Olfactory Coding in Bark Beetles: Understanding morphology and neurophysiology

by

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Field of study: Global Change Forestry

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Ph.D. THESIS ASSIGNMENT

Mayuri Kashinat Shewale

Global Change Forestry

Thesis title

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Objectives of thesis

Bark beetles are significant pests in coniferous forests, relying on olfactory communication to find hosts, mate, and regulate population density. Their host selection process is challenging, as unsuitable choices reduce offspring fitness. Olfactory sensory neurons (OSNs) on their antennae play a crucial role in detecting volatile signals. While I. typographus olfactory physiology is well-researched, the olfactory mechanisms of other Ips species remain largely unknown.

The aims of this research are:

1. To characterize the morphological types of olfactory sensilla in bark beetle Ips species by using scanning electron microscopy and map their distribution on the antennal surface.

2. To characterize the olfactory receptor neurons (ORNs) in Ips species using electrophysiological techniques and compare these findings to the well-studied model species I. typographus.

Methodology

Microscopic techniques will be employed to thoroughly examine the bark beetle antennal morphology and olfactory sensilla distribution. The antennae of selected Ips species will undergo chemical processing and will be examined using a scanning electron microscope. Electrophysiological techniques, such as single sensillum recording, will be performed to gain more information about the olfactory sensory neuron responses in bark beetles. Chemicals from various ecological sources and at different concentrations will be applied to measure neuronal responses, based on their recorded action potentials.

Schedule:

2021-2022: Understanding research problem, finalizing ISP and study objectives, dissertation methodology, literature review defense, coursework submission

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The proposed extent of the thesis

70 pages

Keywords

Bark beetles, Ips species, single sensillum recordings, antennal morphology, olfaction, electrophysiology, olfactory receptor neurons

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Recommended information sources

Andersson et al., 2009 Specificity and redundancy in the olfactory system of the bark beetle Ips typographus: Single-cell responses to ecologically relevant odors. Journal of Insect Physiology 55 (2009) 556–567 doi:10.1016/j.jinsphys.2009.01.018

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2024/25 WS – FFWS – Doctoral Thesis Defense

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Electronic approval: 11. 03. 2025
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Dean

Prague on 11. 03. 2025

Certificate of Authorship

I hereby declare that this dissertation, titled "***Olfactory coding in bark beetles: Understanding the morphology and neurophysiology***", was created independently and ethically. I declare that all the information sources and literature are indicated accordingly, and the thesis was prepared under the direct supervision of my scientific supervisors.

I agree with the disclosure of this Ph.D. Thesis according to Czech Law (Act No. 111/1998 Coll. Sb.) regardless of the Defence of thesis results.

June 2025, Prague

Mayuri Kashinath Shewale

List of publications included in this thesis:

1. Ramakrishnan, R†, **Shewale, M. K**.†, Strádal, J.†, Hani, U., Gershenzon, J., Andersson, M. N., Frühbrodt, T., Doležal, P., Jirošová, A. (2025). Aggregation Pheromones in the Bark Beetle Genus Ips: Advances in Biosynthesis, Sensory Perception, and Forest Management Applications. Manuscript submitted to *Current Forestry Reports,* under revision.

2. **Shewale, M. K.,** Nebesářová, J., Grosse-Wilde, E., & Kalinová, B. (2023). Microscopic morphology and distribution of the antennal sensilla in the double-spined bark beetle, Ips duplicatus (Coleoptera: Curculionidae). *Microscopy Research and Technique*, *86*(12), 1610-1625. https://doi.org/10.1002/jemt.24397

3. **Shewale, M.K.,** Dusek, J., Jirošová, A. (2025) Microscopic morphology and distribution of the antennal sensilla in the larch bark beetle, Ips cembrae (Coleoptera: Curculionidae). Manuscript under preparation.

4. Moliterno, A. A. C. †, **Shewale, M.K. †,** Basile, S.,Synek, J., Jirošová, A.(2025) Size- and dose-dependent behavioral responses to 1,8-cineole and (+)-isopinocamphone: a potential host selection strategy in female Ips typographus L. Submitted to *Annals of Forest Science.* under revision.

5. **Shewale, M. K**., Bláha, J., Synek, J., Schebeck, M., Andersson, M. N., Kandasamy, D., & Jirošová, A. (2025) Comparative analysis of olfactory sensory neurons in two Ips species reveals conserved and species-specific olfactory adaptations. *Frontiers in Forests and Global Change*, *8*, 1588866. doi: 10.3389/ffgc.2025.1588866

†Equal contribution as first author

***Please note that all the manuscripts are attached at the end in the appendix.**

List of publications not included in this thesis:

1. Manjesh, G. N., Kaipa, H., Upreti, K. K., Sharma, D., Puttegowda, M. G. A., Manjunathagowda, D. C., Chinapolaiah, A., **Shewale, M.K.,** & Kusuma, D. K. (2022). Diversity of flavonoids profile in sexually dimorphic clones of betle vine [Piper betle L.] genotypes based on liquid chromatography-mass spectrometry [LCMS/MS]. *Industrial Crops and Products*, *187*, 115363. https://doi.org/10.1016/j.indcrop.2022.115363\

2. Johny, J., Diallo, S., Lukšan, O., **Shewale, M.,** Kalinová, B., Hanus, R., & Große-Wilde, E. (2023). Conserved orthology in termite chemosensory gene families. *Frontiers in Ecology and Evolution*, *10*, 1065947. https://doi.org/10.3389/fevo.2022.1065947

Popular Science Summary

How do bark beetles use their sense of smell to find and attack trees?

Bark beetles are small wood-boring insects that play a natural role in forest ecosystems, helping to recycle and decompose dead trees. However, they can become aggressive pests under certain conditions, causing widespread damage to conifer forests. In recent years, bark beetle infestations have become increasingly problematic. Climate change, particularly rising temperatures and prolonged droughts, has made these forests more vulnerable by weakening tree defenses, creating ideal conditions for beetle outbreaks. While much research has focused on *Ips typographus*, and other species, such as *Ips duplicatus*, *Ips acuminatus*, and *Ips cembrae*, also contribute to forest damage. Yet, much less is known about their peripheral olfactory system. Like many insects, bark beetles rely on their sense of smell to find suitable hosts and communicate with each other. Their antennae are covered with sensilla, specialized hair-like structures that detect chemical signals in the environment. These sensilla house olfactory sensory neurons (OSNs) that recognize and respond to different volatile compounds. Bark beetles use this system to detect stress-related compounds released by weakened trees, indicating suitable colonizing trees. Once a tree is selected, aggregation pheromones are released, attracting more beetles to the site and leading to a mass attack that can overwhelm the tree's defenses. This study investigates how different *Ips* species with diverse hosts and ecology respond to host, non-host tree, and microbial volatiles, by comparing their antennal morphology and OSN function. It also explores whether body size influences olfactory detection, particularly in *Ips typographus* females, where larger and smaller individuals may detect these semiochemicals differently. Using scanning electron microscopy (SEM), electrophysiological recordings, and field experiments, this research aims to map the antennal sensilla and neuronal responses involved in olfactory detection across these species. Understanding the olfactory adaptations of bark beetles is crucial for developing better pest management strategies. Identifying the specific compounds that attract or repel these beetles can help to improve semiochemical-based control methods, such as traps and repellents, to protect forests more effectively. By expanding knowledge beyond *Ips typographus* and considering species-specific and size-dependent olfactory responses, this research provides a broader perspective on how bark beetles locate and infest trees, helping to manage future outbreaks.

Popular science summary (in Czech)

Jak kůrovci využívají čich k nalezení a napadení stromů?

Kůrovci jsou drobní dřevokazní brouci, kteří hrají přirozenou roli v lesních ekosystémech, zejména tím, že se podílejí na rozkladu a recyklaci odumřelých stromů. Za určitých podmínek se však mohou stát závažnými škůdci, kteří způsobují rozsáhlé poškození, zejména v porostech jehličnatých dřevin. V posledních letech představují gradace kůrovců čím dál větší problém. Klimatická změna, zejména rostoucí teploty a prodlužující se období sucha, vede ke snižující se obranyschopnosti lesních porostů, čímž vznikají optimální podmínky pro gradace populací kůrovců. Zatímco velké množství výzkumů se zaměřilo na lýkožrouta smrkového (*Ips typographus),* další druhy, jako *Ips duplicatus*, *Ips acuminatus* a *Ips cembrae*, rovněž významně přispívají k narušování lesních porostů. O jejich periferním čichovém ústrojí však zatím víme podstatně méně.

Stejně jako mnoho jiných druhů hmyzu spoléhají i kůrovci na čichové vjemy při lokalizaci vhodného hostitele a při vzájemné komunikaci. Jejich tykadla jsou pokryta senzilami, specializovanými chloupkovitými strukturami sloužícími k detekci chemických signálů v prostředí. Tyto senzily obsahují čichové smyslové neurony (OSNs), které rozpoznávají a reagují na různé těkavé sloučeniny. Kůrovci využívají tento systém k detekci stresových látek uvolňovaných oslabenými stromy, jež signalizují vhodné jedince k napadení a kolonizaci. Po výběru hostitelského stromu samci začnou produkovat agregační feromon, který přiláká více jedinců a vedou k hromadnému útoku, pomocí něhož jsou kůrovci schopni překonat obranné mechanismy stromu.

Tato studie zkoumá, jak různé druhy z rodu *Ips* s odlišnou ekologickou specializací reagují na těkavé látky hostitelských, nehostitelských a mikrobiálních původů, pomocí porovnání morfologie tykadel a funkce OSN. Součástí výzkumu je i posouzení vlivu tělesné velikosti na schopnost detekce pachových podnětů, zejména u samic kůrovce *Ips typographus*, kde mohou existovat rozdíly mezi menšími a většími jedinci ve vnímání specifických semiochemikálií. Za využití rastrovací elektronové mikroskopie (SEM), elektrofyziologických měření a terénních experimentů se tato práce zaměřuje na mapování tykadlových senzil a neuronální aktivity spojené s čichovým vnímáním u vybraných druhů. Pochopení čichových adaptací kůrovců je zásadní pro vývoj efektivnějších metod ochrany lesů. Identifikace specifických těkavých látek, které kůrovce přitahují nebo odpuzují, může přispět k optimalizaci využití semiochemikálií

v kontrolních metodách ochrany lesa, jako jsou lapače, lapáky či různé repelenty, s cílem lépe chránit lesní ekosystémy. Rozšířením výzkumu i na další druhy rodu *Ips* mimo *Ips typographus* a zahrnutím druhově specifických i velikostně podmíněných čichových odezev nabízí tato práce komplexnější pohled na to, jak kůrovci vyhledávají a napadají hostitelské stromy, což v konečném důsledku přispívá k účinnějšímu zvládání budoucích gradací populací kůrovců.

लोकप्रिय विज्ञान सारांश (मराठी अनुवाद).

झाडांना शोधण्यासाठी आणि त्यांच्यावर हल्ला करण्यासाठी साल भुंगे त्यांच्या गंधाच्या भावनेचा कसा वापर करतात?

साल बीटल हे लहान लाकूड-भोके पाडणारे कीटक आहेत जे वन परिसंस्थांमध्ये नैसर्गिक भूमिका बजावतात, मृत झाडांचा पुनर्वापर आणि विघटन करण्यास मदत करतात. तथापि, विशिष्ट परिस्थितीत, ते आक्रमक कीटक बनू शकतात, ज्यामुळे शंकुधारी जंगलांचे मोठ्या प्रमाणात नुकसान होते. अलीकडच्या वर्षांत, साल बीटलचा प्रादुर्भाव दिवसेंदिवस समस्याग्रस्त बनला आहे. हवामान बदल, विशेषत: वाढते तापमान आणि प्रदीर्घ दुष्काळ यामुळे झाडांचे संरक्षण कमकुवत होऊन ही जंगले अधिक असुरक्षित झाली आहेत, ज्यामुळे बीटलच्या उद्रेकासाठी आदर्श परिस्थिती निर्माण झाली आहे. बरेच संशोधन *Ips typographus*) वर केंद्रित केले गेले आहे, परंतु इतर प्रजाती, जसे की *Ips duplicatus*, *Ips acuminatus*, आणि *Ips cembrae* देखील जंगलाच्या ऱ्हासाची हातभार लावतात. तरीही त्यांच्या परिघीय घ्राण प्रणालीबद्दल फारच कमी माहिती उपलब्ध आहे. इतर बऱ्याच कीटकां प्रमाणे, साल बीटल योग्य यजमान शोधण्यासाठी आणि एकमेकांशी संवाद साधण्यासाठी त्यांच्या गंधज्ञानावर (olfactory sense) अवलंबून असतात. त्यांच्या अँटेना सेन्सिला, विशेष केसांसारख्या रचनांनी झाकलेले असतात, जे वातावरणातील रासायनिक सिग्नल शोधतात. हे सेन्सिला घ्राण संवेदी न्यूरॉन्स (ओएसएन) ठेवतात जे वेगवेगळ्या अस्थिर संयुगे ओळखतात आणि प्रतिसाद देतात. साल बीटल कमकुवत झाडांनी सोडलेल्या तणाव-संबंधित संयुगे शोधण्यासाठी या प्रणालीचा वापर करतात, जे वसाहतीसाठी योग्य झाडाचे संकेत म्हणून काम करतात. एकदा झाड निवडले की, एकत्रीकरण फेरोमोन (aggregation pheromone) सोडले जातात, ज्यामुळे साइटवर अधिक बीटल आकर्षित होतात आणि मोठ्या प्रमाणात एकत्रित हल्ला होतो ज्यामुळे झाडाच्या संरक्षणावर परिणाम होऊ शकतो. हा अभ्यास त्यांच्या अँटेनल मॉर्फोलॉजी आणि ओएसएन फंक्शनची तुलना करून, विविध यजमान आणि पारिस्थितिकी असलेल्या वेगवेगळ्या *Ips* प्रजाती यजमान, नॉन-होस्ट वृक्ष आणि मायक्रोबियल वाष्पशीलांना कसा प्रतिसाद देतात याची तपासणी करतो. विशेषत: *Ips typographus* स्त्रियांमध्ये आकार घ्राण धारणेवर परिणाम करतो की नाही हे देखील शोधते, कारण मोठ्या व लहान व्यक्तींमध्ये सेमिओकेमिकल्स ओळखण्याच्या क्षमतेत फरक असू शकतो. स्कॅनिंग इलेक्ट्रॉन मायक्रोस्कोपी (एसईएम), इलेक्ट्रोफिजिओलॉजिकल रेकॉर्डिंग आणि फील्ड प्रयोगांचा वापर करून, या संशोधनाचे उद्दीष्ट या प्रजातींमध्ये घ्राण शोधण्यात गुंतलेल्या संवेदी संरचना आणि न्यूरोनल प्रतिक्रियांचा नकाशा तयार करणे आहे. चांगल्या कीड व्यवस्थापन रणनीती विकसित करण्यासाठी साल बीटलचे घ्राण अनुकूलन समजून घेणे महत्वाचे आहे. जर आपण या भुंगेंना आकर्षित करणारी किंवा मागे हटविणारी विशिष्ट संयुगे ओळखू शकलो तर आपण जंगलांचे अधिक प्रभावीपणे संरक्षण करण्यासाठी सापळे आणि प्रतिकारक यासारख्या सेमिओकेमिकल-आधारित नियंत्रण पद्धती परिष्कृत करण्यास सक्षम होऊ शकतो. आयपीएस टायपोग्राफसच्या पलीकडे ज्ञानाचा विस्तार करून आणि प्रजाती-विशिष्ट आणि आकार-अवलंबून घ्राण प्रतिक्रियांचा विचार करून, हे संशोधन साल बीटल झाडांना कसे शोधतात आणि संक्रमित करतात याबद्दल विस्तृत दृष्टीकोन प्रदान करतात आणि शेवटी भविष्यातील उद्रेक व्यवस्थापित करण्यास मदत करतात.

Annotation

Bark beetles (Coleoptera: Curculionidae: Scolytinae) are key components of forest ecosystems but can also cause severe economic and ecological damage during population outbreaks. Climate change has worsened bark beetle infestations, resulting in extensive tree mortality. These beetles rely predominantly on their olfactory system to detect suitable host trees, locate conspecifics, and coordinate mass infestations. Their antennae, which serve as the primary olfactory organs, are densely covered with hair-like sensilla that house olfactory sensory neurons (OSNs) responsible for detecting environmental chemical cues. While *Ips typographus* has been extensively studied, little is known about the olfactory adaptations of *Ips duplicatus*, *Ips acuminatus*, and *Ips cembrae* despite their ecological and economic significance. This thesis investigates olfactory coding in *Ips* bark beetles, focusing on antennal morphology, size-dependent olfactory detection, and OSN function. Using scanning electron microscopy (SEM), the antennae of *I. duplicatus, I. acuminatus,* and *I. cembrae* were visualized to observe the general morphology and distribution of sensilla types. We observed six main types of antennal sensilla in *I. duplicatus*. Although males are the pioneers in colonization, females play a critical role in selecting suitable oviposition sites, which directly influences offspring fitness. Electrophysiological experiments in *I. typographus* females also investigated how body size influences semiochemical olfactory detection and host preferences in field. Electrophysiological recordings of OSNs in *I. acuminatus* and *I. cembrae* compared their frequency and responses to beetle-produced compounds, host-, non-host trees, and microbial volatiles with that of existing data from *I. typographus*. Results indicate that the distribution of antennal sensilla is largely conserved across the studied species, suggesting that olfactory adaptations primarily occur at the neuronal level rather than at the morphological level. In *I. typographus*, larger females exhibited stronger responses to synergist compounds.

In contrast, smaller females strongly responded to repellent compounds, revealing a contrasting size-dependent olfactory strategy for host tree choice. OSN characterization in *I. cembrae* and *I. acuminatus* identified shared and species-specific responses, reflecting conserved olfactory strategies related to their host specialization. This research enhances our understanding of bark beetle olfactory coding by integrating

morphological, electrophysiological, and behavioral approaches, offering novel insights for developing semiochemical-based pest management strategies.

Keywords: bark beetles, olfaction, chemical communication, semiochemicals, pheromones, olfactory sensory neurons, host selection

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"What you do makes a difference, and you have to decide what kind of difference you want to make." - Jane Goodall

Dedication

To the incredible women in my family, especially my mother and my sisters, whose love, resilience, and support have shaped my journey. It is also for all the women in STEM, past, present, and future, who challenge barriers, push boundaries, and inspire generations.

"स्वतःवर विश्वास ठेवा आणि सतत मेहनत करा, मग कुठलीही अडचण तुम्हाला रोखू शकत नाही." -सिंधुताई सपकाळ

Translation: *"Believe in yourself and keep working hard, and no obstacle will ever stop you."* **- Sindhutai Sapkal**

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List of tables

List of abbreviations and symbols

µg: micrograms

µm: micrometers

BB: Bark beetles

℃: degree Celsius

EAG: electroantennography

GC-EAD: Gas chromatography coupled with electroantennographic detection

GC-MS: Gas chromatography coupled with mass spectrometry

GRs: gustatory receptor

Hz: Hertz

IAc: *Ips acuminatus*

IC: *Ips cembrae*

ID: *Ips duplicatus*

IR: ionotropic receptors

IT: *Ips typographus*

JHIII: Juvenile Hormone III

mg: milligrams

mL: millilitres

mm: millimeters

mV: millivolts

nm: nanometres

OBP: odorant binding proteins

OR: olfactory receptor

OSN: olfactory sensory neuron

SEM: scanning electron microscopy

SEM: standard error mean

SNMP: single nucleotide membrane proteins

SSR: single sensillum recording

Chapter 1. Introduction

1.1. Research background

Forests cover nearly one-third of Europe's landmass, with coniferous species playing a dominant ecological and economic role across many regions. In Central and Northern Europe, Norway spruce (*Picea abies*), Scots pine (*Pinus sylvestris*), and European larch (*Larix decidua*) constitute the backbone of conifer-dominated forest ecosystems. However, large-scale planting of spruce monocultures outside their natural range forced by historical economic forestry has reduced ecosystem resistance. Combined with climate change-induced stressors such as drought and warming, these simplified stands have become highly susceptible to bark beetle outbreaks. This interaction between past management practices and climate extremes is a key driver of the unprecedented bark beetle epidemics observed in recent decades.

Bark beetles (Coleoptera: Curculionidae: Scolytinae) are diverse wood-boring weevils, comprising around 220 genera and 6000 species distributed worldwide. Many bark beetles contribute positively to forest health by facilitating dead wood's decomposition and nutrient recycling, primarily through their mutualistic associations with wood-degrading fungi that colonize host trees. However, several conifer-infesting species have emerged as serious pests, particularly under climate-driven stress. Trees already weakened by climate-induced stress are particularly vulnerable to beetle infestation. The Eurasian spruce bark beetle, *Ips typographus*, stands out as the most destructive species, especially in mature spruce trees at higher elevations. Other species such as the glossy bark beetle, *Pityogenes chalcographus,* and the double-spined bark beetle, *Ips duplicatus*, also target spruce but are considered less aggressive. In contrast, the pine bark beetle, *Ips acuminatus,* and the larch bark beetle, *Ips cembrae* primarily infest stressed pine and larch trees and are often regarded as secondary pests. The latter species have been less extensively studied, mainly because the damage they cause is typically less severe than that of *I. typographus*. Nonetheless, they often coexist with other bark beetles during infestations, particularly in disturbed or weakened forest stands.

A key feature of bark beetle ecology is their reliance on chemical communication to mediate host selection, aggregation, and reproduction. Volatile organic compounds (VOCs) released by host trees provide crucial information on species identity, physiological condition, and defense status. These chemical cues, in combination with

beetle-produced aggregation pheromones, coordinate mass-attacking behavior which is a critical strategy for overcoming tree defenses. Bark beetles rely on a highly evolved chemosensory system to detect and interpret these complex olfactory environments. Their primary sensory organ for odor detection is the club-shaped antennae, which are densely packed with specialized cuticular sensilla. These sensilla house olfactory sensory neurons (OSNs) within a lymph-filled cavity. Odor molecules first pass through porous sensillum walls, bind to odorant-binding proteins (OBPs), and subsequently interact with specific receptors on the dendritic membrane of OSNs. Individual OSNs can be narrowly or broadly tuned to ecological cues such as host volatiles, pheromones, or inhibitory cues from non-hosts or microbes.

Electrophysiological studies on *I. typographus* have identified at least 26 OSN classes, each with distinct response spectra and distribution patterns on antennae. These findings have deepened our understanding of how chemical information is processed at the peripheral level. Furthermore, detailed morphological studies of antennal sensilla in *I. typographus* and other *Ip*s species have also revealed structural diversity, likely reflecting ecological specializations. Although these species coexist within the same forest ecosystems, they typically prefer different conifer host trees. For example, *I. typographus* and *I. duplicatus* primarily target spruce, *I. acuminatus* prefers pine, and *I. cembrae* specializes in larch trees.

Despite their overlapping distributions and potential ecological interactions in conifer forests, most research have been focused on *I. typographus*, leaving significant knowledge gaps in understanding the olfactory adaptations and sensory ecology of the other *Ips* species. Understanding how these species detect and respond to chemical cues is key to uncovering host adaptation mechanisms and the dynamics of tritrophic interactions involving beetles, host trees, and microbial associates. In addition to interspecific differences, individual-level traits such as body size may influence olfactory sensitivity and host preference, potentially shaping ecological strategies. While *I. typographus* have been intensively studied due to its high economic impact, extending investigations to *I. acuminatus* and *I. cembrae*, and incorporating individual variation, offers valuable opportunities to uncover overlooked sensory adaptations. Such insights are increasingly important as climate change influences host availability, beetle behavior, and pest management challenges.

1.2. Research aims and objectives.

Peripheral olfactory mechanisms are fundamental to key behaviours in bark beetles, such as host recognition and communication. These processes are mediated by OSNs housed within antennal olfactory sensilla, enabling detection of behaviorally relevant chemical cues such as host tree volatiles and aggregation pheromones. Although *Ips typographus* have been extensively studied, the olfactory systems of other *Ips* species, namely *I. duplicatus*, *I. acuminatus*, and *I. cembrae*, remain poorly characterized, despite their differing host preferences and ecological roles.

This thesis adopts a comparative, multi-method approach integrating scanning electron microscopy, electrophysiology, and behavioral assays to address these gaps. The study focuses on mapping antennal morphology, assessing intraspecific factors such as body size in olfactory-mediated behavior, and functionally classifying OSN responses to ecologically relevant chemical cues. The study system consists of four closely related conifer-feeding bark beetles, including the Eurasian spruce bark beetle (*Ips typographus*), the double-spined bark beetle (*Ips duplicatus*), the pine bark beetle (*Ips acuminatus*), and the larch bark beetle (*Ips cembrae*).

1.2.1. Research aims:

The following broad research aims serve as the foundation for this thesis:

1. To broaden understanding of species-specific olfactory adaptations in the studied conifer-feeding bark beetles.
2. To provide insights into the structural and functional organization of the peripheral olfactory system with ecological and behavioral traits in the studied *Ips* species.

1.2.2. Research hypothesis:

Given that these *Ips* species inhabit similar coniferous environments and encounter overlapping chemical landscapes, we hypothesize that these species share similar morphological and functional olfactory systems. Specifically, we expect conserved patterns in antennal morphology, olfactory detection mechanisms, and OSN profiles, with low species-specific differentiation.

1.2.3. Research objectives:

This thesis employed a combination of various techniques, such as scanning electron microscopy and electrophysiological recordings, and literature analysis; this thesis aimed to achieve the following specific objectives:

1) To investigate the antennal morphology and distribution of sensilla types in *I. duplicatus*, *I. acuminatus*, and *I. cembrae* **(Papers II and III)**
2) To examine the influence of body size on the olfactory behavior of *I. typographus* females, particularly focusing on responses to stress-related oxygenated host monoterpenes **(Paper IV)**
3) To identify and classify olfactory sensory neuron (OSN) classes in *Ips acuminatus* (pine host) and *Ips cembrae* (larch host) and compare their response profiles with those of *Ips typographus* (spruce host) **(Paper V)**

1.3. Scope of the thesis

This thesis builds upon both established literature and recent advances to explore comparative functional aspects of olfactory coding in *Ips* bark beetles. The research specifically investigates whether variations exist in antennal morphology, sensilla diversity, and the functional profiles of olfactory sensory neurons (OSNs) among *I. duplicatus*, *I. acuminatus*, and *I. cembrae*. In addition, it examines the presence of size-dependent olfactory perception within populations of *I. typographus* females, with a focus on their antennal responses to oxygenated host monoterpenes. Taken together, these investigations aim to enhance our understanding of peripheral olfactory processing in conifer-specialist bark beetles and contribute to the broader field of insect chemosensory biology.

To establish a strong scientific foundation, the thesis is organized into the following structure:

- **Chapter 2** presents a comprehensive literature review summarizing the current state of knowledge regarding bark beetle pheromone communication, peripheral olfactory mechanisms, and OSN functional organization. This chapter also identifies key research gaps that form the basis for the experimental objectives pursued in the subsequent chapters.

- **Chapter 3** describes the methodological approaches used across the studies, including scanning electron microscopy (SEM) for morphological analysis, single sensillum recordings (SSR) and electroantennography (EAG) for electrophysiological profiling, and gas chromatography–electroantennographic detection (GC-EAD) for chemical stimulus identification. It details the techniques employed to investigate antennal structure, sensilla types, and functional neuronal responses across the target species.
- **Chapter 4** is the results section, organized into five sub-chapters, each addressing specific research questions derived from the literature review in Subchapter 4.1 (corresponding to Paper I):
    - **Subchapter 4.2** focuses on the antennal morphology and spatial distribution of sensilla in *I. duplicatus* (Paper II).
    - **Subchapter 4.3** presents a preliminary morphological analysis of antennal sensilla in *I. acuminatus* and *I. cembrae* (Paper III).
    - **Subchapter 4.4** examines size-dependent antennal sensitivity and behavioral responses to oxygenated monoterpenes in *I. typographus* females (Paper IV).
    - **Subchapter 4.5** characterizes OSNs in *I. acuminatus* and *I. cembrae*, with comparisons to the OSN profiles of *I. typographus* (Paper V).
- **Chapter 5** provides an integrated discussion of the experimental findings, relating them to existing studies and identifying major olfactory system structure and function patterns. The chapter also addresses the ecological relevance of the observed variations and outlines the limitations of the research.
- **Chapter 6** concludes the thesis by summarizing key findings, evaluating the main hypotheses, and offering recommendations for future research directions, particularly in the context of forest pest monitoring and management strategies.

Through the integration of morphological, electrophysiological, and behavioral data, this thesis offers new insights into the diversity and specificity of olfactory coding in *Ips* bark beetles. The work contributes to a deeper understanding of species- and size-related differences in chemosensory function. It supports the development of targeted approaches in applied entomology, particularly in managing bark beetle outbreaks and forest health monitoring.

Chapter 2: Review of literature

2.1. Abiotic disturbances in forest ecosystems

Recent shifts in global climate are having a serious impact on forest ecosystems. Rising temperatures, frequent droughts, and declining precipitation levels have increasingly weakened tree defenses, making forests more susceptible to bark beetle infestations (Jaime et al., 2024*;* Fig.1). Additionally, environmental disturbances such as windthrows, wildfires, and snow damage create large amounts of weakened or dying host material. These disturbances offer ideal breeding grounds for bark beetles and encourage population growth (Allen et al., 2015; Senf et al., 2018; Jakoby et al., 2019).

High temperatures minimize generation times, promote dispersal, and speed up bark beetle development and reproduction, all of which increase the frequency and severity of outbreak incidents (Biedermann et al., 2019; Dobor et al., 2020; Sommerfeld et al., 2021). As climate zones shift, several bark beetle species are expanding into higher elevations and northern latitudes, colonizing previously unsuitable habitats and further altering forest dynamics.

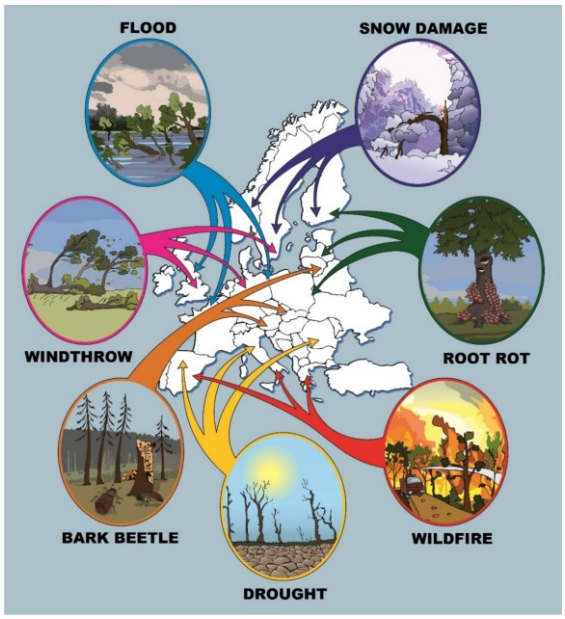

Figure 1: *Map showing key abiotic (wildfire, windstorm, drought, flood, snow damage) and biotic (bark beetle outbreaks, root rot) stressors affecting tree growth in European forests, in the context of global climate change. Adapted from Vacek et al. (2023).*

Long-term environmental stress compromises tree vigor and reduces resistance to herbivore attack. This interaction between abiotic and biotic stressors strongly influences beetle population dynamics (Netherer et al., 2024). Bark beetles usually target weaker or less competitive trees in endemic conditions to maximize offspring success. However, during outbreaks, even healthy or suboptimal trees are colonized. This often results in smaller beetles, lower pheromone

production, and reduced mating success (Pureswaran & Borden, 2003; Sallé & Raffa, 2007; Foelker & Hofstetter, 2014; Dacquin et al., 2024). Coniferous tree species in the *Pinaceae* family, including *Picea* (spruce), *Pinus* (pine), and *Larix* (larch), are especially at risk. These species rely on constitutive defense mechanisms, such as resin production, which can be compromised under prolonged heat and drought stress (McNichol et al., 2021; Netherer et al., 2021). As a result, climate-induced abiotic stressors weaken trees directly and amplify the risks posed by insect herbivores like bark beetles.

2.2. Bark beetle ecology

Bark beetles (Coleoptera: Curculionidae, Scolytinae) play a dual role in forest ecosystems. Under natural conditions, many species contribute positively to forest health by colonizing dead or dying trees. They facilitate nutrient cycling and support habitat creation, helping maintain ecological balance in mature forests (Knížek & Beaver, 2007; Hulcr et al., 2015). However, several bark beetle species, particularly in the Northern Hemisphere, have become serious forest pests. Some of these species can attack living, healthy trees, and during outbreaks, they can cause widespread mortality. Such events disturb forest dynamics, leading to substantial economic losses (Wermelinger, 2004; Hlásny et al., 2021; Jaime et al., 2024; Fig.2).

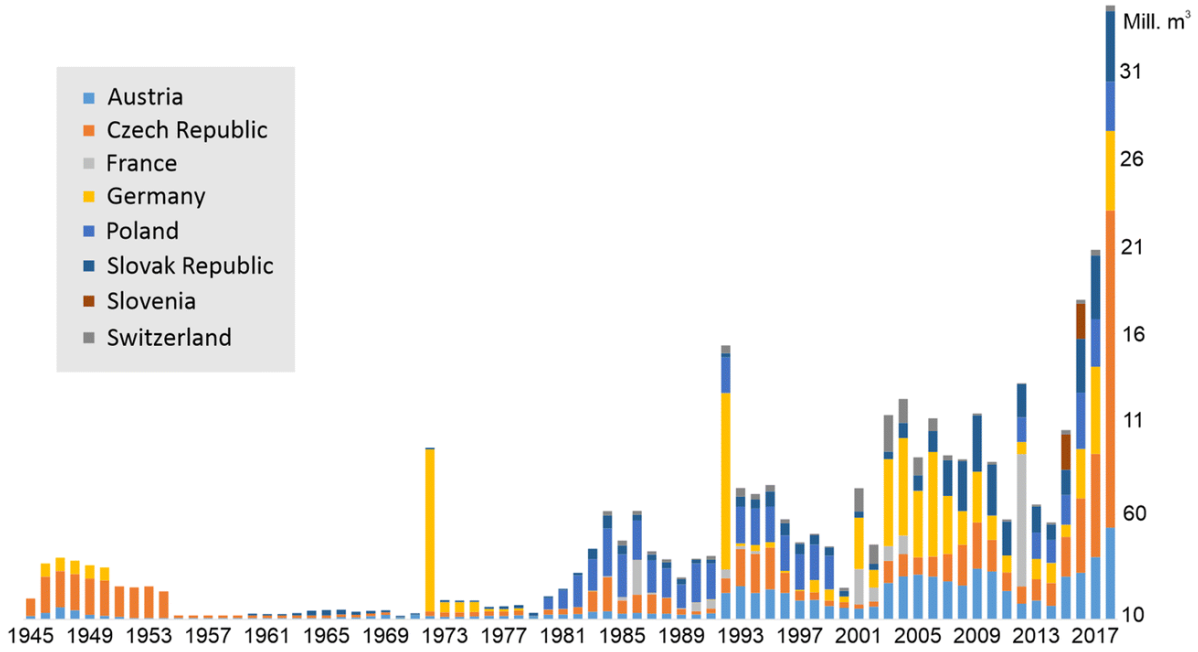

Figure 2. *Cumulative volume of Norway spruce (*Picea abies*) mortality attributed to* Ips typographus *and other bark beetle species across selected European countries over recent decades. Adapted from Hlásny et al., 2021.*

Majority of bark beetle species spend most of their life cycle beneath tree bark, where they bore galleries to feed, reproduce, and develop in the phloem. Some species also carry symbiotic blue-stain fungi, particularly from the *Ophiostomatales* group. These fungi invade the host tree's vascular tissue, reducing water transport and accelerating tree decline (Krokene, 2015). The symbiotic relationship benefits both partners; the beetles gain assistance in overcoming tree defenses while the fungi gain transport and access to new hosts, which weaken tree defenses and contribute to host decline and mortality. Associations between fungi and bark beetles are important for the environment because they can change the dynamics of competition between insect species and accelerate tree mortality. During outbreak seasons, when tree defenses are overwhelmed and both beetles and fungi reproduce quickly, these mutualistic associations are strongest. Therefore, bark beetles have a significant centralized influence on the successional patterns and composition of forests, especially in systems dominated by conifers.

2.3. Impact of bark beetle outbreaks on forest ecosystems

As already described in Section 2.1, climate-induced abiotic stress, such as drought and increased temperatures, creates conditions that increase bark beetle activity. However, the broader ecological impact of outbreaks is influenced by the beetles' behavior, reproductive strategies, and interactions with host trees. When populations shift from endemic to epidemic levels, they can cause extensive mortality in conifer forests, reshaping ecosystem structure, function, and resilience.

