Monoterpenyl esters in juvenile mountain pine beetle and sex-specific release of the aggregation pheromone *trans*-verbenol

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A recent outbreak of mountain pine beetle (MPB) has spread over more than 25 million hectares of pine forests in western North America, affecting pine species of sensitive boreal and mountain ecosystems. During initial host colonization, female MPB produce and release the aggregation pheromone *trans*-verbenol to coordinate a mass attack of individual trees. *trans*-Verbenol is formed by hydroxylation of α-pinene, a monoterpene of the pine oleoresin defense. It is thought that adult females produce and immediately release *trans*-verbenol when encountering α-pinene on a new host tree. Here, we show that both sexes of MPB accumulate the monoterpenyl esters verbenyl oleate and verbenyl palmitate during their development in the brood tree. Verbenyl oleate and verbenyl palmitate were retained in adult female MPB until the time of emergence from brood trees, but were depleted in males. Adult females released *trans*-verbenol in response to treatment with juvenile hormone III (JHIII). While both sexes produced verbenyl esters when exposed to α-pinene, only females responded to JHIII with release of *trans*-verbenol. Accumulation of verbenyl esters at earlier life stages may allow adult females to release the aggregation pheromone *trans*-verbenol upon landing on a new host tree, independent of access to α-pinene. Formation of verbenyl esters may be part of a general detoxification system to overcome host monoterpene defenses in both sexes, from which a specialized and female-specific system of pheromone biosynthesis and release may have evolved.

bark beetle | pheromone | verbenol | pinene | forest health

The current mountain pine beetle (MPB; *Dendroctonus ponderosae*) epidemic has affected over 25 million hectares of pine forests in western North America (1, 2) and is moving into the boreal forest and sensitive high-elevation ecosystems (3, 4). Female MPB produce the aggregation pheromone *trans*-verbenol to coordinate a mass colonization behavior (5, 6) that allows MPB to overcome host defenses such as toxic monoterpenes (7–9). MPB are exposed to host monoterpenes during most of their life cycle from eggs to adults in the bark of the brood tree, followed by a brief period of emergence and dispersal flight and colonization of the bark of a new host where they mate and oviposit.

trans-Verbenol is thought to be produced during host colonization by female MPB through oxidation of the host monoterpene α-pinene (8–14). Female MPB also release *trans*-verbenol when treated with juvenile hormone III (JHIII) without concurrent exposure to α-pinene (15, 16). In other *Dendroctonus* species, exposure of larvae or pupae to high levels of α-pinene vapors caused increased levels of *trans*-verbenol in adults (17). It has been hypothesized that some *Dendroctonus* species accumulate *trans*-verbenol conjugates in preadult life stages or produce *trans*-verbenol de novo (17, 18). However, since these hypotheses were first proposed (15, 17), additional evidence for either the conjugation or de novo biosynthesis of *trans*-verbenol has been lacking. Microbial biosynthesis does not appear to contribute substantially to *trans*-verbenol production (12, 15, 19).

Here, we report the discovery of monoterpenyl esters in MPB larvae and pupae, including verbenyl oleate and verbenyl palmitate, which may be part of a monoterpene detoxification system in young male and female MPB and may also serve as a reservoir for female-specific release of *trans*-verbenol as an aggregation pheromone.

Results

Verbenyl Esters in Female MPB. Gas chromatography/mass spectrometry (GC/MS) metabolite profiling of freshly emerged male and female MPB revealed sex-specific differences in a set of compounds that were present in extracts of females, but not of males (Fig. 1 and *SI Appendix*, Figs. S1 and S2 and Table S1). Silica chromatography and ester hydrolysis, followed by GC/MS analysis, revealed these compounds as fatty acid esters of mostly *trans*-verbenol along with minor amounts of fatty acids of *cis*-verbenol, myrtenol, myrtanol, and an unknown terpene alcohol (*SI Appendix*, Figs. S3 and S4). The female-specific esters were identified by comparison with authentic standards as verbenyl palmitate, verbenyl oleate, myrtanyl oleate, and myrtenyl oleate (Fig. 1 and *SI Appendix*, Fig. S5). *cis*-Verbenyl oleate and *trans*-verbenyl oleate did not separate under the GC conditions and could not be distinguished as these two compounds had very similar mass spectra (*SI Appendix*, Fig. S5). These two compounds were quantified together as "verbenyl oleate" in subsequent analyses of monoterpenyl esters over the life cycle of the beetle and in response to treatments. Monoterpenyl esters were not detected in the phloem collected from the brood trees (*SI Appendix*, Fig. S6).

