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# Sound-Triggered Production of Antiaggregation Pheromone Limits Overcrowding of *Dendroctonus valens* Attacking Pine Trees

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## Abstract

For insects that aggregate on host plants, both attraction and antiaggregation among conspecifics can be important mechanisms for overcoming host resistance and avoiding overcrowding, respectively. These mechanisms can involve multiple sensory modalities, such as sound and pheromones. We explored how acoustic and chemical signals are integrated by the bark beetle *Dendroctonus valens* to limit aggregation in China. In its native North American range, this insect conducts nonlethal attacks on weakened trees at very low densities, but in its introduced zone in China, it uses mixtures of host tree compounds and the pheromone component frontalin to mass attack healthy trees. We found that *exo*-brevicomin was produced by both female and male *D. valens*, and that this pheromone functioned as an antiaggregating signal. Moreover, beetles feeding in pairs or in masses were more likely than were beetles feeding alone to produce *exo*-brevicomin, suggesting a potential role of sound by neighboring beetles in stimulating *exo*-brevicomin production. Sound playback showed that an agreement sound was produced by both sexes when exposed to the aggregation pheromone frontalin and attracts males, and an aggressive sound was produced only by males behaving territorially. These signals triggered the release of *exo*-brevicomin by both females and males, indicating an interplay of chemical and sonic communication. This study demonstrates that the bark beetle *D. valens* uses sounds to regulate the production of an antiaggregation pheromone, which may provide new approaches to pest management of this invasive species.

**Key words:** antiaggregation, bark beetle, *Dendroctonus valens*, *exo*-brevicomin, sound

## Introduction

Among herbivores that aggregate on host plants, mechanisms for attracting and repelling conspecifics can be critical to overcoming host defense and avoiding overcrowding, respectively. Bark beetle (Coleoptera: Curculionidae: Scolytinae) adults construct galleries and oviposit in the phloem under tree bark, where larvae feed and develop (Wood 1982). Most species colonize only dead, diseased, or severely weakened hosts (Raffa et al. 1993), but a few, particularly

in the genera *Scolytus* Geofroy, *Dendroctonus* Erichson, and *Ips* DeGeer, can colonize and kill healthy trees (Wood 1982). Successful colonization of vigorous trees by these species relies on emission of aggregation pheromones that mediate synchronous attacks by numbers that are sufficiently large to overwhelm host defenses that, otherwise, would kill or expel fewer invaders (Raffa and Berryman 1983; Seybold et al. 2006). Host colonization includes 4 phases: initial attack, developing attack, mass attack, and switching (Payne 1980). Switching is a process by which aggregation on one tree terminates, and neighboring trees become foci of aggregation.

Several nonexclusive hypotheses have been proposed for how mass attacks are terminated. One hypothesis is that stridulation by beetles stimulates the production of antiaggregation pheromones (Rudinsky 1969; Pitman and Vite 1974; Rudinsky et al. 1976). Another is that once beetle densities are high, the emission of certain compounds, including some that are attractive in low amounts, increases to concentrations that are inhibitory (Renwick and Vité 1969; Kinzer et al. 1971; Zhang et al. 2006). Pioneering work of by Rudinsky and others launched our understanding of how sound influences beetles' behavior and pheromone production (Barr 1969; Rudinsky 1969; Rudinsky and Michael 1972; Rudinsky and Michael 1974; Rudinsky et al. 1976; Spangler 1987). Rudinsky (1969) showed that sound stimulates the production of methylcyclohexenone (MCH) by the Douglas-fir beetle *Dendroctonus pseudotsugae* Hopkins, which in turn stops the aggregation phase (Rudinsky et al. 1976). Further, *D. pseudotsugae* produce MCH when exposed to sounds, and MCH is attractive at low concentration and repellent at high concentrations (Rudinsky 1969; Rudinsky et al. 1976). No subsequent bark beetle has been reported to use this strategy to terminate aggregation.

Some bark beetle species do not undergo aggregation, and typically colonize trees in single attacks or in only a few attacks in which beetles appear to arrive independently in response to host volatiles. Some examples include the red turpentine beetle (*Dendroctonus valens* LeConte) (Aukema et al. 2010; Owen et al. 2010) and black turpentine beetle (*Dendroctonus terebrans* Olivier) in North America (Wood 1982), and *D. micans* in Europe (Grégoire 1988). *Dendroctonus valens* is of particular interest because it behaves differently in its native range versus its introduced range in China. In North America, this insect often colonizes stumps, or may conduct nonlethal attacks on stressed live trees that are subsequently attacked by lethal, mass-attacking species (Owen et al. 2005; Aukema et al. 2010). Since its introduction into China, however, this beetle has killed over 7 million Chinese pine *Pinus tabuliformis* since its first outbreak in Shanxi Province, China (Yan et al. 2005; Qiu 2013). In its invaded range, *D. valens* adults attack both healthy large trees and freshly cut stumps (Liu et al. 2008; Liu et al. 2011; Xu et al. 2014). In addition to being attracted to the host compound 3-carene, *D. valens* females in China produce frontalin, which is highly attractive to males and moderately attractively to females at some concentrations, and which enhances the attractiveness of 3-carene to both sexes (Liu et al. 2013).

*D. valens* females initiate host colonization and excavate a gallery before being joined by a male (Liu et al. 2006; Owen et al. 2010). As with other *Dendroctonus* beetles (McGhehey 1968; Rudinsky and Michael 1974; Ryker and Rudinsky 1976; Ryker 1988; Lindeman and Yack 2015), both female and male *D. valens* release acoustic signals by rubbing their abdominal files (plectrum) and elytral files together (Michael and Rudinsky 1972; Rudinsky and Michael 1973) and communicate by sound. Males stridulate, which functions as territorial behavior and is termed "aggressive/rivalry chirps" (Rudinsky and Michael 1974; Ryker 1988). Both female and male *D. valens* release "agreement/attractive chirps" when pairs meet in a gallery or are stimulated by pheromones (Ryker and Rudinsky 1976; Ryker 1988). However, the mechanism by which aggregation ceases remains poorly understood.

