiaca*.

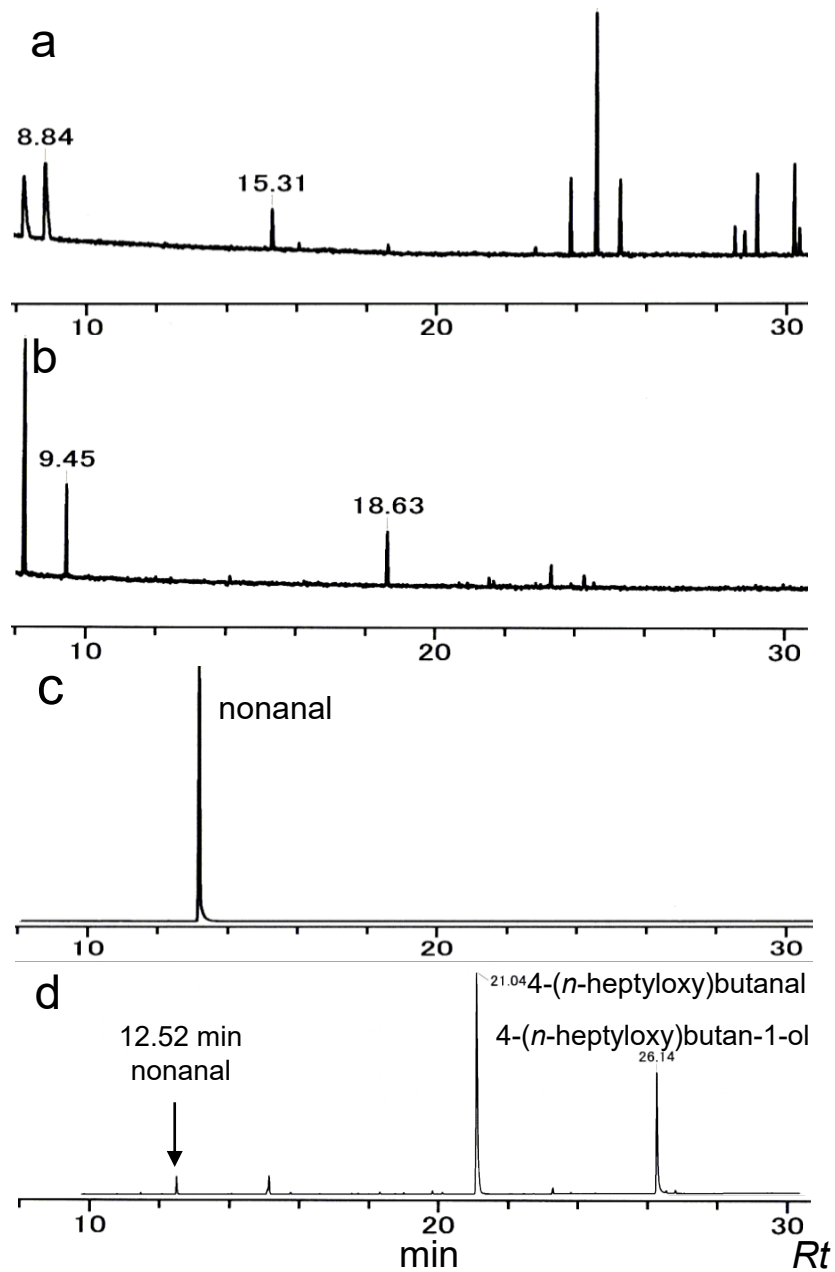


Fig. 2 Gas chromatography-mass spectrometry analysis of willow twig extracts.

