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ORIGINAL ARTICLE

Identification of sex-specific compounds in the invasive four-eyed fir bark beetle *Polygraphus proximus*

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Abstract

Polygraphus proximus, a four-eyed fir bark beetle, is an invasive bark beetle species which has caused extensive damage to forests of *Abies sibirica* in southern and western Siberia and to *Abies* species in the European part of Russia. There is a high risk that the pest insect will spread to areas where it is currently not considered present, such as the European Union. In these areas, it threatens to attack conifer forests of various species which may result in major environmental and economic impact. The aim of this study was to identify pheromone components of *P. proximus* that can be used as pheromone baits. Males and females of *P. proximus* were allowed to bore into the bark of stem sections of *Abies sibirica* at the laboratory, and volatiles were collected with solid-phase microextraction (SPME). Analyses of these extracts with gas chromatography-mass spectrometry (GC–MS) revealed several sex-specific compounds. In total, twelve male-specific compounds and one female-specific compound were identified. The major male-specific compound determined by GC peak area was (*Z*)-2-(3,3-dimethylcyclohexylidene)-ethanol [(*Z*)-DMCHE] and the minor male-specific compounds were 3-methyl-3-buten-1-ol, 3-methyl-2-buten-1-ol, 3-methyl-2-butenal, benzyl alcohol, fragranol, 7-methyl-3-methylene-6-octen-1-ol, (*Z*)- and (*E*)-2-(3,3-dimethylcyclohexylidene)-acetaldehyde, geraniol, geranial and papayanol. The only female-specific compound was identified as 1-hexanol. Two of the male-specific compounds, (*Z*)-DMCHE and 3-methyl-2-buten-1-ol were shown to attract males and females of *P. proximus* in field studies. Thus, we now for the first time can present the structures of two male-specific components that are biologically active parts of *P. proximus* aggregation pheromone. However, some chemical communication overlap between *P. proximus* and *P. subopacus* needs to be further investigated as (*Z*)-DMCHE also attracted males and females of *P. subopacus*.

Keywords SPME · GC–MS analysis · (*Z*)-2-(3,3-dimethylcyclohexylidene)-ethanol · *Abies sibirica* · *Polygraphus proximus* · *Polygraphus subopacus*

Introduction

Large bark beetle outbreaks are a major cause of forest damage in different parts of the world. Some bark beetle species which can cause considerable tree mortality are the mountain pine beetle *Dendroctonus ponderosae* in the US and Canada (Grégoire et al. 2015), the spruce bark beetle *Ips typographus* in Europe and the four-eyed fir bark beetle *Polygraphus proximus* in Russia (Kononov et al. 2016; Krivets et al. 2019; Pavlov et al. 2020). *P. proximus* is an invasive species which has infested more than 660,000 km^2 of forest in southern and western Siberia, mainly *Abies sibirica* (De la Peña et al. 2020). It originates from the Russian Far East and neighboring countries, from where it seems to have spread along the Trans-Siberian Railway into Siberia and the European part of Russia (Kerchev 2014a). First discovered in

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Siberia 13 years ago, it is now considered the most aggressive bark beetle ever found on Siberian fir trees (Baranchikov et al. 2010). There is a high risk that *P. proximus* will eventually spread to the European Union and if it does, a major economic and environmental impact can be expected, as has already happened in Russia. *P. proximus* threatens to attack species of the genera *Abies, Pinus, Picea, Larix* and *Tsuga* (EPPO 2014; De la Peña et al. 2020). Efficient pheromone traps would facilitate early detection of *P. proximus* if they were available but, up to now, the pheromone of *P. proximus* is not known.

In 2016, it was suggested that females of *P. proximus* produce an aggregation pheromone based on olfactometer studies, however, no specific pheromone compounds were identified (Kerchev and Pousheva 2016). Other studies have indicated that the males produce a pheromone which attracts females (Kerchev 2014b) or that both males and females of *P. proximus* can secrete aggregation pheromones (Krivets et al. 2019). *P. proximus* is a monogamous species, unlike most other species in the *Polygraphus* genus (Kerchev 2014b; Köbayashi and Takagi 2020). In four other *Polygraphus* species, male-specific aggregation pheromones have been found. *Polygraphus rufipennis* has been shown to use 3-methyl-3-buten-1-ol as their aggregation pheromone (Bowers et al. 1991), *Polygraphus poligraphus* use (−)-(*R*)-terpinen-4-ol (Schurig et al. 1985; Rahmani et al. 2015), *Polygraphus punctifrons* use (+)-(1*R*,2*S*)-grandisol and (–)-(*R*)-terpinen-4-ol as the main components of their aggregation pheromone (Rahmani et al. 2019) and *Polygraphus subopacus* use (*Z*)-2-(3,3-dimethylcyclohexylidene)-ethanol as their main aggregation pheromone component (Viklund et al. 2021). It has been shown that bark beetles of the same genus often have overlapping pheromone components (Blomquist et al. 2010).

In addition to pheromones, beetles of *P. proximus* use acoustic signals to communicate with each other. Males of *P. proximus* have been shown to produce acoustic signals which are species-specific when compared to the signals produced by *P. subopacus* males and *Polygraphus nigrielytris* males (Kerchev 2020). Similarly to other bark beetles, *P. proximus* has been associated with phytopathogenic fungi, mainly *Grosmannia aoshimae*, *Ophiostoma subalpinum* and *O. nikkoense* (Pashenova et al. 2018), and infestation of fir logs with *G. aoshimae* increases their colonization by *P. proximus* in the field (Baranchikov et al. 2017). Previously it has been shown that fungal symbionts of bark beetles can produce volatiles which increase the attraction of the beetles to attacked trees (Kandasamy et al. 2016). *P. proximus* has been caught in traps baited with synthetic pheromones of *Ips sexdentatus* and *Ips acuminatus*, although the number of caught *P. proximus* was not reported (Kerchev et al. 2019). Pheromone baits for *I. sexdentatus* and *I. acuminatus* often contain ipsenol, ipsdienol, *cis*-verbenol and 2-methyl-3-buten-2-ol, as these are produced by the males of these species or have been shown to increase the beetles' attraction to the baits (Knizek et al. 2022).

The aim of this work was to identify pheromone components of *P. proximus* using solid-phase micro extraction together with gas chromatography and mass spectrometry (SPME–GC–MS). These methods have previously been shown to be useful for pheromone identification in *Polygraphus* bark beetles (Rahmani et al. 2015, 2019; Viklund et al. 2021).

Materials and methods

In this study, volatiles released by boring *P. proximus* were collected with SPME at the Sukachev Institute of Forest in Krasnoyarsk, Russia by a researcher from the Eco-Chemistry group, Mid Sweden University, Sundsvall Sweden. GC–MS-analyses of the collected samples were then conducted at the Mid Sweden University in Sundsvall, Sweden.

