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p-Mentha-1,3-dien-9-ol: A novel aggregation-sex pheromone for monitoring longhorn beetles (Cerambycidae) in Eurasia and North America

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Abstract

Longhorn beetles (Cerambycidae) are a diverse family of beetles that can cause considerable damage as forest pests and vectors of pathogens, as well as being important components of forest food webs and ecosystem functionality. In recent years, numerous cerambycid pheromones have been identified, revealing some broad general patterns in functionality in terms of sex or aggregation-sex pheromones in different subfamilies and different types of compounds characterizing the pheromones of various cerambycid taxa. Here, we describe the identification of the aggregation-sex pheromones of the Eurasian longhorn beetle *Aromia moschata moschata* (L.) (Cerambycinae; tribe Callichromatini) and the North American species *Holopleura marginata* LeConte (Cerambycinae; Holopleurini), as part of an ongoing effort to extend the taxonomic coverage of identified cerambycid pheromones and to expand the prospects for cerambycid monitoring into the study of biodiversity and ecosystem services. Both species were found to use the novel pheromone compound *p*-mentha-1,3-dien-9-ol, which also attracted significant numbers of the longhorn beetle *Xestoleptura crassipes* (LeConte) (Lepturinae; Lepturini) in trials in California. *p*-Mentha-1,3-dien-9-ol represents a class of pheromone compounds novel to both tribes (Callichromatini and Holopleurini), further increasing the chemical space of identified pheromones within the subfamily Cerambycinae. This compound is also noteworthy because it represents an entirely different chemical class of pheromones than the monoepoxide (*E*)-2-*cis*-6,7-epoxynonenal, previously reported as an aggregation-sex pheromone for the invasive Asian congener *Aromia bungii* (Faldermann).

KEYWORDS
conservation, Douglas fir, monitoring, musk beetle, *p*-cymen-9-ol, willow

1 | INTRODUCTION

Longhorn beetles (Coleoptera: Cerambycidae) are mainly xylophagous insects, developing in living or dead wood of numerous trees and shrubs and thus assisting in the natural recycling of woody biomass (Švácha & Lawrence, 2014). Due to their taxonomic diversity (>36,000 described species), global distribution and association with woody plants, cerambycids are economically and biologically important insects (Haack, 2017; Linsley, 1959; Nearns, 2013). It is now known that many longhorn beetle species produce long-distance sex or aggregation-sex pheromones to facilitate mate finding (Hanks & Millar, 2016). Because many species are cryptic and difficult to monitor, which can be compounded by annual flight periods of only a few weeks, monitoring populations with traps baited with synthetic pheromone lures is an effective methodology for quantitative studies of both pest species (e.g., Rassati et al., 2012; Sweeney et al., 2010; Xu et al., 2017) and non-pest species of concern for environmental management (Molander, 2019; Ray et al., 2014; Žunič-Kosi et al., 2017). Recent research has increased the available data on the specific compounds that serve as pheromones in a number of cerambycid species and subfamilies (Millar & Hanks, 2017). However, to date, fewer than 1% of all cerambycid species have been studied in terms of their pheromone chemistry.

As part of ongoing efforts to explore the chemical space occupied by cerambycid pheromones and extend the applications of pheromone-based monitoring of these insects, we examined the pheromone chemistry of two non-pest species, the musk beetle *Aromia moschata moschata* (L.), native to Europe and Central Asia, and *Holopleura marginata* LeConte, native to western North America. Both species are members of the subfamily Cerambycinae, in the tribes Callichromatini and Holopleurini, respectively (Danilevsky, 2019; Monné & Hovore, 2005). *Aromia m. moschata* is well known for the musky scent produced by adults of both sexes, consisting of the monoterpenoids rose oxide and iridodial that are presumed to be defensive secretions (Vidari et al., 1973), but neither species has been studied previously with respect to their use of attractant pheromones.

In northern Europe, larvae of *A. m. moschata* develop in woody tissues of living willow trees (*Salix* spp.) for 3 years, feeding subcortically and in the xylem of larger branches and stems (~ ≥10 cm diameter; [Bense, 1994; Ehnström & Axelsson, 2002; Ehnström & Holmer, 2007]). In continental Europe, thinner branches are also frequently used (Klausnitzer et al., 2016). Despite developing in living trees, creating deep galleries, *A. m. moschata* is of minor economic importance within its native range; occasional damage has been reported in *Salix* plantations grown for production of baskets and cricket bats (Anonymous, 1936; Duffy, 1949; Waleryś & Sądej, 2009). The species is of greater significance from a biodiversity standpoint. Larvae of *A. m. moschata* often develop alongside larvae of the goat moth (*Cossus cossus* [L.]), and the two species together may weaken trees and create sections of dead wood that are utilized by other saproxylic insects, including species considered rare (Duffy, 1949; Ehnström & Holmer, 2007, 2011). Further, the sap runs that often form on infested trees are a source of nourishment for a diversity of insect species. Thus, *A. m. moschata* may to some extent serve as a microecosystem engineer, similar to what has been proposed for the great capricorn longhorn beetle (*Cerambyx cerdo* L.), the larvae of which develop in mature oak trees (cf. Buse et al., 2008). Although *A. m. moschata* is widespread and relatively common throughout Europe (Bense, 1994; Bilý & Mehl, 1989; Jeniš, 2001), population declines have been noted in certain areas (Baumann, 1997; Ehnström & Holmer, 2007; Lindhe et al., 2010). In Sweden, where our study areas were located, *A. m. moschata* was nationally red-listed as Near Threatened during 2000–2005 (Gärdenfors, 2000).

There is little published information on the biology of *H. marginata*, the only species in the tribe Holopleurini of the Cerambycinae (Monné & Hovore, 2005). Its known range stretches from British Columbia to California on the western coast of North America, and reported hosts include twigs and branches of deciduous trees such as California bay laurel (*Umbellularia californica* [Hook. & Arn.] Nutt.), toyon (*Heteromeles arbutifolia* [Lindl.]) and conifers such as bigcone spruce (*Pseudotsuga macrocarpa* [Vasey]), Douglas fir (*Pseudotsuga menziesii* [Mirb.] Franco) and bristlecone fir (*Abies bracteata* [D. Don.] A. Poit.) (Cope, 1984; Linsley, 1962). Larvae feed of the inner bark and outer sapwood, but pupate in the heartwood (Grant, 1963; Penrose & Westcott, 1974). Adults are reported to be active from May to July. *Holopleura marginata* is not of economic importance within its native range because it develops in dead wood, but its limited distribution could make it vulnerable to environmental disturbance.

