A recent outbreak of mountain pine beetle (MPB; *Dendroctonus ponderosae*) epidemic has affected over 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 monoterpnyl 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 monoterpenes defenses in both sexes, from which a specialized and female-specific system of pheromone biosynthesis and release may have evolved.

**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, myrtenyl oleate, and myrtenyl palmitate (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...
myrtyl 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, myrtyl, and myrtyl 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. 2A), 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 larval stages, they significantly increased in teneral adult females and remained high in emerged females. In contrast to the verbenyl and myrtyl esters, the levels of other monoterpenyl esters, specifically perillyl and caryyl oleate, were independent of sex and decreased over the life cycle from early instar larvae to emerged adults (Fig. 2 E and F).

Levels of trans-Verbenol and Verbenyl Esters Increased in Females and Males Exposed to α-Pinene. Following the observation that exposure to α-pinene vapor resulted in increased levels of verbenyl and myrtyl 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 monoterpenes exposure. Females and males were individually exposed to α-pinene, (−)-β-pinene, (−)-limonene, and (−)-β-phellandrene. Exposure to all of these monoterpenes resulted in the production of monoterpenic alcohols and the corresponding monoterpenyl esters in the beetles (Fig. 6). Often, multiple products were produced from the treatment with a single monoterpen. 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). The presence of C. funebralis and I. pini produce the monoterpenol pheromones ispenol 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).
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 oleoresin 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, 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 4).
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 monoterpentyl esters have also been proposed to act as a reservoir for the subsequent generation, 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-bistrimethylsilyl trifluoroacetamide (BSTFA, catalog no. 15209), (+)-α-pinene (CAS 7785-70-8), (−)-α-pinene (CAS no. 7785-26-4), (−)-carveol (catalog no. 192384), (−)-myrtenol (catalog no. W343900), oleic acid (catalog no. D1008), palmitic acid (catalog no. P0500), (5)-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 (−)-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. 51345) was obtained from Extrasynthese, and (−)-β-phellandrene was obtained by purification from lodgepole pine (Pinus contorta) turpentine by Synergy Semichemicals.

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 (α = 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).

Fig. 5. The presence of monoterpolyester 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 (α = 0.05). (A) Verbenyl oleate, (B) verbenyl palmitate, and (C) myrtenyl 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).
Figure 6. GC/MS chromatograms of extracts of female and male beetles treated with acetone, (--)limonene, (--)pinene, and (--)α-phellandrene. The monoterpene esters, (A) carvyl oleate and perillyl oleate, were identified in both sexes of limonene-treated MPB; (B) pinocarvyl oleate and myrtyl oleate were identified in both sexes of pinene-treated MPB; and (C) a monoterpene ester was present in α-phellandrene-treated MPB, but could not be identified. The mass spectra of peaks labeled 1–6 are shown in SI Appendix, Fig. S8.

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 [4α(+)-3β(--)] of α-pinene was used in treatments, of which 2 μL was applied to a 1-cm² 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 monoterpane treatments were done with 2 μL of (--)α-phellandrene, 2 μL of (--)pinene, or 1 μL of (--)limonene applied to a 1-cm² Whatman filter. Beetles were treated for 24 h and then removed from the vial, frozen in liquid N₂, 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/mL tridecane as internal standard. Small amounts of tridecane (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 M sodium 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 extracts were treated 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 beetles 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 0.5 mL pentane was added to 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 fractions 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 CH₃Cl₂ and placed on ice, and 0.2 mL of an ice-cold CH₃Cl₂ 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 MgSO₄. The CH₃Cl₂ 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 mentha- trienes, cymenes, and verbenes (decomposition products of verbenol) were detected in the GCMS 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).
GCMS Analysis. Monoterpenyl esters were separated and analyzed using an Agilent VF-5 column (5% phenyl methyl silicone, 27.4 m length, 0.25 μm i.d., 0.25-μm film thickness) on an Agilent 7890A system GC, Agilent 78938 series GC Sampler, and a 7000A GCMS 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 silicone, 25 m length, 0.25 μm i.d., 0.25-μm film thickness) at 0.9 mL·min⁻¹. He on an Agilent 6890A system GC, Agilent 7893 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. Enzyme-catalyzed purification of trans- and cis-β-geranial extracted from beetles was analyzed on an Agilent Cyclodex® column (10.5% β-cyclodextrin, 25.7 μm i.d., 0.25-μm film thickness) at 0.9 mL·min⁻¹. He on an Agilent 7890A system GC, Agilent GC Sampler 800, and a 7000A GCMS triple quad MS5975C 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 Bartlett 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.

Acknowledgments. We thank Mr. Stirling Angus (UC Forestry Ltd.), Mr. Peter Ackhurst (Cheekamusk Community Forests), Mr. David Ehrhardt (Wedgewoods Estates), Dr. Justin G. A. Whithell [University of British Columbia (UBC)], and Ms. Judith K. Booth (UBC) for technical and logistic assistance, including access and help with beetle collections and bioassays; Ms. Lina Madilao (UBC) for expert GCMS analysis; and Dr. Erika Plettner (Simon Fraser University). Dr. Murry H. J. F. Huang (UBC) for advice and discussions. The research was supported by the Natural Sciences and Engineering Research Council of Canada (J.B.) through the TRIA Net Project and a Discovery Grant. J.B. is a Distinquished University Scholar.