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

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

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

Introduction

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

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

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

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

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

Materials and methods

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

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

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

Insect-produced volatile collection by PorapakQ for coupled

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

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

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

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

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

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

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

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

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

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

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

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

level.

Results

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

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

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

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

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

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

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

Discussion

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

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

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

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

It was not clear whether adding 4-(*n*-heptyloxy)butan-1-ol to nonanal had a synergistic effect on the female orientation responses, but there was clearly no synergistic effect on male responses (Fig. 5a). One possible reason was a low nonanal 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-

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

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

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

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

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Figure legends

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

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

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

Fig. 4 Behavioral responses of *A. malasiaca* adults to synthetic pheromone 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 × 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 × 2 chi-square test and subsequent paired chi-squared test
508 with Bonferroni's-corrected p-values). n.s.: not significant.
509
510

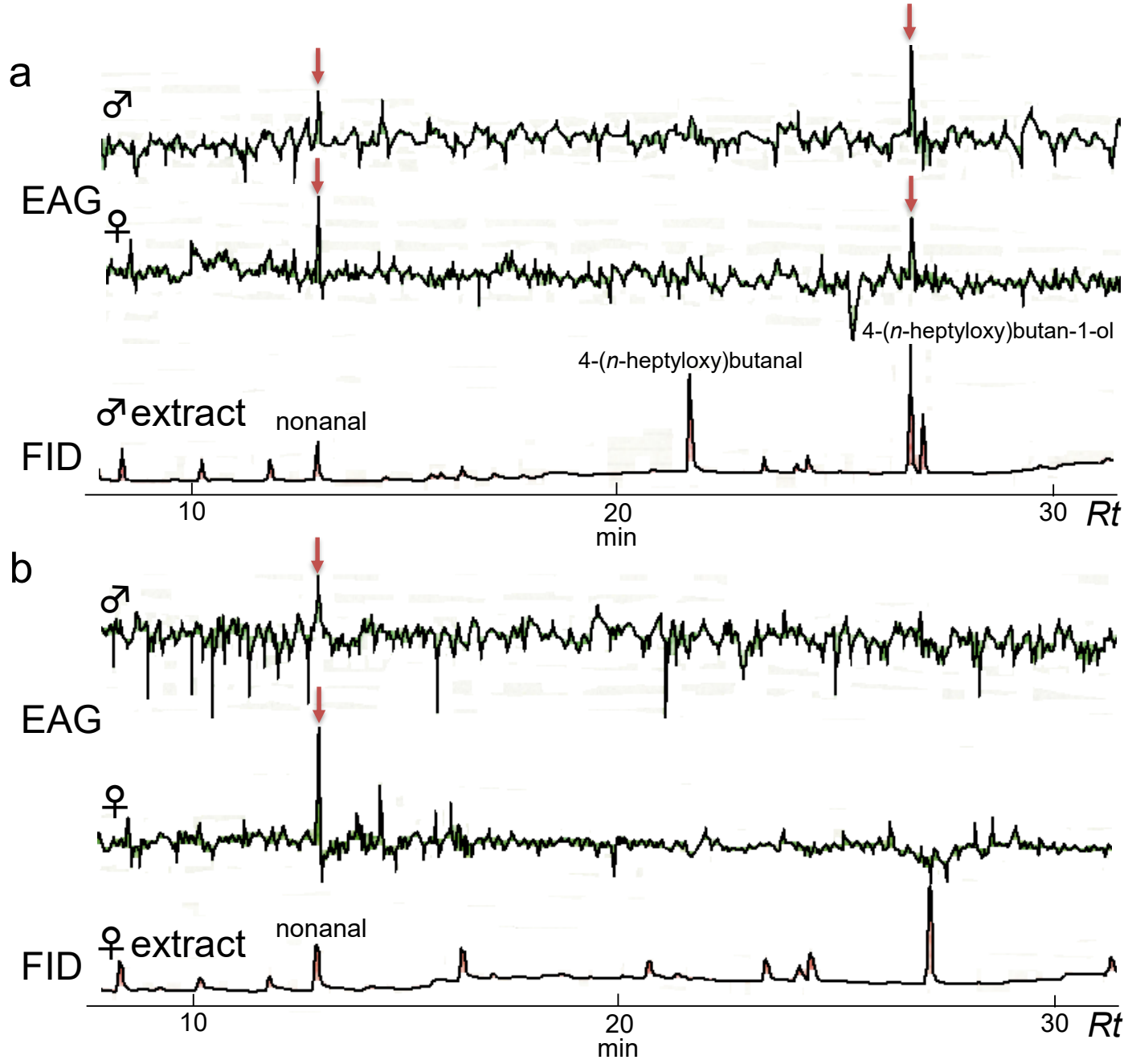

Fig. 1 Coupled gas chromatography-electroantennogram detection of headspace volatile extracts produced by *A. malasiaca*.

