Chemoecology (2017) 27:91–99
DOI 10.1007/s00049-017-0235-3

CHEMOECOLOGY

REVIEW

Electrophysiological and behavioral responses of *Dendroctonus armandi* (Coleoptera: Curculionidae: Scolytinae) to two candidate pheromone components: frontalin and *exo*-brevicomin

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Received: 21 June 2016 / Accepted: 4 April 2017 / Published online: 10 April 2017

Abstract The Chinese white pine beetle, *Dendroctonus armandi* Tsai and Li, is considered a serious native pest in the Qinling and Bashan Mountains of China. Relatively few information is available regarding its pheromone characterization, and the functions of its pheromones have not yet been identified. Gas chromatographic and mass spectrometry (GC–MS) analyses of volatiles collected from live *D. armandi* revealed that (1) virgin female and mated male but not mated female *D. armandi* produce frontalin and (2) female but not male beetles produce *exo*-brevicomin. Electroantennography (EAG) and Y-tube laboratory assays indicated that male *D. armandi* are more sensitive to frontalin and frontalin + α-pinene, whereas female *D. armandi* are more sensitive to frontalin + α-pinene and *exo*-brevicomin. These results support frontalin as a virgin female-produced sex pheromone, and frontalin + α-pinene as a virgin female and mated male-produced aggregation pheromone. Furthermore, different concentrations of *exo*-brevicomin have aggregation and anti-aggregation roles in female *D. armandi*. This study provides evidence for classifying the two compounds as certain types of pheromones and indicates that these pheromones have clear potential value for monitoring and controlling the outbreak of this serious pest.

Keywords *Dendroctonus armandi* · Pheromone · Gas chromatography-mass spectrometry (GC–MS) · Electrophysiological (EAG) · Y-tube assays

Introduction

Bark beetles (Curculionidae: Scolytinae) are considered destructive pests in natural and managed coniferous forests and have caused considerable economic losses (Coulson and Stark 1982; Furniss and Carolin 1977). The Chinese white pine beetle (*Dendroctonus armandi* Tsai and Li) kills mature *Pinus armandi* Franch, and has caused serious damage to mature *P. armandi* forests in the Qinling and Bashan Mountains of China (Yin et al. 1984; Chen and Yuan 2000). More than 3×10^8 m^3 of Chinese white pines in China have been killed by *D. armandi* since the 1970s (Xie and Lv 2012). *D. armandi* mainly attacks healthy Chinese white pines older than 30 years (Chen and Tang 2007), and in recent years, *D. armandi* has begun to attack younger *P. armandi* (10–30 years) and another pine species, *Pinus tabulaeformis* (Chen et al. 2015). *Dendroctonus armandi* completes its entire lifespan under the bark of *P. armandi* with the exception of a brief dispersal period, during which adults fly out to find new host trees. Similarly to most *Dendroctonus* species, *D. armandi* females primarily attack a tree and use certain semiochemicals to attract both males and females (Wood 1982; Borden et al. 1986; Raffa et al. 1993; Liu et al. 2006; Pureswaran et al. 2008; Xie and Lv 2012). The semiochemical communication of bark beetles enables host and mate location, aggregation, and resource partitioning (Wood 1982; Borden et al. 1986). The aggregation pheromone is required to ensure the successful colonization and reproduction of bark beetles. Therefore, the study of semiochemicals could aid

Handling Editor: Michael Heethoff.

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the protection of pine species and lower the primary beetle attack rate (Stephen 2001; Faccoli and Stergulc 2008; Blazenec and Jakus 2009).

The EAG responses of *D. armandi* to blended volatiles extracted from a host and certain synthetic terpenes showed significant variation with respect to different compound concentrations and the sex of the beetles (Wang et al. 2011a, b; Zhang et al. 2010, 2011). The effects of host volatiles from Chinese white pine, non-host volatiles and hindgut extracts of *D. armandi* on *D. armandi* were tested through laboratory olfactometer trials and field trapping experiments (Wu et al. 2012; Xie and Lv 2012; Zhao et al. 2014; Zhang et al. 2015; Chen et al. 2015). Some *Dendroctonus* species possess common pheromone components, although the sex of the species producing the pheromones and the functions of the components vary (Pitman et al. 1969). The semiochemical frontalin was first identified in male western pine beetles, *Dendroctonus brevicomis*, and was shown to serve as an aggregation pheromone component of the southern pine beetle, *Dendroctonus frontalis*, and the Douglas-fir beetle, *Dendroctonus pseudotsugae* (Silverstein et al. 1968; Bedard et al. 1970; Kinzer et al. 1969; Pitman and Vité 1970). Another semiochemical, *exo*-brevicomin, is produced by other *Dendroctonus* species (El Sayed 2013), some wood-boring Curculionidae (Fatzinger 1985; Perez et al. 1996) and Scolytinae (Francke et al. 1979; Camacho et al. 1993; Lindgren and Miller 2002), some *Thanasimus* predators (Zhou et al. 2001) and even the African elephant (Goodwin et al. 2006).