We investigated antiaggregation signals used by *D. valens* and how these chemical and acoustic signals are regulated. First, we investigated the activity of *exo*-brevicomin in the laboratory and in the field. Second, we examined how production of this pheromone is regulated in nature. Third, we tested whether production of *exo*-brevicomin is stimulated by acoustic signals, and whether such acoustic signals are produced by both sexes.

## Materials and methods

### Insects

Eight-unit Lindgren funnel traps baited with the host tree kairomonal attractant 3-carene (Sun et al. 2004; Erbilgin et al. 2007) were used to catch adult *D. valens* in flight shortly after they emerged from their over-wintering sites, from early May to early June during 2011–2014. The 3-carene (10 mL load) (Lvzhou Biocontrol Company, Taiyuan, Shanxi, China) was released from 15 mL polyethylene bottles (Hongzhi Plastic Plant, Taiyuan, China) and a mean release rate of 100 mg/day was measured under field conditions (mean temperature 27 °C) (Zhang and Sun 2006). Field trapping was conducted in a natural stand of *P. tabuliformis* at Beishe Mountain, located at the base of the Luliang Mountains (37 °48′N, 111 °57′ E, mean elevation 1400 m), west of Gujiao City, Shanxi Province, China. All necessary permits were obtained from Forest Pests Quarantine and Control Bureau of Shanxi Province. Traps were sampled every other day; *D. valens* were collected alive, and the sexes were separated by listening for stridulation produced by males (McGhehey 1968). Insects were stored in plastic boxes with holes for ventilation and provided with fresh phloem for food; the boxes were kept in an environmental chamber at 25 °C, 55% RH under a photoperiod of L14:D10 for later bioassay in the laboratory.

### Pheromone *exo*-brevicomin identification and bioassay

#### Pheromone identification

To simulate beetle attack and pheromone production in nature, trees of 30 cm diameter at breast height (DBH) were selected randomly and fell in a valley within the Beishe Mountains mentioned above. Each log was cut unto two 100-cm long bolts and transported to the laboratory. Bolts were placed upright at 20 °C in a temperature-controlled room with natural light from a window, and their cut ends were coated with melted wax to avoid moisture loss.

Following procedures described by Pureswaran and Borden (2003), beetles used for pheromone analysis were sampled according to 5 treatments: 1) newly trapped females (controls); 2) newly trapped males (controls); 3) individual females feeding alone in galleries for 72 h; 4) paired females joined by males feeding in galleries for 72 h; and 5) males with feeding females in galleries for 72 h. The treatments were administered as follows: First, after beetles were collected, 40 newly trapped females and males were each sampled to collect volatiles as controls. Second, 50 females were introduced singly into holes predrilled with a 1.0-cm-diameter cork borer, secured with wire mesh (mesh hole size, 2 × 2 mm), and allowed to feed for 72 h. Third, 50 females were initially introduced singly into the predrilled holes on the bolts, and secured with wire mesh, and checked twice a day to determine when the female bored into the bark. After females had bored into the bark, males were placed at the entrance of the tunnel and allowed to join the female in the gallery.

Seventy-two hours after the male entered, galleries were dissected to remove beetles for volatile extraction; these beetles were termed a paired female feeding and paired male feeding, accordingly.

Volatile extraction was conducted using the method described in Liu et al. (2013). Once beetles were excavated from galleries, they were immediately placed into insert vials (250 µL glass with polymer feet), whose tips were filled with about 10 mg of adsorbent Tenax (80–100 mesh) (Restek Corporation). The insert vial was placed into a 2-mL screw cap vial. Individual beetles were inserted abdomen first into the vials and immobilized by using silane-treated glass wool so that the tip of the abdomen was 1–2 mm from the absorbent. Vials were closed loosely with polytetrafluoroethylene (PTFE) lined caps to allow adequate gas exchange for beetle respiration. Volatiles released from the beetles were passively collected on the adsorbent Tenax for 72 h. Beetles were removed and reared on fresh phloem until death. Fifty microlitres of redistilled hexane spiked with 5.0 ng/µL heptyl acetate (internal standard) were added to the adsorbent, vortexed, and kept at –20 °C for further analysis.

All extracts were analyzed on a Hewlett-Packard (HP) 6890 gas chromatograph-mass spectrometer (GC-MS) equipped with a DB-Wax column (30 m length × 0.25 mm i.d. × 0.25 mm film thickness) (J&W Scientific). The temperature program was set at 40 °C for 1 min, 5 °C/min to 240 °C and held for a final 5 min. The flow of helium (carrier gas) was 1.0 mL/min. Aliquots of extracts (1 µL) were injected in splitless mode at 250 °C. Pheromones were identified by comparing retention times and mass spectra to those of synthetic standards. The detected volatiles were quantified by comparing the relative abundance of diagnostic ions to the internal standard.