Sex-Specific Presence of Verbenyl Esters in Freshly Emerged Females. Verbenyl esters, which represent the total of *cis*- and *trans*-verbenyl oleates and palmitates, as well as myrtenyl oleate and

Significance

***trans*-Verbenol is a critical aggregation pheromone of the female mountain pine beetle. It is derived from a compound of the host defense, the monoterpene α-pinene. We found that beetles accumulate monoterpenyl esters during early life stages. These may serve as a previously unknown reservoir for the sex-specific release of aggregation pheromone when female beetles synchronize the mass colonization of a new host tree. The improved knowledge of the mountain pine beetle pheromone system can inform prediction of bark beetle outbreaks and invasion of new habitats.**

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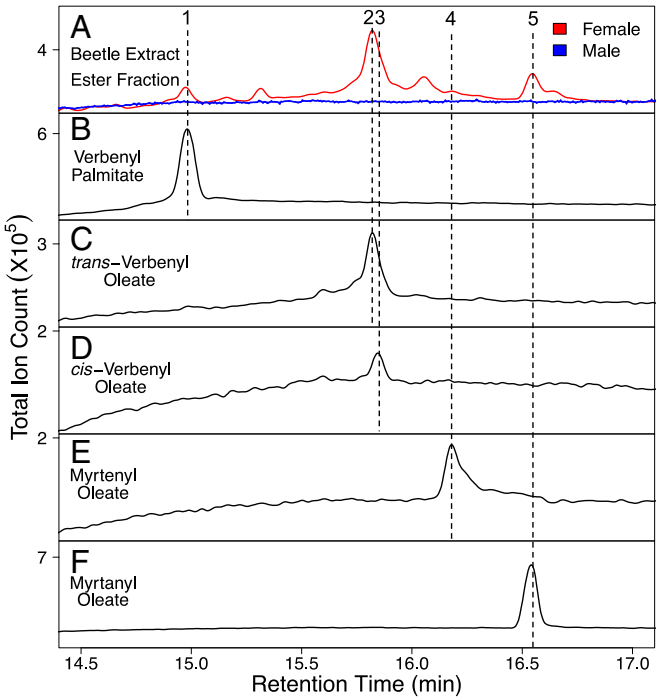

Fig. 1. GC/MS chromatogram of the ester fraction of freshly emerged adult beetles (*A*) and monoterpenyl ester standards (*B–F*). The standards and ester fraction were injected onto an HP-5 column at 30 °C using a cool on-column injector. Verbenyl palmitate, *trans*- and *cis*-verbenyl oleate, myrtenyl oleate, and myrtanyl oleate were all present in the emerging female ester fraction (red) but were not present in the emerging male ester fraction (blue). The mass spectra of peaks labeled 1–5 are shown in *SI Appendix*, Fig. S5.

myrtanyl oleate, were present with similar amounts in females and males in early life stages of first instar larvae until pupation (Fig. 2 *A–D*). In contrast, significant sex-specific differences appeared in the freshly emerged adult beetles that were no longer in contact with tissues of the brood tree. In the emerged adults, only females contained substantial amounts of verbenyl, myrtenyl, and myrtanyl esters (Fig. 2 *A–D*). During the early life stages until pupation, verbenyl oleate was the most abundant of the monoterpenyl esters in both sexes with 250–1,500 ng/mg of beetle body weight (Fig. 2*A*), which was at least one order of magnitude higher than any of the other monoterpenyl esters. Levels of verbenyl esters significantly increased in male pupae compared with the larvae stages (Fig. 2 *A* and *B*) and then declined and were absent in freshly emerged adult males. In contrast, while levels of verbenyl esters in females were similar to those of males during the four larvae stages, they significantly increased in teneral adult females and remained high in emerged females. In contrast to the verbenyl and myrtanyl esters, the levels of other monoterpenyl esters, specifically perillyl and carvyl oleate, were independent of sex and decreased over the life cycle from early instar larvae to emerged adults (Fig. 2 *E* and *F*).

Verbenyl Oleate Accumulated in Freshly Emerged Females with Highest Abundance in the Abdomen and Fat Body. Verbenyl oleate was present in freshly emerged females in the three major body parts—head, thorax and abdomen—where the alimentary canal with the fat body was removed from the abdomen (Fig. 3). Dissection of the alimentary canal showed that verbenyl oleate was most abundant in the layer of perivisceral fat body that surrounds the alimentary canal (Fig. 3), with much lower amounts in the midgut, Malpighian tubules, and hindgut.

Levels of *trans*-Verbenol Increased and Levels of Verbenyl Esters Decreased in Females Treated with JHIII. Following the identification of verbenyl esters in preadult males and females and their sex-specific abundance in freshly emerged females, we tested to see if their levels were affected by JHIII, which regulates pheromone release in MPB. We also tested if JHIII treatment affected levels of free verbenol and other monoterpene alcohols in female and male emerged adults. Levels of *trans*-verbenol increased in JHIII-treated females compared with acetone-treated controls (Fig. 4*A*), while levels of verbenyl esters decreased in JHIII-treated females compared with controls (Fig. 5*A*). Levels of *cis*-verbenol and myrtenol also significantly increased in females in response to JHIII (Fig. 4 *B* and *C*), while the corresponding esters decreased (Fig. 5 *B* and *C*). JHIII treatment did not induce elevated levels of monoterpenols in males, which lacked substantial amounts of the corresponding fatty acid esters (Figs. 4*D* and 5*D*).

Levels of *trans*-Verbenol and Verbenyl Esters Increased in Females and Males Exposed to α-Pinene. We tested the effect of α-pinene vapor, which is the presumed host-derived monoterpene precursor of verbenol, on levels of monoterpenols and monoterpenyl esters in freshly emerged males and females. Exposure to a nearly racemic blend of α-pinene caused increased levels of *trans*-verbenol, *cis*-verbenol, and myrtenol, as well as increased accumulation of the corresponding esters, in both sexes (Figs. 4 *A–C* and 5 *A–C*). Exposure to α-pinene did not induce a significant increase in the levels of myrtanol, a β-pinene–derived monoterpenol, or accumulation of myrtanyl oleate in either sex (Figs. 4*D* and 5*D*).

