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# Pilot Study of 3D Spatial Distribution of α-Pinene Emitted by Norway Spruce (L.) Karst Recently Infested by *Ips typographus (L. 1758) (Coleoptera: Scolytinae)*

**Barbora Stříbrská, Antonioni Acacio Campos Moliterno, Tereza Hüttnerová, Martin Leiner, Peter Surový and Anna Jirošová ***

Faculty of Forestry and Wood Sciences, Czech University of Life Sciences Prague, 16500 Prague, Czech Republic; stribrska@fld.czu.cz (B.S.); moliterno@fld.czu.cz (A.A.C.M.); huttnerovat@fld.czu.cz (T.H.); leiner@fld.czu.cz (M.L.); surovy@fld.czu.cz (P.S.)
* Correspondence: jirosovaa@fld.czu.cz

**Abstract:** The Eurasian Spruce Bark Beetle (*Ips typographus*) (L. 1758) (Coleoptera: Scolytinae) poses a significant threat to Eurasia's Norway spruce (*Picea abies*) (L.) Karst, forests. Early detection of infested trees is crucial to control beetle outbreaks and allow salvage logging before the next generation emerges. Besides traditional methods, new approaches focus on monitoring volatile organic compounds, mainly monoterpenes, emitted by infested trees. Using analytical chemistry, we studied the distribution of these compounds, particularly α-pinene, around infested trees. In lab trials, we optimized α-pinene detection using dynamic absorption and solid-phase microextraction (SPME), analyzed by gas chromatography with flame ionization detection (GC-FID). We conducted forest trials, revealing varying α-pinene abundance due to changing conditions. However, consistent trends emerged: levels were highest near the infested tree stem and 1.3 m above ground in the first trial and at a 1 m distance from the infested stem in the second. We generated a three-dimensional cloud depicting the distribution of α-pinene around infested trees in their natural habitat. These findings open avenues for detecting bark beetles on a large scale by mapping elevated concentrations of volatile organic compounds emitted by infested trees, potentially leading to alternative pest management methods. Scanning methods, such as electronic sensors combined with remote sensing, hold promise for this application.

**Keywords:** early attack detection; bark beetle; VOC; α-pinene; *Picea abies*; SPME; Eurasian Spruce Bark Beetle



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## 1. Introduction

The Eurasian Spruce Bark Beetle *Ips typographus* (L. 1758) (Coleoptera: Scolytinae) is the main pest of Norway spruce, *Picea abies* (L.) Karst. forests in the Central European region. Over the past decade, the combination of ongoing climate change, economically driven silvicultural practices, and the presence of spruce stands in areas beyond their natural range have weakened the natural defense mechanisms of trees and resulted in the occurrence of severe bark beetle spreading [1]. In the Czech Republic, outbreaks started after severe drought events in 2015 and 2018 [2] and led to an exponential increase in salvage logging volume from 2017 to 2020, with the volume rising from approximately 5.9 million $m^3$ in 2017 to 26.2 million $m^3$ in 2020 [3,4] (Figure S1).

The initial step in managing a bark beetle outbreak is early detection of newly infested trees to enable timely salvage before the emergence of offspring [4]. Forest keepers typically rely on traditional methods, which involve personally observing the boring dust produced by infesting beetles [5]. However, during bark beetle outbreaks in large, forested areas, this approach has severe limitations, often resulting in the exponential spread of the beetles.

Hence, an alternative method for early detection on a large scale is needed. Remote sensing techniques have been extensively investigated, using the detection of different indicators from spectral features to temperature [6] and recently also involving chemical substances (Sentinel SP) [7].

Recent research by [8] has proposed measuring the emission of volatile organic compounds (VOCs) from infested spruce as an indicator of bark beetle attacks. Furthermore, various methods for detecting these VOCs at different developmental stages have been introduced. Current research is investigating the utilization of an electronic nose equipped with nonspecific sensors for VOC detection [9]. Likewise, nonspecific metal oxide sensors have been mounted on UAVs to assess the concentration of $\alpha$-pinene in forest environments [10]. Notably, natural olfaction systems of dogs trained to detect bark beetle pheromones have proven more effective in finding infested trees in cooperation with their dog handler compared to human experts only [11,12].

The VOC emitted by conifer mainly consists of hemi- and monoterpenes, which are produced as defense secondary metabolites. The conifer's immediate defense mechanism against wood-boring insects is the exudation of constitutive resin, which has a toxic and immobilizing effect on beetles [13]. In the later stages of a bark beetle attack, the production of resin is induced in the newly formed resin ducts in the phloem, xylem, and bark [14]. The resin is a mixture of terpenic compounds. The monoterpenes are volatile and form the main content of VOCs emitted by conifers. In spruce, $\alpha$-pinene, $\beta$-pinene, $\Delta$-carene, limonene, $\beta$-phellandrene, camphene, and myrcene dominate [15] but resin also contains sesquiterpenes and a high content of highly viscous diterpenes [16,17]. Oxidized forms of all terpenes are also present.

In addition to resin emissions from the stem, volatile terpenes are also emitted from the needles in the canopy of conifers [18,19]. The emission rate of volatile terpenes from healthy trees is influenced by various macro- and microclimatic conditions, such as temperature and humidity [17,20]. Different temperatures, and consequently varying VOC emissions, are observed in clearings and forest edges within fragmented forests [17,21]. Furthermore, VOC emissions in conifer forests exhibit vertical variations [22] and follow a diurnal rhythm dependent on tree physiological processes [19]. The terpenes emissions from conifer forests are widely discussed in the context of terpenes as a free radical source in the atmosphere [23–25], because hemi and monoterpenes are photochemically reactive compounds that affect ozone and carbon monoxide concentrations and their oxidation products can participate in the formation of secondary organic aerosol and cloud condensation nuclei [26].

When Norway spruces are attacked by bark beetles, the content of emitted terpenes from the stem significantly increases during the first two weeks of infestation. This growth is primarily attributed to the opening of constitutive resin storage and is quantified in the close vicinity of the stem. Different methods used for quantification have yielded a wide range of results, ranging from a 10 to 100-fold upturn [8,27,28]. The dominant compound in emissions was always $\alpha$-pinene, representing the time and spatial distribution of the other main monoterpenes [8]. The bouquet of infested trees also includes the aggregation pheromone produced by bark beetles. Beetles use this scent for navigation to aggregate, allowing them to overcome the tree's defense during the infestation [29]. The content of bark beetle pheromones is several orders of magnitude lower than that of $\alpha$-pinene in forests [30]. However, beetles are capable of discerning this signal from the background of host odors thanks to specialized receptors on their antennae, the organs responsible for perceiving smells [31]. Furthermore, beetles may orient themselves by detecting host compounds, primarily terpenes, when choosing a suitable host tree or habitat [32]. They also have specialized receptors for host compounds [33].

Numerous studies have investigated monoterpenes emitted by conifers in both laboratory and natural conditions. In the laboratory, collection systems can be readily optimized, as detailed in a comprehensive review [34]. In field conditions, VOC collection is more complex, and various techniques have been employed to address specific research questions [35].

The most common approach involves dynamic headspace sampling with compound collection onto sorbents, followed by extraction into solvents or thermal desorption. Additionally, solid-phase microextraction methods have been utilized (as shown in Table 1) [36,37].

**Table 1.** Methods of VOC collection from conifers in forests.

| Tree Species/ Stress Occasions | Sampling Specification | Compound (Unit) | Technical Parameters (Sorbent; Amount; Flow Rate) | Time of Sorption | Analytical Method | Source |
|---|---|---|---|---|---|---|
| *Picea abies*/ intact forest | Stem (not specify)<br>5 m above the ground; stainless steel TD tubes | Individual monoterpenes<br>α-pinene 3.07 ± 0.25 ppbv | Tenax TA, (35/60)<br>200 mg; 200 mL/min | 30 min | GC-MS | [38] |
| *Picea abies*/ attacked trees | Stem<br>1.3 m above the ground; surrounded PET (25 × 38 cm) encloser | Individual monoterpenes<br>α-pinene 62.8 ± 23.6 μg $h^{-1}$ $m^{-2}$ bark area | Tenax-TA a Carbopack-B, (60/80)<br>100/100 mg; 200 mL/min | 60 min | GC-MS | [28] |
| *Picea engelmannii*/ attacked trees | Stem<br>0.5 to 1.5 m above the ground; the trunk by dynamic sampling <1 cm from stem (sorbent trap) | Individual monoterpenes<br>α-pinene 8.5 ± 2.1 ng $L^{-1}$ | Porapak Q<br>110 mg; 400 mL/min | 120 min | GC-MS | [39] |
| *Picea abies*/ attacked trees on forest edge | Stem 3 h<br>1–2 m above the ground; sanitized T gllas tube | Verbenone (ng/3 h): α-pinene (μg/3 h)<br>0.6 (ng/3 h): (μg/3 h) | Porapak Q, (80/100)<br>70 mg; 20 mL/min | 180 min | GC-MS | [40] |
| *Pseudotsuga menziesii*/ attacked trees | Branch<br>1.5 m above the ground; Teflon bag (50 × 75 cm) | Individual VOCs<br>α-pinene 813.9 ± 482.29 ng $h^{-1}$ $g^{-1}$ fresh weight | HayeSep-Q<br>30 mg; 500 mL/min | 30 min | GC-MS | [41] |
| *Pinus rigida* and *Pinus koraiensis*/intact forest | Branch<br>20 L Tedlar bag | Total monoterpenes emission (μgC $gdw^{-1}$ $h^{-1}$)<br>*Pinus rigida* 0.9 μgC $gdw^{-1}$ $h^{-1}$ *Pinus koraiensis* 0.4 μgC $gdw^{-1}$ $h^{-1}$ | Tenax TA, (60/80) and Carbotrap, (20/40)<br>200 mg; 100–200 mL/min | 15–60 min | GC-MS GC-FID | [42] |
| *Pinus sylvestris*/ intact forest | Branch<br>canopy height; (FEP) copolymer foil (50 μm thickness) mounted in cylindrical frames | Individual monoterpenes<br>α-pinene 917 ± 58 ng $h^{-1}$ $g^{-1}$ (April) α-pinene 75 ± 12 ng $h^{-1}$ $g^{-1}$ (July) | Tenax TA, (60/80) and Carbotrap, (20/40)<br>50–100 mg; 100 mL/min | 60 min | GC-MS GC-FID | [43] |
| *Pinus sylvestris* and *Picea abies*/intact forest | Branch<br>18 L all-Teflon chamber made of 0.05 mm transparent FEP-Teflon film enclosing a 20–30 cm branch segment | Acetone and α-pinene (ng $gdw^{-1}$ $h^{-1}$)<br>α-pinene 80 ng $gdw^{-1}$ $h^{-1}$<br>Monoterpene emission 900 ± 640 ng C $gdw^{-1}$ $h^{-1}$ | Tenax TA<br>200 mg; 100 mL/min | 12–40 min | GC-MS GC-FID | [44] |
| *Picea abies*/ attacked trees | Stem<br>1.3 m above the ground; tree trunk chamber connected with PTFE tubing | Individual VOCs (μg $m^{-2}$ $h^{-1}$)<br>α-pinene 911.14 μg $m^{-2}$ $h^{-1}$ | Tenax TA and Carbograph 1TD<br>200 mL/min | 30 min | GC-MS | [27] |
| *Picea abies*/stress from sun irradiation | Stem<br>3.5 m above the ground; aluminum chamber | Individual monoterpenes<br>sum of eight main MT 8.5 log10 VOC | SPME | 60 min (from 1 to 2 p.m.) | GC-MS | [17] |
| *Picea abies*/attacked trees | Stem<br>3.5 m above the ground; aluminum chamber | Individual monoterpenes<br>α-pinene 9.5 $\log_{10}$ sum peak area | SPME | 60 min (from 1 to 2 p.m.) | GC-MS | [8] |
| *Lerosa* NP forest/conifer forest; 6 different plots | open air | Individual VOCs 894 abundance relative to hexanal (%) | SPME | 300 min (from 10 a.m. to 3 p.m.) | GC-MS | [45] |

To study emissions on an ecosystem scale, researchers have employed various instrumental techniques, such as proton-transfer-reaction-time-of-flight (PTR-TOF) [46–48] mass spectrometry for quantifying monoterpene fluxes [19] or specialized gas chromatographs installed in situ at collection sites. However, these instruments can be expensive and lack portability, restricting data collection to a limited number of sites [49].

Hypothesis and objectives: This study is founded on a previously proposed concept suggesting that the number of volatile organic compounds emitted by trees, particularly monoterpenes, significantly increases within two weeks of bark beetle infestation [8]. This increase can serve as a measurable characteristic upon which the foundation of a newly developed alternative early bark beetle detection method may be based.

Our objectives were to optimize the collection of α-pinene emissions from spruce logs subjected to a controlled simulative bark beetle infestation in the laboratory and consequently detect the outdoor 3D dispersion of monoterpenes, represented by α-pinene, in the surroundings of freshly infested spruce trees at horizontal and vertical distances. We employed analytical chemistry methods for collecting VOCs, which are not typically used for VOC collection in open environments.

Particular objectives were:

1. Assess the distribution of α-pinene at different distances from and heights within a simulatively infested log pile in the laboratory, considering specific temperature conditions.
2. Validate the feasibility of measuring the distribution of α-pinene under actual field conditions, specifically focusing on naturally *Ips typographus*-infested trees within a forest environment.
3. Consider other influencing factors, such as temperature, wind speed, and the immediate surroundings of the forest.
4. Compare the effectiveness of the Solid-Phase Microextraction (SPME) method with HayeSep-Q® sorbent cartridges, which involve drawing air through them using air pumps.

## 2. Materials and Methods

### *2.1. Optimization of VOC Collection from Simulatively Infested Spruce Logs in a Laboratory*

The experiments were conducted under laboratory conditions (25 ± 1 °C, humidity 60% ± 10%) using three fresh-cut logs (height of 40, diameter of 25 cm) of *Picea abies* previously stored in a cold room (−5 °C). The experiment was repeated three times at one-week intervals. Prior of all repetitions, logs were acclimated for 24 h before volatile collection in the conditions described above. Logs were arranged vertically in piles from the floor: The lowest log represented level 1—L1 (20 cm), the middle log level 2—L2 (60 cm), and the upper log level 3—L3 (100 cm) (Figure 1a). To prevent unwanted VOC emissions from fresh-cut logs, the upper and lower exposed surfaces were covered with PE plastic film.

The bark beetle attack was simulated by drilling holes (Ø 2 mm × 0.3 cm deep) into the bark and phloem on the surface of the logs (100 holes per log, spaced in 5 cm spin) to mimic the production of VOC emissions during bark beetle infestation.

VOCs were collected from each of the three levels (L1, L2, and L3) at two distances, 5 cm and 30 cm from the bark surface, using Solid-Phase Microextraction (SPME) fibers (PDMS/CAR/DVB, Supelco, PA, USA) as shown in Figure 1b. The SPME fibers were fixed in protective hard plastic cylindrical chambers hung in an open space (7.2 cm high, Ø 5.2 cm, material PP, Merci, CZE), with an open bottom and a hole covered by septa in the upper lid to hold the fiber. To follow the distance and cardinal directions described above, the chambers in the experimental apparatus were fixed by metal wires.

SPME collection was conducted for 15 min, beginning 2 h after drilling, under controlled conditions (25 ± 1 °C, humidity 60% ± 10%). A total of 24 samples were taken, with 8 samples per level ($n$ = 24).

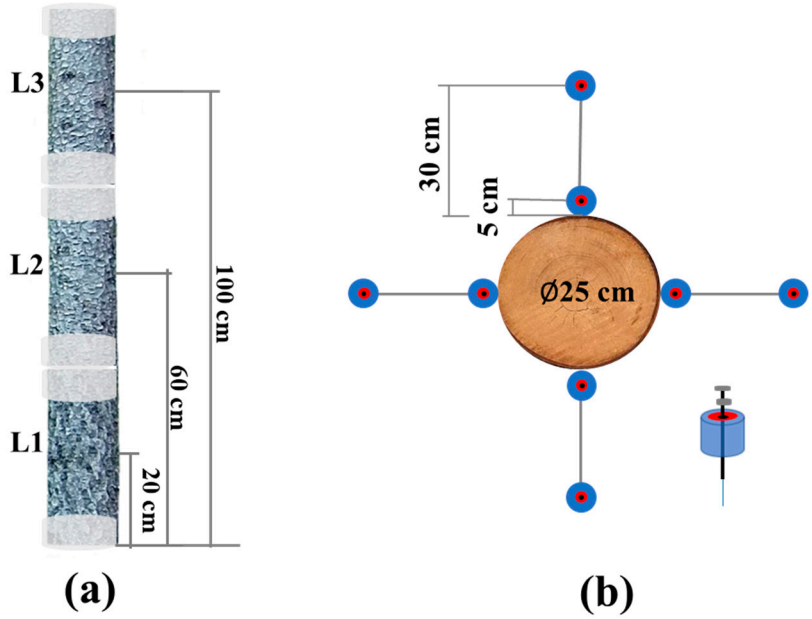


**Figure 1.** Volatile collection of α-pinene under laboratory conditions from fresh drilled logs of *Picea abies*. (**a**) The collection of α-pinene occurred across three height levels, namely L1, L2, and L3. (**b**) Top view of the log pile; black dots indicate distances between the bark surface and the point of collection of α-pinene for each level.

### *2.2. Field Collection of Distributed VOCs Emitted by the Ips typographus Naturally Infested Picea abies*

We conducted two collections of VOCs from naturally infested trees in the field at different locations and during different periods of the 2022 growing season. In this study, we did not measure VOCs from non-infested trees as controls. This decision stems from the continuation of our prior study [8] and existing literature reports (Table 1), which have consistently demonstrated several-fold increases in emissions from infested trees using similar techniques. Both collections took place on the Forests ČZU property near Kostelec nad Černými lesy, the Czech Republic. The area is characterized by a mature forest primarily composed of a 90-year-old Norway spruce (*P. abies*) plantation, situated at an altitude of 400–450 m above sea level (Figures 2 and 3).

The distributed VOCs were collected within 900 m around the freshly *Ips typographus*-infested individual Norway spruces. The infestation status was found by the occurrence of fresh frass at the stem's base, and infestation stadia were specified by assessing the beetle attack density exhibited by the sampled tree. Both sampled trees were in the nuptial chamber building attack stadia, approximately two weeks from the beginning of the mass attack.

The VOCs were collected from infested trees at three different height levels from the ground (1.3 m, 2.6 m, and 4 m). Collection chambers were placed at three different distances from the tree trunk (5 cm, 100 cm, and 900 cm away from the tree) (Figure 3). The row of collection chambers was oriented in four cardinal directions: north (N), east (E), south (S), and west (W) (see Figures 2–4 for reference). Dataloggers were used throughout the experiment to measure the temperature and humidity (Tables S1 and S2), and an anemometer was used to measure the wind speed (Figures 2 and 3).