#### Bioassay of exo-brevicomin

Bioassays were conducted in a glass Y-tube olfactometer using the method described by Liu et al. (2006). One chamber contained filter paper that had been treated with hexane (control), and the other chamber contained filter paper that had been treated with 10 µL 1 of 4 concentrations of *exo*-brevicomin diluted in hexane (0.4, 4, 40 and 400 ng/µL). The olfactometer was maintained at 25 °C and RH 70%. Thirty minutes before each trial, single *D. valens* adults were introduced into a separate holding container, so they would not be exposed to any test odors before their release. At the beginning of each trial, a beetle was released at the downwind end of the Y tube, and given 10 min to respond to the odors. Choice for the left or right arm of the olfactometer was noted when the beetle walked 5 cm past the Y junction. If the beetle failed to select one of the chambers, a new individual was introduced. To eliminate directional bias, the right and left branches of the olfactometer were switched after each trial. The Y-tubes were changed after each trial and cleaned with 100% alcohol before reuse. For each trial, 30 individuals of each sex were tested.

Field trapping experiments with 3-carene and *exo*-brevicomin were conducted from 1 May to 1 June in 2011, 2012 and 2013 in the same plantation where beetles were collected originally (see above). 3-Carene was formulated as described above; that is, 10 mL 3-carene in 15 mL-polyethylene bottle (Hongzhi Plastic Plant), and the release rate of 3-carene under field conditions (mean temperature 27 °C) was 100 mg/day (Zhang and Sun 2006). Racemate *exo*-brevicomin dissolved in hexane was added to the 3-carene in various concentrations to evaluate its function. Four treatments were tested: 1) 10 mL 3-carene alone (control); 2) *exo*-brevicomin in 10 mL 3-carene with the concentration of 4 ng/µL in the 10 mL of 3-carene; 3) *exo*-brevicomin in 10 mL 3-carene with the concentration of 40 ng/µL; 4) *exo*-brevicomin in 10 mL 3-carene with the concentration of 400 ng/µL. Lures lasted 40 days in the field. Eight-unit multiple-funnel traps were suspended on randomly selected host trees (about DBH 15 cm) which appeared to be relatively resistant to *D. valens* attack based on few symptoms (pitch tubes, frass) relative to neighboring trees (Liu et al. 2008). Trees were spaced at least 50 m apart and treatments were organized in a randomized design. The collection cup was positioned about 20 cm above the ground to match the typical height of *D. valens* attacks. Then traps were checked every 3 days and beetles were counted and sexed.

### Effect of beetle density on the production of *exo*-brevicomin

To evaluate the potential influence of beetle density, and hence resulting sounds, on the production of *exo*-brevicomin, we set up the following treatments: 1) single female feeding alone in a tube with phloem for 72 h; 2) single male feeding alone in a tube with phloem for 72 h; 3) paired female and male feeding together in tube with phloem for 72 h; 4) 50 females feeding in masses with fresh phloem in Petri dishes for 72 h, and 5) 50 males feeding in masses with fresh phloem in Petri dishes for 72 h. Volatile extraction was conducted using the same method described above. Fifty micrlitres of redistilled hexane spiked with 5.0 ng/µL heptyl acetate (internal standard) were added to the adsorbent, vortexed, and kept at –20 °C for further analysis.

All extracts were analyzed on the Hewlett-Packard (HP) 6890 gas chromatograph-mass spectrometer (GC-MS) equipped with the DB-Wax column (30 m length × 0.25 mm i.d. × 0.25 mm film thickness) (J&W Scientific), with the same temperature program above. *exo*-Brevicomin was identified by comparing its retention times and mass spectra to those of synthetic standards. Beetles that produced *exo*-brevicomin were counted, and the concentration of each was calculated to the internal standard.

### Effects of sounds on behavioral responses and *exo*-brevicomin production by *D. valens*

#### Acoustic sounds and behavioral functions

Male aggressive chirps (male stridulation), and female and male agreement chirps, were recorded as follows. Test beetles were set up individually in acoustically isolated 4 mL tubes (Agilent Company) and provided with cotton beds at the bottom of the tubes. An omnidirectional condenser microphone (AT803, audio-technica U.S., Inc, 30–20 000 Hz) was mounted on the tube (3 cm from the beetle), which was placed in the glass container (diameter 15 cm × height 20 cm). Then the container was put in a sound-proof box (length 1 m × width 0.5 m and height 0.8 m). Sound was recorded using the omnidirectional condenser microphone connected to a solid-state recorder (Marantz PMD 661, 16 bit, 44.1 kHz). When the agreement chips were recorded, 10 µL frontalin (5 ng/µL) was added to the cotton bed to stimulate sound production using the method of Rudinsky and Michael (1972). Sound pressure levels were corrected using a calibrator (Aihua, Aihua Inc.) that produces a 1 kHz tone at 94 dB. Data were saved as WAV files and analyzed (fast Fourier transform, 1024 points), and displayed using Raven Pro 1.3 software (Cornell Laboratory of Ornithology, www.birds.cornell.edu/raven). Each beetle was recorded for 1 min and 10 replicates for each sound were recorded.

Acoustic playback experiments were performed for both sex beetles using one of each recorded chirp produced by each sex as the stimulus. These sounds were stored as digital files on a laptop computer (Thinkpad T400) from which they were broadcast through an earphone (Sennheisser CX300III). This playback system has the ability to adjust the level of acoustic playback to 80 dB SPL measured at 3 cm from the earphone, which was recorded again for validation.

Playback experiments were conducted by using a glass Y-tube under dark in the sound-proof box mentioned above. During the phonotaxis experiments, each male or female beetle was released at the stem end and earbuds were fixed to the 2 branch-ends of Y-tube that were covered by ferric mesh to prevent the tested beetle from reaching the earphone. Sound present versus sound absent comparisons were conducted. The stimulus was played on repeat for 10 min at 1 branch end by earphone; the other earphone without sound was the control. There were 30 beetles of each sex per replicate, and 3 replicates each.