One of the marking attributes of bark beetle outbreaks is the ability of some species to coordinate mass attacks through aggregation pheromones. Pioneering male individuals release species-specific pheromones that attract conspecifics to the same host, facilitating them to overcome the tree's defense mechanisms. This strategy is particularly effective in physiologically stressed trees but can also work in otherwise healthy trees under high beetle pressure (Christiansen & Bakke, 1988; Byers, 2007; Raffa et al., 2016; Keeling et al., 2021). Large-scale bark beetle infestations can have great ecological consequences (Fig. 3). Outbreaks can lead to extensive dieback, significantly lowering forest biodiversity, altering population dynamics, and disrupting nutrient flow and carbon cycles. Accumulated deadwood after outbreaks often increases wildfire risk by acting as fuel, intensifying the ecological disturbance (Allen et al., 2010).

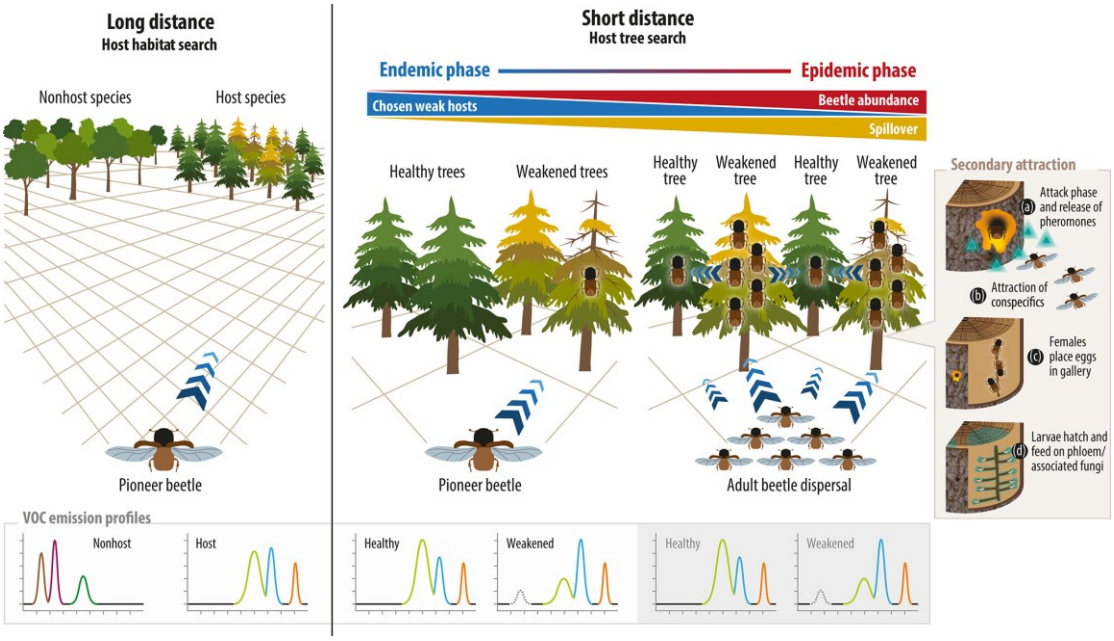

Figure 3. *Host selection behavior of* Ips typographus *on Norway spruce. Long-range attraction involves visual and VOC cues; short-range selection depends on tree stress signals. During outbreaks, aggregation pheromones trigger mass attacks (a–b), followed by egg laying and fungal inoculation in the phloem (c–d), adapted from Lehmanski et al., 2023.*

Some of the bark beetle species in the *Ips* genus are considered among the most aggressive pests, with a strong preference for conifers. Many *Ips* species show significant flexibility in their reproductive strategies, altering their voltinism (the number of generations per year) depending on environmental conditions. While some species are univoltine or bivoltine, others, particularly in North and Central America, are polyvoltine and may produce up to five generations annually (Wood, 1982; Byers, 2007). This reproductive plasticity gives them a strong advantage in warmer climates, where longer growing seasons and higher temperatures support faster development and higher population growth (Christiansen & Bakke, 1988; Raffa et al., 2016).

Recent observations support this pattern. In parts of Central Europe, rising temperatures have enabled *I. cembrae*, which was previously restricted to low-elevation forests, to produce up to two generations per year (Byers, 2007). At the same time, *I. typographus* is expanding its range into higher-elevation forests, formerly less suitable for its development. Warmer temperatures facilitate increased voltinism, which is closely associated with this ascending trend (Keeling et al., 2021). These variations imply that, in addition to making bark beetle outbreaks more severe, climate change is also making

it possible for them to spread into previously unsuitable environments, which could have long-term effects on the management and health of forests.

2.4. Bark beetle chemical communication

Chemical communication is central to bark beetle ecology, mediating crucial behaviors such as host selection, aggregation, mating, and avoidance of unsuitable host trees. These beetles primarily use volatile organic compounds (VOCs) emitted by trees to assess host identity, physiological conditions, and stress status (Jirošová et al., 2022a; Moliterno et al., 2023). Host-emitted volatiles act as attractants, guiding beetles toward weakened or susceptible trees. In contrast, non-host volatiles (NHVs), typically released by deciduous trees, function as repellents, helping beetles avoid unsuitable trees and boosting host specificity in mixed-species forests *(*Zhang and Schlyter, 2004, Fig.4*)*.

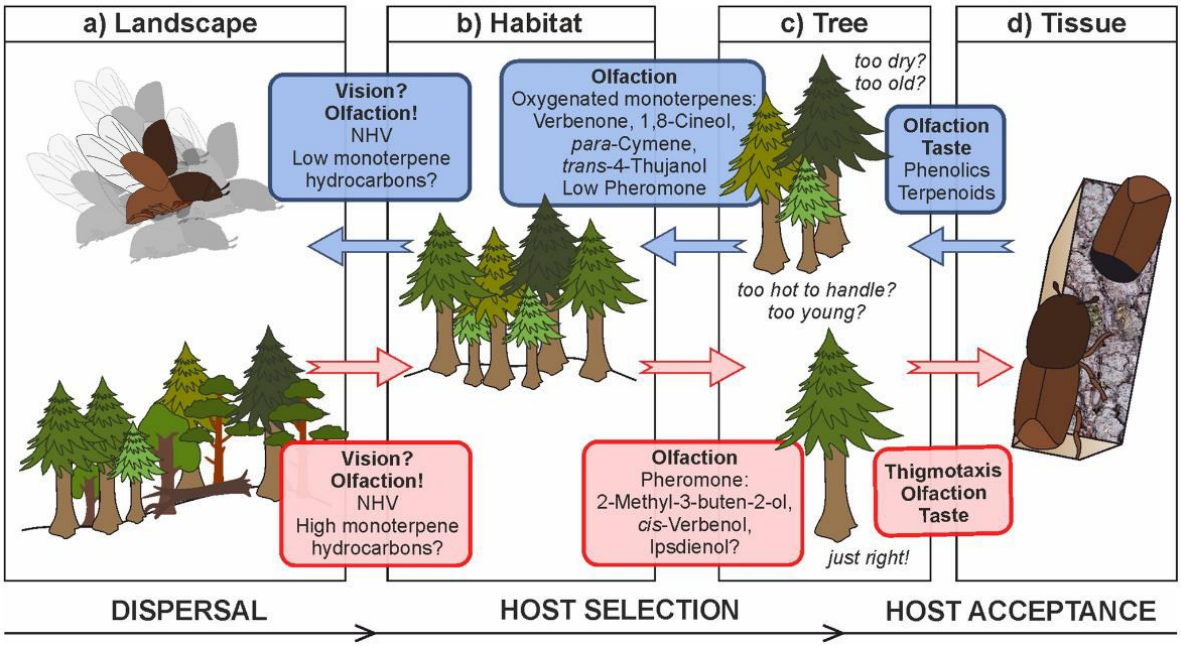

Figure 4. *Schematic representation of* I. typographus *host location and acceptance behaviour. The process shows from long-range dispersal to host entry, integrating olfactory signals from host volatiles, non-host volatiles (NHVs), beetle pheromones, and fungal associates. Adapted from Netherer et al. (2021).*

In addition to host- and non-host volatiles, bark beetles respond to chemical signals from their symbiotic fungi, mainly ophiostomatoid species, which colonize host tissues following beetle entry. These microbial VOCs influence beetle behavior by enhancing aggregation and signaling successful colonization, further supporting beetle development within the host (Jirošová et al., 2022b; Kandasamy et al., 2019, 2023). At the same time,

anti-aggregation pheromones help regulate colonization density by signaling resource saturation, thus reducing competition and overexploitation of host resources (Frühbrodt et al., 2024).

Beetles also respond to volatiles produced by other bark beetle species and their associated microbes. This broader and complex network of interspecific chemical signaling likely reflects the ecological complexity of forest environments, where multiple species interact and compete within shared habitats (Andersson et al., 2009; Schiebe et al., 2019; Yuvaraj et al., 2024).

2.4.1. Aggregation Pheromones in *Ips* Bark Beetles

Aggregation pheromones are central to the successful colonization strategy of *Ips* bark beetles. In *Ips* bark beetles, pioneering males release aggregation pheromones during the initial phase of host colonization. These compounds attract both sexes to the same tree, enabling coordinated mass attacks to overcome the tree's defenses. These same pheromones also act as mating signals, enhancing reproductive success alongside colonization efficiency.

The first bark beetle pheromones identified, i.e., ipsenol, ipsdienol, and *cis*-verbenol, were isolated from *I. paraconfusus* (Silverstein et al., 1966). Their structures are similar to host-derived monoterpenes such as myrcene and α-pinene, suggesting beetles may synthesize these pheromones from tree-derived precursors (Hughes, 1973, 1974, Fig.5). This metabolic link reflects the close ecological association between pheromone signaling and host volatile chemistry.

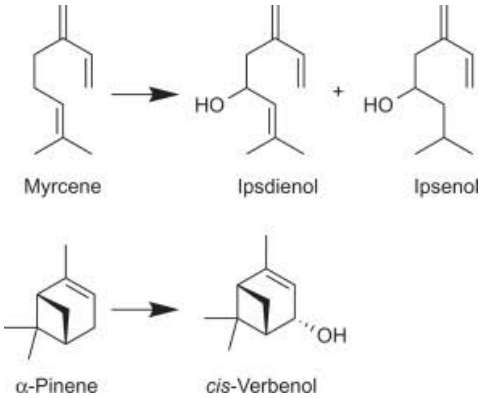

Figure 5. *Chemical structures of major* Ips *pheromones (ipsenol, ipsdienol, and cis-verbenol) and their proposed precursors myrcene and α-pinene from conifer monoterpenes. Adapted from Keeling et al. (2021).*

Most *Ips* pheromones are oxygenated hemi- and monoterpenes that closely resemble the resin compounds of their conifer hosts. Some of the components include ipsdienol, ipsenol, *E*-myrcenol, amitinol, and *cis*-verbenol, as well as byproducts like lanierone and 3-methyl-3-buten-1-ol (Byers, 2007; Cognato, 2015). Despite the limited number of structural components, species-specific pheromone blends have variations through differences in overall composition, relative concentration, and stereochemistry, especially enantiomeric ratios (Cognato, 2015; Keeling et al., 2021). Even minor variations in enantiomeric ratios minimize cross-attraction between sympatric species and contribute to reproductive isolation within the *Ips* genus (Table 3).

2.4.2. Enantiomeric Specificity of *Ips* aggregation pheromones

Many pheromone components used by *Ips* spp. exist in enantiomeric forms, with species often showing strict preferences for specific stereoisomers. These preferences are critical for maintaining species recognition and reproductive isolation, especially in habitats where multiple *Ips* species co-occur and where similar compounds may be shared. For example, most *Ips* species rely predominantly on (*S*)-(−)-*cis*-verbenol and (*S*)-(−)-ipsenol as main aggregation pheromone components (Byers, 2007), while their response to the opposite enantiomers is much weaker or entirely absent. Ipsdienol, another component, shows significant variability in its enantiomeric ratios between different species (Table 3). In particular, the enantiomeric ratio of ipsdienol varies among species and sometimes even in populations within the same species, highlighting its role in prezygotic isolation (Byers & Levi-Zada, 2022).

2.4.3. Host Tree Volatiles and Bark Beetle Attraction

The volatile organic compounds (VOCs) emitted by host trees play a fundamental role in guiding bark beetles towards suitable habitats. These airborne signals can help beetles navigate in the environment. In *Ips typographus*, for example, host trees such as Norway spruce (*Picea abies*) emit common monoterpenes, including α-pinene, β-pinene, limonene, and β-phellandrene, all of which are known attractants (Hulcr et al., 2006; Netherer et al., 2021). Recently, studies have identified oxygenated monoterpenes, though present in trace amounts (less than 1% of total emissions), for their exceptionally strong influence on beetle behavior. Compounds like isopinocamphone and 1,8-cineole elicit strong antennal responses and appear central as short-range host acceptance cues (Kalinová et al., 2014; Schiebe et al., 2019). These volatiles are regularly linked with

stressed or recently felled trees, making them markers for host targeting. They are produced not only from the host tree but also from fungal symbionts, adding further complexity to the chemical interface between beetles and their environment (Celedon & Bohlmann, 2019; Kandasamy et al., 2023).

2.5. Bark beetle outbreak control and management

One of the main challenges is controlling bark beetle outbreaks across large forest landscapes. Traditional techniques, such as applying pesticides, are typically used cautiously because of their impracticality. Environmental problems, legal limits (particularly in the EU), and potential effects on non-target organisms render them unsuitable for landscape-level control, despite their potential effectiveness in high-value or local small-scale environments (Hlásny et al., 2021).

On the other hand, semiochemical-based approaches, especially those involving pheromones, offer more directed and eco-friendly alternatives. Aggregation pheromone composition has been identified in more than 20 *Ips* species, which include some of the most economically important pests (El-Sayed, 2025). Nonetheless, most pheromone-based approaches have been directed at monitoring rather than active population control.

Semiochemical-based techniques often yield inconsistent results when used alone, especially under outbreak conditions. Their efficacy varies greatly depending on location characteristics, beetle population density, and the presence of competing attractants or host material. To improve efficiency, pheromone-based methods can be combined with silvicultural practices, such as thinning and sanitation harvesting, that reduce host availability and improve forest resistance (Lubojacký et al., 2014; Gallo et al., 2020; Table 1). This integrated approach is increasingly favored in forest health strategies, balancing ecological considerations with operational feasibility.

Modern forest pest management is based on this integrated approach, representing an increasing trend toward multidimensional, environmentally based tactics. These techniques seek to strike a compromise between environmental sustainability and controlling outcomes, especially because of the growing rate of climate change and the rise in disturbance regimes.

Table 1: Overview of current control strategies: effectiveness and limitations

Strategy	Effectiveness	Challenges	References
Silviculture (thinning, sanitation)	Moderate	Requires timely detection and proactive action	Gallo et al., 2020; Holuša and Fiala 2025
Pheromone-based (mass trapping, push–pull)	Variable	Site- and species-specific outcomes; deployment complexity	Lubojacký et al., 2014, Jakuš and Zhang, 2003, Deganutti et al., 2024
Biological control (predators, entomopathogens)	Limited	Not fully scalable; mixed success in field trials	Hajek and Delalibera, 2010, Mann and Davis, 2021
Insecticides (Last option, banned in Europe)	Localized effectiveness	Environmental impact; non-target risk	Gillete and Fettig et al., 2021

2.5.1. Mass Trapping ("Attract-and-Kill")

Pheromone-baited traps or trap trees are used in mass trapping to draw in and catch bark beetles before they infest living hosts. This approach can lower local beetle populations, but its effectiveness has been inconsistent, especially in small-scale applications or the early stages of epidemics (Byers, 2007; Lubojacký et al., 2014). Its efficiency, however, varies greatly depending on the situation and is affected by beetle pressure, trap density, and spatial arrangement. Inadequate implementation may unintentionally draw in additional beetles without successfully eliminating them, raising the possibility of infestation. Another issue is non-target by-catch, since traps can collect beneficial insects like pollinators and predators (Brockerhoff et al., 2023). For large-scale control, mass trapping is therefore rarely enough. It works best when combined with insecticides, repellents or silvicultural techniques as part of an integrated pest management (IPM) approach to improve overall efficacy and ecological compatibility.

2.5.3. Anti-Aggregation Signals

Anti-aggregation pheromone derivatives, such as verbenone, non-host compounds, or defensive compounds from conifers (Schiebe 2011), serve as chemical repellents, preventing beetles from further colonization of already attacked or healthy trees. These "push" cues are useful in controlling beetle density and protecting uninfested hosts. While such compounds have shown promising efficacy in some *Dendroctonus* species, their use in *Ips* beetles is still under development (Schebeck et al., 2024). One main limitation is that push-only strategies can relocate beetles to untreated areas, especially in fragmented forests or under high beetle pressure. Improving cost-effectiveness and application precision will be important steps toward broader implementation. Nonetheless, anti-aggregation signals are a promising part of integrated pest management. Ongoing research aims to improve utilization strategies, improve compound formulations, and evaluate context-specific outcomes to support more reliable and sustainable bark beetle control (Frühbrodt et al., 2024).

2.5.4. Push-Pull Strategy

The push–pull strategy combines repellent cues ("push") with attractive pheromones ("pull") to divert bark beetles away from vulnerable trees and toward traps or baited trap trees. This technique has proven effective in North American species like *Ips paraconfusus* and *Ips pini* by disrupting host colonization (Byers & Levi-Zada, 2022). In Europe, similar approaches using anti-attractants with pheromone traps or trap trees show promise for *Ips typographus* management (Jakuš et al., 2022; Lindmark et al., 2022). However, their efficacy declines under high beetle pressure or during severe drought, when stressed trees become more attractive despite repellent cues (Deganutti et al., 2024; Keeling et al., 2021). Additionally, success depends on factors such as the timing, spatial deployment, and release rates of semiochemicals and the surrounding landscape structure.

Despite these drawbacks, push-pull tactics are valued for their flexibility and low environmental impact. Together with silvicultural practices, they improve the overall resilience of forests and could provide effective alternatives for chemical pesticides in environmentally vulnerable locations (Keeling et al., 2021).

2.5.5. Toward Integrated Pest Management (IPM)

Multilayered approaches are needed to manage bark beetle epidemics effectively over the long run. IPM aims to minimize environmental damage while achieving sustainable control by combining ecological, behavioral, and silvicultural techniques. Pheromone-based strategies, including mass trapping or push-pull techniques, work best when combined with habitat changes, thinning, or sanitation harvesting (Gallo et al., 2020).

Biological control remains limited despite its potential due to its uneven field results and application issues. Similarly, insecticides are now rarely used in Europe due to environmental regulations and concerns over non-target effects (Gillete and Fettig et al., 2021). IPM acknowledges that no single strategy works alone. Early identification, site-specific adaptability, and the capacity to combine complementary approaches are ultimately essential for success. Continued research into species-specific behavior, olfactory ecology, and ecosystem interactions will be essential for developing resilient, adaptive forest pest management strategies.

2.6. Insect olfactory system

Olfaction plays a fundamental role in the lives of insects, guiding them in essential behaviors like finding hosts, locating mates, avoiding predators, and selecting habitats. These tiny creatures depend on volatile chemical signals to navigate in their complex ecological landscapes.

These signals can come from various sources, including plant-emitted volatiles, pheromones, associated microbes, and even volatiles released by their predators or natural enemies (Visser, 1986; Bruce & Pickett, 2011). The process of olfactory detection starts at the periphery (Fig. 6). Airborne odor molecules diffuse via porous, specialized structures called sensilla. These sensilla are predominantly located on the antennae (Hallberg, 1982a) and sometimes on mouthparts and other appendages (Hallberg, 1982b). Inside each sensillum, olfactory sensory neurons (OSNs) detect and process these odorants. The dendritic membranes of OSNs contain membrane-bound chemoreceptors: primarily odorant receptors (ORs) (Clyne et al., 1999), ionotropic receptors (IRs) (Benton et al., 2009), and occasionally gustatory receptors (GRs) (Wicher, 2018). These receptors convert chemical signals into neural impulses that travel to the antennal lobes and are further interpreted by the brain (Martin et al., 2011), leading to behavior (Andersson et al., 2015).

The insect olfactory system is both evolutionarily conserved and ecologically diverse. The insect olfactory system is often finely tuned to meet the specific needs and behaviors of different insects. Given the ecological importance of olfaction, insect olfactory systems have evolved remarkable diversity and sensitivity, adapting to the specific needs and habitats of different species (Hansson & Stensmyr, 2011; Carraher, 2015).

2.6.1. Olfactory Organization in Bark Beetles

Bark beetles rely heavily on their sense of smell to find suitable hosts and coordinate aggregation and mass-attack. Their primary olfactory organs, the club-shaped antennae (Payne et al., 1973), are densely covered with porous sensilla, each housing OSNs tuned to detect volatiles from host trees, conspecifics, and symbiotic fungi (Hansson & Stensmyr, 2011; Fig. 6). These sensilla contain one to three olfactory sensory neurons (OSNs) placed in a lymph-filled chamber. Odorant molecules pass through tiny pores on the sensillum surface and are transported by odorant-binding proteins (OBPs) to receptors on the dendritic membranes of olfactory sensory neurons (OSNs).

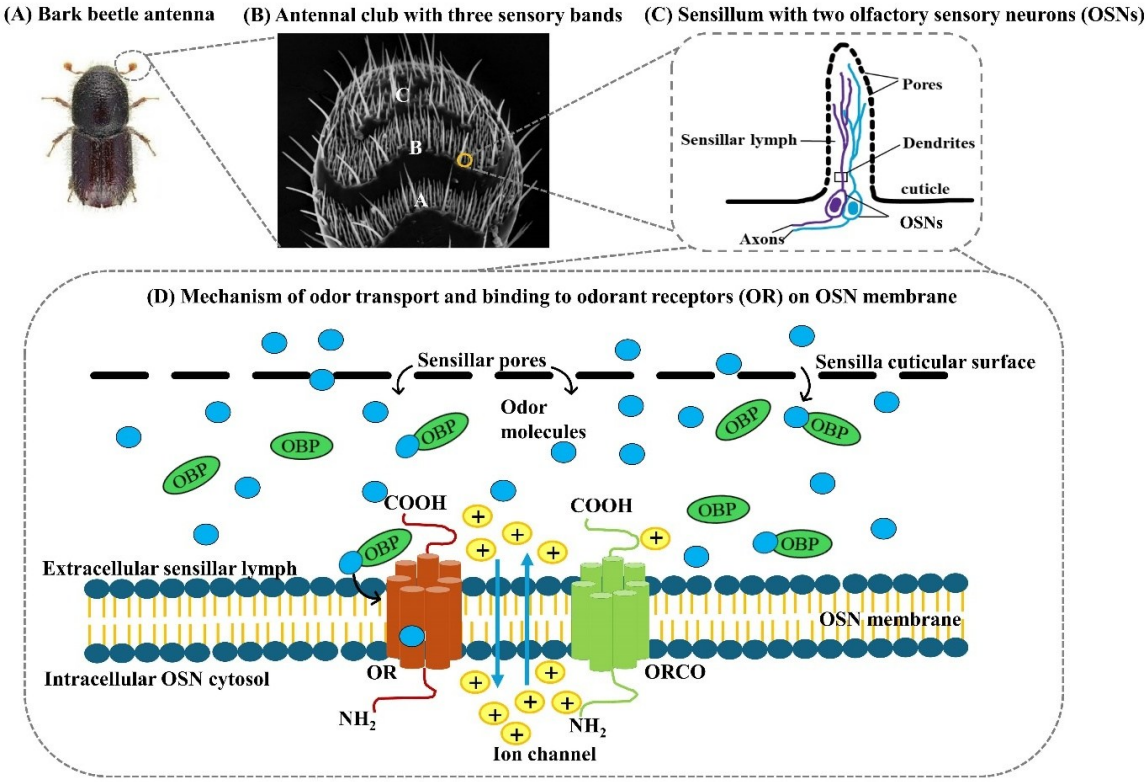

Figure 6. *Peripheral olfactory system in* I. typographus. *(A) Adult beetle with antennae. (B) SEM image of antennal club showing sensory bands A–C. (C) Structure of an olfactory sensillum with two OSNs. (D) Schematic of odor detection: odorants enter via*

pores, are transported by OBPs, and activate OR-ORCO complexes to trigger neural signals. Adapted from Ramakrishnan et al., unpublished.

Each OSN is functionally tuned, with some acting as specialists, responding only to a narrow range of compounds (e.g., specific pheromone enantiomers), while others function as generalists, reacting to broader sets of structurally related volatiles (Hallem & Carlson, 2006; Binyameen et al., 2014). This balance between precision and flexibility allows beetles to discriminate among complex odor blends in dynamic environments. OSNs express different classes of chemoreceptors, including odorant receptors (ORs) (Clyne et al., 1999) that detect host volatiles and pheromones, ionotropic receptors (IRs) that respond to acids and amines, and gustatory receptors (GRs) which are typically associated with taste but also implicated in CO_2 and bitter odorant detection (Clyne et al., 2000; Wicher, 2018; Fig. 7). ORs tend to function together with a co-receptor, ORCO, to form ligand-gated ion channels. These receptors generate action potentials that initiate the olfactory signal cascade upon activation.

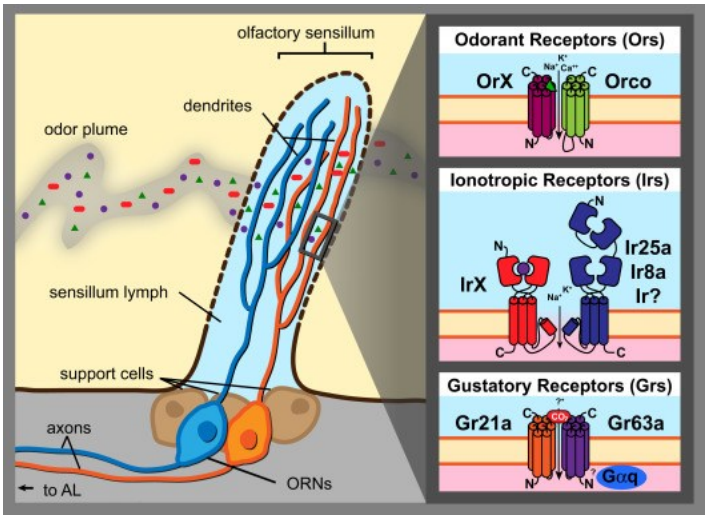

Figure 7. *Schematic overview of insect chemosensory receptor classes and peripheral olfactory signal transduction. ORs, IRs, and GRs form the molecular basis of odor detection, translating chemical signals into electrical activity in OSNs. Adapted from Pask & Ray (2016).*

OSNs in bark beetles exhibit variable specificity, ranging from specialists that are highly selective neurons tuned to specific pheromones or enantiomers to generalists that respond to broader environmental cues (Hallem & Carlson, 2006; Carey et al., 2010). Specialist OSNs detect sex pheromones at extremely low concentrations, capable of enantiomeric discrimination (Wojtasek et al., 1998). Generalist OSNs are typically tuned to host

volatiles and non-pheromonal cues, playing a role in host selection (Andersson et al., 2010; Binyameen et al., 2014). Together, this diversity supports both precise pheromone-mediated behaviors and flexible environmental sensing.

The organization of sensilla and OSNs is not random. In *Ips* species, the antennal club contains three distinct sensory bands (A, B, and C), each having specific sensillum types (Hallberg, 1982a; Shewale et al., 2023). These bands are densely populated with olfactory sensilla. OSNs within these sensilla vary in tuning breadth, with narrowly tuned OSNs responding selectively to host volatiles or pheromones, while some neurons exhibit broader tuning, reacting to structurally related compounds (Andersson et al., 2009; Kandasamy et al., 2019, 2023). This highly tuned system enables bark beetles to respond with great specificity to environmental cues, discriminating between tree species and physiological status and between pheromone enantiomers and microbial volatiles.

2.6.2. Structure and Function of the Antennae

Antennae are the main olfactory sensory organs in *Ips* bark beetles (Hallberg, 1982a; Faucheux, 1989, 1994). The appendages are abundantly equipped with sensilla-carrying sensory neurons that detect chemical, mechanical, thermal, and humidity stimuli (Hallberg, 1982; Faucheux, 1989, 1994; Hallberg et al., 2003). The antennal surface is dominated by olfactory sensilla responsible for host volatile and pheromone detection. Additional sensory modalities are supported by mechanoreceptors and thermoreceptors within the antennae. In certain behavioral contexts, particularly during host evaluation or oviposition, secondary sensory organs such as maxillary palps and ovipositors may also contribute to chemical perception (Payne et al., 1973; Hallberg, 1982b).

2.6.3. Sensilla types and functional diversity

The diversity of sensilla on bark beetle antennae reflects their ecological specialization and chemical sensitivity. Each sensillum is a small, morphologically distinct structure with specific functional roles, enabling beetles to detect a wide range of semiochemicals. Olfactory sensilla are central to odor detection (Schneider, 1964). Their morphological diversity is closely linked to functional specialization, enabling insects to discriminate among the various chemical cues (Hallberg et al., 2003). Among the most common sensilla are sensilla trichodea, elongated, hair-like, which are involved in pheromone detection. Sensilla basiconica are generally shorter and peg-like, highly sensitive to host

and food-related volatiles. These two types dominate the antennal surfaces in many *Ips* species and house most OSNs (Hallberg et al., 2003; Shewale et al., 2023; Fig. 8).

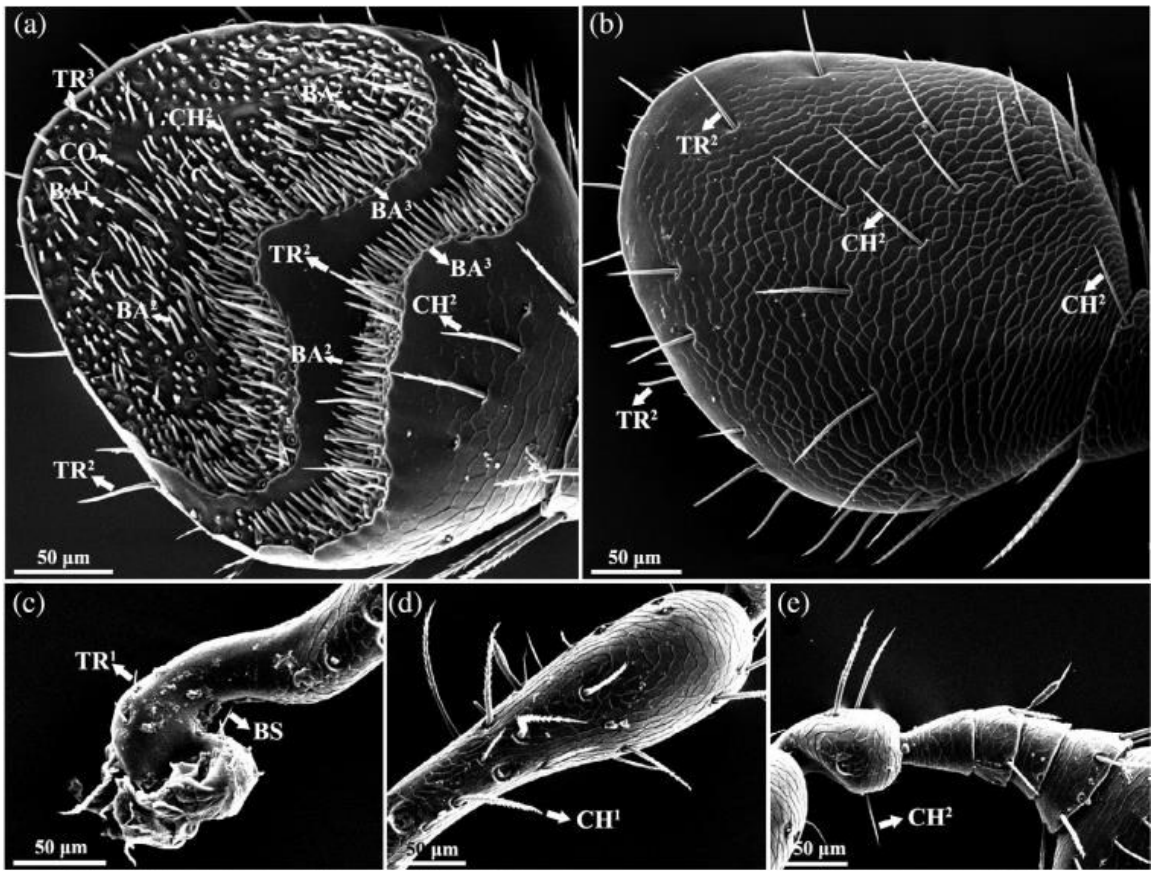

Figure 8. *Scanning electron micrograph showing* I. typographus *antennae showing major types of olfactory sensilla. Labeled structures include sensilla trichodea (TR), sensilla basiconica (BA), and sensilla chaetica (CH). Adapted from Shi et al. (2021).*

Other sensilla types, although not strictly olfactory, contribute to the insect's broader sensory perception. Sensilla coeloconica are peg-like, double-walled structures that detect amines, carboxylic acids, ammonia, and humidity (Yao et al., 2005; Prieto-Godino et al., 2017). Sensilla chaetica are mechanosensory and gustatory, largely involved in tactile and taste perception. Sensilla styloconica and sensilla ampullacea are typically associated with thermoreception and hygroreception, allowing the insect to sense temperature and humidity changes (Ruchty et al., 2010; Schneider et al., 2018). Böhm's bristles are located near the antennal base, show mechanoreceptive function, and respond to antennal movement and wind flow. This structural and functional diversity allows bark beetles to process complex chemical landscapes with high specificity, combining cues related to host tree identity, condition, and interspecies interactions (Suh et al., 2014; Pelosi et al., 2018).

While antennal sensilla have been well-characterized in species like *I. typographus*, *I. sexdentatus*, *I. pini*, and *I. subelongatus*, detailed studies on *I. duplicatus* remain limited (Payne et al., 1973; Hallberg, 1982a; Faucheux, 1989). Investigating sensilla distribution and morphology in this species is critical for understanding its olfactory perception and host selection mechanisms.

2.6.4. Central Olfactory Processing in Bark beetles

When odor molecules are detected by OSNs on the antennae, they are transmitted to the antennal lobes (ALs), the primary olfactory center of the insect brain. There, OSN axons target specialized structures called glomeruli, which are separate processing units for individual odorants (Vosshall et al., 2000; Gao et al., 2000). OSNs that share a receptor type send input to a given glomerulus, and the antennal lobe thereby spatially translating odor quality and intensity. Output from the processed information in the antennal lobes is sent via projection neurons (PNs) to higher-order brain regions. These include the mushroom bodies (MBs), which mediate learning, memory, and decision-making, and the lateral horn (LH), which controls more reflex and instinctual behaviors (Galizia, 2014; Clark & Ray, 2016).

This neural structure allows bark beetles to combine olfaction-based data with other sensory modalities, like visual or mechanosensory information, thereby allowing context-dependent behavioral responses. It also enhances the capacity for odor discrimination with high accuracy, showing the capacity to discriminate between pheromone enantiomers or slight differences in the volatile organic compound profiles of the host trees. Understanding this critical processing system is crucial to understanding how to correlate peripheral olfactory perception with behavioral response. Additionally, it provides valuable insights into the mechanisms by which bark beetles make rapid, ecologically relevant decisions in chemically complex environments (Raffa et al., 2016).

2.7. Influence of Beetle Size on Behavior

Bark beetle body size is a key trait that determines behavior, reproductive success, and olfactory sensitivity. It is determined by developmental conditions, especially resource quality and competition in the host, and is both interspecific and intraspecific, sometimes even brood-specific. In *I. typographus*, it can influence the beetle host location, responses to pheromone, and reproductive success (Foelker & Hofstetter, 2014; Dacquin et al., 2024).

Small males, often the products of inadequate larval nutrition or high brood densities, are typically associated with diminished aggregation pheromone production. This also consequently lowers their attractiveness to females and their capacity for inducing mass attack, thereby removing mating success and offspring quality (Anderbrant et al., 1985; Pureswaran & Borden, 2003). Dominant males, on the other hand, produce more pheromones and have better chances of acquiring mates in polygynous systems like that of *I. typographus* (Schebeck et al., 2023).

Female beetles also have size-dependent differences in behavior. The larger females are more selective in host and mate choice and respond more strongly to semiochemicals (Müller et al., 2020). As the primary task of constructing maternal galleries relies on females, their host selection choice directly affects larval survival and development. Overall, intraspecific size variation adds yet another aspect of complexity to bark beetle olfactory ecology. Besides impacting signal production and detection, it influences ecological strategies, including competition, reproduction, and host use.

2.8. Olfactory Systems and Sensory Perception in *Ips typographus*

Ips typographus is the most economically important forest pest in Europe, being the primary pest of *P. abies* (Norway spruce). In the past decade alone, it has been responsible for losing over 70 million cubic meters of spruce timber on the continent (Hlásny et al., 2021).

Male beetles are pioneers, using a combination of visual and chemical cues to locate physiologically stressed trees. Once a host is located, the male bores into the bark and releases aggregation pheromones, such as (−)-*cis*-verbenol and 2-methyl-3-buten-2-ol, which attract conspecifics and lead to mass attacks (Franceschi et al., 2005; Raffa et al., 2016). This strategy enables beetles to overcome tree defenses and colonize (Schebeck et al., 2023). The role of the female is also equally crucial. Females construct maternal galleries and inoculate symbiotic ophiostomatoid fungi, which assist in overcoming tree defenses and provide a source of nutrition to the developing larvae (Paynter et al., 1990; Kandasamy et al., 2023).