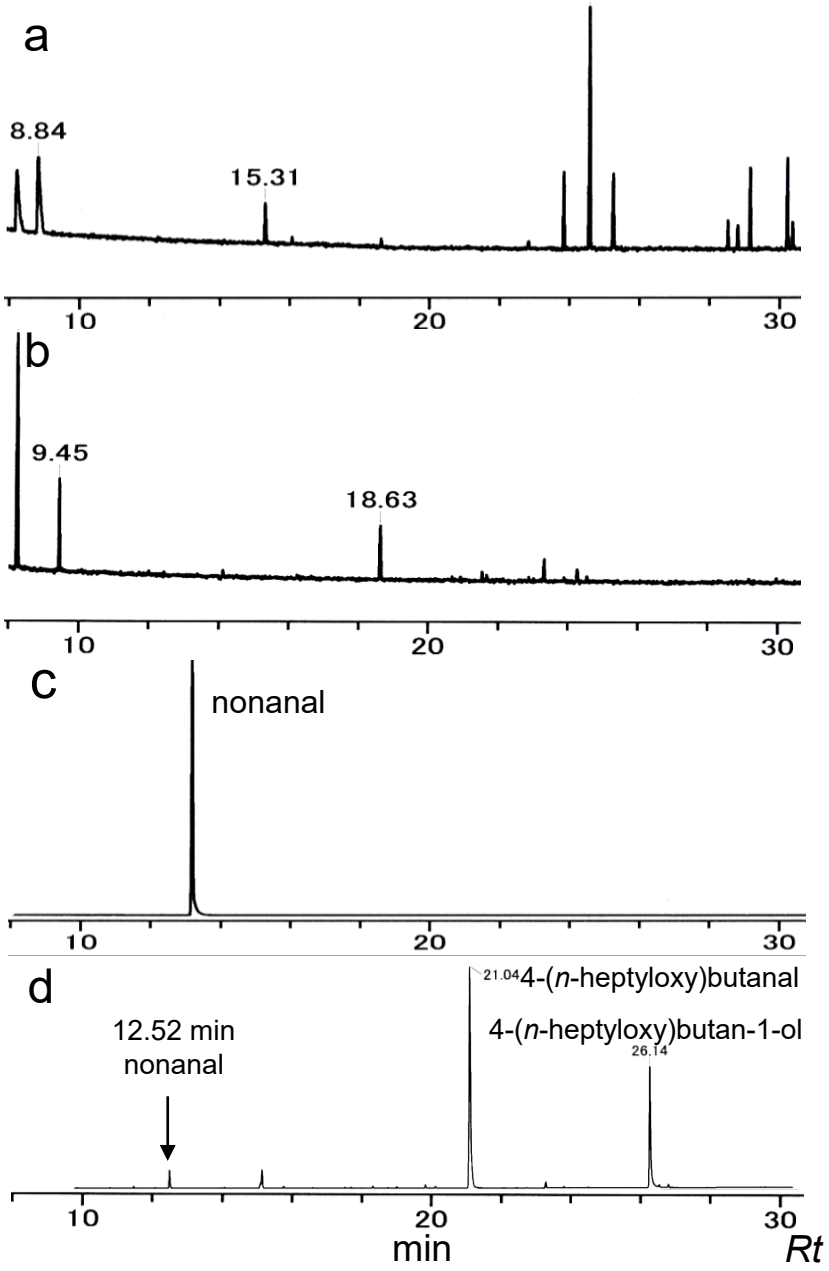

Fig. 2 Gas chromatography-mass spectrometry analysis of willow twig extracts.