Here, we show that male beetles of *A. m. moschata* and *H. marginata* both emit the unstable monoterpene alcohol *p*-mentha-1,3-dien-9-ol, not previously known as a cerambycid pheromone component. Synthetic *p*-mentha-1,3-dien-9-ol, combined with a stabilizer, was significantly attractive to both species in field bioassays in Sweden and California, respectively. Because both females and males were attracted, the novel compound functions as an aggregation-sex pheromone (sensu Cardé, 2014). We discuss the potential to use the newly identified pheromone in an applied context during field studies of *A. m. moschata* and *H. marginata*, and the need for further bioassays of this compound in other countries and continents to assess how prevalent this particular structure, and more generally other analogues with the same 1-isopropyl-4-methylcyclohexane structural motif, may be as pheromones among the Cerambycidae.

2 | MATERIALS AND METHODS

2.1 | Source of study animals

Adults of *A. m. moschata* were reared from branches and stems of goat willow (*Salix caprea* L.) cut from several large, mature willow trees at various locations within Ecopark Hornsö, southern Sweden (approximate centre coordinates of the park, WGS 84: DD 57.0120 N, 16.0897 E). Many of the trees showed evidence of recent feeding by larvae of *A. m. moschata*, with fresh galleries and emergence holes. In total, ~0.25 m^3 of wood (diameter ~ 10–15 cm)

was collected in mid-December 2015. In mid-January 2016, the logs were cut into ~50 cm-long pieces that were placed in transparent plastic boxes with part of the lid replaced with a fine plastic mesh for ventilation. The boxes were stored in a greenhouse at the SLU campus (daily mean temperature ~ 15°C), and checked at least once per day for emerged insects. Logs were periodically misted with water to prevent desiccation.

Adults of *A. m. moschata* began to emerge after 7 weeks and continued to emerge for nearly a week, yielding four males and five females (sex determined by antennal length relative to body length, longer in males; (Bilý & Mehl, 1989)). The adult beetles were caged separately by sex in smaller plastic containers with lids partially covered by plastic mesh for ventilation. Pieces of paper, smeared with honey water, were added to the containers to provide nourishment and replaced every 2–3 d. While adults were continuing to emerge, and in between headspace collections (see below), the containers were kept in a refrigerator at 8°C to extend longevity.

Adults of *H. marginata* used for collection of headspace odours were reared from infested branches of Douglas fir collected by ABR on 29 March 2013 at Nelson Ravine in Tehama Co., California (Highway 32 near mile 32.48; 39.98111, −121.63889, 955 m). The rearing chamber was a plastic 19 L bucket with a mesh-covered hole in the lid for ventilation, held in an unheated garage (see site #1 below) to approximate seasonal temperatures. Five adults of *H. marginata* (and a number of other species) emerged from the wood from 13 to 26 March 2015. Although the flight period for this species is May–July (Linsley, 1962), adults can be reared outside the normal emergence period (ABR, pers. obs.). The beetles were removed from the rearing chamber before they could mate and placed individually into glass vials. Two living males were shipped by overnight courier to JGM at UC Riverside for collection of headspace odours.

2.2 | Collection of volatiles

Volatile compounds emitted by adults of *A. m. moschata* and *H. marginata* were sampled using headspace collections. Procedures for *A. m. moschata* have been described before (Molander & Larsson, 2018). Three males and three females were sampled in 1 L gas washing bottles (Lenz Laborglas Gmbh, Wertheim, Germany), with an empty bottle used as a system control. To reduce the risk of aggression between same-sex individuals, strips of metal mesh were added to the bottles to allow beetles to distribute themselves.

Ambient air was pulled through the glass bottles with a pump (model PM 10879 NMP 03, KNF Neuberger, Freiburg, Germany) at 0.2 L min^{-1}. A volatiles trap consisting of a bed of Porapak™ Q adsorbent (25 mg, mesh size 50–80, Supelco/Sigma-Aldrich, Munich, Germany), enclosed in polytetrafluoroethylene (PTFE) tubing was placed in line between each glass bottle and the pump. A second Porapak™ Q trap was connected to the bottle inlet to purify the incoming air. Headspace collections were performed from ~10 AM–4 PM and lasted for ~5–6 h. In total, four headspace collections were performed, each resulting in one extract of volatiles from males, females and the control. Collectors were eluted immediately after each headspace collection with 300 µl of hexane and reconditioned with 3 × 300 µl of hexane followed by 3 × 300 µl of acetone before reuse. Extracts of volatiles were stored at −18°C until analyses.

Analogous headspace extracts were prepared from *H. marginata*, using beetles shipped by overnight courier to UC Riverside. For collection of volatiles, beetles were held in modified 0.5 L glass canning jars, with volatiles collected on activated charcoal traps and eluted with methylene chloride. Details of the materials and procedures have been previously described (Millar et al., 2019).

2.3 | Identification of insect-produced compounds

Extracts of volatiles from *A. m. moschata* were analysed at SLU Alnarp using a gas chromatograph (model 7890B; Agilent Technologies, Palo Alto, CA, USA), interfaced to a mass spectrometer (model 5977A; Agilent). The GC was equipped with a DB-WAX capillary column (polyethylene glycol, 60 m × 0.25 mm inner ø, 0.25 µm film thickness; J&W Scientific, Folsom, CA, USA). Injections of 2 µl of each extract of volatiles were made manually in splitless mode (split vent opened after 0.5 min, injector temperature 225°C). Blank control injections (2 µl of hexane) were performed before and in between analyses of headspace samples. The carrier gas was helium with a constant flow rate of 1.9 ml min^{-1} (front inlet pressure 182 kPa). The oven programme started at 30°C, with a 3 min hold, thereafter rising by 8°C/min to 230°C, hold 10 min. The mass spectrometer was set with a solvent delay of 6 min, and spectra were taken in electron impact ionization (EI) mode at 70 eV, with a scan range of 29–400 amu. Calculations of the ratios of compounds for males of *A. m. moschata* males were performed with the data from Sweden.