Stereochemistry of Monoterpenols Induced by JHIII and α-Pinene Treatment. The stereochemistry of the monoterpenols detected in MPB is expected to be dependent upon the stereochemistry of the proposed precursor monoterpenes (*SI Appendix*, Fig. S7). We compared the enantiomeric ratio of the monoterpenols induced by treatment with JHIII or α-pinene to the enantiomeric ratio of the presumed monoterpene precursors.

The enantiomeric ratio of *trans*-verbenol detected in beetles differed between the JHIII and α-pinene treatments. The average enantiomeric ratio of *trans*-verbenol in JHIII-treated females was 3(+):97(−) for *trans*-verbenol (Fig. 4*A*). This ratio was similar to the ratio 4(+):96(−) of the *trans*-verbenol that comprised the verbenyl esters in freshly emergent females (*SI Appendix*, Fig. S3). The enantiomeric ratio of *trans*-verbenol found in freshly emerged beetles exposed to a nearly racemic α-pinene vapor was 34(+):66(−) in females (Fig. 4*A*) and 35(+):65(−) in males (Fig. 4*B*), which was closer to the 44(+):56(−) ratio of α-pinene used for treatment.

Monoterpenyl Esters in MPB Exposed to Other Monoterpenes. Following the observation that exposure to α-pinene vapor resulted in increased levels of verbenyl and myrtenyl esters in both females and males, we also exposed freshly emerged adult beetles to vapors of other monoterpenes that are present in host trees to test if the occurrence of monoterpenyl esters was specific to α-pinene or a general effect of monoterpene exposure. Females and males were individually exposed to (−)-β-pinene, (−)-limonene and (−)-β-phellandrene. Exposure to all of these monoterpenes resulted in the production of monoterpene alcohols and the corresponding monoterpenyl esters in the beetles (Fig. 6). Often, multiple products were produced from the treatment with a single monoterpene. However, the identity of only a few of the esters from (−)-β-pinene and (−)-limonene could be verified by our synthetic standards (Fig. 6 and *SI Appendix*, Fig. S8).

Discussion

Bark beetles (Scolytidae) commonly use monoterpenol pheromones. Their biosynthesis has been studied in MPB and *Ips* species using genomic (20), transcriptomic (21–23), and proteomic (16) approaches and functional characterization of genes (24–28). *Ips paraconfusus* and *I. pini* produce the monoterpenol pheromones ipsenol and ipsdienol de novo (29–31) in a process regulated by JHIII (32). In general, strategies for pheromone production in bark beetles have been hypothesized to involve detoxification of host-derived monoterpenes (33, 34), which may include conjugation of monoterpenes (17), microbial production (35), and de novo biosynthesis (36).