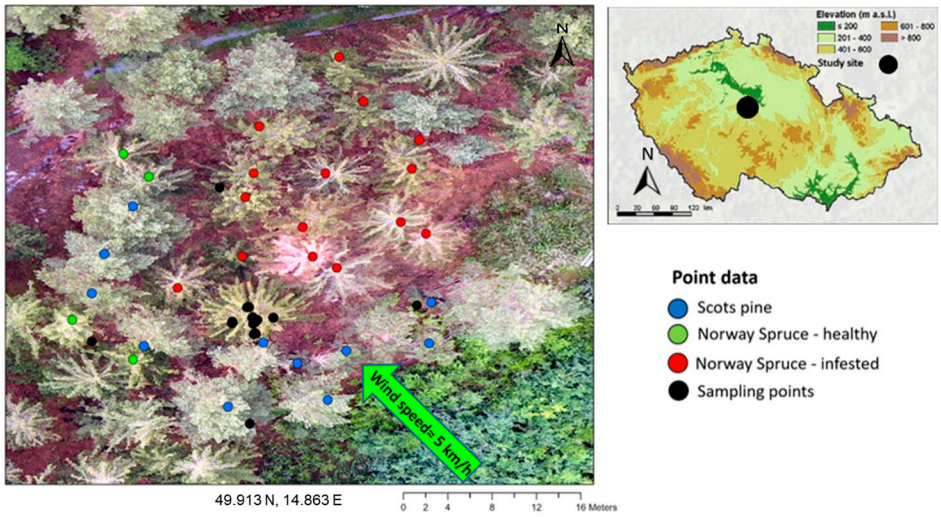


**Figure 2.** Study site for the first forest spatial VOC measurement around the infested tree (30 June 2022). Locality Forests ČZU close to Stříbrná Skalice, the Czech Republic (49.913 N, 14.863 E). Black points—sampling points around the sampled bark beetle-infested tree, in nuptial chamber infestation stadia; red points—Norway spruce infested trees in later stadia of infestation or dead; green—healthy Norway Spruce trees; blue points—Scot pines.

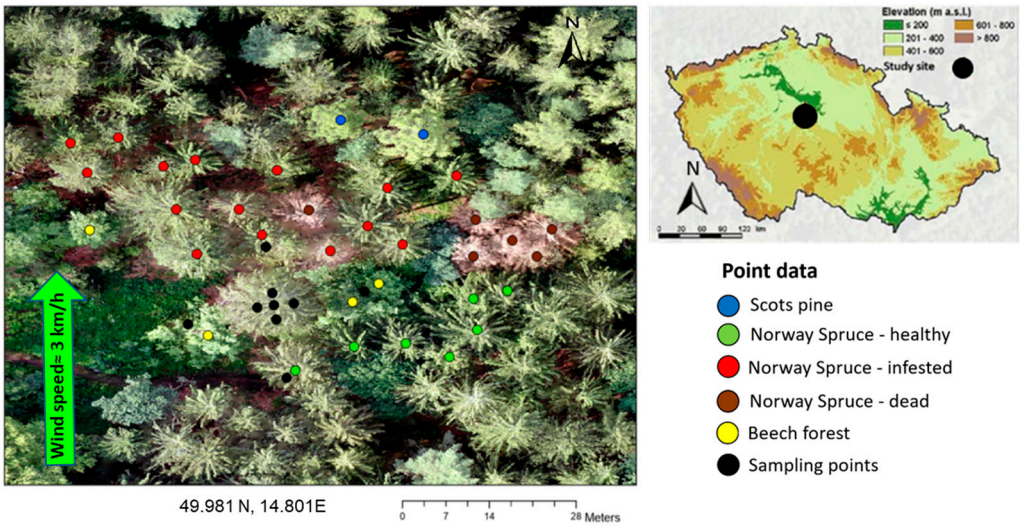


**Figure 3.** Study site for the second forest spatial VOC measurement around the infested tree (24 August 2022) for data in the ČZU Forests close to Vyžlovka, the Czech Republic (49.981 N, 14.801 E). Black points—sampling points; red points—Norway spruce infested trees; green—healthy Norway Spruce trees; brown points—dead Norway Spruce trees; blue points—Scot pines; yellow points—beech trees.

Two analytical approaches were employed to collect VOCs from infested trees in natural forest conditions [27,28,45]:

Sorption onto SPME fibers (PDMS/CAR/DVB, Supelco, PA, USA): Eight SPMEs fixed in protective chambers were positioned at three height levels: L1 (130 cm), L2 (240 cm), and L3 (400 cm). They were placed at two distances from the tree (5 cm and 100 cm) in the immediate proximity of the tree in four cardinal directions: north, south, west, and east. Additionally, four fibers were positioned 900 cm away from the tree in the same direction. These SPMEs were exposed for 60 min to collect volatile organic compounds (VOCs) from the forest air. After collection, the fibers were sealed in vials with septa in the same manner as in the laboratory. They were then placed on dry ice and subsequently stored in a freezer at −18 °C before undergoing measurement using Gas Chromatography-Flame Ionization Detection GC-FID, Agilent 8890 (Agilent, CA, USA).

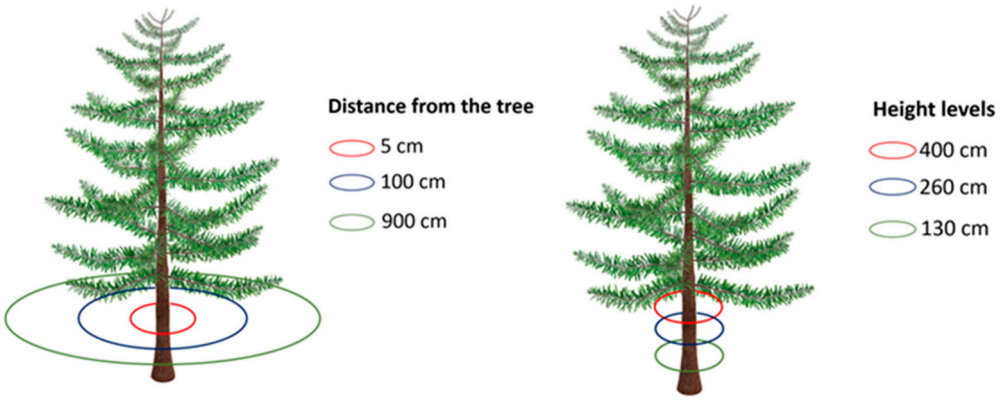


**Figure 4.** Established collection directions for VOCs around trees newly infested by bark beetles.

Sorption using cartridges (inner Ø 3 mm) filled with HayeSep-Q® sorbent (30 mg, Sigma-Aldrich, St. Louis, MO, USA,): This approach involved filtering the surrounding air through sorbent in cartridges sucked by sampling pumps (Pocket Pump Touch; Serie 220–1000, Eighty Four, PA, USA). The pumps were calibrated to operate at a flow rate of 100 mL/min for 60 min. Of the 12 air pumps, the chosen measurements were established along the first height level (130 cm from the ground) in all cardinal directions and different distance levels, and two cartridges were set up at the second height level in north and south directions at a 5 cm distance from the bark surface.

### *2.3. Chemical Analyses of SMPE Fiber and Cartridges via Gas Chromatography-Flame Ionization Detection (GC-FID)*

Cartridges were washed with 1 mL of GC-grade hexane (GC-capillary grade; Avantor, PA, USA) and stored at −18 °C for further chemical analysis.

The SPME and cartridge analyses were carried out via Gas Chromatography-Flame Ionization Detection (GC-FID) Agilent 8890 (Agilent, CA, USA). The GC-FID was equipped with a DB-WAX capillary column (30 m × 320 µm × 0.25 µm film thickness; Agilent, CA, USA). The GC oven program followed a temperature profile of the initial temperature at 40 °C for 2 min, followed by a ramping rate of 10 °C per minute to reach 230 °C, where it was held for 2 min. The carrier gas He flow was 1.5 mL·min$^{-1}$. The inlet operated in spitless mode and the inlet temperature was 220 °C. For the desorption of SPME fibers, an SPME liner was used (n°5190-4048, 78.5 × 0.75 mm id, Agilent, CA, USA). The extracts in hexane (1 µL) from the cartridge collection were analyzed in spitless mode.

### *2.4. Determination of Relative Quantities of α-Pinene via SPME and Absolute Quantities Sorbed to Cartridges*

On the chromatogram, peaks of the main spruce monoterpenes and other volatile organic compounds (VOCs) from the forest air near *P. abies* were observed. However, to describe their 3D distribution, only the most abundant *α*-pinene was chosen, as it adequately represents the trend of the other main monoterpenes [8].

In both collection methods, *α*-pinene's peak identity was confirmed by comparing its retention time with the commercial standard (*α*-pinene, Sigma-Aldrich, St. Louis, MO, USA).

The abundance of *α*-pinene collected via SPME fiber was determined by measuring the peak area of *α*-pinene divided by the sum of areas of the peaks of the five main monoterpenes chosen (*α*-pinene, *β*-pinene, Δ-carene, limonene, camphene, and myrcene, 1.8 cineole) comparing it across individual samples. The quantification of *α*-pinene in the cartridge extracts was based on a calibration curve (Figure S2) constructed using the commercial standard of *α*-pinene diluted in hexane at 0.1, 0.5, 1, 10, 25, 50, and 100 µg/mL. The amount of *α*-pinene in one cartridge, expressed here as µg/mL, means µg/(6 L of air) in the vicinity of an infested tree.

### *2.5. Statistical Analyses*

Statistical analyses were performed using Statistica (version 14.0.0.15). The normality assumption was tested using the Shapiro–Wilk test, and in each case, the null hypothesis (H0) was rejected, indicating the need for nonparametric testing. The Kruskal–Wallis test was used to compare individual levels, distances, and cardinal directions. When the test showed statistical differences, post hoc tests were conducted to examine differences between repetitions.

For SPME analysis, the dependent variable was the relative peak area of α-pinene collected. For cartridges, the amount of α-pinene quantified by the calibration curve was the dependent variable. In both analyses, the independent variables were the distance and height measurements.

## 3. Results

### *3.1. Optimization of VOC Collection to SPME in a Laboratory*

The three VOC collections in the laboratory, taken at different times, were considered three repetitions since they were kept under the same experimental conditions (temperature and method of log drilling). Statistical analysis was conducted on all of them together ($n$ = 72).

The abundance of α-pinene, the main monoterpene representing trends of other MT, was statistically highest at a 5 cm distance from the drilled stems compared to a 30 cm distance from the drilled stems ($p$ = 0.0362) (Figure 5a).

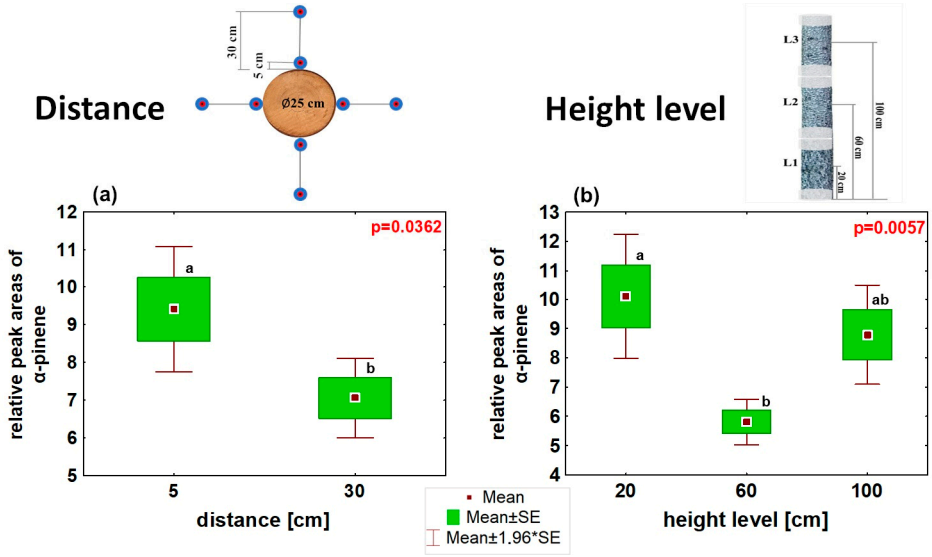


**Figure 5.** SPME laboratory data collections of α-pinene, three replications. (**a**) Abundance of α-pinene for lab experiment at different distances from drilled stem (5 cm and 30 cm); (**b**) abundance of α-pinene for lab experiment at different height levels from the ground (L1—20 cm; L2—60 cm; L3—100 cm). Small red squares—Means; green boxes—Means ± SE; Whiskers—Means ±1.96*SE. Lowercase letters above columns indicate significant differences between different distance or different height level. The $p$-values result from Kruskal–Wallis test; $n$ = 72.

In the vertical direction, the distribution of α-pinene was statistically different ($p$ = 0.006). The lowest abundance was observed at the medium level L2 (60 cm from the ground), and it was significantly different from the abundance at the bottom level L1 (20 cm from the ground) (post-hoc; $p$ = 0.007) (Figure 5b).

### *3.2. α-Pinene Spatial Distribution around Norway Spruce Infested by Ips typographus for Two Weeks*

The conditions in the first forest spatial VOC measurement conducted on 30 June 2022 on two-week naturally infested trees were an average temperature of 25.1 °C, average humidity of 72.6%, wind speed of 5 km/h, wind direction from the SE, and sunshine. The abundance of α-pinene emitted from the naturally infested tree was upregulated at a 5 cm distance from the stem. This upregulation was detected by both collection methods, with a significant increase observed using SPME ($p$ = 0.036), where 5 cm and 100 cm distances significantly differed ($p$ = 0.036 post hoc) (Figure 6a) and a non-significant increase was observed using sorption to cartridges ($p$ = 0.0585). A trend of a decreasing α-pinene concentration at a 900 cm distance from the infested tree was observed in the collection involving cartridges (Figure 6d), but not in the collection using SPME (Figure 6a).

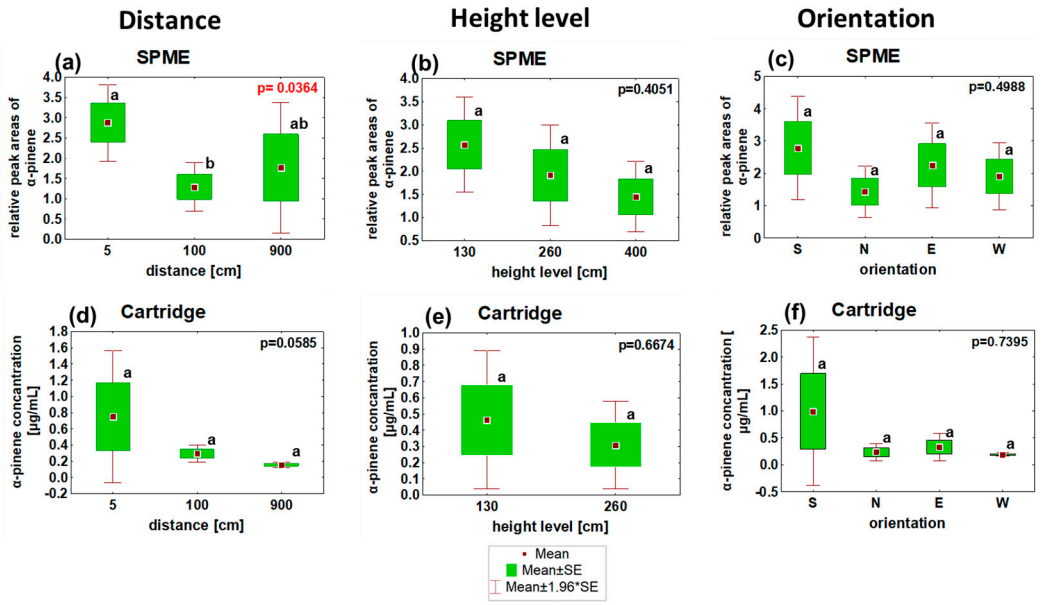


**Figure 6.** First field detection of α-pinene distribution around *Ips typographus*-infested spruce (30 June 2022). (**a**) Abundance of α-pinene collected by SPME in different distances from stem (5 cm; 100 cm; 900 cm); (**b**) abundance of α-pinene collected by SPME at different height levels from ground (130 cm; 260 cm; 400 cm); (**c**) abundance of α-pinene collected by SPME in cardinal directions (S—south; N—north; E—east; W—west); (**d**) abundance of α-pinene collected by HayeSep-Q® cartridges in different distances from stem (5 cm; 100 cm; 900 cm); (**e**) abundance of α-pinene collected by HayeSep-Q® cartridges at different height levels from ground (130 cm; 260 cm); (**f**) abundance of α-pinene collected by HayeSep-Q® cartridges in cardinal directions (S—south; N—north; E—east; W—west). Small red squares—Means; green boxes—Means ± SE; Whiskers—Means ±1.96*SE. Lowercase letters above columns indicate significant differences between different distance different height level, and different orientation. The $p$-values result from Kruskal–Wallis test. ($n$ = 27 SPME samples) (cartridges $n$ = 12).

In the vertical direction, a weak, non-significant trend was observed in the accumulation of α-pinene in the bottom level of the stem at 130 cm above the ground, decreasing with altitude up to 400 cm above the ground. This trend was consistent across both collection methods (Figure 6b,e).

Regarding cardinal orientation, there was no significant accumulation of α-pinene on any of the measured sides. However, considering the high variability of the data, there was a trend of increased α-pinene abundance on the south side of the infested stem, as observed with both SPME fiber and cartridge collection methods (Figure 6c,f).

A 3D depiction of the α-pinene collection using SPME fiber on 30 June 2022 is shown in Figure 7. Increased color saturation in the visualization corresponds to higher accumulated

α-pinene levels. The accumulation aligns with Figure 6, indicating elevated concentrations at the first level (130 cm). In terms of distance, there is a notable increase at 5 cm from the stem. Beyond 900 cm, there is considerable variation in α-pinene amounts, making it difficult to observe a clear trend. Regarding the orientation, slightly higher concentrations are observed on the south and east sides, though are not significantly dominant.

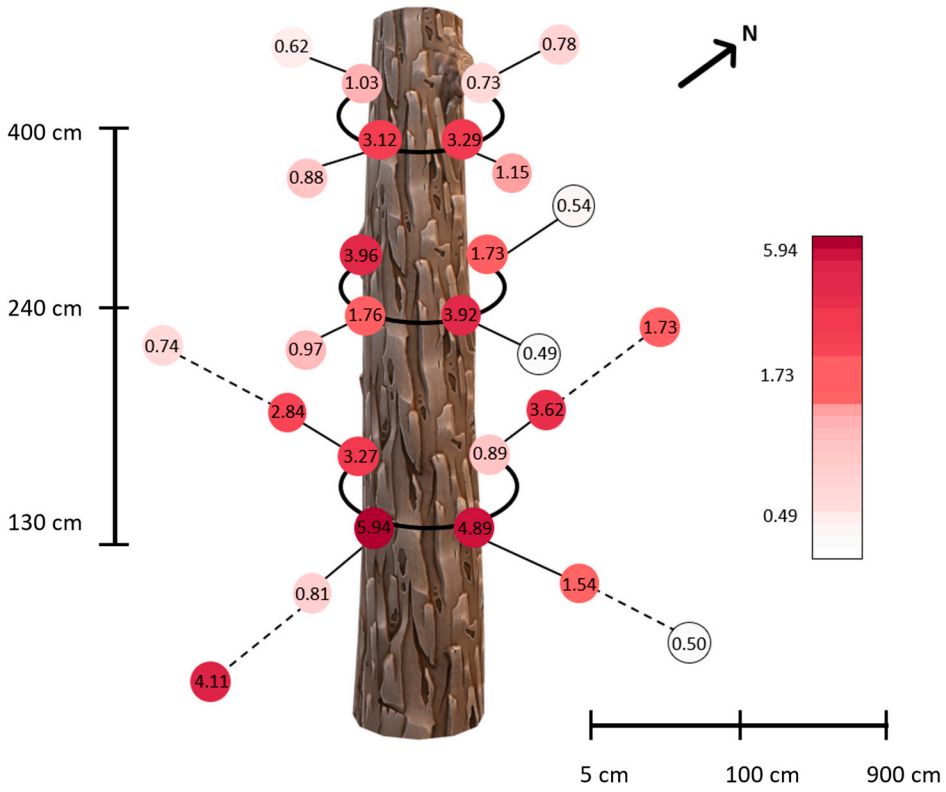


**Figure 7.** 3D distribution of α-pinene collected by SPME in first field data collection (30 June 2022). More saturated color means a higher abundance of α-pinene. The numbers in circles are relative peak areas of α-pinene.