Extracts of *H. marginata* were analysed by GC–MS at UC Riverside, with an Agilent 7820A GC coupled to a 5977E mass selective detector. Samples were run in splitless mode on an HP-5 column (30 m × 0.25 mm inner ø × 0.25 µm film; Agilent) using helium as carrier gas, at a linear velocity of 37 cm/sec. The GC oven was programmed from 40°C/1 min, 10°C/min to 280°C, hold for 10 min. Spectra were taken with electron impact ionization (70 eV). The transfer line, GC inlet, ion source and quadrupole temperatures were 280, 250, 150 and 200°C, respectively. Extracts of *A. m. moschata* shipped from Sweden were analysed under the same conditions.

2.4 | Chemicals for field trials

The synthesis of the pheromone chemicals is described in the Online Supplement.

2.5 | Field bioassays

Adults of *A. m. moschata* were trapped with custom-built, cross-vane, flight-intercept traps with an underhanging funnel protruding into a collecting jar and a top cover for rain protection (detailed description in Molander et al., 2019). The traps were wired to steel reinforcing bars that were driven partway into the ground, with the central part of the trap hanging at ~1.5 m above ground. The cross-vane panels, and the inside of the funnel, were coated with Fluon® (polytetrafluoroethylene dispersion, 60 wt % in H_2O, Sigma-Aldrich, St. Louis, Missouri, USA, further diluted 1:1 with water) just before trap deployment, to increase trap efficiency (see e.g., Graham & Poland, 2012). Propylene glycol (~0.25 L per trap) was used as a killing and preservative agent in the collecting jars.

Traps were deployed with 20 spatial replicates dispersed in two areas directly west (13 replicates) and east (seven replicates) of Lake Krankesjön in southern Sweden in 2018 (approximate centre coordinates, WGS 84: DD 55.6996 N, 13.4765 E). Willows (*Salix* spp.) are common in this area and often grow in open, or semi-open, sun-exposed conditions along the edges of wetlands and grasslands where adults of *A. m. moschata* can sometimes be observed on flowering herbs (Anonymous, 2018; MAM personal observations). The replicates were mainly situated in sun-exposed locations along woodland fringes, or within glades, typically in the vicinity of willow trees and bushes, sometimes with evidence of recent activity by *A. m. moschata* larvae.

The replicates were on average spaced ~200 m apart (range 30–568 m, mean 182 ± 28 m). Each replicate consisted of one trap with a lure of *p*-mentha-1,3-dien-9-ol and one control trap (neat solvent). Within the replicates, the two traps were deployed ~10 m apart, and the lure treatment was assigned at random.

Lures consisted of Grippie® zip-lock polyethylene bags (6.5 × 5.5 cm × 40 μm, Grippie Light Nr-02, b.n.t. Scandinavia AB, Arlöv, Sweden) with 50 mg racemic *p*-mentha-1,3-dien-9-ol and 2.5 mg of butylated hydroxytoluene stabilizer, dissolved in 0.5 ml of isopropanol. The control consisted of 0.5 ml of isopropanol alone. The bag was attached with metal wire (without piercing the bag) to the central (south-facing) part of the trap, just below the top cover. Traps were deployed from 11–30 July, with the 19 d trapping period covering the main peak of flight activity for *A. m. moschata* in this area of Sweden (see Anonymous, 2018; Lindhe et al., 2010).

All longhorn beetles were counted and identified to species using the key by Ehnström and Holmer (2007). The trapped cerambycids were preserved in 70% ethanol and stored at the Department of Plant Protection Biology, SLU Alnarp Campus. Voucher specimens will be transferred to the public entomological collections in the Biological Museum, Lund University, Sweden.

Attraction of *H. marginata* to synthesized candidate pheromones was tested with a field bioassay conducted during 2018 at four sites in northern California chosen for the presence of the host species and accessibility, as follows: (1) Slaughterhouse Ravine, Magalia, Butte Co. (39.83861, −121.61694, 730 m elevation), trapping period 21 April–27 July. Site dominated by black oak (*Quercus kelloggii* Newb.), with incense cedar (*Calocedrus decurrens* Torr.), Ponderosa pine (*Pinus ponderosa* Douglas ex. C. Lawson) and Douglas fir. (2) Rattlesnake Creek at Forest Road 27N12, ~2.8 km N Colby Mt., Tehama Co. (40.17205, −121.51889, 1322 m), trapping period 22 April–12 August. Site dominated by Douglas fir, Ponderosa pine, big leaf maple (*Acer macrophyllum* Pursch) and canyon live oak (*Quercus chrysolepis* Liebm.) and white fir. (3) Junction Forest Roads 27 N06 and 27N12Y, ~2.7 km NW Colby Mt., Tehama Co. (40.16209, −121.54632, 1245 m), 6 May–12 August. Site dominated by Douglas fir, black oak, Ponderosa pine and big leaf maple. (4) Whispering Pines Pet Clinic property, Magalia, Butte Co. (39.84055, −121.59600, 767 m), 11 May–19 July.

In field bioassays with *H. marginata*, black plastic cross-vane traps coated with Fluon® (Alpha Scents, Inc., West Linn, OR, USA) were used. Trap collection buckets were partly filled with propylene glycol as a killing agent and preservative. Traps were hung from tree branches at all four sites at heights of 3–5 m to prevent tampering by bears and other wildlife. Lures consisted of 5 × 7.5 cm low density polyethylene resealable baggies each with a cotton dental wick loaded with solutions of test compounds in isopropanol as described above. At each site four traps were deployed (~10 m apart), one baited with *p*-mentha-1,3-dien-9-ol as a single component, *p*-cymen-9-ol as a single component, a 1:1 blend of the two and a control lure (neat isopropanol). Traps were serviced at intervals of ~1 wk and fresh lures were deployed every other week. Trap servicing included transferring insect catches into 70% ethanol, recharging propylene glycol and cleaning dust and debris from the traps. The beetles trapped in this study are in the personal collection of ABR. Voucher specimens will be deposited into the collection of the UC Riverside Entomology Museum.

2.6 | Statistical analysis

Differences among treatment means in numbers of adult beetles captured were tested separately for all species represented by at least 10 specimens with the nonparametric Friedman's test (PROC FREQ, option CMH; SAS Institute 2011), with replicates defined by number of traps per treatment within transects and collection date. Replicates that contained no specimens in any treatment of the beetle species in question (e.g., due to inclement weather) were dropped from analyses. Pairs of treatments were compared using the REGWQ test (controlling experiment-wise error rates; SAS Institute 2011) and were protected (i.e., assuming a significant overall Friedman's test).