However, frontalin has not yet been detected in *D. armandi*, and a research study on frontalin in *D. armandi* is not found in the literature. Although *exo*-brevicomin has been detected in the aeration product of a *P. armandi* log subjected to natural *D. armandi* attacks and a hindgut extract of *D. armandi* females (mated and unmated) (Chen et al. 2015), the function of *exo*-brevicomin in *D. armandi* remains unclear. Moreover, only information regarding the details of the release and mechanisms of these semiochemicals is currently available. We identified two semiochemicals, frontalin and *exo*-brevicomin, by GC–MS and studied their biological roles in *D. armandi* by electrophysiological responses and laboratory olfactometer trials. These tests could provide a basis for future studies, suggesting that these compounds might be useful for beetle biocontrol.

Materials and methods

Insects

Two groups of bark beetles were prepared, and the bark beetles belonging to Group 1 were subjected to GC–MS analysis. The beetles belonging to Group 2 were subjected to electroantennography (EAG) and Y-tube assays in the laboratory.

Group 1: Beetles were collected from newly invaded Chinese white pines from the Qinling Mountains, Shaanxi, China (33°18′–33°28′N, 108°21′–108°39′E, elevation = 1450–1800 m) from June 25 to July 10, 2015 during a search for new invasive beetles from new invasive trees. The intrusion locations were marked, and the time points at which the beetle's trunk could be observed from outside as the beetles started to invade (but had not completely invaded) the bark of Chinese white pine were recorded. Forty-eight hours after the recording start time, half of these beetles were collected. If only one female beetle was included in the gallery, it was collected and recorded as a virgin female. If the tunnel included two or three beetles, the beetles were not collected. Ninety-six hours after the recording start time, the other half of the beetles were collected from the gallery. If two beetles were found in the gallery and were mating, they were recorded as a mated female beetle and a mated male beetle after their sexes were distinguished by listening for the stimulation produced by males (Xu et al. 2014). If only one female was found in the gallery, the beetle was not collected. The three types of bark beetles (virgin female, mated female and mated male) were divided into eight 50-mL glass vials. Each glass vial accommodated three bark beetles, and the heads of the glass vials were covered with plastic wrap, which was perforated to allow the bark beetles to breathe. The glass vials were immediately transported to the laboratory, and GC–MS tests were performed on the same day. Moreover, the frass at the entrance of the gallery was collected and divided into six glass vials (each vial contained 0.1 g).

Group 2: Logs of Chinese white pine infested with *D. armandi* from the southern slope of the middle Qinling Mountains (33°26′53.0″N, 108°28′48.3″E, at a mean altitude of 1500 m) were collected in November 2015. These logs were placed in a thin stainless-steel net (bore diameter ≤0.8 mm) in a greenhouse to allow the emergence of adult beetles and were watered daily to keep the log surfaces moist. Upon emergence, adult beetles were collected daily from these logs, transported to the laboratory, sorted by sex and stored at 4 °C on moist paper. Active beetles with intact antennae and legs were subjected to electrophysiological and Y-tube assays (Light 1983; Wood 1982).

Chemicals

The chemicals used for the EAG and laboratory bioassays were (±)-*exo*-brevicomin (>95% chemical purity) and frontalin (>95% c. p.), which were obtained from Contech Enterprises Inc., Delta, BC, Canada, and hexane (HPLC-certified), which was obtained from Sigma-Aldrich Co.

Collection and identification of volatiles

According to the procedures described by Pureswaran and Borden (2003) and Liu et al. (2013), the following three sets of beetles were sampled for pheromone analysis: (1) virgin females, (2) mated females, and (3) mated males. The volatiles released from these beetles were passively collected by headspace solid-phase microextraction (HS–SPME), which is the technique used for the extraction and concentration of volatile compounds (Chai et al. 2012; Keenan et al. 2012). Three beetles in a 50-mL vial were transported to the laboratory for the collection of volatile compounds. A SPME fiber coated with a 75-μm film thickness of divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) obtained from Supelco (Sigma-Aldrich, Bellefonte, PA, USA) was exposed to the headspace of each vial for 10 min. This fiber was selected for its suitability for gasses and compounds with a low molecular mass. Prior to use, the fiber was preconditioned in the injection port of the gas chromatography at 270 °C for 60 min according to the manufacturer's instructions.