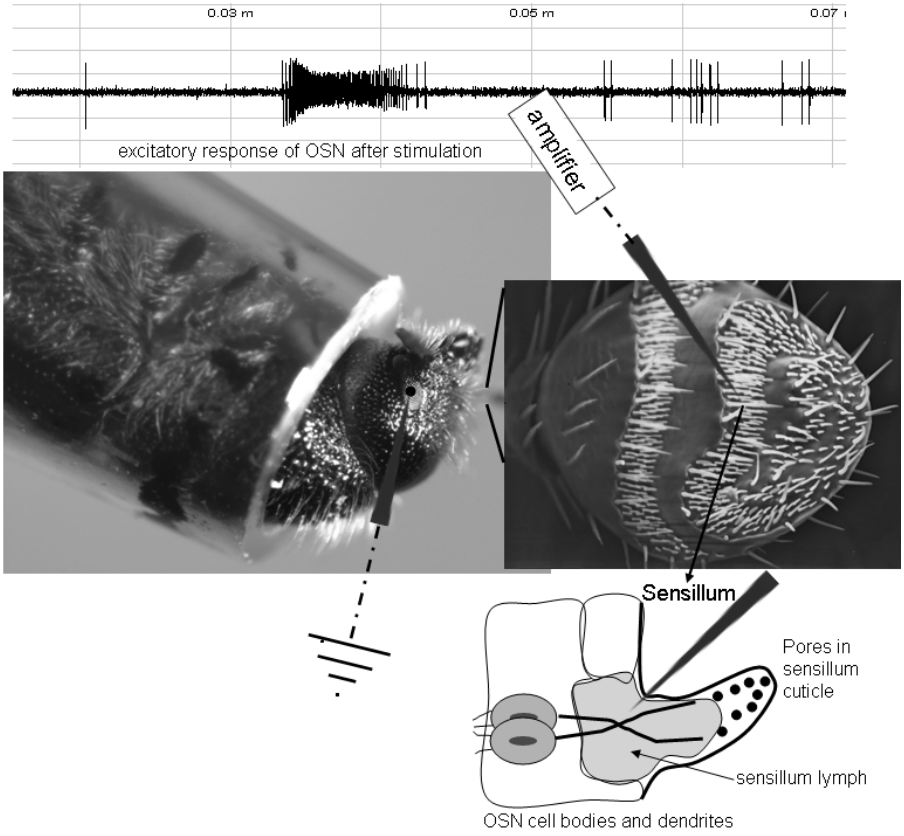

Figure 9. *Basic electrophysiological setup for recording bark beetle OSN responses to semiochemicals using SSR or EAG. Adapted from Schiebe (2012).*

Electrophysiological studies have revealed a remarkably varied repertoire of olfactory sensory neurons (OSNs) in *I. typographus*, comprising at least 26 functionally different classes of OSNs (Fig. 9; Table 2 for identified OSN classes in *I. typographus*). They are spatially distributed across the entire antennal club and are selectively tuned to various stimuli. Some neurons respond strongly to oxygenated monoterpenes such as 1,8-cineole and (+)-isopinocamphone, which are linked to host stress and may enhance pheromone signaling. Others are tuned to fungal volatiles or green leaf volatiles (GLVs), supporting host selection and reproductive isolation. Refer to Table 1: OSN classes in *I. typographus* and their response profiles. One of the first electrophysiological studies suggests that *I. typographus* can distinguish between enantiomers of key compounds, supporting high olfactory decision-making (Tömmerås 1985). This capacity likely underpins both mating specificity and host discrimination. Field experiments have further demonstrated that *I. typographus* can spatially distinguish between these compounds, highlighting the beetle's refined olfactory discrimination abilities (Binyameen et al., 2014). Its thoroughly characterized OSN map and behavioral responses offer a standard against which to

compare other *Ips* species and determine both conserved and species-specific features of bark beetle chemoreception.

Table 2: Identified OSN classes in *I. typographus* and their responses to ecologically relevant chemical compounds from different sources (Modified from Raffa et al., 2016 and updated to recent findings)

OSN classes	Biological sources	OSN class (Primary responses)	OSN class (Secondary responses)	References
1.	Beetle	(−)-*cis*-Verbenol	(−)-Verbenone (±) camphor	(Andersson et al. 2009; Schiebe et al. 2019)
2.	Beetle	Ipsenol	(±) ipsdienol	(Tömmerås 1985)
3.	Beetle	Ipsdienol A and B	(±) ipsenol, aminitol	(Andersson et al., 2009)
4.	Beetle	2-methyl-3-buten-2-ol	Aminitol 2-methyl-1-butanol pinocarvone	(Andersson et al., 2009; Kandasamy et al., 2019)
5.	Beetle	Aminitol	(±)ipsdienol	(Andersson et al., 2009)
6.	Beetle	Lanierone	-	(Yuvaraj et al., 2024)
7.	Beetle/fungi	(−)-Verbenone	(−)-*trans*-verbenol α-isophorone	(Andersson et al., 2009; Kandasamy et al., 2023)
8.	Beetle/fungi	2-Phenylethanol	2-phenethyl acetate 3-methyl-1-butanol Chavicol Benzyl alcohol	(Kandasamy et al., 2019)
9.	Host	(+)-α-Pinene	(−)-β-pinene, (−)-*cis*-verbenol	(Andersson et al., 2009)
10.	Host	Myrcene	Terpinolene, 4-terpineol Phenethyl acetate Isoamyl acetate	(Andersson et al., 2009; Kandasamy et al., 2019; Schiebe et al., 2019)
11.	Host	p-cymene	(±) limonene, Δ3-carene (+) borneol Terpinolene γ-terpinene (±)-Carvone	(Andersson et al., 2009; Schiebe et al., 2019)

12.	Host	1,8-cineole	(±)chalcogran, *trans*-conophthorin	(Andersson et al., 2009)
13.	Host	Δ3-carene	*trans*-conophthorin, (±)*exo*-brevicomin, methyl-2,4-decadienoate	(Andersson et al., 2009)
14.	Host	Pinocarvone	(−)-β-pinene (±) camphor 4-allylanisole (estragole)	(Schiebe et al., 2019)
15.	Host/fungi	Estragole	?	(Raffa et al., 2016)
16.	Host/fungi	(+)-*trans*-4-thujanol	Terpine-4-ol 3-octanol	(Kandasamy et al., 2023; Schiebe et al., 2019)
17.	Host/fungi	(+) isopinocamphone	(+)-pinocamphone	(Kandasamy et al., 2023)
18.	Non-host	GLV-OHs (green leaf volatile alcohols) 1-Hexanol (*E*)-2-Hexenol (*Z*)-3-Hexenol	2-methyl-3-buten-2-ol Hexanal (±)-1-Octen-3-ol *E*2-Hexenol	(Andersson et al., 2009)
19.	Non-host/fungi	3-octanol	(−)Bornyl acetate (±)-1-Octen-3-ol	(Andersson et al., 2009)
20.	Non-host/fungi	1-octen-3-ol	(±)chalcogran 3-octanol	(Andersson et al., 2009)
21.	Non-host/fungi	(5*S*, 7*S*)-*trans*-conophthorin	(±)chalcogran (±)*exo*-brevicomin (*R*,*R*)-trans-conophthorin Dehydro-conophthorin	(Andersson et al., 2009; Unelius et al., 2014)
22.	Non-host/ fungi	Geranyl acetone	Geranyl acetate	(Kandasamy et al., 2019)
23.	Fungi	3-Methyl-1-butanol	?	?
24.	Fungi	2-Methyl-1-butanol	?	?
25.	Fungi	3-Methyl-1-butyl acetate	?	?
26.	Fungi	Styrene	2-phenylethanol	(Schiebe et al., 2019; Kandasamy et al., 2023)
27.	Fungi	2-Phenethyl acetate	?	?

?: Responses not yet identified/unknown.

2.9. Study species

2.9.1. *Ips duplicatus* (Double-Spined Bark Beetle)

Ips duplicatus is a secondary bark beetle species primarily associated with Norway spruce (*P. abies*), though it occasionally occurs in other coniferous hosts. Originally distributed in areas like Fennoscandia, Siberia, and East Asia, its range has extended southward over time into Central Europe, where it often occurs together with *I. typographus*, particularly in spruce stands at higher elevations (Holuša et al., 2010; Wermelinger et al., 2020).

Unlike *I. typographus*, *I. duplicatus* generally infests the upper sections of affected trees (Schlyter and Anderbrant, 1993) or residual wood debris after harvest. Its subtle infestation patterns, in shaded locations or inner parts of stands, often preclude early detection and confuse management strategies (Davídková et al., 2023). However, it can substantially contribute to spruce mortality under outbreak conditions, especially when present with *I. typographus* in mixed infestations (Kasák & Foit, 2015; Knízek et al., 2019).

Males produce aggregation pheromones with a structure similar to other *Ips* species, and beetles utilize host volatiles of stressed trees (Schlyter et al., 1992; Zhang et al., 2007). The species remains underinvestigated in terms of antennal morphology and OSN diversity. Little has been reported regarding its sensilla organization or functional olfactory tuning, highlighting the primary knowledge gap. Knowledge of *I. duplicatus* olfactory biology is critical for developing species-specific monitoring tools and elucidating olfactory adaptations among sympatric *Ips* species that occupy equivalent ecological niches.

2.9.2. *Ips acuminatus* (Pine Bark Beetle)

The pine bark beetle, *Ips acuminatus*, is a common secondary pest of Scots pine (*Pinus sylvestris*) across European forests (Liška et al., 2021; Papek et al., 2024). While it mostly colonizes stressed or felled trees, climate change, especially heat stress and droughts, has increased the number of susceptible hosts, adding outbreak potential (Wermelinger et al., 2008; Thabeet et al., 2009). This species coexists with other pine bark beetles, including *I. sexdentatus*, *Tomicus piniperda*, and *Tomicus minor*, but exhibits distinct microhabitat preferences.

Ips acuminatus inhabits the upper sections of tree trunks and crowns, while *I. sexdentatus* prefers thicker lower trunks (Pfeffer, 1955; Petterson, 2000). Upon host location, males produce aggregation pheromones composed of *S*-(−)-ipsenol, *S*-(+)-ipsdienol, and (4*S*)-*cis*-verbenol, which attract both sexes for mass colonization (Bakke, 1978; Francke et al., 1986). Like its relatives, *I. acuminatus* is polygynous (Kirkendall, 1989, 1990) and maintains close associations with blue-stain fungi (*ophiostomatoid fungi*), which may aid in nutrition and host degradation (Francke-Grosmann, 1965; Villari et al., 2012).

Despite its ecological importance, *I. acuminatus* is understudied regarding olfactory morphology and physiology. Its antennal sensilla structure and OSN responses to host or fungal volatiles have not been fully characterized. This gap hinders our understanding of how this species navigates chemically complex pine forests and responds to environmental change. In this thesis, *I. acuminatus* will serve as a comparison to the well-studied *I. typographus* and the less characterized *I. cembrae* to uncover patterns of olfactory adaptation across host-specialized bark beetles.

2.9.3. *Ips cembrae* (Larch Bark Beetle)

The larch bark beetle, *Ips cembrae*, usually infests European larch (*Larix decidua*) and Japanese larch (*L. kaempferi*); it can also attack Norway spruce (Postner, 1974). *Ips cembrae* is generally described as a secondary pest because it infests weakened and felled trees, but when conditions are suitable, it can infest healthy larches as well (Grodzki, 2008; EFSA, 2017). This bark beetle species often colonizes the entire trunk, including the canopy, with widespread co-occurrence with *Pityogenes*, *Pityophthorus*, and *Cryphalus* species (Pfeffer, 1955; Postner, 1974). Unlike *I. acuminatus,* which limits colonization to the crown part of the tree, *I. cembrae* utilizes a larger portion of its host and is more destructive during outbreaks. The aggregation pheromone of *I. cembrae* consists of a mix of *S*-(−)-ipsenol, *S*-(+)-ipsdienol, and 3-methyl-3-buten-1-ol that attracts both sexes to weakened hosts (Stoakley et al., 1978; Kohnle et al., 1988). This species is also known to vector the pathogenic fungus *Endoconiophora laricola,* which could accelerate host decline (Redfern et al., 1987; Kiristis, 2004; Jankowiak et al., 2007).

Studying the olfactory system of *I. cembrae* is ecologically relevant, but very little is known about this species. The antennal sensillum types and their OSN responses have

not been reported, making *I. cembrae* one of the least studied Ips species in chemosensory biology. Given the increasing importance of this species in larch forest dynamics, this species presents a valuable model for studying the divergence and specificity of olfactory coding in bark beetles (Table 3 for details on selected species).

Table 3: *Ips* species aggregation pheromone blends compositions including enantiomeric ratio of components and host distribution. ***From Ramakrishnan et al., unpublished manuscript.***

Species	**Pheromone composition**	**Enantiomeric ratio of pheromone components**	**Host/ Distribution region**
Ips duplicatus (C.R. Sahlberg, 1836)	ipsdienol:*E*-myrcenol **5:1:0.01**	ipsdienol (*S*)-(+)-:(*R*) -(−)- **50:50**	*Picea abies (L.) H. Karst.* Central Europe
Ips typographus (Linnaeus, 1758)	2-methyl-3-buten-2-ol *cis*-verbenol ipsdienol **9:1:0.1**	ipsdienol (*S*)-(+) :(*R*) -(−)- **5:95** (*S*)-(−)-*cis*-verbenol **100**	*Picea abies (L.) H. Karst.* Europe and Asia
Ips acuminatus (Gyllenhal, 1827)	*cis*-verbenol:ipsdienol:ipsenol **2:5:3**	ipsdienol (*S*)-(+)-:(*R*) -(−)- **95:5**	*Pinus spp. (Pinus nigra J.F. Arnold; Pinus sylvestris L.)*, Europe and Asia
Ips cembrae (Heer, 1836)	ipsenol:ipsdienol: 3-methyl-3-buten-1-ol ~ **68:28:4**	ipsenol (*S*)-(−)-:(*R*) -(+)- **99:1** ipsdienol (*S*)-(+)-:(*R*) -(−)- **96:4**	*Larix spp. (L. decidua Mill.; L. kaempferi (Lamb.), Piea abies (Karst.)* Europe

2.9.4. Current Research Gaps

While there has been substantial investigation regarding *I. typographus*, there are still critical knowledge gaps for the other *Ips* species. The antennal morphology and olfactory sensory neuron profiles for *I. duplicatus*, *I. acuminatus*, and *I. cembrae* are understudied, limiting our knowledge of interspecific sensory adaptations. The role of intraspecific variability, such as body size, on olfactory perception is also largely unstudied. These knowledge gaps limit our ability to develop species-specific monitoring techniques and also limit potential ecological comparisons. This thesis aims to address these issues through a comparative study that includes morphology, electrophysiology, and behaviour across three *Ips* species that feed on conifers.

2.9.5. Study species

Our study mainly focused on four species of bark beetles, *I. typographus, I. duplicatus, I. acuminatus*, and *I. cembrae,* which are key conifer pests in European forests.

Ips typographus

Ips duplicatus

Ips acuminatus

Ips cembrae

Chapter 3: Summarized workflow of methodology

3.1. Study Species (Collection, maintenance, and rearing)

For Paper II, *I. duplicatus* were collected from infested *Picea abies* logs near Kostelec nad Černými lesy (49°59'39", 14°51'33", Czech Republic), maintained in rearing chambers at the Czech University of Life Sciences, Prague until beetles developed. After debarking, adult beetles were collected and stored at 4°C. Five males and five females were selected for SEM.

For Paper III, *I. acuminatus* and *I. cembrae* were collected from forests near Rouchovany (Czech Republic) in late spring 2024. Beetles were identified in the field, sexed in the laboratory (Pfeffer, 1955; Zhang & Niemeyer, 1992), and reared in butterfly cages under controlled conditions until development (25°C Day, 19°C night, 60% RH, 16/8 light/dark). Five males and five females from each species were selected for SEM.

For Paper IV, *I. typographus* adults were obtained from ethanol-stored specimens and from newly emerged beetles reared under controlled conditions. For body size measurements, 50 undamaged beetles per replicate were randomly selected, dried at 25°C for two hours, sexed, and measured for body length. A total of eight groups (each with 50 beetles) were selected from ethanol-stored individuals captured using pheromone alone or pheromone combined with either 1,8-cineole or (+)-isopinocamphone during field experiments conducted in 2019 and 2021. For antennal club measurements and electroantennography (EAG), only females of the F0 generation, aged approximately three days, were used. These individuals emerged from naturally infested *Picea abies* logs (n = 12; ~50 × 28 cm) collected in Kostelec nad Černými lesy between June and July 2024. Logs were placed in fine-mesh emergence cages monitored daily, and freshly emerged beetles were collected by hand. Only undamaged females were selected for further analyses**.**

For Paper V, *I. acuminatus* and *I. cembrae* were again collected in 2024 from pine and larch hosts, respectively, in Rouchovany. Beetles were reared under identical lab conditions at CULS. A separate *I. acuminatus* population from Schönberg am Kamp (Austria) was reared at BOKU University and shipped to Lund University for SSR recordings. Adults were stored at 4°C and tested across a standard odor panel. Beetles from both locations were used for single sensillum recordings (SSR) and dose-dependent

response experiments. Each individual was screened across ten odorant stimuli during SSR, while separate individuals were used for each dose-response trial.

3.2. Morphological Studies

3.2.1. Scanning electron microscopy (SEM) analyses and sensilla categorization (Paper II, III)

Scanning electron microscopy (SEM) was used to investigate the antennal morphology and sensilla structures of beetles.

Beetles were cleaned with an air blower to remove surface dirt. Antennae were dissected under an optical microscope (NIKON, Japan), fixed in 2.5% glutaraldehyde for 24 hours, post-fixed in 2% OsO4 for 4 hours, and washed twice with distilled water. The antennae were then dehydrated through an ethanol series (35%, 50%, 70%, 96%, 100%) for 10 minutes at each step, and dried using a critical point dryer (Bal-Tec CPD 030). The samples were sputter-coated with gold (thickness: 20 nm) using a Bal-Tec SCD 050 ion sputter coater and observed under a JEOL JSM-IT200 scanning electron microscope and JEOL IT800 high-resolution SEM at 3, 5, 10, and 15 kV with a working distance of 3-5 mm at the University of Karlova, Prague. Images were obtained using a JEOL SU3500 scanning electron microscope at 5 kV at the FFWS Microscopy Facility, CZU Prague. The antennae and sensilla types, their numbers, and distribution were examined on five antennae from both sexes.

The general antennal morphology of *I. duplicatus*, *I. cembrae* and *I. acuminatus* was described as per Hulcr et al. (2015). Sensilla classification was based on external morphological criteria such as size, shape, presence or absence of pores (Schneider, 1964), and attachment to the cuticle (flexible vs. inflexible socket) (Nowińska & Brożek, 2017). Further classification followed the guidelines of Chen et al. (2010), Shewale et al. (2023), and Schneider (1964).

3.2.2. Morphometric analysis (Paper IV)

The total body length of adult female *I. typographus* was measured from the pronotum to the distal end of the elytra. Females ranged from 4.2 to 5.3 mm in size, with two categories established for further analysis:

1. Large females: ≥ 4.80 mm (n = 30)
2. Small females: ≤ 4.70 mm (n = 30)

Excised antennae were mounted on borosilicate glass slides for antennal club measurements and imaged using a Nikon DFK 33UX250 camera attached to a Nikon SMZ800N stereomicroscope. Antennal club length was measured from the apical end (ventral view) to the distal tip of the final antennomere, and width was determined at the midpoint of the club (ventral view). Measurements were conducted using IC Capture - Image Acquisition 4.0 software. For each individual, the average value from both the left and right antennae was calculated and recorded in micrometres.

3.3. Electrophysiological studies

3.3.1. Chemical stimuli (Papers IV & V)

For Paper IV, electroantennography (EAG) experiments were conducted using an aggregation pheromone mix of 2-methyl-3-buten-2-ol (MB) and *cis*-verbenol (cV) in a 10:1 ratio, along with individual compounds such as 1,8-cineole and (+)-isopinocamphone. All chemicals were procured from Sigma Aldrich, except (+)-isopinocamphone, which was a gift from Prof. Unelius from Linnaeus University, Sweden. The compounds were presented in seven doses ranging from 0.001 µg to 1000 µg in decadic concentrations. For odor cartridge preparation, 10 µL of each odor solution, 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 minute before the strip was placed into a glass Pasteur pipette, which served as the odor delivery cartridge for stimulation.

For Paper V, a broader odor panel was used for screening experiments using single sensillum recordings, including 57 ecologically relevant compounds such as beetle pheromones, host and non-host volatiles, and microbial-related compounds. These compounds were selected based on previous studies of *Ips* beetles, including *I. typographus*. Stock odor solutions were prepared at 10 µg/µL in paraffin oil and diluted as needed. A 10 µL volume of each solution was applied to filter paper inside glass Pasteur pipettes. Control stimuli consisted of paraffin oil alone. Pipettes were stored at -18°C between experiments and replaced regularly to prevent odor depletion. The essential oils of *P. sylvestris* and *L. decidua* were acquired from Oshadhi Ltd. (United Kingdom) for the GC-EAD studies. For use, the stock odor solutions (10 µg/µL) were

made in hexane and then further diluted. GC was directly injected with 1 µL of the solution for GC-EAD studies.

3.3.2. Electroantennographic detection (EAD) Experiments (Paper IV)

In Paper IV, electrophysiological analyses were performed on F0 female *Ips typographus* beetles, selected for their representative status in wild populations from natural spruce forests. Females were classified into two size categories: large (≥ 4.80 mm) and small (≤ 4.70 mm). The beetles were immobilized at 4 °C for 5 minutes before dissection.

Electroantennograms (EAGs) were recorded by excising the head and connecting two capillary electrodes filled with Ringer's solution: one electrode was placed on the antennal club, and the other served as a reference inserted into the excised head. The EAG probe was connected to a pre-amplifier, and a constant stream of humidified air (200 mL/min) was directed over the antenna. Odor cartridges were used to deliver the stimuli, and responses were recorded using EAG Pro software (Syntech, IDAC-4). Control and odor stimuli were presented sequentially with a 1-minute interval between stimulations. The EAG probe was configured with a 0–32 Hz filter and a sampling rate of 100 Hz. Antennal responses were recorded as downward deflections in millivolts (mV), with response amplitudes representing antennal peak depolarizations. Ten biological replicates were conducted for each stimulus, and mean response amplitudes were calculated to assess antennal sensitivity.

3.3.3. Gas chromatography coupled with electroantennographic detection (GC-EAD) Experiments (Paper V)

For Paper V, Gas chromatography was carried out using an Agilent 7890B GC system with an HP-5 column (Agilent Technologies, Inc.), measuring 30 m in length, 0.32 mm in diameter, and with a 0.25 µm film thickness. GC setup was combined with standard EAD setup (Syntech, IDAC-4). For the GC-EAD analysis, beetle heads with antennae were mounted between glass microelectrodes filled with Ringer's solution, following the procedure described by Olsson and Hansson (2013). Antennal signals were captured using a Universal probe (Syntech) and processed through the IDAC 2 data acquisition system (Syntech). Data were analyzed using GcEad software version 4.6.1 (Syntech). A minimum of five replicates per sample was conducted. A volatile compound was

considered electrophysiologically active if at least two antennal responses were detected in *Ips acuminatus* and *I. cembrae*.

The column was split, with 5 m directed toward the flame ionization detector (FID) and 1 m toward the insect antenna. At the end of the column, effluents were mixed with humidified air flowing at 2 L/min before being delivered to the antenna. Samples were introduced in splitless mode, using helium as the carrier gas at a constant flow rate of 3 mL/min. The GC oven was programmed to start at 40 °C (held for 1 minute), ramping at 10 °C/min to 100 °C (held for 0.5 minutes), then at 20 °C/min to 150 °C, and finally at 40 °C/min to a final temperature of 300 °C, held for 3 minutes. The FID temperature was maintained at 300 °C.

3.3.4. Single-Sensillum Recordings (SSR) (Paper V)

To characterize the olfactory sensory neuron (OSN) response profiles in *I. acuminatus* and *I. cembrae*, single-sensillum recordings were performed on live adult beetles using established electrophysiological protocols. Individual insects were immobilized in modified pipette tips, exposing the head and antennae. One antenna was fixed onto a microscope slide using dental wax to allow stable electrode access and optimal light transmission. Under a light microscope at 500× magnification (NIKON), recordings were carried out using electrolytically sharpened tungsten microelectrodes.

The reference electrode was inserted into the pronotum, and the recording electrode was precisely positioned at the base of an olfactory sensillum using a micromanipulator. Neural signals were amplified and digitized using an IDAC4 system (Syntech) and visualized in real time with AutoSpike software. A continuous flow of humidified, charcoal-filtered air (1.2 L/min) was directed toward the antenna, and odor stimuli were introduced as brief (0.5 s) pulses via a stimulus controller, mixed into the airstream at 0.3 L/min. Odor pipettes used in the screening phase were reused under controlled conditions, while dose-response pipettes were freshly prepared daily to maintain stimulus integrity. Screening was conducted with a high-dose application (10 µg) to identify OSN classes based on differential spike activity. Compounds were stimulated in randomized order, and sufficient time was allowed between stimulations to avoid adaptation. Selected OSNs from each species (five classes in *I. acuminatus*, three in *I. cembrae*) were subsequently subjected to dose–response assays using increasing concentrations (10 pg

to 10 µg). This approach allowed precise assessment of OSN sensitivity and tuning breadth for key odorant stimuli.

3.4. Field experiments and pheromone traps

Field experiments for **Paper IV** were conducted in 2019 and 2022 to investigate the behavioral responses of *I. typographus* to semiochemical treatments under natural conditions. Both studies were carried out in a mature (~100-year-old) Norway spruce forest at the Czech University of Life Sciences research site in Kostelec nad Černými lesy, Czech Republic (600 m a.s.l.), a natural habitat of the target species. The 2019 experiment took place at coordinates 49°56′02″N, 14°52′21″E, and the 2022 experiment at 49°55′57″N, 14°55′13″E. Each trial spanned from early June to late July, coinciding with peak beetle flight activity.

Traps were deployed approximately 30 meters inside a two-year-old forest clearing and positioned 1.5 meters above ground on wooden poles, with a minimum spacing of 15 meters to reduce inter-trap interference. In 2019, seven cross-vane Ecotraps (Fytofarm, Slovak Republic) were used in a Latin square design. Six traps were baited with three concentration levels (low, medium, high) of either 1,8-cineole or (+)-isopinocamphone, each combined with a standard pheromone blend; one trap with pheromone only served as a control (See Paper IV). Trap positions were rotated seven times to control for location effects.

In 2022, a randomized complete block design was employed separately for the two compounds. Each block included four traps: three baited with varying doses of the test compound plus pheromone, and one control trap with pheromone alone. Trap positions within each block were rotated four times, and each block was replicated twice, yielding eight replicates per treatment.

Captured beetles were preserved in ethanol for post-collection analyses, including species confirmation, sex determination, and morphometric measurements.

3.5. Data interpretation and statistical analysis

For Paper II,

Morphometric measurements of antennal sensilla were performed using ImageJ v1.53q (Schneider et al., 2012). The software enabled calibration to a defined scale, allowing precise quantification of sensilla length and basal width (n = 10 per sensilla type per sex). Data were analyzed using Bonferroni multiple comparison tests in GraphPad Prism v9.0 to assess sex-based differences in sensilla dimensions and abundance.

In Paper IV,

Normality and variance were tested using Shapiro–Wilk and Levene's tests. ANOVA with Tukey's post hoc tests analyzed year-wise treatment effects. Chi-square tests (Yates' correction) compared female proportions. Wilcoxon signed-rank tests were used for paired morphometric comparisons.

Standardized major axis (SMA) regression (R package "smatr") was applied to log-transformed antennal data to test allometric relationships (Jolicoeur, 1990). EAG dose-response differences between size classes were tested using Wilcoxon tests. All analyses used $p = 0.05$.

For Paper V,

Neuronal activity was analyzed offline using AutoSpike v3.9 by measuring spike rates during the initial 0.5 seconds of odorant exposure, from which the baseline (pre-stimulus) activity was subtracted. Any activity recorded in response to the paraffin oil control was also deducted. At the screening concentration, responses below 20 Hz were deemed biologically insignificant. Excitatory responses were classified into intermediate (40–60 Hz) and strong (>80 Hz) categories. Recordings of insufficient quality or neurons that were not fully assessed were omitted from further analysis. Data visualizations, including graphs and heatmaps, were produced with GraphPad Prism version 10.1.2 (GraphPad Software, San Diego, CA, USA). The Venn diagram was generated using InteractiVenn (Heberle et al., 2015).

Chapter 4: Results

Subchapter 4.1: Review of aggregation pheromones and olfactory mechanisms in *Ips* bark beetles (Paper I)

Article type and status:

Review article **(***Submitted to Current Forestry Reports* (*under revision))*

Based on: Ramakrishnan, R.†, **Shewale, M.K.†,** Strádal, J.†, Hani, U., Gershenzon, J., Andersson, M.N., Frühbrodt, T., Doležal, P., Jirošová, A. (2025). Aggregation Pheromones in the Bark Beetle Genus *Ips*: Advances in Biosynthesis, Sensory Perception, and Forest Management Applications.

† *Equal contribution as first author*

My contribution: Compiled literature on olfactory perception in *Ips* species, interpreted pheromone composition data, prepared figures and tables, and authored sections on antennal morphology and pheromone-based management.

Article Summary

This review provides an in-depth synthesis of the current understanding of aggregation pheromones in *Ips* bark beetles. It emphasizes their chemistry, biosynthetic origins, olfactory detection, and application in forest pest control. It includes key pest species such as *Ips typographus*, *I. duplicatus*, and *I. cembrae*, alongside others of ecological and economic relevance. Although this thesis did not directly investigate pheromone biosynthesis, this section reviews essential pathways relevant for interpreting species-specific blends studied in Paper V.

The review outlines the molecular structures of pheromone compounds, their enantiomeric specificity, and the biochemical pathways involved in their production. Furthermore, it discusses how olfactory sensory neurons (OSNs) detect these semiochemicals and their use in integrated pest management. The article also highlights several knowledge gaps and suggests directions for future research to improve species-specific monitoring and control strategies. This review directly supports the primary aims of the dissertation by providing the theoretical framework for understanding species-specific olfactory adaptations in conifer-feeding *Ips* bark beetles. It bridges the literature

analysis in Chapter 2 and the experimental findings presented in Chapters 4.2 to 4.5, guiding both the species selection and methodological choices in this thesis. Moreover, the insights gathered here inform future research on semiochemical-based management and underscore the ecological complexity of pheromone-mediated behaviors in bark beetles.

4.1.1. Chemical composition of aggregation pheromones in *Ips* species

Key message: *Ips* species use species-specific blends of structurally similar pheromones, with enantiomeric variation ensuring clear communication and reproductive isolation.

In *Ips* bark beetles, the aggregation pheromone blends consist predominantly of a few structurally related compounds. However, they exhibit species-specificity through subtle pheromone blend ratios and enantiomeric composition variations. The most common pheromone components include ipsenol and ipsdienol, which are produced exclusively within the genus, alongside compounds such as amitinol, *E*-myrcenol, lanierone, and hemiterpenes like 2-methyl-3-buten-2-ol. Additionally, monoterpenoid alcohols such as *cis*-verbenol (a derivative of host compound α-pinene) also frequently contribute to aggregation pheromone blends.

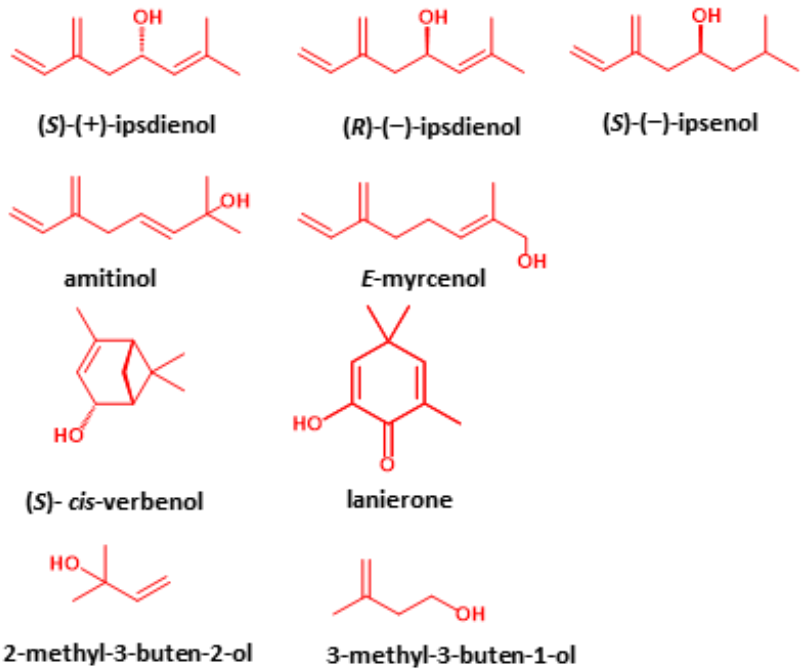

Figure 10: *Structures of pheromone compounds from* Ips *species. Adapted from Ramakrishnan et al. unpublished manuscript.*

Despite their structural similarities (Fig. 10), these compounds' specific combinations and enantiomeric configurations contribute to distinct pheromone blends for each species, giving them a distinct olfactory signature. These variations support reproductive isolation and reduce cross-attraction in sympatric environments where multiple *Ips* species co-occur. A comparative overview of pheromone compositions for nine *Ips* species is provided in Table 4 (also more details in section 2.5).

Table 4: Aggregation pheromone blends compositions including enantiomeric ratio of components in selected *Ips* species. *From Ramakrishnan et al., unpublished manuscript.*

Species	**Composition of pheromone**	**Enantiomeric ratio of pheromone components**
Ips amitinus (Eickhoff, 1872)	ipsdienol:ipsenol:amitinol **4:2:4**	ipsdienol (*S*)-(+)-:(*R*) -(−)- **5:95**
Ips duplicatus (C.R. Sahlberg, 1836)	ipsdienol:*E*-myrcenol **5:1:0,01**	ipsdienol (*S*)-(+)-:(*R*) -(−)- **50:50**
Ips hauseri (Reitter, 1895)	ipsenol: *cis*-verbenol **95:5**	(*S*)-(−)-ipsenol **100** (*S*)-(−)-*cis*-verbenol **100**
Ips nitidus (Eggers, 1933)	2- methyl-3-buten-2-ol: ipsdienol: (*S*)- (−)-*cis*-verbenol **7:2:1**	ipsdienol (*S*)-(+)-:(*R*) -(−)- **74:26**
Ips perturbatus (Eichhoff, 1869)	ipsdienol: *cis*-verbenol: ipsenol **1:0,8:1**	ipsenol (*S*)-(−)-:(*R*) -(+)- **99:1** ipsdienol (*S*)-(+)-:(*R*) -(−)- **90:10**
Ips shangrila (Cognato & Sun, 2007)	ipsenol:ipsdienol: *cis*-verbenol **1:5:4**	ipsdienol (*S*)-(+)-:(*R*) -(−)- **99:1** (*S*)-(−)-*cis*-verbenol **100**
Ips typographus (Linnaeus, 1758)	2-methyl-3-buten-2-ol *cis*-verbenol ipsdienol **9:1:0,1**	ipsdienol (*S*)-(+) :(*R*) -(−)- **5:95** (*S*)-(−)-*cis*-verbenol **100**
Ips acuminatus (Gyllenhal, 1827)	*cis*-verbenol:ipsdienol:ipsenol **2:5:3**	ipsdienol (*S*)-(+)-:(*R*) -(−)- **95:5**
Ips confusus (LeConte, 1876)	ipsenol:ipsdienol **9:1**	ipsenol (*S*)-(−)-:(*R*) -(+)- **99:1** ipsdienol (*S*)-(+)-:(*R*) -(−)- **95:5**
Ips grandicollis (Eichhoff, 1868)	ipsenol	ipsenol (*S*)-(−)-:(*R*) -(+)- **99:1**
Ips lecontei (Swaine, 1924)	ipsdienol:ipsenol **2:1**	Ipsdienol (*S*)-(+)-:(*R*) -(−)- **95:5** ipsenol (*S*)-(−)-:(*R*) -(+)- **99:1**

Ips paraconfusus (Lanier, 1970)	ipsenol:ipsdienol: *cis*-verbenol **1:1:0,1**	(*S*)-(−)-*cis*-verbenol **100** ipsenol (*S*)-(−)-:(*R*) -(+)- **99:1** ipsdienol (*S*)-(+)-:(*R*) -(−)- **90:10**
Ips pini (Say, 1826)	ipsdienol: lanierone **99:1**	Ipsdienol (*S*)-(+)-:(*R*) -(−)- **35:65** ipsdienol† (*S*)-(+)-:(*R*) -(−)- **95:5**
Ips sexdentatus (Börner, 1776)	ipsdienol:ipsenol **1:0,5**	Ipsdienol (*S*)-(+)-:(*R*) -(−)- **50:50**
Ips cembrae (Heer, 1836)	ipsenol:ipsdienol: 3-methyl-3-buten-1-ol ~ **68:28:4**	ipsenol (*S*)-(−)-:(*R*) -(+)- **99:1** ipsdienol (*S*)-(+)-:(*R*) -(−)- **96:4**
Ips subelongatus (Motschulsky, 1860)	ipsenol: ipsdienol:3-methyl-3-buten-1-ol **3:1**	ipsenol (*S*)-(−)- **100** ipsdienol (*S*)-(+)-:(*R*) -(−)- **96:4**
Ips avulsus (Eichhoff, 1868*)*	ipsdienol:lanierone **10:1** [91]	Ipsdienol (*S*)-(+)-:(*R*) -(−)- **96:4** (Texas) Ipsdienol (*S*)-(+)-:(*R*) -(−)- **75:25** (Alabama)

†Ratio varies within eastern and western populations

4.1.2. Pheromone biosynthesis and regulatory mechanisms

Key message: Pheromone production is hormonally regulated and involves both beetle enzymes and microbial symbionts, reflecting a complex biosynthetic network.