**Sound playback and pheromone production**

Two sounds, the male's aggressive chirps and female's agreement chirps, were played back to test their relationship to pheromone production since both sexes produce the same kind of agreement chirp (no sound as control). Acoustic playback experiments were performed as above, that is, male's stridulation and the common agreement sounds of both females and males were played back at 80 dB SPL, which was recorded again for validation, with the distance of 3 cm over tested beetles for 72 h. This experiment was performed in the dark inside the cooler mentioned above. Newly trapped females and males were individually reared in 1.5 mL Eppendorf tube provided with fresh *P. tabuliformis* phloem for 3 days. Then, these beetles were placed individually, abdomen first, into insert vials (250 µL glass with polymer feet). The insert vials were filled with about 10 mg of adsorbent Tenax (80–100 mesh) in the tip, and then the insert vial was placed into a 2-mL screw cap vial without cap to collect volatiles as the method mentioned above during the experiment of sound playback (40, 20 each sex, replicates per treatment). These 20 vials of same sex were placed in a plastic container (diameter 6 cm × height 7 cm) with lid closed. A hole was made in the center of the lid of the plastic container to hold the earphone, which was 3 cm from tested beetles. The plastic container was individually placed in the sound-proof glass container (diameter 15 cm × height 13 cm) and the lid closed, with the cable of earphone through the hole in the lid and sealed with cotton. Three days later, the sound was stopped and the beetle was removed, 50 µL of redistilled hexane spiked with 5.0 ng/µL heptyl acetate (internal standard) was added to the adsorbent and kept at −20 °C for further analysis. Extracts were analyzed by the same protocol mentioned above to evaluate the sounds on pheromone production.

### Statistical analysis

The amounts of identified potential pheromone components were compared between treatments by using 1-way ANOVA in SPSS (1999). Data from the olfactometer bioassays comparing beetle response to *exo*-brevicomin versus hexane were analyzed by a chi-squared test in SPSS. The 3 years of trapping data were assumed to have an over-dispersed Poisson distribution and analyzed using a generalized linear model (GLM), with year, treatment, and sex as fixed factors. For trapping data from each year, a separate GLM was used with treatment and sex as fixed factors, followed by maximum likelihood tests with Bonferroni correction for multiple comparisons. Behavioral responses to sound playback were analyzed using paired *t* tests between sound present and sound absent for each sex to evaluate effects of male and female sounds on male and female choice in the Y-tube arena.

## Results

### Identification and amount of pheromone produced by *D. valens*

Single feeding females, paired feeding females, and paired feeding males released *trans*-verbenol, *cis*-verbenol, myrtenal, and verbenone (Table 1). Besides frontalin produced by females, which we had identified earlier, both females and males produced *exo*-brevicomin. Few newly trapped beetles produced detectable or substantial quantities of pheromones.

### Function of *exo*-brevicomin

Laboratory bioassays indicated that males were significantly repelled by all concentrations of *exo*-brevicomin tested, from the trace amount 0.4 to 400 ng/µL in Y-tube trails (Table 2). However, females were not sensitive to tested concentrations of *exo*-brevicomin in the Y-tube assay.

In each of 3 years of field trapping, addition of *exo*-brevicomin to traps baited with 3-carene significantly reduced the number of *D. valens* captured (Figure 1). The inhibition of attraction to 3-carene increased with increasing concentrations of *exo*-brevicomin. GLM analysis showed beetle catches were influenced by year, treatment, and sex (year, $F = 24.08$, df = 2, $P < 0.0001$; treatment, $F = 38.66$, df = 3, $P < 0.0001$; sex, $F = 68.61$, df = 1, $P < 0.0001$; year × treatment, $F = 0.96$, df = 6, $P = 0.453$; year × sex, $F = 1.02$, df = 2, $P = 0.363$; treatment × sex, $F = 1.24$, df = 3, $P = 0.294$; year × treatment × sex, $F = 0.30$, df = 6, $P = 0.935$). In each year, the catches were significantly affected by both treatment and sex (2011: treatment, $F = 13.90$, df = 3, $P < 0.0001$, sex, $F = 23.43$, df = 1, $P < 0.0001$, treatment × sex, $F = 0.48$, df = 3, $P = 0.70$; 2012: treatment, $F = 14.17$, df = 3, $P < 0.0001$, sex, $F = 25.62$, df = 1, $P < 0.0001$, treatment × sex, $F = 0.89$, df = 3, $P = 0.450$; 2013: treatment, $F = 11.39$, df = 3, $P < 0.0001$, sex, $F = 18.83$, df = 1, $P < 0.0001$, treatment × sex, $F = 0.26$, df = 3, $P = 0.855$).

**Table 1.** Concentration (ng/µL) of pheromone detected in feeding *Dendroctonus valens*[a]

| | *n* | Frontalin | *trans*-Verbenol | *exo*-Brevicomin | *cis*-Verbenol | Myrtenal | Verbenone |
|---|---|---|---|---|---|---|---|
| Newly trapped female | 10 | ND | 0.10 ± 0.00 (1) | ND | 0.01 (1) | 0.03 (2) | 0.02 (1) |
| Newly trapped male | 10 | ND | ND | ND | ND | ND | ND |
| Single feeding female | 38 | 0.08 ± 0.02 (8) | 2.20 ± 1.00 (35) | 0.07 ± 0.02 (3) | 16.70 ± 5.08 (31) | 377.41 ± 92.84 (38) | 25.62 ± 11.69 (38) |
| Paired feeding female | 36 | 1.64 ± 0.79 (10) | 0.38 ± 0.09 (29) | 0.05 ± 0.01 (14) | 5.14 ± 1.67 (36) | 177.54 ± 44.88 (36) | 9.46 ± 2.07 (36) |
| Paired feeding male | 36 | — | 0.62 ± 0.27 (29) | 0.27 ± 0.09 (17) | 8.06 ± 1.64 (28) | 519.51 ± 212.73 (34) | 29.54 ± 16.91 (33) |
| *F* | | 3.11 | 1.53 | 1.67 | 2.53 | 1.25 | 0.59 |
| *P* | | 0.097 | 0.213 | 0.232 | 0.062 | 0.294 | 0.625 |

[a]Sampled female and male beetles had fed in bolts for 48 and 24 h, respectively. Values shown as mean ± SE and the values in the parentheses are the number of samples that component identified.