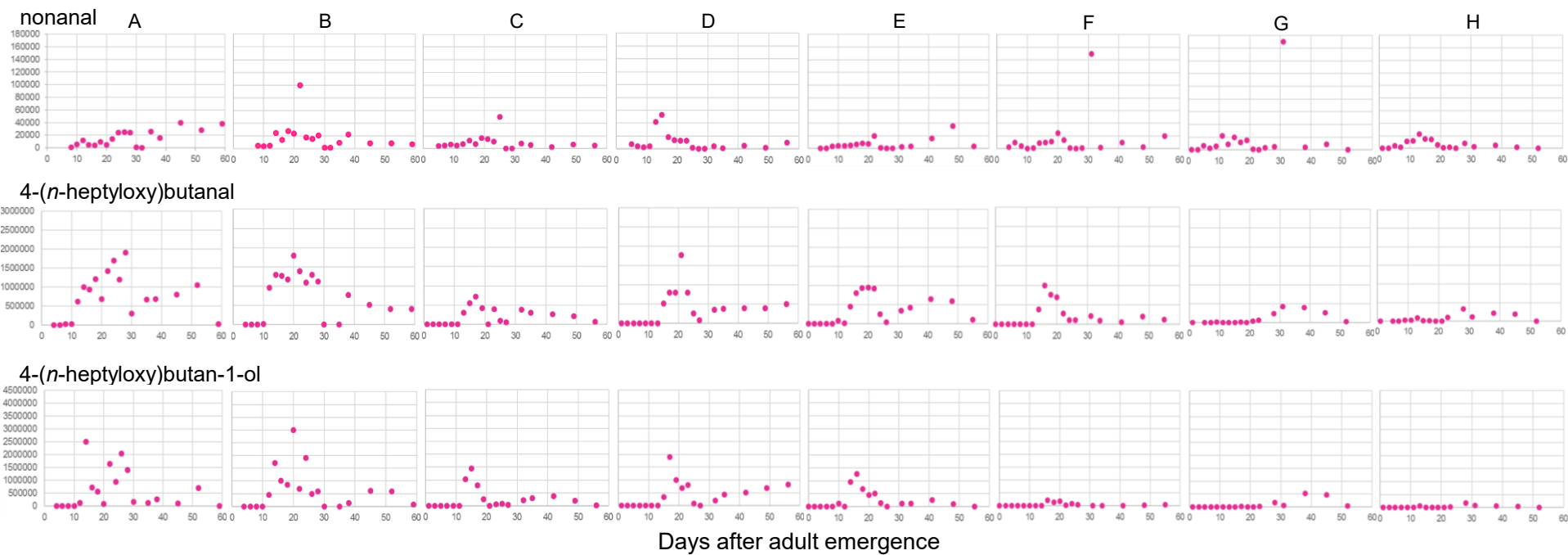


Fig. 3 Time course analysis of pheromone candidates in the individual male volatiles.