This section discusses the biochemical and molecular pathways through which *Ips* beetles produce aggregation pheromones. Two primary biosynthetic origins are described as follows: 1) *de novo* biosynthesis through the mevalonate pathway, and 2) from host compounds, like α-pinene. The former occurs mainly in the gut and fat body, with hormonal regulation through juvenile hormone III and several enzymes.

Hormonal regulation of this pathway permits species- and sex-specific pheromone output. In most *Ips* species, the site of pheromone production is limited to the gut where biosynthetic activity is initiated when the insect feeds. At the same time, juvenile hormone III (JH III) appears to be the primary regulator of pheromone synthesis and has a major role in triggering pheromone biosynthesis, metabolism, and release.

Interestingly, some of the pheromone compounds, such as (*S*)-*cis*-verbenol, which is part of several species' pheromone signaling systems, is not produced *de novo*, but is instead formed through the hydroxylation of α-pinene. This is done by cytochrome P450 monooxygenases (CYP450s), indicating that pheromone production may have developed as an evolutionary extension of host terpenes detoxification (Figure 10, see earlier section 4.1.1).

Beyond endogenous biosynthesis, there is also the potential for both gut-resident microbes and exosymbiotic fungal symbionts to influence or contribute to the pheromone profile produced by bark beetles. For example, antibiotic inhibition studies have suggested that gut microbiota can convert host compounds into active pheromones. Examples of fungal associates, *Grosmannia penicillata* and *Endoconidiophora polonica*, were shown to produce compounds such as 2-methyl-3-buten-2-ol and brevicomin from wood substrates (i.e., implicating fungi in semiochemical signaling).

4.1.3. Olfactory detection and sensory specialization in *Ips*

Key message: *Ips* bark beetles detect pheromones using finely tuned sensory systems that enable species-specific mate and host recognition under complex forest environments.

This subsection summarizes advances in our understanding of how *Ips* beetles detect pheromone signals using specialized olfactory sensory neurons (OSNs) located in antennal sensilla. While antennal morphology in Coleoptera has generally received less focused research attention than in Lepidoptera, recent investigations of *I. typographus* have greatly advanced our understanding of beetle chemosensation. Multiple classes of OSNs have been identified, with responses selective to particular key pheromone components, including their enantiomers. These OSNs are organized into two functionally distinct classes: some are narrowly tuned to specific pheromone compounds, and the other class is much more broadly tuned. This OSN function reflects the ecological needs and diversity of the semiochemicals of the species.

Recent molecular and electrophysiological data suggest that detection occurs through a conserved set of similar odorant receptors across *Ips* species. These may partly represent evolutionary balance and also species-level adaptation. The results presented here complement the literature reviewed in Chapter 2 (specifically Section 2.6 and Fig. 6),

which contribute toward the ecological role of antennal coding in the expression of aggregation behavior and patterns of reproductive isolation.

4.1.4. Pheromone-based management approaches

Key message: Semiochemical tools offer targeted and eco-friendly alternatives, but optimizing their effectiveness requires species-specific strategies.

Aggregation pheromones have been widely employed in *Ips* bark beetle management, particularly for population monitoring and outbreak control. Some common methods include mass trapping and push–pull systems, and there are several options for using anti-aggregation compounds, such as verbenone, but effectiveness varies because of beetle pressure, design of the trap, release rates of the lure, and forest condition. In reviewing this chapter, we have focused on best practices in semiochemical use, and it is critical that there is increased species specificity, particularly in forestry situations where there are multiple co-occurring *Ips* species. Future directions that show promise include making region-specific blends, using volatiles of the host or fungus to increase lure attractiveness, and exploring pheromone disruption as a novel pest control strategy (section 2.4).

Subchapter 4.2: Antennal morphology is highly conserved across *Ips* Species, with minor differences in sensilla frequency and distribution (Paper II)

Article type and status:

Original research article (Published in *Microscopy Research and Technique*)

Based on: *Shewale et al., 2023 – Microscopy Research and Technique***:**

Shewale, M. K., Nebesářová, J., Grosse-Wilde, E., & Kalinová, B. (2023). Microscopic morphology and distribution of the antennal sensilla in the double-spined bark beetle, Ips duplicatus (Coleoptera: Curculionidae). *Microscopy Research and Technique*, 86(12), 1610–1625. https://doi.org/10.1002/jemt.24397

My contribution: Conceptualization, data curation, investigation, funding acquisition, methodology, formal analysis, data visualization, original drafting, and editing.

Article Summary

This study directly addresses the second objective of the thesis, which was to examine antennal morphology and sensilla distribution across important *Ips* species, as the first detailed description of the antennal morphology of *Ips duplicatus*. As an important pest of *Picea abies* in Central Europe, *I. duplicatus* acts as comparison species to consider the conservation and divergence of olfactory sensilla at the genus-level.

Using scanning electron microscopy (SEM), the five major sensilla chaetica, basiconica, trichodea, coeloconica, and Böhm's sensilla were characterized on the antennal clubs in both sexes. The five types of sensilla occurred across the three sensory bands (A–C) found across all *Ips* species. *Ips duplicatus* also possesses a richer diversity with more subtypes of observed sensilla, with some subtle patterns of sexual dimorphism of sensilla distributions. These results provide further support for the hypothesis that general olfactory architecture is largely conserved across *Ips* species with slight variations that show elements of the respective ecological niches. These results also provide a morpho-functional framework for a future study investigating olfactory detection in *I. duplicatus* and its close relatives, as well as the structural framework for comparative analysis in subchapter 4.3 with *I. acuminatus* and *I. cembrae*.

4.2.1. General antennal morphology of *Ips duplicatus*

Key message: The antennal structure of *I. duplicatus* follows the conserved *Ips* species morphological pattern yet displays subtle differences in sensilla density and organization.

The antennae of *I. duplicatus* are made up of seven segments: the scape (proximal), the five funicular segments (the pedicel is represented by F1 and is followed by F2 - F5), and club-shaped terminal segment (Fig. 11a). The five funicular segments are bowl-shaped and flexibly attached to one another, whose depth gradually increases while the diameter decreases toward the distal end of the antenna. The pedicel (F1) is the largest segment with slight lateral curvature, opposite in both antennae.

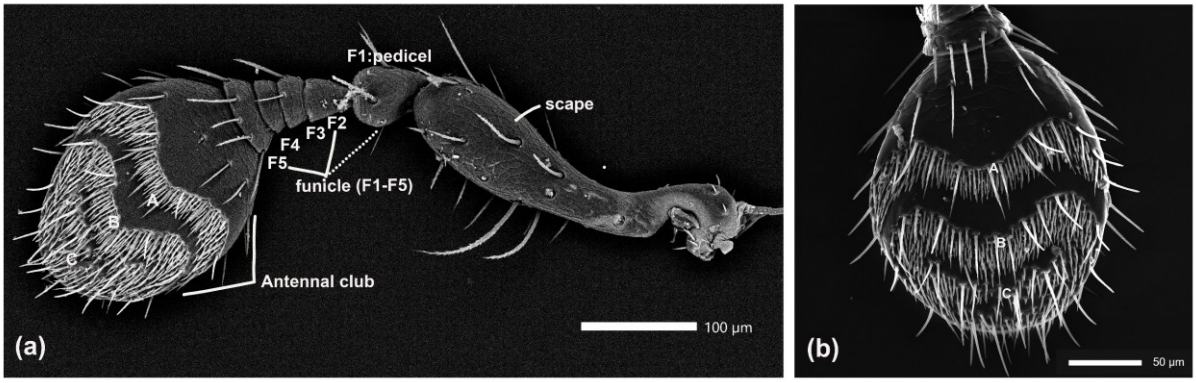

Figure 11. *General antennal morphology of* Ips duplicatus *(female). (a) Ventral view showing scape, pedicel (F1), funicle (F2–F5), and club. (b) Distinct sensory bands (A, B, C) on the ventral side of the antennal club. Shewale et al.,2023*

The club, along with the anterior face, are oval in shape (Fig. 11b), slightly convex on each side, and covered with scale-like structures, particularly prominent on the scape, funicle and proximal club surface. Most of the olfactory sensilla occur on the ventral side of the antennal club concentrated on the distal three-fourths of the club's surface (Figure 11b). These sensilla are organized in three distinct sensory bands (A, B, and C) which are previously described in other species of *Ips*, including *I. typographus*, *I. pini*. Bands A and B were arranged in a wave pattern (stripes) with a plain cuticle in between these bands; this was used to indicate band C because characteristics in the distal area were partly fused to band B. Surprisingly, oval-shaped surface pore (SP) structures were present over the dorsal and ventral surfaces of the club and other segments, distributed among sensilla.

Ips duplicatus has a higher number of sensilla basiconica, coeloconica, and trichodea compared to other *Ips* species. Additionally, *I. duplicatus* has two types of sensilla coeloconica, a feature not found in the other examined *Ips* species. It is impossible to determine whether these distinctions reflect true species-specific adaptations or simply methodological differences; however, this could represent ecological or behavioral specializations unique to *I. duplicatus*.

4.2.2. Sensilla types and distribution on the antennal surface

Key message: *Ips duplicatus* antennae exhibit a rich diversity of sensilla subtypes, reflecting a complex sensory landscape that likely supports chemical detection.

Five main sensilla categories were identified on the antennal club of *I. duplicatus*, including chaetica, basiconica, trichodea, coeloconica, and Böhm's sensilla, which had further subtypes. These sensilla appeared primarily on the ventral surface and were arranged into three sensory bands. Some sensilla (e.g. the sensilla chaetica and sensilla trichodea) were also located on the dorsal areas. Sensilla chaetica were long, aporous, and branched and likely act as mechanosensory or gustatory sensilla members. There were two identifiable subtypes based on their length and surface texture (SchI and SchII), along with their branching pattern (Table 5).

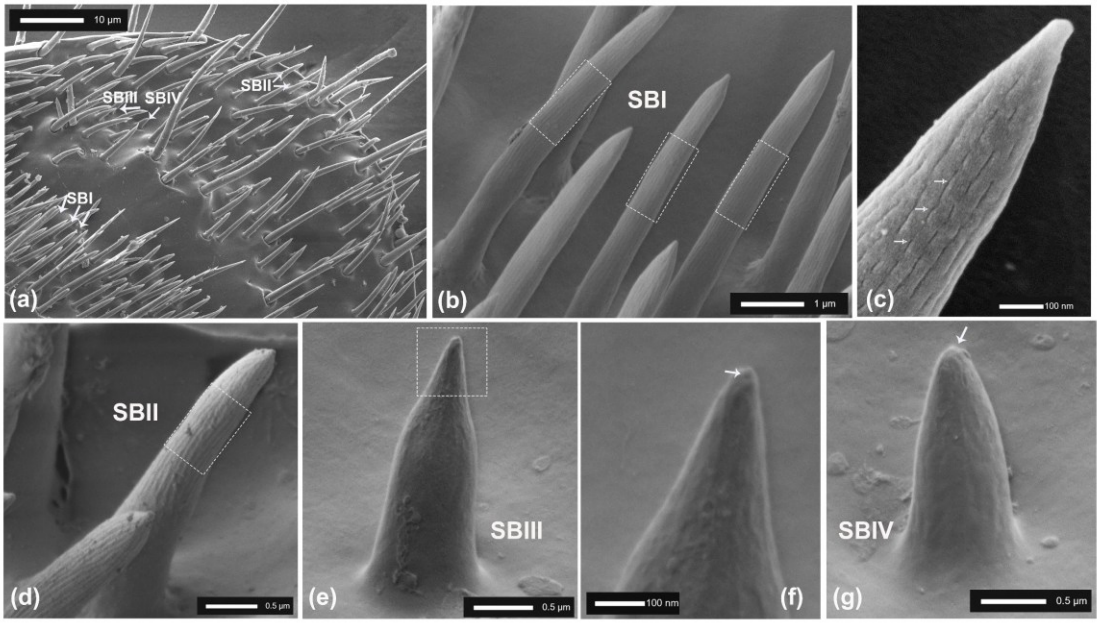

Figure 12. *Sensilla basiconica subtypes on* Ips duplicatus *antennae: (a) Clustered distribution on band C; (b–g) Morphological details of SBI–SBIV showing differences in shape, wall texture, and pore structures. Shewale et al., 2023.*

Basiconica represented the most abundant group of antennal sensilla and was composed of four morphologically distinct porous subtypes, mainly SBI, SBII, SBIII, and SBIV (Fig. 12). The presence of multiple pores across their cuticular walls strongly indicates an olfactory role, likely involved in detecting a wide range of odor molecules. These subtypes varied slightly in size and distribution but shared the common feature of being structurally adapted for chemoreception.

Sensilla trichodea were classified into three subtypes, mainly STrII, STrIII, and STrIV (Fig. 13), based on differences in socket structure and the presence or absence of wall pores. These morphological distinctions suggest functional specialization, with some subtypes likely acting as generalist olfactory sensilla capable of detecting broad odor profiles, while others may serve more specialized roles in recognizing specific pheromones or environmental cues.

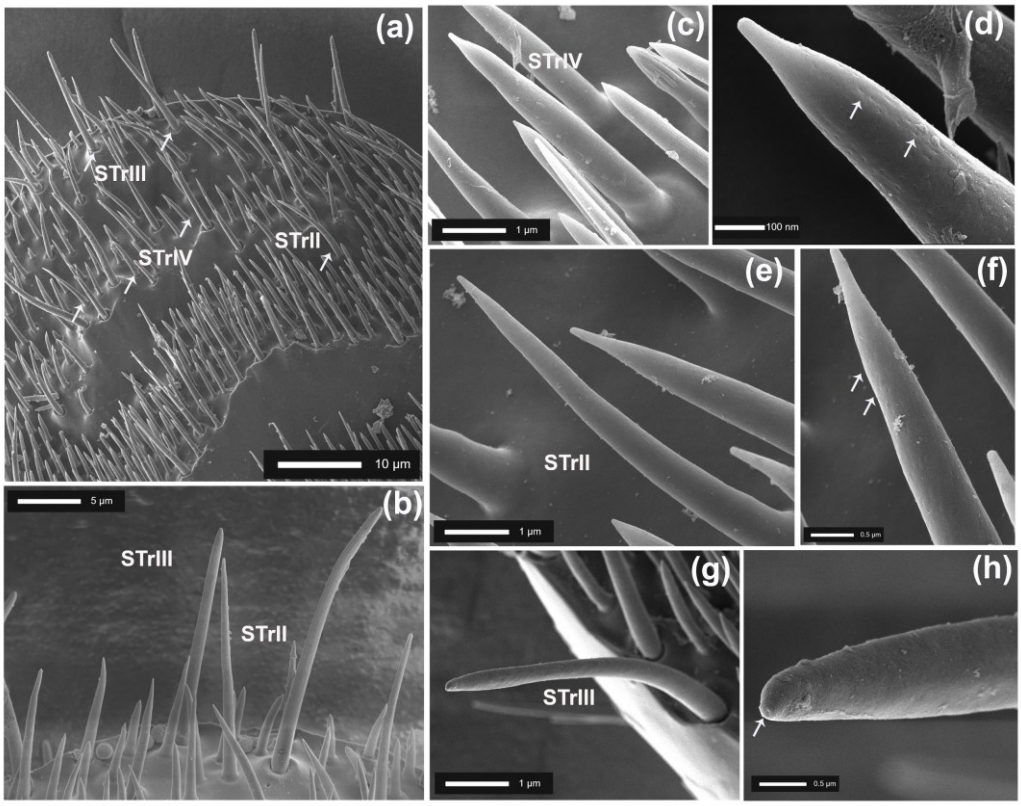

Figure 13. *Sensilla trichodea subtypes on* Ips duplicatus *antennae: (a) Grouped distribution; (b–h) STrII–STrIV showing variations in socket type, wall porosity, and tip morphology. Shewale et al., 2023.*

Two of the more interesting sensory types identified in *I. duplicatus* were shallow coeloconica with two mostly distinct subtypes: fluted with either pointed or rounded tips (ScoI and ScoII), both of which were newly described in *Ips* and could suggest some

form of sensory specialization. On the other hand, Böhm's sensilla (BS), portal structures appear mechanoreceptive and were only located at the antennal base. Shallow surface pores (SP) were found in diffuse distribution and were predominantly associated with flexible socket sensilla, although their exact function remains uncertain. Overall, these results reveal both conserved and localized components of the antennal morphology of *I. duplicatus*. Table 5 shows sensilla types and subtypes, their locations across antennal regions, and external features.

Table 5. Morphological characteristics and distribution of sensilla types on the antennae of *Ips duplicatus.* Shewale et al.,2023

Sensilla type	Distribution	Pores	Wall structure	Tip	Shape	Socket
SchI	Antennal club (A, B and C), funicular segments (F1-F5) and scape	Aporous	Longitudinal grooved wall, bilateral branching	Sharp	Straight	Flexible
SChII	Antennal club (A), funicular segments (F1-F5) and scape	Aporous	Longitudinal grooved wall, multi-branching	Sharp	Curved	Flexible
SBI	Antennal club (A, B, C)	Multiporous	Pitted	Blunt	Straight	Inflexible
SBII	Antennal club (A, B and C)	Multiporous	Grooved	Blunt	Straight	Inflexible
SBIII	Antennal club (B and C)	Uniporous	Smooth	Blunt and round	Peg shaped	Inflexible
SBIV	Antennal club I	Uniporous	Smooth	Round	Straight	Inflexible
StrII	Antennal club (B and C)	Multiporous	Smooth	Pointed	Slightly curved	Inflexible
STrIII	Antennal club (A, B and C)	Terminal pore	Smooth	Blunt	Long and curved	Flexible
StrIV	Antennal club (A, B and C)	Multiporous	Pitted	Pointed	Straight	Inflexible
Sco I	Antennal club (A, B and C)	Aporous	Grooved	Round	Cone-shaped	Inflexible

Sco II	Antennal club (A, B and C)	Aporous	Grooved	Sharp	Cone-shaped	Inflexible
BB	Scape	Aporous	Smooth	Blunt and round	Short and straight	Flexible
SP?	Club (A, B and C), funicle segments (F1-F5) and scape	?	Pit on the club surface	-	Oval	-

SchI: sensilla chaetica type I, SChII: sensilla chaetica type II, SBI: sensilla basiconica type I, SBII: sensilla basiconica type I, SBIII: sensilla basiconica type III, SBIV: sensilla basiconica type IV, StrII: sensilla trichodea type II, STrIII: sensilla trichodea type III, StrIV: sensilla trichodea type IV, ScoI: sensilla coeloconica type I, ScoII: sensilla coeloconica type II, BB: Böhm's bristles, and SP: Surface Pores

4.2.3. Distribution, dimensions, and sex-based differences in sensilla

Key message: While overall antennal structure is similar in both sexes of *I. duplicatus*, minor differences in sensilla size and abundance suggest subtle sexual dimorphism.

The organization of sensilla on the male and female *I. duplicatus* antennae was relatively similar, although statistical analyses indicated slight but significant sexual dimorphism in several sensilla characteristics. While total length and major segment dimensions showed no significant differences between sexes, SChII and BS had greater width in females, and some SBI and STrIV showed different abundance in either sex (Fig. 14).

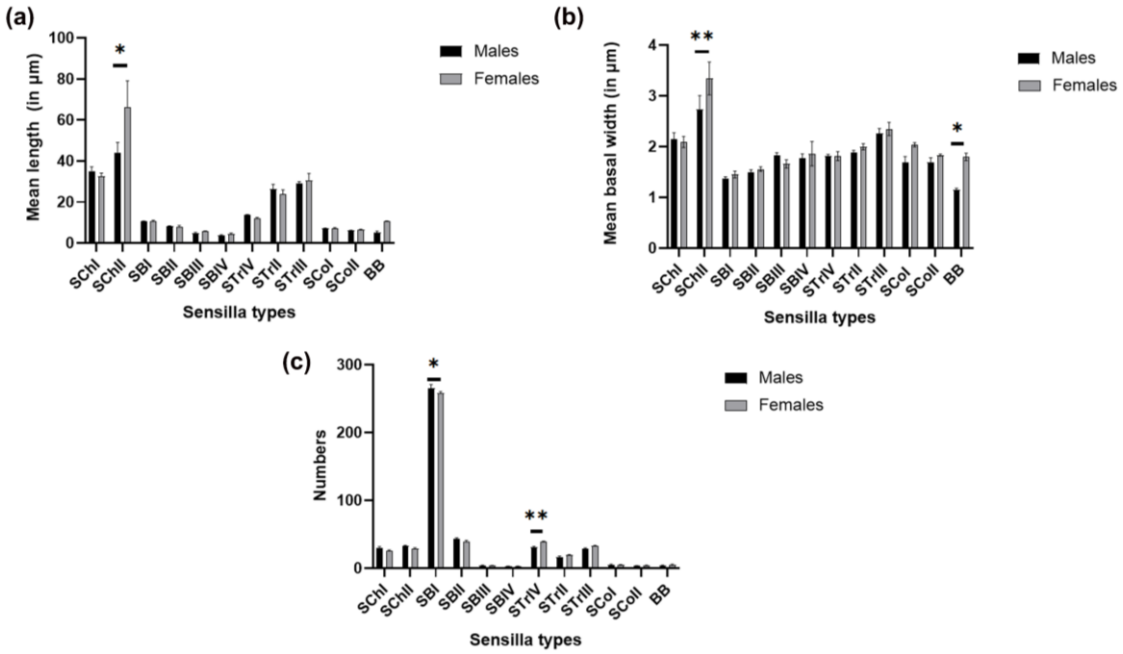

Figure 14. *Comparative bar graphs of sensilla length (a), width (b), and abundance (c) between sexes in* I. duplicatus *(Bonferroni test,* n = *5 per sex). Shewale et al., 2023.*

Sensilla mapping revealed that SChI and SChII were primarily distributed along the outer edges of sensory bands A, B, and C (Fig. 15a). Böhm sensilla (BS) were localized exclusively to the scape and pedicel. Long basiconica sensilla types SBI and SBII were widely distributed across sensory bands A and B. In contrast, the shorter basiconica types SBIII and SBIV were restricted to the distal sensory band C (Fig. 15e). Trichoid sensilla showed subtype-specific patterns, with STrIII notably concentrated near the margins of bands A and B (Fig. 15d).

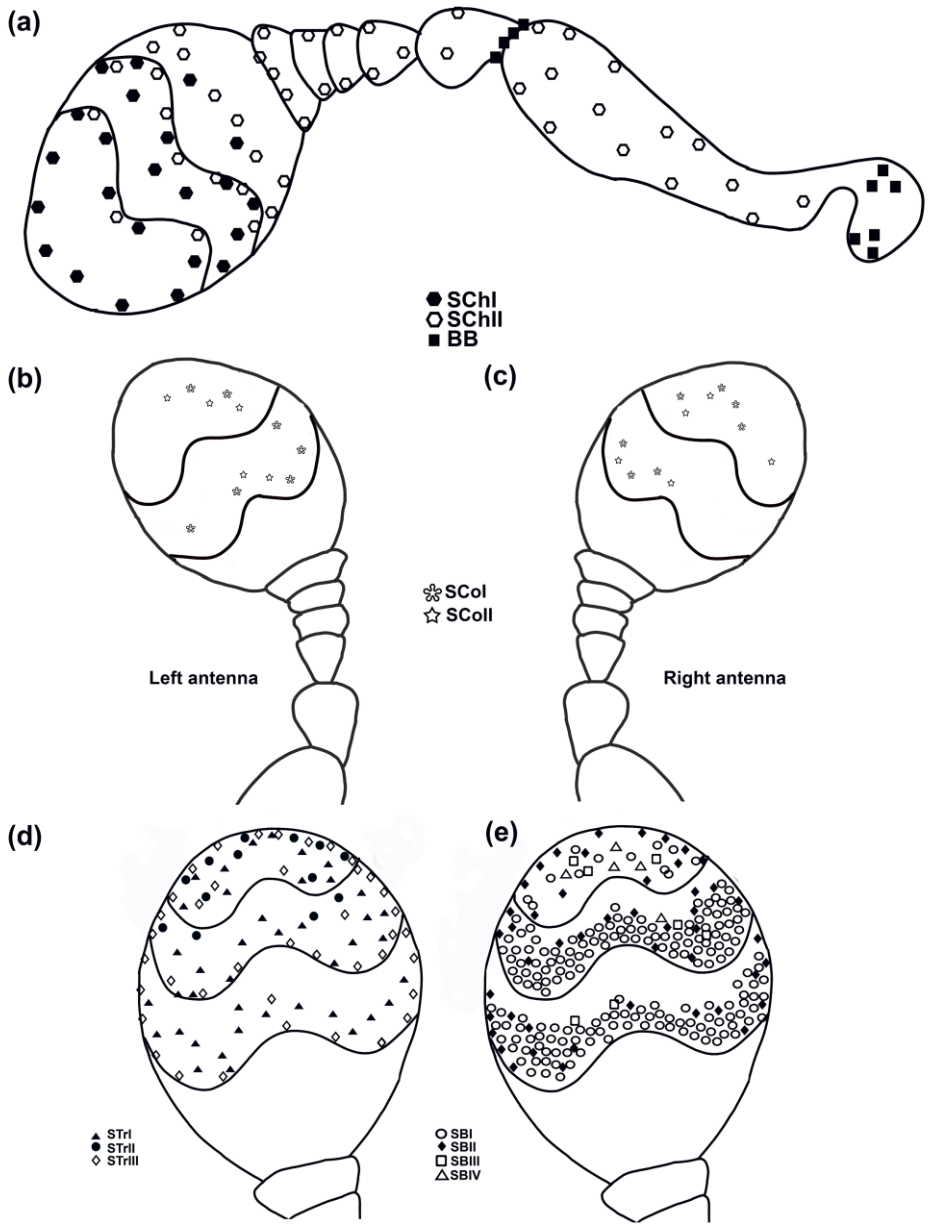

Figure 15. *a–e. Sensilla distribution maps across the antennal club in* I. duplicatus*: chaetica (SchI and SchII) and Bohm sensilla (BB) (a); coeloconica (Sco I and II) (b); trichodea (STr II, I and III) (c), and basiconica (SBI, II, III and IV)***.** *Shewale et al., 2023.*

Interestingly, both types of coeloconic sensilla (SCoI and SCoII) were more consistently distributed in bands B and C and were often present in pairs. SCoI and SCoII also exhibited asymmetric lateral distributions occurring more frequently on one side or the other, more often on the side (right or left) of the antennal club, depending on the antenna side (Fig. 15 b,c). Why there is an asymmetry of SCoI and SCoII is unknown and may suggest some lateralized processing, indicating there is different processing based on where the sensory information is received.

Subchapter 4.3: Comparative descriptive morphology of antennal sensilla in *Ips cembrae* and *Ips acuminatus* (*Paper III*)

Based on:

Shewale, M.K., Dusek, J., Jirošová, A. (2025). Microscopic morphology and distribution of the antennal sensilla in the larch bark beetle, *Ips cembrae,* and pine bark beetle, *Ips acuminatus* (Coleoptera: Curculionidae). Manuscript in preparation.

***Note:**

This chapter presents preliminary results from ongoing research. Quantitative morphometric data and statistical analyses will be incorporated into the final manuscript.

My contribution: Conceptualization, sample preparation, SEM imaging, data curation, morphological classification, figure and table preparation.

Key message:

Despite species-specific host preferences, *Ips cembrae* and *Ips acuminatus* display a conserved antennal sensilla architecture, providing a foundational map for future electrophysiological studies.

Article Summary

This study is the first comparative account of antennal sensilla morphology in *I. cembrae* and *I. acuminatus* conifer-associated bark beetles of ecological significance within European forests. A scanning electron microscope (SEM) was used to view the antennal club of each species to examine sensilla types and their distribution patterns. These preliminary findings showed that antennal architecture is conserved within the genus *Ips*. The major sensilla types included in each species are sensilla chaetica, trichodea, basiconica, coeloconica and Böhm's sensilla, and all were present in both species. These morphological sensilla map will help to inform future electrophysiological studies and advance our understanding of olfactory specialization in the genus. The general observations are consistent with the conserved antennal architecture observed in other *Ips* species, including *I. typographus* and *I. duplicatus* (see Chapter 4.2).

4.3.1. General antennal morphology

Key message: Both species display a conserved antennal architecture, supporting genus-level patterns in sensilla distribution across *Ips* bark beetles.

Both *I. cembrae* and *I. acuminatus* exhibit the characteristic scolytine antennal structure, consisting of four main segments: the scape, pedicel, funiculus, and the club or terminal segment. The antennal club serves as the primary olfactory organ and is structurally organized into three distinct ventral sensory bands, designated as bands A, B, and C. This organization is consistent with previous descriptions in other *Ips* species, such as *I. typographus* and *I. duplicatus* (Hallberg, 1982a; Shewale et al., 2023).

The majority of sensilla are located on the anterior (ventral) surface of the antennal club and show a clear pattern of distribution that aligns with the defined sensory bands. Scanning electron microscopy (SEM) images (Figs. 16 and 17) provide detailed views of the surface morphology and the arrangement of sensilla within each band. These initial images highlight the grouping and structural characteristics of sensilla across the club surface. At this stage, evaluation of sexual dimorphism was not possible, as detailed quantitative morphometric analyses have not yet been conducted.

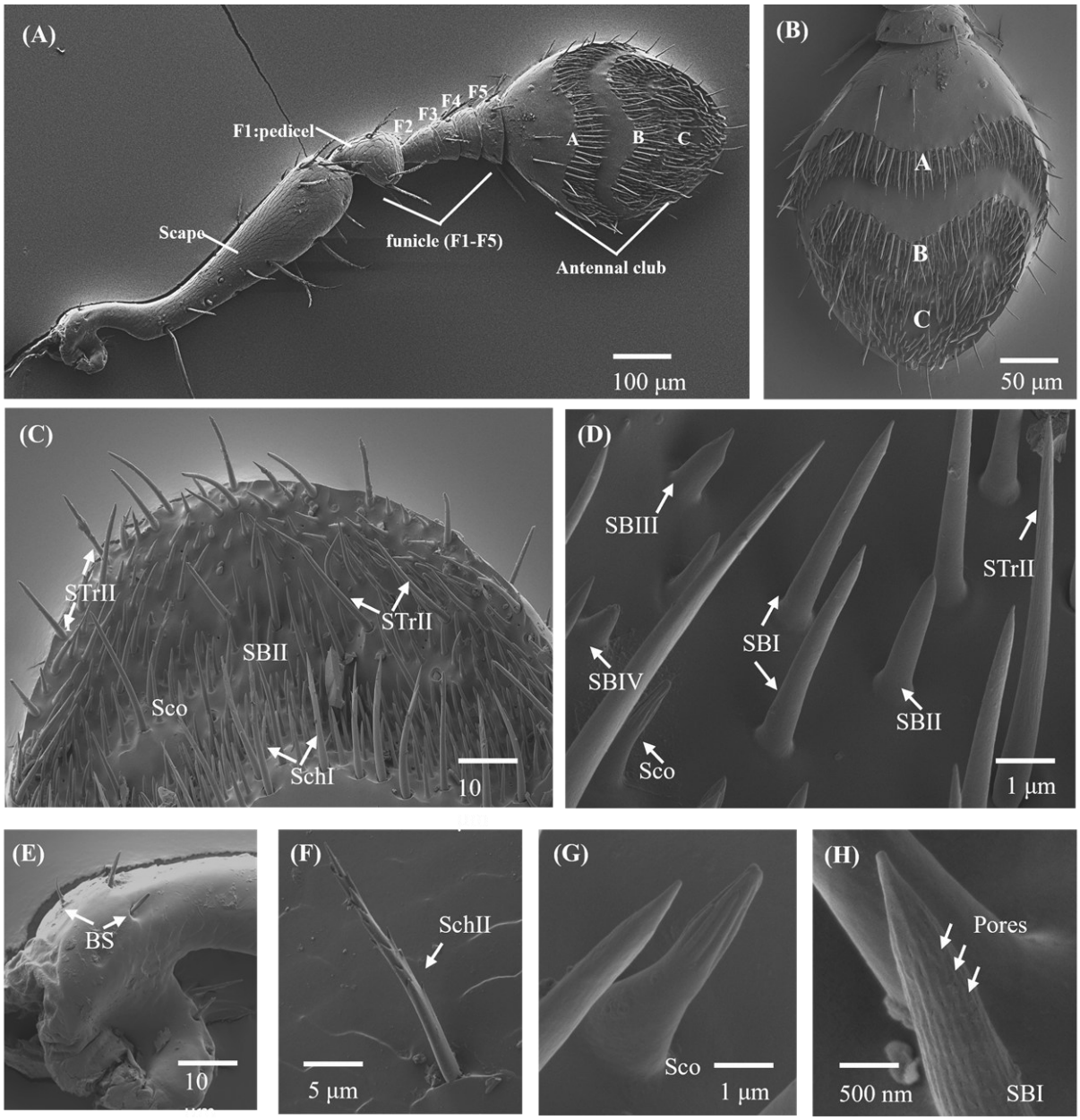

Figure 16. *SEM image of* Ips cembrae *antennal club indicating general structure (A)and the three bands (A–C) with sample types of sensilla labeled (B); sensilla trichodea* (STrIII & IV) *(C); sensilla basiconica* (SBI, II, III & IV) *(D); Bohm sensilla* (BS) *(E); sensilla chaetica* (SchI & II) *(F); sensilla coeloconica* (Sco)*(G); and wall pores on sensilla basiconica type I* (SBI) *(I).Shewale et al., unpublished.*

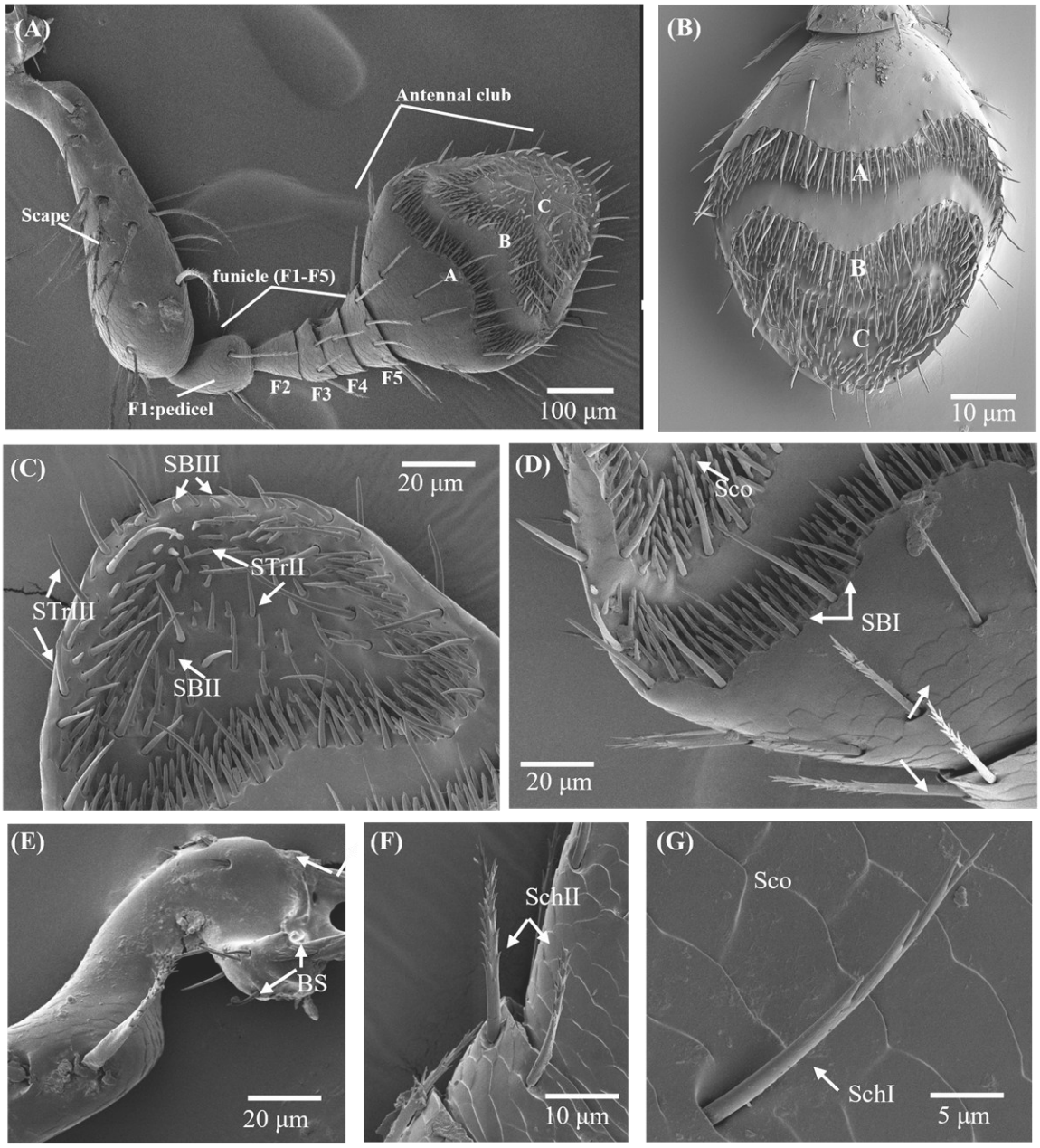

Figure 17. *SEM image of* Ips acuminatus *antennal club showing general structure (A)and the three bands (A–C) with sample types of sensilla labeled surface(B); topography and distribution of important sensilla types sensilla trichodea and sensilla basiconica (C, D); Bohm sensilla (E); sensilla chaetica (F); sensilla coeloconica (G). Shewale et al., unpublished.*

4.3.2. Classification of sensilla types

Key message: The sensilla types and their arrangement that we have characterized further illustrate the conserved structure of the peripheral antennal morphology in *Ips*.