The conditions for the second VOC measurement in the forest open space around a naturally infested tree, conducted on 24 August 2022, differed from the first collection in terms of location and environmental factors (average temperature of 20.7 °C, average humidity of 78.8%, wind speed of 3 km/h, wind direction from S, and sunshine).

α-pinene abundance significantly increased at a 100 cm distance from the stem, as detected by SPME ($p = 0.0037$) (Figure 8a) and sorption to cartridges ($p = 0.0244$) (Figure 8d), compared to both the 5 cm and 900 cm distances considered as controls.

A non-significant, very weak trend was observed for the accumulation of α-pinene at a height of 260 cm from the ground measured by SPME (Figure 8b). No significant differences were found in α-pinene accumulation in any cardinal direction (Figure 8c,e).

The 3D depiction of α-pinene collection using SPME fiber during the second field collection on 24 August 2022 is shown in Figure 9. Notably, there is an increase 100 cm from the stem in terms of distance and higher accumulation at the second level, 260 cm from the ground. This aligns with Figure 8.

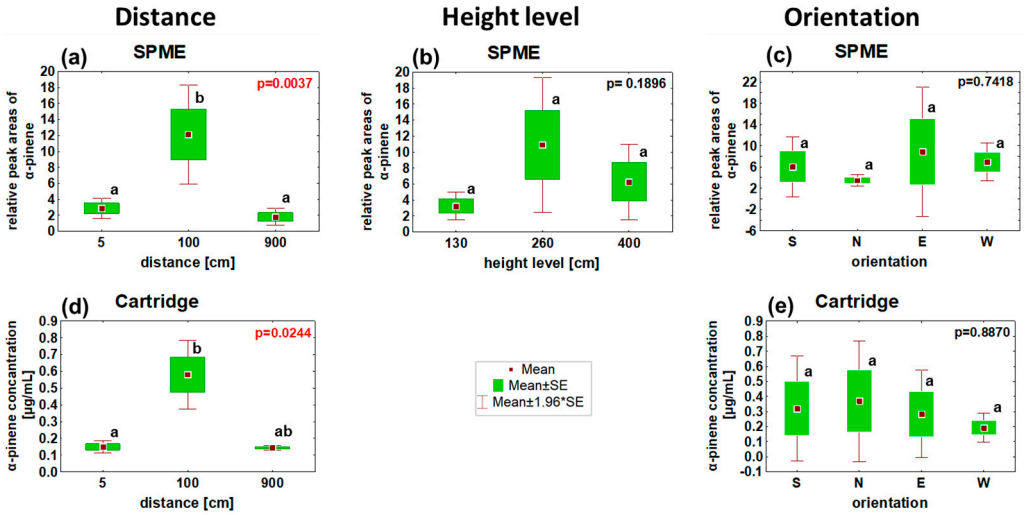


**Figure 8.** The second field detection of α-pinene distribution around *Ips typographus*-infested spruce (24 August 2022). (**a**) Abundance of α-pinene collected by SPME at different distances from stem (5 cm; 100 cm; 900 cm); (**b**) abundance of α-pinene collected by SPME at different height levels from ground (130 cm; 260 cm; 400 cm); (**c**) abundance of α-pinene collected by SPME in cardinal directions (S—south; N—north; E—east; W—west); (**d**) abundance of α-pinene collected by HayeSep-Q® cartridges at different distances from stem (5 cm; 100 cm; 900 cm); (**e**) abundance of α-pinene collected by HayeSep-Q® cartridges at different height levels from ground (130 cm; 260 cm), abundance of α-pinene collected by HayeSep-Q® cartridges in cardinal directions (S—south; N—north; E—east; W—west). Small red squares—Means; green boxes—Means ± SE; Whiskers—Means ±1.96*SE. Lowercase letters above columns indicate significant differences between different distance, different height level, and different orientation. The *p*-values result from Kruskal–Wallis test. (*n* = 22 SPME samples) (cartridges *n* = 12).

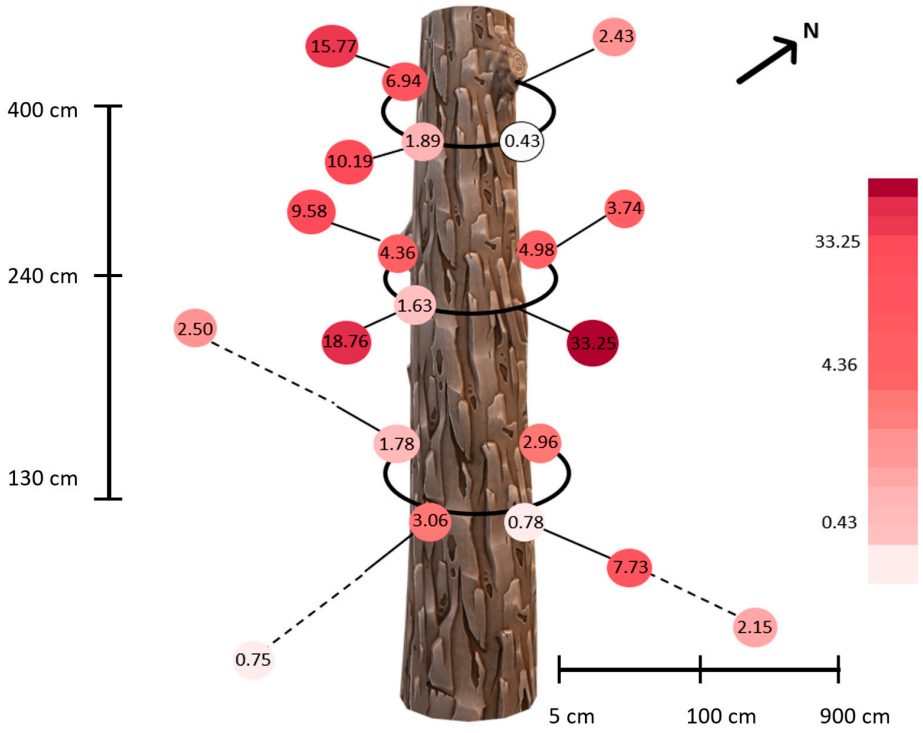


**Figure 9.** 3D distribution of α-pinene collected by SPME in second field data collection (24 August 2022). More saturated color means a higher amount of collected α-pinene. The numbers in circles are relative peak areas of α-pinene.

## 4. Discussion

The volatile compounds emitted by infested trees have been studied in two main contexts: atmospheric chemistry processes, with a focus on reactive particle formation [50], and ecological roles related to bark beetles and their predators [27,28,32,51]. Most reports in atmospheric chemistry measured volatiles at the canopy or twig level [52]. Emission measurements of biogenic volatile organic compounds (BVOC) from infested trees appeared in only a few studies investigating the potential use of these emissions as early attack detection markers [9,10,39]. Our study is unique as it examines emissions within a distance of 10 m of the infested tree, specifically at ground level and up to 5 m, where $\alpha$-pinene accumulates at an average infestation season temperature of 20 to 25 °C in central European forests [53]. This novel approach contributes new insights to our understanding of volatiles emitted by bark beetle-infested trees.

Primarily, in our study, we employed laboratory optimization techniques to collect volatiles from freshly infested trees. The main emitted compound, $\alpha$-pinene, was selected as a representative of the primary monoterpenes. It reflects the trends observed in other monoterpenes emitted by spruce trees infested by bark beetles [54].

In the laboratory, where the temperature was 25 °C, we observed a higher abundance of $\alpha$-pinene in the open space in the closest proximity to logs, particularly in the lowest and highest positions. This increase was likely caused by the proximity of open log cuts and exposed bark edges, despite our attempts to prevent them by covering them with PE foil.

The first detection of $\alpha$-pinene in the surroundings of a naturally infested tree in the field was conducted approximately two weeks after the beginning of a mass attack on the tree, corresponding with the time the monoterpene emission peaks [8,50]. This detection also took place at a temperature of 25 °C, when, expectedly, $\alpha$-pinene vapors are heavier than air (with a vapor pressure of 4.9 mm Hg at 27 °C) [55].

Similar to our laboratory findings and reports from the literature [27,28], we observed the highest abundance of $\alpha$-pinene in the immediate vicinity of the infested tree. Vertically, $\alpha$-pinene accumulated at the lowest level, approximately 1.3 m above the ground, which was confirmed by two different collection methods:

The lower-level accumulation of the monoterpenes under similar conditions was previously observed as the output of the continual measuring campaign of BVOC in the forest. It has been reported that monoterpenes are emitted by healthy trees during the daytime, but they are also more susceptible to degradation in the atmosphere during the same period [53]. Monoterpenes are known to be unstable in the atmosphere, with a relatively short lifetime, often lasting only hours or even minutes, forming atmospheric secondary organic aerosol particles, followed by reactions with ozone and radicals like NO, OH, and $NO_3$ [56]. As a result of their decomposition, methanol and acetone, both of which are highly abundant in the forest atmosphere, are produced, alongside other products. Additionally, at higher elevations in the forest, strong air streams are present, resulting in monoterpenes having a higher 'mixing ratio,' which implies a lower concentration. The monoterpenes accumulate at lower altitudes in the forest, particularly during the nighttime [57].

The abundance of $\alpha$-pinene in the controls placed at a further distance from the tree (9 m) exhibited greater variability [10].

This variability was most likely influenced by the surrounding trees. There were spruces, some of them in more progressed infestation stadia, or even dead and intact pines. In naturally infested trees, we also assessed an $\alpha$-pinene orientation related to the infested stem's cardinal direction. During the first field collection, we noticed a non-significant trend of higher $\alpha$-pinene levels on the south side of the stem, despite the prevailing wind coming from the opposite direction. This was attributed to the tree's location near the forest edge, allowing for direct afternoon sunlight exposure during collection [17,21].

The second collection, conducted later in the season, took place at a lower temperature of 22 °C. Interestingly, the highest abundance of monoterpenes was not observed at the lowest position, but rather at a height of 2.6 m, Additionally, it was not closest to the tree

stem, but rather 1 m away. Results were confirmed again by two collection methods, Solid-Phase Microextraction (SPME) and HayeSep-Q® Cartridge sampling, conducted at two different time intervals. This sampled tree, despite being in a similar stage of infestation as the first tree, was fully surrounded by other trees, including some broadleaf varieties in the vicinity, resulting in no direct irradiation of the stem.

During this collection, we did not observe any significant influence of sunlight exposure or wind speed on the orientation of increased $\alpha$-pinene abundance.

The temperature and humidity differences in altitude were recorded on the same experimental plot [9]. The highest temperatures were observed approximately 1 m above the ground with an upward gradient. This temperature variation may impact the higher abundance of $\alpha$-pinene at an altitude of 2.6 m compared to ground level due to the physical properties of pinene vapors.

The variation in outcomes between the first and second field collections was attributed to differences in the surroundings of the measured trees, distinct parts of the season, and, primarily, variations in temperature and humidity conditions [36,37].

In forest practices, our 3D distribution data of $\alpha$-pinene emitted by freshly infested spruces may serve as a foundation for scanning techniques, demonstrating promising potential for managing bark beetle populations by facilitating early attack detection.

The most advanced technique for volatile scanning in situ can be based on field and portable Gas Chromatography-Mass Spectrometry (GC-MS) instruments, which are possible to mount on UAV. These instruments have the capability to separate, identify, and quantify VOC compounds in situ and collect data at short intervals. Existing instruments on the market have been tested for various analytes, primarily for military applications [58]. However, reliable devices for detecting terpenes and other infestation marker VOCs are still lacking.

To specifically select markers of infestation by *Ips typographus* and exclude infestations of different herbivores (as relevant bark species or defoliators) from the complex odor environment of a natural forest, such as bark beetle pheromones and spruce host compounds, an olfactory perception system of the bark beetles, antennae can be used as a specific detector working via electroantennography principles [59,60]. Ongoing research is exploring the application of this method in insect pest detection, with future directions aiming to target insect protein odorant receptors for specific compounds indicative of infestation [31].

However, the most potentially promising novel device is a non-specific electronic nose, designed for real-time chemical substance detection (e.g., detecting dangerous gas leaks or measuring concentrations near landfills, monitoring volcanic activity, etc.). We previously conducted tests to explore the feasibility of early-stage stress detection in forest stands using an electronic nose, specifically the Sniffer4D [9].

Due to limitations in measuring VOCs with non-specific sensors in forests, especially in areas with higher concentrations of compounds from fresh clearings, debris, and broken trees, an ideal approach would involve integrating environmental VOC scanning with other data collected through different scanning methods. This integration could be achieved, ideally using UAV vehicles equipped with various sensors for high-resolution data collection [61]. The map of VOC abundance can be automatically overlaid with aerial maps of the area, changes in reflectance, dedicated vegetation indices [6], or temperature fluctuations [62].

*Limitation of the Study*

The reported study is considered a pilot trial investigating the distribution of monoterpenes around infested trees at ground level and up to 5 m. To enhance the study, future research employing classical collection and analytical techniques should result in confirmation of the quantitative monoterpene distribution. The expanded study will involve a large-scale tree group, multiple replications for robust statistical analyses, and a focus on genetically relevant trees. To ensure control, infested and non-infested trees will be monitored simultaneously, maintaining consistent bark beetle infestation density. Standardized

environmental conditions will be implemented to minimize variability, with the potential to explore environmental conditions as a variable.

## 5. Conclusions

In summary of this pilot study, VOCs, represented here by α-pinene, emitted by freshly attacked trees may act as detectable markers for timely infestation. The spatial distribution of their concentration in an open space follows a gradient pattern that can be analyzed using various collection techniques. However, this distribution is notably influenced by environmental factors. With further optimization and integration with other scanning methods, these VOCs emitted by freshly infested trees can be used to develop effective "early detection methods" for bark beetles. Real-time data distribution provides a strong foundation for implementing crucial scanning techniques in early attack detection strategies.

**Supplementary Materials:** The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/f15010010/s1, Table S1. The averages of the temperature and humidity in the first data collection 30 June 2022 in two periods of collections (SPME and cartridge collection). Table S2. The averages of the temperature and humidity in the second data collection 24 August 2022 in two periods of collections (SPME and cartridge collection). Figure S1. The map of development of Norway spruce logging due to bark beetle infestation in the Czech Republic in the period 9/2021–7/2022. Figure S2. Calibration curve of α-pinene. Constructed using commercial standard of α-pinene diluted in hexane to 0.1; 0.5; 1; 10; 25; 50 and 100 µg/mL.

**Author Contributions:** B.S.: experimental work, data curation, statistical data processing, and writing draft. A.A.C.M.: experimental work, GC-FID analysis, and writing draft and editing. T.H.: experimental work, figures processing. M.L.: experimental work and attack expertise. P.S.: editing manuscript and supervision. A.J.: conceptualization, data sampling, formal analysis, writing, editing, and supervision. All authors contributed to the article and approved the submitted version. All authors have read and agreed to the published version of the manuscript.

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**Data Availability Statement:** Data are available in the article and in the Supplementary Materials.

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**Conflicts of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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EDITED BY
Davide Rassati,
University of Padua, Italy

REVIEWED BY
Nadir Erbilgin,
University of Alberta, Canada
Brian Sullivan,
Southern Research Station, Forest Service (USDA), United States

*CORRESPONDENCE
Anna Jirošová
✉ jirosovaa@fld.czu.cz

†These authors have contributed equally to this work

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# Field effects of oxygenated monoterpenes and estragole combined with pheromone on attraction of *Ips typographus* and its natural enemies

Antonioni Acacio Campos Moliterno[1†], Rastislav Jakuš[1,2], Roman Modlinger[1], C. Rikard Unelius[3], Fredrik Schlyter[1,4] and Anna Jirošová[1*†]

[1]Faculty of Forestry and Wood Sciences, Czech University of Life Sciences Prague, Prague, Czechia, [2]Institute of Forest Ecology, Slovak Academy of Sciences, Zvolen, Slovakia, [3]Faculty of Health and Life Sciences, Linnaeus University, Kalmar, Sweden, [4]Department of Plant Protection Biology, Chemical Ecology, Swedish University of Agricultural Sciences, Alnarp, Sweden

**Introduction:** Central European Norway spruce monocultures face *Ips typographus* outbreaks due to decreasing resistance. These beetles use volatile compounds to communicate and select suitable host trees. Spruce trees, beetles, and their symbiotic ophiostomatoid fungi emit oxygenated monoterpenes, including 1,8-cineole, α-terpineol, camphor, carvone, terpinen-4-ol, isopinocamphone, and pinocamphone, and the phenylpropanoid estragole, particularly in the infestation phase. These compounds trigger strong responses in *I. typographus* antennae, motivating our field study.

**Objective:** This study aimed to assess how adding these compounds to the aggregation pheromone of *Ips typographus* modulates the attraction of this bark beetle and its natural enemies.

**Methods:** In combination with *I. typographus* pheromone, estragole, 1,8-cineole, (±)-camphor, (−)-carvone, *alpha*-terpineol, (−)-terpinen-4-ol, (+)-pinocamphone, and (+)-isopinocamphone at low, medium, and high doses were tested in pheromone traps at two sites in the Czech Republic.

**Results:** All 1,8-cineole doses and the high estragole dose acted as anti-attractants for *I. typographus*, whereas all (+)-isopinocamphone doses enhanced their attraction to pheromone. Catches of natural enemies, the Staphylinidae and Pteromalidae, varied by location.

**Conclusion:** 1,8-cineole, isopinocamphone, and estragole may play vital roles in tritrophic interactions among spruce trees, and *I. typographus* and its natural enemies, and these compounds may be developed into new or enhanced semiochemical-based pest control methods.

KEYWORDS

**Eurasian spruce bark beetle, host compounds, Pteromalidae, Staphylinidae, Norway spruce, *Picea abies*, spruce kairomone, pheromone traps**

# 1 Introduction

In Central Europe, Norway spruce (*Picea abies*) (L.) Karst. (Pinales: Pinaceae) has been severely affected by infestations of the spruce bark beetle, *Ips typographus* (L.) (Coleoptera: Curculionidae: Scolytinae), that in the Czech Republic have resulted in timber losses of 5.9 mil. $m^3$ in 2017 and 26.2 mil. $m^3$ in 2020 (Hlásny et al., 2022). In outbreak regions, managing bark beetles often involves applying insecticides to *P. abies* trunks or stored timber to eliminate the emerging beetles (Fettig and Hilszczański, 2015). However, the use of pesticides can negatively impact the forest ecosystem, including beneficial bark beetle predator species (Hlásny et al., 2019). The development of alternative, eco-friendly strategies in forestry is a logical progression. One such strategy involves utilizing semiochemicals, compounds that mediate the interactions of beetles with each other and other organisms. These signals enable beetles to locate a mate or host tree by providing intraspecific and interspecific chemical information (Bergström, 2007). Aggregation pheromone components, produced by male beetles after successful colonization (Birgersson et al., 1984; Ramakrishnan et al., 2022), have been employed for monitoring and controlling *I. typographus* populations (Heber et al., 2021). Furthermore, recent research has focused on the management potential of kairomones, compounds originating from both host spruce trees and non-host trees, e.g., broadleaf trees (Zhang and Schlyter, 2004; Jakuš et al., 2022). Bark beetles possess a sophisticated olfactory system that enables them to detect and distinguish the chemical composition and quantity of these odors (Andersson, 2012).