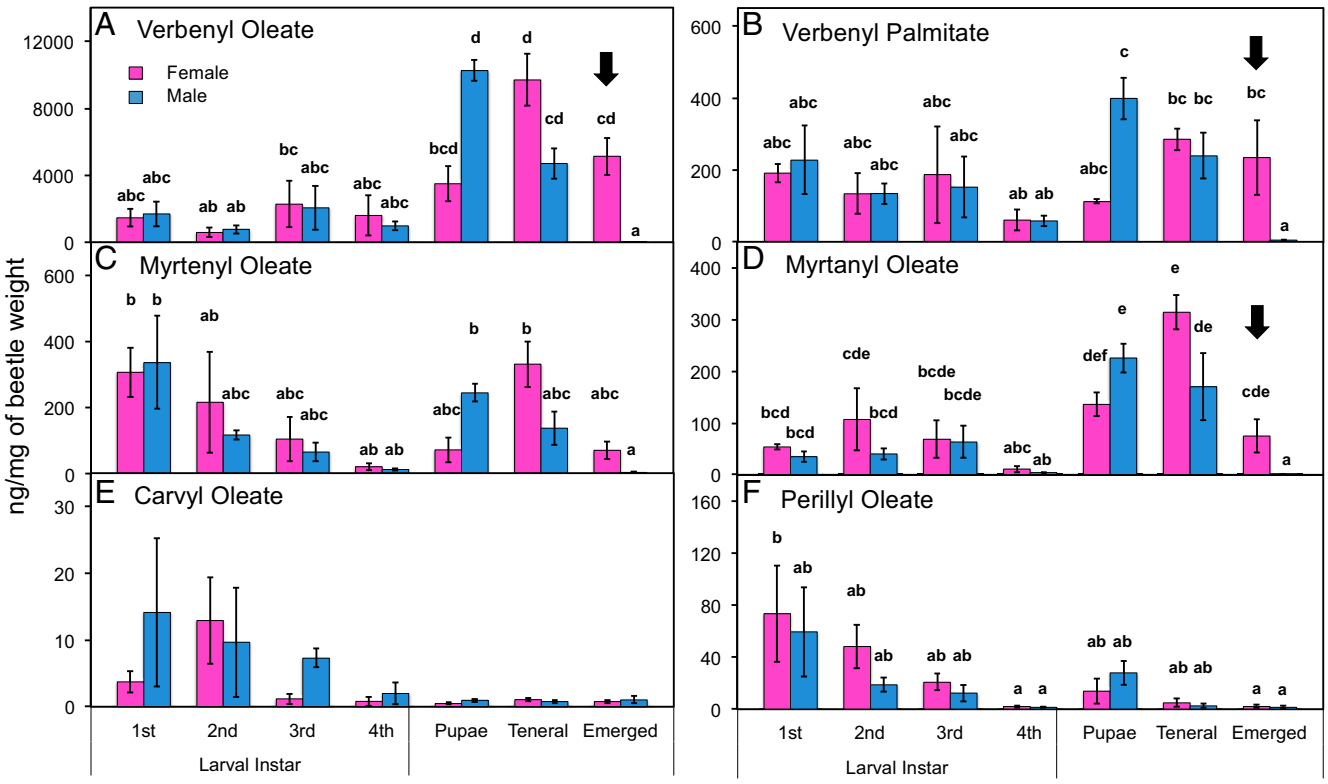

Fig. 2. The abundance of monoterpenyl esters in females (pink) and males (blue) over the life cycle of MPB. Columns in the same graph with the same letter were not significantly different by Conover's test ($\alpha \geq 0.05$). During the larval instar to the teneral adult stage, developing beetles remain in the brood tree. Tenerals are adult beetles that do not have fully sclerotized cuticle, a stage that lasts 7–14 d. Emerged beetles are fully mature adults, which leave their brood tree in search of a new host. The amounts of (*A*) verbenyl oleate, (*B*) verbenyl palmitate, and (*D*) myrtanyl oleate were significantly different between female and male emerged beetles as indicated by the arrows, but not for (*C*) myrtenyl oleate or (*F*) perillyl oleate. No significant differences were found for (*E*) carvyl oleate ($n = 4$).

In the context of the present geographic and host-range expansion of MPB, it is important to understand how MPB produce and release the female-specific aggregation pheromone *trans*-verbenol (11, 37). This involves testing the capacity of MPB to accumulate host monoterpenes as pheromone precursors when they are most exposed to pine oleoresion during their life cycle before adult emergence from brood trees.

In support of the accumulation via conjugation hypothesis of *trans*-verbenol (15, 17, 18), we identified the monoterpenyl esters verbenyl oleate and verbenyl palmitate, along with myrtenyl oleate and myrtanyl oleate, in developing MPB. The effect of JHIII in females on the decrease of verbenyl oleate with the simultaneous increase of *trans*-verbenol in the absence of direct contact with α-pinene supports the conclusion that verbenyl esters can serve as a metabolite pool for the female-specific pheromone release. JHIII triggers the sex-specific up-regulation of gene expression, including the female midgut-specific up-regulation of an esterase gene as observed in transcriptome data (16).

The ability of MPB to accumulate host monoterpenes via esterification of hydroxylated intermediates may be a general mechanism of detoxification of pine monoterpenes that is not sex-specific. Such a detoxification system would be similar to those of various lepidopteran species that conjugate phytoecdysteroids with fatty acids as a detoxification mechanism (38). In the MPB, the increased polarity of the monoterpenyl fatty acid ester, compared with the monoterpene hydrocarbon, along with the lipophilic fatty acid side chain, may facilitate the transport and localization of the monoterpene derivative into the abdomen and specifically the fat body as a long-term storage site.

In female MPB, the accumulation of verbenyl esters as a detoxification mechanism may have further evolved into a sex-specific pheromone system, by which the release of *trans*-verbenol may have become independent of immediate contact with α-pinene. The presence of verbenyl esters becomes a sex-specific trait at the end of MPB development in the brood tree and when beetles emerge. At this stage of their life cycle, MPB cease being in direct contact with the monoterpenes of the brood trees and have not yet come in contact with monoterpenes of a new host. Females retain their verbenyl esters until the stage when they emerge and disperse in search of a new host, while emerged males appear to have metabolized or otherwise lost these compounds.

Monoterpenyl esters of short chain fatty acids such as bornyl acetate have been identified in related *Dendroctonus* species (39, 40). Monoterpenyl esters of longer chain fatty acids similar to verbenyl oleate have not been found previously in *Dendroctonus* but do exist in plants, such as chrysanthenyl hexanoate and

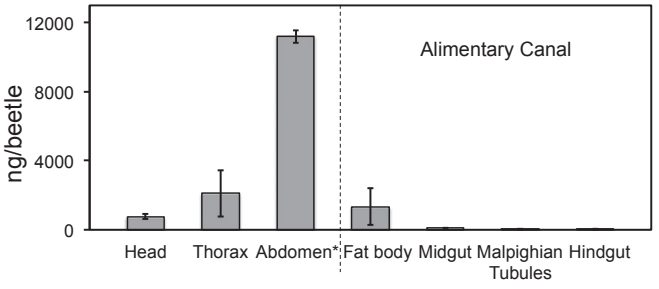

Fig. 3. The abundance of verbenyl oleate in dissected tissues of emerging female MPB. The head, thorax, and abdomen were separated, and then the alimentary canal with Malpighian tubules and perivisceral fat body were removed from the abdomen for further dissection into the fat body, midgut, Malpighian tubules, and hindgut. The asterisk denotes the abdomen with the alimentary canal removed ($n = 4$).