Sampling of volatiles from boring beetles

Overwintering beetles were reared from the bark of Siberian fir (*Abies sibirica*), collected in the mixed coniferous taiga forest near the city of Krasnoyarsk, Siberia. 43 males and 34 females of *P. proximus* beetles were placed on 50 cm long stem sections of *A. sibirica* (10–15 cm diameter) and were allowed to bore into the bark. Eight stem sections were placed horizontally at the laboratory in room temperature (20–22 °C). Each insect was placed in an Eppendorf tube which had been pinned to the stem and where the end had been cut off and covered with aluminium foil. Insects were placed in the Eppendorf tube on the stem sections between Jan 19–24, 2017. Out of the tested beetles 26 females and 36 males started boring into the bark within 0–4 days. Volatiles from the beetles were sampled with SPME 3–7 days after the beetles had been placed on the bark. The SPME fiber was introduced into the cut end of the Eppendorf tube and the opening was sealed with aluminium foil. Sampling time was 1 h, except for two red fibers which were left over night to sample one male and one female. The most active beetles were chosen for sampling based on the amount of frass they produced. In total, 15 females and 15 males were sampled and 11 samples were taken from the fir background where a hole had been drilled manually 0–2, 24 or 48 h before sampling. Males, females and the fir background were sampled individually in separate Eppendorf tubes. As sex-specific compounds were in focus, males and females also served as background references for each other, to account for compounds which may have been produced by the tree in response to herbivorous activity. 17 red fibers, 6 yellow

fibers, 6 black fibers, 6 orange fibers and 6 pink fibers were used. 2 males, 2 females and 2 fir backgrounds were sampled with each type of fiber, except for the red fibers where 7 males, 7 females and 3 backgrounds were sampled.

The stationary phases of the SPME fibers were PDMS (polydimethylsiloxane) 100 µm for red fibers and 30 µm for yellow fibers, PDMS/DVB (divinylbenzene) 65 µm for pink fibers, carboxen/PDMS 75 µm for black fibers and carbowax/DVB 65 µm for orange fibers. All fibers were from Supelco (Bellefonte, PA, USA). The SPME fibers were conditioned in Sweden in the GC inlet at 250 or 280 ºC until no peaks were seen on the chromatograms. Directly after conditioning the fibers were placed through septa into 2 ml glass vials which had been prefilled with argon and were then transported to Russia. After sampling of volatiles between Jan 24 and 30, 2017 each SPME fiber was again placed into glass vials with argon and transported to the Mid Sweden University. They were kept in the vials at room temperature until GC–MS analyses, which were conducted between Feb 1 and March 23, 2017.

Analysis of collected volatiles

A mid-polar HP5-MS GC column (30 m × 0.25 mm × 0.25 µm, Agilent J&W Scientific, Folsom, USA) was used and the temperature was set to 50 °C for 2 min, then increased by 10 °C/min up to 230 °C and then increased by 15 °C/min to 280 °C, where it was held for 5 min. A polar VF23-MS column was also used (30 m × 0.25 mm × 0.25 µm, Agilent J&W Scientific, Folsom, USA) and the temperature was set to 50 °C for 2 min and then increased by 5 °C/min up to 250 °C and held there for 10 min.

GC peaks which were found in at least three samples from either males or females, but not in samples from both sexes or from the fir background, were identified by comparing retention times and MS spectra to synthetic references. The synthetic references were chosen based on top suggestions from the NIST 14 MS library, except for one case where no good match could be found in the MS library and where the compound was eventually identified based on a mass spectrum published in the literature.

The gas chromatograph was a Hewlett-Packard 6890 from Agilent Technologies (Santa Clara, CA, USA) and the mass spectrometer was a HP 5973 N, operating in electron impact ionization mode (EI, 70 eV). Helium was used as mobile phase (flow rate 1 mL/min) and the injector was set to 250 °C, splitless mode. Transfer line temperature was set to 230 °C. Desorption time for each SPME fiber was 5 min. The software used for analysis of the raw MS data was Workstation 7.0.0 (Agilent) together with NIST 14 mass spectral library.

EAG studies of *P. subopacus*

Many of the sex-specific compounds which were identified in *P. proximus* have previously been tested for activity on *P. subopacus* antennae (Viklund et al. 2021). Three additional compounds, fragranol, geraniol and 1-hexanol were also tested on *P. subopacus*, using the same methodology as described by Viklund et al (2021). Unfortunately, *P. proximus* was not available for EAG studies as it is a quarantine species in the European Union (De la Peña et al. 2020) and could not be brought to Sweden, where the EAG studies were conducted.

Chemicals

3-Methyl-3-buten-1-ol, 3-methyl-2-buten-1-ol, 3-methyl-2-butenal, benzaldehyde and benzyl alcohol were purchased from Sigma-Aldrich (Schnelldorf, Germany). (*Z*)- and (*E*)-2-(3,3-dimethylcyclohexylidene)-ethanol (Grandlure II and (*E*)-isomer of Grandlure II), racemic grandisol (Grandlure I) and a 1:1 mixture of (*Z*)- and (*E*)-2-(3,3-dimethylcyclohexylidene)-acetaldehyde (Grandlure III and IV) were from Bedoukian Research (Danbury, CT, USA). Citral was purchased from Acros Organics (Geel, Belgium). Racemic fragranol and 7-methyl-3-methylene-6-octen-1-ol (γ-isogeraniol) were synthesized at our laboratory. These syntheses have been described previously (Rahmani et al. 2019; Viklund et al. 2021; Yong et al. 2001). Papayanol was synthesized at our laboratory starting from racemic grandisol, according to the method described by Zarbin et al. (2010). (*Z*)- and (*E*)-2-(3,3-dimethylcyclohexylidene)-acetaldehyde were separated by flash chromatography (straight-phase silica gel, 60 Å, 230–400 mesh) using a gradient technique with an increasing concentration of ether (0–100%) in pentane. The (*Z*)- and (*E*)-isomers could be easily identified after separation based on their mass spectra. Mass spectra for these compounds have been published previously in Viklund et al. (2021).

Field experiments

Three field studies were conducted in 2017, 2018 and 2019 in an outbreak area of *P. proximus* in Siberian fir (*Abies sibirica*) dominated mixed coniferous forest near Krasnoyarsk, Russia. According to dendrochronological analysis, fir dieback from *P. proximus* attack at this locality has started in the middle of 1970's (Baranchikov et al. 2014). Until 2019, approximately 60% of firs were killed there although spruce (*Picea abovata*) and pines (*Pinus sibirica*) were not attacked.

Experiment 1

The major male-specific compound identified in the GC–MS analysis was used in a field experiment in 2017 to assess its attractiveness to *P. proximus*. Traps baited with (*Z*)-DMCHE in a solvent, *n*-nonane, were alternated with control traps containing only *n*-nonane. They were placed along lines with 20–25 m between the traps and the treatments were rotated (moved one trap position forward) each time the traps were emptied. Two sites were used with approximately 1–2 km between them. There were 25 traps per treatment, of which 15 were at the first site and 10 were at the second site. The experiment was conducted between June 18, 2017 and September 4, 2017. Traps were emptied at irregular intervals depending on the flight activity of the beetles.

Experiment 2

In 2018, nine treatments were tested in the field by the same methodological approach as presented above. There were 10 traps per treatment and one site was used. The tested treatments included (*Z*)-DMCHE, (*E*)-DMCHE, γ-isogeraniol and racemic grandisol, alone and in combinations. Dispensers with *n*-nonane and traps without dispensers were used as controls. Treatments were rotated when the traps were emptied. The experiment was conducted between May 27, 2018 and July 27, 2018.