3 | RESULTS

3.1 | Identification of insect-produced compounds

The headspace volatiles of *H. marginata* were dominated by two compounds (ratio 100:75 ± 31, $n = 6$; Figure 1) with apparent molecular ions at *m/z* 152 and 150, respectively, for possible molecular

formulae of $C_{10}H_{16}O$ and $C_{10}H_{14}O$, respectively. There were no good matches for either compound in the NIST 14 mass spectral database, so the compounds were identified by interpretation of their spectra in combination with biosynthetic considerations, as follows:

The mass spectrum of the compound with a molecular ion of *m/z* 150 (Figure 2, unknown B) exhibited limited fragmentation, which, along with an ion at *m/z* 91, suggested a methyl-substituted benzene ring with an additional substituent. The base peak at *m/z* 119, from a possible loss of 31 amu (=CH_2OH), suggested the presence of a primary alcohol. Furthermore, the 119 ion was 28 mass units larger than the *m/z* 91 ion, indicating that the CH_2OH group had likely been cleaved from a methyl-substituted benzene with an additional alkyl substitution consisting of either $-CH_2CH_2CH_2OH$ or $-CH(CH_3)CH_2OH$. The latter seemed more likely given the base peak at *m/z* 119 because the secondary benzylic ion resulting from loss of the CH_2OH group would be stabilized by resonance, whereas the corresponding ion from the isomer with an unbranched alkyl chain would not. Furthermore, from biosynthetic considerations, numerous monoterpenoids are characterized by a six-membered ring with methyl and isopropyl groups at the 1 and 4 positions, respectively, and so unknown B was tentatively identified as 2-(4-methylphenyl)-1-propanol (=*p*-cymen-9-ol). The tentative identification was confirmed by synthesis of an authentic standard from the corresponding acid (see Online Supplement). The GC retention time and mass spectrum of the insect-produced compound were excellent matches with those of the authentic standard.

It seemed probable that the second compound, with molecular weight 152, was a diene analog of compound B because the mass spectrum (Figure 3) exhibited several fragment ions analogous to those in the spectrum of B. Thus, the base peak at *m/z* 121 suggested loss of CH_2OH to form a stabilized allylic carbocation, and the fragment at *m/z* 93 was tentatively identified as a methylated cyclohexadiene resulting from cleavage of the alkyl group. There were five possible structures with two double bonds in the ring (three conjugated and two unconjugated, **I-V** in Figure 4). Of these, a search of Chemical Abstracts revealed that structures **III** and **V** were apparently unknown. Thus, we first focused our attention on structures **I**, **II** and **IV**. Structure **II** was readily available by Birch reduction of structure B, but its mass spectrum and retention time did not match those of the insect-produced compound. However, base-catalysed isomerization of **II** gave **I**, the retention time and mass spectrum of which were excellent matches for the insect-produced compound, confirming the structure of unknown A as the conjugated cyclohexadienol *p*-mentha-1,3-dien-9-ol.

Having identified these two structures in extracts from *H. marginata*, it was straightforward to identify them in analogous extracts from males of *A. m. moschata*. Analyses by GC-MS showed that all four extracts from males were dominated by *p*-mentha-1,3-dien-9-ol, with lesser amounts of *p*-cymen-9-ol (12.4 ± 3.2%, $n = 4$; Figure 5). As with *H. marginata*, the GC retention times and mass spectra of the two compounds in the *A. m. moschata* extracts were excellent matches with those of the authentic standards. Neither compound was detected in any of the corresponding extracts of volatiles from female beetles or controls. In addition to the two most abundant male-specific compounds, we also observed small and variable amounts of three male-specific compounds eluting after *p*-mentha-1,3-dien-9-ol, which, based on their apparent molecular ions at *m/z* 152, were likely to be isomers. The identity of one of these was confirmed as *p*-mentha-1,4-dien-9-ol, because a standard was available as an intermediate in the synthesis of the main