A total of five main categories of antennal sensilla were identified and characterized in both *Ips cembrae* and *Ips acuminatus* (Table 6). Sensilla chaetica (SCh), identified as long, uniporous mechanosensory hairs, were predominantly located along the peripheral edges and outer margins of the antennal club. Two distinct subtypes were differentiated based on the presence or absence of lateral branching structures.

Sensilla basiconica (SB) were observed as a short, thick, multiporous sensilla with four morphologically distinct subtypes. These were primarily arranged within sensory bands A and B. Among them, subtype SBI was the most numerous and densely distributed, appearing across all three sensory bands (A, B, and C).

Sensilla trichodea (STr) were slender, hair-like, porous structures, and three subtypes were recognized. These were mainly confined to the sensory band C. Sensilla coeloconica (SCo), recognized by their characteristic peg-in-pit morphology, appeared in low numbers and were sparsely distributed across the antennal surface.

Lastly, Böhm's sensilla (BS), known for their mechanosensory function, were identified at the articulation between the scape and pedicel. Their presence and morphology were consistent with those previously reported in other species of the *Ips* genus.

Table 6: Morphological characteristics and distribution of sensilla types on the antennae of *Ips acuminatus and I. cembrae.* Shewale et al., unpublished.

Sensilla type	Distribution	Pores	Wall structure	Tip	Shape	Socket
SchI	Antennal club (A, B and C), funicular segments (F1-F5) and scape	Aporous	Longitudinal grooved wall, bilateral branching	Sharp	Straight	Flexible
SChII	Antennal club (A), funicular segments (F1-F5) and scape	Aporous	Longitudinal grooved wall, multi-branching	Sharp	Curved	Flexible
SBI	Antennal club (A, B, C)	Multiporous	Pitted	Blunt	Straight	Inflexible
SBII	Antennal club (A, B and C)	Multiporous	Grooved	Blunt	Straight	Inflexible
SBIII	Antennal club (B and C)	Uniporous	Smooth	Blunt and round	Peg shaped	Inflexible
SBIV	Antennal club I	Uniporous	Smooth	Round	Straight	Inflexible
STrIII	Antennal club (A, B and C)	Terminal pore	Smooth	Blunt	Long and curved	Flexible
Sco	Antennal club (A, B and C)	Aporous	Grooved	Round	Cone-shaped	Inflexible
BB	Scape	Aporous	Smooth	Blunt and round	Short and straight	Flexible
SP?	Club (A, B and C), funicle segments (F1-F5) and scape	?	Pit on the club surface	-	Oval	-

4.3.3. Current scope and future directions of this study

Key message: This preliminary map presents a significant starting point for SSR-based OSN classification and future comparative sensory ecology studies.

In this section, we have set the foundational map morphology for subsequent SSR studies set to functionally characterize OSN classes in *I. cembrae* and *I. acuminatus* (see Chapter 4.5). While only qualitative data are presented here, future analyses will explore more detailed morphometrics, i.e., sensillum length, socket type, and sex differences, and they will be submitted as a separate manuscript.

The consistency of the sensory band patterns and sensilla types across the species supports the hypothesis that antennal morphology is a conserved trait across the genus *Ips*. This data give a better understanding of how bark beetles have evolved olfactory structures related to their ecological characteristics in conifer forests.

Subchapter 4.4: Size-dependent olfactory responses in female *Ips typographus* (Paper IV)

Article type and status: Original Research Article (Submitted to *Annals of Forest Science*, under revision)

Based on: Moliterno, A. A. C. †, **Shewale, M.K**. †, Basile, S., Synek, J., Jirošová, A. (2025). Size- and dose-dependent behavioral responses to 1,8-cineole and (+)-isopinocamphone: a potential host selection strategy in female *Ips typographus*. Manuscript submitted to *Annals of Forest Science*.

† *Equal contribution as first author*

My contribution: Conceptualization, electroantennography experiments, data analysis and visualization, writing of original draft, and review and editing of manuscript.

Key message: Body size in female *Ips typographus* significantly influences antennal sensitivity and behavioral responsiveness to host-related semiochemicals, suggesting size-linked adaptive roles in host selection.

Article summary:

This research investigated whether body size variation among female *I. typographus* may influence their olfactory sensitivity to two chemically related oxygenated monoterpenes: 1,8-cineole and (+)-isopinocamphone. These two compounds are ecologically relevant because they are both emitted by a drought-stressed host and symbiotic fungi, respectively. Field experiments conducted using pheromone-baited traps added with varying doses of either monoterpene resulted in size-dependent reaction in females, which may indicate greater behavioral sensitivity and possible preference during host selection.

Along with the behavioral experiments, complementary electroantennography (EAG) recordings confirmed these patterns at the physiological level. The antennae of large females demonstrated significantly stronger responses to (+)-isopinocamphone than small females when responding to the same stimuli and with respect to the dose gradient. In contrast, smaller females showed stronger responses to a high dose of 1,8-cineole than larger females. Morphometric data also confirmed size-related variation in the antennal club structure, providing further evidence of a relationship between morphology and

olfactory function. Taken together, these findings illustrate intraspecific variability in chemical perception and evidence suggesting that female body size can be a criterion for olfactory responsiveness and ecological role when colonizing hosts.

4.4.1 Prevalence and body size differences in female trap captures

Key message: Female *I. typographus* of varying sizes respond differently to semiochemical treatments, suggesting a size-linked preference or sensitivity in host cue recognition.

Field trapping data collected in 2019 and 2022 demonstrated significant variation in female body size across different chemical treatment groups. Traps baited with higher concentrations of 1,8-cineole consistently captured smaller females (Fig. 18A). In contrast, traps containing elevated doses of (+)-isopinocamphone were associated with the capture of larger females (Fig. 18B). These observations indicate a measurable, compound-specific difference in the size distribution of captured females depending on the type and dose of the chemical lure.

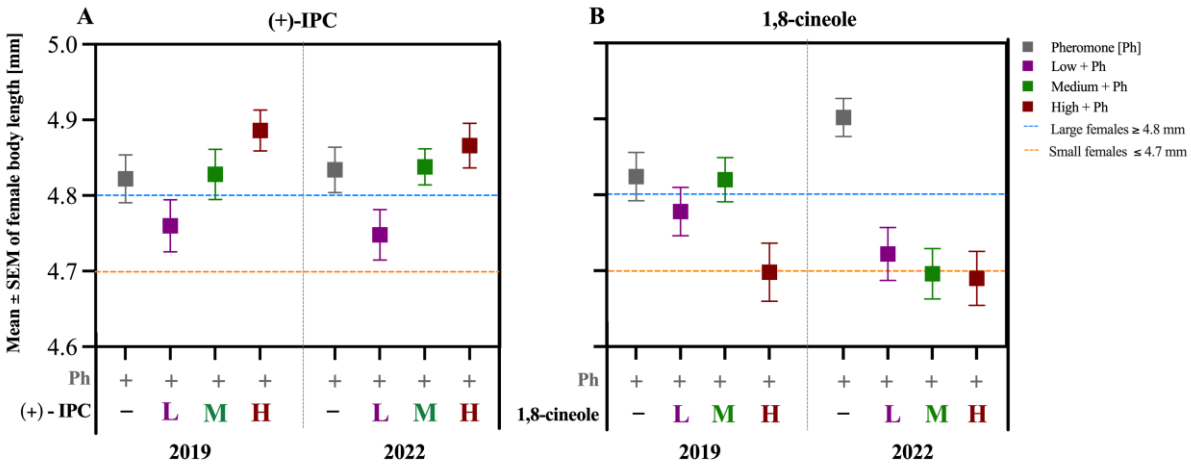

Figure 18. *Body length of female* I. typographus *captured with different doses of (A) 1,8-cineole and (B) (+)-isopinocamphone vs. pheromone-only controls in 2019 and 2022. Moliterno et al., unpublished.*

4.4.2 Antennal club dimensions scale with body size

Key message: Antennal club morphology scales isometrically with body size in female *I. typographus*, preserving proportional structure regardless of individual size class.

Morphometric analysis comparing large and small *Ips typographus* females revealed that antennal club dimensions, specifically length and width, scaled isometrically with total

body length in both size groups. Statistical comparisons confirmed significant differences in overall body and antennal size between the large and small individuals. However, the relationship between antennal length and width remained consistent, indicating isometric scaling. This finding shows that while absolute sizes varied, the proportional dimensions of the antennal club were maintained across individuals of different sizes.

4.4.3 Size-dependent antennal sensitivity to oxygenated monoterpenes

Key message: Female body size in *I. typographus* correlates with differential antennal sensitivity to specific semiochemicals, possibly affecting individual-level host selection strategies.

Electroantennography (EAG) recordings revealed size-dependent differences in antennal responses among *Ips typographus* females. No significant variation in pheromone sensitivity was observed between large and small females across size classes (Fig. 19A). However, larger females exhibited significantly higher antennal responses to increasing concentrations of (+)-isopinocamphone (Fig. 19B). In contrast, smaller females showed stronger antennal responses to higher doses of 1,8-cineole (Fig. 19C). These data demonstrate a compound- and size-specific variation in olfactory response intensity.

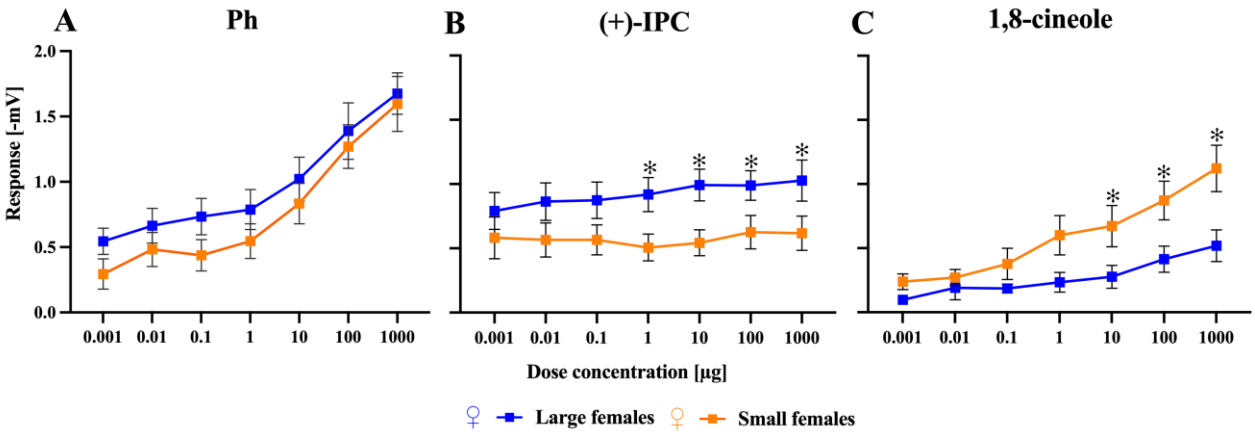

Figure 19. *EAG responses of large and small* I. typographus *females to (A) pheromone blend (MB:cV, 10:1), MB: 2-methyl-3-buten-2-ol and cV: cis-Verbenol, (B) (+)-isopinocamphone* (IPC)*, and (C) 1,8-cineole across increasing doses. Asterisks denote significant differences (Wilcoxon test, $p < 0.05$). Moliterno et al., unpublished.*

Subchapter 4.5: Electrophysiological characterization of Olfactory Sensory Neurons in *I. acuminatus* and *I. cembrae* (Paper V)

Article type and status: Original research article (Accepted for publication)

Authors: Shewale, M. K., Bláha, J., Synek, J., Schebeck, M., Andersson, M. N., Kandasamy, D., & Jirošová, A. (2025) Comparative analysis of olfactory sensory neurons in two Ips species reveals conserved and species-specific olfactory adaptations. *Frontiers in Forests and Global Change*, *8*, 1588866.

doi: 10.3389/ffgc.2025.1588866

My contribution: Conceptualisation, data curation, investigation, formal analysis, writing original draft, visualisation, methodology, review and editing.

Article summary

This work is the first detailed electrophysiological mapping of olfactory sensory neurons (OSNs) in *Ips acuminatus* and *Ips cembrae* using single sensillum recordings (SSR), and details 19 OSN classes between the two species that responded to a variety of ecological odorants, including pheromones, host-, non-host, and microbial volatiles.

The findings reveal conserved and species-specific olfactory adaptations across the compared *Ips* bark beetles, deepening our understanding of their peripheral olfactory coding systems and identifying OSN in pheromone and host volatile detection. This study presents a comprehensive olfactory profile for two economically important bark beetle species, establishing a reference for their peripheral sensory systems.

Despite morphological similarities, their OSN tuning diversity highlights ecological specialization and evolutionary divergence. These results provide essential groundwork for designing semiochemical-based pest management strategies and future neurogenetic research into *Ips* olfactory mechanisms.

4.5.1 General classification of OSN types

Key message: *I. acuminatus* and *I. cembrae* exhibit complex and highly responsive peripheral olfactory systems, with most antennal sensilla housing multiple OSNs tuned to ecologically relevant volatiles.

Using single sensillum recordings (SSR), we characterized 19 classes each of olfactory sensory neurons (OSNs) in *I. acuminatus* and *I. cembrae* and identified different response profiles to a wide range of 57 ecologically relevant odorants (Fig. 21A and 24A).

In both species, most sensilla had two OSNs, distinguished by different spike amplitudes: A neuron with larger spike amplitudes and B neuron with smaller ones. Occasionally, a few sensilla housed only one or at most three neurons. Responses exhibited tonic or phasic-tonic response patterns, with firing rates consistently above 80 Hz for their primary ligands (Fig. 20A and B).

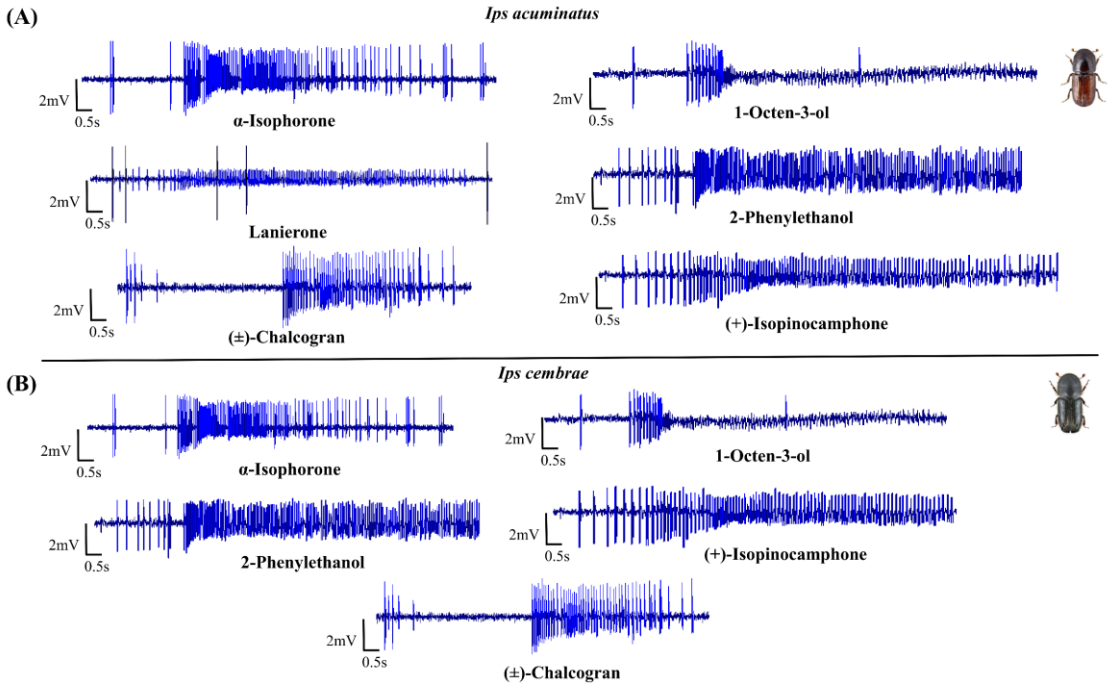

Figure 20. *Olfactory sensory neurons (OSNs) exhibit distinct phasic-tonic responses to 10 µg of each compound. Two OSNs (A and B), differing in spike amplitude, are typically present in one sensillum. Panel (A) shows* I. acuminatus *responses to six odorants; panel (B) shows* I. cembrae *responses to four.*

OSNs generally responded to multiple compounds, but primary ligands consistently triggered the highest firing rates, often exceeding 80 Hz, with secondary responses typically elicited by structurally similar compounds. Maximum response frequencies reached 150 Hz in *I. acuminatus* and 200 Hz in *I. cembrae*. Compounds that exhibited the strongest responses also displayed the lowest detection thresholds.

In both *Ips* species, a good percentage of sensilla responded to at least one of the compounds, with 84% of sensilla in *I. acuminatus* and 73% of sensilla in *I. cembrae*. Strongly responsive OSNs (>80 Hz) were grouped into distinct OSN classes based on their tuning profiles. In contrast, OSNs with moderate responses (20–80 Hz) remained unclassified, as we could not clearly identify the ligand specificity. These results provide the first foundational OSN map for peripheral olfactory detection for these species and allow for comparative study with existing data for *I. typographus* (see Section 4.5.4).

4.5.2 OSN responses in *Ips acuminatus*

Key message: *Ips acuminatus* exhibits specialized OSNs that selectively respond to pheromones, host-, non-host and microbial volatiles, highlighting its complex chemosensory adaptations for host selection and intraspecific communication.

OSNs responding to aggregation pheromone components in *I. acuminatus*

In *I. acuminatus*, at least five OSN classes responded strongly to aggregation pheromone components, each demonstrating high ligand specificity and dose-dependent activity. IAc1 neurons were strongly responsive to (4*S*)-*cis*-verbenol, the major pheromone component. This class also showed weak responses to secondary compounds such as *trans*-verbenol isomers, verbenone, and chalcogran (Fig.22C). These A neurons were co-ocalized with IAc2 B neurons, which responded to the host volatile 1,8-cineole. Both were primarily located in sensory band C of the distal antennal club (Fig. 21A).

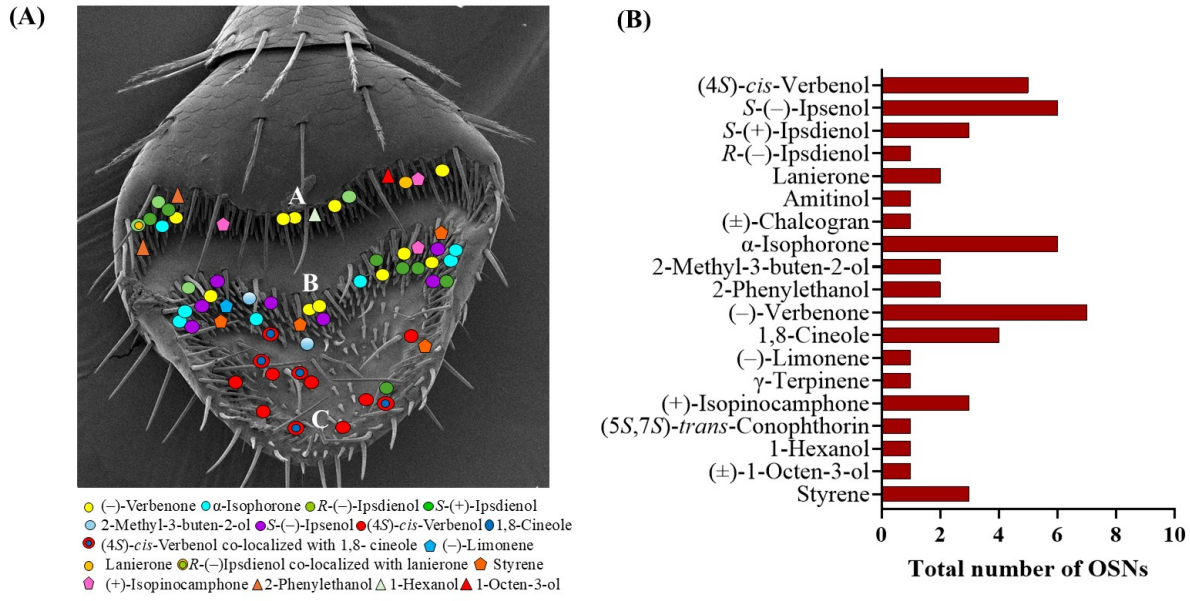

Figure 21: *(A) Spatial distribution of olfactory sensory neuron (OSN) classes across sensory bands A, B, and C on the antennae of* Ips acuminatus. *(B) Total counts of 19 identified OSN classes, categorized by primary response to ecologically relevant compounds. Shewale et al., 2025.*

Class IAc3 had a strong response to *S*-(−)-ipsenol and weak responses to *R*-(+)-ipsenol and ipsdienol (Fig.22G). The sensitivity of this neuron was confirmed at 100 pg (Fig.23E). These neurons were localized in sensory band B. IAc4 neurons were exclusively found only in females, responded strongly to *R*-(−)-ipsdienol, with weaker responses to its *S*-(+) enantiomer, racemic ipsdienol, and amitinol (Fig.22E). These were co-localized with IAc9 B neurons, which responded to lanierone, and were distributed in bands A and B. Dose-response tests later revealed stronger tuning to *S*-(+)-ipsdienol, suggesting the presence of two enantiomer-selective OSN classes (Fig.23D). OSN class IAc13 responded most to racemic ipsdienol, with secondary responses to amitinol, *E*-myrcenol, and ipsdienol enantiomers. Another OSN class IAc14 showed strong activation by amitinol, followed by weaker responses to racemic ipsdienol and its enantiomers.

OSN responses to other beetle-produced compounds in *I. acuminatus*

Five OSN classes in *I. acuminatus* were specifically tuned to beetle-produced semiochemicals beyond its own aggregation pheromones. IAc5 was the most abundant class and responded strongly to (−)-verbenone, with a 1 ng threshold (Fig. 23B). This class was also exclusively found in females.

Furthermore, another very specific OSN class, IAc6, was tuned to α-isophorone and was very sensitive, with detection thresholds at the picogram level (Fig. 23C). IAc9 responded strongly to lanierone, co-localized with either *R*-(−)-ipsdienol-responsive or non-responsive A neurons. IAc10 strongly responded to 2-phenylethanol, and IAc11 to 2-methyl-3-buten-2-ol, with weaker responses to 3-methyl-3-buten-1-ol. IAc17 strongly responded to chalcogran, with intermediate responses to (±)-*exo*-brevicomin and weaker activity to *trans*-conophthorin. These OSNs were broadly distributed across sensory bands A, B, and C.

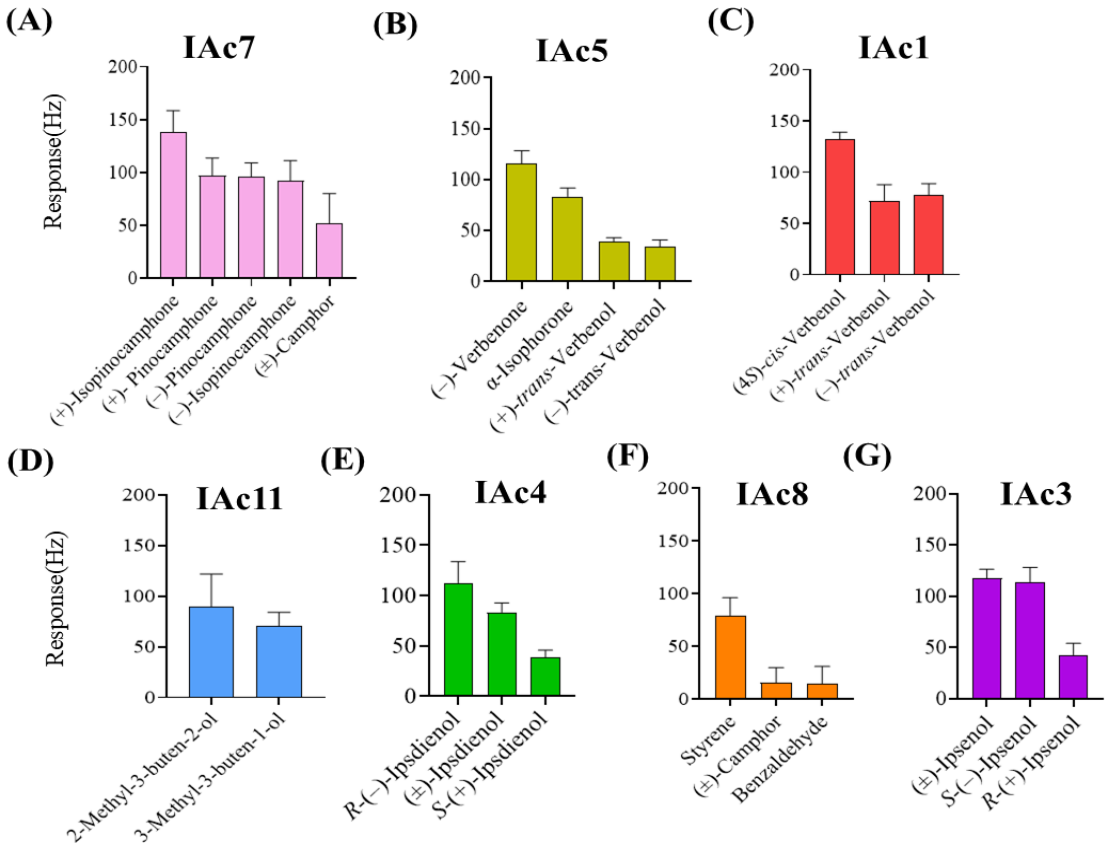

Figure 22. *Number of OSNs uniquely identified in* I. acuminatus*, indicating primary and secondary responses. Primary OSN classes (A-G) labeled IAc7, IAc5, IAc1, IAc11, IAc4, IAc8 and IAc3 correspond to compounds (+)-isopinocamphone, (−)-verbenone, (4*S*)-*cis*-verbenol,2-methyl-3-buten-2-ol,* R-*(−)-ipsdienol,styrene and racemic ipsenol. Shewale et al.,2025.*

OSN responses to host-, non-host, and microbial volatiles

Three OSN classes were highly responsive to host tree-derived volatiles. The B neuron of class IAc2 responded exclusively and robustly to 1,8-cineole. The A neuron class IAc15 was activated by both (−)- and (+)-limonene, with additional secondary responses to myrcene, p-cymene, terpinolene, and Δ-3-carene, and weaker activity to (+)-terpine-4-ol and (−)-β-pinene. Another A neuron class, IAc18, responded strongly to γ-terpinene and showed secondary responses to several structurally related oxygenated monoterpenes, including both isomers of isopinocamphone and pinocamphone, as well as racemic camphor. These OSNs were predominantly located in sensory band B of the antennal club. While most pheromone- and host-volatile-responsive neurons were not spatially segregated, (4*S*)-*cis*-verbenol-sensitive neurons (IAc1) were always located exclusively to sensory band C.

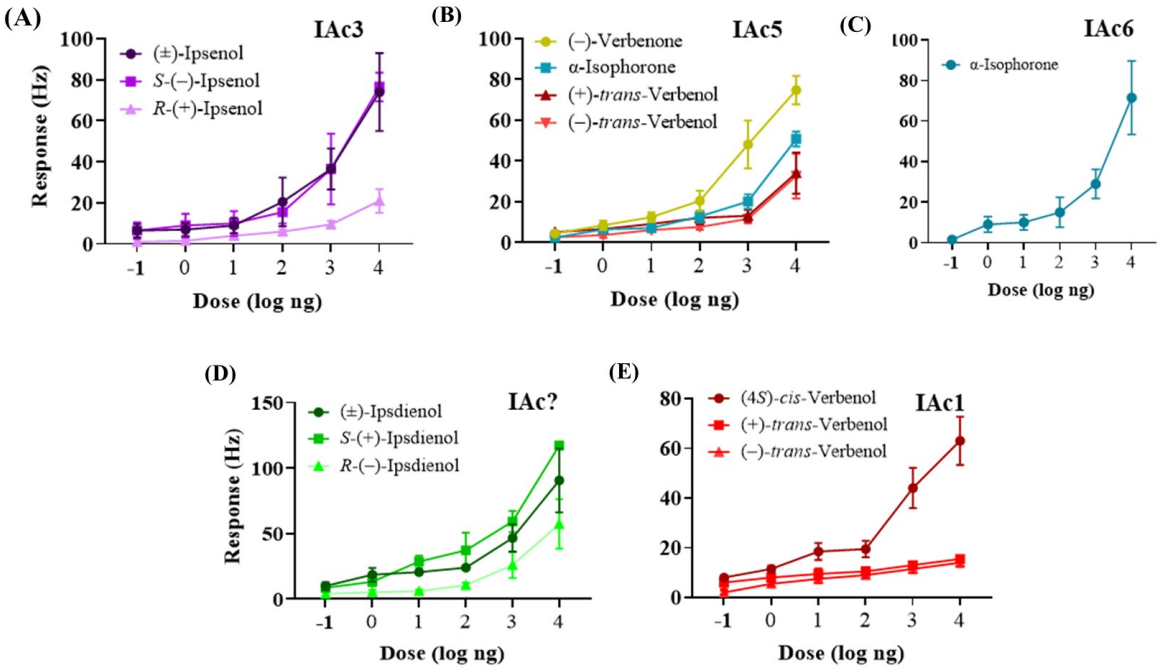

Figure 23. *Mean dose responses (Hz) of selected OSN classes in* I. acuminatus*, showing both primary and secondary responses: IAc3:* S-*(−)-ipsenol, IAc5: (−)-verbenone, IAc6: α-isophorone, IAc?:* R-*(−)-ipsdienol, and IAc1: (4*S*)-*cis*-verbenol. Shewale et al.,2025.*

In response to non-host volatiles, three OSN classes were identified. OSN class IAc12 responded strongly to 1-hexanol, with weaker secondary responses to racemic 1-octen-3-ol and chalcogran. IAc16 was activated by racemic 1-octen-3-ol and showed minor responses to racemic 3-octanol. The IAc19 class responded specifically to the non-host volatile (5*S*,7*S*)-*trans*-conophthorin.

Two OSN classes showed strong tuning to microbial volatiles. The A neuron class IAc7 was specifically activated by (+)-isopinocamphone and showed moderate responses to structurally related compounds such as (−)-isopinocamphone, (+)- and (−)-pinocamphone, and racemic camphor. Meanwhile, IAc8, also an A neuron, responded selectively to styrene and exhibited weaker responses to benzaldehyde and racemic camphor.

4.5.3. OSN responses in *Ips cembrae*

Key message: In *I. cembrae*, 19 olfactory sensory neuron (OSN) classes were identified, revealing a highly conserved and partially species-specific peripheral olfactory system.

OSNs responding to aggregation pheromone of *I. cembrae*

Two classes of OSNs responded to aggregation pheromone components of this species. OSN class IC1, an A neuron, responded strongly to (4*S*)-*cis*-verbenol with dose-dependent activity and a sensitivity threshold of 100 pg (Fig.26C) . Secondary responses to (+)- and (−)-*trans*-verbenol were moderate (Fig. 25D). (4*S*)-*cis*-verbenol OSNs were co-localized with IC2, a B neuron class responsive to 1,8-cineole. These OSNs were predominantly located in sensory band C of the distal antennal club, with a few located in band B (Fig. 24A).

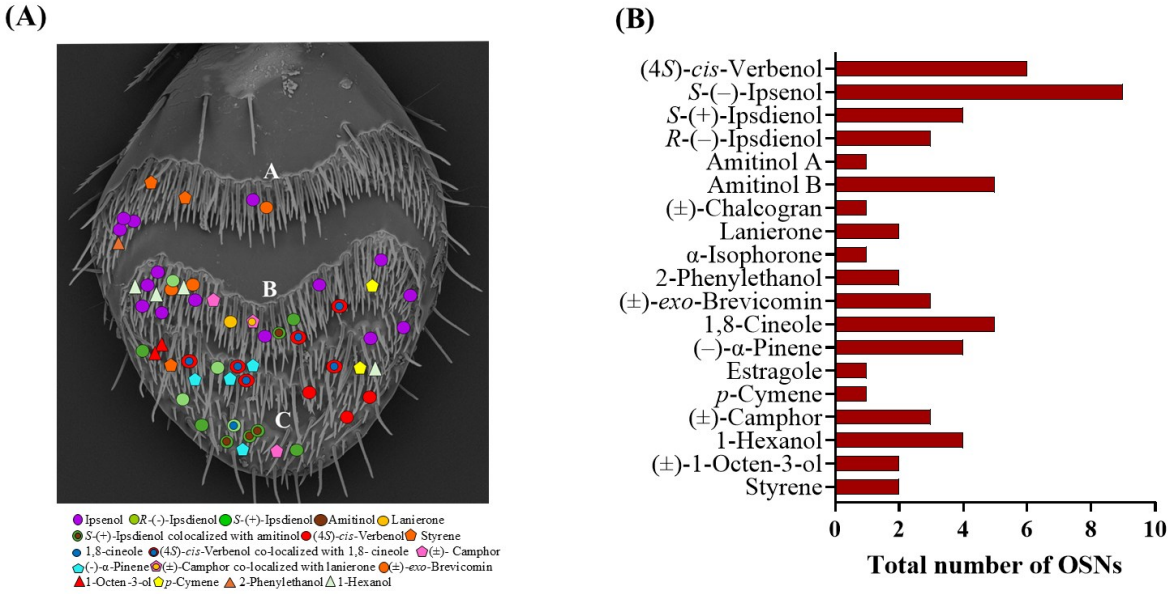

Figure 24. *(A) Distribution of olfactory sensory neuron (OSN) classes across sensory bands A, B, and C on the antenna of* Ips cembrae*. (B) Total counts of the 19 OSN classes, grouped by primary responses to compounds from various ecological origins. Shewale et al.,2025.*

The most frequently encountered OSN class was IC3, tuned to racemic ipsenol and *S-(−)*-ipsenol, the major component of the species' pheromone blend. These neurons showed high specificity, low thresholds (~100 pg), and minimal responses to *R*-(+)-ipsenol, aligning with its absence in the natural pheromone mix (Fig. 25A). IC3 neurons were uniformly distributed across sensory bands A and B. Another class, IC4, responded to *R*-(−)-ipsdienol, with weaker responses to the corresponding *S*-enantiomer and racemic form of ipsdienol, but was not observed in dose-response studies. In contrast, IC5 neurons were specifically tuned to *S*-(+)-ipsdienol, with a response threshold of 1 ng (Fig. 25B). These were consistently co-localized with IC6, a B neuron class responsive to amitinol. Both IC5 and IC4 classes mainly were localized in sensory band C and rarely in band B (Fig. 24A). Interestingly, no OSNs were detected that responded to 3-methyl-3-buten-1-ol, which is a pheromone component of *I. cembrae.*

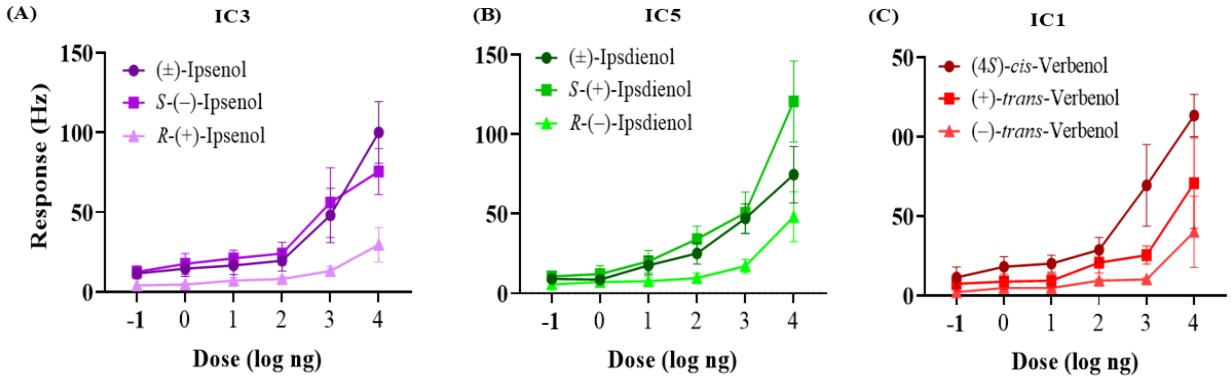

Figure 25. *Dose-response profiles of three pheromone-specific OSN classes in* I. cembrae*: IC3 responding to* S-*(−)-ipsenol, IC5 to* S-*(+)-ipsdienol, and IC1 to (4*S*)-*cis*-verbenol. Mean responses are presented with SEM error bars. Shewale et al., 2025.*

Other OSNs responding to other beetle-produced pheromones

Four additional OSN classes in *I. cembrae* responded strongly to various beetle-produced volatiles. OSN class IC7 was activated by (±)-*exo*-brevicomin, with weaker responses to chalcogran and (5*S*,7*S*)-*trans*-conophthorin (Fig. 26C). IC14 was tuned to lanierone and was co-localized with either IC9 or a non-responsive A neuron. IC12 responded specifically and strongly to 2-phenylethanol, while IC15 was primarily activated by chalcogran and secondarily by 1-hexanol, 1-octen-3-ol, and *trans*-conophthorin. OSN class IC16 responded strongly to amitinol with secondary responses to racemic ipsdienol, and IC17 was tuned to *α*-isophorone, with weaker responses to verbenone and both enantiomers of *trans*-verbenol. These OSNs were primarily located in the distal region

of the antennal club, indicating a localized sensory specialization for these beetle-produced cues.