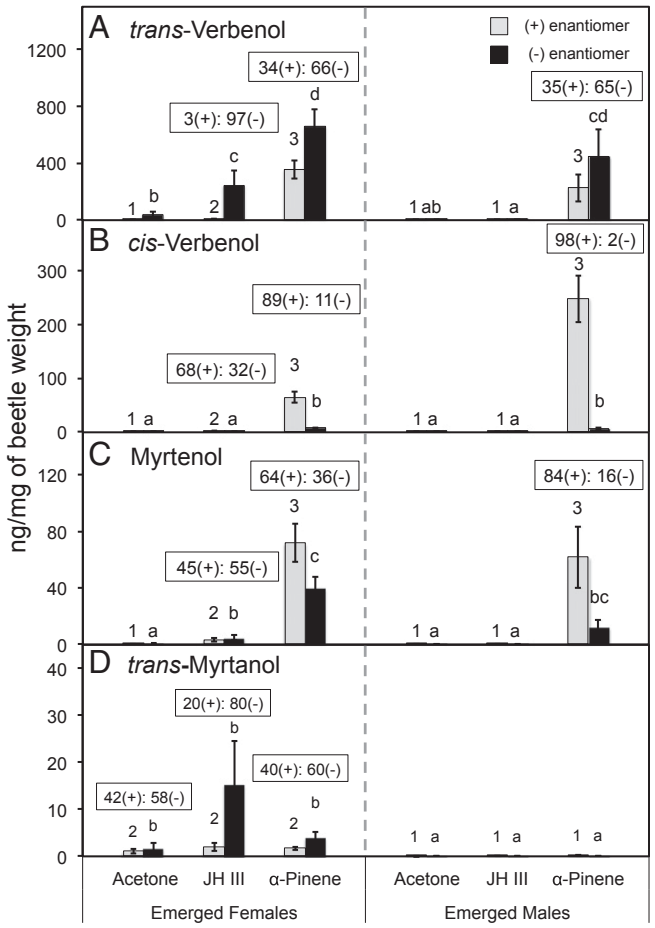

Fig. 4. The presence of monoterpene alcohol pheromone components in MPB after treatment with acetone, JHIII, or 44(+):56(–) α-pinene. Columns in the same graph with the same number [(+) enantiomer] or same letter [(–) enantiomer] were not significantly different by Conover's test ($\alpha \geq 0.05$). Compared with the acetone control, JHIII treatment significantly increased (*A*) *trans*-verbenol, (*B*) *cis*-verbenol, and (*C*) myrtenol production in female, but not male beetles. α-Pinene treatment increased (*A*) *trans*-verbenol, (*B*) *cis*-verbenol, and (*C*) myrtenol production in both sexes. (*D*) α-Pinene did not increase *trans*-myrtanol, a product of β-pinene metabolism. Enantiomeric ratios are shown where relevant ($n = 6$).

octanoate in flowers of the flat sea holly (*Eryngium planum*) (41) and geranyl, neryl, and citronellyl palmitate and stearate in rose (*Rosa* × *hybrida*) petals (42). Interestingly, these monoterpenyl esters have also been proposed to act as a reservoir for the subsequent release of the volatile monoterpenols as part of the floral scent (42).

Similar to the present study, previous work (12, 15, 19) also showed that male and female MPB can produce similar amounts of *trans*-verbenol upon treatment with α-pinene. However, males produced very little or no *trans*-verbenol when in contact with a new host tree, although both males and females come in contact with host-derived α-pinene (8, 10). These observations suggest that *trans*-verbenol released by the female MPB at the new host tree may be released, at least in part, from the female-specific reservoir of verbenyl esters perhaps triggered by exposure to host cues and regulated by JHIII.

A previous study (11) showed that the enantiomeric ratio of *trans*-verbenol released by attacking females does not correspond to the enantiomeric ratio of the α-pinene supplied by the attacked tree. Our results show that there was a small preference toward the (–) enantiomer in the conversion of α-pinene to *trans*-verbenol in adults. However, there appears to be a greater preference for the (–) enantiomer during the hydroxylation and esterification steps in earlier life stages, as evidenced by the enantiomeric excess of (–)-*trans*-verbenol in the accumulated esters and released by the female adults.

The results reported here may have broader implications for understanding pheromone production in other destructive *Dendroctonus* species that produce *trans*-verbenol as an aggregation, antiaggregation, or synergist component in their communication systems. These include the southern pine beetle (*Dendroctonus frontalis*) (43), western pine beetle (*D. brevicomis*) (44), and the red turpentine beetle (*D. valens*) (33), which cause damage across forests in North America and Asia.