The principal olfactory stimulants for *I. typographus* emitted by *P. abies* are primarily composed of high-abundance monoterpenes such as $\alpha$-pinene (23–39%), $\beta$-pinene (25–58%), $\beta$-phellandrene (5–19%), limonene (1.5–4%), myrcene (1.6–3.4%), Δ-3-carene (0.6–1.1%), and camphene (0.2–1.1%; Netherer et al., 2021). However, recent comparative analysis utilizing *I. typographus* antennae as biological detectors (gas chromatography coupled with electroantennography, GC-EAD) has identified several novel compounds present in relatively small amounts but exhibiting high activity with the beetles' antennae (Kalinová et al., 2014; Schiebe et al., 2019). These compounds include oxygenated monoterpenes, 1,8-cineole (eucalyptol), *trans*-4-thujanol (sabinene hydrate), camphor, pinocarvone, pinocamphone, isopinocamphone, terpinen-4-ol, alpha-terpineol, carvone, and phenylpropanoid estragole (4-allylanisole and methyl chavicol). In single-cell electrophysiological studies, researchers identified 24 classes of olfactory sensory neurons (OSN) within olfactory sensillae for *I. typographus* (Hallberg, 1982). Plant odor-responding OSNs exhibit a variety of response specificities from broadly tuned OSNs for host monoterpene hydrocarbons to several highly specific OSN classes responding mainly to oxygenated monoterpenes (1,8-cineole, isopinocamphone, *trans*-4-thujanol, or verbenone; Andersson et al., 2009; Schiebe et al., 2019; Kandasamy et al., 2023).

In Norway spruce, oxygenated monoterpenes are minor compounds (∼1% representation), and their content is influenced by tree health and stress levels (Netherer et al., 2021). The production of oxygenated monoterpenes in trees naturally occurs through the cytochrome P450-catalyzed oxidation of monoterpene hydrocarbons or by cyclization of oxygenated intermediates (Celedon and Bohlmann, 2019). The release rate of oxygenated monoterpenes, including 1,8-cineol, camphor, pinocarvone, terpinen-4-ol, and $\alpha$-terpineol, from healthy trees at 24°C ranges from 0.1 to 7 $\mu g/m^2/h$ of stem surface area (Ghimire et al., 2016). In infested trees, these rates increased 10–100 times (Jaakkola et al., 2022), and in cut trees, they increased 10 times (Schiebe et al., 2019).

Bark beetle symbiotic ophiostomatoid fungi generate oxygenated terpenes in laboratory conditions when they are inoculated onto a wood substrate (Kandasamy et al., 2023). In the forest, fungi may assist beetles in colonizing healthy trees by being involved in detoxifying host defense terpenes (Krokene, 2015; Kandasamy et al., 2021). Additionally, the beetles themselves generate oxygenated monoterpenes, as they metabolize toxic terpenes while feeding on the spruce tree's phloem (Blomquist et al., 2021). The detoxification process of terpenes involves a series of steps. In the first step, a hydroxyl group is introduced to a terpene molecule by cytochrome P450 catalysis. This modification increases the molecule's polarity and solubility in water, enabling beetles to eliminate it (Blomquist et al., 2021). In subsequent steps, the resulting terpenic alcohols are either excreted from the body or bound to detoxification conjugative molecules, such as fatty acid esters (Chiu et al., 2018) or glycosylates (Dai et al., 2021). This mechanism was studied in *Dendroctonus ponderosae* (Chiu, 2018; Chiu et al., 2019), *Dendroctonus armandi* (Dai et al., 2021), as well as in *Ips* species (Blomquist et al., 2021; Ramakrishnan et al., 2022) that feed on conifer trees, as these trees possess terpenes as a defense trait. This adaptation allows the beetles to overcome the tree's defenses and successfully colonize it. It is theorized that during evolution, some of these detoxification products, such as *cis*-verbenol in *I. typographus*, started to serve as aggregation pheromones for the bark beetles (Blomquist et al., 2021; Schebeck et al., 2023).

The exact behavioral role of all host-produced oxygenated monoterpene semiochemicals in bark beetles is not fully understood. However, according to the primary attraction theory proposed by Lehmanski et al. (2023), these compounds may play a role in helping male bark beetles detect weakened host trees, thereby facilitating successful colonization. Other compounds, e.g., 1,8-cineole and *trans*-4-thujanol, have proved to be potent anti-attractive compounds inhibiting beetle attraction to their pheromone (Jirošová et al., 2022b). 1,8-cineole has been identified as a potential predictor of bark beetle-resistant trees, along with several other specialized metabolites (Schiebe et al., 2012). Higher levels of *trans*-4-thujanol were detected in younger Norway spruces. Given that this compound has demonstrated repellency in high doses in laboratory olfactometer studies, it provides a potential explanation for the reduced attraction of *I. typographus* to trees below the age of 60 years (BlaŽyte-Cereškiene et al., 2016). The activity of 1,8-cineole and *trans*-4-thujanol for *I. typographus* has been evaluated in a field trapping experiment using different doses in combination with pheromones. Both compounds demonstrated a similar level of dose-dependent anti-attractant activity, with *trans*-4-thujanol inhibiting more the captures of females than males (Jirošová et al., 2022b). These compounds have been tested in combination with other anti-attractants for the protection of spruce trees in various forest environments, such as fresh forest

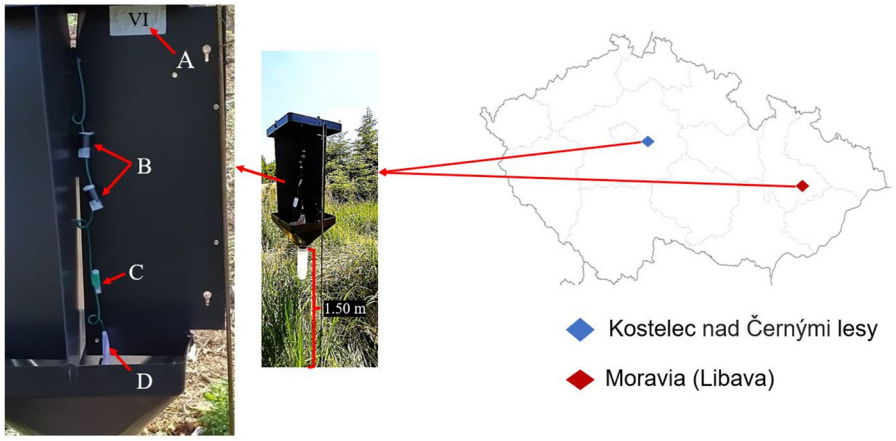


FIGURE 1
Field experiment sites and cross-panel field traps in the Czech Republic: **(A)** Trap label, **(B)** synthetic aggregation pheromone dispensers, **(C)** synthetic host tree odor dispenser, and **(D)** bait label for treatment identification.

edges or fragmented forests (Jakuš et al., 2022; Jirošová et al., 2022a). Furthermore, individual compounds *trans*-4-thujanol, (+)-isopinocamphone, camphor, and terpinen-4-ol were tested in different doses in two-choice Petri dish walking laboratory tests to assess their attractiveness to *I. typographus*. The activity of these compounds was largely insignificant, and only *trans*-4-thujanol and camphor at a high dose were attractive. In contrast, a more complex mixture of oxygenated monoterpenes, generated through the metabolization of (−)-$\beta$-pinene and (−)-bornyl acetate by the ophiostomatoid fungus *Grosmania polonica*, exhibited dose-dependent attractivity in the test (Kandasamy et al., 2023).

In multi-trophic interactions, the bark beetle predator-prey relationships are influenced by qualitative and quantitative aspects of host tree compounds and prey pheromones (Erbilgin and Raffa, 2001; Netherer et al., 2021). The natural enemies associated with *I. typographus* include Hymenoptera: Pteromalidae (*Rhopalicus* spp.), Diptera: Dolichopodidae (*Medetera* spp.), Coleoptera: Cleridae (*Thanasimus* spp.), and Coleoptera: Staphylinidae (*Nudobius* sp. and *Quedius* sp.; Wegensteiner et al., 2015). Laboratory bioassays demonstrated the strong attraction of female *Rhopalicus* spp. to infested logs, with antennal responses to oxygenated monoterpenes including pinocamphone, pinocarvone, and the phenylpropanoid estragole (Pettersson, 2001; Pettersson and Boland, 2003). Similarly, camphor, pinocamphone, and terpinen-4-ol derived from bark beetle-associated microorganisms in infested spruce logs exhibited strong attraction with *Medetera signaticornis* Loew (Sousa et al., 2023). Staphylinidae feeds on a wide range of prey, and some species are hypothesized to be associated with bark beetles (Pelto-Arvo, 2020). However, their specific association with host tree volatiles, such as oxygenated monoterpenes and *I. typographus* pheromone, remains unexplored.

The foraging strategies of some *I. typographus* predators, *Medetera* spp. and *Thanasimus* spp., could be governed by a more complete blend of the aggregation pheromone and host volatiles (represented by oxygenated monoterpenes; Hulcr et al., 2006). The hemiterpene pheromone component 2-methyl-3-buten-2-ol by itself does not increase predator catch, while the minor component ipsdienol does (Bakke and Kvamme, 1981; Hulcr et al., 2006; Raffa, 2014).

Further research is necessary to achieve a more comprehensive understanding of the mechanisms and functions of oxygenated monoterpenes and estragole in the behavior of bark beetles and their predators.

In this study, we aimed to address the following specific questions:

1. Can individual host tree-oxygenated monoterpenes and estragole enhance or reduce the attractiveness of *I. typographus* pheromones in field traps for capturing bark beetles?
2. If so, which of the three tested decadic steps in release rates, namely, low (representing conditions closest to natural levels), medium, and high (representing doses used in management), exhibits the highest efficacy?
3. What is the species composition and abundance of predatory insects attracted to the tested compounds using the methodology described?
4. Can we propose a specific role for the tested compounds in bark beetle ecology?

We conducted a field trapping experiment to investigate the activity of eight synthetic host tree compounds (estragole, 1,8-cineole, (±)-camphor, (−)-carvone, (−)-α-terpineol, (−)-terpinen-4-ol, (+)-pinocamphone, and (+)-isopinocamphone) for *I. typographus* and some of its natural enemies. The compounds were tested at low, medium, and high release rates, in combination with components in pheromone barrier traps.

## 2 Materials and methods

### 2.1 Experimental area and design

For field experiments, we chose two locations in the Czech Republic. The first location was in North Moravia in Libavá

TABLE 1 The gravimetric establishment of released rates of tested compounds performed in the laboratory and field.

| Compounds | Sources | Purity (%) | Doses | Release rates (mg/day) | | | Dispenser design |
|---|---|---|---|---|---|---|---|
| | | | | Nominal | Lab. ± SEM $N = 3$ | Field ± SEM $N = 3$ | |
| Estragole | Sigma-Aldrich | 98 | L<br>M<br>H | 0.1 | 0.13 ± 0.04 | 0.14 ± 017 | Glass vial (2 ml), lid hole (1 mm) |
| | | | | 1 | 1.19 ± 0.17 | 1.72 ± 0.94 | †Foil sachet: hole of 2 mm |
| | | | | 10 | 8.78 ± 1.71 | 1.86 ± 0.4 | †Foil sachet: hole of 9 mm |
| 1,8-Cineol | Sigma-Aldrich | 98 | L<br>M<br>H | 0.1 | 0.06 ± 0.07 | 0.11 ± 0.01 | ††PE-vial (Kartell 731), without hole with paraffin oil (1 ml) |
| | | | | 1 | 0.66 ± 0.12 | 0.92 ± 0.12 | Glass vial (2 ml), lid hole (1 mm) |
| | | | | 10 | 5.20 ± 0.30 | 5.70 ± 6.7 | ††Kartell 730 with hole (2 mm) |
| (–)-terpinen-4-ol | Sigma-Aldrich | 98 | L<br>M<br>H | 0.1 | 0.07 ± 0.04 | 0.35 ± 0.06 | Glass vial (2 ml), lid hole (1 mm) |
| | | | | 1 | 0.41 ± 0.21 | 0.52 ± 0.49 | ††Kartell 731 without hole |
| | | | | 10 | 9 ± 2.34 | 7.8 ± 10.16 | †Foil sachet: hole of 18 mm |
| (–)-carvone | Sigma-Aldrich | 95 | L<br>M<br>H | 0.1 | 0.13 ± 0.05 | 0.27 ± 0.13 | Glass vial (2 ml), lid hole (1 mm) |
| | | | | 1 | 0.66 ± 0.28 | 0.22 ± 0.16 | ††Kartell 730 without hole |
| | | | | 10 | 9.1 ± 2 | 4.92 ± 3.45 | †Foil sachet: hole of 18 mm |
| ( ± )-camphor | Alfa Aesar | 95 | L<br>M<br>H | 0.1 | 0.09 ± 0.05 | 0.14 ± 0.9 | Glass vial (2 ml), lid hole (1 mm) |
| | | | | 1 | 0.58 ± 0.06 | 1.71 ± 0.77 | ††Kartell 730: hole of 2 mm |
| | | | | 10 | 7.33 ± 0.94 | 0.67 ± 10 | PE-sachet without hole |

*(Continued)*





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TABLE 1 (Continued)

| Compounds | Sources | Purity (%) | Doses | Release rates (mg/day) | | | Dispenser design |
|---|---|---|---|---|---|---|---|
| | | | | Nominal | Lab. ± SEM $N = 3$ | Field ± SEM $N = 3$ | |
| (-)-α-terpineol | Sigma-Aldrich | 90 | L<br>M<br>H | 0.1 | 0.11 ± 0.05 | 0.05 ± 0.02 | ††Kartell 730 without hole |
| | | | | 1 | 1.37 ± 0.27 | 0.14 ± 0.07 | †Foil sachet: hole of 9 mm |
| | | | | 10 | 4.01 ± 0.4 | 1.11 ± 0.48 | PE-sachet without hole |
| (+)-isopinocamphone | ††† | 99 | L<br>M<br>H | 0.1 | 0.37 ± 0.40 | 0.40 ± 0.11 | †Foil sachet: hole of 1 mm |
| | | | | 1 | 1.47 ± 0.08 | 1.87-0.67 | ††Kartell 730 without hole |
| | | | | 10 | 8.39 ± 0.98 | 8.21 ± 1.41 | ††Kartell 731, lid hole (2 mm) |
| (+)-pinocamphone | ††† | †††† | L<br>M<br>H | 0.1 | 0.4 ± 0.11 | 0.3 ± 0.6 | †Foil sachet: hole of 1 mm |
| | | | | 1 | 1.01 ± 0.34 | 1.32 ± 0.83 | ††Kartell 730 without hole |
| | | | | 10 | 9.1 ± 0.59 | 7.91-2.14 | ††Kartell 731, lid hole (2 mm) |
| 2-methyl-3-buten-2-ol | Across | 97 | H | 50 | 32.2 ± 20.5 | 9.10 ± 16.1 | ††Kartell 731, lid hole (1 mm) |
| (*S*)-*cis*-Verbenol | Sigma-Aldrich | 95 | H | 1 | 1.53 ± 0.15 | 0.85 ± 1.34 | ††Kartell 731, lid hole (9 mm) |

†Cellulose sponge square 7.5 × 3.5 × 0.25 cm sealed in PE foil thickness 0.1 mm, loaded with 200 μl of compounds, finally sealed in an outer layer made of aluminum/PE foil with the hole with a given diameter in the middle of one side of the dispenser.
††PE vials Kartell (Labware-Italy) size 731 and 730.
†††Compounds synthesized in Unelius laboratory (Ganji et al., 2020).
††††The (+)-pinocamphone contained 29% (+)-isopinocamphone.





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(Military Forests, latitude 49°38′49 "N, longitude 017°33′50" E, 350 m above sea level). It consisted of a 40-year-old Norway spruce forest that has been heavily impacted by a bark beetle outbreak since 2015 (Brázdil et al., 2022). The experiment in Moravia was conducted from 18 May to 3 June 2022. The second location, Kostelec nad Cernými Lesy (Forests CZU; latitude: 49°55′57 "N, longitude: 014°55′13" E, 600 m above sea level), consisted of a 70–90-year-old Norway spruce forest. Traps were placed in a 2-year-old clearing measuring ~200 m × 300 m. The experiment was carried out from 3 June to 28 July 2022. The experiment was designed identically in both locations (Figure 1).

The activity of estragole, 1,8-cineole, (±)-camphor, (−)-carvone, (-)-α-terpineol, (−)-terpinen-4-ol, (+)-pinocamphone, and (+)-isopinocamphone was tested at three different doses, represented by their release rates evaporated/sublimated from the dispenser (nominal 0.1, 1, and 10 mg/day, Table 1). The doses were determined based on the published releases of oxygenated monoterpenes from healthy trees at 24°C, which varied from 0.1 to 7 μg/m²/h of stem surface (Ghimire et al., 2016). When considering a tree stem with a 50 cm diameter and an exposed surface area of ~24 m² (representing 15 m of stem height vulnerable to bark beetle attack), the estimated daily release rate of these oxygenated monoterpenes over a 24-h period would be ~0.5–4 mg/day. We used the pure enantiomers of (−)-carvone, (−)-terpinen-4-ol, (+)-pinocamphone, and (+)-isopinocamphone, which triggered a higher response on the *I. typographus* antennae (Schiebe et al., 2012; Hou et al., 2021; Kandasamy et al., 2023) and were commercially available or synthesized in the laboratory (Ganji et al., 2020). Experimental dispensers were designed in the laboratory, and their exact laboratory and field release rates were established using the gravimetric method and measured six times (Jirošová et al., 2022b) in a laboratory fume hood (temperature 25°C and airflow 0.5 m/s) and in the field under the same weather conditions as the experiments (Table 1).

In the field, the intercept pheromone traps (Ecotrap/Fytofarm, Ltd., Bratislava; Slovakia) were mounted on poles 1.5 m above the ground in rows >30 m from any forest edge. The distance between traps was >15 m (Supplementary Figure 1). In each field location, 32 intercept pheromone traps were baited with dispensers with *I. typographus* pheromone (2-methyl-3-buten-2-ol at 9.1 mg/day and (*S*)-*cis*-verbenol at 0.9 mg/day). In 24 of these traps, an additional dispenser was placed with one of the eight test compounds in one of three doses.

For each compound, one block represented four traps arranged in a row: three traps with different doses in combination with pheromone and one trap with pheromone-only (Control). The position of the tested baits among these four traps was changed four times according to the randomization scheme in a Latin square design (Evans et al., 2020). These four rotations were repeated twice for each compound, resulting in a total of eight collections of catches for each treatment. Insects collected during the field experiment in both localities were preserved in ethanol for further analysis. Predators and parasitoids were sorted by family and identified at the genus level. The identification of Pteromalidae wasps followed the methods described by Peck et al. (1964), Graham (1969), and Bouček and Rasplus (1991). For Staphylinidae (rove beetles), the identification followed the guidelines provided by Arnett and Thomas (2000) and Navarrete and Newton (2003).

TABLE 2 Testing of relative catches of *I. typographus* in the tested compounds in different doses.

| Compound | Model family | AIC | Pr(>Chi) | | *t*-test contrast comparison to pheromone-only | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | Low | | Medium | | High | |
| Estragole | genpois | 521.63 | 0.003476 | ** | 0.4973 | | 0.2991 | | 0.0206 | * |
| 1,8-Cineol | genpois | 557.89 | 8.03E-08 | *** | 0.0003 | *** | 0.0051 | ** | 3.58E-09 | *** |
| (−)-terpinen-4-ol | nbinom2 | 532.27 | 0.2849 | | 0.106 | | 0.667 | | 0.81 | |
| (−)-carvone | nbinom2 | 509.67 | 0.8852 | | 0.869 | | 0.64 | | 0.782 | |
| (±)-camphor | nbinom2 | 537.5 | 0.5298 | | 0.19 | | 0.345 | | 0.221 | |
| (−)-α-terpineol | nbinom2 | 526.26 | 0.8826 | | 0.655 | | 0.903 | | 0.721 | |
| (+)-isopinocamphone | genpois | 550.17 | 0.003014 | ** | 0.0785 | . | 0.00079 | *** | 0.003142 | ** |
| (+)-pinocamphone | genpois | 521.03 | 3.32E-07 | *** | 0.924 | | 1.3E-06 | *** | 0.483 | |

Asterisks mark a significant difference (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$) of the dose from pheromone alone.