Experiment 3

In 2019, 12 treatments were tested in the field by the same methodological approach as presented above and there were 5 traps per treatment. The tested compounds were (*Z*)-DMCHE, 3-methyl-3-buten-1-ol, 3-methyl-2-buten-1-ol, 3-methyl-2-butenal, geraniol and citral (a 1:1 mixture of geranial and neral), alone and in combinations. *n*-Nonane and unbaited traps were used as controls. The experiment was conducted between May 27, 2019 and July 31, 2019.

Traps and dispensers

The traps used in all field experiments were standard pheromone traps used by forest and quarantine services in Russia (Fig. 1a). Each trap is made of plastic and has four wings (45 × 15 cm each), a funnel (25 cm high, upper diameter 30 cm) and a 500 ml collection jar (12 cm high). The dispensers used for the field experiments were wick baits (Fig. 1b; Birgersson et al. 2012). In experiment 1, 50 mg of (*Z*)-DMCHE was dissolved in 8 mL of *n*-nonane and contained in a 12 mL glass vial. The dissolved compound was released through a teflon tube, 8 cm × 15 mm i.d. which was lined with cotton yarn and inserted through a drilled hole in the lid of the vial. The solvent, *n*-nonane, was used to control the release rate. Release rates from the dispensers were not measured, but based on previous laboratory studies in Sweden with similar compounds, the expected release rate of (*Z*)-DMCHE in a fume hood (22–25 °C, air

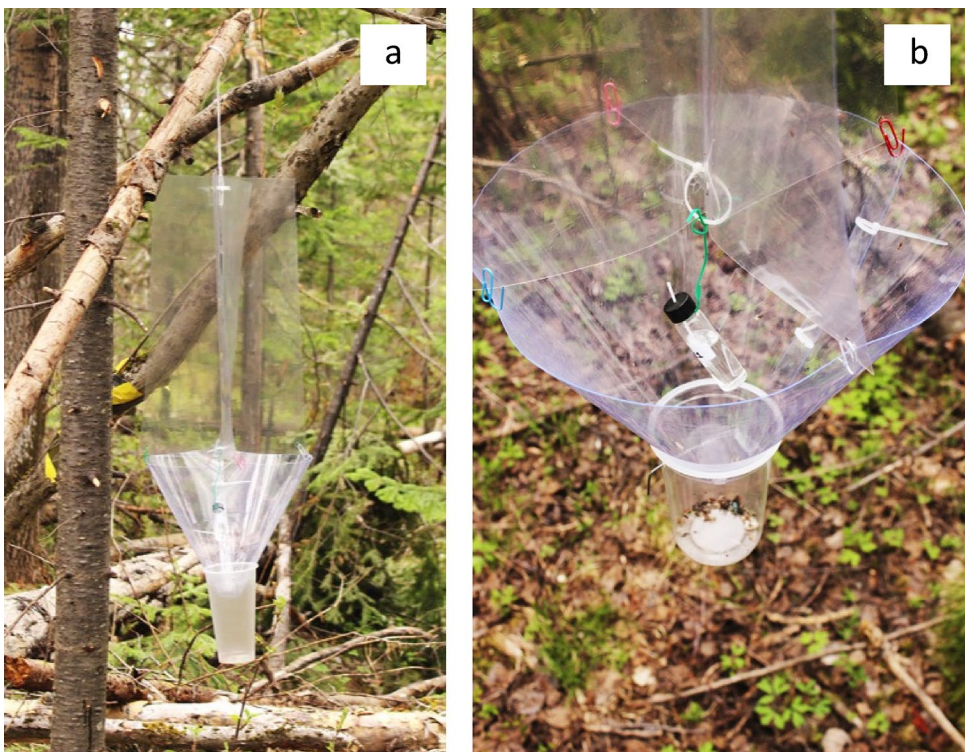

Fig. 1 Photos of **a** the trap type used in experiment 1–3 and **b** a wick-bait dispenser in one of the traps

flow 0.5–0.6 m/s) was 0.8 mg/day (Viklund et al. 2019). In experiment 2 and 3, 50 mg of (*Z*)-DMCHE was combined with 5 mg of an additional compound in 8 mL of *n*–nonane. Each compound was also used alone. The additional compounds were (*E*)-DMCHE, γ-isogeraniol, *rac*-grandisol, 3-methyl-3-buten-1-ol, 3-methyl-2-buten-1-ol, 3-methyl-2-butenal, geraniol and citral. Release rates were expected to be 0.08 mg/day for each of these compounds.

Statistical analyses

As treatment positions were changed when the traps were emptied, each rotation was considered a replicate. Relative catches were used in the statistical analyses since the beetles' flight activity was expected to vary with time. The number of beetles caught by each treatment was converted to proportions of the total catch within that replicate. The arcsine square root transformation was used to better fit the assumption of normality. Data was analyzed with Welch's analysis of variance (ANOVA) and a significance level of $\alpha = 0.05$ together with Games-Howell post hoc test due to unequal variances (Welch, 1951). In Experiment 1, Welch's two sample *T* test was used instead of ANOVA as only two treatments were compared. Missing data were handled using the average number of insects caught per trap for that treatment in that replicate (i.e., if there were ten traps baited with (*Z*)-DMCHE and insects could not be counted in two of the traps due to predators, the average number of insects per trap for that specific date was calculated from the other eight traps). Statistical tests were performed in R.

Results

Sampling and analysis of volatile organic compounds from boring bark beetles

In total, twelve male-specific compounds and one female-specific compound were seen in the SPME extractions from *P. proximus* when the HP5-MS column was used (Fig. 2 and Supplementary Information, Figs. S1–S12).

The major male-specific compound (GC-peak at 10.39 min in Fig. 2) was identified as (*Z*)-2-(3,3-dimethylcyclohexylidene)-ethanol [(*Z*)-DMCHE)]. This compound was found in SPME extracts from all males in which sex-specific compounds were observed (13 males), but not from any of the females or the background samples which were analyzed. The second largest male-specific compound was 7-methyl-3-methylene-6-octen-1-ol (γ-isogeraniol, GC peak at 10.25 min in Fig. 2). These two compounds were identified from all types of SPME fibers which were used in the analysis.