Extracts were analyzed using a DB-5 MS column (30 m × 0.25 mm × 0.25 μm) (Thermo Fisher Scientific Company). The SPME fiber was injected directly into the injection port at a temperature of 250 °C for 3 min. The temperature of the GC-oven was maintained at 40 °C for 2.5 min, increased to 240 °C at a rate of 6 °C/min and maintained at 240 °C for 10 min. The flow of helium (carrier gas) was 1.0 mL/min. Compounds were identified by a comparison of their retention times and mass spectra to those in the NIST and Varian libraries. Furthermore, the retention times and mass spectra of *exo*-brevicomin and frontalin detected from the GC–MS experiment were also compared with the purchased standards.

EAG assays

The test method used was that described by Zhang et al. (2010). Antennae were excised from the heads of *D. armandi* and mounted between two electrodes. The recording electrode was inserted into the distal edge of the club, and the indifferent electrode was inserted into the scape of the antenna. In the experiments, 20 μL of each test solution was absorbed onto a filter paper strip (5 × 50 mm) and placed into a Pasteur pipette (with a diameter of 10 mm and a length of 15 cm) to serve as an odor cartridge after the solvent (hexane) had completely evaporated from the paper. Controls consisting of a filter paper strip treated with 20 μL of hexane alone were prepared. A Pasteur pipette with a test compound was inserted into a small hole in the wall of a steel tube (with a diameter of 15 mm diameter and a length of 15 cm), which was connected to a stimulus air controller (model CS-05b, Syntech, the Netherlands) to deliver a constant flow of humidified air at a rate of 40 mL/min. The open end of the steel tube was positioned 10 mm from the antenna. During the stimulation, air at a rate of 20 mL/min was applied through the pipette into the main airflow for 0.2 s. An interval of at least 1 min between puffs was used to ensure complete antenna recovery. Serial dilutions of each compound from the lowest to the highest concentration were included (Delorme and Payne 1990; Den Otter et al. 1996). A hexane-only control was tested before and after each test compound. A male or female antenna was only tested with one type of compound (Zhang et al. 2010). A hexane-only control and a standard solution (1-hexanol at 1 ng/μL in hexane) were tested before and after each test pheromone. Each pheromone was tested on five male and five female beetles, and the trials with each EAG preparation were repeated at least five times.

Laboratory olfactometer trials

Bioassays were conducted in a glass Y-tube olfactometer (with a diameter of 35 mm, a length of 40 cm, and an inside angle of 120°) with airflow at a rate of 200 mL/min through each branch using the method described by Liu et al. (2013). Four sets of tests were performed. In the first set of tests, one chamber contained a filter paper that had been treated with hexane, and the other chamber contained a filter paper that had been treated with frontalin diluted in hexane (0.0025, 0.05, 0.5, or 5 ng/μL, 10 μL). In the second set of tests, one chamber contained a filter paper that had been treated with hexane, and the other chamber contained a filter paper that had been treated with *exo*-brevicomin diluted in hexane (0.0025, 0.05, 0.5, or 5 ng/μL, 10 μL). The third set of tests investigated the attraction of beetles to frontalin in hexane (0.05 or 0.5 ng/μL, 10 μL) compared with α-pinene, to frontalin in hexane (0.05 ng/μL, 10 μL) compared with frontalin in α-pinene (0.05 ng/μL, 10 μL), to frontalin in hexane (0.5 ng/μL, 10 μL) compared with frontalin in α-pinene (0.5 ng/μL, 10 μL), and to frontalin in α-pinene (0.05 or 0.5 ng/μL, 10 μL) compared with α-pinene. The above-mentioned α-pinene compound was used without prior dilution. The fourth set of tests investigated the attraction of beetles to *exo*-brevicomin in hexane (0.05 or 0.5 ng/μL, 10 μL) compared with α-pinene, to *exo*-brevicomin in hexane (0.05 ng/μL, 10 μL) compared with *exo*-brevicomin in α-pinene (0.05 ng/μL, 10 μL), to *exo*-brevicomin in hexane (0.5 ng/μL, 10 μL) compared with *exo*-brevicomin in α-pinene (0.5 ng/μL, 10 μL), and to *exo*-brevicomin in α-pinene (0.05 or 0.5 ng/μL, 10 μL) compared with α-pinene. The above-mentioned α-pinene compound was used without dilution, and as the control at the dose of 10 μL. Approximately 30 min before each trial, adult beetles were

introduced into a separate holding tube and exposed to the test odors before being released. Moreover, 10 μL of each test chemical was placed on a filter paper strip (5 × 50 mm) and allowed to evaporate for 20 s, and the filter paper was then placed into a holding chamber. In each trial, a solitary beetle was released into the stem of the Y-tube and was given 10 min to respond. When the beetle walked 5 cm past the Y junction, its selection of the left or right branch of the olfactometer was noted. To eliminate directional bias, the treatments associated with the right and left branches of the olfactometer were exchanged after every trial, and the Y tubes were cleaned with 100% alcohol before reuse. The olfactometer was maintained at a temperature of 25 °C and a relative humidity of 70% during the trials. At least 30 females and 30 males were tested in each trial.