Materials and Methods

Chemicals. The following chemicals were obtained from Sigma-Aldrich: racemic JHIII [catalog no. J2000, Chemical Abstracts Service (CAS) no. 24198–95-6], *N*,*N*′-dicyclohexylcarbodiimide (DCC, catalog no. D8002), 4-(dimethylamino)pyridine (DMAP, catalog no. 39405), *N*,*O*-*bis*(trimethylsilyl)trifluoroacetamide (BSTFA, catalog no. 15209), (+)-α-pinene (CAS no. 7785–70-8), (–)-α-pinene (CAS no. 7785–26-4), (–)-carveol (catalog no. 192384), (–)-myrtenol (catalog no. W343900), oleic acid (catalog no. O1008), palmitic acid (catalog no. P0500), (*S*)-(–)-perillyl alcohol (catalog no. 218391), stearic acid (catalog no. 85679), (–)-transpinocarveol (catalog no. 80613), (–)-β-pinene (catalog no. 112089), (+)-3-carene (catalog no. 441619), (–)-limonene (catalog no. 218367), and myrcene (catalog no. M100005). *trans*-Verbenol [~20(+):80(–) optical purity, lot no. W06-00141], and *cis*-Verbenol [~20(+):80(–) optical purity, lot no. CV001129] were obtained from PheroTech. The (–)-*trans*-myrtanol (catalog no. 5134 S) was obtained from Extrasynthese, and (–)-β-phellandrene was obtained by purification from lodgepole pine (*Pinus contorta*) turpentine by Synergy Semiochemicals.

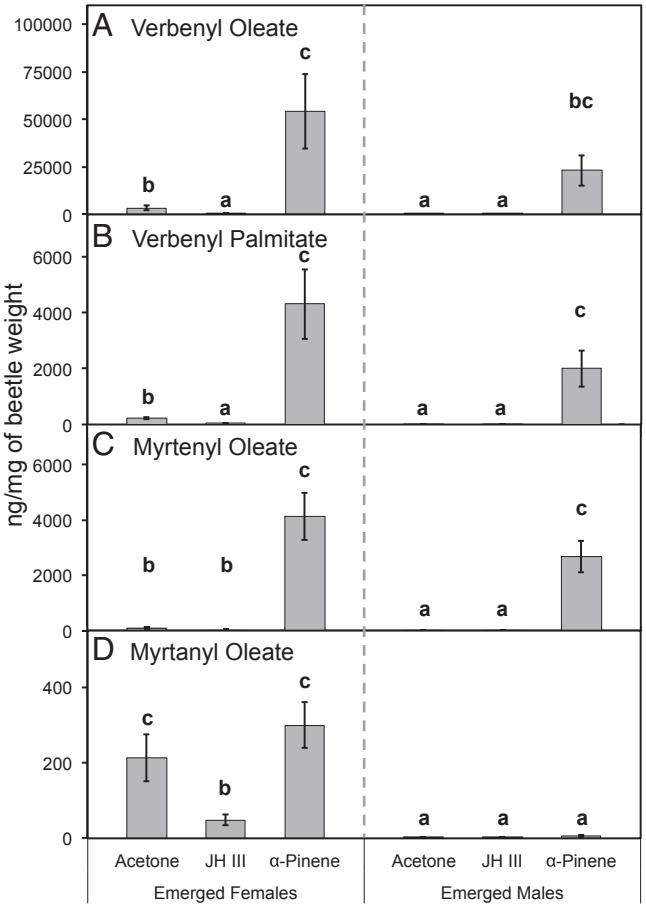

Fig. 5. The presence of monoterpenyl esters in MPB after treatment with acetone, JHIII, or α-pinene. Columns in the same graph with the same letter were not significantly different by Conover's test ($\alpha \geq 0.05$). (*A*) Verbenyl oleate, (*B*) verbenyl palmitate, and (*D*) myrtanyl oleate declined significantly with JHIII treatment in females. (*A*) Verbenyl oleate, (*B*) verbenyl palmitate, and (*C*) myrtenyl oleate increased significantly with α-pinene treatment in both sexes ($n = 6$).