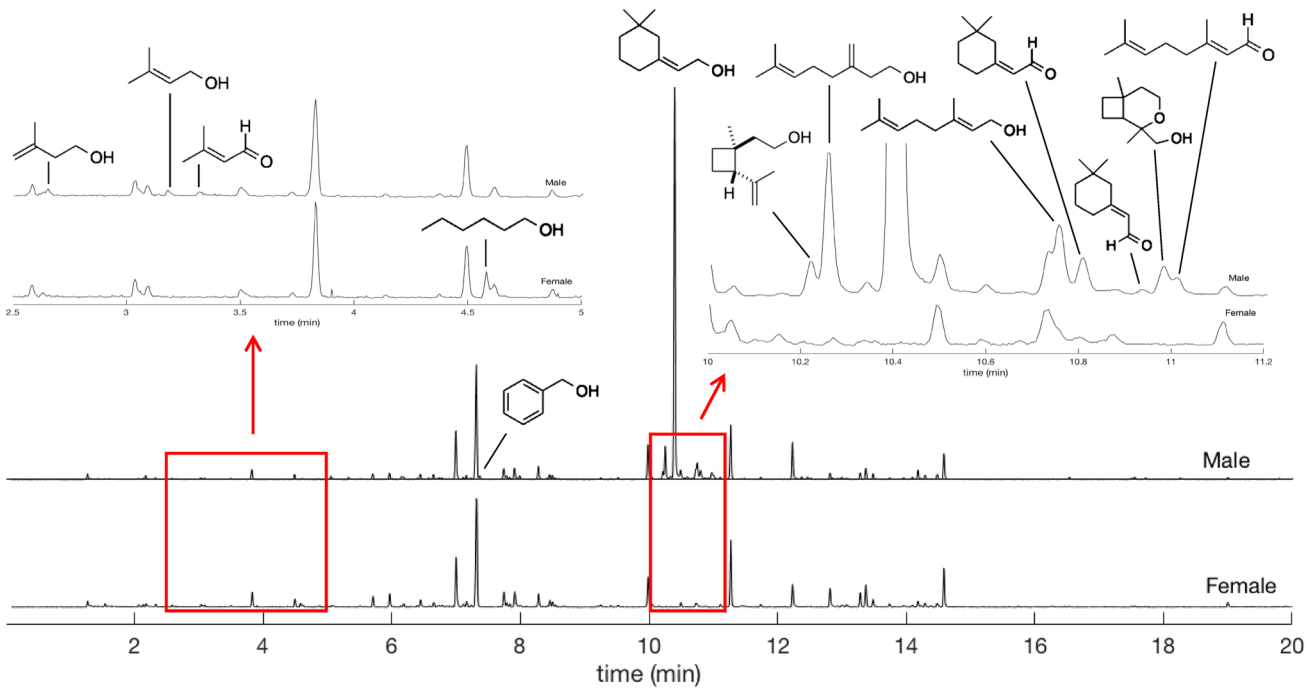

Fig. 2 GC chromatograms showing volatile organic compounds from a *P. proximus* male and female. Insects were sampled with orange SPME fibers which were kept in vials with argon for 5 days before analysis. The GC column was an HP5-MS and the temperature was set to 50 °C for 2 min, then increased by 10 °C/min until 230 °C and after that increased by 15 °C up to 280 °C where it was held for 5 min. The chromatograms have been zoomed in on 0–20 min

The other male-specific compounds were mainly seen when using the orange (Fig. 2), pink and black SPME fibers, and they were identified as 3-methyl-3-buten-1-ol (rt 2.64 min), 3-methyl-2-buten-1-ol (rt 3.17 min), 3-methyl-2-butenal (rt 3.32 min), benzyl alcohol (rt 7.36 min), fragranol (rt 10.22 min), (*Z*)-2-(3,3-dimethylcyclohexylidene)-acetaldehyde (rt 10.80 min, (*Z*)-DMCHA), (*E*)-2-(3,3-dimethylcyclohexylidene)-acetaldehyde (rt 10.92 min, (*E*)-DMCHA), geraniol (rt 10.75 min), geranial (rt 11.01 min) and papayanol (rt 10.97 min). The only female-specific compound was identified as 1-hexanol (rt 4.58 min).

Benzaldehyde (rt 6.15 min) was seen in samples from males of *P. proximus,* but also from the females and the fir background. As it coeluted with other compounds from the fir background, it was not possible to determine whether the GC peak of benzaldehyde was larger in males than in females.

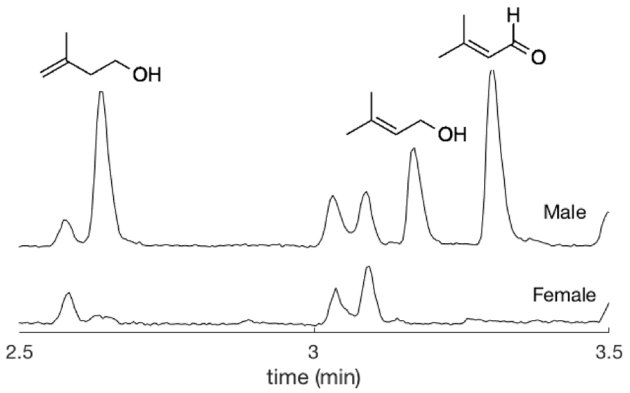

Fig. 3 GC chromatograms showing male-specific compounds 3-methyl-3-buten-1-ol (rt 2.64 min), 3-methyl-2-buten-1-ol (rt 3.17 min) and 3-methyl-2-butenal (rt 3.32 min). These compounds were clearly seen when a pink SPME fiber was used. The GC column and temperature program was the same as in Fig. 2. The chromatograms have been zoomed in on 2.5–3.5 min

The compounds with the shortest retention times, 3-methyl-3-buten-1-ol, 3-methyl-2-buten-1-ol and 3-methyl-2-butenal, generated very small GC peaks when the orange SPME fiber was used (Fig. 2), however, GC peaks were larger when the pink SPME fiber was used (Fig. 3), making the identification of these compounds easier.

As the mass spectra of (*Z*)-DMCHE and (*E*)-DMCHE are quite similar (Fig. 4), and since the retention times of these compounds are close on an HP5-MS column, a VF23-MS column was also used to determine whether it was only the (*Z*)-isomer which was present in *P. proximus* males, or if there was also an amount of the (*E*)-isomer. A slower program was used, starting at 50 °C for 2 min and then increasing by 5 °C per minute until the temperature reached 250 °C where it was held for 10 min. These chromatograms showed that *P. proximus* males emit (*Z*)-DMCHE but not (*E*)-DMCHE (Fig. 5 and Supplementary Information, Fig. S13).

Another challenge was the identification of a GC-peak close to the GC-peak of γ-isogeraniol, showing a similar mass spectra to grandisol. The mass spectra of grandisol and fragranol are near identical, but retention times differ slightly on an HP5-MS column, as shown previously in our study of *P. subopacus* (Viklund et al. 2021). Grandisol and fragranol had previously been synthesized at our laboratory, and these references could be used to identify fragranol as the male-specific compound in *P. proximus*. Fragranol eluted just before γ-isogeraniol and was identified by comparing the unknown peak at retention time 10.22 min to a synthetic sample of fragranol, giving an overlapping retention time and identical mass spectrum (Figs. 6, 7).