OSNs responding to host-, non-host, and microbial volatiles

In *I. cembrae*, five OSN classes were specifically tuned to host volatiles. Among them, IC2 class was specific for 1,8-cineole, while IC9 responded strongly to camphor and (+)-isopinocamphone, with additional weaker responses to structurally related oxygenated monoterpenes including isopinocamphone, pinocamphone, and borneol. IC9 was co-localized with a B neuron responsive to lanierone (IC14). The IC10 class was tuned to (−)-α-pinene and showed lower responses to several related terpenoids such as *cis*-verbenol and β-pinene. OSN classes IC18 and IC19 responded strongly to *p*-cymene and estragole, respectively. These host-volatile-responsive neurons were primarily located in sensory bands B and C (Fig. 23A).

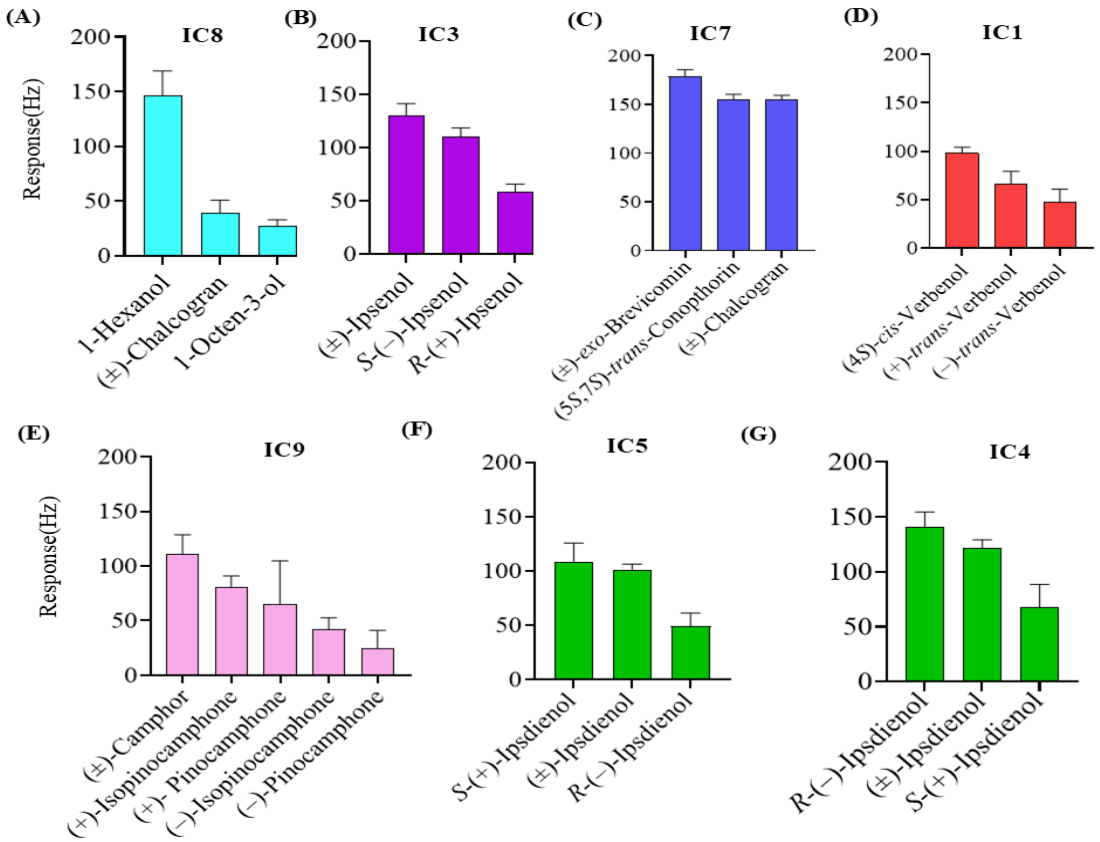

Figure 26. *Mean response rates (Hz) of selected OSN classes in* I. cembrae*, including secondary responses: IC8 responding to 1-hexanol, IC3 to* S-*(−)-ipsenol, IAc7 to (±)-*exo-*brevicomin, IC9 to racemic camphor, IC1 to (4*S)-cis-*verbenol, IC5 to* S-*(+)-ipsdienol, and IC4 to* R- *(−)-ipsdienol. Error bars indicate the standard error of the mean (SEM). Shewale et al.,2025.*

Two OSN classes strongly responded to non-host volatiles. IC8 responded primarily to 1-hexanol, with secondary responses to chalcogran, 1-octen-3-ol, and 2-phenylethanol (Fig. 26A). IC13 was activated by 1-octen-3-ol, with weaker responses to 3-octanol. Both classes were found in sensory bands B and C.

One additional class, IC11, responded to the microbial volatile styrene, showing weak secondary activity to benzaldehyde. These results indicate a well-distributed and chemically diverse OSN system in *I. cembrae*, capable of detecting key volatiles from host trees, non-hosts, and microbial sources.

4.5.4 Comparative analysis of OSN profiles and distribution among *Ips acuminatus*, *I. cembrae*, and *I. typographus*

Key message: *Ips acuminatus*, *I. cembrae*, and *I. typographus* share a conserved set of OSN classes tuned to ecologically relevant volatiles, yet each species also possesses unique OSN types reflecting distinct host preferences.

Comparison across the three *Ips* species: *I. acuminatus*, *I. cembrae*, and *I. typographus* revealed 11 OSN classes shared by all three, predominantly those tuned to aggregation pheromones and host volatiles (Fig.27; Table 7). However, unique OSNs were observed in each species: four in *I. cembrae* (e.g., α-pinene, *exo*-brevicomin) and two in *I. acuminatus* (e.g., γ-terpinene, limonene). The comparison indicates both evolutionary conservation and species-specific tuning of peripheral olfactory systems.

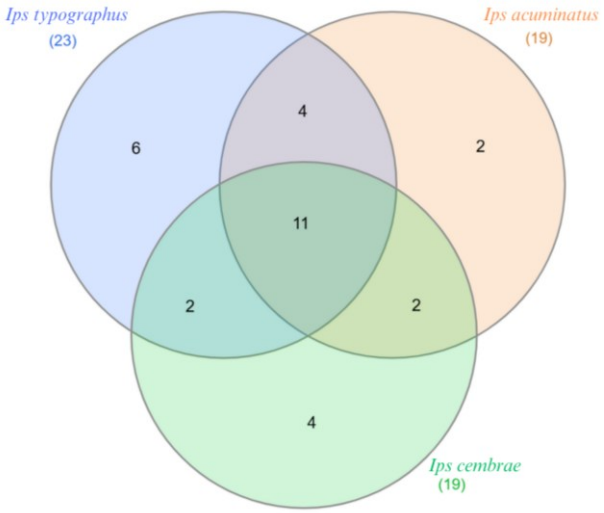

Figure 27. *Venn diagram illustrating the overlap of identified olfactory sensory neuron (OSN) classes in* Ips acuminatus *and* Ips cembrae *compared to previously reported OSN classes in* Ips typographus*. Shewale et al., 2025.*

Table 7. Olfactory sensory neurons (OSNs) classified based on their response profiles at a 10 µg screening dose in *I. acuminatus* and *I. cembrae* and their comparison to previously characterized OSN classes in *I. typographus.* Shewale et al., 2025.

Biological origin	OSN class↓/Species→	*I. typographus* (IT)	*I. acuminatus* (IAc)	*I. cembrae* (IC)
Beetle	(4*S*)-*cis*-Verbenol	✓[1,2]	✓	✓
Beetle	*S*- (+)-Ipsdienol	✓[1]	✓	✓
Beetle	*R*- (−)-Ipsdienol	✓[1]	✓	✓
Beetle	*S*- (−)-Ipsenol	✓[6]	✓	✓
Beetle	*R*- (+)-Ipsenol	-	-	-
Beetle	Amitinol	✓[1]	✓	✓(A and B neuron)
Beetle	*2*-Methyl-3-buten-2-ol	✓[1,3] (B neuron)	✓ (B neuron)	-
Beetle	3-Methyl-3-buten-1-ol	-	-	-
Beetle	Lanierone	✓ [5] (B neuron)	✓ (B neuron)	✓ (B neuron)
Beetle	(±)-Chalcogran	-	✓	✓
Beetle	α-isophorone	-	✓	✓
Beetle/fungi	(−)-Verbenone	✓[1,4]	✓	-
Beetle/ fungi	(±)-*exo*-Brevicomin	-	-	✓
Beetle/fungi	2-Phenylethanol	✓[3]	✓	✓
Host	(+)-3-Carene	✓[1]	-	-
Host	Myrcene	✓[1,2,3]	-	-
Host	(+)-α-Pinene	✓[1]	-	-
Host	(-)-α-Pinene	-	-	✓
Host	*p*-Cymene	✓[1]	-	✓
Host	(−)-Limonene	-	✓	-
Host	γ-Terpinene	-	✓	-
Host	1,8-Cineole	✓[1] (B neuron)	✓(B neuron)	✓(B neuron)
Host/fungi	(±)-Camphor	-	-	✓
Host/fungi	(+)-Isopinocamphone	✓[4]	✓	-
Host/fungi	Estragole	✓[7]	-	✓
Host/fungi	(+)-*trans*-4-Thujanol	✓[2,4]	-	-
Non-host	1-Hexanol	✓[1]	✓	✓
Non-host/fungi	(±)-3-Octanol	✓[1]	-	-

Non-host/fungi	(±)-1-Octen-3-ol	✓[1]	✓	✓
Non-host/fungi	Geranyl acetone	✓[3]	-	-
Non-host/fungi	(5S,7S)-*trans*-Conophthorin	✓[1]	✓	-
Fungi	Styrene	✓[2,4]	✓	✓

✓ OSN class identified; OSN class not found yet

[1]Andersson et al. 2009; [2] Schiebe et al. 2019; [3] Kandasamy et al. 2019; [4] Kandasamy et a.l 2023; [5] Yuvaraj et al. 2024; [6] Tömmerås 1985; [7] Raffa et al. 2016

4.5.5 Antennal responses to host essential oils for further validation (GC-EAD)

Key message: GC-EAD confirms that *Ips* species display species-specific antennal sensitivity to conifer volatiles.

To complement the single sensillum recording (SSR) data, gas chromatography coupled with electroantennographic detection (GC-EAD) was employed to assess antennal sensitivity to host-derived essential oil compounds. In *Ips acuminatus*, GC-EAD recordings revealed four electroantennographically active peaks (1-4), corresponding to α-pinene, limonene, linalool, and isobornyl acetate (Fig. 28A). These compounds elicited clear and reproducible antennal responses across multiple replicates.

In *Ips cembrae*, five distinct EAD-active peaks (1-5) were detected, identified as β-pinene, p-cymene, camphor, linalool, and terpinen-4-ol (Fig. 28B). Each of these compounds produced distinct deflections in the EAD signal, indicating activation of olfactory receptor neurons.

Several other host-emitted monoterpenes, despite being abundant in the essential oil blends, produced only weak or inconsistent antennal responses in both species. This pattern of antennal activation was consistent with SSR results and provided additional evidence of differential sensitivity across individual host volatiles.

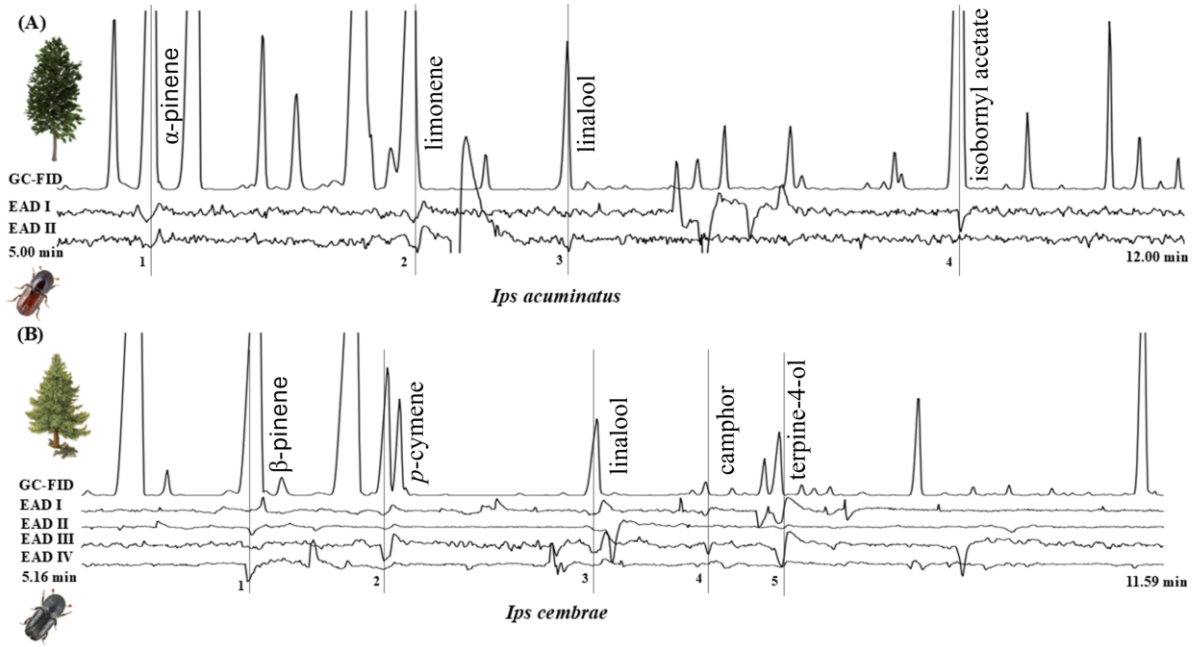

Figure 28. *GC-EAD traces showing antennal responses of* Ips acuminatus *and* Ips cembrae *to pine and larch essential oils at a dose of 10 µg. Shewale et al.,2025.*

Chapter 5: Discussion

5.1 Overview

The central objective of this thesis was to examine how *Ips* bark beetles perceive and respond to chemical cues in their environment, with a particular focus on variations in olfactory detection and sensitivity across species, sexes, and individual traits such as body size. By investigating both morphological and physiological aspects of the peripheral olfactory system, this work aimed to provide a more comprehensive understanding of the sensory mechanisms underlying semiochemical communication in *Ips* species.

A multidisciplinary methodology was employed to address this objective. This included scanning electron microscopy (SEM) for high-resolution morphological characterization of antennal sensilla, single sensillum recordings (SSR) and electroantennography (EAG) for functional analysis of sensory neuron activity, and gas chromatography coupled with electroantennographic detection (GC-EAD) for the identification of behaviorally and physiologically active odorants. Together, these techniques enabled a detailed investigation of antennal sensory systems' structure and function across four ecologically important *Ips* species: *I. typographus*, *I. duplicatus*, *I. cembrae*, and *I. acuminatus*.

The experimental result sub-chapters presented novel findings based on the background outlined in the first review article (**Paper I**). **Papers II and III** addressed the diversity and spatial distribution of antennal sensilla, offering the first comparative morphological descriptions for *I. duplicatus, I. cembrae* and *I. acuminatus*, species that have been understudied in the context of olfactory biology and antennal morphology. **Paper IV** examined how body size influences antennal sensitivity to semiochemicals, revealing distinct response patterns that suggest intraspecific variation in olfactory function. **Paper V** focused on electrophysiological recordings from individual olfactory sensory neurons, enabling classification based on response profiles to known pheromone components, host-, non-host and microbial volatiles. Overall, these studies contribute new insights into the chemosensory biology of *Ips* beetles. By integrating structural and functional data across multiple species and individual traits, this thesis establishes a comparative framework for understanding how bark beetles detect and process ecologically relevant chemical signals in their environment.

5.2. Conserved antennal morphology and potential function of sensilla types in *Ips* bark beetles

(Synthesizing Subchapters 4.2 & 4.3)

Our direct comparison of *I. duplicatus*, *I. acuminatus*, and *I. cembrae* reveals a highly conserved antennal morphology among *Ips* species, confirming earlier reports for *I. typographus* and *I. sexdentatus* (Payne et al., 1973; Hallberg, 1982a; Faucheux, 1989). Each of the three species possesses a seven-segmented antenna with a club-shaped terminal segment that houses sensilla in three sensory bands (A, B, and C), a characteristic at the genus level for scolytine bark beetles.

Five principal sensilla types, mainly, sensilla chaetica, basiconica, trichodea, coeloconica, and Böhm's sensilla observed across species. While this typology is largely conserved, detailed morphological distinctions were observed, with *I. duplicatus* displaying two different subtypes of coeloconica and a novel subtype of trichodea (STrIV). These distinctions could be a reflection of species-specific adaptations to different ecological niches, or they could reflect the increased imaging resolution afforded by HR-SEM. For example, the lateralized mirror-like pattern of coeloconica sensilla in *I. duplicatus* can suggest fine-scale thermohygrosensory tuning for habitat microclimate detection.

Functionally, sensilla chaetica are likely mechanosensory, supporting antennal positioning and perhaps acoustic communication, particularly in females, in which multibranched SChII subtypes were found to be longer. Such subtle sexual dimorphism correspond with that described in other bark beetles such as *I. sexdentatus* and *T. lineatum* (Moeck, 1968; Faucheux, 1989) and may be linked to oviposition behavior or bark navigation (Moeck, 1968; Rudinsky, 1979; Hofstetter et al., 2019). Similar can be possibly true in *I. acuminatus* and *I. cembrae,* although not yet quantified and confirmed.

Multiporous sensilla basiconica (especially SBI) are the most prevalent and abundant sensilla, occurring in clustered densities in sensory bands A and B, and are considered central to pheromone and host volatile detection (Hallberg, 1982a; Shi et al., 2021), consistent with electrophysiological data reported in *I. typographus* (Andersson et al., 2009; Kandasamy et al., 2019). Sensilla trichodea are structurally diverse based on species, with all multiporous walls indicating their role in olfactory function. One of these trichodea sensilla (STrIV) was observed specifically in *I. duplicatus* and has not

been previously documented in the *Ips* genus, representing a morphological variation from a general form. These sensilla are likely used to detect airborne pheromones and volatiles from hosts (Hallberg, 1982a; Shi et al., 2021).

Sensilla coeloconica reportedly arise from a double-walled or grooved structure, and have been implicated in thermo-, hygro-, and chemoreception (Altner et al., 1977; Hallberg, 1982a). Sensilla coeloconica, typically associated with thermo- and hygroreception or specific volatiles of hosts (ketones and aldehydes), were comprised of two morphological subtypes in *I. duplicatus,* a distinct novelty from other *Ips* species. Böhm's sensilla on the scape base and pedicel are species-conserved and likely serve as proprioceptors signaling antennal movement (Merivee et al., 1999). Finally, surface pores (SPs) found along the antennal club in *I. duplicatus* may have glandular or mechanosensory in function, but are otherwise speculative in purpose (Hallberg, 1982a; Faucheux, 1989).

In general, while overall antennal organization is evolutionarily conserved in *Ips*, sensilla subunit diversity and subtle dimorphisms suggest a hierarchical olfactory system, insensitive to widespread semiochemicals, but flexible enough for species-specific behavior and ecological specialization.

5.3 Size-dependent olfactory perception and host selection in *I. typographus*

(Synthesizing Subchapter 4.4)

This study provides compelling evidence that female *Ips typographus* exhibit body size-dependent variation in both antennal morphology and olfactory response to specific host-emitted volatiles. The findings highlight distinct patterns in antennal sensitivity and semiochemical-guided behavior related to body size, particularly in response to two oxygenated monoterpenes: (+)-isopinocamphone and 1,8-cineole.

Larger females had proportionally longer and wider antennal clubs, which scaled isometrically with overall body length. These structural morphometrics were functionally associated with significantly stronger antennal responses, as measured by electroantennography (EAG), particularly toward higher doses of (+)-isopinocamphone. This compound, commonly released during host degradation by symbiotic fungi, is associated with advanced stages of tree colonization and fungal metabolism (Kandasamy et al., 2023). The greater antennal surface area in large females likely facilitates enhanced

odorant capture and detection, supporting previous observations that antennal size correlates with increased olfactory detection (Spaethe et al., 2007; Makarova et al., 2022). The behavioral implications of this higher sensitivity were reflected in field trapping results, where large females showed increased attraction to isopinocamphone-baited traps, suggesting a preference for trees exhibiting signs of fungal activity, environments that may offer higher reproductive success (Sallé & Raffa, 2007; Foelker & Hofstetter, 2014).

In contrast, smaller females exhibited a different olfactory profile. Notably, they demonstrated higher antennal sensitivity to 1,8-cineole, an oxygenated monoterpene generally associated with host resistance and considered an anti-attractant for *I. typographus* (Schiebe et al.,2019; Jirošová et al., 2022a). Field trap data supported this physiological sensitivity, showing that smaller females were more frequently captured in traps baited with 1,8-cineole. These findings were unexpected but may suggest altered neural processing or behavioral strategies in smaller individuals (Martin et al., 2011). It is possible that smaller females, with potentially lower competitive abilities, may engage in a risk-tolerant or avoidance-based strategy by selecting suboptimal hosts to reduce intraspecific competition. This idea aligns with prior work suggesting that body size can influence colonization strategy and habitat choice under varying ecological pressures (Anton et al., 2007; Wiesel et al., 2022).

Seasonal field data further support the role of body size in host selection behavior. During epidemic conditions in 2022, a significantly higher number of large females were captured, compared to 2019 when populations were at endemic levels. This seasonal contrast supports density-dependent behavioral plasticity in host selection, where larger females may more successfully exploit resources under high-density conditions (Sallé et al., 2005).

Although this study focused exclusively on females, the olfactory sensory neurons (OSNs) responsive to both 1,8-cineole and (+)-isopinocamphone are known to occur in both sexes (Andersson et al., 2009; Kandasamy et al., 2023). Therefore, similar size-related olfactory patterns may also exist in males, and future studies could usefully explore the extent of sex-specific and size-dependent chemosensory variation in male *I. typographus*.

In summary, the data presented here demonstrate that olfactory perception in *I. typographus* is influenced by body size, which in turn affects antennal sensitivity and semiochemical-guided behavior. These size-dependent differences have potential ecological consequences for host selection, intraspecific competition, and outbreak dynamics. They also emphasize the importance of considering individual morphological traits when interpreting bark beetle behavior in the context of forest pest management and chemical communication.

5.4 Conserved OSN classes and evolutionary constraints across *Ips* species

(Comparative synthesis with I. typographus, Subchapter 4.5)

This study presents the first electrophysiological profiling of olfactory sensory neurons (OSNs) in *Ips acuminatus* and *Ips cembrae*, revealing 19 OSN classes in both species. Most OSNs were narrowly tuned to single compounds or structurally similar analogues, while few OSNs were broadly responsive. At lower stimulus doses, OSNs exhibited high specificity, which is consistent with similar findings in *I. typographus* (Andersson et al., 2009; Kandasamy et al., 2019, 2023).

Several OSNs showed conserved tuning, particularly to the enantiomers of aggregation pheromones such as ipsenol and ipsdienol (Renwick & Dickens, 1979; Francke & Vité, 1983). For both species, OSNs showed enantiomer-specific responses, often with heightened specificity to the natural form (*S*-(−)-ipsenol). This enantiomeric selectivity aligns with previous studies on *Ips* species like *I. typographus*, *I. pini*, and *I. paraconfusus* (Mustaparta et al., 1979, 1980; Tømmerås, 1985).

(4*S*)-*cis*-Verbenol was detected by specific OSNs in both species, but only functions as an aggregation pheromone in *I. acuminatus*. In *I. cembrae*, it appears to act as a disruptive interspecific signal, likely mediated by *I. typographus* (Schlyter et al., 1989). OSNs responsive to amitinol, lanierone, and (−)-verbenone were also identified in both species, mirroring patterns seen in *I. typographus* (Andersson et al., 2009, 2012b; Yuvaraj et al., 2024). Co-localization of OSNs within single sensilla, such as ipsdienol with amitinol or lanierone, supports mechanisms for blend discrimination (Baker et al., 1998; Bruce et al., 2005).

Responses to fungal volatiles (e.g., 2-phenylethanol, 1-octen-3-ol, and *trans*-conophthorin) and minor oxygenated host monoterpenes (e.g., camphor and

isopinocamphone) indicate that both species detect chemical cues associated with microbial activity and host stress, as observed in *I. typographus* (Kandasamy et al., 2019, 2023; Moliterno et al., 2023). Responses to non-host volatiles (NHVs) such as 1-hexanol and 1,8-cineole suggest shared avoidance mechanisms in conifer-feeding bark beetles (Schlyter et al., 1999, 2000).

Electrophysiological responses were consistent with a broader pattern across insects, where OSNs are often finely tuned to ecologically relevant odorants (Hallem et al., 2004; de Bruyne & Baker, 2008). Comparative evidence from other beetle genera (e.g., *Protapion* and *Pachnoda*) supports the idea of conserved OSN classes with a subset of species-specific specializations (Bengtsson et al., 2011; Carrasco et al., 2019). While OSN responses observed here parallel known patterns in *I. typographus*, molecular data such as the functionally characterized odorant receptors (ORs) in that species suggest potential conserved OR orthologs also underline responses in *I. acuminatus* and *I. cembrae* (Hou et al., 2021; Yuvaraj et al., 2021, 2024; Biswas et al., 2024). These conserved OSN profiles suggest strong stabilizing selection for detecting key semiochemicals involved in mating, aggregation, and host discrimination.

In sum, this subchapter demonstrates that while core elements of the olfactory system are conserved across *Ips* species, likely due to shared ancestral traits and ecological overlap, species-specific sensilla structures and OSN classes have evolved in response to niche partitioning and chemical specialization. These findings underscore the delicate balance between evolutionary conservation and adaptive divergence in the peripheral olfactory systems of conifer-feeding bark beetles.

5.5 Species-specific OSN tuning in *I. acuminatus* and *I. cembrae*

(Synthesizing Subchapter 4.5)

Despite predominant similarities, notable species-specific OSN features were identified. Only *I. acuminatus* showed strong, female-specific responses to (−)-verbenone, a bark beetle anti-attractant. This sex-specific OSN distribution, including others tuned to lanierone and *R*-(−)-ipsdienol in females and 2-methyl-3-buten-2-ol in males, may reflect its polygynous mating system and sex-specific behavioral roles (Kirkendall, 1989, 1990). In *I. cembrae*, OSNs specific to (−)-α-pinene and styrene were observed exclusively in males, suggesting a role in host tree detection.

OSN responses to heterospecific pheromones, such as chalcogran and *exo*-brevicomin (Francke, 1977; Zhao et al., 2019), were also more prominent in *I. cembrae*, potentially linked to its ability to colonize a broader range of hosts and interact with other bark beetle genera (Pfeffer, 1955; Postner, 1974). These findings imply a capacity for interspecific signal detection, likely facilitating coexistence or competition avoidance in overlapping habitats. Such heterospecific olfactory recognition is also reported in other Coleoptera and suggests a broader ecological role for olfaction beyond conspecific communication (Andersson et al., 2009; Kandasamy et al., 2023).

Finally, differences in monoterpene detection were evident. OSNs for monoterpenes were rare in both species and the compounds and their response strengths differed. *I. acuminatus* OSNs primarily responded to (−)-limonene and γ-terpinene, while *I. cembrae* responded to (−)-α-pinene and p-cymene. GC-EAD results supported these trends and indicated that monoterpenes may play a minor role in host tree location, especially in *I. acuminatus*, which does not show strong host attraction in the field (Brattli et al., 1998).

5.6 Integration of morphological and functional insights into bark beetle olfaction *(Synthesizing Subchapters 4.2 to 4.5)*

This study demonstrates that the peripheral olfactory system in *Ips duplicatus*, *I. acuminatus*, and *I. cembrae* is built upon a structurally conserved antennal basis, with consistent sensilla organization across species. However, subtle morphological differences, such as distinct sensilla subtypes and species-specific olfactory sensory neuron (OSN) classes, reveal adaptations linked to ecological specialization.

Antennal mapping revealed differences in the spatial distribution of OSNs across species. For instance, ipsenol OSNs were restricted to band B in *I. acuminatus* but were present in bands A and B in *I. cembrae*. On the contrary, ipsdienol OSNs occurred mostly in bands A and B in *I. acuminatus*, and in bands B and C in *I. cembrae*. These species-specific differences may reflect unique olfactory adaptations related to their respective host detection and pheromone communication shaped by distinct ecological pressures.

Single sensillum recordings confirmed the presence of both conserved and species-specific OSN classes. Conserved classes were generally tuned to shared pheromone components and host volatiles, while species-specific OSNs—such as those responsive to γ-terpinene in *I. acuminatus* or estragole in *I. cembrae*. These patterns demonstrate functional divergence tailored to particular ecological contexts. Apart from coleopteran

insects (Larsson et al., 2001), this pattern is also observed in other groups such as Drosophila and various Lepidoptera, where species exhibit both conserved OSNs and the evolution of narrowly tuned, species-specific neurons (Hallem et al., 2004; de Bruyne & Baker, 2008; Andersson et al., 2015).

At the molecular level, recent studies have linked odorant receptor (OR) repertoires in *I. typographus* to specific OSN classes (Hou et al., 2021; Roberts et al., 2021, 2022; Yuvaraj et al., 2021, 2024; Biswas et al., 2024). The presence of OR orthologs between *I. typographus* and *I. duplicatus* (Johny et al., 2024) suggests that similar conservation patterns likely extend to *I. cembrae* and *I. acuminatus*. This molecular parallel supports the idea that functional similarities in OSN tuning are supported by conserved genetic mechanisms. Taken together, these findings indicate that the olfactory system in *Ips* beetles is shaped by a dynamic interplay between evolutionary conservation and ecological diversification. Conserved OSN classes facilitate the detection of broadly relevant cues, while species-specific neurons provide the flexibility required for ecological specialization and reproductive isolation. Genomic and functional approaches, including receptor-ligand characterization and comparative transcriptomics, will be essential in elucidating the molecular basis of these olfactory adaptations.

Furthermore, results from *I. typographus* highlight the influence of antennal morphology and body size on olfactory performance and behavior. These observations suggest that both interspecific differences and intraspecific variation contribute to shaping olfactory function. Overall, the balance between phylogenetic constraints and adaptive flexibility enables bark beetles to efficiently navigate complex olfactory landscapes and maintain ecological success across diverse environments.

5.7. Methodological considerations and study limitations

While this study provides valuable insights into the sensilla equipment and olfactory adaptations of *Ips* species, particularly *I. typographus*, *I. duplicatus*, *I. acuminatus,* and *I. cembrae*, several challenges were encountered. One of the main difficulties was maintaining live beetles for experiments year-round, as some species are challenging to rear in laboratory conditions. Many bark beetles require specific environmental factors for successful breeding, and attempts to rear them under control conditions were often

unsuccessful. Additionally, since these beetles emerge seasonally, specimen collection was limited to specific periods, reducing the number of individuals available for study.
While highly sensitive, the single sensillum recording (SSR) technique has several challenges. Accurate recording from olfactory sensory neurons (OSNs) required precise electrode placement, and accessing certain antennal regions was difficult. Similarly, scanning electron microscopy (SEM) presented technical challenges, including sample preparation, imaging resolution, and classification of sensilla located in challenging areas, potentially affecting the accuracy of morphological analyses.
Furthermore, comparisons with *I. typographus* relied on previously published data, which may have been generated under slightly different experimental conditions. Variations in odor panels, methodologies, and environmental factors influencing beetle populations could introduce biases, making distinguishing species-specific adaptations from shared traits difficult. Despite these limitations, the findings of this study remain significant. However, future research could benefit from expanding sampling efforts, refining rearing techniques, and integrating behavioral and molecular studies.

5.8. Recommendations for future research and applied perspectives in forest pest management

Based on the findings of this study, a number of directions are proposed to advance our understanding of bark beetle olfaction and to improve semiochemical-based control strategies:

1. **Expand OSN and OR characterization across *Ips* species**: Future work should prioritize the functional mapping of olfactory sensory neurons (OSNs) and their associated odorant receptors (ORs) in underexplored *Ips* species and geographic populations.
2. **Incorporate sex and size-specific olfactory sensitivity in other species:** Future research should explore whether similar size-dependent olfactory adaptations exist in males and other *Ips* species. Management tools such as pheromone traps could be refined to selectively target individuals based on sex and size, especially those contributing disproportionately to reproduction and outbreak potential.
3. **Integrate fungal symbionts and host-derived volatiles into lure design:** Combine fungal-symbionts and host-derived oxygenated monoterpenes in lures

to enhance trap performance and species selectivity in mixed-beetle environments.

4. **Validate species-specific OSNs through behavioral assays:** Compounds such as estragole, chalcogran, and camphor, which activate specific OSN classes, represent promising targets for future behavioral validation to assess their ecological roles and potential in pest management. Their role in species recognition, interspecific interactions, or host selection warrants further ecological testing before potential inclusion in monitoring or control strategies.
5. **Develop smart trapping and monitoring systems:** Integrate biosensors mimicking beetle olfaction into traps for real-time semiochemical detection, enhancing early outbreak forecasting and surveillance.

   Collectively, these recommendations aim to bridge fundamental olfactory research with applied forest entomology, contributing to the development of more ecologically informed pest management systems.

Chapter 6: Concluding remarks

This thesis provides an in-depth investigation of antennal morphology and olfactory sensory neuron function in three *Ips* species: *I. duplicatus*, *I. acuminatus*, and *I. cembrae*. It also provides a focused analysis of size-dependent olfactory detection in *Ips typographus*. By combining high-resolution morphological techniques with advanced electrophysiological recordings and behavioral assays, this work significantly contributes to our understanding of chemosensory diversity and specialization in *Ips* bark beetles.

The first detailed morphological characterization of antennal sensilla in *I. duplicatus* revealed diverse sensillum types and identified subtle sexual dimorphism in their distribution and structure. It also reported descriptive analysis of antennal sensilla types in *I. cembrae* and *I. acuminatus.* These findings align with patterns observed in other *Ips* species and offer a valuable comparative framework. The sensilla classification proposed here contributes to a more standardized and reproducible nomenclature for future studies on antennal morphology and sensory system organization in bark beetles.

Electrophysiological and behavioral experiments in *I. typographus* females demonstrated clear size-dependent differences in antennal sensitivity and attraction to host-emitted oxygenated monoterpenes. Larger females showed increased antennal responses to (+)-isopinocamphone, a fungal-associated compound, while smaller females were more responsive to the anti-attractant 1,8-cineole. These results indicate that body size influences olfactory perception and semiochemical-guided behavior, potentially affecting host selection, dispersal capacity, and reproductive success. Such findings add an important individual-level perspective to population-level bark beetle dynamics.

This thesis presents the first functional classification of OSNs in *I. acuminatus* and *I. cembrae* through single sensillum recordings, identifying 19 distinct OSN classes in each species. Most OSNs were selectively tuned to key semiochemicals, including aggregation pheromones, host volatiles, non-host cues, and microbial metabolites, reflecting specialized olfactory roles. Comparative analysis with *I. typographus* revealed a shared set of OSNs, suggesting evolutionary conservation of key chemosensory functions across *Ips* species. At the same time, several OSNs were species-specific, likely representing adaptations to different ecological conditions or chemical landscapes. A

subset of OSNs remained unresponsive to the test panel, highlighting the need to expand odorant libraries and include a broader range of semiochemicals for future screening.