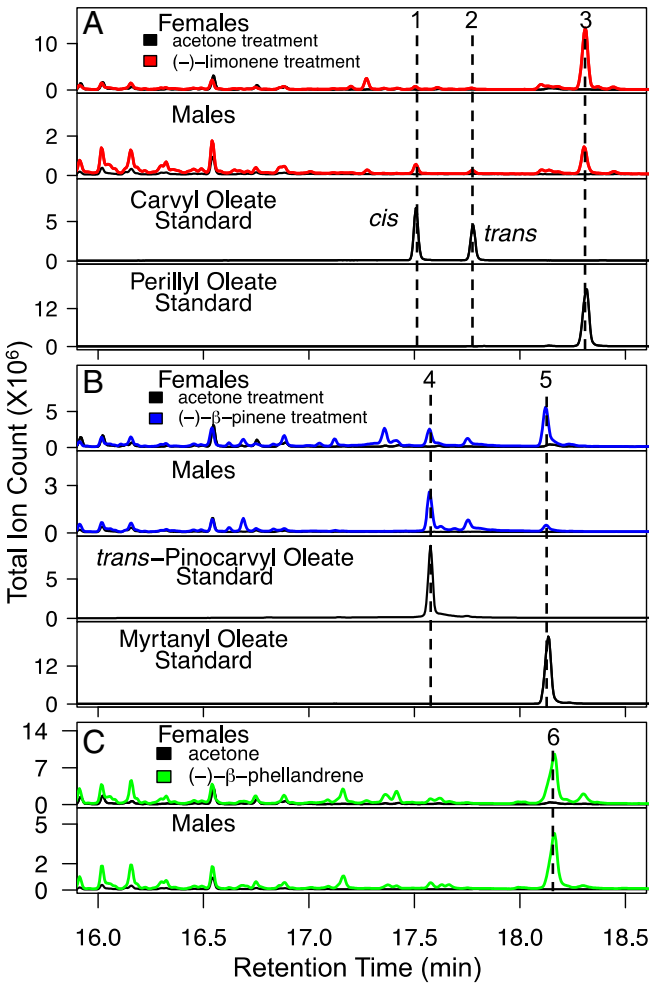

Fig. 6. GC/MS chromatograms of extracts of female and male beetles treated with acetone, (–)-limonene, (–)-β-pinene, and (–)-β-phellandrene. The monoterpenyl esters, (*A*) carvyl oleate and perillyl oleate, were identified in both sexes of limonene-treated MPB; (*B*) pinocarvyl oleate and myrtanyl oleate were identified in both sexes of β-pinene–treated MPB; and (*C*) a monoterpenyl ester was present in β-phellandrene–treated MPB, but could not be identified. The mass spectra of peaks labeled 1–6 are shown in *SI Appendix*, Fig. S8.

MPB and Phloem Collection. MPB-infested lodgepole pine (*P. contorta*) stems were collected near Whistler, British Columbia, Canada (50°12′46.6″ N, 122°53′ 20.8″ W). In June 2016, MPB pheromone baits (Contech) were attached to trees and removed after attack 4 wk later. In September 2016, infested trees were felled and the logs placed in screened cages at room temperature. Phloem samples were collected from the infested logs. Larvae, pupae, and teneral adults were dissected from infested logs and stored at –80 °C. Larval instar was estimated by head capsule width according to Bleiker and Régnière (45). In addition, emerging adults were collected every 3–4 d from inside the screened cages. Adult MPB were sexed using the shape of the seventh abdominal tergite according to Lyon (46), and larvae and pupae were sexed by PCR.

MPB Sexing by PCR. Sex-specific PCR primers were designed to identify the sex of MPB larvae and pupae (*SI Appendix*, Figs. S9 and S10 and Table S2). Similar to a method developed to identify the sex in *Tribolium casteneum* (47), these primers amplify regions of the neo-X and neo-Y chromosomes in a multiplex PCR. PCR of males (neo-X and neo-Y) gives two major DNA products, and PCR of females (neo-X and neo-X) gives one major DNA product as detected on an agarose gel. Primers were validated with 60 individual male and female adults that were sexed using morphological characteristics (46). DNA was extracted from larval and pupal samples that had previously been extracted with pentane for *trans*-verbenyl ester analysis. A volume of about 1 mm^3 MPB sample was used to produce a 20-μL DNA extract using the prepGEM insect kit (ZyGEM) according to the manufacturer's instructions.

MPB Treatment. Emerging adult female and male MPB were separately treated with acetone, JHIII, α-pinene, and other monoterpenes to test for the production of *trans*-verbenol. For treatment with JHIII, 0.5 μL of JHIII (20 mg/mL in acetone) was topically applied to the abdomen. Topical application of 0.5 μL acetone served as a control. Treated beetles were placed individually into sealed 20-mL glass vials. Both enantiomers of α-pinene were used to treat beetles, as both enantiomers are present in the pine hosts of MPB. A nearly racemic mixture [44(+):56(–)] of α-pinene was used in treatments, of which 2 μL was applied to a 1-cm^2 Whatman filter paper and placed into a sealed 20-mL glass vial with an individual beetle. Beetles were allowed to come in contact with the α-pinene carrying filter paper. Other monoterpene treatments were done with 2 μL of (–)-β-phellandrene, 2 μL of (–)-β-pinene, or 1 μL of (–)-limonene applied to a 1-cm^2 Whatman filter. Beetles were treated for 24 h and then removed from the vial, frozen in liquid N_2, and stored at –80 °C until extraction.

MPB Dissection. Emerging females were dissected into head, thorax, and abdomen. The alimentary canal was removed from the dissected abdomen and separated into the fat body (perivisceral layer), midgut, Malpighian tubules, and hindgut. The perivisceral layer of the fat body surrounds the alimentary canal and was removed cleanly without contamination from other tissues. The rest of the fat body (parietal layer), located between the muscles in the abdomen, thorax, and head, could not be removed cleanly or completely and was left in place.

Metabolite Extraction. Frozen beetles were crushed in a 2-mL Safe-Lock tube (Eppendorf) on dry ice using a cold glass stir rod and extracted with 0.5 mL pentane containing 1 ng/μL of tridecane as internal standard. Smaller specimens (first and second instar larvae and dissected alimentary canal) were extracted with 0.1–0.25 mL pentane. A single larva, pupa, or adult was extracted per tube. Samples were removed from dry ice, allowed to thaw for a few minutes, and centrifuged for 20 s at 2,000 × *g*. Samples were frozen again on dry ice, and the pentane supernatant was transferred into an amber 2-mL glass vial (Agilent). Beetles were extracted a second time with 0.5 mL pentane, and the two extracts were combined. To remove excess amounts of fatty acids from the sample, 400 μL of 1 mM ammonium carbonate (pH 8) was added to the combined pentane extract and vortexed. The sample was centrifuged for 10 min at 3,000 × *g*, and the pentane layer was removed for analysis by GC/MS. Phloem samples were extracted with 1.5 mL of methyl tertiary butyl ether (MTBE) (Sigma-Aldrich).