Statistical analysis

The EAG responses were corrected for solvent and other background effects by subtracting the mean response to the solvent-only controls before and after exposure to each sample from the response to the test compound. To compensate for the decline in the sensitivity of the antennae during the experiment and individual differences in the test insects, the EAG data were standardized by calculating the EAGs as percentages relative to the response to the standard solution, which allowed direct comparisons of the responses obtained from different preparations. Mann–Whitney tests were performed using SPSS (1999) to determine the significance of differences in the relative EAG responses between the sexes. Chi square tests were performed using SPSS (1999) to assess the differences in the responses to the different compounds measured through the olfactometer bioassays.

Results

Collection and identification of volatiles

As identified by GC–MS, eight major volatile compounds (α-pinene, frontalin, *exo*-brevicomin, *trans*-verbenol, verbenone, myrtenal, myrtenol, and myrtanol) were released by *D. armandi* (Table 1). The experimental results showed that the presence/absence of frontalin, *exo*-brevicomin and myrtenal significantly differed between the sexes. Frontalin was detected in three of eight groups of virgin females and in all eight tested groups of mated males. Frontalin was not detected in any of the eight groups of mated females tested. *exo*-Brevicomin was detected in six of eight groups of virgin females and in seven of eight groups of mated females. *exo*-Brevicomin was not detected in any of the eight groups of mated males tested. Myrtenal was detected in three of eight groups of virgin females and in one of eight groups of mated females. Myrtenal was not detected in any of the eight groups of mated males tested. In addition, α-pinene, *trans*-verbenol, verbenone, myrtenol and myrtenol were emitted by both female and male beetles might function as pheromones.

EAG assays

The EAG results revealed that frontalin and *exo*-brevicomin elicited olfactory responses from *D. armandi* as follows (Fig. 1). The antennae of male adult *D. armandi* were more sensitive to frontalin at dose of 0.05, 1, 10 and 100 ng (0.0025, 0.05, 0.5 and 5 ng/μL, 20 μL). Mann–Whitney tests showed significant differences ($p \leq 0.01$) between the sexes in the antennal responses to 10 ng frontalin (0.5 ng/μL, 20 μL) ($P_{0.0025\ ng/\mu L} = 0.117$, $P_{0.05\ ng/\mu L} = 0.076$, $P_{0.5\ ng/\mu L} = 0.009$, $P_{5\ ng/\mu L} = 0.117$).

Table 1 Percentage composition of identified volatiles released by *D. armandi*

	Virgin females ($n = 8$)	Mated females ($n = 8$)	Mated males ($n = 8$)	Frass[b] ($n = 6$)
α-Pinene	44.92 ± 16.47 (8)	43.53 ± 9.45 (8)	36.85 ± 16.50 (8)	93.01 ± 5.14 (6)
Frontalin	2.18 ± 2.54 (3)	ND[a]	5.31 ± 5.18 (8)	0.73 ± 0.59 (2)
exo-Brevicomin	17.42 ± 8.83 (6)	18.84 ± 5.2 9 (7)	ND[a]	ND[a]
trans-verbenol	8.68 ± 7.06 (2)	14.56 ± 1.96 (5)	14.23 (1)	1.03 ± 0.38 (4)
Verbenone	30.76 ± 5.52 (5)	30.23 ± 4.71 (2)	34.44 ± 15.60 (8)	7.27 ± 5.36 (5)
Myrtenal	4.73 ± 4.10 (3)	0.29 (1)	ND[a]	ND[a]
Myrtenol	21.09 ± 5.18 (6)	21.66 ± 5.00 (8)	20.11 ± 6.96 (8)	ND[a]
Myrtanol	3.53 ± 0.52 (5)	3.28 ± 0.33 (4)	4.06 ± 1.19 (3)	ND[a]

The values shown are the mean ± SE, and the values in parentheses are the number of samples in which the component was identified