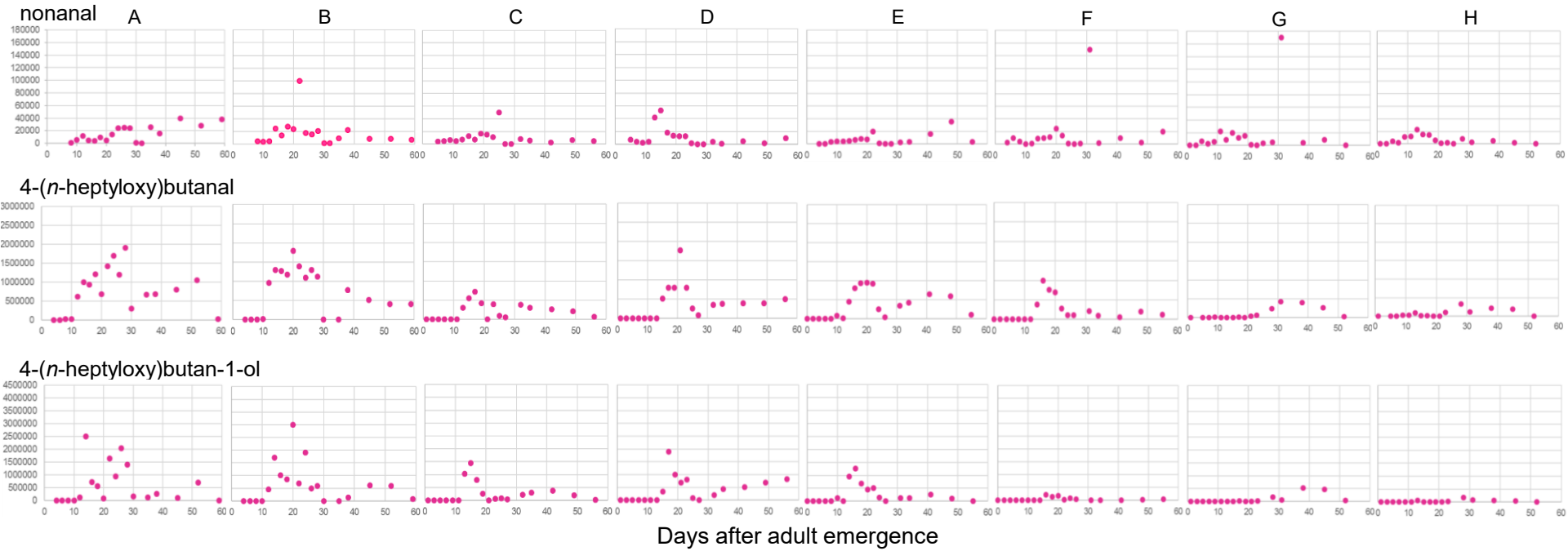

Fig. 3 Time course analysis of pheromone candidates in the individual male volatiles.