## 2.2 Statistical analysis

For the evaluation of the effects of each individual compound, a separate regression model was fitted with the relative number of *I. typographus* as the dependent variable. The relative number was expressed as the number of insects of a single taxon captured by a treatment within a block divided by the total catches by the block for a single catch collection. Due to the experimental design, we utilized a mixed-effects model approach (Zuur et al., 2009). The random part of the model was, in all cases, trap-nested in the locality. During model building and validation, an appropriate distribution function was selected by minimizing the Akaike information criterion (AIC), and the significance of the model was tested by the likelihood ratio test ($\chi^2$). Between the best models, only two distribution functions were selected (Table 2): generalized poison distribution (Joe and Zhu, 2005) and negative binomial distribution in quadratic parameterization, according to Hardin and Hilbe (2007). We used a *t*-test to compare the response to each compound dose against the pheromone-only control. The model formulation was performed in R version 4.3.1 (R Core Team, 2023) in the package *glmmTMB* following the procedures described by Brooks et al. (2017).

# 3 Results

## 3.1 *Ips typographus* response to tested compounds in combination with aggregation pheromone

During field experiments, a total of 39,650 *I. typographus* adults were caught. The number of adults captured in Kostelec ($N$ = 28,931) was 2.7 times higher than in Moravia ($N$ = 10,719). However, the pattern of catches for the tested compounds was almost the same. Compounds with significantly different catches in the treatments: estragole ($\chi^2$; $p < 0.01$), 1,8-cineole ($\chi^2$; $p < 0.001$), (+)-pinocamphone ($\chi^2$; $p < 0.01$), and (+)-isopinocamphone ($\chi^2$; $p < 0.01$; Table 1, Figure 2). For 1,8-cineol, all three doses in combination with pheromones resulted in significantly fewer beetles caught than the pheromone-only control, with stronger effects observed for the high dose (*t*-test; $p < 0.001$, $p < 0.01$, $p < 0.001$; Table 2, Figure 2). For estragole, the inhibitory effect was significant only for the high dose (*t*-test; $p < 0.05$), while for pinocamphone, it was observed at the medium dose (*t*-test; $p < 0.001$). However, regarding the medium dose of pinocamphone, there were different catch rates in Libava and Kostelec (Figure 2), suggesting a potential problem with the dispenser used in Kostelec. Conversely, isopinocamphone resulted in statistically higher catch rates at both high and medium doses (*t*-test; $p < 0.01$ and $p < 0.001$, respectively) compared to the pheromone-only treatment (Figure 2). A low dose of isopinocamphone showed the same nearly significant trend (*t*-test; $p = 0.08$; Table 2). Catches of beetles of remaining tested compounds in combination with pheromone, including (±)-camphor, (–)-carvone, (-)-α-terpineol, (-)-terpinen-4-ol, and pheromone-only, did not exhibit significant differences (Table 2). Additionally, there were no significant differences in catches between the individual doses and the pheromone-only control group for any of these compounds (Supplementary Figure 2).

For each compound, a separate model (GLMM) was created with the formula: *Relative count of I. typographus* ~ *Compound dose* + *(1|Locality)*. The appropriate distribution function (model family: genpois—Generalized poison; nbinom2—Negative binomial), Akaike information criterion (AIC), and significance test ($\chi^2$) are stated for each compound. The results of the t-test contrast comparison given for each compound and dose combination against pheromone-alone (control). Asterisks mark a significant difference ($p < 0.05$ *; $p < 0.01$ **; $p < 0.001$ ***) of the dose from pheromone alone.

## 3.2 Predatory insect response to tested compounds in combination with aggregation pheromone

The catch of natural enemies was three times higher in Kostelec compared to Moravia, which corresponds to a higher number of bark beetle catches in Kostelec. Four families of natural enemies of bark beetles were identified with a prevalence of Pteromalidae and Staphylinidae (Supplementary Tables 1, 2). In Kostelec, there were 93 specimens of parasitoid wasps belonging to the genus *Rhopalicus* sp. (Hymenoptera: Pteromalidae), while in Moravia, there were only 13 specimens. Additionally, in Kostelec, 19 specimens of rove beetles (Coleoptera: Staphylinidae) were caught, and in Moravia, 23 specimens (Table 3).

The ratio between these two groups appeared different between the two locations, with Kostelec having a higher proportion of Pteromalid wasps compared to Staphylinids, while in Moravia, we observed the opposite trend. There were a few catches recorded for *Medetera* sp. (Diptera: Dolichopodidae), with five specimens in Kostelec and three specimens in Moravia. There were four catches of *Lonchaea* sp. (Diptera: Lonchaeidae), three in Kostelec, and one in Moravia. For *Thanasimus* sp. (Coleoptera: Cleridae), there were two catches in Kostelec and three catches in Moravia.

The catches of *Rhopalicus* sp. wasps did not show significant differences between compounds and their doses. The number of caught specimens in Moravia and Kostelec is listed in the Supplementary Tables 1, 2.

# 4 Discussion

## 4.1 Response of *Ips typographus* to tested compounds

The variability in total catches of *I. typographus* and its predators, as well as their varying distributions across different treatments in the experimental locations of Kostelec and Moravia, could be attributed to the unique weather conditions experienced during each field experiment, as described in Supplementary Figure 3. Furthermore, this variation may have been influenced by the season impacting the flight activity of the beetles. The variation between catches in blocks of traps may have been due to different locations of blocks within the clearing, in terms of their distance to the forest edge and wind speed and

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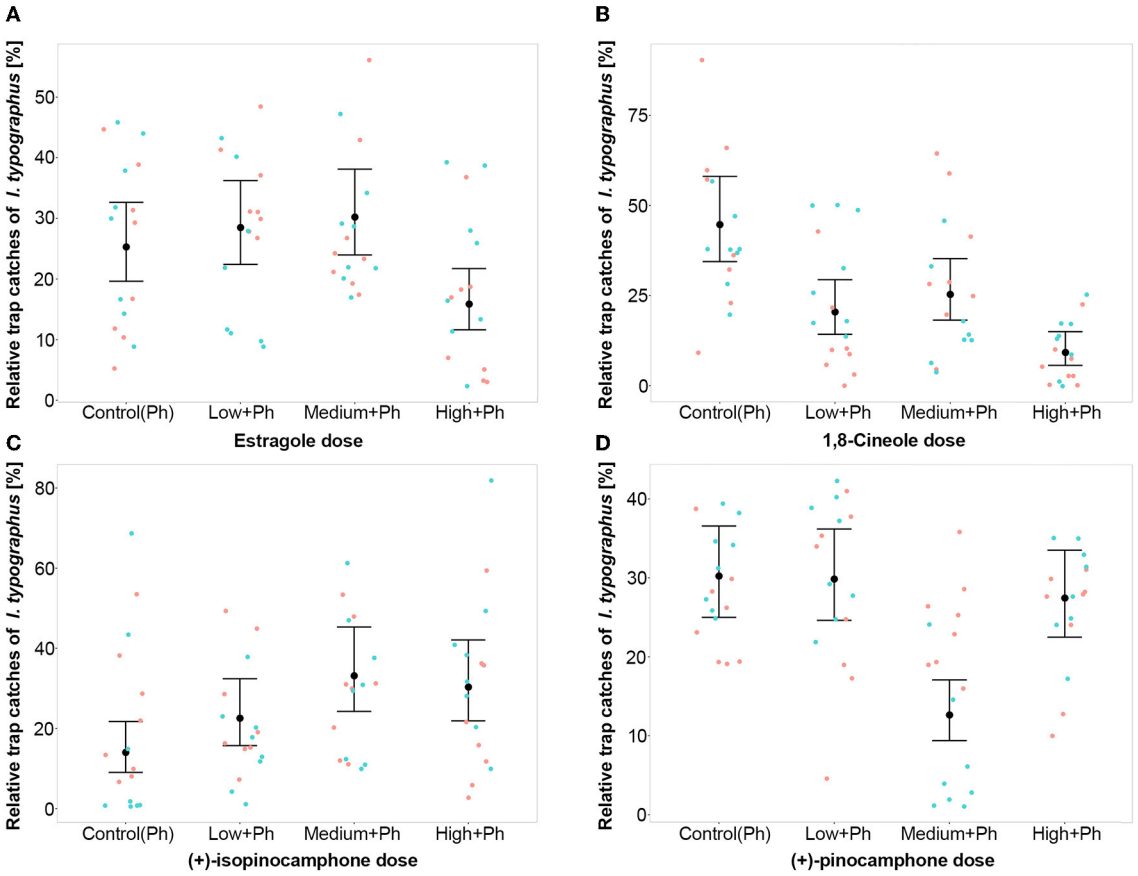


FIGURE 2
Relative number of *I. typographus* captured in traps baited with pheromone attractant (Ph) alone or attractant with host volatiles **(A)** estragole, **(B)** 1,8-cineole, **(C)** (+)-pinocamphone, and **(D)** (+)-isopinocamphone in a low-medium, high-release dose combined with pheromone (Ph) and control pheromone-only. The bigger black dot in the middle of the vertical line is a posterior mean value and whiskers. Error bars represent the 95% confidence interval, and colored points represent the raw catches. The smaller dots are original values: blue Kostelec and red Libavá.

direction. The trap catches could also have been influenced by different ages of spruce stands near the experiments.

In many cases, the beetles exhibited weak differences in response to the tested compounds in combination with the pheromone compared to the pheromone alone, and the observed effects were only marginally significant. However, there was a noticeable anti-attractive effect observed with 1,8-cineole, a compound for which similar findings and similar trends have been reported previously (Andersson et al., 2010; Binyameen et al., 2014; Jirošová et al., 2022b).

New findings were the anti-attractive effect of a high dose of estragole and the effect of (+)-isopinocamphone on enhancing the attractive activity of the pheromone for *I. typographus*. There was also a less clear inhibition effect of a medium dose of pinocamphone, probably caused by a defective test dispenser for a medium dose at Kostelec. In contrast, when α-terpineol, camphor, carvone, or terpinen-4-ol were added to the pheromone, they did not alter its attractiveness, despite these compounds eliciting strong responses from *I. typographus* antennae (Kalinová et al., 2014; Schiebe et al., 2019).

The ecological role of estragole, 1,8-cineole, and isopinocamphone in the interaction between *I. typographus* and Norway spruce trees was investigated by Schiebe (2012, 2019). The amount of these compounds, along with other oxygenated monoterpenes found in spruce, increased in felled trees and in standing trees after the application of the plant hormone analogue methyl jasmonate. The quantity of these compounds was negatively correlated with the density of bark beetle attacks when the beetles infested the felled trees, and the standing trees that exhibited a higher induction of these compounds were able to survive a natural bark beetle infestation.

The effect of estragole has been tested on several bark beetle species (Coleoptera: Curculionidae: Scolytinae), including *Dendroctonus brevicomis* LeConte (Hayes and Strom, 1994), *Ips pini* (Say), *Ips latidens* (Leconte) (Joseph et al., 2001), and *Tomicus piniperda* (Curculionidae: Scolytinae) (Haack et al., 2004). Its inclusion in their host odor blend resulted in reduced trap catches. Estragole was also reported to interrupt the responses of bark beetle species *Dedroctonus simplex* (Le Conte) and *D. ruffipenis* (Kirby) to their attractive pheromone components (Werner, 1995).

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TABLE 3 Natural enemies of bark beetles (Staphylinidae and Pteromalidae) captured in synthetic host tree compounds: estragole, 1,8 cineole, camphor, carvone, alpha-terpineol, terpinene-4-ol, (+)-pinocamphone, and (+)-isopinocamphone, in combination with pheromone and pheromone-only controls.

| Doses/location | Estragole+Ph | 1,8-cineole+Ph | a-terpineol+Ph | Camphor+Ph | Carvone +Ph | terpinen-4-ol+Ph | (+)-isopinocamphone +Ph | (+)-pinocamphone +Ph | Pheromone-only Ph |
|---|---|---|---|---|---|---|---|---|---|
| *Doses/Moravia* **Pteromalidae** | *Rhopalicus* | *Rhopalicus* (*N* = 4) | *Rhopalicus* (*N* = 2) | *Rhopalicus* | 0 | *Rhopalicus* | *Rhopalicus* (*N* = 3) | 0 | *Rhopalicus* |
| *Doses/Moravia* **Staphylinidae** | *Gyrohypnus* (*N* = 2) | *Nudobius* (*N* = 4) | *Gambrinus* | *Bisnius Nudobius Anotylus* | *Bisnius* (*N* = 2) *Anotylus* | *Nudobius Gabrius* | *Nudobius* | *Bisnius; Gyrohypnus* | *Nudobius* (*N* = 2); *Gyrohypnus Anotylus Rugilus* |
| *Doses/Kostelec* **Pteromalidae** | *Rhopalicus* (*N* = 16) | *Rhopalicus* (*N* = 9) | *Rhopalicus* (*N* = 8) | *Rhopalicus* (*N* = 2) | *Rhopalicus* (*N* = 10) | *Rhopalicus* (*N* = 5) | *Rhopalicus* (*N* = 14) | *Rhopalicus* (*N* = 7) | *Rhopalicus* (*N* = 22) |
| *Doses/Kostelec* **Staphylinidae** | *Nudobius Anotylus Gyrohypnus* | *Nudobius* (*N* = 2); *Stenus Gyrohypnus Heterothops* | *Rugilus Quedius* | *Aleochara Nudobius Gyrohypnus* | *Bisnius Nudobius* | *Aleochara* | *Nudobius* | *Nudobius* | *Anotylus* |

Doses: The sum of total captures in all three tested release rates of compounds or pheromone-only controls summed together for both localities, Kostelec and Moravia.

However, recent research has revealed that the addition of estragole increased catches of both *Dendroctonus frontalis* (Zimmermann) and *D. terebrans* (Olivier) on their pheromone lures (Munro et al., 2020). Based on these findings, we suggest that this semiochemical has variable ecological roles for these different species.

In our study, (+)-isopinocamphone caused a synergistic increase in beetle catches when added to the pheromone. This observation, combined with the fact that bark beetles possess specialized sensilla on their antennae (Hou et al., 2021) to detect it, suggests the potential role of (+)-isopinocamphone in the selection of host trees. Kandasamy et al. (2023) tested in a short-range two-choice test in a Petri dish synthetically prepared (+)-isopinocamphone added as a solution in mineral oil to spruce bark agar, which did not exhibit significant attractivity for *I. typographus* bark beetles in tested doses. This further indicates that the effect of (+)-isopinocamphone we see in trap catches may be a long-range attraction (in accordance with the known long-range attraction of *cis*-verbenol; Schlyter and Birgersson, 1999).

## 4.2 Response of bark beetle insect natural enemies to tested compounds

The anticipated captures of the common predatory beetle *Thanasimus* sp. were relatively low. This could be attributed to the fact that our pheromone bait only contained the two major pheromone components, 2-methyl-3-buten-2-ol and (*S*)-*cis*-verbenol, and not ipsdienol, an *I. typographus* pheromone component emitted in smaller amounts in the later attack states (Birgersson et al., 1984; Hulcr et al., 2006). Furthermore, we observed only a few captures of *Medetera* sp. and *Lonchaea* sp. flies, likely due to the use of a trap optimized for Coleoptera that lacked sticky surfaces.

Although there is limited information on Staphylinidae predators using host tree volatiles for locating bark beetles (Wegensteiner et al., 2015), it has been reported that they are attracted to pheromone traps used for monitoring *Ips typographus* (El-Sayed, 2023). Additionally, commercial pheromone traps tested in combination with host tree logs (*P. abies*) caught ∼38% more predatory Staphylinidae than traps without logs, in comparison to a 32% increase in catches of *Thanasimus formicarius* (L.) (Zumr, 1983). Hence, host tree compounds may mediate staphylinid prey location.

The pteromalid parasitoid wasp *Rhopalicus* sp. was the most abundant among the captured bark beetle natural enemies, but there were no significant preferences for any of the tested compounds due to the low number of caught specimens. In the literature, an electroantennographic study of bark beetle gallery smell was tested on the antennae of *Rhopalicus tutela* (Walker) females. The antennae showed sensitivity to oxygenated monoterpenes and estragole (Pettersson, 2001). Additionally, the olfactory response to estragole was reported in other species of parasitoid wasps, *Spathius pallidus* Ashmead, 1893,

and *Roptrocerus xylophagorum* (Hymenoptera: Pteromalidae; Sullivan et al., 1997).

## 5 Conclusion

The effect of 1,8-cineole, estragole, and (+)-isopinocamphone, as observed in our field experiments, provides evidence that these oxygenated monoterpenes and estragole can exhibit biological activity for *I. typographus* and their natural enemies when combined with *I. typographus* aggregation pheromone. This suggests that their long-range activity is not solely dependent on a complex mixture, such as that emitted by symbiotic fungi inoculated on wood substrates (Kandasamy et al., 2023).

The discovery of new attraction inhibitors or adjuvants for attractants can be applied to the development of integrated pest management methods for controlling *I. typographus*. Anti-attractants, a term broadly used for attraction inhibitors, have already been tested to deter various pest bark beetles, such as *Dendroctonus ponderosae*, *D. rufipennis*, *D. pseudotsugae*, *Ips pini*, and *Dryocoetes confusus*, from attraction to their pheromone or to the host tree (Schlyter, 2012). These anti-attractants can originate from host trees, non-host trees, associated microorganisms, or the beetles themselves (Borden et al., 2000; Munro et al., 2020).

In the protection of Norway spruce trees against *I. typographus*, verbenone, a well-established repellent for bark beetles, was tested with varying success (Jakuš et al., 2003; Frühbrodt et al., 2023). In nature, verbenone signals an old and over-exploited host. The synergistic blend effect of verbenone mixed with green leaf volatiles (C6 alcohols) and C8 alcohols (3-octanol and 1-octen-3-ol) and the angiosperm and fungal spiroacetal conophthorin (Zhang and Schlyter, 2004) was also evaluated for tree protection against *I. typographus* (Schiebe et al., 2011), resulting in a reduction of tree killing ranging from 35 to 76% in protected areas.

The recently tested anti-attractant mixture also includes, besides the 3-octanol, 1-octen-3-ol, hexanol and conophthorin, 1,8-cineole and *trans*-4-thujanol from spruce and excludes verbenone (Jirošová et al., 2022a). Anti-attractant blends offer partial protection for standing trees but are ineffective for windfallen trees. Adding new anti-attractants, e.g., geranyl acetone (Lindmark et al., 2023), to the mixture may enhance tree protection effects.

A comprehensive approach to semiochemical tree protection against *I. typographus* attacks could employ the push-pull strategy. Trees are protected by anti-attractants, and repelled beetles are caught in pheromone traps baited with attractive *I. typographus* pheromones. Both the push and pull might be enhanced by the addition of new semiochemicals (Jakuš et al., 2022; Deganutti et al., 2023). The addition of (+)-isopinocamphone to the trapping lure could increase beetle attraction away from trees while simultaneously protecting them from an estragole-enhanced beetle repellant.

## Data availability statement

The original contributions presented in the study are included in the article/Supplementary material, further inquiries can be directed to the corresponding author.