[a] ND indicates that the pheromone was not detected

[b] Frass samples were collected at the entrance of the gallery

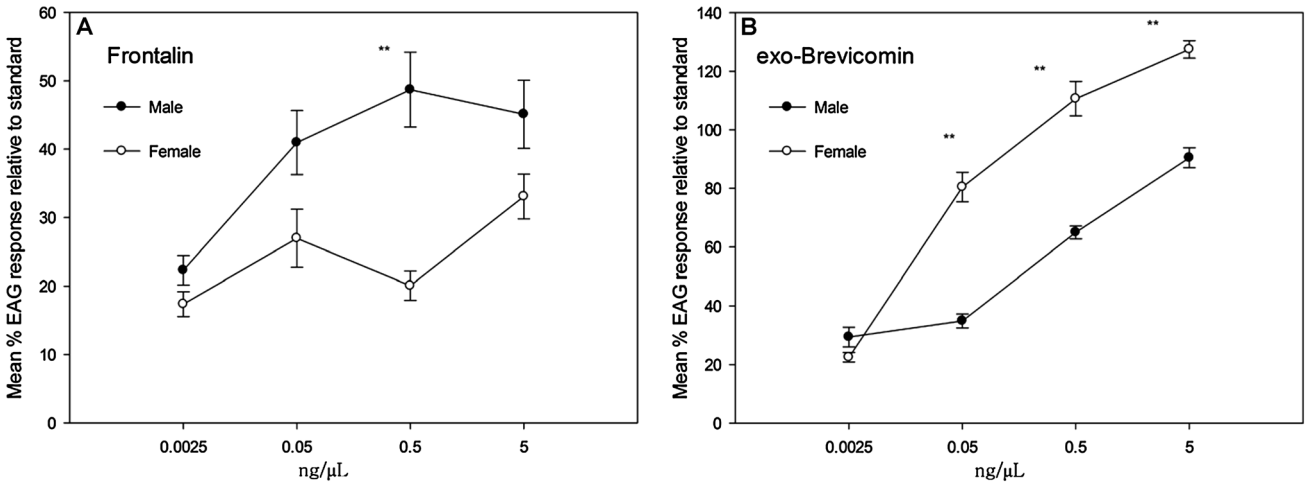

Fig. 1 EAG responses (mean ± SE) of *D. armandi* to frontalin and *exo*-brevicomin. The *error bars* represents the standard errors of five replicates. *Asterisks* indicate a significant difference between male and female beetles at the same concentration (*t* test; $*p \leq 0.05$; $**p \leq 0.01$)

The antennae of female adult *D. armandi* were more sensitive to *exo*-brevicomin at dose of 1, 10 and 100 ng (0.05, 0.5 and 5 ng/μL, 20 μL). ($p \leq 0.01$) ($P_{0.0025\ ng/\mu L} = 0.175$, $P_{0.05\ ng/\mu L} = 0.009$, $P_{0.5\ ng/\mu L} = 0.009$, $P_{5\ ng/\mu L} = 0.009$).

Laboratory olfactometer trials

Y-tube olfactometer assays with frontalin showed that males were more attracted to frontalin at certain concentrations (0.05 and 0.5 ng/μL, 10 μL) than to the hexane control (Table 2). Instead, females were not attracted to frontalin at any concentration tested. The comparison of α-pinene and frontalin as stimuli in the choice assay revealed that males were significantly more attracted to frontalin than α-pinene (Table 3), and females were not. The mixture of frontalin with α-pinene showed that both females and males preferred the frontalin + α-pinene blend over α-pinene alone.

The Y-tube olfactometer assays with various concentrations of *exo*-brevicomin showed that females were more attracted to certain concentrations of *exo*-brevicomin (0.05 ng/μL, 10 μL) and repelled it when the concentration

Table 2 Attraction of male and female *D. armandi* to various concentrations of frontalin in Y-tube assays

		Males	Females	χ^2
$F_{0.0025\ ng/\mu L}$ × Hexane	$F_{0.0025\ ng/\mu}$	25	15	
	Hexane	25	16	
	χ^2	0	0.032	0.02
$F_{0.05\ ng/\mu L}$ × Hexane	$F_{0.05\ ng/\mu L}$	36	17	
	Hexane	21	14	
	χ^2	3.947*	0.29	0.58
$F_{0.5\ ng/\mu L}$ × Hexane	$F_{0.5\ ng/\mu L}$	27	13	
	Hexane	14	18	
	χ^2	4.122*	0.806	4.090*
$F_{5\ ng/\mu L}$ × Hexane	$F_{5\ ng/\mu L}$	16	24	
	Hexane	28	21	
	χ^2	3.273	0.200	2.589

The table entries represent the numbers of *D. armandi* of each sex that responded to either the control or treatment stimulus

The asterisks indicate significant differences (* $p \leq 0.05$, ** $p \leq 0.01$, Chi-squared test)

Table 3 Attraction of male and female *D. armandi* to frontalin + α-pinene blend determined through Y-tube assays