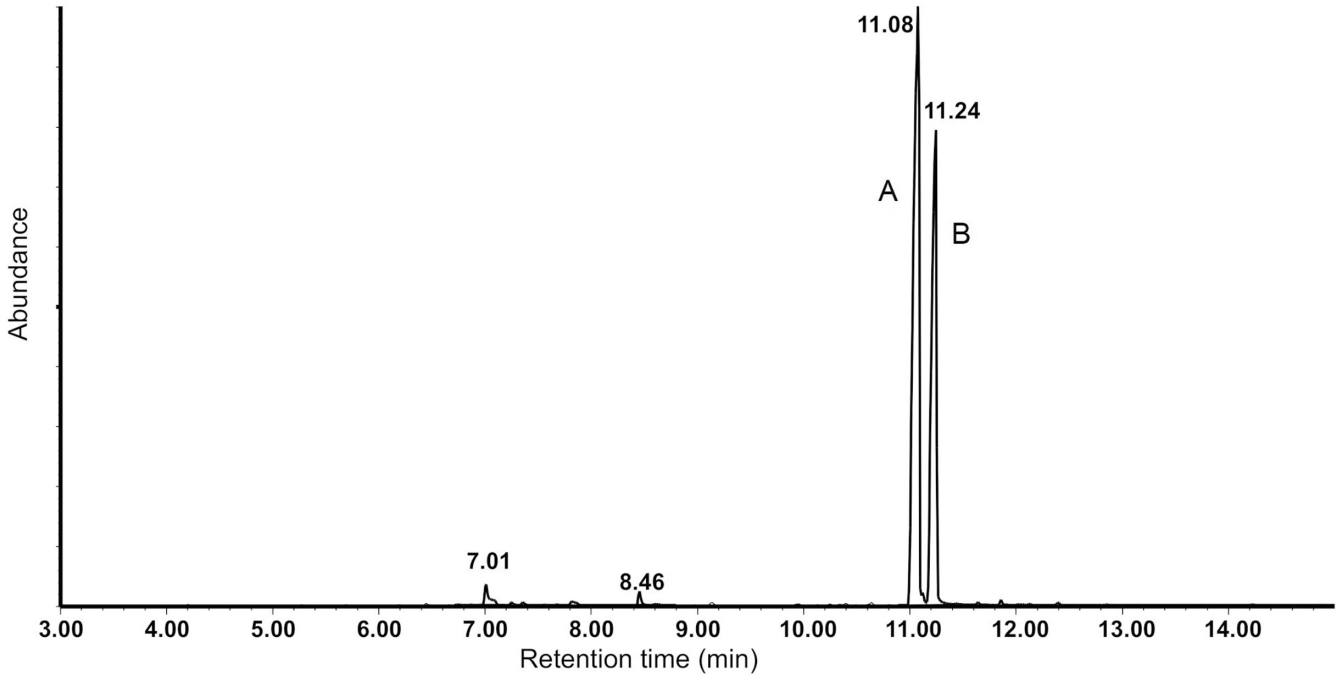

FIGURE 1 Representative total ion chromatogram of a headspace extract from a male of *Holopleura marginata*, using an HP-5 GC column. Labels (A) and (B) denote the two unknown compounds, later identified as *p*-mentha-1,3-dien-9-ol and *p*-cymen-9-ol, respectively