## Ethics statement

Ethical approval was not required for the study involving animals in accordance with the local legislation and institutional requirements because Ethical review and approval were not required for the study on animals in accordance with the local legislation and institutional requirements. We have performed all beetle experiments that comply with the ARRIVE guidelines and were carried out in accordance with (Scientific Procedures) Act, 1986 and associated guidelines, EU Directive 2010/63/EU for animal experiments.

## Author contributions

AACM: Data curation, Formal analysis, Investigation, Validation, Writing—original draft. RJ: Data curation, Investigation, Writing—review & editing. RM: Formal analysis, Writing—review & editing. CRU: Methodology, Writing—review & editing. FS: Conceptualization, Methodology, Supervision, Validation, Writing—review & editing. AJ: Conceptualization, Formal analysis, Investigation, Methodology, Supervision, Validation, Visualization, Writing—original draft, Writing—review & editing.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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## Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/ffgc.2023.1292581/full#supplementary-material

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RESEARCH PAPER

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# Size-dependent behavioral and antennal responses to doses of (+)-isopinocamphone and 1,8-cineole mixed with pheromone: a potential host selection strategy in female *Ips typographus* L

Antonioni Acacio Campos Moliterno[1†], Mayuri Kashinath Shewale[1†], Sara Basile[1], Jiří Synek[1] and Anna Jirošová[1*]

## Abstract

***Key message*** This study revealed differing behavioral and antennal responses between large and small female Ips typographus to two bioactive oxygenated monoterpenoids, ( +)-isopinocamphone and 1,8-cineole, which serve contrasting ecological roles as aggregation pheromone synergist and inhibitor. Larger females were more attracted to ( +)-isopinocamphone and had larger antennal clubs leading to enhanced antennal sensitivity, potentially improving their ability to select suitable host trees. In contrast, smaller females were less repelled by 1,8-cineole but had higher antennal sensitivity despite smaller antennae. This discrepancy can be explained by behavioral decisions made after downstream olfactory signal processing in the central nervous system (CNS) and by the co-localization of 1,8-cineole with pheromone-sensitive neurons. Ecologically, small females may avoid competition with larger females by selecting less suitable trees. In conclusion, females' body size influences olfactory-driven responseto potential host selection decisive volatiles, which can impact reproductive success and bark beetle population dynamics.

**Context** *Ips typographus*, a major pest of Norway spruce (*Picea abies* L.) in Europe, is experiencing more frequent outbreaks due to climate change. These outbreaks involve shifts in population dynamics and phenotypic traits, influencing beetle responses to olfactory cues from stressed host trees.

**Aims** The study examines the size-dependent behavioral and antennal responses of female *I. typographus* to two host selection–deciding volatiles with contrasting ecological roles: 1,8-cineole, which inhibits attraction to unsuitable trees, and (+)-isopinocamphone, a pheromone synergist. Size-linked morphological and olfactory adaptations may influence females' ability to select suitable host trees for reproduction.

**Methods** In field trap experiments conducted in 2019 and 2022, the body size of *I. typographus* females caught in response to different doses of (+)-isopinocamphone or 1,8-cineole in combination with pheromone

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†Antonioni Acacio Campos Moliterno and Mayuri Kashinath Shewale contributed equally to this work.

*Correspondence:
Anna Jirošová
jirosovaa@fld.czu.cz

was compared. Female *Ips typographus* were sorted based on body length. In females from the laboratory, the size of the antennal club was measured, and size-dependent antennal responses to these volatiles were analyzed using electroantennography.

**Results** Larger females were more attracted to (+)-isopinocamphone in combination with pheromone in the field, showed stronger antennal response, and had larger antennal clubs than smaller females proportionally to their body size. In contrast, smaller females were less repelled by 1,8-cineole added to pheromone, but, in contradiction, antennally responded to it more strongly than larger females despite having smaller antennal clubs.

**Conclusion** Body length significantly influenced semiochemical detection in *I. typographus* females. (+)-Isopinocamphone was detected more effectively by larger females, implying an advantage in the selection of suitable host trees. In contrast, the discrepancy between behavioral and antennal responses to 1,8-cineole in smaller females suggests involvement of not only peripheral responses but also central nervous processing of olfactory signals that influence behavior. This adaptation may enable smaller females to reduce competition with large ones by selecting less suitable trees. These findings provide new insights into the ecological relationship between beetle phenotype and olfactory cues, with implications for tree–bark beetle interactions.

**Keywords** Bark beetle, Olfaction, Pheromone, Oxygenated spruce monoterpenoids, Phenotypic variation, Host choice

## 1 Introduction

The Eurasian spruce bark beetle, *Ips typographus* L. 1758 (Coleoptera: Curculionidae), is a major pest associated with Norway spruce (*Picea abies* L.) in Europe (Hlásny et al. 2021; Powell et al. 2021). Outbreaks of this species have intensified in frequency and severity, mainly due to climate change, and are facilitated by its complex and sophisticated chemical communication system (Biedermann et al. 2019). Male *I. typographus* play a pivotal role in locating weakened or stressed spruce trees using a combination of visual and chemical cues across both long and short distances (Birgersson and Bergström 1989; Netherer et al. 2021; Lehmanski et al. 2023). After initiating attack by boring into the bark (Wermelinger 2004), males produce aggregation pheromone (Birgersson et al. 1984; Ramakrishnan et al. 2022) to attract conspecifics for coordinating mass attacks to colonize the host tree and overcome tree defenses (Franceschi et al. 2005; Raffa et al. 2016). The success of this colonization process is highly influenced by the host tree's organic compounds.

Norway spruce releases a range of monoterpenoids, including highly abundant compounds such as α-pinene, β-pinene, β-phellandrene, and limonene (Netherer et al. 2021). These compounds have been shown to enhance the attraction of *I. typographus* (Erbilgin et al. 2007). In addition to these dominant volatiles, many studies have identified several low-abundance compounds emitted by spruce, comprising approximately 1% of the total volatile profile. While present in low amounts, these compounds elicit strong antennal responses in beetles (Kalinová et al. 2014; Schiebe et al. 2019), highlighting their ecological significance in tri-trophic interactions with beetles, its symbiotic microbiota, and the host tree (Netherer et al. 2021). Most of these minor yet biologically active volatiles are oxygenated spruce monoterpenoids, with a few exceptions such as estragole and styrene, which have phenolic character. Oxygenated monoterpenoids are produced within the spruce–bark beetle–symbiotic microorganism niche through multiple mechanisms. They can be formed via the oxidation of major spruce monoterpene hydrocarbons, either by autooxidation or enzymatically by the spruce microbiome. This transformation becomes especially prominent when trees experience stress, such as after being cut, windthrown, or infested by bark beetles (Netherer et al., 2021); Schiebe et al. 2019). Under these conditions, the levels of compounds such as isopinocamphone, camphor, pinocarvone, terpinen-4-ol, and terpineol significantly increase. However, they remain minor components of spruce volatile profile compared to the main terpenic hydrocarbons.

Additionally, monoterpene hydrocarbons can be hydroxylated (introducing oxygen to molecules) by the beetles' enzymatic detoxification systems. For example, α-pinene can be converted into myrtenol or *cis*-verbenol (Blomquist and Vogt 2021), while limonene may be transformed into carvone (Duetz et al. 2001). Over evolutionary time, several of these oxidation products have been co-opted by bark beetles as pheromonal compounds, e.g., *cis*-verbenol in *I. typographus* (Francke et al., 1983). Moreover, the beetle-associated intestinal microbiome also plays a key role in modifying host volatiles. It contributes not only to the oxidation of tree hydrocarbons but also to the further oxidation of *cis*-verbenol into verbenone, a potent bark beetle anti-aggregation signal

(Frühbrodt et al., 2023). In parallel, beetle-exosymbiotic ophiostomatoid fungi, which are inoculated into trees by boring beetles during colonization, also metabolize monoterpenoids to their oxidative forms (Kandasamy et al. 2023). On the other hand, some oxygenated monoterpenoids, namely 1,8-cineole, are directly de novo formed through the cyclization of oxygenated intermediates within the spruce tree enzymatic system and not by oxidation of hydrocarbon precursors (Celedon and Bohlmann 2019).

Like many insects, bark beetles depend on highly specialized olfactory systems located in their antennae to navigate and interact with their environment (Hansson and Stensmyr 2011). In *I. typographus*, olfactory sensory neurons (OSNs) are housed within hair-like sensilla on the antennal surface. These neurons enable precise discrimination among a wide array of odor cues, including aggregation pheromone, host- and nonhost-derived tree volatiles, and volatiles produced by symbiotic microorganisms (Andersson et al. 2009). OSNs differ in their specificity. Some range from highly selective specialists detecting specific pheromones (Wojtasek et al. 1998) while some are broadly tuned generalists responsive to diverse environmental cues like host volatiles (Andersson et al. 2010; Binyameen et al. 2014). This specificity is determined by odorant receptors (ORs) located on their dendrites (Carey et al. 2010; Hallem and Carlson 2006). Upon odor detection, signals are transmitted to the antennal lobes (ALs), where glomeruli integrate input (Vosshall et al. 2000), and projection neurons relay this information to the mushroom bodies, which are involved in learning and memory, and the lateral horn, associated with innate behavioral responses (Galizia 2014; Clark and Ray 2016). This finely tuned chemosensory system plays a critical role in mediating behaviors such as host location, mate finding, and avoidance of unsuitable environments (Andersson et al. 2009; Zhang and Schlyter 2004).

Functional mapping of these neurons in *I. typographus* has identified specific OSN classes that respond to oxygenated spruce monoterpenoids, including 1,8-cineole and (+)-isopinocamphone ((+)-IPC) (Andersson 2012; Kandasamy et al. 2023). Interestingly, OSNs activated by 1,8-cineole are consistently co-localized within the same sensilla as those tuned to the pheromonal component *cis*-verbenol (Andersson et al. 2009, 2010). This arrangement of OSNs enables peripheral-level signal integration, where exposure to high concentrations of 1,8-cineole suppresses the neural response to *cis*-verbenol (Andersson et al. 2009; Binyameen et al. 2014). In contrast, OSNs responsive to (+)-isopinocamphone in *I. typographus* are individually localized and have not been observed in co-localization with neurons detecting other compounds. The specificity of this response is attributed to the olfactory receptor ItypOR29, located on the OSN membrane, which binds selectively to (+)-isopinocamphone, as confirmed through receptor expression and functional characterization in *I. typographus* (Hou et al. 2021).

Behavioral studies further support the ecological relevance of these olfactory interactions of oxygenated spruce monoterpenoids. Field experiments have shown that 1,8-cineole, when added to pheromone blends containing *cis*-verbenol, inhibits beetle attraction in a clear dose-dependent manner (Andersson et al. 2010; Jirošová et al. 2022). Moreover, studies on the functional role of neuronal co-localization, where one neuron within the same sensillum responds to an attractant and another to an inhibitor, have demonstrated that 1,8-cineole induces more precise spatial avoidance of beetles from the pheromone source than verbenone. Verbenone is a known anti-attractant (Frühbrodt et al., 2023), yet its corresponding neuron has never been found to be co-localized with those for pheromonal compounds (Binyameen et al. 2014). Interestingly, a significantly higher content of 1,8-cineole has been found in spruce trees that are less susceptible to bark beetle attacks or that survived infestations more successfully (Schiebe et al. 2012). Additionally, preliminary feeding studies further indicate that 1,8-cineole exhibits greater toxicity to female *I. typographus* than to males (Zaman et al. 2024). This suggests that 1,8-cineole could serve as a potential chemical marker of bark beetle-resistant trees. In contrast to the inhibitory effects of 1,8-cineole, field studies showed that (+)-isopinocamphone significantly enhanced *I. typographus* captures at pheromone-baited traps and acted as a synergist with pheromone activity (Moliterno et al. 2023). Among the several tested compounds including estragole, 1,8-cineole, (±)-camphor, (−)-carvone, α-terpineol, (−)-terpinen-4-ol, (+)-pinocamphone, and (+)-isopinocamphone, each evaluated at three different doses; (+)-isopinocamphone was the only one to exhibit this relatively rare synergistic effect with pheromone. Additionally, (+)-isopinocamphone was also identified as a substantial component of the volatile bouquet produced by *Grosmannia penicillata*, *Leptographium europhioides*, and *Ophiostoma bicolor* which are beetle-associated fungi when cultured on spruce phloem media. This fungal volatile blend was shown to attract beetles in short-range Petri-dish bioassays (Kandasamy et al. 2023).

Variations in abiotic and biotic factors significantly influence tree physiology, which directly affects host suitability and selection by bark beetles (Netherer et al. 2024). During endemic population stages, beetles prefer high-quality trees with low competition, conditions that favor offspring growth and fitness. However, during epidemic outbreaks, beetles are often forced to colonize suboptimal hosts, leading to reduced offspring

vigor, including smaller body size (Foelker and Hofstetter 2014; Sallé and Raffa 2007). This decline in body size has cascading effects, as it can negatively influence pheromone production (Anderbrant et al. 1985; Pureswaran and Borden 2003), ultimately reducing mating success (Dacquin et al. 2024). The reproductive biology of *I. typographus* is closely linked to chemical signals. Males produce pheromone that serve not only for aggregation but also function partially as sexual attractants. As polygynous species, males typically mate with up to four females, increasing their mating success and overall fecundity (Schebeck et al. 2023). Female beetles are central to reproductive success, as they are responsible for gallery construction and oviposition. Consequently, females play a selective role in reproduction, evaluating both mate quality and host tree suitability to optimize offspring survival and fitness (Schlyter and Zhang 1996; Paynter et al. 1990), relying on signals from both male-produced pheromones and tree-emitted volatile cues. The precision of pheromone-based recognition is well-documented at the interspecific level among *Ips* beetles, suggesting strong selective pressures on olfactory systems (Schlyter et al. 2015). Variations in female total body length, combined with pheromonal and host tree chemical cues, are crucial for understanding ecological adaptations, such as host selection strategies (Müller et al., 2020); Schlyter and Anderbrant 1993).

This study explores size-specific behavior in female *I. typographus*, with a focus on their olfactory assessment of host tree quality, which is a critical factor for survival and reproductive success. We examine whether large and small females respond differently to two oxygenated spruce monoterpenoids, 1,8-cineole and (+)-isopinocamphone, tested in combination with aggregation pheromones in a field trapping experiment. Additionally, we investigate if their antennae exhibit size-dependent differences in sensitivity to these compounds using electroantennographic responses and whether the antennal club parameters differ between large and small females. Building on these objectives and the current understanding of bark beetle chemical ecology, this study is guided by the core hypothesis: "Larger and smaller *I. typographus* females will exhibit distinct behavioral and electrophysiological responses to the two oxygenated spruce monoterpenoids with contrasting ecological roles. Particularly, 1,8-cineole inhibits attraction to unsuitable trees, and (+)-isopinocamphone enhances the attraction to aggregation pheromones. These behavioral differences could be caused by size-dependent variations in antennal sensitivity for these two compounds, which is potentially influenced by differences in the antennal club parameters."

The ecological impact of these phenotypic variation-dependent differences, driven by morphological and olfactory adaptations, may affect *I. typographus* females' ability to select high-quality host trees. This, in turn, could have broader implications for the beetles' reproductive strategies and, consequently, their population dynamics.

## 2 Material and methods

### 2.1 Experimental approach

We evaluated the responses of *I. typographus* females using two complementary assays. The first was a field assay involving traps baited with either 1,8-cineole or (+)-isopinocamphone at three different doses in combination with a pheromone, and we compared the sex ratio and body length of beetle captures to those from traps baited with pheromone alone. The second assay included electroantennography (EAG) responses to measure the antennal responses of small and large females to varying doses of 1,8-cineole or (+)-isopinocamphone. We also conducted a morphometric analysis of antennal club size in these two groups.

### 2.2 Field experiment area and pheromone traps

The trapping experiments were conducted in 2019 and 2022 at the Forest CZU property in Kostelec nad Černými lesy, Czech Republic. The experiments took place in a mature, 100-year-old Norway spruce forest, a natural habitat for *I. typographus*, located at 600 m above sea level. In 2019, the experiment was conducted at coordinates (49°56′02″N, 14°52′21″E), while the 2022 experiment took place at (49°55′57″N, 14°55′13″E). Both experiments were conducted during the same time frame: June 3 to July 28 of each year. In both the 2019 and 2022 experiments, traps were set up approximately 30 m from the forest edge in a 2-year-old clearing. The average temperature was 2019 16 °C (June), 18 °C (July), and 2022 17 °C (June), 19 °C (July). They were arranged in a row, with a minimum distance of 15 m between each trap, and were installed on wooden poles 1.5 m above the ground.

In 2019, seven cross-vane Ecotraps (Fytofarm, Slovak Republic) were used for collecting data for this experiment: three traps were baited with three different doses (low, medium, high) of 1,8-cineole or (+)-isopinocamphone, respectively, in combination with the synthetic pheromone. One trap was baited with pheromone alone and served as a control (for bait composition, see Table 1 for details). To minimize positional bias, the positions of the tested baits among these seven traps were changed seven times according to a randomization scheme based on a Latin square design (Evans et al. 2020).

In 2022, for each compound (1,8-cineole and (+)-isopinocamphone), one block was set up, consisting of four

**Table 1** Description of treatment bait characteristics used in the experiments conducted in 2019 and 2022

| Chemical | Source | Purity (%) | Dose | Nominal | Field 2019 mg/day ± SEM ($n=3$)† | Field 2022 mg/day ± SEM ($n=3$)† | Dispenser design |
|---|---|---|---|---|---|---|---|
| **1,8-Cineole** | Sigma-Aldrich | 98 | L | 0.1 | 0.1 ± 0.04 | 0.1 ± 0.01 | Kartell 731 without hole plus 1 mL of paraffin oil |
| | | | M | 1 | 0.84 ± 0.09 | 0.92 ± 0.12 | Glass vial of 2 mL, lid hole by syringe (1 mm) |
| | | | H | 10 | 6.03 ± 4.78 | 5.7 ± 6.7 | Kartell 730, lid hole by syringe (2 mm) |
| **(+)-Isopinocamphone** | * | 99 | L | 0.1 | 0.61 ± 0.18 | 0.40 ± 0.11 | Foil sachet: hole by syringe (1 mm) |
| | | | M | 1 | 1.95 ± 0.41 | 1.87 ± 0.67 | Kartell 730 without hole |
| | | | H | 10 | 7.82 ± 1.63 | 8.21 ± 1.41 | Kartell 731: 2 mm lid hole |
| **2-Methyl-3-buten-2-ol** | Across | 97 | H | 10 | 11.31 ± 8.9 | 9.10 ± 16.1 | PE-vial (Kartell 731): 1 mm lid hole |
| ***Cis*-verbenol** | Sigma-Aldrich | 95 | H | 1 | 0.93 ± 1.17 | 0.85 ± 1.34 | PE-vial (Kartell 731): 9 mm lid hole |

Doses are represented by low dose (L), medium dose (M), and high dose (H). For further details, see Moliterno et al. (2023 )

*Synthesized compound by Dr. Prof. Unelius from Linnaeus University, Sweden. †Established by gravimetric analysis. SEM indicates standard error mean

traps arranged in a row: three traps baited with different doses of the tested compounds in combination with pheromone and one trap with pheromone only (control). The positions of the tested baits among these four traps were changed four times according to a randomization scheme based on a Latin square design (Evans et al. 2020). These four rotations were repeated twice for each compound, resulting in a total of eight collections of catches for each treatment (Moliterno et al. 2023). Insects collected during the field experiment in both localities were preserved in ethanol for further analysis, including counting, sex separation, and measurement.

### 2.3 Source and selection of beetles used for body length and antennal size measurement and electroantennographic responses

For further measurement, 50 beetles were randomly selected from the ethanol-stored beetles caught in one of three doses of 1,8-cineole or (+)-isopinocamphone combined with pheromone, or caught with pheromone alone (a total of 8 groups each consisting of 50 randomly selected beetles). These beetles were selected from each replication of the experiments conducted in 2019 and 2022. Selected beetles were dried on tissue paper at 25 °C for 2 h, sorted by sex, and measured for body length. Damaged specimens were excluded from the analysis.