Taken together, the findings of this thesis offer important insights into the structure and function of the peripheral olfactory system in *Ips* bark beetles. These results not only advance fundamental knowledge in insect sensory biology but also provide practical implications for improving pest detection and control strategies. The demonstrated importance of oxygenated host volatiles and non-host cues suggests that integrating these compounds into pheromone-based traps may enhance their effectiveness and selectivity in field monitoring programs.

Lastly, this work establishes a foundation for future interdisciplinary research. Promising avenues include linking OSN functionality with olfactory receptor gene expression, exploring sex-specific differences in olfactory coding and behavior, and investigating how changing environmental conditions, including host stress and microbial interactions under climate change may influence bark beetle chemosensory ecology. Addressing these questions will further clarify the role of chemical communication in bark beetle population dynamics and inform more adaptive and sustainable forest pest management strategies.

7. References

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APPENDIX

Paper I

Ramakrishnan, R†, **Shewale, M. K.†,** Strádal, J.†, Hani, U., Gershenzon, J., Andersson, M. N., Frühbrodt, T., Doležal, P., Jirošová, A. (2025). Aggregation Pheromones in the Bark Beetle Genus Ips: Advances in Biosynthesis, Sensory Perception, and Forest Management Applications. Manuscript submitted to *Current Forestry Reports,* under revision.

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Aggregation Pheromones in the Bark Beetle genus *Ips*: Advances in Biosynthesis, Sensory Perception, and Forest Management Applications

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Conflict of Interest

Rajarajan Ramakrishnan, Mayuri Shewale K., Jaroslav Strádal, Um-e-Hani, Jonathan Gershenzon, Martin N. Andersson, Tobias Frühbrodt, Petr Doležal and Anna Jirošová declare that they have no conflict of interest**.**

Author Contributions

Conceptualization: RR and AJ; Literature search and data collection: PD, TF, MNA JS, RR, and MKS; Writing original draft: RR, MKS, JS, AJ, MNA, PD, TF, ; Data visualization: MKS, JS, and RR, AJ; Funding acquisition: MKS, JS, UH and AJ; Supervision and final editing: JG and AJ.

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Abstract:

Purpose of Review

This review synthesizes current knowledge on the aggregation pheromones of *Ips* bark beetles, major conifer forest pests worldwide whose outbreaks have intensified due to climate change. Their high pest potential arises from coordinated mass attacks on trees facilitated by male-released pheromones.

Bringing together expertise from various fields, this review integrates pheromone-based *Ips* management strategies with laboratory research on pheromone biosynthesis and detection at the neuronal and genetic levels, framed within the ecological context of selected species. By linking traditional forestry perspectives with new molecular insights, we aim to foster productive discussions and inspire innovative control approaches that can be integrated into existing management methods.

Recent Findings

- With global warming, the plasticity in voltinism allows *Ips* pest species to produce more generations per year, even at higher altitudes. Combined with weakened tree defenses, this increases their pest potential.
- Several key genes involved in the final steps of pheromone biosynthesis have been identified and characterized, enabling potential suppression of aggregation pheromone production. A new pheromone storage conjugate within the beetle body has also been proposed.
- Genes encoding olfactory pheromone receptors have been functionally characterized in *Ips typographus* as potential targets for interference, aiming to disrupt the aggregation pheromone perception of bark beetles.
- Manipulation of pheromone production and detection on the genetic level is supported by the published *I. typographus* and *I. nitidus* genomes.
- Pheromone-based population monitoring remains a key strategy in the *Ips* beetle management, while trap-and-kill methods are being underscored. Efforts are underway to develop new lure formulations and optimize the push-and-pull strategies involving anti-aggregation signals, with varying degrees of success.

Summary

Seventeen *Ips* species from diverse geographical regions, colonizing one of three conifer hosts, were selected for this review based on economic impact and biological significance. Their global distribution, preferred hosts, ecology, and biology provide a foundation for discussing pheromone composition, including advanced insights into its chemical basis.

The review details pheromone production mechanisms, biosynthetic pathways, and genetic regulation. It also explores the olfactory mechanisms on the antennae of *Ips* species, focusing on the selectivity of pheromone detection, which has been unraveled through the functional characterization of pheromone receptors and sensory neurons.

Pheromone-based management methods, including monitoring, attract-and-kill, and push-and-pull strategies, are reviewed.

Knowledge gaps in each area are highlighted, and the final section addresses these gaps while proposing future directions for innovative bark beetle management strategies.

Keywords:

Bark beetle; *Ips* genus; pheromone biosynthesis; pheromone receptor; pheromone derived application; pest management.

1. Introduction

Bark beetle (*Coleoptera: Curculionidae, Scolytinae*) outbreaks have intensified worldwide, primarily due to climate change. Rising temperatures and prolonged droughts weaken tree defenses [1], while warmer conditions accelerate beetle development and expand their ranges [2], fueling larger infestations [3] . With projected global warming of 2–4°C this century, these trends are expected to worsen, with climate-driven beetle surges potentially causing increasingly severe ecological and economic losses [4,5].

Trees in the pine family (Pinaceae), particularly *Pinus* (pine), *Picea* (spruce), and *Larix* (larch), are highly susceptible to drought and rising temperatures worldwide [6]. A major threat to their health is the bark beetle genus *Ips*, which comprises 37 known species—23 in North America,13 in Eurasia and one in Australia [7]. Several *Ips* species can infest and kill living trees during mass outbreaks triggered by favorable abiotic conditions, with the most economically significant species in Europe, Asia, and North America causing severe forest damage. Their management primarily relies on silvicultural practices [8], with insecticides used as a last resort [9].

Pheromone-based methods offer significant advantages over conventional insecticide approaches. Pheromones have been identified in at least 20 *Ips* species, including several economically significant tree-killing species targeted for control. However, their application remains largely restricted to trap-based monitoring of beetle activity.

This review promotes a deeper and more comprehensive understanding of *Ips* pheromones. By examining their role within the ecology, physiology, and management of *Ips* beetles, we provide new insights while also exploring the biochemical and genetic mechanisms that regulate pheromone production and detection. A more integrated understanding of these aspects could pave the way for innovative, more effective, and sustainable pest management strategies.

2. Distribution, economic importance, and preferred host trees for selected *Ips* species

All *Ips* bark beetles reproduce in conifer trees, with the most aggressive species in Europe, America, and Asia capable of killing trees, particularly when forests are weakened by climatic stressors such as drought or windstorms (Figure1) [9].

Among them, *Ips grandicollis* stands out as the only invasive species. Originally native to North America, it was introduced to Australia in the 1940s, causing significant damage to *Pinus radiata* plantations. Today, *I. grandicollis* is considered a major exotic forest pest in Australia [10].

Most *Ips* species exhibit considerable plasticity in their voltinism, except for North and Central American *Ips* species that infest pine trees. These species are strictly polyvoltine, producing two to five generations yearly [11,12]. Populations of other *Ips* species can be univoltine, bivoltine, or produce up to three generations per year, depending on temperature variations along altitudinal, latitudinal, or both gradients.[13–17]. This plasticity in responding positively to warmer temperatures is likely a key factor contributing to the pest potential of *Ips* bark beetles.

For example, *I. cembrae*, primarily inhabiting low-altitude regions, has recently benefited from rising temperatures and climate change, accelerating its development and completing up to two generations in Central Europe [18]. Similarly, *I. typographus*, already responsible for significant damage at lower altitudes, exhibits an increased outbreak potential at higher elevations due to a larger number of generations per year [19].

Ips bark beetles on *Picea* sp.

Regarded as one of the main dangers to conifer stands in Eurasia, *I. typographus* is a prominent pest of Norway spruce (*Picea abies* (L.) H. Karst). Recent heat waves, droughts, and overall climate shifts, together with the widespread planting of Norway spruce, driven by its economic importance, have intensified the severity of its outbreaks [20,21]. In the last ten years, *I. typographus* has affected approximately 70.1 million cubic meters of spruce wood across Europe [22]. During recent outbreaks, *I. typographus* often infested the same trees as *I. duplicatus*. This species originating from Eurasian north boreal forests, has quickly spread across Europe [23] and become a significant local pest [24]. It prefers the upper stem below the canopy of shaded trees inside the stand, which complicates their timely detection and removal. Although *I. duplicatus* competes with *I. typographus* for space and resources on the same tree [25,26], both species differ in several aspects, e.g. overwintering biology, rate of development, and flight activity [27,28]. *Ips amitinus* is economically less important but often colonizes thicker branches and the upper parts of the trees infested by *Ips typographus*. Its range and bionomy are also very similar, with the number of generations per year depending on altitude and the occurrence of sister broods. In the last ten years, it has spread rapidly in the Nordic countries and in Siberia, where it causes significant damage [29]

Two species that mirror the ecological and economic impacts of *I. typographus* and *I. duplicatus* on other spruce species in Central Asia are *I. hauseri* and *I. nitidus*. *Ips hauseri* primarily attacks Schrenk spruce (*Picea schrenkiana* Fisch. & C.A. Mey.) and Siberian spruce (*Picea obovata* Ledeb.), weakened by abiotic factors in mountainous regions [14] whereas large outbreaks of *I. nitidus* together with *I. shangrila* occurred on *Picea crassifolia* [30].

The only member of the genus *Ips* involved in spruce mortality on the North American continent is *I. perturbatus*. The hosts of this bark beetle are mainly *Picea glauca*, but also *P. engelmannii* and *P. lutzii*.

Ips bark beetles on *Pinus* sp.

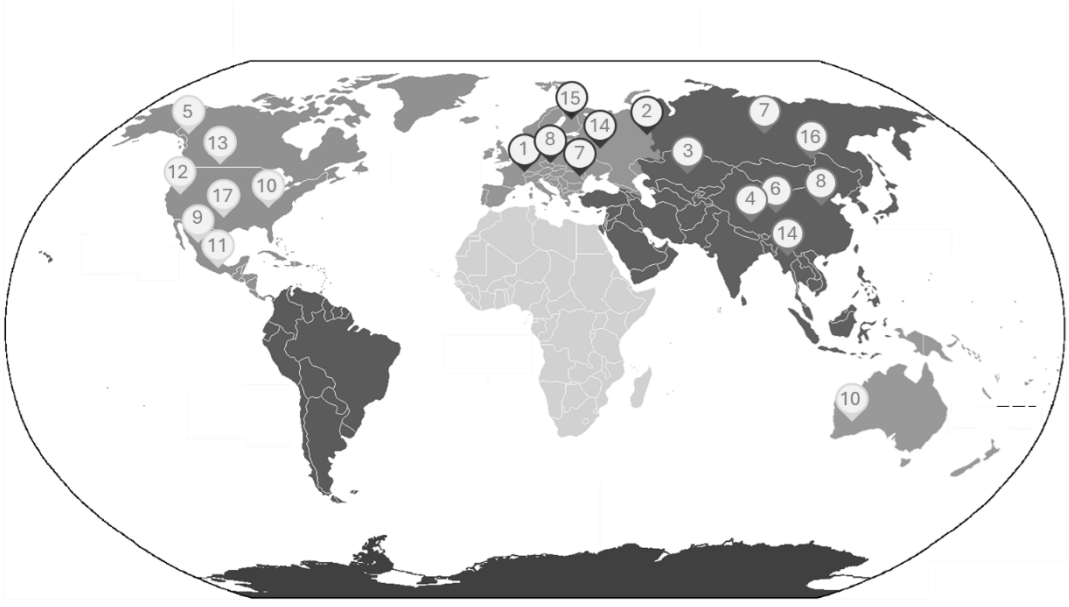
Several *Ips* bark beetles also colonize pines (*Pinus* spp.) as their primary hosts. The climatic extremes of recent decades have also weakened pine stands in many areas (*Pinus* sp.), making them more susceptible to bark beetle attacks. Many bark beetle species have spread beyond their original range, which has led to an increase in economic losses [31]. The effects of rising temperatures can be well illustrated by the example of *I. sexdentatus*, a species with a Eurasian distribution range that causes great damage not only to *Pinus* sp. but also to spruce (*Picea*) trees, especially in the Mediterranean region. It tends to colonize the lower and middle parts of the trunk with thick bark. In Central Europe, an intensive spread of this species has been observed in recent decades. In Scandinavia, the abundance of the species varies from decade to decade, depending on the minimum temperatures in winter, which determine the success of overwintering. In the warmest areas, the gradation process is very intense, and up to five generations per yearcomplete their development. At the northern limit of its range, however, it is univoltine [32]. *Ips acuminatus*, on the other hand, is an example of a species with the same distribution range that can cope rather well even with the lowest temperatures. It tends to colonize the treetops and branches, where it also partially hibernates and where it can survive temperatures below -35 °C [33].

In North and Central America, several *Ips* species, which differ in their biology and host preferences, can lead to the death of pine trees on a large scale. *Ips avulsus* is a smaller species which, like *I. acuminatus* mentioned above, prefers the thin bark of branches in the crowns of

large trees, most frequently *Pinus palustris*, *P. taeda* and *P. serotina*. Unlike *I. acuminatus*, however, it occurs exclusively in the warm southern part of the United States, where high temperatures accelerate development, resulting in up to 10 generations per year [34,35].A species that feeds on a variety of pines, including red pine (*Pinus resinosa* Aiton), jack pine (*Pinus banksiana* Lamb.) and white pine (*Pinus strobus* L.) [36] and occasionally larch (*Larix laricina*), is *I. pini*. It infests weakened or recently dead trees but can also infest healthy trees [11,37]. Other species that play an important role in pine mortality in North American forests include *I. confusus* and its sibling species *I. lecontei*, *I. paraconfusus* and *I. grandicollis* [38–40]. These species differ in their host plant spectrum. *Ips confusus* prefers the pinyon pines *P. edulis* and *P. monophyla* [38], but other pines are only rarely infested. *Ips lecontei*, *I. grandicollis* and *I. paraconfusus* are important pests of *P. ponderosa*, *P. radiata*, *P. concorta*, and several other pine species [40].

***Ips* bark beetles on *Larix* sp.**

Larches (*Larix* sp.) are also hosts for *Ips* bark beetles. The most important pest in larch stands is *I. cembrae*, which was only recently derived from a closely related species, *Ips subelongatus*. These two species are geographically separated, with *I. cembrae* occurring in Europe and *I. subelongatus* in Asia [41]. The host trees include not only various larch species, e.g. the European larch (*Larix decidua* Mill.) and the Japanese larch (*L. kaempferi* (Lamb.)), but also the common spruce *Picea abies* (Karst.) [42]. The ability of *I. cembrae* to attack healthy trees following abiotic disturbance has led to severe outbreaks, particularly in reforested areas and outside the natural geographic range of *L. decidua*, highlighting the need for robust management measures [25,43]. *I. subelongatus* is reported to be even more damaging to healthy larch stands in Asia than *I. cembrae* in Europe [44].



225

Figure 1. *Global distribution of selected Ips species* [host trees]*:* ***Ips* bark beetles on *Picea* sp.: 1**- *Ips amitinus* [*Picea* spp. (*Picea abies, Picea pungens*)]; **2**- *Ips duplicatus* [*Picea abies*]; **3**- *Ips hauseri* [*Picea schrenkiana, Picea obovata*]; **4**- *Ips nitidus* [*Picea crassifolia*]; **5**- *Ips perturbatus* [*Picea glauca, P. engelmannii, P. lutzii*]; **6**- *Ips shangrila* [*Picea crassifolia*]; **7**- *Ips typographus* [*Picea abies*]; ***Ips* bark beetles on *Pinus* sp: 8**- *Ips acuminatus* [*Pinus* spp. (*Pinus nigra, Pinus sylvestris*)]; 9- *Ips*

confusus [*Pinus edulis, P. monophylla*]; 10- *Ips grandicollis* [*Pinus* spp. (*P. ponderosa, P. radiata, P. contorta*)]; **11**- *Ips lecontei* [*Pinus* spp. (*P. ponderosa, P. radiata, P. contorta*)]; **12**- *Ips paraconfusus* [*Pinus* spp. (*P. ponderosa, P. radiata, P. contorta*)]; **13**- *Ips pini* [*Pinus resinosa, Pinus banksiana, Pinus strobus, Larix laricina*]; **14**- *Ips sexdentatus* [*Pinus* spp. (*Pinus sylvestris, Pinus pinaster*), *Spruce* spp.]; ***Ips* bark beetles on *Larix* sp.: 15**- *Ips cembrae* [*Larix* spp. (*L. decidua, L. kaempferi*), *Picea abies*]; **16**- *Ips subelongatus* [*Larix* spp. (*L. decidua, L. kaempferi*), *Picea abies*]; **17**- *Ips avulsus* [*Pinus* spp. (*P. sylvestris*)].

3. Chemical structures and compositions of aggregation pheromones in *Ips* species

The aggregation pheromones of *Ips* species are produced exclusively by males to gather conspecifics and overcome tree defense, as well as to attract females for mating. The structural repertoire of biologically active pheromonal compounds in this genus is relatively limited, primarily consisting of oxygenated hemi- or monoterpenes [45]. These compounds are volatile and structurally resemble the defense compounds (resin) found in their conifer host trees (Figure 2, Table 1.).

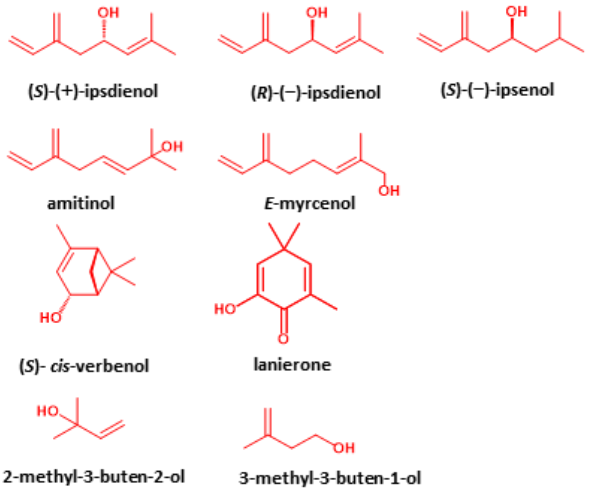


Figure 2: *Structures of pheromone compounds from Ips species with the known biological activity*

In most *Ips* species, the primary pheromone components consist of ipsdienol and ipsenol, compounds exclusively synthesized by this beetle genus [46,47]. Some species also produce additional linear hydroxylated monoterpenes, such as amitinol and *E*-myrcenol, along with the quinone derivative lanierone [48–50]. Other identified active pheromone compounds include the hemiterpenes 2-methyl-3-buten-2-ol and 3-methyl-3-buten-1-ol, as well as the monoterpene *cis*-verbenol, which originates from the host-derived compound α-pinene (Figure 2). Despite the relatively limited number of structural components, the resulting pheromone blends are species-specific, driven by variations in the relative proportions of each compound and differences in enantiomeric composition (Table 1).

Table 1: *Ips* species aggregation pheromone blends compositions including enantiomeric ratio of components.

Species	***Composition of pheromone***	***Enantiomeric ratio of pheromone components***	***Literature***
***Ips* bark beetles on *Picea* sp.**			
Ips amitinus (Eichhoff, 1872)	ipsdienol:ipsenol:amitinol **4:2:4**	ipsdienol (*S*)-(+)-:(*R*)-(−)- **5:95**	[51]
Ips duplicatus (C.R. Sahlberg, 1836)	ipsdienol:*E*-myrcenol **5:1:0,01** [52]	ipsdienol (*S*)-(+)-:(*R*)-(−)- **50:50**	[53,54]
Ips hauseri Reitter, 1895	ipsenol *cis*-verbenol **95:5**	(*S*)-(−)-ipsenol **100** (*S*)-(−)-*cis*-verbenol **100**	[55]
Ips nitidus Eggers, 1933	2- methyl-3-buten-2-ol: ipsdienol: (*S*)-(−)-*cis*-verbenol **7:2:1**	ipsdienol (*S*)-(+)-:(*R*)-(−)- **74:26**	[30,56]
Ips perturbatus (Eichhoff, 1869)	ipsdienol: *cis*-verbenol: ipsenol **1:0,8:1** [57]	ipsenol (*S*)-(−)-:(*R*)-(+)- **99:1** ipsdienol (*S*)-(+)-:(*R*)-(−)- **90:10**	[58–60]
Ips shangrila Cognato & Sun, 2007	ipsenol:ipsdienol: *cis*-verbenol **1:5:4** [61]	ipsdienol (*S*)-(+)-:(*R*)-(−)- **99:1** (*S*)-(−)-*cis*-verbenol **100**	[30,62]
Ips typographus (Linnaeus, 1758)	2-methyl-3-buten-2-ol *cis*-verbenol ipsdienol **9:1:0,1** [63]	ipsdienol (*S*)-(+):(*R*)-(−)- **5:95** (*S*)-(−)-*cis*-verbenol **100**	[64,65]
***Ips* bark beetles on *Pinus* sp.**			
Ips acuminatus (Gyllenhal, 1827)	*cis*-verbenol:ipsdienol:ipsenol **2:5:3** [66]	ipsdienol (*S*)-(+)-:(*R*)-(−)- **95:5** [67]	[68]
Ips confusus (LeConte, 1876)	ipsenol:ipsdienol **9:1** [69]	ipsenol (*S*)-(−)-:(*R*)-(+)- **99:1** [70] ipsdienol (*S*)-(+)-:(*R*)-(−)- **95:5** [71]	[50,69,70,72]
Ips grandicollis (Eichhoff, 1868)	ipsenol [73]	ipsenol (*S*)-(−)-:(*R*)-(+)- **99:1** [74]	[75]
Ips lecontei Swaine, 1924	ipsdienol:ipsenol **2:1** [76]	ipsdienol (*S*)-(+)-:(*R*)-(−)- **95:5** ipsenol (*S*)-(−)-:(*R*)-(+)- **99:1**	[67]
Ips paraconfusus Lanier, 1970	ipsenol:ipsdienol: *cis*-verbenol **1:1:0,1** [77]	(*S*)-(−)-*cis*-verbenol **100** [78] ipsenol (*S*)-(−)-:(*R*)-(+)- **99:1** [45] ipsdienol (*S*)-(+)-:(*R*)-(−)- **90:10**	[79–83]
Ips pini (Say, 1826)	ipsdienol: lanierone **99:1** [75]	ipsdienol (*S*)-(+)-:(*R*)-(−)- **35:65** [46] ipsdienol† (*S*)-(+)-:(*R*)-(−)- **95:5** [46]	[46,48,72,75,83,84]

Ips sexdentatus (Börner, 1776)	ipsdienol:ipsenol **1:0,5**	ipsdienol (*S*)-(+)-:(*R*)-(−)- **50:50** [85]	[67,68]
***Ips* bark beetles on *Larix* sp.**			
Ips cembrae (Heer, 1836)	ipsenol:ipsdienol: 3-methyl-3-buten-1-ol ~ **68:28:4**	ipsenol (*S*)-(−)-:(*R*)-(+)- **99:1** ipsdienol (*S*)-(+)-:(*R*)-(−)- **96:4** [86]	[87–89]
Ips subelongatus (Motschulsky, 1860)	ipsenol:ipsdienol:3-methyl-3-buten-1-ol **3:1**	ipsenol (*S*)-(−)- **100** ipsdienol (*S*)-(+)-:(*R*)-(−)- **96:4**	[90]
Ips avulsus (Eichhoff, 1868)	ipsdienol:lanierone **10:1** [91]	ipsdienol (*S*)-(+)-:(*R*)-(−)- **96:4** (Texas) [92] ipsdienol (*S*)-(+)-:(*R*)-(−)- **75:25** (Alabama) [93]	[68,75,91]

† The ratio varies for eastern and western populations of *I. pini* in the USA.

Among *Ips* aggregation pheromones are three chiral compounds: (*S*)-(+)- and (*R*)-(−)-ipsdienol; (*S*)-(−)- and (*R*)-(+)-ipsenol, and (*S*)-(−)- and (*R*)-(+)-*cis*-verbenol (each structure possesses at least two asymmetrical mirror-image forms called enantiomers). However, *Ips* males predominantly utilize only the enantiomeric forms (*S*)-(−)-*cis*-verbenol and (*S*)-(−)-ipsenol as their pheromonal signals [94]. In contrast, ipsdienol is the only chiral compound whose enantiomeric ratio varies, not only between *Ips* species (Table 1) but also among spatially distinct populations within the same species [71]. This enantiomeric specificity, along with the unique composition and ratio of pheromonal molecules (Table 1), helps minimize cross-attraction between related species and likely reflects an important mechanism of prezygotic reproductive isolation within the genus *Ips* [95].

4. Production of the aggregation pheromones by *Ips* bark beetles

4.1. Sequence of host tree attack by *Ips* bark beetles

As mentioned earlier, *Ips* pioneer males first select a host tree and use chemical signals to coordinate attacks on trees. In the terminal phase of the attack, as tree resources deplete and wood decomposition begins due to fungal growth and other factors, the profile of volatile changes and compounds with anti-attractive functions are released [96–100]. Conifer bark beetles often release a universal anti-attractant known as verbenone, acting as a switch signal that guides beetles to other nutrient resources [101] (Figure 3).

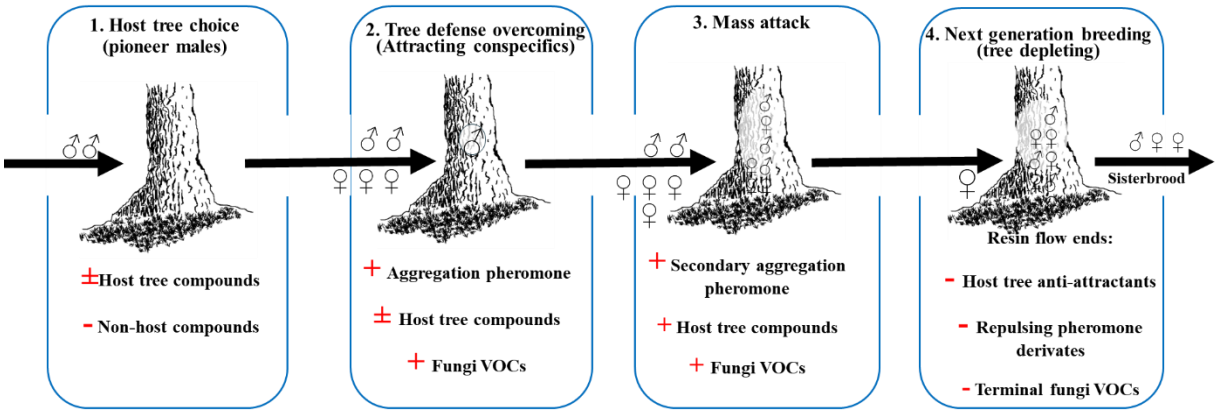


Figure 3: *The attack dynamics of Ips bark beetles on host trees. Description:* ***Step 1:*** *Pioneer males locate the host tree hypothetically using host and non-host compounds.* ***Step 2:*** *Attraction to a male-released aggregation pheromone blend along with host compounds.* ***Step 3:*** *The chemical blend attracts more conspecific beetles to the host tree. After mating, males can release additional compounds with attractive activity, resulting in a secondary aggregation blend.* ***Step 4:*** *The tree defence is depleted with low resin flow, and other compounds, such as anti-attractants are released to direct further conspecific beetles towards new hosts.*

4.2. **Biosynthesis of main aggregation pheromone components**

The pheromone production of bark beetles has been studied for decades [47,70,102–104]. Pheromone-resulting biosynthetic pathways have likely co-evolved with tree defense detoxification mechanisms possessed by beetles to enable successful host tree attacks [105]. Aggregation pheromones are formed in the beetle's body either via *de novo* synthesis from basic metabolic units or by modifying host-derived precursors. With aggregation pheromones released in feces [106], the gut tissue is the primary site of biosynthesis for most pheromonal compounds in *Ips* species.

Pheromone biosynthesis in bark beetles is naturally induced during feeding on a suitable host tree, which triggers a hormonal cascade involving juvenile hormone III (JH III), an insect hormone regulating different metabolic pathways, from metamorphosis to pheromone production. [47,107–109].

4.2.1. Role of the mevalonate pathway in *de novo* biosynthesis

The major *Ips* aggregation pheromones are all isoprenoids (Figure 2), with some synthesized *de novo* in beetles via the mevalonate pathway (Figure 4), a process shared by most eukaryotes.

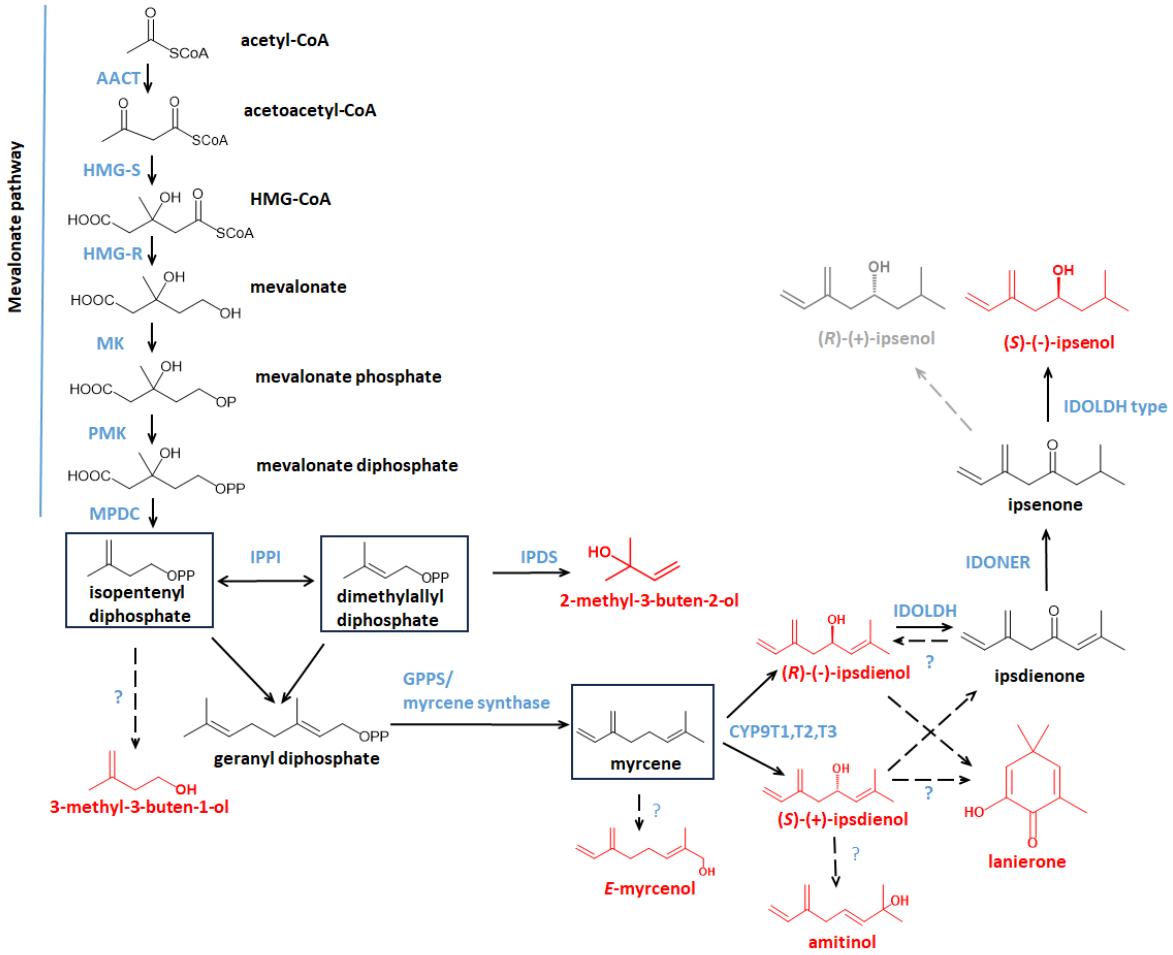


Figure 4*: Overview of the mevalonate pathway and subsequent steps in the biosynthesis of aggregation pheromones in* Ips bark beetles. *The diagram illustrates the reaction sequences (precursors - black, pheromone compounds -red), and the enzymes or proposed enzymes that catalyze each step (blue).*

The mevalonate pathway starts with acetyl-CoA condensation, subsequently forming hydroxymethylglutaryl-CoA (HMG-CoA) and mevalonic acid. These steps are catalyzed by the enzymes HMG-CoA synthase (*HMGS*) and HMG-CoA reductase (*HMGR*), found as key regulators of *Ips* pheromone biosynthesis in *I. pini* males [47,72,110]. Mevalonic acid is then converted by isopentenyl diphosphate isomerase (*IPPI*) to isopentenyl diphosphate (IPP), which isomerizes to dimethylallyl diphosphate (DMADP), the universal C5 building blocks of all isoprenoids (Figure 4).

In *Ips* males, when ipsdienol is produced, IPP and DMADP condense into geranyl diphosphate (GPP), which is then converted to myrcene by an insect-unique enzyme first identified in *I. pini* [111] and later characterized as GPPS/myrcene synthase[112] ; Figure 4).

Recent studies also identified genes coding these mevalonate pathway enzymes in the guts of male *I. typographus* and *I. hauseri*. HMGS, HMGR, and IPPI transcripts were upregulated after pheromone induction via feeding or JH III treatment, aligning with HMGS's and HMGR's

regulatory roles. Additionally, these species also possess GPPS, which is involved in myrcene-ipsdienol synthesis [55,65,109]. Exclusively in *I. typographus*, a newly identified isoprenyl diphosphate synthase gene (IPDS) has been reported, suggesting its role in synthesizing the hemiterpene 2-methyl-3-buten-2-ol [65,109]. On the other hand, in females, transcripts of mevalonate genes did not respond to any of the pheromone induction methods [55,109].

4.2.2. Specific steps in the formation of *de novo*-produced *Ips* pheromones

In *Ips* species, myrcene produced by males is hydroxylated to ipsdienol by cytochrome P 450 (CYP9T) enzymes, identified previously in *I. paraconfusus* [113], and in *I. pini* and *I. confuses* [114–116]. Recently, CYP9Ts were reported in *I. hauseri* [55] and *I. typographus* [65,109]. The mechanism underlying inter- and intraspecific variation in the enantiomeric composition of ipsdienol, defined by the ratio of (4*R*)-(−)-ipsdienol to (4*S*)-(+)-ipsdienol (Table 1), remains a key focus of research. However, this variation is not driven by CYP9T-mediated hydroxylation, but is more likely influenced by subsequent steps in the ipsdienol-to-ipsenol conversion, a process occurring across all *Ips* species [117,118]; (Figure 4) Responsible are two enzymes: ipsdienol dehydrogenase (*IDOLDH*), which selectively oxidizes only (4*R*)-(−)-ipsdienol to ipsdienone[65,71,119] and ipsdienone reductase (*IDONER*) converting ipsdienone to ipsenone [120]. Further characterization of these enzymes will give us new insights into what creates pheromone differences among *Ips* species and populations, knowledge that could improve trapping success.

Amitinol (Figure 2), a second linear monoterpene alcohol, is typically a minor component alongside ipsdienol, except in *I. amitinus*, where it acts as the primary pheromone. Trace amounts also appear in the aggregation pheromones of *I. paraconfusus* [45,89] and *I. duplicatus* [90]. Amitinol is hypothesized to form either through ipsdienol allylic rearrangement [45] or cytochrome P450-mediated site-specific oxidation of myrcene (Figure 4).

E-myrcenol, structurally related to ipsdienol and amitinol (Figure 2), is a pheromone component in *I. duplicatus* [121]. It likely forms *de novo* via myrcene hydroxylation, similar to ipsdienol [122] (Figure 4), as its production was suppressed by the HMGR inhibitor compactin [52,123].

Lanierone, a cyclic keto-enol compound (Figure 2), is found in the hindgut extracts of male *I. pini* [84]and functions as a pheromone in eastern U.S. populations [50]. It was found also in *I. avulsus* [75]. It is thought to originate from ipsdienol or ipsenol through cyclization, decarboxylation, and oxidation reactions (Figure 4). However, its biosynthetic pathway remains uncharacterized [103].

The hemiterpene 2-methyl-3-buten-2-ol is a pheromone component of *I. typographus*. Though hemiterpenoid biosynthesis was previously unknown in insects, it was hypothesized to form *de novo* via the mevalonate pathway, where DMADP is converted through a carbocation intermediate by the novel IPDS enzyme [65,109].

Another hemiterpene, 3-methyl-3-buten-1-ol, functions as an aggregation pheromone in male *I. cembrae* and *I. subelongatus* [88,89]. It is proposed to form *de novo* from IPP via dephosphorylation and double-bond rearrangement [87] (Figure 4), differing from 2-methyl-3-buten-2-ol biosynthesis by not involving IPDS.