[b]ND means pheromone not detected.

**Table 2.** Behavioral responses of male and female *D. valens* to various concentrations of *exo*-brevicomin (B) in Y-tube assay[a]

| | CK[b] × $B_{0.4\,ng/\mu L}$ | | | CK × $B_{4\,ng/\mu L}$ | | | CK × $B_{40\,ng/\mu L}$ | | | CK × $B_{400\,ng/\mu L}$ | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Sex | CK | $B_{0.4\,ng/\mu L}$ | $\chi^2$ | CK | $B_{4\,ng/\mu L}$ | $\chi^2$ | CK | $B_{40\,ng/\mu L}$ | $\chi^2$ | CK | $B_{400\,ng/\mu L}$ | $\chi^2$ |
| Male | 22 | 8 | 6.53* | 24 | 6 | 10.80* | 25 | 5 | 13.33** | 23 | 7 | 8.33** |
| Female | 16 | 14 | 0.72 | 16 | 14 | 0.13 | 17 | 13 | 0.53 | 16 | 14 | 0.13 |

[a]Table entries represent the numbers of *D. valens* of either sex that responded positively to either the control or treatment stimulus. CK indicates hexane. Significant differences at $*P \le 0.05$ and $**P \le 0.01$, respectively (Chi-squared test).

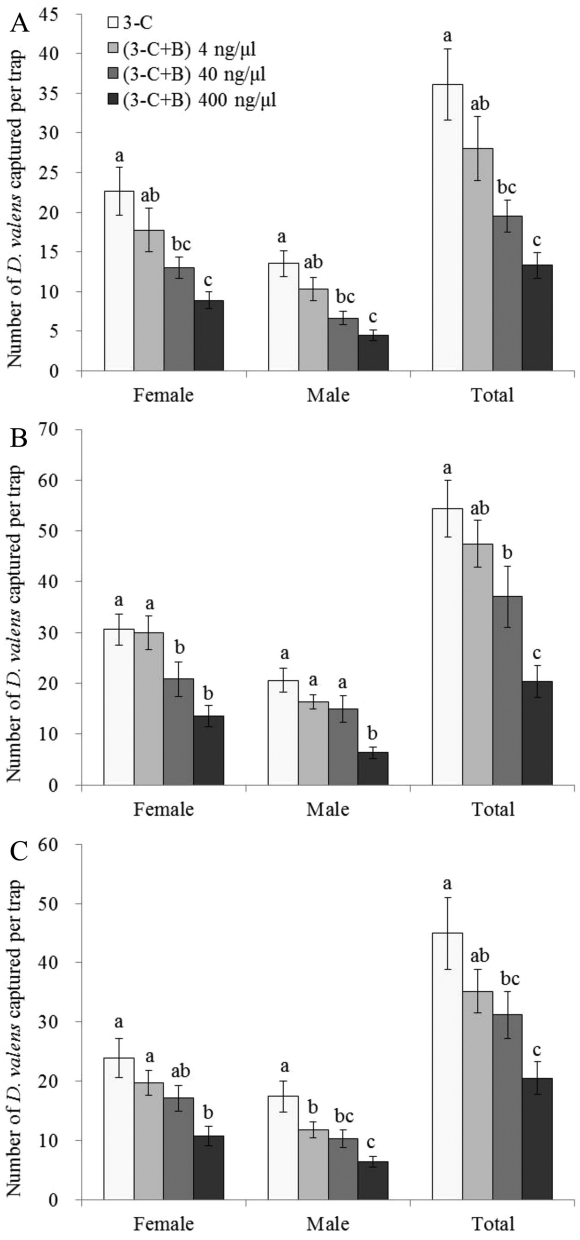


**Figure 1.** Field trapping with various concentrations of *exo*-brevicomin in 3-carene over 3 years. **A**–**C** indicate 2011, 2012, and 2013, respectively. Bars indicate mean responses and standard errors, and different letters above bars indicate significant differences at $P \le 0.05$ with Bonferroni Multiple Comparison (ANOVA).

## Effect of beetle density on *exo*-brevicomin production

The frequency of beetles that produced *exo*-brevicomin differed significantly among beetles feeding alone, feeding in pairs and feeding in masses (Figure 2A) (Female, Chi-square = 35.58, df = 2, $P < 0.0001$; Male, Chi-square = 54.15, df = 2, $P < 0.0001$; Total, Chi-square = 88.27, df = 2, $P < 0.0001$). However, the amount of *exo*-brevicomin produced did not differ among treatments (Figure 2B) (Female, $F = 0.55$; df = 2, 33; $P = 0.580$; Male, $F = 0.79$; df = 2, 43; $P = 0.458$; Total, $F = 0.71$; df = 2, 79; $P = 0.497$). More beetles feeding in pairs (50%) and in masses (84%) produced *exo*-brevicomin than did those feeding alone (10%) (Figure 2).