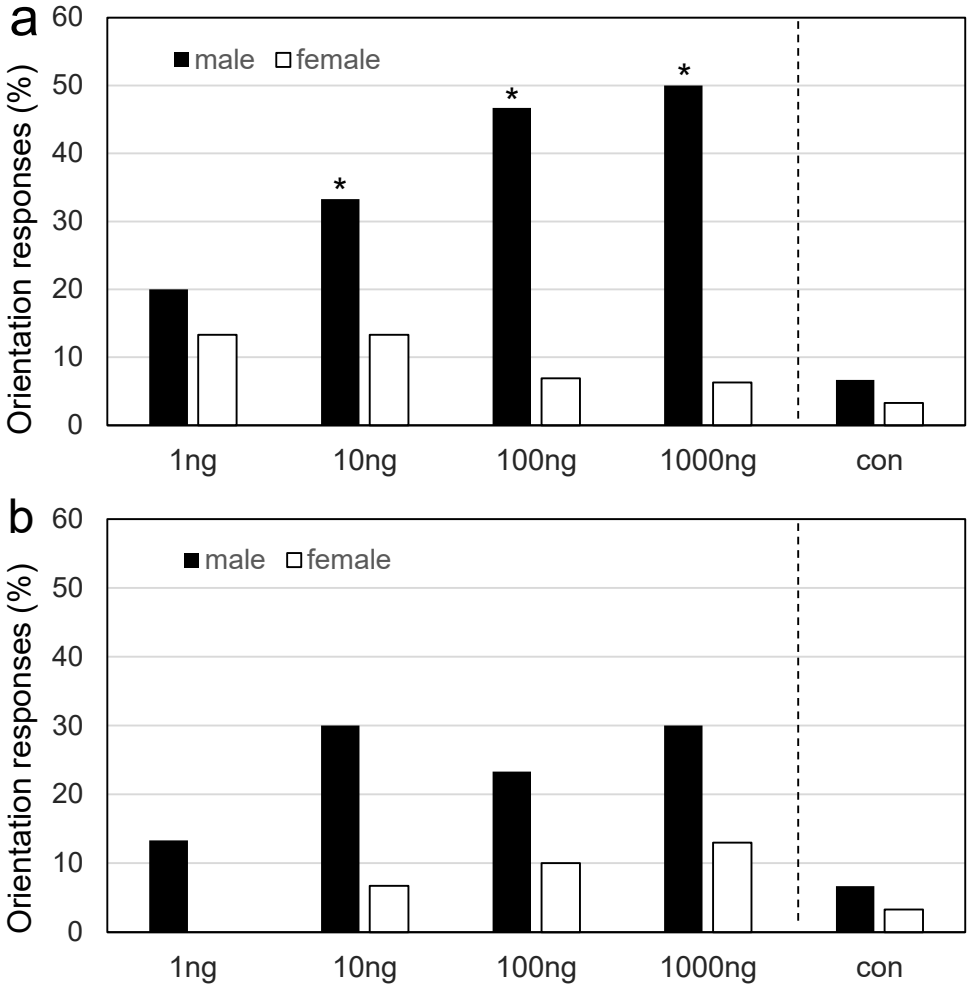

Fig. 4 Behavioral responses of *A. malasiaca* adults to synthetic pheromone candidates.

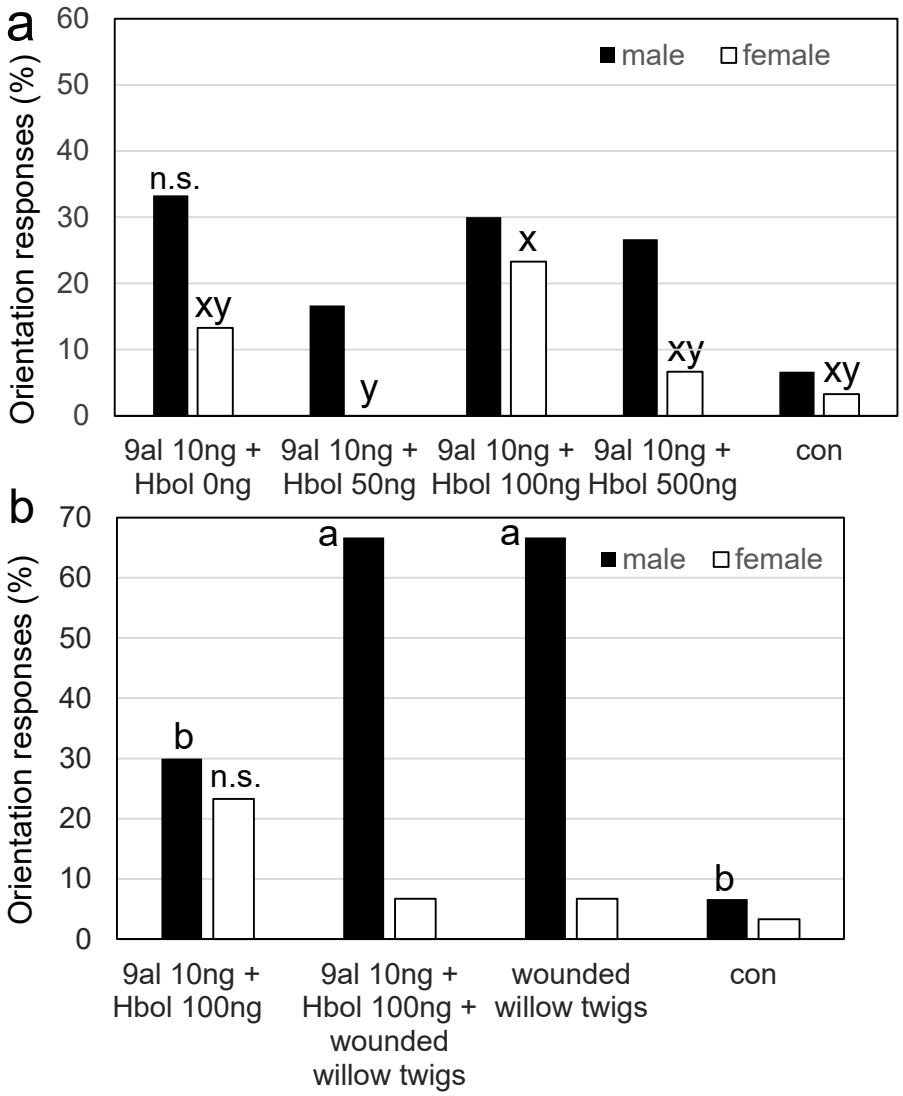

Fig. 5 Behavioral responses of *A. malasiaca* adults to volatile blends of synthetic pheromone candidates, nonanal (9al) and 4-(*n*-heptyloxy)butan-1-ol (Hbol), and wounded willow twigs.