All compounds, except for papayanol, were identified using suggestions from the NIST14 MS library and then comparing the MS spectra and retention times to those of synthetic references. A compound was considered male-specific if it was found in at least three males and not in any of the females or background samples which were analyzed. For papayanol, the NIST14 MS library did not yield

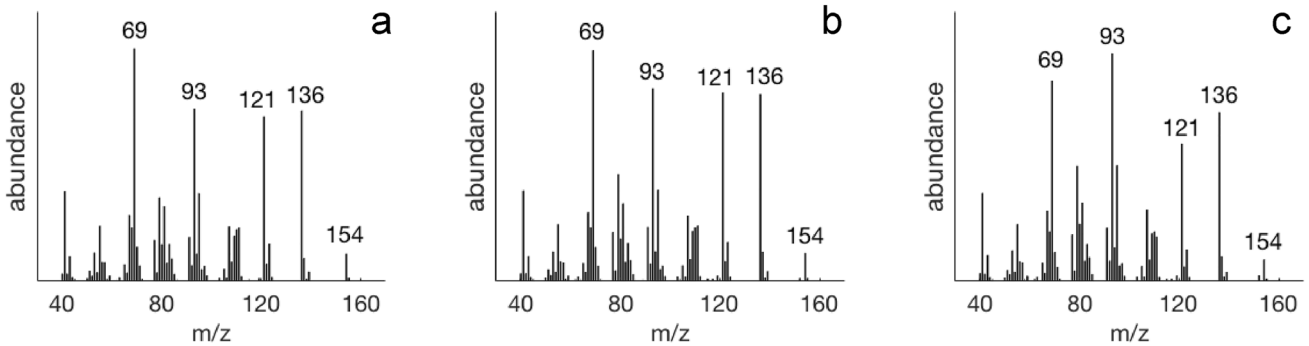

Fig. 4 MS-spectra (EI) from **a** the major male-specific compound at retention time 10.39 min on the HP5-MS column, **b** a synthetic reference of (*Z*)-DMCHE and **c** a synthetic reference of (*E*)-DMCHE

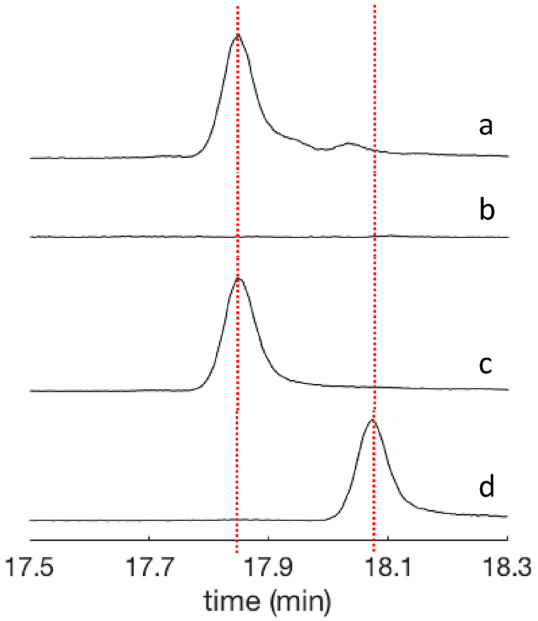

Fig. 5 GC chromatograms showing a) the major male-specific compound in *P. proximus*, **b** the absence of this compound in the female, **c** a synthetic reference of (*Z*)-DMCHE and **d** a synthetic reference of (*E*)-DMCHE. Insects were sampled with pink SPME fibers and the GC column was a VF23-MS. The temperature was set to 50 °C for 2 min, then increased by 5 °C/min until 250 °C where it was held for 10 min. The chromatograms have been zoomed in on 17.5–18.3 min. The minor, male-specific GC peaks which elute after (*Z*)-DMCHE were identified as fragranol and geraniol based on mass spectral analysis as well as retention times

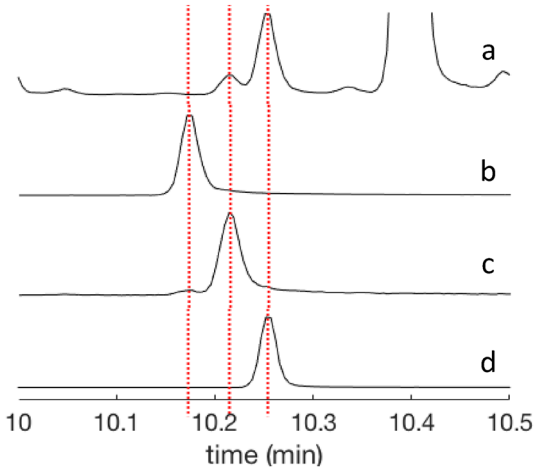

Fig. 6 GC chromatograms showing **a** male-specific compounds in *P. proximus*, **b** a synthetic reference of grandisol, **c** a synthetic reference of fragranol and **d** a synthetic reference of γ-isogeraniol. The insect was sampled with an orange SPME fiber and the GC column was an HP5-MS. The temperature started at 50 °C for 2 min, increased by 10 °C/min until 230 °C and then by 15 °C/min up to 280 °C where it was held for 5 min. The chromatograms have been zoomed in on 10.0–10.5 min

any good quality suggestions. Instead, a suggestion of the structure of this compound came up from a literature search where insects with similar pheromone systems were investigated. We found a compound produced by the papaya borer,

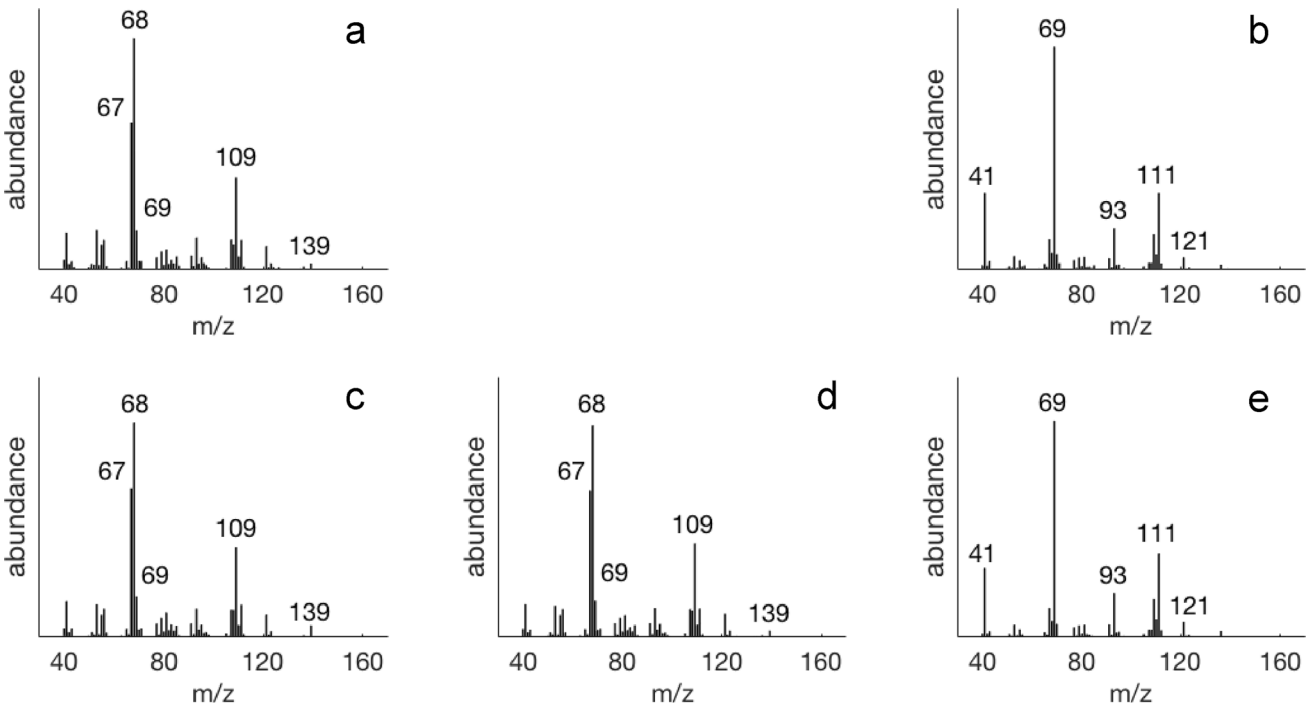

Fig. 7 MS-spectra (EI) from **a** the male-specific compound at retention time 10.22 min in Fig. 6, **b** the male-specific compound at retention time 10.25 min in Fig. 6, **c** a synthetic reference of grandisol, **d** a synthetic reference of fragranol and **e** a synthetic reference of γ-isogeraniol