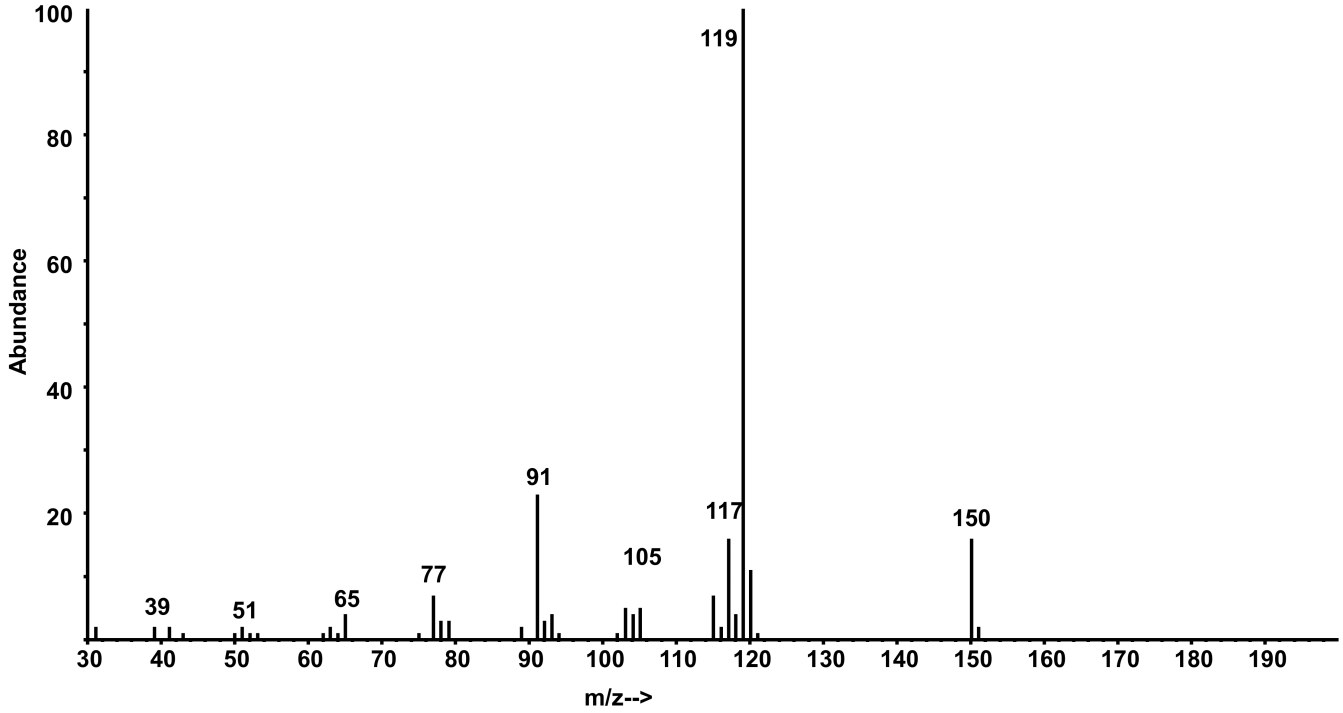

FIGURE 2 EI mass spectrum of unknown B, identified as *p*-cymen-9-ol

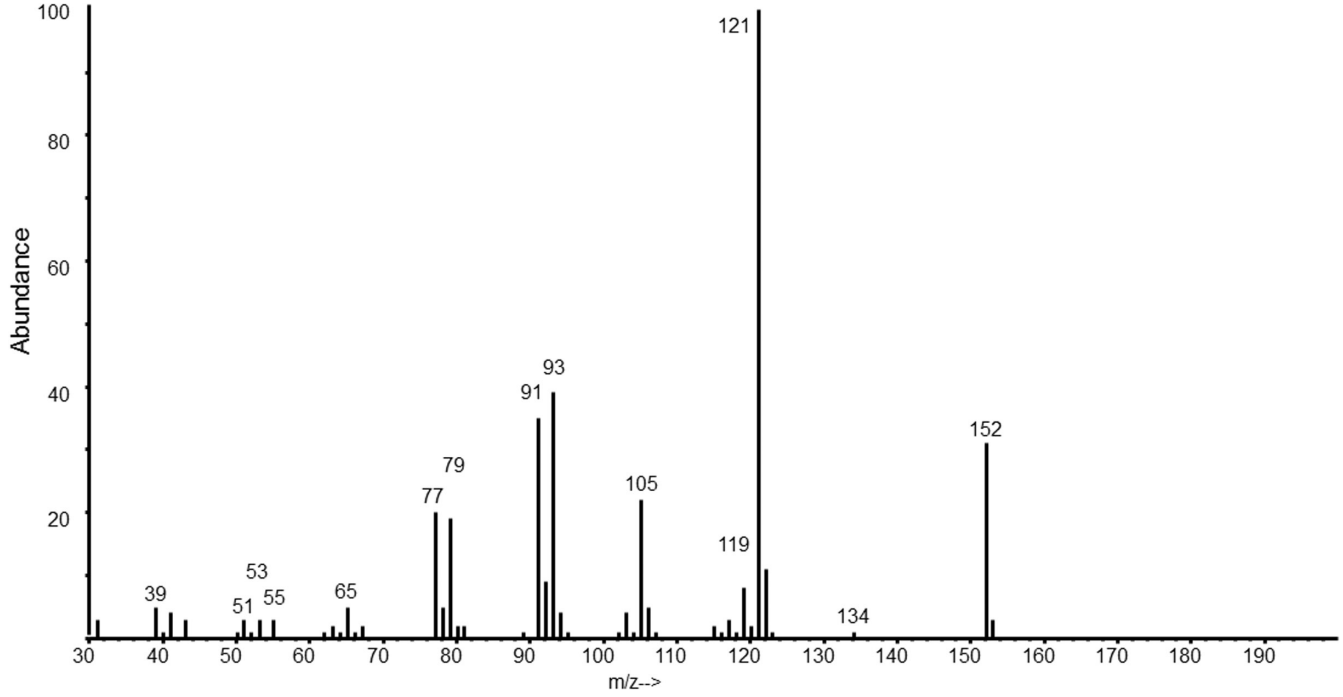

FIGURE 3 EI mass spectrum of unknown A, identified as *p*-mentha-1,3-dien-9-ol

component *p*-mentha-1,3-dien-9-ol. The three other isomers were present at an average of 1.7% per isomer (range: 1.0–2.3%) relative to the *p*-mentha-1,3-dien-9-ol.

We did not observe any sex-specific compounds in extracts from female *A. m. moschata*. However, we did observe several previously reported defensive compounds in extracts from both sexes (Vidari et al., 1973), including compounds tentatively identified as *cis*- and *trans*-rose oxide based on their mass spectra and an iridodial isomer.

p-Cymen-9-ol was readily available by reduction of commercially available 2-(4-methylphenyl)-propanoic acid with lithium aluminium hydride. Several further steps, including Birch reduction of *p*-cymen-9-ol followed by base-catalysed isomerization of the nonconjugated double bonds into conjugation (Shvartsbart & Smith, 2015) and separation of the conjugated *p*-mentha-1,3-dien-9-ol from nonconjugated isomers by reversible formation of a Diels-Alder adduct produced pure samples of the desired compound (see Online Supplement).

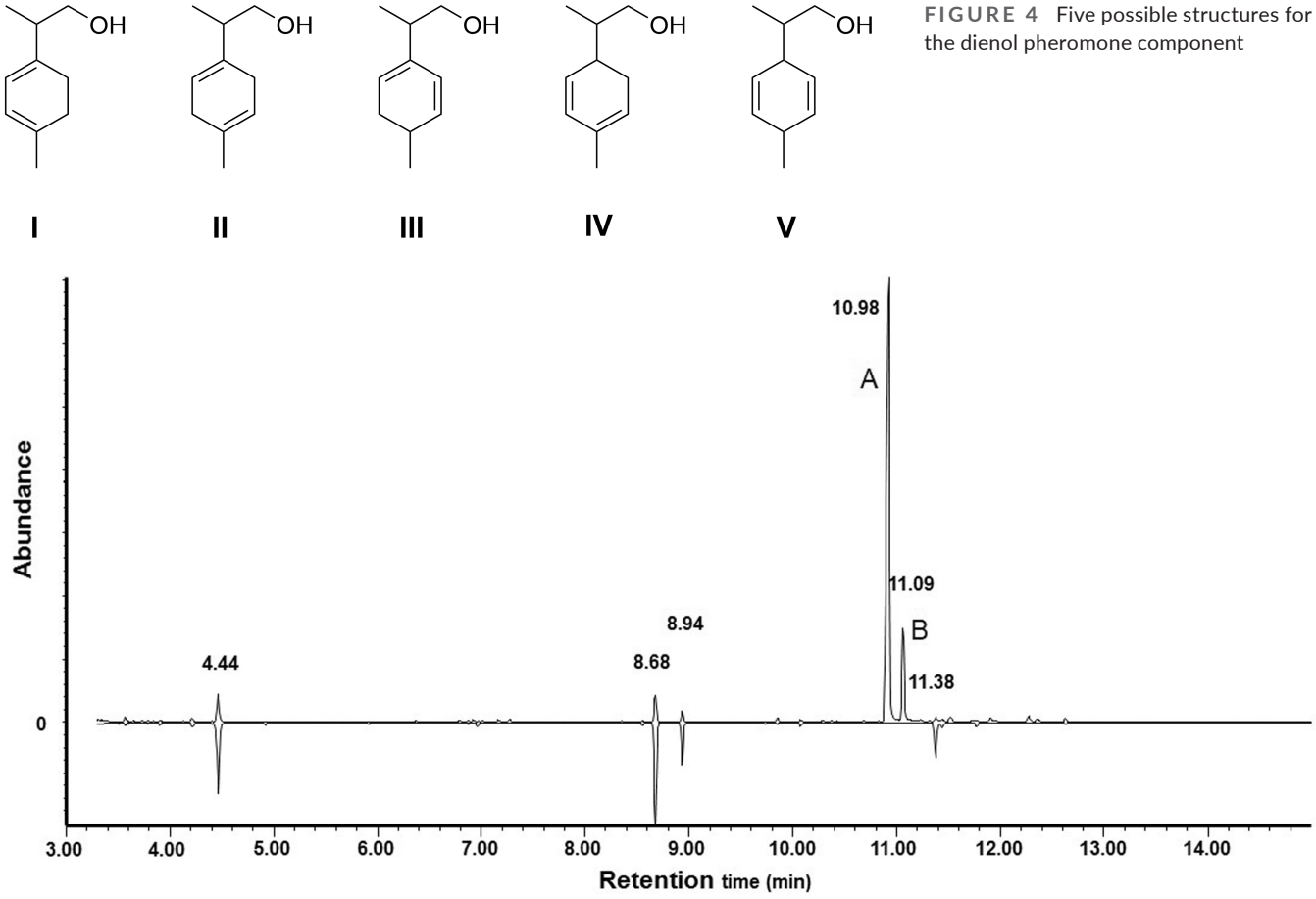

FIGURE 4 Five possible structures for the dienol pheromone component

FIGURE 5 Representative total ion chromatograms of headspace extracts from a male and female of *Aromia moschata moschata*, using a DB-wax column. Upper trace is from a male beetle, lower, inverted trace is from a female. Labels (A) and (B) denote the two unknown compounds, later identified as *p*-mentha-1,3-dien-9-ol and *p*-cymen-9-ol, respectively

3.2 | Field bioassays

In Sweden, a total of 76 adults of *A. m. moschata* were captured. The species was significantly attracted to *p*-mentha-1,3-dien-9-ol compared with the solvent control (Figure 6a; Friedman's test: $Q_{1,38} = 30.5$, $p < 0.001$). Traps with lures of *p*-mentha-1,3-dien-9-ol captured a total of 75 individuals, of which 38 were females 37 were males (51% female). The controls contained a single male beetle (0.05 ± 0.05 beetles/trap). One additional cerambycid species was captured during the bioassay, *Leptura quadrifasciata quadrifasciata* L. of the subfamily Lepturinae (tribe Lepturini). Three individuals were captured in traps with lures of *p*-mentha-1,3-dien-9-ol, compared with zero individuals in control traps, but the low number of beetles prevent any meaningful statistical analysis.