		Males	Females	χ^2
$F/H_{0.05\ ng/\mu L}$ × α-p	$F_{0.05\ ng/\mu L}$	23	23	
	α-p	10	14	
	χ^2	5.121*	2.189	0.440
$F/H_{0.5\ ng/\mu L}$ × α-p	$F_{0.5\ ng/\mu L}$	27	19	
	α-p	8	13	
	χ^2	10.314**	1.125	2.452
$F/\alpha\text{-}p_{0.05\ ng/\mu L}$ × α-p	$F/\alpha\text{-}p_{0.05\ ng/\mu L}$	26	23	
	α-p	10	11	
	χ^2	7.111**	4.235*	0.174
$F/\alpha\text{-}p_{0.5\ ng/\mu L}$ × α-p	$F/\alpha\text{-}p_{0.5\ ng/\mu L}$	24	31	
	α-p	7	16	
	χ^2	9.323**	4.787*	1.180

The table entries represent the numbers of *D. armandi* of either sex that responded positively to either the positive control (α-p) or treatment stimulus. The data show the bioassay results for frontalin in α-p at concentrations of 0.05 and 0.005 ng/μL. α-p and F represent α-pinene and frontalin, respectively. $F/H_{0.05\ ng/\mu L}$ and $F/H_{0.5\ ng/\mu L}$ refer to frontalin in hexane at concentrations of 0.05 and 0.005 ng/μL, respectively, and $F/\alpha\text{-}p_{0.05\ ng/\mu L}$ and $F/\alpha\text{-}p_{0.5\ ng/\mu L}$ refer to frontalin in α-p at concentrations of 0.05 and 0.005 ng/μL, respectively

The asterisks indicate significant differences (* $p \leq 0.05$, ** $p \leq 0.01$, Chi-squared test)

Table 4 Attraction of male and female *D. armandi* to various concentrations of *exo*-brevicomin in Y-tube assays

		Males	Females	χ^2
$B_{0.0025\ ng/\mu L}$ × Hexane	$B_{0.0025\ ng/\mu}$	16	15	
	Hexane	17	18	
	χ^2	0.030	0.273	0.061
$B_{0.05\ ng/\mu L}$ × Hexane	$B_{0.05\ ng/\mu L}$	15	27	
	Hexane	19	13	
	χ^2	0.471	4.900*	4.094*
$B_{0.5\ ng/\mu L}$ × Hexane	$B_{0.5\ ng/\mu L}$	18	10	
	Hexane	14	23	
	χ^2	0.500	5.121*	4.461*
$B_{5\ ng/\mu L}$ × Hexane	$B_{5\ ng/\mu L}$	13	16	
	Hexane	19	16	
	χ^2	1.125	0	0.567

The table entries represent the numbers of *D. armandi* of either sex that responded to either the control or treatment stimulus

The single and double asterisks (* and **) indicate significant differences at $p \leq 0.05$ and $p \leq 0.01$, respectively (Chi-squared test)

Table 5 Attraction of male and female *D. armandi* to *exo*-brevicomin + α-pinene blend as determined through Y-tube assays

		Males	Females	χ^2
$B/H_{0.05\ ng/\mu L}$ × α-p	$B/H_{0.05\ ng/\mu L}$	20	25	
	α-p	12	9	
	χ^2	2.000	7.529**	0.924
$B/H_{0.5\ ng/\mu L}$ × α-p	$B/H_{0.5\ ng/\mu L}$	22	24	
	α-p	9	6	
	χ^2	5.452*	10.800**	0.671
$B/\alpha\text{-}p_{0.05\ ng/\mu L}$ × α-p	$B/\alpha\text{-}p_{0.05\ ng/\mu L}$	19	20	
	α-p	12	13	
	χ^2	1.581	1.485	3.065
$B/\alpha\text{-}p_{0.5\ ng/\mu L}$ × α-p	$B/\alpha\text{-}p_{0.5\ ng/\mu L}$	17	11	
	α-p	14	23	
	χ^2	0.290	4.235*	3.344

The table entries represent the numbers of *D. armandi* of either sex that responded positively to either the positive control (α-p) or treatment stimulus. The data show the bioassay results for *exo*-brevicomin in α-p at concentrations of 0.05 and 0.005 ng/μL. α-p and B represent α-pinene and *exo*-brevicomin, respectively. $B/H_{0.05\ ng/\mu L}$ and $B/H_{0.5\ ng/\mu L}$ refer to *exo*-brevicomin in hexane at concentrations of 0.05 and 0.005 ng/μL, respectively, and $B/\alpha\text{-}p_{0.05\ ng/\mu L}$ and $B/\alpha\text{-}p_{0.5\ ng/\mu L}$ refer to *exo*-brevicomin in α-p at concentrations of 0.05 and 0.005 ng/μL, respectively

The asterisks indicate significant differences (* $p \leq 0.05$, ** $p \leq 0.01$, Chi-squared test)

of *exo*-brevicomin raised to 0.5 ng/μL, 10 μL (Table 4), whereas males were not attracted to any concentration of *exo*-brevicomin tested. The comparison of α-pinene and *exo*-brevicomin as stimuli in the choice assay showed that females reacted significantly more to *exo*-brevicomin (0.05 and 0.5 ng/μL, 10 μL) than α-pinene (Table 5), males were more attracted to *exo*-brevicomin at 0.5 ng/μL, 10 μL. The mixture of *exo*-brevicomin with α-pinene revealed that the reactions of the males to the *exo*-brevicomin + α-pinene blend (0.05 and 0.5 ng/μL, 10 μL) and α-pinene were not significantly different, whereas females preferred α-pinene compared with the *exo*-brevicomin + α-pinene blend at a concentration of 0.5 ng/μL, 10 μL.