Table 1 Trap catches of *P. proximus* and *P. subopacus* in Experiment 1, June 18–September 4, 2017

Treatment	Number of *P. proximus*		Number of *P. subopacus*	
	Total	Mean per trap per rotation, (95% CI)	Total	Mean per trap per rotation, (95% CI)
(*Z*)-DMCHE	6562	33 (±8)	18,423	93 (±30)
Control (*n*-nonane)	472	2 (±1)	10	0 (±0)

Catches are shown as a total from the entire trapping period, and as a mean per trap per rotation with a 95% confidence interval (95% CI). There were 25 traps per treatment and 8 rotations in the experiment

Pseudopiazurus obesus (Zarbin et al. 2010) with an identical mass spectrum and when this compound (papayanol) was synthesized in our laboratory, its mass spectrum and retention time matched the male-specific compound in *P. proximus*. The stereochemistry of fragranol and papayanol in *P. proximus* was not determined in this study.

Field experiments

Experiment 1

(*Z*)-DMCHE caught significantly more *P. proximus* than the control traps with *n*-nonane (Welch's *T* test, two-tailed, $T = -13.0$; $df = 14$; $P < 0.001$). However, *P. subopacus* were also caught in these traps and in much larger numbers than in the control traps ($T = -122.8$; $df = 14$; $P < 0.001$). Total catches of each species, as well as a mean per trap per rotation are presented in Table 1.

Experiment 2

Treatment effects were compared in two groups. First, trap catches by (*Z*)-DMCHE was compared to catches by (*E*)-DMCHE, γ-isogeraniol, grandisol, control traps with *n*-nonane and the unbaited control traps. According to Welch's ANOVA, there were significant differences between at least two of these treatments ($F = 9.6$; $df = 5$; $P < 0.001$ for *P. proximus* and $F = 37.0$; $df = 5$; $P < 0.001$ for *P. subopacus*). Then, trap catches by (*Z*)-DMCHE was compared to the combinations of (*Z*)-DMCHE with (*E*)-DMCHE, γ-isogeraniol or grandisol, as well as to the control traps baited with *n*-nonane. There were significant differences between these treatments ($F = 9.9$; $df = 4$; $P < 0.001$ for *P. proximus* and $F = 46.4$; $df = 4$; $P < 0.001$ for *P. subopacus*).

According to Games-Howell's post hoc test, (*Z*)-DMCHE caught significantly more *P. proximus* than the control traps with *n*-nonane ($P = 0.003$). (*E*)-DMCHE did not catch significantly more (or less) *P. proximus* than the control traps with *n*-nonane and neither did γ-isogeraniol or grandisol. The combination of (*Z*)- and (*E*)-DMCHE caught significantly fewer *P. proximus* than (*Z*)-DMCHE alone ($P = 0.008$) and the catches of the combination were not significantly different from the catches in control traps with *n*-nonane. γ-Isogeraniol or grandisol in combination with (*Z*)-DMCHE did not increase or decrease the catches compared to (*Z*)-DMCHE alone. *P. proximus* did not seem to be attracted to *n*-nonane, as the catches in control traps with *n*-nonane were not significantly different from the unbaited control traps. For *P. subopacus*, (*Z*)-DMCHE caught more beetles than the control traps with *n*-nonane ($P < 0.001$). Catches were not significantly altered when γ-isogeraniol, grandisol or (*E*)-DMCHE was added to (*Z*)-DMCHE. For both species, total catches as well as a mean per trap per rotation are presented in Table 2.

Experiment 3

Once again, treatment effects were compared in two groups where (*Z*)-DMCHE was first compared to 3-methyl-3-buten-1-ol, 3-methyl-2-buten-1-ol, 3-methyl-2-butenal, geraniol, citral and the control traps, and then compared to the combinations of (*Z*)-DMCHE with these compounds. There were significant differences between treatments in both comparisons and for both species according to Welch's ANOVA ($F = 17.3$; $df = 6$; $P < 0.001$ and $F = 6.3$; $df = 6$; $P < 0.001$ for *P. proximus*; $F = 62.4$; $df = 6$; $P < 0.001$ and $F = 68.9$; $df = 6$; $P < 0.001$ for *P. subopacus*). The ANOVAs were followed by Games-Howell's post hoc test.

Also now (*Z*)-DMCHE caught significantly more *P. proximus* than the traps baited with only *n*-nonane and so did 3-methyl-2-buten-1-ol ($P = 0.001$ and $P < 0.001$). 3-Methyl-3-buten-1-ol, 3-methyl-2-butenal, geraniol and citral did not catch more beetles than *n*-nonane on its own. When 3-methyl-2-buten-1-ol, 3-methyl-3-buten-1-ol, 3-methyl-2-butenal, geraniol or citral were combined with (*Z*)-DMCHE, catches were not higher than when only (*Z*)-DMCHE was used. For *P. subopacus*, (*Z*)-DMCHE caught more beetles than *n*-nonane ($P < 0.001$) but combining (*Z*)-DMCHE with 3-methyl-3-buten-1-ol, 3-methyl-2-buten-1-ol, 3-methyl-2-butenal, geraniol or citral did not increase or decrease the catches of *P. subopacus* as compared to only (*Z*)-DMCHE. A summary of the experiment is presented in Table 3.

The proportion of beetles caught by each treatment in each replicate were used for the statistical analysis (Supplementary Information, Figs. S14–S23). Before the analysis, these proportions were transformed with the arcsine square root transformation.

Table 2 Trap catches of *P. proximus* and *P. subopacus* in Experiment 2, May 27–July 27, 2018

Treatment	Number of *P. proximus*		Number of *P. subopacus*	
	Total	Mean per trap per rotation, (95% CI)	Total	Mean per trap per rotation, (95% CI)
(*Z*)-DMCHE	2602	34 (±17)	1155	15 (±6)
(*E*)-DMCHE	176	2 (±1)	13	0 (±0)
γ-isogeraniol	549	7 (±4)	83	1 (±2)
Grandisol	232	3 (±1)	10	0 (±0)
(Z)-DMCHE and (*E*)-DMCHE	432	6 (±2)	2233	29 (±17)
(Z)-DMCHE and γ-isogeraniol	1406	18 (±8)	1392	18 (±8)
(Z)-DMCHE and grandisol	1110	15 (±5)	1873	25 (±12)
Control (*n*-nonane)	421	6 (±4)	26	0 (±0)
Control (unbaited)	339	4 (±3)	18	0 (±0)

Catches are shown as a total from the entire trapping period, and as a mean per trap per rotation with a 95% confidence interval (95% CI). There were 9 traps per treatment and 10 rotations in the experiment