Identification of Monoterpenyl Fatty Acid Esters in Female MPB. To identify female-specific compounds found as esters in emerged beetles, male and female beetle extracts were separately processed by silica chromatography and ester hydrolysis. Beetle extracts were evaporated to dryness, redissolved in 0.2 mL hexane, loaded onto a silica column (300 mg) (catalog no. S2509; Sigma), and washed with 4 mL hexane to remove alkanes. Esters were eluted with 6 mL of 1% (vol/vol) MTBE in hexane. To hydrolyze the esters, 3 mL of the ester fraction was evaporated to dryness and hydrolyzed by redissolving in 0.5 mL of 0.3 M methanolic potassium hydroxide and incubation at 75 °C for 1 h in a sealed amber glass 2-mL vial (catalog no. 5182–0716; Agilent). The released alcohols were then extracted twice with 0.5 mL pentane. The ester fraction and the alcohols obtained by hydrolysis were analyzed using GC/MS.

Synthesis of Monoterpenyl Ester Standards. Monoterpenyl esters were synthesized by Steglich esterification (48) for the identification of the female-specific esters. Amounts of 35 mmol of fatty acid and 105 mmol of monoterpenol were combined in 1 mL of CH_2Cl_2 and placed on ice, and 0.2 mL of an ice-cold CH_2Cl_2 solution containing 39 mmol of DCC and 3.5 mmol of DMAP was added. Amber GC vials (Agilent) containing the reaction mixture were briefly shaken and then held on ice for 10 min before being placed on a shaker at room temperature for 2 h. The subsequent work-up involved washing with 1 M sodium acetate (pH 5.2), washing with saturated NaCl, and then drying over anhydrous $MgSO_4$. The CH_2Cl_2 solution was evaporated under a flow of nitrogen gas, and the product was redissolved in 0.5 mL hexane. The product was purified on a 300-mg silica column by first passing 4 mL hexane through the column and then eluting the ester product with 6 mL of 1% (vol/vol) MTBE in hexane. Monoterpenyl esters were analyzed using GC/MS. However, verbenyl oleate was thermally unstable at the high-temperature conditions of the GC inlet (250 °C), resulting in several breakdown products detected with multiple peaks in the chromatogram of standards. In addition, the menthatrienes, cymenes, and verbenes (decomposition products of verbenol) were detected in the GC/MS of beetle extracts whenever the esters were present, even when hydrocarbons had been removed by silica chromatography (*SI Appendix*, Fig. S1). Cool (30 °C) on-column injection of ester standards and beetle ester fractions prevented the decomposition of the esters (Fig. 1).

GC/MS Analysis. Monoterpenyl esters were separated and analyzed using an Agilent VF-5 column (5% phenyl methyl siloxane, 27.4 m length, 250 μm i.d., 0.25-μm film thickness) at 0.9 mL·min^{-1} He on an Agilent 7890A system GC, Agilent 7683B series GC Sampler, and a 7000A GC/MS triple quad MS detector at 70 eV. The GC temperature program was as follows: 40 °C for 2 min, increase at 18 °C min^{-1} to 300 °C, hold for 7 min, using a pulsed splitless injector held at 250 °C. Fatty acids were derivatized with BSTFA before analysis.

Due to their thermal instability, the monoterpenyl ester standards were also analyzed using a cool-on column injector on an Agilent HP-5 column (5% phenyl methyl siloxane, 30 m length, 250 μm i.d., 0.25-μm film thickness) at 0.9 mL·min^{-1} He on an Agilent 6890A system GC, Agilent 7683 series GC Sampler, and an Agilent 5973 Mass Selective Detector at 70 eV. The GC temperature program was as follows: 30 °C for 1 min, increase at 20 °C min^{-1} to 300 °C, and hold for 7 min. The temperature of the cool on-column injector increased at 20 °C min^{-1}, tracking the temperature of the column.

Enantiomeric purity of *trans*- and *cis*-verbenol extracted from beetles was analyzed on an Agilent CyclodexB column (10.5% β-cyclodextrin, 25.7 m length, 250 μm i.d., 0.25-μm film thickness) at 0.9 mL·min^{-1} He on an Agilent 7890A system GC, Agilent GC Sampler 80, and a 7000A GC/MS triple quad M5975C inert XL MSD with triple axis detector at 70 eV. The GC temperature program was as follows: 40 °C for 2 min, increase at 10 °C min^{-1} to 100 °C, 20 °C min^{-1} to 230 °C, hold for 7 min with pulsed splitless injector held at 250 °C.

Statistical Analysis. Compound quantities were analyzed using nonparametric tests because they failed tests for normality and homogeneous variances (Shapiro–Wilk normality test and Barlett test of homogeneity of variances). We used the Kruskal–Wallis rank sum test followed by the Conover's test for pairwise comparisons with the *P* values adjusted by the Benjamini, Hochberg, and Yekutieli correction method for multiple comparisons.

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