During the field bioassay in California, traps captured a total of 592 cerambycid beetles of 39 species of five subfamilies (Table S1). Of species that were represented by at least 10 specimens, only two species showed significant differences between treatment means, the target species *H. marginata*, and *Xestoleptura crassipes* (LeConte) of the subfamily Lepturinae (Lepturini). A total of 42 adults of *H. marginata* were captured (36F:6M), and the beetles were similarly attracted to the dienol alone and to a 1:1 blend of the dienol with *p*-cymen-9-ol (Figure 6b; $Q_{3,36} = 25.6$, $p < 0.001$). *p*-Cymen-9-ol was not significantly attractive as a single component, nor did it appear to influence attraction to the dienol.

The lepturine *X. crassipes* was significantly attracted to traps baited with the blend of *p*-mentha-1,3-dien-9-ol and *p*-cymen-9-ol (Table S1), with both sexes being attracted (sex ratio of all trapped beetles, 20M:5F). However, there was no significant attraction to *p*-mentha-1,3-dien-9-ol or *p*-cymen-9-ol as a single compounds, compared with the control.

4 | DISCUSSION

The consistent and sex-specific presence of *p*-mentha-1,3-dien-9-ol in headspace extracts of males of *A. moschata* and *H. marginata* and the significant attraction of both sexes to synthetic *p*-mentha-1,3-dien-9-ol demonstrate that this compound functions as an aggregation-sex pheromone for both species. Recently, Collignon et al. (2019) reported the isomeric *p*-mentha-1,3-dien-8-ol as a pheromone component of at least one (but possibly two) North American species in the subfamily Cerambycinae and tribe Oemini. The two compounds share the same carbon skeleton and

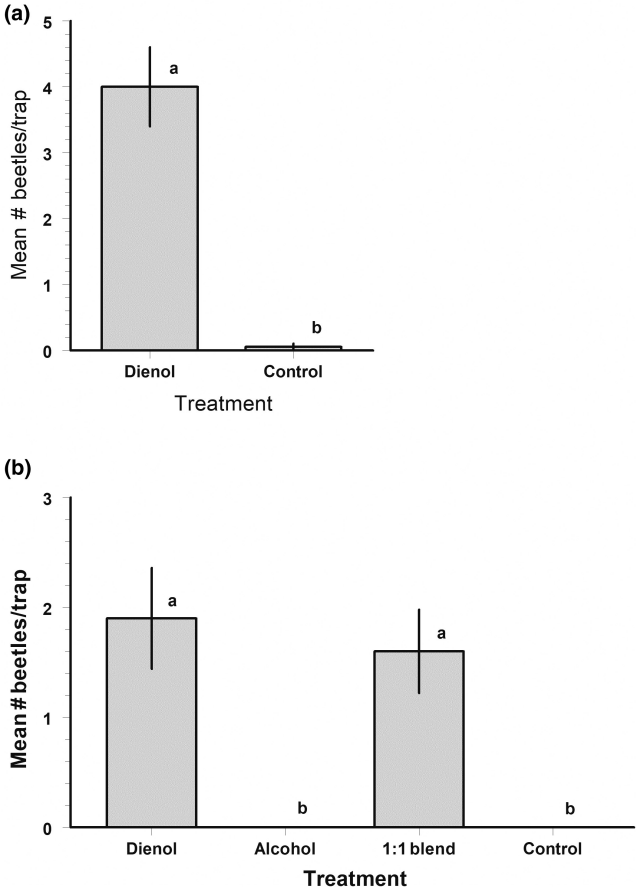

FIGURE 6 Mean (±*SE*) number of beetles captured by panel traps baited with test chemicals for the species (a) *Aromia moschata moschata* in southern Sweden, and (b) *Holopleura marginata* in northern California, USA. Compound abbreviations: Dienol = *p*-mentha-1,3-dien-9-ol, alcohol = *p*-cymen-9-ol, control = neat isopropanol. Bars with different letters are significantly different (REGWQ test, $p < 0.05$)

conjugated diene system, but the placement of the hydroxyl group is different. In addition to being a previously unknown variant of the 1-alkyl-4-methylcyclohexane structural motif, emission of *p*-mentha-1,3-dien-9-ol by males of *A. moschata* and *H. marginata* is noteworthy for two reasons. First, our results reinforce the hypothesis that the 1-isopropyl-4-methylcyclohexane structural motif may be used to form pheromones for a number of species in the Cerambycinae. Besides the two species studied by Collignon et al. (2019) that emit *p*-mentha-1,3-dien-8-ol, two *Megacyllene* species in the tribe Clytini are known to emit three compounds with the same carbon skeleton, of which α-terpineol is an important pheromone component for both species (Lacey et al., 2008; Mitchell et al., 2018). All species mentioned above (and *H. marginata*) are native to the Nearctic ecozone, but *A. moschata* demonstrates that the 1-alkyl-4-methylcyclohexane motif also occurs among species of the Palearctic region. While *H. marginata* is the only member of its tribe (Holopleurini), the tribes Calliochromatini, Oemini and Clytini comprise altogether nearly 400 genera and occur in all biogeographic regions (Monné et al., 2017). Thus, screening bioassays testing compounds with this structural motif, carried out in different geographic regions and habitats and at different times of the year, may show attraction of additional species. This approach has the potential to expedite identification of the pheromones of further species in the Cerambycinae, as has been the case in analogous field trials with compounds such as short-chain 3-hydroxyalkan-2-ones (Bobadoye et al., 2019; Hanks & Millar, 2013; Sweeney et al., 2014). Whereas only one other cerambycid (the lepturine *X. crassipes* mentioned above) was significantly attracted to the pheromone treatments in our bioassays in Sweden and California, the field trials described here were limited both spatially and temporally.