Discussion

We identified frontalin and *exo*-brevicomin in the volatile compounds produced by male and female *D. armandi* by GC–MS and demonstrated that the antennae of both sexes showed an electrophysiological response to frontalin and *exo*-brevicomin. We also confirmed that males exhibited a dose-dependent behavioral response to frontalin and frontalin + α-pinene, and females to frontalin + α-pinene and *exo*-brevicomin in olfactometer bioassays. *Dendroctonus* spp. are known to use semiochemicals and host volatiles to colonize hosts and attract mates (Wood 1982; Schlyter and Birgersson 1999; Byers and Zhang 2012). Semiochemicals might be a key characteristic trait that allows beetles to overcome the defense system of host trees, making it a necessary trait for the success of mass attacks by *Dendroctonus* spp, such as the synergism between semiochemicals and host volatiles in *D. valens, D. ponderosae* and *D. brevicomis* (Chen et al. 2015). The bicyclic acetals frontalin, *exo*- and *endo*-brevicomin and the hydroxylation products of α-pinene are often identified as pheromones of *Dendroctonus* spp. (Gary et al. 2010).

Recent GC–MS analyses have mainly focused on the hindgut, fat body, insect odor collection and host logs odor collection (Chen et al. 2015; Xie and Lv 2012; Liu et al. 2013; Song et al. 2014), and the beetles are usually collected after long-term indoor feeding; as a result, the experimental results might yield differences compared with beetles collected from the field. This manuscript describes the first experimental GC–MS and HS-SPME analysis of invaded *Dendroctonus* spp. collected from the field to more accurately reveal the pheromones released by *D. armandi* in nature. The GC–MS experiment aimed to compare the differences in the presence or absence of pheromones between the sexes. The results of the GC–MS analysis of *D. armandi* pheromones show that the release of frontalin and *exo*-brevicomin differs between sexes and over time. Frontalin and *exo*-brevicomin might play a significant role in aiding the invasion of *D. armandi*. What is more, because frontalin and *exo*-brevicomin have similar material structures and play an important role as pheromones in *Dendroctonus* spp., so this study focused on exploring the

functions and roles of frontalin and *exo*-brevicomin in *D. armandi*. The identity and function of *trans*-verbenol and verbenone were characterized by EAG, Y-tube assays and field trapping (Zhao et al. 2017). The properties and functions of myrtenal, myrtenol and myrtanol need to be further studied.

Frontalin was first identified in a virgin female and was not identified in mated females. After mating, females constructed egg galleries, and males were found to be responsible for the release of frontalin + α-pinene to attract more beetles (Table 1). For convenience, we divided the process of semiochemical release into two stages: (1) after a successful attack, females released semiochemicals to attract more female and male beetles, and (2) after mating, females and males released semiochemicals to adjust the population density. The EAG dose–response curve and laboratory olfactometer trials of *D. armandi* to frontalin showed that male beetles were more sensitive to frontalin than female beetles (Fig. 1; Tables 2, 3). Although females were not attracted to frontalin at all tested concentrations (Table 2), the response of females and the control group to frontalin supplemented with α-pinene presented significant differences ($p \leq 0.05$; Table 3). Females were attracted to the mixture of frontalin and α-pinene. α-Pinene was the main volatile (accounting for 63.18%) in host Chinese white pine (Chen et al. 2015), which was also accounting for >30% in the volatile of *D. armandi* and accounting for >90% in the frass (Table 1). So frontalin will have chance to co-work with α-pinene in a natural environment. The Y-tube experimental results (Table 3) revealed that frontalin + α-pinene played an important role in attracting females and males. Collectively, these lines of evidence supported our conclusion that frontalin worked together with α-pinene as an aggregation pheromone released by virgin females to attract females and males during the first stage. Males were more attracted to low concentration frontalin (Table 2) and no response to high concentration frontalin. Virgin females released low concentration frontalin (2.18 ± 2.54%) (Table 1), low concentration frontalin single might play a role as a sex pheromone to attract males during the first stage. Still frontalin + α-pinene became an aggregation pheromone released by mated males to attract more females and males in the second stage. High concentration frontalin (5.31 ± 5.18%) (Table 1) was released by mated males, which lost its attraction for males at this time. Frontalin has been identified as an anti-aggregation pheromone in certain *Dendroctonus* species and an aggregation pheromone in others (Borden 1985). In *D. valens,* frontalin is only produced by females as an aggregation and sex pheromone (Liu et al. 2013), and frontalin is an anti-aggregation pheromone in *D. ponderosae* (Conn et al. 1984; Hunt et al. 1986; Greis et al. 1990; Pureswaran et al. 2000). The experimental results reveal that the role of frontalin in *D. armandi* is adverse to that in *D. ponderosae.* In contrast with *D. valens*, mated male *D. armandi* also release frontalin during the second stage. *D. armandi* beetles are the pioneering invaders of healthy Chinese white pine and might, therefore, need more companions to overcome the defense of host trees than *D. valens. D. armandi* males might release the aggregation pheromone frontalin + α-pinene to attract more males and females, which might help them overcome the defense reactions of the host. The biggest difference maybe was that frontalin worked together with α-pinene as an aggregation pheromone in *D. armandi,* while frontalin alone played its role in the *D. valens* and *D. ponderosae.* The function and presence/absence of frontalin in *D. armandi, D. valens* and *D. ponderosae* are different, and these differences might be related to the physiological adaptations of these species to different habitats, as well as their specific life cycles and specific behavior habits.