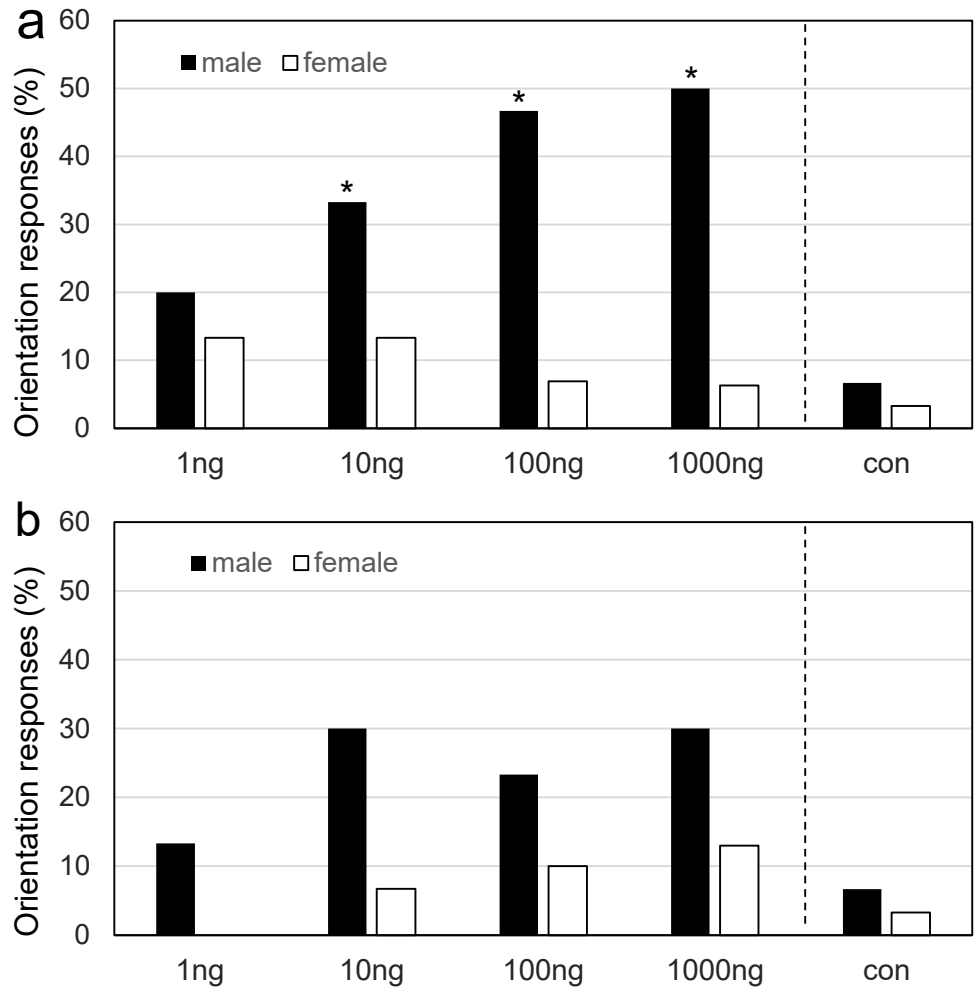


Fig. 4 Behavioral responses of *A. malasiaca* adults to synthetic pheromone candidates.

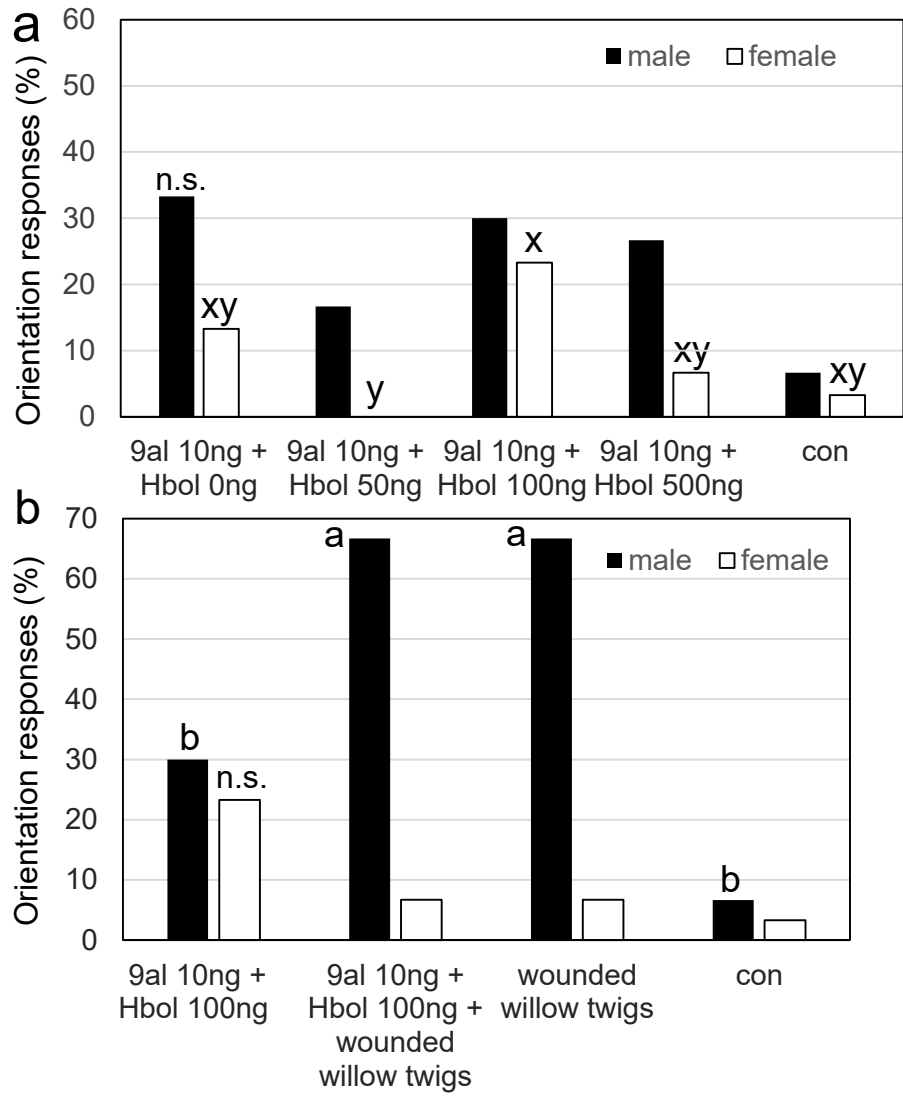


Fig. 5 Behavioral responses of *A. malasiaca* adults to volatile blends of synthetic pheromone candidates, nonanal (9al) and 4-(*n*-heptyloxy)butan-1-ol (Hbol), and wounded willow twigs.