Table 3 Trap catches of *P. proximus* and *P. subopacus* in Experiment 3, May 27, 2019 until July 31, 2019

Treatment	Number of *P. proximus*		Number of *P. subopacus*	
	Total	Mean per trap per rotation, (95% CI)	Total	Mean per trap per rotation, (95% CI)
(*Z*)-DMCHE	1534	22 (±12)	1894	27 (±14)
3-methyl-3-buten-1-ol	155	2 (±2)	5	0 (±0)
3-methyl-2-buten-1-ol	3050	45 (±21)	9	0 (±0)
3-methyl-2-butenal	425	6 (±6)	4	0 (±0)
Geraniol	298	4 (±2)	2	0 (±0)
Citral	202	3 (±2)	4	0 (±0)
(*Z*)-DMCHE and 3-methyl-3-buten-1-ol	2047	29 (±24)	1257	18 (±10)
(*Z*)-DMCHE and 3-methyl-2-buten-1-ol	1070	15 (±8)	1929	28 (±13)
(*Z*)-DMCHE and 3-methyl-2-butenal	498	7 (±3)	1767	25 (±13)
(*Z*)-DMCHE and geraniol	518	7 (±3)	1246	18 (±8)
(*Z*)-DMCHE and citral	572	8 (±4)	2162	31 (±40)
Control (*n*-nonane)	296	4 (±3)	2	0 (±0)
Control (unbaited)	183	3 (±1)	3	0 (±0)

Catches are shown as a total from the entire trapping period, and as a mean per trap per rotation with a 95% confidence interval (95% CI). There were 5 traps per treatment and 14 rotations in the experiment

Comparison of insect emitted compounds from *P. subopacus* and *P. proximus*

In total, 12 male-specific compounds and one female-specific compound were identified in *P. proximus*. Of these 13 compounds, 10 are also present in *P. subopacus* although the amounts appear to differ (Supplementary Information, Figs. S24–S27), a notable example was γ-isogeraniol which generated a large GC peak in *P. proximus* but a barley noticeable GC peak in *P. subopacus*. For fragranol and papayanol, the stereochemistry was not determined in our study. Thus, it is possible that *P. proximus* and *P. subopacus* use different enantiomers of fragranol. Two compounds which appeared to be specific for *P. proximus*, geraniol and 1-hexanol, were tested for activity on *P. subopacus* antennae with EAG. However, none of these compounds generated a strong response. Papayanol was also specific to *P. proximus,* but could unfortunately not be tested on *P. subopacus* antennae due to a lack of insects when the compound was available. The compounds which were specific to *P. subopacus* were (*E*)-DMCHE and grandisol. Both compounds elicited a strong to medium response in *P. subopacus* antennae. The method used for the EAG analysis of *P. subopacus* has been described previously (Viklund et al. 2021). As EAG studies were conducted in Sweden, *P. proximus* antennae could not be used since restrictions prevented us from bringing live insects to Sweden. *P. proximus* is a quarantine species in the European Union (De la Peña et al. 2020). All compounds

Table 4 Volatile organic compounds identified in *P. proximus* and in *P. subopacus* (Viklund et al. 2021) and their EAG response on *P. subopacus* antennae

Compound	*P. proximus*		*P. subopacus*		*P. subopacus*
	Male	Female	Male	Female	EAG response
3-methyl-3-buten-1-ol	X	–	X	–	None
3-methyl-2-buten-1-ol	X	–	X	–	None
3-methyl-2-butenal	X	–	X	–	None
Benzaldehyde	X	X	X	(X)	None
Benzyl alcohol	X	–	X	–	None/weak
Grandisol	–	–	X	–	Strong*
Fragranol	X	–	X	–	None/weak**
γ-isogeraniol	X	–	X	–	None
(*Z*)-DMCHE	X	–	X	–	Strong
(*E*)-DMCHE	–	–	X	–	Weak/strong
Geraniol	X	–	–	–	None
(*Z*)-DMCHA	X	–	X	–	None/weak
(*E*)-DMCHA	X	–	X	–	Weak
Papayanol	X	–	–	–	ND
Geranial	X	–	X	–	None***
1-hexanol	–	X	–	–	Weak

X Present. (X) Possibly present/seen in single individual(s).– Not present/not identified

ND not determined

*Racemic grandisol, **racemic fragranol and ***citral were used in EAG analyses

which were detected in *P. proximus* and *P. subopacus* are presented in Table 4. Most of the EAG results relating to *P. subopacus* have been published previously (Viklund et al. 2021).

Discussion

In our study, twelve male-specific compounds were identified in the SPME collections from *P. proximus*; (*Z*)-DMCHE, γ-isogeraniol, 3-methyl-3-buten-1-ol, 3-methyl-2-buten-1-ol, 3-methyl-2-butenal, benzyl alcohol, fragranol, (*Z*)- and (*E*)-2-(3,3-dimethylcyclohexylidene)-acetaldehyde, geraniol, geranial and papayanol. In all males, (*Z*)-DMCHE was identified as the largest male-specific GC peak. In the females, only one sex-specific compound was found and it was identified as 1-hexanol. Two of the male-specific compounds appeared to attract males and females of *P. proximus* in the field; (*Z*)-DMCHE and 3-methyl-2-buten-1-ol. These two compounds are, however, not likely to make up the complete pheromone, as catches were smaller than expected and not species-specific. The main male-specific compound (*Z*)-DMCHE has also recently been identified as the main male pheromone component of a closely related species, *P. subopacus* (Viklund et al. 2021). The composition of *P. proximus'* pheromone appears to be strikingly similar to the pheromone of *P. subopacus*, as most of the male-specific compounds were found in both species; 3-methyl-3-buten-1-ol, 3-methyl-2-buten-1-ol, 3-methyl-2-butenal, benzyl alcohol, fragranol, γ-isogeraniol, (*Z*)-DMCHE, (*Z*)- and (*E*)-DMCHA and geranial. But, geraniol and papayanol in males and 1-hexanol in females appeared to be specific to *P. proximus,* while grandisol and (*E*)-DMCHE were specific to *P. subopacus*. Another notable difference was the GC peak size of γ-isogeraniol. This compound generated the second largest GC peak in *P. proximus* males whereas it was barely noticeable in *P. subopacus* males. Although several of these compounds were tested in field experiments, no species-specific composition was identified for *P. proximus*. However, the combination of (*Z*)- and (*E*)-DMCHE at a ratio of 10:1 caught significantly fewer *P. proximus* than traps baited with only (*Z*)-DMCHE. In fact, adding the (*E*)-isomer to (*Z*)-DMCHE appeared to reduce catches of *P. proximus* to the same level as in the control traps with *n*-nonane (Experiment 2). It is thus possible that (*E*)-DMCHE is part of *P. subopacus'* pheromone.