For antennal club size measurements and electroantennography studies, *I. typographus* (F0 generation) emerged from naturally infested Norway spruce logs ($n=12$; ± 50 × 28 cm) collected in Kostelec nad Černými Lesy from June to July 2024 were used. The beetles were reared by placing naturally infested Norway spruce logs into fine mesh emergence cages under controlled laboratory conditions. The logs were monitored daily, and newly emerged adult beetles were collected manually from the mesh enclosures. Only females, ± 3 days old, were selected after sorting by sex for further measurements and experiments.

### 2.4 Morphometric analysis

The total body length of adult female *I. typographus* collected from field traps was measured in millimeters from the apical margin of the pronotum to the distal end of the elytra, using traditional linear morphometric analysis. The body size of the captured females was measured using a graticule (1–10 mm) integrated into a Nikon SMZ800N stereomicroscope at 30 × magnification. Measurements were taken by the same researcher to ensure consistency, with recorded sizes ranging from 4.2 to 5.3 mm. Based on this size range, individuals were classified into two distinct size categories that were established for further analysis. Two groups of female specimens selected for antennal club measurements and electroantennography were divided into:

Large-sized females: body length ≥ 4.80 mm ($n=30$)
Small-sized females: body length ≤ 4.70 mm ($n=30$)

To measure the antennal club parameters, excised antennae were mounted on borosilicate glass and imaged using a Nikon DFK 33UX250 camera (Imaging Source®, Germany) attached to a Nikon SMZ800N stereomicroscope. The antennal club length was measured from the apical end (ventral side) to the tip of the last antennomere, while the width was measured at the midpoint of the antennal club (ventral side). Measurements were obtained using IC Capture—Image Acquisition 4.0 software. The average measurements, calculated from the left

and right antennae of each individual, were recorded in micrometers.

### 2.5 Electroantennographic (EAG) responses

The sources and purity of the chemicals used for electroantennography (EAG) experiments were the same as described in Table 1. Dose–response tests were conducted using an aggregation pheromone in a 10:1 ratio of 2-methyl-3-buten-2-ol (MB) to *cis*-verbenol (cV), as well as the individual compounds 1,8-cineole and (+)-isopinocamphone. Antennae from large and small females were stimulated with odor stimuli at seven doses: 0.001 µg, 0.01 µg, 0.1 µg, 1 µg, 10 µg, 100 µg, and 1000 µg. For odor cartridge preparation, 10 µl of each odor stimuli solution at the corresponding concentration (diluted in hexane) was applied to a 1 × 1 cm strip of Whatman No. 1 filter paper. The solvent was allowed to evaporate for 1 min before the strip was inserted into a glass Pasteur pipette (10 cm in length, 6 mm outer diameter), which was then used as an odor delivery cartridge for stimulation. Electrophysiological analyses were conducted using *I. typographus* females (F0 generation), as previously described. The F0 generation was chosen to directly represent the wild population of beetles originating from natural spruce forests. Prior to analysis, insects were immobilized by cooling at 4 °C for 5 min. This approach ensured the selection of morphometrically classified females within two size categories: large (≥ 4.80 mm, $n = 10$) and small (≤ 4.70 mm, $n = 10$).

Electroantennogram (EAG) responses were conducted as described (Zhang et al. 2000). The sex of the beetles was determined through dissection, and the heads of female beetles were excised using a microblade. Two capillary glass electrodes filled with Ringer's solution were used: one electrode was connected to the antennal club, while the other served as a reference by being inserted into the excised beetle head. The electrodes were attached to holders with an EAG probe (Syntech, Germany) and connected to a pre-amplifier. A constant stream of humidified air (200 ml/min) was directed over the antenna using a Syntech stimulus controller.

Odor cartridges (prepared as described above) were used to stimulate the antenna, and responses were recorded using EAG Pro software (Syntech, IDAC-4). Each stimulus (odor or control) was delivered as a 0.5-s pulse into the airstream directed at the antennal preparation, ensuring brief and consistent exposure. Control and odor stimuli were presented sequentially, with a 1-min interval between stimulations, allowing for antennal recovery and avoiding adaptation. The EAG probe was configured with a 0–32 Hz filter and a sampling rate of 100 Hz. Antennal responses were recorded as downward deflection signals in millivolts (mV), with response amplitudes defined as the peak depolarization of the olfactory sensilla of antennae measured during the 0.5-s odor stimulation. For each female beetle, recordings were made starting with the control stimulus and followed by sequential doses of the respective compound, increasing from the lowest to the highest concentration (0.001 to 1000 µg) applied to the filter paper to minimize sensory adaptation. Each biological replicate consisted of a single female beetle tested once per stimulus ($n = 10$ individuals per tested compound). The mean peak response amplitude across all replicates was calculated to assess antennal response to each compound.

### 2.6 Statistical analysis

The body length of female *Ips typographus* was examined utilizing a generalized linear model (GLM). In this analysis, body length served as the continuous response variable, while compound served as the explanatory variable. Each compound corresponded to a specific dose, and analyses were conducted without separating the effects of the compound and dose. The model employed a gamma distribution with a log link function. Additionally, a nested model incorporating dose within treatment was evaluated; however, no improvement in model fit was observed. The model performance was tested against the null model using an *F*-distribution and Akaike information criterion (AIC). Post hoc comparisons were conducted via the R package "multcomp" (Horthon et al. 2016), with Tukey test followed by multiplicity correction Bonferroni method.

A Pearson's chi-squared test of independence (Zar 2014) was conducted to assess whether the sex ratio, calculated as the proportion of females (number of females divided by the total number of individuals, i.e., females + males), differed significantly between treatments and their respective doses. To facilitate this analysis, separate contingency tables were constructed for each dose comparison, and chi-squared tests were performed independently for each table.

The data obtained from small and large female *I. typographus* were compared using the *t*-test. The two-sample *t*-test analysis assessed differences in:

1. Total body length between large and small females
2. Antennal club length between large and small females
3. Antennal club width between large and small females

The posterior analysis evaluated whether antennal club growth follows an isometric or allometric pattern relative to body size. We employed the standardized major axis (SMA) regression using the "smatr" package in R (Warton et al., 2012). Before conducting an SMA regression, the

length and width were log-transformed (ln = log natural), providing comparability, addressing potential scale issues, and making the relationship linear for better interpretation (Legendre and Legendre 1998). SMA regression was selected because it accounts for measurement errors in body size and antennal club length. SMA evaluates slopes > 1 as indicated isometric relationships, while deviations from < 1 indicate allometry (Jolicoeur 1990; Warton et al. 2006).

The one-tailed two-sample *t*-test (Zar 2014) was employed to assess the dose–response in electroantennography (EAG) responses between large ($n = 10$) and small ($n = 10$) females at individual doses of 1,8-cineole or isopinocamphone. A one-tailed test was used because our hypothesis, based on field experiments, predicted a specific directional effect: the behavioral responses of large females to high doses of 1,8-cineole and (+)-isopinocamphone, respectively. In contrast, for the dose–response to the pheromone blend, where both sizes exhibited equal reactions, a two-tailed test was adopted. Both analyses confirmed equal variance using the *F*-test. All statistical analyses were performed using R Studio version 4.1.1 (Core R Team 2015), with a significance level (alpha) 0.05. The dataset and R script used for the analysis are publicly available in the Dryad Digital Repository: https://doi.org/10.5061/dryad.rxwdbrvn1 (Moliterno et al. 2025). All figures were created using GraphPad Prism (version 9.5.0) software for macOS.

## 3 Results

We analyzed the sex ratio of beetles caught in the field using pheromone traps baited with three doses of 1,8-cineole and (+)-isopinocamphone in combination with pheromone, collected in 2019 ($N = 7$) and 2022 ($N = 8$) (Moliterno et al. 2023). In both years, females comprised 70–85% of the captures across treatments and pheromone-only groups (Fig. 1 in the Appendices). In 2022, the proportion of females was significantly higher for all three doses of (+)-isopinocamphone combined with pheromone compared to the appropriate doses of 1,8-cineole combined with pheromone (Table 2).

### 3.1 Prevalence and total body length differences in female captures across treatments and years

Females captured in control traps containing only pheromones had an average body length of 4.82 mm (SD = 0.22) in 2019 and 4.90 mm (SD = 0.17) in 2022.

In pheromone traps baited with (+)-isopinocamphone, statistical analyses revealed a significant effect of dose on the body size of captured insects in both 2019 ($F = 2.89$, $p = 0.036$) and 2022 ($F = 3.03$, $p = 0.030$). In 2019, females captured in traps baited with a low dose of (+)-isopinocamphone had an average body length of 4.75 mm (SD = 0.24), while in 2022, the average was 4.75 mm (SD = 0.23). Conversely, traps baited with a high dose of (+)-isopinocamphone attracted larger females, with average body lengths of 4.88 mm (SD = 0.19; $Z = -2.934$, $p = 0.020$) in 2019 and 4.86 mm (SD = 0.20; $Z = -2.850$, $p = 0.023$) in 2022 (Fig. 1A).

In pheromone traps baited with 1,8-cineole, the results from 2019 revealed a significant effect of dose on the body size of captured insects ($F = 3.29$, $p = 0.021$; Fig. 1B). A similar pattern was observed in 2022, with a more pronounced effect ($F = 9.40$, $p < 0.001$; Fig. 1B). Females captured in traps baited with the high dose of 1,8-cineole were significantly smaller than those captured in the control traps. At the high dose, the average body length was 4.69 mm (SD = 0.26; $Z = -2.77$, $p = 0.034$) in 2019 and 4.69 mm (SD = 0.23; $Z = -4.56$, $p < 0.001$) in 2022 (Fig. 1B).

**Table 2** Total beetle catches and percentage of females captured in response to three dose levels of treatments: (+)-isopinocamphone ((+)-IPC) and 1,8-cineole, each combined with pheromone, during field experiments in 2019 and 2022. Includes statistical comparisons of sex ratios between the two compared treatments

| Year/compound dose | (+)-IPC | | 1,8-Cineole | | Chi-sq | *df* | *p* value/Bonferroni |
|---|---|---|---|---|---|---|---|
| | Absolute catches | % of females | Absolute catches | % of females | | | |
| 2019/low | 1508 | 84 | 1059 | 84 | 0.002 | 1 | 1 |
| 2019/medium | 2875 | 81 | 2103 | 80 | 0.819 | 1 | 1 |
| 2019/high | 2477 | 82 | 370 | 79 | 1.382 | 1 | 0.71 |
| 2022/low | 1267 | 83 | 651 | 74 | 21.71 | 1 | < 0.001** |
| 2022/medium | 1406 | 80 | 519 | 75 | 5.78 | 1 | 0.05* |
| 2022/high | 1917 | 78 | 258 | 71 | 6.42 | 1 | 0.03* |

Data represents absolute beetle catches pooled from the respective number of trap rotations per year (2019: 7 rotations; 2022: 8 rotations). Pearson's chi-squared test analysis: chi-squared values indicate the results of contingency tests comparing female/male ratios across treatments. df represents degrees of freedom

*P* values indicate the significance level of the observed proportion between caught females versus males for respective compound and dose, with significance considered at $p < 0.05$ (*) and $p < 0.001$ (**) under the Bonferroni correction method per year

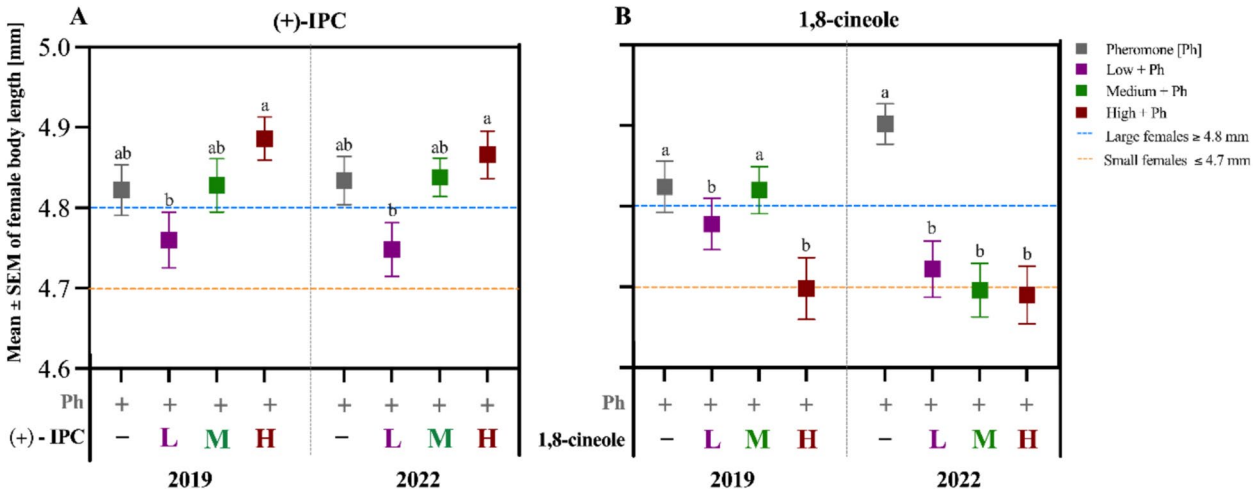


**Fig. 1** Mean body size of female *I. typographus* captured in response to three doses (low, medium, high) of **A** (+)-IPC is (+)-isopinocamphone and **B** 1,8-cineole, along with a control (pheromone only = Ph), in 2019 and 2022. Colors represent different doses or the control, with a sample size of $n = 50$ per group. Vertical bars show the standard error of the mean. Letters indicate significant differences based on Tukey's with Bonferroni adjustment ($p = 0.05$). Equal letters means no significative difference

**Table 3** Means of measurements in total body length and antennal club (length and width) of large and small females of *Ips typographus*; mean ± SD

| Parameters (mean ± SD) | Large females ($N = 30$) | Small females ($N = 30$) |
|---|---|---|
| Body length (mm) | 4.88 ± 0.079 | 4.59 ± 0.105 |
| Antennal club length (μm) | 258.72 ± 20.05 | 233.59 ± 17.35 |
| Antennal club width (μm) | 222.76 ± 16.11 | 198.75 ± 14.94 |

Total body length data from females of *Ips typographus* were defined as large ≥ 4.80 mm ($n = 30$) versus small ≤ 4.70 mm ($n = 30$) and their respective antennal club measurements

### 3.2 Antennal club size of large and small females is isometric to total body length

Comparison of phenotypic parameters revealed significant differences between large and small females in total body size ($t = 12.10$, $df = 58$, $p < 0.001$), antennal club length ($t = 5.20$, $df = 58$, $p < 0.001$), and antennal club width ($t = 5.99$, $df = 58$, $p < 0.001$) (Table 3).

Significant positive correlations were found between antennal club length and width in both size groups: for large females ($R^2 = 0.43$, $p \leq 0.001$) and for small females ($R^2 = 0.32$, $p = 0.001$). The relationship between log-transformed antennal club length and width indicated isometric scaling in both size groups, with regression slopes close to 1 (large females: 0.99; small females: 1.00) (Table 4 in the Appendices). This suggests a proportional relationship between length and width in both groups, where the two dimensions increase at similar rates (Fig. 2).

### 3.3 *Larger females are more responsive to (+)-isopinocamphone, whereas smaller females have higher antennal sensitivity to 1,8-cineole*

Electroantennogram (EAG) responses to the pheromone blend (MB:cV at a 10:1 ratio) did not show significant differences between large ($n = 10$) and small ($n = 10$) females at any of the tested doses ($t$-test; $p > 0.05$; Fig. 3A). However, large females exhibited significantly stronger responses to four higher concentrations of (+)-isopinocamphone: 1 μg ($t = 2.45$, $df = 18$, $p = 0.012$), 10 μg ($t = 2.83$, $df = 18$, $p < 0.01$), 100 μg ($t = 2.10$, $df = 18$, $p = 0.025$), and 1000 μg ($t = 1.96$, $df = 18$, $p = 0.033$) (Fig. 3B, Table 5 in the Appendices).

In contrast, large females showed significantly weaker EAG responses than small females to various concentrations of 1,8-cineole: 1 μg ($t = -2.14$, $df = 18$, $p = 0.023$), 10 μg ($t = -2.15$, $df = 18$, $p = 0.023$), 100 μg ($t = -2.52$, $df = 18$, $p = 0.011$), and 1000 μg ($t = -2.75$, $df = 18$, $p = 0.007$) (Fig. 3C).

## 4 Discussion

Our findings demonstrate that large and small phenotypes of female *I. typographus* respond differently to two ecologically relevant oxygenated spruce monoterpenoids, 1,8-cineole and (+)-isopinocamphone, which serve as a pheromone inhibitor and a pheromone synergist, respectively. These semiochemicals influence female attraction and decision-making, with clear size-dependent variation in both antennal responses and field behavior.