## Effects of sounds on behavioral responses and *exo*-brevicomin production by *D. valens*

### Acoustic sounds and behavioral functions

Both females and males released the same kind of agreement sounds, which consisted of a single chirp with a frequency of 689.1 Hz and a duration of 23.1 ± 0.1 ms (Figure 3A-a–c, Supplementary Audio File S1; Figure 3 B-a–c, Supplementary Audio File S2). The male-produced aggressive sounds had a single frequency of 861.3 Hz and duration of 37 ± 0.5 ms (Figure 3C-a~c, Supplementary Audio File S3).

Males, but not females, actively responded to the sound playback of female agreement sounds (Figure 4A) (male, $t = 22.09$, df = 2, $P = 0.002$; female, $t = 4.00$, df = 2, $P = 0.057$). When male agreement sounds were played back, the behavioral assay showed the same results as with female agreement sounds (Figure 4B) (male, $t = 11.75$, df = 2, $P = 0.007$; female, $t = -1.70$, df = 2, $P = 0.231$). This suggests the agreement sound is attractive to males but is not used to discriminate females from males. Moreover, both males and females negatively responded to male aggressive sounds when these were played back (Figure 4C) (male, $t = -18.52$, df = 2, $P = 0.003$; female, $t = -5.48$, df = 2, $P = 0.032$).

### Sound playback and pheromone production

Beetles to which the agreement sounds and male aggressive sounds had been played back were significantly more likely to produce *exo*-brevicomin than beetles not treated with sound playback (Figure 5A) (Female, Chi-square = 7.83, df = 2, $P = 0.02$; Male, Chi-square = 12.06, df = 2, $P = 0.002$; Total, Chi-square = 21.44, df = 2, $P < 0.0001$). However, similar to the results with beetle feeding density, the amounts of *exo*-brevicomin produced were not significantly different among control, female agreement sounds playback, and male aggressive sounds playback (Figure 5B) (Female, $F = 1.34$; df = 2, 16; $P = 0.291$; Male, $F = 0.16$; df = 2, 17; $P = 0.852$; Total, $F = 0.35$; df = 2, 37; $P = 0.706$).

## Discussion

Both female and male *D. valens* feeding in galleries, but not newly trapped beetles, released trans-verbenol, cis-verbenol, myrtenal, and verbenone, which were common pheromones that were produced at larval and adult stage by *D. valens* (Shi et al. 2010; Liu et al. 2013; Xu et al. 2014; Xu et al. 2015). Besides these common pheromones, females were found to produce frontalin and both sexes produced *exo*-brevicomin. Frontalin functions as an aggregation pheromone

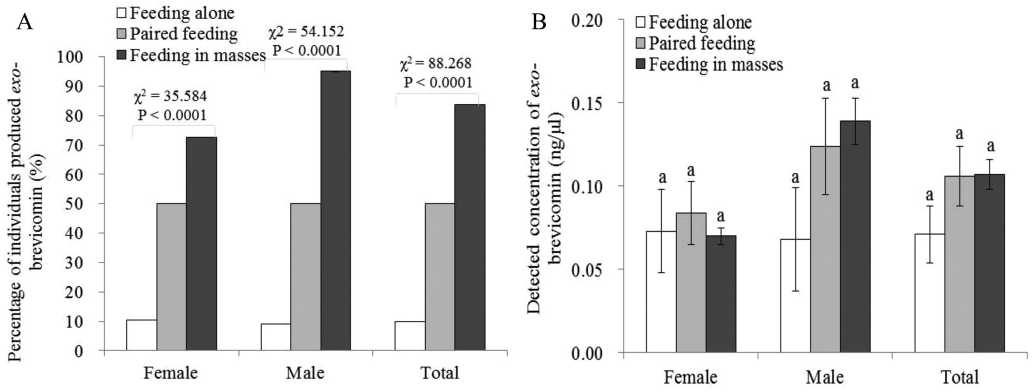


**Figure 2.** *Exo*-Brevicomin production of *D. valens* with varied beetle density. Chi-square test was conducted, and the percentage of individuals who produced *exo*-brevicomin is shown in (**A**). Same letters above bars in (**B**) indicate not significant differences at $P \leq 0.05$ (1-way ANOVA).