Second, although pheromone motifs often show a high degree of conservation within the subfamily Cerambycinae, with members of the same genus or tribe often using the same or similar structures as pheromones, such as different variations of 2,3-hydroxyketones and the corresponding 2,3-alkanediols (Millar & Hanks, 2017), this does not appear to be the case among *Aromia* species. Unexpectedly, *A. m. moschata* produces a pheromone compound of a completely different chemical class (an oxygenated monoterpenoid) than the East Asian species *A. bungii* (Faldermann), which emits the monoepoxide (*E*)-2-*cis*-6,7-epoxynonenal (Xu et al., 2017), clearly not a monoterpenoid. The natural selection processes which have resulted in this divergence in pheromone chemistry between congeners are unknown. This underlines the importance of continued efforts to study the pheromone chemistry of a variety of cerambycids, even closely related species, to provide a better understanding of the biochemical pathways underlying pheromone communication in this beetle family.

It should be noted that we studied the nominate subspecies of *A. moschata*, which is the most widespread, and the only subspecies present in Northern and Central Europe. However, the systematics within the *Aromia* genus are partially uncertain, because *A. moschata* exhibits considerable variation in external morphology, particularly in colour. Danilevsky (2019) recognizes four separate species, including *A. orientalis* (native to East Asia) which has sometimes been treated as a subspecies of *A. moschata*, in addition to seven other subspecies of *A. moschata* native to various parts of southern Europe, northern Africa and central Asia. Özdikmen et al. (2014) argued that subspecies of *A. moschata* with a partially red pronotum should be treated as a separate species (*A. ambrosiaca* [Steven], with several subspecies). Given the marked difference between the pheromone compounds of *A. m. moschata* and *A. bungii*, it could be interesting to conduct screening bioassays with these two components in areas where the other congeners and subspecies occur, particularly because pheromones can act as chemotaxonomic characters to separate species which may be morphologically very similar (Lassance et al., 2019; Tolasch et al., 2013).

It is unclear why both sexes of the lepturine species *X. crassipes* were attracted to the blend of *p*-mentha-1,3-dien-9-ol and *p*-cymen-9-ol, but showed no attraction to either compound as single treatments. It seems unlikely that either of these two compounds might be a pheromone for this species because all the pheromones identified to date from the subfamily Lepturinae have been

female-produced sex pheromones, attracting only males (Hanks & Millar, 2016). To our knowledge, the dienol has not been conclusively identified from any natural source to date. However, given that the dienol is unstable, it may have been missed in analyses of volatiles from plants. Adults of *X. crassipes*, like most species of lepturines, are flower feeders (Frost, 1979; Linsley, 1959), suggesting that the dienol may be an unreported component of floral volatiles or act as a mimic of a floral volatile.

For both study species, the data suggest that the dienol as a single component is sufficient to elicit significant attraction to lures. We did not undertake a careful examination of the other male-specific compounds that were present at low and variable levels for either species (except *p*-cymen-9-ol for *H. marginata* in California), some of which are likely to be degradation products of the unstable *p*-mentha-1,3-dien-9-ol. In particular, our first bioassays with the synthetic compound in Sweden failed (data not shown) because of the rapid degradation of the compound under natural conditions (particularly oxidation to *p*-cymen-9-ol). Following this failure, all lure formulations were stabilized with butylated hydroxytoluene, which prolonged the field lifetime of lures. In a previous study with the cerambycine *Paranoplium gracile* (LeConte) which produces the similarly unstable *p*-mentha-1,3-dien-8-ol as a pheromone, field trials which tested several minor components found in the extracts of volatiles from male beetles did not result in enhancement of attraction when combined with the main component (Collignon et al., 2019). Having captured *A. moschata* at 19 of the 20 sites in the bioassay, and *H. marginata* at all four sites used, the major component alone appears to be adequate in terms of detecting each species. Further studies of release rates under field conditions could be important to define effective field lifetimes of lures under different climatic conditions, particularly with different stabilizers to minimize degradation of the active component.

Pheromone-based monitoring systems represent a potential game-changer for monitoring the distribution and abundance of rare or elusive insects (Larsson, 2016). *p*-Mentha-1,3-dien-9-ol has considerable potential as a practical tool to study the range and seasonal dynamics of *A. moschata* and *H. marginata* under field conditions. As for most cerambycids, it is typically difficult to sample or monitor populations of these species with a standardized, quantitative method. Surveys rely mainly on manual techniques such as searches for larval galleries or adult beetles visiting flowers. These methods are likely more labour-intensive and difficult to standardize compared with pheromone-based trapping. Monitoring of either species with pheromone traps could not only be useful for biological studies of these beetles, but also as an indirect method to monitor and quantify ecosystem health. For example, the abundance of the large, old willow trees that *A. moschata* use as hosts could be quantified indirectly at the local and landscape levels, by using the presence and abundance of *A. moschata* as a proxy. Old willow trees in particular are important ecological resources not only for saproxylic taxa, but also for many generalist and specialist pollinators active in spring. A similar pheromone-based monitoring system for multiple non-pest cerambycids, including threatened species, is already available for species associated with recently dead oak wood in Northern Europe (Molander, 2019). Thus, the pheromone of *A. moschata* could be part of a parallel system for species dependent on willow. Similarly, in the case of the poorly known *H. marginata*, pheromone-based surveys could be a useful tool to determine the abundance and distribution of this rarely observed species and to begin studies of its environmental requirements.

AUTHOR CONTRIBUTIONS

Mikael A. Molander, Björn Eriksson, Lawrence M. Hanks, Mattias C. Larsson, Jocelyn G. Millar conceived the research. Mikael A. Molander, Björn Eriksson and Austin B. Richards conducted the field experiments. Jocelyn G. Millar conducted the chemical identification. Kyle Arriola, Jocelyn G. Millar conducted the chemical synthesis. Lawrence M. Hanks analysed field data and conducted statistical analyses. Mikael A. Molander, Kyle Arriola and Jocelyn G. Millar wrote the first draft of the manuscript. All authors approved and contributed in writing the final version of the manuscript. Lawrence M. Hanks, Mattias C. Larsson and Jocelyn G. Millar secured funding.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are openly available at https://doi.org/10.5878/zfpv-3f84.

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