The release of semiochemicals by bark beetles usually occurs through frass, as has been observed for *D. brevicomis*, *Dendroctonus micans*, *D. ponderosae, D. valens* and *Dendroctonus rufipennis* (Silverstein et al. 1968; Meurisse et al. 2008; Nancy et al. 2006; Borden et al. 1996). The present experimental results, frontalin was detected in frass collected from galleries of *D. armandi* (Table 1), supported frontalin as one semiochemical of *D. armandi*.

The collection and identification of volatiles revealed that *exo*-brevicomin was released by females. *endo*-Brevicomin is produced by male *D. ponderosae* following a pattern parallel to that found for *exo*-brevicomin (Pureswaran et al. 2000), but GC–MS experiments have not detected *endo*-brevicomin in the volatiles from *D. armandi*. The similar results were detected in Chen's experiment (Chen et al. 2015). Moreover, α-pinene, a host monoterpene, preferred over *exo*-brevicomin. α-Pinene has been found to be attractive for female and male *D. armandi*, as determined through electroantennography, and has been used as a lure pheromone for trapping *D. armandi* (Zhang et al. 2010; Xie and Lv 2012). The response of both sexes to *exo*-brevicomin determined through olfactometer bioassays and EAG experiments revealed that females were more sensitive to *exo*-brevicomin. Our research study provides evidence that *exo*-brevicomin plays a role as an aggregation pheromone in females or as spacing factor signaling that "the tree is blank" or "the tree is full" to other females to adjust the population numbers. In addition, *exo*-brevicomin is an aggregation pheromone component in *D. ponderosae* (Conn et al. 1984; Hunt et al. 1986; Greis et al. 1990; Pureswaran et al. 2000). Prior to our study, *exo*-brevicomin was detected in the aeration product of *P. armandi* logs subjected to natural *D. armandi* attacks and the hindgut extract of females (Chen et al. 2015). However,

the mating status of females was not distinguished, and the hindgut extract of males was not investigated, resulting in an inexplicit role of *exo*-brevicomin. This conclusion could be inferred because only female *D. armandi* were thought to produce volatiles as pheromone candidates, and the mating status of female beetles were not considered. However, male beetles could also release the pheromone, and the difference due to mating status was found to be significant. A similar pattern was found for frontalin, which is released by males and not released by mated females. Moreover, field-trapping experiments of *D. armandi* were performed (Chen et al. 2015), and the role of *exo*-brevicomin in un-distinguished sexes remains unclear. Further field-trapping experiments are needed to determine the role of *exo*-brevicomin.

In summary, this manuscript describes the first detection and behavioral analysis of frontalin and *exo*-brevicomin as possible semiochemicals of *D. armandi,* shedding light on the biological activities and roles of frontalin and *exo*-brevicomin through electrophysiological and laboratory olfactometer trials. Our results demonstrate that frontalin might play a role as a sex semiochemical and work together with α-pinene as aggregation pheromone, and provide evidence that *exo*-brevicomin serves as an aggregation and anti-aggregation pheromone in female *D. armandi*. Further studies should be performed to confirm the behavioral activity of both sexes of *D. armandi* to these two semiochemicals.

Acknowledgements We acknowledge the financial support provided by the National Natural Science Foundation of China (31670658) and the Program for Changjiang Scholars and Innovative Research Team at the University of China (IRT1035).

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