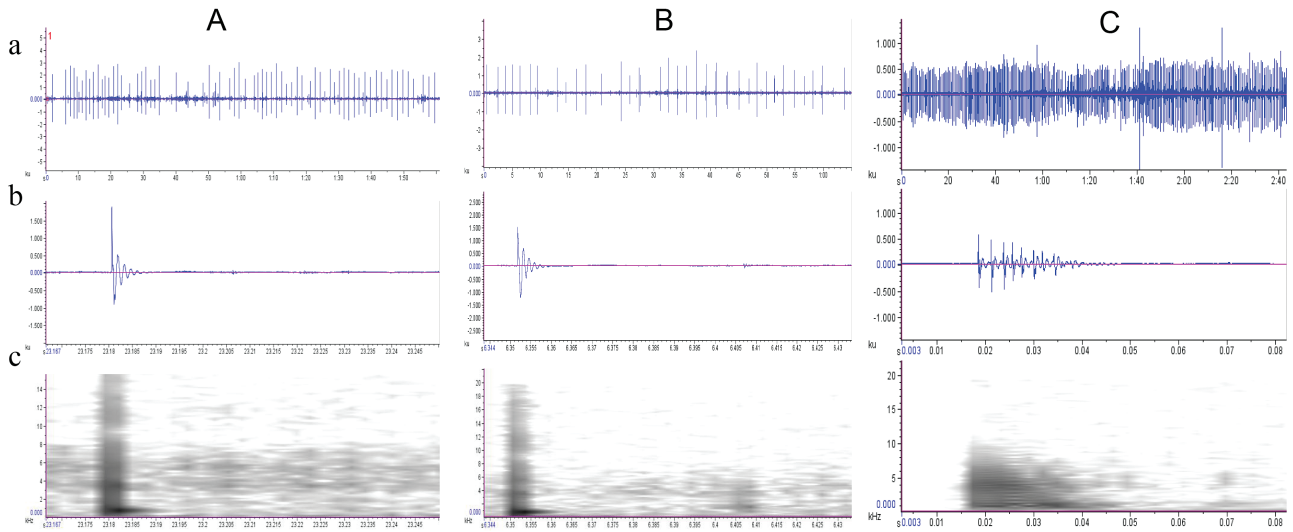


**Figure 3.** Acoustic signals recorded of female and male *D. valens*. (**A**) Female-produced agreement sounds, (**B**) male-produced agreement sounds, and (**C**) male-produced aggressive sounds. (a) a waveform of each type of sound recorded; (b) a waveform of a single chirp of each type of sound; (c) a spectrogram of each type of sound.

component of *D. brevicomis* and *D. frontalis* (Kinzer et al. 1969; Renwick and Vité 1969). In *D. valens*, frontalin functions primarily as a sex pheromone, but is also attractive to females (Liu et al. 2013). In our study, *exo*-brevicomin was released by both sexes of *D. valens*, and functioned as an antiaggregation pheromone, similar to this function in some other *Dendroctonus* species such as *D. ponderosae* (Rudinsky et al. 1974; Ryker and Rudinsky 1982). On the contrary, *exo*-brevicomin is an attractant in the closely related *D. terebrans* (Phillips et al. 1989, 1990). The use of *exo*-brevicomin as an antiattractant by *D. valens* may be important in maintaining species separation between *D. valens* and *D. terebrans*. Furthermore, beetles in masses were more likely to produce *exo*-brevicomin, indicating the production of this pheromone is density-dependent, and may be elicited by beetles' sounds. Both *D. valens* females and males release the same type of agreement sounds, in addition to male aggressive sounds. Both kinds of sounds trigger the production of *exo*-brevicomin to avoid overcrowding. This study provides the first clear evidence that sounds exert an important role in reducing attraction in *D. valens*. Further, this finding demonstrates an interaction between both auditory and olfactory stimuli (Rudinsky 1969; Rudinsky and Michael 1972; Anton et al. 2011).

Rudinsky (1969) proposed that stridulation and increased production of the pheromone MCH terminated aggregation by *D. pseudotsugae* (Rudinsky et al. 1976). In that system, the function of MCH varies with concentration, being attractive at low and repellant at high quantities (Rudinsky and Michael 1973). Moreover, both sexes of *D. pseudotsugae* produced MCH in response to sound, and considerably greater quantities were obtained from beetles exposed to sound (Rudinsky et al. 1976). In *D. valens*, *exo*-brevicomin was likewise released by both sexes, but in contrast to *D. pseudotsugae* (Rudinsky and Michael 1973), was repellent at all concentrations tested, down to the level of nanograms. In the field, *exo*-brevicomin decreased attraction to 3-carene by 50% (Figure 1). A second difference from *D. pseudotsugae* was that only the frequency of individuals that produced *exo*-brevicomin, increasing from 10% to 84%, but not the amount of exo-brevicomin produced, increased when the density of *D. valens* increased. However, *D. pseudotsugae* increased the amount of MCH when treated by sounds (Rudinsky et al. 1976). The high level of repellence elicited by nanogram quantities in *D. valens* indicated emission of effective antiaggregation signals as soon as individuals encountered each other. These differences may reflect different lifestyles between *D. pseudotsugae* and *D. valens*, in

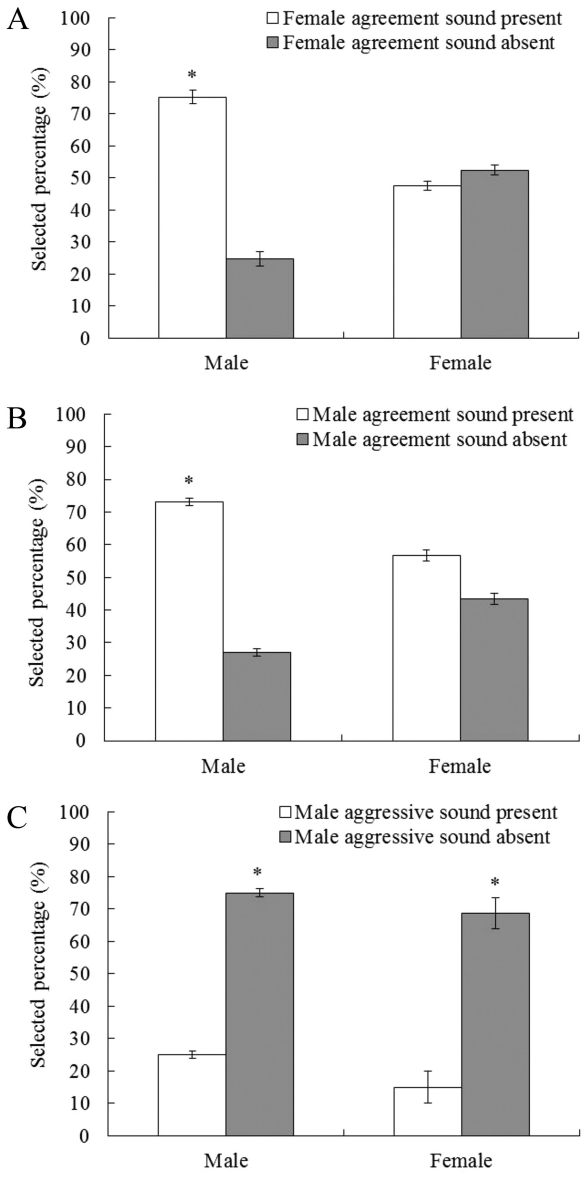


**Figure 4.** Phonotaxis of *D. valens* male and female beetles to sounds present and absent. Data were analyzed by paired *t* test, and the percentage was presented. Asterisks indicate significant difference at $P \leq 0.05$.

that the former engages in rapid mass attacks that kill trees, whereas the latter colonizes trees as solitary or just a few individuals and typically does not kill trees, in the native range where it evolved. However, the ability of *D. valens* to produce such diverse and multifunctional pheromones as frontalin and *exo*-brevicomin may have served as a preadaptation for its role as a mass-attacking, tree-killing outbreak species under the different selection regime it encountered following its introduction into China.