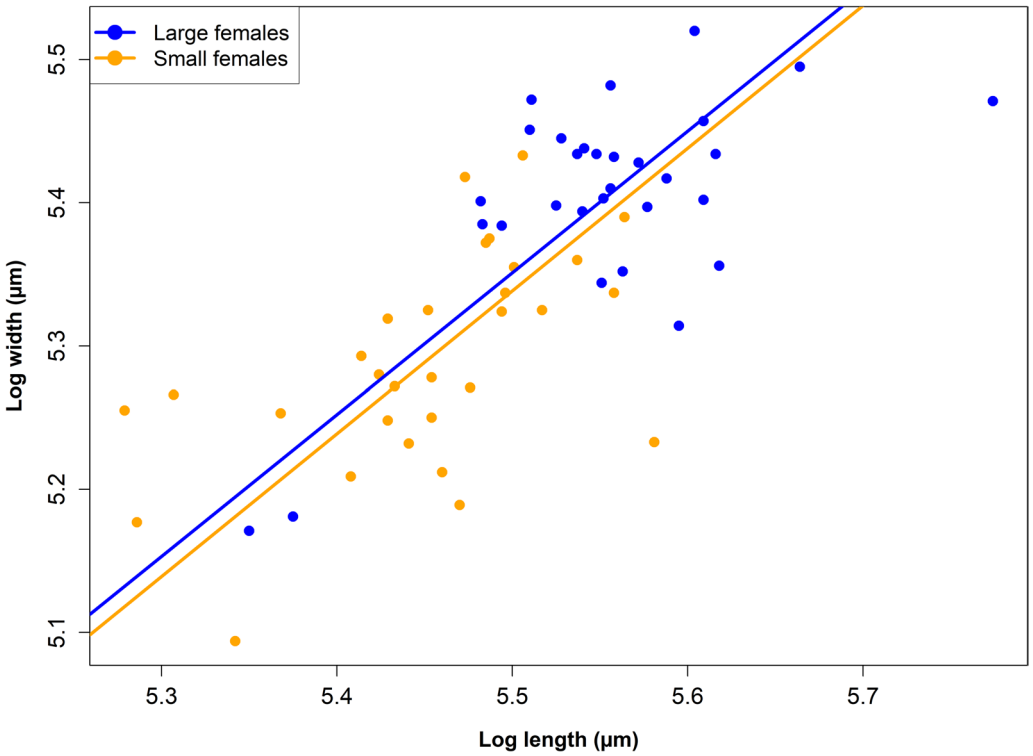


**Fig. 2** Standardized major axis (SMA) regression analysis representing positive trend in log (ln) length and width of categorized as "large females ≥ 4.80 mm" ($n = 30$) and "small females ≤ 4.70 mm" ($n = 30$) from antennal club of females of *I. typographus*. Each color represents the measurements taken in length and width expressed in micrometers (µm). Blue line and dots represent the "large females" group. Orange line and dots represent the "small females" group

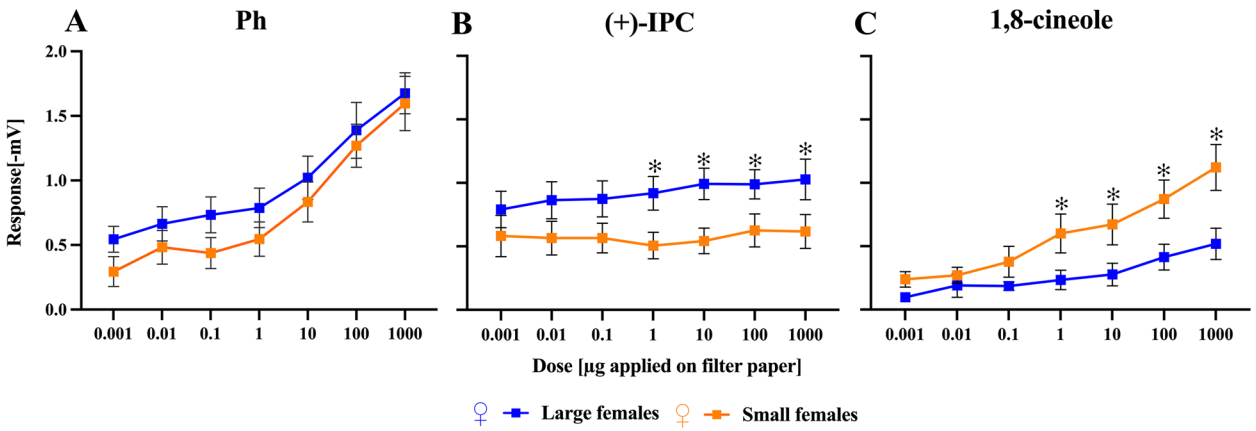


**Fig. 3** Dose–response curves based on electroantennographic (EAG) responses in female *Ips typographus*, categorized by body size: "large" (≥ 4.80 mm, $n = 10$) and "small" (≤ 4.70 mm, $n = 10$). Responses are shown for **A** pheromone, **B** (+)-isopinocamphone, and **C** 1,8-cineole. Each compound was tested across a concentration range of 0.001 to 1000 µg applied on the source filter paper, with hexane serving as the solvent control. EAG responses are expressed as the mean amplitude of antennal depolarizations (in millivolts), normalized by subtracting the response to hexane (blank). Error bars represent the standard error of the mean (SEM). Asterisks (*) indicate statistically significant differences between large versus small at individual doses, based on the one-tailed *t*-test ($p < 0.05$)

Enhanced sensitivity and attraction of larger *Ips typographus* females to (+)-isopinocamphone may facilitate the selection of higher-quality host trees.

In our field experiments, larger female phenotype was significantly more attracted to high doses of (+)-isopinocamphone, a compound known to synergize pheromone

attraction, when it was presented alongside the synthetic aggregation pheromone. This behavioral pattern was supported by electroantennography (EAG) analyses, which showed that the larger female phenotype exhibited stronger olfactory responses to high doses of (+)-isopinocamphone compared to the smaller female phenotype. The morphometric analysis further revealed that larger females possess isometrically larger antennal clubs. This increased antennal surface area likely improves their odor detection capabilities. Across various insect taxa, a correlation between antennal size and odor sensitivity has been widely documented (Makarova et al. 2022; Spaethe et al. 2007; Elgar et al. 2018; Lockey and Willis 2015). Longer antennae can house longer sensilla with greater pore density, which enhances the detection of odorants and the resulting neural activation (Mohebbi et al. 2022; Steinbrecht 2007; Liu et al. 2021). Miniaturized insects often display reductions in the antennomere number and sensilla count, as well as have shorter sensilla (Makarova et al. 2022; Steinbrecht 2007), although the diversity of sensilla types is typically maintained, allowing the detection of ecologically relevant odors (Polilov 2015; Diakova and Polilov 2020). These structural traits likely contribute to the higher sensitivity to (+)-isopinocamphone observed in larger females in our study.

The ecological implications of stronger attraction to (+)-isopinocamphone in larger females are somewhat speculative but may confer adaptive advantages. Notably, several symbiotic ophiostomatoid fungi associated with bark beetles, *G. penicillata*, *L. europhioides*, and *O. bicolor*, can metabolize host tree monoterpenoids into substantial quantities of (+)-isopinocamphone (Kandasamy et al. 2023). An increased olfactory response to this compound could help larger, dominant females locate trees where fungal symbionts have already detoxified monoterpenoids, thereby increasing the likelihood of successful colonization. This relationship may also benefit the fungi. Larger females tend to excavate longer galleries and transport greater fungal spore loads, potentially enhancing both fungal dispersal and establishment (Foelker and Hofstetter 2014; Sallé and Raffa 2007; Sallé et al. 2005). These dynamics suggest a potential feedback loop in which fungal metabolites selectively attract the most fecund or competitive beetles, reinforcing mutualistic interactions. Future research should investigate whether larger *I. typographus* females exhibit specific preferences for fungal species producing (+)-isopinocamphone and how this might shape the evolution of beetle–fungus mutualisms. Additionally, natural enemies of *I. typographus* are responsive to isopinocamphone (Pettersson and Boland 2003), suggesting potential, yet unexplored, tri-trophic interactions linking beetle body phenotypic variation, fungal volatiles, and predator attraction (Souza et al. 2024; Wegensteiner et al. 2015). Trap data also indicated a higher proportion of large females in 2022 compared to 2019, caught in treatments, even though the mean size of all females caught in pheromone-only treatments was the same in both years. We attribute a shift in the proportion of large female phenotype caught to treatments to the transition from the endemic bark beetle population in 2019 to the epidemic population that occurred in 2022. During endemic periods, selective pressure on host quality increases, potentially favoring larger females that can better discriminate among semiochemical cues (Sallé et al. 2005). These findings suggest that total body length-linked behavioral strategies are modulated by population density.

The unexpectedly elevated antennal sensitivity of smaller *Ips typographus* females to 1,8-cineole contrasts with the lower repellency of this anti-attractant for them.

An interesting contradiction was observed in the response of smaller females to 1,8-cineole. Although this compound is well-documented as an anti-attractant and toxic to *I. typographus* (Andersson et al. 2010; Jirošová et al. 2022; Zaman et al. 2024), and its addition significantly reduced the overall number of beetles captured in our field experiment with pheromone (Moliterno et al. 2024), a size-dependent pattern in females was observed. Specifically, we observed a higher proportion of smaller female phenotype in catches when exposed to high doses of 1,8-cineole combined with pheromone, compared to larger females. This pattern could still align with the antennal size hypothesis discussed earlier: those larger females with larger antennae may detect the anti-attractant more effectively and are, therefore, more strongly repelled. However, contrary to expectations, electroantennographic (EAG) data showed that smaller females exhibited greater antennal sensitivity to 1,8-cineole than larger females despite having shorter and narrower antennal clubs.

One explanation is that while differences in peripheral sensitivity at the antennal surface between small and large females are influenced by antennal size, their host choice and decision-making may be shaped by higher-order processing in CNS regions, such as the mushroom bodies and lateral horns. These central brain areas integrate olfactory input with learning, memory, and behavioral context. Insects' age, mating status, and energy reserves can influence both odor detection and the downstream processing of olfactory signals (Wiesel et al. 2022, Anton et al. 2007; Bodin et al. 2008; Martin et al. 2011). Consequently, the same odor may trigger

different or even opposite behaviors within the same species, depending on the individual's neuronal factors. Smaller females may have stronger neural connections mediating responses between cineole-sensitive OSNs and central brain regions, such as the lateral horn, which controls avoidance behaviors and suppresses their repulsive responses.

A potential ecological rationale for this pattern is linked to findings that trees with higher levels of 1,8-cineole are generally more resistant to bark beetle attacks (Schiebe et al. 2012). Larger *I. typographus* females, typically associated with higher fitness and greater capacity to kill trees (Grodzki 2004), may actively avoid such trees, recognizing them as poor-quality hosts. Doing so may increase their chances of successful colonization and reproduction (Raffa et al. 2016). In contrast, smaller females, who may have reduced competitive success during outbreaks, might tolerate trees with high levels of 1,8-cineole as a strategy for competitive escape. This strategy allows them to occupy less suitable trees while avoiding competition from larger females despite the higher risks posed by the compound's toxicity. Since our experiments were conducted in June–July, when the nutritional feeding of beetles and sister brood females from the first generation may overlap with the emergence of second-generation beetles searching for new hosts, we cannot precisely narrow down the ecological explanation solely to females seeking mates alongside suitable trees. However, we expect that the ecological principle of finding suitable host trees, where large females prefer trees with compromised defense, while smaller females avoid competition, will also apply to secondary-emerging and sister-brooding females.

### 4.1 Expanding future research framework to include males

Our analysis focused exclusively on females due to their central role in reproduction and host colonization. Additionally, field captures showed a female-biased sex ratio in traps baited with either oxygenated monoterpenoids combined with pheromone or pheromone alone. This pattern is consistent with earlier reports of female-biased attraction to both aggregation pheromone (Franklin et al. 2000; Schlyter et al. 1987) and 1,8-cineole (Jirošová et al. 2022).

However, it is also important to consider the potential implications for males. As the pioneer sex, males initiate host colonization and benefit from detecting semiochemicals related to the host tree's nutritional quality and the tree's defense ability. Unfortunately, in our catches of beetles with (+)-isopinocamphone and 1,8-cineole, there were not enough males for body length measurements after sorting by sex through dissection, making it impossible to obtain a statistically significant dataset. However, similarly to the total beetle catches, we observed that more males were attracted to the combination of (+)-isopinocamphone and pheromone (Moliterno et al. 2023) than to pheromone alone. In contrast, fewer males were attracted to the mixture of cineole and pheromone compared to pheromone alone.

Interestingly, when focusing on the sex ratio, we found a lower proportion of males attracted to (+)-isopinocamphone than 1,8-cineole. However, previous studies have identified olfactory sensory neuron classes in males and females of *Ips typographus* that are primarily tuned to both (+)-isopinocamphone and 1,8-cineole (Andersson et al. 2009; Kandasamy et al. 2023), suggesting that males are equally sensitive on the periphery to both compounds as females. To better understand the signal processing in the beetle's olfactory system and the ecological relevance of our findings, further research on sex-specific electrophysiological responses, the single cell recordings data showing numbers of sensilla types on differently sized clubs, as well as phenotypic variation-dependent behavior and detection abilities in males, is needed.

## 5 Conclusion

Our study demonstrates how size phenotypic variation influences adaptive responses in semiochemical-mediated host selection among female bark beetles. We report clear size-dependent olfactory and behavioral strategies in female *I. typographus*, linking antennal size, olfactory sensitivity, and host-selection behavior. The de novo spruce-derived oxygenated monoterpenoids 1,8-cineole and the multisource-derived hydroxylated (+)-isopinocamphone, with their contrasting ecological roles as pheromone synergist or inhibitor, respectively, may significantly influence responses in *I. typographus* females based on their size. Larger females exhibited greater olfactory sensitivity and attraction to (+)-isopinocamphone, allowing them to more effectively discriminate between suitable, more stressed, and/or fungus-colonized hosts. In contrast, smaller females were less repelled but, surprisingly, more antennally sensitive to 1,8-cineole, possibly reflecting an alternative strategy to avoid competition with larger females by exploiting lower-quality or riskier habitats. These findings suggest that body size can influence olfactory detection and subsequent CNS processing, leading to behavioral decision-making that may impact the reproductive success and population dynamics of bark beetles. While our focus was on females due to their crucial role in reproduction and colonization, future research should investigate whether similar size-dependent responses occur in pioneer males.

## Appendix

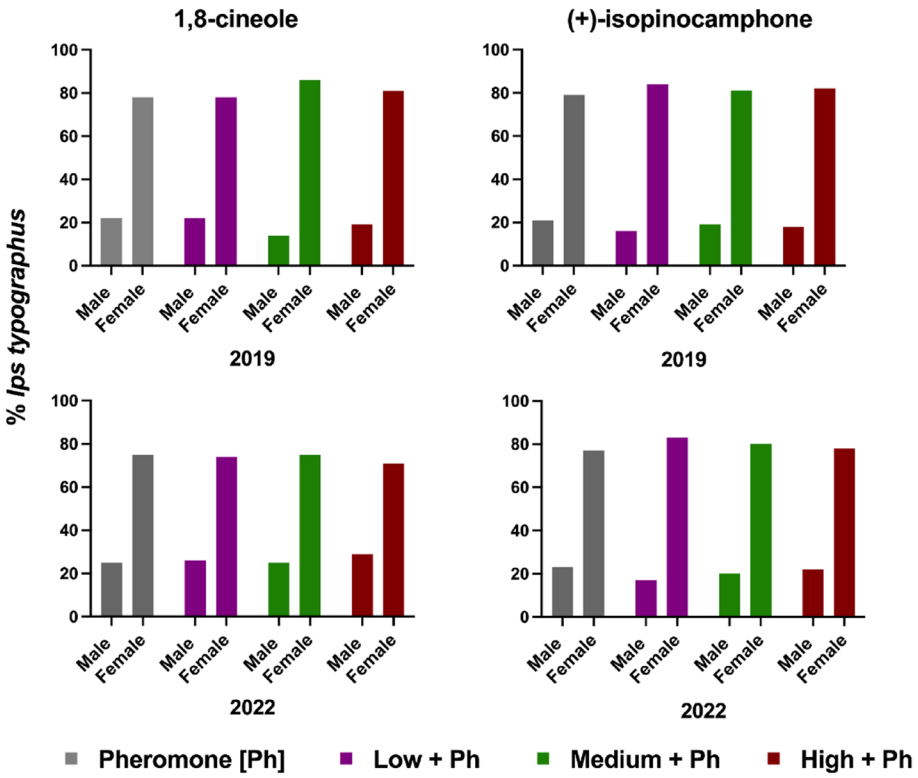


**Figure Appendix 1.** Relative catches and sex ratio of *Ips typographus* in two-year experiment of pheromone traps baited with oxygenated host tree compounds 1,8-cineole and (+)-isopinocamphone in 2019 and 2022. Low, Medium and High represent relative dose trapped in combination with pheromone

**Table 4** Standardized major axis (SMA) regression between antennal club length and width log- transformed. In bold indicating slopes close to 1 (Large: 0.99, Small: 1.0), isometrical development. R-squared showing the correlation between length and widthindicated significant and positive correlations ($p \leq 0.001$) and (Small= $R^2$= 0.32, $p$= 0.001)

| SMA_Group_antennal club_size | Parameter | Estimate | Lower Limit | Upper Limit | P-value |
|---|---|---|---|---|---|
| Large_females | elevation | -0.09334208 | -1.70663684 | 1.51995268 | |
| | ***slope*** | **0.99** | 0.741138 | 1.3221391 | |
| | R-squared | 0.43 | | | 0.0001 |
| Small_females | elevation | -0.1440909 | -1.8781666 | 1.5899849 | |
| | ***slope*** | **1.00** | 0.7282489 | 1.3644331 | |
| | R-squared | 0.32 | | | 0.001 |

**Table 5** Responses are shown for Pheromone, 1,8-cineole, (+)-isopinocamphone, and. Each compound was tested across a concentration range of 0.001 µg to 1000 µg applied on the source filter paper, with hexane serving as the solvent control. EAG responses are expressed as the mean amplitude of antennal depolarizations (in millivolts), normalized by subtracting the response to hexane (blank). Bold marks indicate statistically significant differences between large versus small at individual doses, based on the t-test ($p < 0.05$)

| **Dose responses** *Pheromone* (MB:cV; 10:1) | **Female size** | **Average** (Standard deviation) | **Two sample *t*- test** (two.sided) | **N = Small** | **N = Large** |
|---|---|---|---|---|---|
| *1ng* | Large | 0.544 (SD= 0.32) | t = 1.635, p = 0.119 | | |
| | Small | 0.294 (SD= 0.37) | | | |
| *10ng* | Large | 0.664 (SD= 0.42) | t = 0.975, p = 0.343 | | |
| | Small | 0.483 (SD= 0.41) | | | |
| *100ng* | Large | 0.734 (SD= 0.44) | t = 1.619, p = 0.123 | | |
| | Small | 0.438 (SD= 0.38) | | | |
| *1µg* | Large | 0.788 (SD= 0.49) | t = 1.191, p = 0.249 | 10 | 10 |
| | Small | 0.546 (SD= 0.42) | | | |
| *10µg* | Large | 1.021 (SD= 0.53) | t = 0.818, p = 0.424 | | |
| | Small | 0.834 (SD= 0.49) | | | |
| *100µg* | Large | 1.389 (SD=0.68) | t = 0.437, p = 0.667 | | |
| | Small | 1.270 (SD=0.53) | | | |
| *1mg* | Large | 1.675 (SD= 0.50) | t = 0.299, p = 0.767 | | |
| | Small | 1.597 (SD= 0.66) | | | |
| **Dose responses** *(+) -isopinocamphone* | **Female size** | **Average** (Standard deviation) | **Two-sample *t*- test** (one.sided) | **N = Small** | **N = Large** |
| *1ng* | Large | 0.791 (SD= 0.45) | t = 0.967 , p = 0.173 | | |
| | Small | 0.582 (SD= 0.51) | | | |
| *10ng* | Large | 0.863 (SD= 0.46) | t = 1.52, p = 0.074 | | |
| | Small | 0.565 (SD= 0.42) | | | |
| *100ng* | Large | 0.874 (SD= 0.45) | t = 1.673, p = 0.056 | | |
| | Small | 0.565 (SD= 0.37) | | | |
| *1µg* | Large | 0.918 (SD= 0.41) | **t = 2.450, p = 0.012** | 10 | 10 |
| | Small | 0.506 (SD= 0.32) | | | |
| *10µg* | Large | 0.992 (SD= 0.38) | **t = 2.825, p = 0.006** | | |
| | Small | 0.543 (SD= 0.32) | | | |
| *100µg* | Large | 0.989 (SD=0.36) | **t = 2.093, p = 0.025** | | |
| | Small | 0.626 (SD=0.41) | | | |
| *1mg* | Large | 1.027 (SD=0.50) | **t = 1.962, p = 0.033** | | |
| | Small | 0.618 (SD=0.42) | | | |

Legend: Dose–response curves based on electroantennographic (EAG) responses in female *Ips typographus*, categorized by body size:"large"(≥ 4.80 mm, $n = 10$) and"small"(≤ 4.70 mm, $n = 10$)

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**Authors' contributions**
AACM conceived and designed the study experiments. JS assisted in insect maintenance and rearing. AACM and MKS performed morphometric and electrophysiological analyses and collected data. AACM performed modeling work and statistical analysis of output data. SB prepared the figures and tables. AACM wrote the first draft. AACM, MKS, SB, and JS edited the draft. AJ and JH supervised the work, edited text, and provided valuable feedback.

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**Data availability**
The data is available in the Dryad Digital Repository: https://doi.org/10.5061/dryad.rxwdbrvn1.

## Declarations

**Ethics approval and consent to participate**
Ethical approval was not required for this study. We have performed all beetle experiments that comply with the ARRIVE guidelines and were carried out in

accordance with Scientific Procedures Act, 1986 and associated guidelines, EU Directive 2010/63/EU for animal experiments.

**Consent for publication**
All authors gave their informed consent to this publication and its content.

**Competing interests**
The authors declare no competing interests.

**Author details**
[1]Faculty of Forestry and Wood Sciences, Czech University of Life Sciences Prague, Prague, Czech Republic.

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