Several species of *Anthonomus* weevils also use (*Z*)-DMCHE, (*Z*)-DMCHA and (*E*)-DMCHA as parts of their aggregation pheromones. In these beetles, species-specificity seems to be achieved by different relative abundances of the pheromone components or by species-specific compounds such as grandisol, (*E*)-DMCHE or lavandulol, which are necessary for attraction in some species and repellants in other species (Tumlinson et al. 1969; Eller et al. 1994; Szendrei et al. 2011; Innocenzi et al. 2001; Rodriguez-Saona et al. 2020). A similar situation is seen in the bark beetles *Pityogenes quadridens* and *Pityogenes bidentatus*, whose pheromone blends are similar but where (*E*)-DMCHE, chalcogran and grandisol are species-specific pheromone components which repel one species from the pheromone blend of the other (Byers et al. 2013). In the pecan weevil *Curculio caryae*, the stereochemistry of grandisol may play a role in the species-specificity of their pheromone. These beetles emit two isomers of grandisol, although it is not clearly described whether these are enantiomers of grandisol, or if they are grandisol and its *trans*-isomer fragranol (Hedin et al. 1997). In *P. proximus* and *P. subopacus*, the stereochemistry of fragranol was not investigated but if these two species use different enantiomers of fragranol, it may be a species-specific component of their pheromone blends. Geraniol and γ-isogeraniol were seen among the volatiles from *P. proximus* in our studies, but were not active on *P. subopacus* antennae in EAG studies and had no significant effect in our field studies. It has been suggested by other authors that γ-isogeraniol and geraniol are biosynthetic precursors of (*Z*)-DMCHE and grandisol (Byers et al. 2013;

Thompson and Mitlin 1979). The compound γ-isogeraniol was identified in our study as the second largest GC peak emitted from boring *P. proximus* males. Earlier it has been found among the volatiles from several other beetle species, but it is not known to be behaviorally active in bark beetles (Ambrogi et al. 2012; Byers et al. 2013).

We found that for *P. proximus,* 3-methyl-2-buten-1-ol was attractive in the field even though a small amount of this compound was collected from males of *P. proximus* in our SPME–GC–MS study. The combination of this compound and (*Z*)-DMCHE did not increase the catches of *P. proximus,* nor decrease the number of *P. subopacus* caught in the traps as compared to only (*Z*)-DMCHE (Experiment 3). The effect of 3-methyl-2-buten-1-ol on *P. proximus* should be further examined in future field studies, but it is not likely that the compund will repel *P. subopacus* from traps baited with (*Z*)-DMCHE as *P. subopacus* antennae could not detect this compound in our EAG studies. This despite the fact that males of *P. subopacus* appears to produce 3-methyl-2-buten-1-ol themselves. However, it is possible that 3-methyl-2-buten-1-ol is part of *P. proximus* pheromone since a similar compound, 3-methyl-3-buten-1-ol, is the aggregation pheromone of the closely related species *Polygraphus rufipennis* (Bowers et al. 1991) and as 3-methyl-2-buten-1-ol is a major aggregation pheromone component in the walnut twig beetle, *Pityophtorus juglandis* (Seybold et al. 2015). The results of our field studies indicate that 3-methyl-2-buten-1-ol can be used in itself to monitor *P. proximus* and in this case, without interference of *P. subopacus* caught in the traps (Table 3).

In our first field experiment, Experiment 1, the catches of *P. subopacus* on (*Z*)-DMCHE were nearly three times larger than the catches of *P. proximus*, although the latter species was expected to be present in larger numbers at the sites used for the field studies since the chosen locations were outbreak areas of *P. proximus*. The high catches of *P. proximus* (but not *P. subopacus*) in control traps also confirms that *P. proximus* was far more common than *P. subopacus* at these locations. *P. poligraphus* was not present in the area where field studies were conducted, but we know from previous studies that they are also attracted to (*Z*)-DMCHE (Viklund et al. 2021). Thus, it seems clear that the complete compostition of *P. proximus* pheromone has yet to be determined. The difference in catches of *P. proximus* by (*Z*)-DMCHE compared to the control traps was also relatively small, since the baited traps caught only 5–14 times more *P. proximus* than the control traps with *n*-nonane. In field experiments in Sweden using pheromone traps with similar release rates of aggregation pheromones for *P. poligraphus* and *P. punctifrons,* we caught at least 250 times more beetles than the unbaited control traps (Viklund et al. 2019; Rahmani et al. 2019). Thus, we think that there should be other compound(s) in *P. proximus* pheromone which would increase catches and at the same time make the pheromone species-specific and for that EAG studies of *P. proximus* would be useful along with more field trials. However, such EAG studies are difficult to conduct at our laboratory in Sweden as *P. proximus* is a quarantine pest species in the European Union (De la Peña et al. 2020). Our EAG studies of *P. subopacus* can partly serve as replacement as there should be compound(s) in *P. proximus* pheromone which repels *P. subopacus*. Based on these EAG studies, it would be interesting to test (*Z*)- and (*E*)-DMCHE but also the stereoisomers of grandisol and fragranol, (*Z*)-DMCHA, (*E*)-DMCHA and the female-specific compound 1-hexanol on the antennae of *P. proximus* in the future. Papayanol and its stereoisomers may also be of interest, as it was not tested in our EAG studies and as it is a pheromone in other species of beetles such as the Papaya borer *Pseudopazurus obesus* (Zarbin et al. 2010) and the guava weevil, *Conotrachelus psidii* (Palacio-Cortés et al. 2015).

If species-specific pheromone traps were developed, they could be used to detect *P. proximus* at an early stage, delimit the current distribution area and demonstrate further spread. They could also be used in containment efforts, together with sanitation cuttings of attacked trees, as a way of reducing populations in outbreak areas by mass trapping or by transmission of insect pathogens (Kreutz et al. 2004). Without species-specific traps, a major challenge will be to differentiate between the different *Polygraphus* species which may be caught in the traps. Many *Polygraphus* species are very similar and can only be distinguished under a microscope, making identification difficult and time-consuming if catches consist of more than one species and large numbers of native species.

This study has, in addition to giving us new insights relating to sex-specific compounds in *P. proximus*, also shown that SPME–GC–MS can be used with a delay between SPME sampling and GC–MS analysis and that compounds are retained on the fiber when they are stored inert under argon. The pink, orange and black SPME fibers were considered most useful in our experiment as they collect the largest number of compounds. Red fibers could collect approximately half of the sex-specific compounds in *P. proximus* whereas the yellow SPME fibers only collected three of the male-specific compounds. These results gives that it is of utmost importance to use several fiber-types when looking for new substances using SPME.

To summarize, this work provides evidence of several male-specific compounds which are emitted from the four-eyed fir bark beetle *P. proximus* as well as one female-specific compound. (*Z*)-DMCHE was found to be the main compound released by boring *P. proximus* males according to the GC peak areas in our SPME–GC–MS analyses, and we have shown that (*Z*)-DMCHE attracts both males and females of *P. proximus* in the field. Thus, we suggest that (*Z*)-DMCHE is a component of *P. proximus* aggregation

pheromone. The compound can be used as bait to catch *P. proximus* but then together with *P. subopacus* in the traps. However, 3-methyl-2-buten-1-ol can also be used as bait to catch only *P. proximus,* without interference of *P. subopacus* caught in the traps.

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Author contributions LV, YB, MS and EH contributed to the study conception and design. SPME–GC–MS analyses, material preparation and statistical analysis were performed by LV. YB contributed with insects and stem sections for the SPME-analysis. AE and DD conducted the field studies in Russia. The first draft of the manuscript was written by LV. EH, YB and MS commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Declarations

Conflict of interest The authors have no relevant financial or non-financial interests to disclose.

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