The release of *exo*-brevicomin triggered by sound also provides a means by which males can avoid competition for females. *D. valens* is apparently monogamous and exhibits a high level of cooperative biparental care (Wood 1982; Kirkendall 1983; Liu et al. 2006). If a male in a gallery with a female encounters another male, he might be disturbed by this rival, produce the territorial stridulation sounds and even engage in fighting (McGhehey 1968; Rudinsky and Michael 1974). Thus, male stridulation may not be enough to maintain monogamy. However, both sexes produce *exo*-brevicomin when pairs join and experience their counterpart sounds, conspecific male landing on the bark decreases. In general, the sounds produced in Scolytinae are short-range signals, whereas chemical signals can be more long-range. Thus, *exo*-brevicomin triggered by sounds may provide a complementary mechanism to maintain monogamy and avoid potential male competition.

Bark beetles show high plasticity in their colonization behaviors (Wallin and Raffa 2004), and such plasticity may be reflected in how integrated audio-chemo communication functions for *D. valens* in a new region. In its native range, *D. valens* can complete reproduction in trees that remain alive, by tolerating their resins (Aukema et al. 2010). However, in its invaded areas of China, aggregation of *D. valens* may help to overcome host resistance in a similar fashion to native North American outbreak species such as *D. ponderosae* (Liu et al. 2013; Qiu 2013; Xu et al. 2014). For example, in Wisconsin, 57% of attacked *P. resinosa* had only 1 pair of *D. valens*, and these reproduced despite resin production (Aukema et al. 2010). However, in China, *D. valens* that enter standing *P. tabuliformis* trees in low numbers are often confined and killed within pitch tubes. Differences between North American *Pinus* and Chinese *P. tabuliformis* physiology might contribute to these contrasting behaviors. For example, *P. tabuliformis* has very prominent resin flow and had been used for commercial resin collection. Facing a new host species in a new environment may require *D. valens* to evolve new strategies to overcome tree defense, such as adapting mating pheromones to aggregate both sexes, and likewise avoiding over-crowding by using antiaggregant signals. Combined with drought in the north central China in late

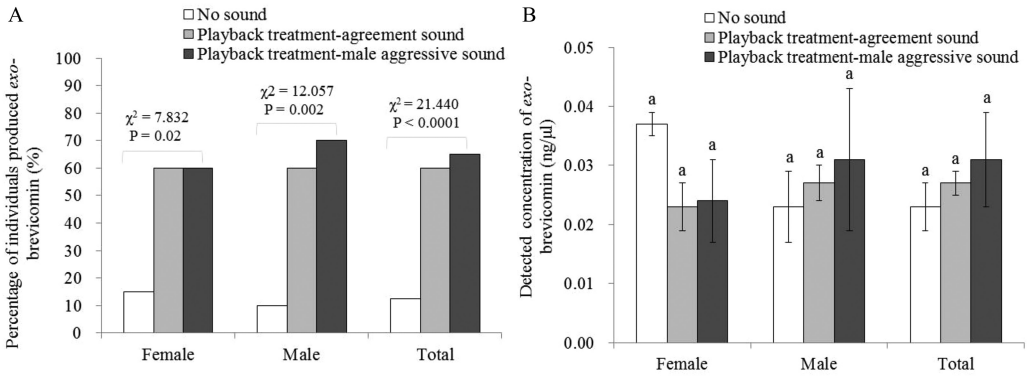


**Figure 5.** *Exo*-brevicomin production of *D. valens* with sounds played back. Chi-square test was conducted, and the percentage of individuals who produced *exo*-brevicomin was shown in (**A**). Same letters above bars in (**B**) indicate not significant differences at $P \leq 0.05$ (1-way ANOVA).

20th century (Yan et al. 2005; Xu et al. 2006), this challenge may explain why *D. valens* resided in China for 20 years before undergoing its first outbreak.

The interplay between sound and chemicals suggests some potential opportunities for management of invasive populations of *D. valens*. For example, acoustic devices can be used to detect pests in trees and wood materials (Mankin and Moore 2010). Further, sounds (or vibrations) may potentially be used to protect plants from insects (Polajnar and Čokl 2008; Eriksson et al 2012; Aflitto and Hofstetter 2014; Hofstetter et al. 2014).

In conclusion, this study indicates that acoustic communication among *D. valens* elicits production of an antiaggregation pheromone. This tends to distribute the available males evenly, prevent overcrowding, and contribute to the maintenance of monogamy, all of which favor establishment and reproduction, and associated tree mortality, following human transport of this insect. Studies comparing and contrasting the interaction of acoustic and chemical signals in the introduced and native ranges of *D. valens* can facilitate our understanding of the invasion dynamics, postintroduction evolutionary adaptation, and management of *D. valens* and other invasive bark beetles.

## Supplementary material

Supplementary material can be found at http://www.chemse.oxfordjournals.org/

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