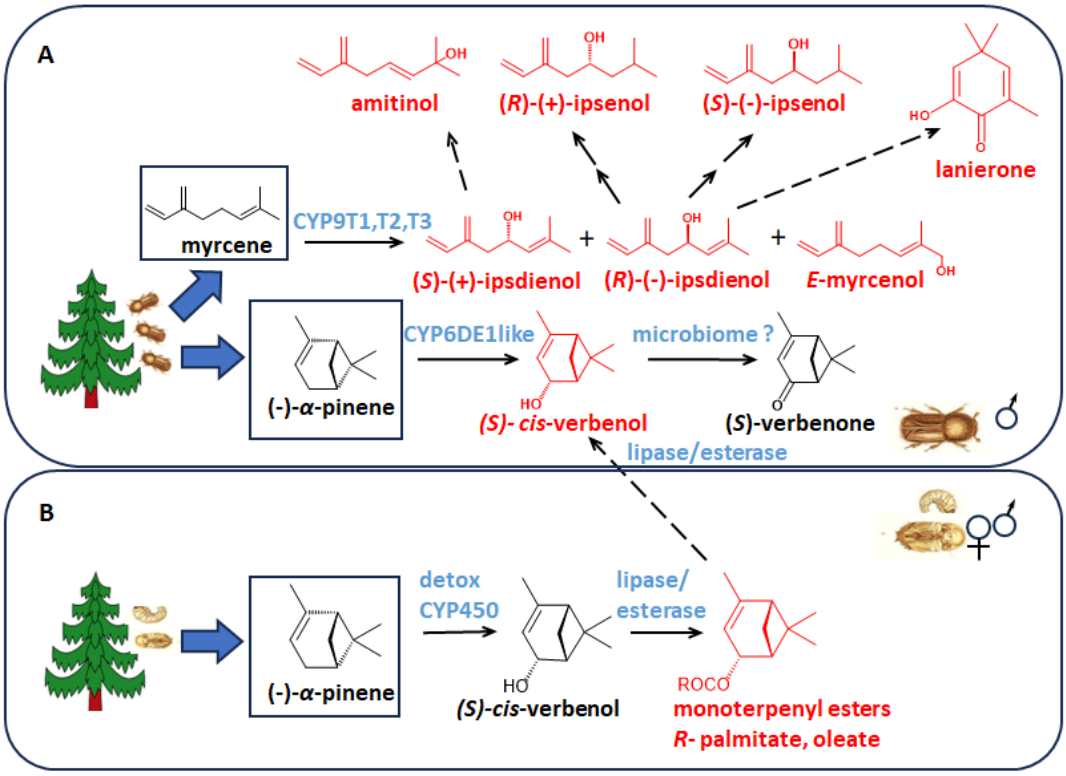


***Figure 5**: A. Pathways of pheromone biosynthesis in Ips using precursors sequestered from host conifers: (-)-α-pinene or myrcene. B. Storage of cis-verbenol as fatty acid esters in the fat bodies of beetles for later pheromone supply.*

4.2.3 Pheromones made from host tree precursors

Many *Ips* species use the cyclic hydroxylated monoterpene (*S*)-*cis*-verbenol (Figure 5) as an aggregation pheromone. However, neither *cis*-verbenol nor its pheromonally inactive stereoisomer, *trans*-verbenol, is produced *de novo*. Instead, beetles hydroxylate α-pinene, sequestered from spruce trees, via *CYP450* enzymes [82] ; Figure 5. Since insects frequently hydroxylate host terpenes for detoxification, *Ips* bark beetles may have evolved to utilize *cis*-verbenol, originally the detoxification product of α-pinene, as a pheromone [104].

The distinction between pheromone biosynthesis and detoxification CYP450 genes remains unclear, as both likely function in gut-adjacent cells. Recently, CYPP450 genes responsible for α-pinene hydroxylation were identified in the guts of *I. hauseri* and *I. typographus* [55,65]. As expected, the transcripts of these genes are induced by feeding on host trees [65,124] and, interestingly, also by topical treatment with JH III [55,109].

The ketone verbenone (Figure 2, 5A) co-occurs with *cis*-verbenol but increases in concentration late in the attack phase, acting as an anti-attractant for many bark beetles [101]. Its production is attributed to gut microbiota (fungi, yeast, bacteria), external symbionts [63,125–127], or autooxidation, with no beetle enzyme identified for this conversion. Therefore, verbenone should not be classified as a pheromone.

The monoterpenyl lipid conjugates (Figure 5B), namely *cis*-verbenol fatty acid esters, were detected in the fat body of *I. typographus* across various life stages, peaking in young, pre-sclerotized adults before emergence [65]. In young beetles, ester formation likely serves as

monoterpene detoxification, catalyzed by multipurpose lipase/esterase enzymes. However, in mature beetles, these esters persist only in males and decrease during aggregation pheromone production. In calling males, they are proposed to function as pheromone precursors hydrolyzed into pheromonal *cis*-verbenol by a male-specific lipase/esterase enzyme when the α-pinene source is insufficient. This mechanism ensures a continuous pheromone supply during mass attacks [65,104]. The cleaving of esters, likely regulated by JH III, may explain how beetles release *cis*-verbenol even before feeding, facilitating rapid attack buildup [109] (Figure 5B).

Identified candidate genes for these enzymes in *I. typographus* could serve as other potential targets for controlling beetle infestations.

4.2.4. Microbial involvement in *Ips* aggregation pheromone production

Bark beetle microbial symbionts may contribute to pheromone production in various ways [128]. Labeling experiments indicated that gut microbiomes produce pheromones when exposed to precursors but not when inhibited by antibiotics [129,130]. More recently, exosymbiotic ophiostomatoid fungi, *Grosmannia penicillata* and *Endoconidiophora polonica* were found to synthesize pheromones like 2-methyl-3-buten-2-ol [131] and brevicomin [132] when growing on wood. These compounds, along with fungi metabolites of host tree terpenes [133–135], may influence beetle attraction [100]. Further research is needed to clarify microbes' role in *Ips* aggregation pheromone production, which could offer alternative targets for beetle management.

5. Detection of aggregation pheromones by the bark beetle olfactory system

5.1. Insect olfaction

Like other insects, *Ips* bark beetles rely on highly specialized olfactory systems for precise odor detection, a critical function for locating food, mates, and suitable reproduction sites [136]. Bark beetles can detect and assess the concentrations of not only pheromones but also volatiles from host and non-host trees, as well as other components of their environmental niche, such as microbiota [133,134,137–139].

The main olfactory organ is the antennae (Figure 6A), which harbour sensilla in which olfactory sensory neurons (OSNs) reside (Figure 6B) [137,140]. The OSN dendrites house odorant receptors (ORs) [141], which transduce olfactory information in the environment into electrical signals that can be interpreted by the brain [142] (Figure 6D). The odor specificity of an OSN depends on which OR gene(s) it expresses [143]. Pheromone responsive ORs and OSNs are typically highly specific in their response, ensuring high-fidelity detection of mating signals [142,144,145]. Traditionally, it has been believed that each OSN expresses a single OR gene together with the conserved co-receptor ORCO [146] (Figure 6D); however, recent studies have found some exceptions to this rule [147–149]. The ORCO is necessary for signal transduction by forming a cation ion channel when the OR binds odour molecules [150–154] (Figure 6).

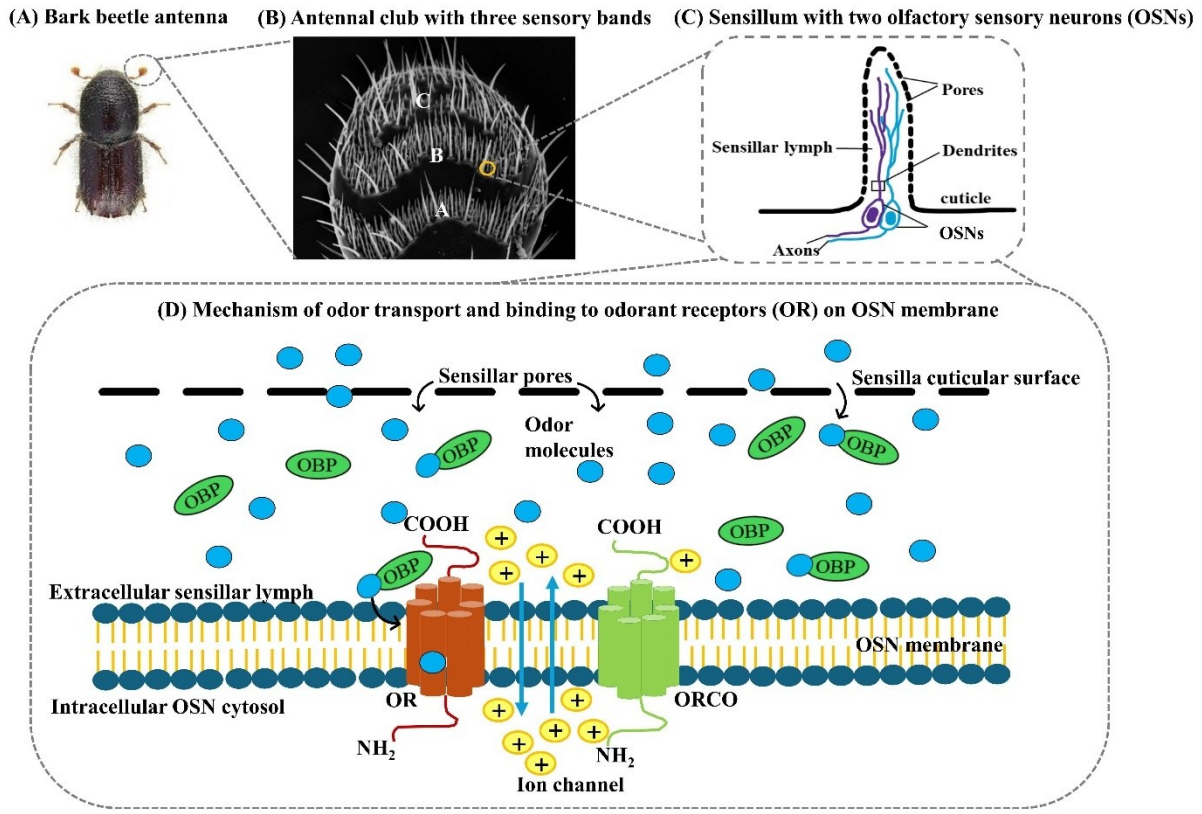


Figure 6. *The peripheral olfactory system in bark beetles and its anatomical and molecular features. (A) Typical bark beetle with its antenna (here I. typographus), (B). Scanning electron micrograph of the antennal club of I. typographus antenna showing three sensory bands, A, B, and C with olfactory sensilla (C). Illustration of an olfactory sensillum with pores on its cuticular wall and the internal cellular arrangement with two OSNs and their cell bodies at the base (D) Schematic representation of olfactory detection including the entry of odor molecules through wall-pores, transport of odor molecules through the sensillar lymph facilitated by odorant binding proteins (OBPs), which release the odor molecules near the odorant receptor (OR) complexes. The cell membrane is depolarized through the opening of the non-selective cation channel of the OR-ORCO receptor complex upon ligand binding. The ORs and ORCOs are seven transmembrane domain proteins.*

5.2. Specific pheromone detection by OSNs in *Ips*

The antennal club of *Ips* beetles is flattened, and the olfactory sensilla are located on its anterior surface, arranged as three undulating bands, labelled A, B, and C [137,140,155–159]. (Figure 6B). Olfactory sensilla of *Ips* beetles include the single-walled hair-like sensilla trichodea and basiconica, which are the most abundant, and the peg-like double-walled sensillum coeloconicum [140,157]. Each single-walled sensillum generally contains two OSNs with different odor specificities [137,140,160,161].

Pioneering studies revealed OSNs with primary responses to (*S*)-(+)-ipsdienol, (*R*)-(–)-ipsdienol, and (*S*)-(–)-ipsenol, respectively, in *I. paraconfusus*, *I. pini*, and *I. typographus* [162–166]. Furthermore, OSNs responding to (racemic) ipsdienol and ipsenol were found in *I. grandicollis* [160]. The (*S*)-(+)- and (*R*)-(–)-ipsdienol-responsive OSNs are highly specific for their key enantiomer [167]. Additionally, OSNs responding to *cis*-verbenol, *trans*-verbenol, and

verbenone, respectively, have been identified [160,163]. In *I. typographus*, these OSNs respond to the enantiomers (*S*)-*cis*-verbenol, (+)-*trans*-verbenol, and (–)-verbenone [165]. Whereas the OSN class tuned to (*S*)-*cis*-verbenol is narrowly tuned [137], the OSN class responding to (–)-verbenone responds strongly also to α- and β-isophorone [134]. β-isophorone was identified in mated female *I. typographus*, but its behavioral effects have not been elucidated [63]. The neurons that in early studies were reported to respond primarily to (+)-*trans*-verbenol in *I. typographus* [165] were, however, not recovered in a recent study that screened >200 sensilla with a large test odor panel [134]. *Ips typographus* also has OSNs that primarily respond to amitinol, lanierone, and 2-methyl-3-buten-2-ol, respectively [137,161,165]. The lanierone-responsive OSNs respond exclusively to lanierone, rendering this OSN class the most specific one in *I. typographus* [161].

5.3. Abundance and distribution of pheromone-specific OSNs in *Ips*

Pheromone-responsive OSNs occupy a large proportion of the antennal sensilla of *Ips* beetles [137]. The abundance and spatial distribution of such OSNs have been mapped in *I. typographus* [133,137]. Neurons tuned to (*S*)-*cis*-verbenol are almost exclusively found in the distal sensory area C [137] (Figure 6B), whereas OSNs tuned to (–)-verbenone, ipsdienol, ipsenol, and amitinol are all found in both areas A and B, but not in C. Neurons for 2-methyl-3-buten-2-ol are found at the border between areas B and C [137]. In contrast, sensilla housing the lanierone OSN class are present in all three areas, which is unique among the described OSN classes in *I. typographus* [137,161].

In the study by Andersson et al. (2009) in which 150 *I. typographus* sensilla were randomly screened, the neurons tuned to the aggregation pheromone component (*S*)-*cis*-verbenol were reported as the most abundant, occupying 22 (15%) of the contacted sensilla. Similar patterns were found in *I. pini* and *I. paraconfusus* for OSNs tuned to components or their respective aggregation pheromones, that is, different enantiomers of ipsdienol [164,168]. However, a recent SSR study on *I. typographus*, which for the first time included lanierone, showed that the OSNs tuned to this compound are even more abundant than the OSNs for (*S*)-*cis*-verbenol; these neurons were present inside 42% of the contacted sensilla [161]. Lanierone elicits sex- and context-dependent behavioral effects in *I. typographus* but it has never been shown to be produced by this species [63,161]. However, the beetles analyzed by Yuvaraj and colleagues also did not produce ipsdienol [161]. If ipsdienol indeed is the precursor for lanierone [103] (Figure 4), the lack of ipsdienol would explain the lack of lanierone in the investigated specimens. In contrast to the high abundance of OSNs tuned to lanierone and (*S*)-*cis*-verbenol, neurons for the more abundant aggregation pheromone component in *I. typographus*, 2-methyl-3-buten-2-ol, were only encountered in 2% of the sensilla [137].

5.4. Function and evolution of pheromone receptors in *Ips*

Genes encoding the OR proteins that underlie the responses of the OSNs have been identified in several scolytines [169–178], but only from two species in the *Ips* genus. Seventy-three ORs were identified from antennal transcriptomes of *I. typographus* and 69 ORs in *I. duplicatus* [169,173,178]. A large proportion of these ORs show conserved orthology between the two species, potentially suggesting conserved olfactory functions [179].

Pheromone receptors (PRs) are ORs with specificity for pheromone compounds. Functionally characterizing PRs is crucial for understanding the specificity and evolution of pheromone communication. Additionally, PRs may also be candidates for chemoreceptor-targeted insect control, thus forest management [180]; see also Chapters 7.2 and 7.3). To identify the PRs, the receptor genes need to be functionally tested in heterologous expression systems. To date, approximately 30 coleopteran ORs have determined functions [181]. Of these, 14 ORs belong to the bark beetles *D. ponderosae* (2 ORs) and *I. typographus* (12 ORs) [161,178,179,181–183]. In fact, *I. typographus* ('Ityp') has the highest number of functionally characterized ORs of all coleopterans investigated to date, including PRs.

Functional showed that ItypOR46 responds to (*S*)-(−)-ipsenol, and ItypOR49 to (*R*)-(−)-ipsdienol [178,183], with impressive discrimination between structurally related compounds and enantiomers, similar to the corresponding OSNs [165]. The ItypOR46 gene has the third highest antennal expression of all ItypOR, which is consistent with the abundance of OSNs that respond to (*S*)-(−)-ipsenol [137,178]. A third pheromone receptor (ItypOR28) was subsequently characterized, with primary responses to *E*-myrcenol [182] – an aggregation pheromone component in *I. duplicatus* [184]. Interestingly, OSNs responding to *E*-myrcenol have so far not been identified in *I. typographus* [137]. ItypOR28 belongs to the same *Ips*-specific OR clade as ItypOR46 and ItypOR49 [178,182], demonstrating a shared evolutionary origin of these PRs. However, this OR clade contains four additional ORs that respond to monoterpenoids produced by spruce trees and/or the symbiotic fungi of *I. typographus*. Hence, PRs in *Ips* bark beetles also share close relatednessith ORs detecting compounds with different ecological origins [182].

Two recent studies focused on the ItypORs with the highest and second-highest antennal expression [178], namely ItypOR36 and ItypOR41, respectively. The ItypOR36 responds exclusively to lanierone [161]. ItypOR41 responds primarily to the aggregation pheromone component (*S*)-*cis*-verbenol, with minor responses elicited by (−)-verbenone and *trans*-verbenol enantiomers [181]. Both the high expression of these PR genes and their responses match with the antennal abundances and responses of the corresponding OSNs, suggesting that these ORs underlie the neuronal responses. ItypOR36 and ItypOR41 are phylogenetically well separated from each other and also from the clade that houses ItypOR28, ItypOR46, and ItypOR49, suggesting that bark beetle PRs have evolved on several independent occasions [181]. It is likely that the recently identified conserved receptor orthologues in *I. duplicatus* detect the same pheromones in this species [173,179].

To understand the molecular interactions between PRs and their ligands, 3D models have been generated for ItypOR41 and ItypOR46, and ligand docking simulations with the active pheromones have been performed [178,181]. Two amino acid residues (Tyr84 and Thr205) were shown to be important for the interaction between (*S*)-(−)-ipsenol and ItypOR46 [178]. In ItypOR41, two other residues (Gln179 and Trp310) were predicted to be key for the binding of (*S*)-*cis*-verbenol in this receptor [181]. In both studies, the predicted binding residues were confirmed experimentally.

In summary, our understanding of pheromone detection mechanisms in *Ips* bark beetles has progressed extensively in recent years, especially in *I. typographus*. The identification of pheromone binding sites in the PRs that are tightly linked to the reproductive success and mass attacks of bark beetles is essential for identifying receptor antagonists or agonists that could possibly be designed to interfere with bark beetle pheromone communication and hence contribute to the toolkit of bark beetle management strategies (see also sections 7.3 and 7.4).

6. Pheromone-Based Strategies for Managing *Ips* Bark Beetle Outbreaks: Current Approaches and Future Prospects

6.1. Mass trapping

The primary use of aggregation pheromones is to attract and eliminate target populations [185,241]. Attract-and-kill strategies, such as mass trapping, employ pheromone traps or natural/poisoned, baited/non-baited trap trees [8,126,186–189]. However, these methods remain controversial for several reasons. Treatment success varies widely from no effect [190] to significant infestation reduction [191]. Even without insecticides, non-target species, including predators and parasitoids of bark beetles, are frequently caught, potentially disrupting natural biological control [192,193]. Poor trap placement can also inadvertently trigger infestations in nearby trees, causing so-called spillover effect [194,195]. Additionally, mass trapping can be labor-intensive. Non-insecticide-treated trees must be removed before brood development, and traps require frequent emptying, as bark beetles are deterred by the scent of dead conspecifics [196].

A major limitation of mass trapping is the lack of standardized protocols [194]. Research should establish guidelines for trap density based on infestation levels [197] and optimal inter-trap distances. Many studies focus on beetle capture rates without assessing impacts on surrounding stands [198,199]. Additionally, the conditions under which mass trapping is effective remain poorly understood, with population size likely playing a key role [200]. Further research is needed to determine environmental and population-related factors affecting trapping efficiency.

6.2. Anti-aggregation signal

Several formulations of anti-aggregation blends for tree protection are commercially available in North America, but their use is largely restricted to *Dendroctonus* bark beetles [187]. Although multiple attraction inhibitors exist for *Ips* species—such as the anti-aggregation signal verbenone [101] or plant-derived compounds like *trans*-4-thujanol, 1,8-cineole, C_6 green leaf volatiles, C_8-alcohols, and *trans*-conophthorin [100,201–204] no commercial formulation is currently available for *Ips* bark beetle management. A major limitation of push-only approaches is the uncertain fate of repelled beetles. Whereas reduced aggregation on suitable hosts may increase mortality due to exhaustion [205], it could also lead to spillover infestations in untreated areas ([206]. Additionally, the high cost of anti-attractants, including verbenone, poses a challenge [207], especially since inhibition is strongest in the first weeks, requiring frequent dispenser replacements in multivoltine populations [205].

To improve anti-aggregation strategies, more effective and cost-efficient bait formulations are needed to ensure widespread application and reduce spillover risk. Additionally, like mass trapping, further research is required to understand the environmental factors influencing treatment success and to define optimal management scenarios where anti-aggregation pheromones can complement existing control methods [101].

6.3. Push-and-pull

A proposed solution to the limitations of the push-only approach with anti-aggregation signal is the push-pull strategy, which combines aggregation and anti-attractant cues [208]. Here, anti-attractants "push" bark beetles away from healthy stands, while traps baited with aggregation pheromones "pull" them in [209]. In North America, this method locally reduced *I. paraconfusus*

populations [208] and showed promise for protecting pine trees from *I. pini* [210,211]. In Europe, push-pull has been tested to protect forest edges from *I. typographus*. In Swedish boreal spruce forests, baited trap trees served as the pull component [199], while in Czech spruce forests, pheromone dispensers were used [206]. However, this method proved ineffective under severe drought and extreme beetle population densities [212].

Interestingly, many studies reporting successful push-and-pull strategies only find a significant effect from either the push or pull component when tested individually [207,213–215]. It remains unclear whether anti-aggregation signal actively repel beetles or merely mask attractant cues (Byers and Levi-Zada, 2022), although a recent study showed clear avoidance of verbenone or *trans*-4-thujanol by *I. typographus* in short-range laboratory walking bioassays[161,217]. If they only obscure attraction signals, their contribution to push-and-pull success may be limited. A deeper understanding of these mechanisms is essential to enhance future push-and-pull strategies. Despite its promise, evidence for successful tree protection remains limited for *Ips* species compared to *Dendroctonus* bark beetles [209].

7. Future Perspectives on *Ips* Bark Beetle Aggregation Pheromones: Advances in Research and Pest Management

Based on the above-reviewed informations, we have identified key knowledge gaps in pheromone research that future studies should address. These gaps span from laboratory research utilizing advanced post-genomic tools to field studies directly applicable to practical forest pest management.

7.1. Knowledge Gaps

Intervention in Pheromone Production and Detection on Genetic Level

- Identifying and characterizing additional genes involved in pheromone biosynthesis, including their regulatory mechanisms and genetic underpinnings.
- Investigating additional genes encoding pheromone receptors (PRs) to understand their role in pheromone detection, especially in species other than *I. typographus* where information is entirely lacking.
- Applying genetic manipulation techniques (e.g., RNAi-mediated silencing or CRISPR-Cas knockdown) to disrupt male pheromone production or alter pheromone detection in conspecifics.

Development of Novel Techniques for Early Attack Detection

- Utilizing OR-based or whole-antenna biosensors to detect pheromone release and identify early bark beetle infestations.

Optimization of Pheromonal Lures and Push-pull strategy in Forest Management

- Improving lure effectiveness by enhancing attraction efficiency, increasing selectivity for bark beetles while minimizing non-target captures, and adjusting the sex ratio of trapped individuals toward males.
- Developing more effective and optimized push-pull strategies for bark beetle management.
- Identifying additional olfactory receptors on bark beetle antennae that detect specific ecological compounds and integrating these compounds into synthetic lures to enhance attraction and control efficiency.

7.2. Intervention in Pheromone Production and Perception on Genetic Level

Manipulating pheromone production in *Ips* bark beetles offers a promising strategy for disrupting their communication without removing them from ecosystems. This approach helps preserve their ecological roles in forest renewal and nutrient cycling while mitigating large-scale tree infestations [95,218]. Additionally, targeting their PRs using antagonists or highly potent agonists can prevent the beetles from detecting aggregation pheromones, thereby reducing their ability to locate and attack specific trees[180].

The key advantage of these approaches—though still largely speculative—lies in their species specificity and non-lethal nature. However, manipulating beetle behavior at the genetic level requires functional characterization of selected genes, which has only been completed for a few. Access to a complete genome of a target beetle significantly facilitates gene selection. Yet, to date, only the genomes of *I. typographus* and *I. nitidus* have been published [15,219].

Species-specific targeting of pheromone biosynthetic pathways depends on the careful selection of genes, particularly those involved in the terminal steps of biosynthesis. Several candidate genes for ipsdienol and ipsenol biosynthesis have already been characterized and could be studied for genetic manipulation in species where these compounds play a crucial role in attraction (Table 1). These genes include GPP/myrcene synthase [112], IDOLDH [220], IDONER [120]; Chapter 4.2.1), and CYP450 myrcene hydroxylase [71]; Chapter 4.2.3.

In *I. typographus*, additional key genes require further characterization, including isoprenyl diphosphate synthase (IPDS) (Chapter 4.2.1), which is involved in 2-methyl-3-buten-2-ol biosynthesis, and lipases/carboxylesterases (Chapter 4.2.2), which are involved in verbenyl fatty acid ester metabolism. Characterizing these genes involves expressing them in bacterial, eukaryotic, or coleopteran cell lines to produce the relevant enzymes, followed by functional assays to validate their roles.

Another knowledge gap lies in understanding the regulatory cascades that control pheromone production in bark beetles, offering a potential alternative for intervention. The roles of hormones like ecdysteroids and the specific receptors for JH III, which are known to induce de novo pheromone biosynthesis (Chapter 4.2), remain unclear.

Regarding pheromone receptors of *Ips* species, five receptors have been functionally characterized from *I. typographus*, with specific responses to (*S*)-(−)-ipsenol, (*R*)-(−)-ipsdienol, *E*-myrcenol, lanierone, and (*S*)-*cis*-verbenol, respectively (Chapter 5.4) [171,178,181,182] . However, receptors for other pheromone compounds, including hemiterpenes and (*S*)-(+)-ipsdienol, as well as those in other *Ips* species, remain unidentified.

When a gene's full sequence and function are known, its expression can be regulated through genetic manipulation. This can be achieved temporarily via RNA interference (RNAi), which degrades target mRNA using double-stranded RNA (dsRNA), or permanently through CRISPR-Cas genome editing [221]. RNAi, offering greater specificity and environmental safety than traditional insecticides, has gained traction in agricultural pest control [222,223]. However, bark beetle outbreaks in forestry pose unique challenges, including vast forested areas, wide beetle dispersal, multiple generations per year, and delivery method limitations [224,225]. Despite these obstacles, progress has been made in RNAi-based bark beetle control [218,226]. Research into dsRNA delivery for coleopteran wood-feeding beetles includes methods such as spraying tree trunks, injecting dsRNA into the sap stream [227] or using polymer carriers [228]. These approaches could effectively silence pheromone biosynthetic genes during bark beetle feeding [229]

Additionally, the genetic approach to preventing bark beetle attacks may target their olfactory system to impair pheromone detection, conspecific recognition, and mass attack coordination [230]. RNAi silencing and CRISPR-Cas may also target OR and ORCO genes in *Ips* species, even though both have mainly been used to study receptor roles in pheromone-driven behaviour so far [231,232].

7.3. Development of Novel Techniques for Early Attack Detection employing biosensors based on bark beetle olfactory system

Early detection of bark beetle-infested trees is crucial for timely salvage logging before beetles spread. The most effective method remains visual inspection for boring dust [233], but the vastness of forests limits it, and new approaches are being sought. To improve efficiency, UAVs equipped with various sensors are being tested for faster, large-scale detection.

Properly characterized insect olfactory receptors (ORs) or entire antennae can be used to develop species-specific biosensors that convert pheromone-receptor interactions into readable signals [234–236]. While this has been applied to lepidopteran antennae, it remains unexplored for bark beetles. Potential instrumentation includes portable electroantennography (EAG) devices with insect antennae on plastic chips [237] or lighter BioFETs combining antennae with field-effect transistors [238].

7.4. Optimization of Pheromonal Lures and Push-pull strategy in Forest Management

To optimize pheromone lures, electrophysiological studies should identify new pheromonally active compounds [200,239], while improved dispenser designs and optimized blend compositions should be tested in pheromone traps to reduce non-target captures and adjust the trapped sex ratio toward males. Also, beetle-derived synergistic compounds can be added to lures to enhance dispenser effectiveness and maximize beetle capture [189,240]. Additionally, incorporating ecosystem-based attractants, such as high monoterpene concentrations from host trees [97,189] or symbiotic fungal compounds (e.g., fusel alcohols, fusel acetates, or oxygenated terpenes with synergistic properties) may further increase bait specificity [100,134,135]. More precise enantiomeric composition and purity of chiral compounds can also improve species specificity and overall efficacy (Table 1). Enhanced lures could unlock the hidden potential of trap-and-kill strategies or serve as more selective monitoring tools.

Push-and-pull strategies can be improved by optimizing both push (anti-attractants) and pull (aggregation pheromone) components. More than for mass trapping, practical spatial arrangements for these components need to be developed.

8. Concluding remark.

Recent research on *Ips* bark beetle aggregation pheromones has been driven by the increasing frequency and severity of outbreaks, as well as their expansion into new habitats. This review synthesizes insights from multiple disciplines, linking pheromone-based *Ips* management strategies with laboratory research on pheromone biosynthesis and detection at neuronal and genetic levels. Framed within the ecological and behavioral context of selected *Ips* species, it provides a comprehensive perspective on aggregation pheromones and their applications.

While the initial promise of using aggregation pheromones for mass trapping in *Ips* pest management has diminished due to limited effectiveness and unintended consequences, these

pheromones remain valuable for monitoring bark beetle populations. Moreover, emerging research continues to explore novel applications, making it worthwhile to investigate their potential further.

On the other hand, advancements in molecular and genomic techniques in the post-genomic era have significantly enhanced our understanding of pheromone biosynthesis, its regulation, and the olfactory mechanisms underlying pheromone detection. In the future, gene manipulation techniques—already applied in agricultural pest management—may offer innovative approaches to influence pheromone production and perception in *Ips* bark beetles.

By bridging traditional forestry perspectives with cutting-edge molecular insights, this review aims to stimulate productive discussions and inspire novel control strategies—not to replace traditional, effective bark beetle management practices but to integrate new approaches that enhance their effectiveness in an eco-friendly manner.

Key Recent References:

The key references published after 2020 that support this review are listed here.

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Hlásny et al. (2021) provide a comprehensive overview of bark beetle outbreaks in European conifer forests, highlighting the increasing impact of climate change on these calamities and outlining management strategies.

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Blomquist et al. (2021) provide a comprehensive overview of the biosynthesis of pheromonal compounds in bark beetles, highlighting the essential role of terminal hydroxylation catalyzed by Cyp450 enzymes. Their study details the genetic basis of this process and discusses the overlap between pheromone production and the beetles' detoxification of plant-derived compounds.

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Ramakrishnan et al. (2022, 2024) unraveled the biosynthetic pathways of pheromonal compounds in *Ips typographus*, including the identification of transcripts of key terminal genes suitable for manipulation. Their research covered ipsdienol, *cis*-verbenol, and the newly studied 2-methyl-3-buten-2-ol. They also investigated the production dynamics of pheromone storage conjugates and identified genes involved in their synthesis and cleavage. In *Ramakrishnan et al. (2024)*, these findings were further detailed by studying the regulation of these processes by juvenile hormone III.

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Yuvaraj et al. (2021) offers significant insights into the molecular mechanisms of odorant detection in bark beetles by identifying and characterizing the two first pheromone receptors in bark beetles (*I. typographus*). Their work also highlights the ligand-binding sites important for pheromone recognition and bark beetle communication.

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Biswas et al. (2024) identified the first aggregation pheromone receptor in *I. typographus* and its molecular interactions with the ligand (*S*)-*cis*-verbenol. This PR could be a prime target for pheromone receptor-targeted bark beetle control.

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Sweeny et al. (2023) provide a more general summary on the management of forest pests (also including, but not specifically referring to bark beetles) covering also the application of pheromones for monitoring purposes and in “semiochemical tactics”.

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Singewar and Fladung (2023) placed an overview of studies on dsRNA applications for forest insect pests and pathogens, addressing current challenges and opportunities in their use for forest protection. Key aspects discussed include target selection, delivery methods, potential impacts on non-target species, and the need for collaboration among multidisciplinary experts to advance research in this field.

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Paper II

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RESEARCH ARTICLE

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Microscopic morphology and distribution of the antennal sensilla in the double-spined bark beetle, *Ips duplicatus* (Coleoptera: Curculionidae)

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Abstract

The double-spined spruce bark beetle, *Ips duplicatus*, has become an infamous secondary pest of Norway spruce, causing extensive ecological and economic destruction in many Central European countries. Antennae are the primary olfactory organs that play a fundamental role in insect-host chemical communication; therefore, understanding morphology is crucial before conducting electrophysiological investigations. Here, we present our analysis of sensilla types on the antennal surface of *I. duplicatus* for the first time, using high-resolution-scanning electron microscopy. We studied the external morphological characteristics of antennae and the types, numbers, and distribution of the antennal sensilla in males and females. Our results revealed the presence of five different types of morphologically distinct sensilla: sensilla chaetica, sensilla basiconica, sensilla trichodea, sensilla coeloconica, and Böhm's sensilla. We observed two subtypes of sensilla chaetica (SChI and SChII), four subtypes of sensilla basiconica (SBI, SBII, SBIII, and SBIV), three subtypes of sensilla trichodea (STrII, STrIII, and STrIV) and two subtypes of sensilla coeloconica (SCoI and SCoII), respectively in *I. duplicatus* males and females. Minor differences in length and numbers between the sexes for some sensilla types were found. Distribution maps for different sensillar types were constructed, and specific areas for the respective sensilla were found. Possible functions of observed sensilla types are discussed. The present study provides a basis for future electrophysiological studies to understand how *I. duplicatus* detects ecologically important olfactory cues.

Research Highlights

- The first report of morphology and distribution pattern of the antennal sensilla in *Ips* duplicatus is discussed.

- A total of 6 main types and 11 antennal sensilla subtypes were observed in male and female *Ips duplicatus*.
- Minor sex-specific differences were seen in the length and numbers in several sensilla types.

KEYWORDS
antenna, *Ips duplicatus*, morphology, scanning electron microscopy, sensilla

1 | INTRODUCTION

Most bark beetles (Coleoptera: Curculionidae, Scolytinae) are natural decomposers of dead and dying trees in forests, and several species are considered economically significant conifer pests that also attack living trees. The double-spined bark beetle, *Ips duplicatus* (Sahlberg, 1836), originally native to Fennoscandia, Siberia, and East Asia, has spread recently to Central Europe and is expanding southward (Wermelinger et al., 2020). The primary host tree of *I. duplicatus* is Norway spruce (*Picea abies* (L.) Karst.), the most cultivated conifer in Europe (Grodzki, 2012; Holuša et al., 2010). In endemic phases, *I. duplicatus* colonizes the uppermost stem and the crown of the weakened or dying spruce or spruce trees, often attacked by European spruce bark beetle (*Ips typographus* [Linnaeus, 1758; Schlyter & Olle, 1993]). However, in outbreaks, *I. duplicatus* can also infest living trees (Kašák & Foit, 2015; Knížek et al., 2019). The current *I. duplicatus* population increases in Central Europe, and its southwest expansion has worsened the already problematic situation in spruce forests (Wermelinger et al., 2020). *I. duplicatus* shares similar biology as *I. typographus*; however, due to its specific host preferences and different bionomy, its management is different and less effective than that for *I. typographus* (Holuša et al., 2010).

In bark beetles, antennae are the primary sensory organ (Faucheux, 1989, 1994; Hallberg, 1982; Shi, Zhang, Liu, Zhang, et al., 2021). The antennal surface is covered with many hair-like structures called sensilla, which are small sensory organs protruding from the cuticle of the exoskeleton. The sensillum is the antenna's basic functional unit and each one houses sensory receptor neurons. Thus, sensilla are distinct sensory units defined by their shape, size, wall ultrastructure, internal arrangements, and the number and modality of sensory neurons present. Usually, the olfactory sensilla are the most numerous on insect antennae (Schneider, 1964). The morphology of the antennal sensilla is very diverse. It often includes the hair- or peg-shaped types (sensilla trichodea and basiconica, respectively), types with pegs recessed in pits or surrounded by various cuticular protrusions (sensilla coeloconica), pegs set at the bottom of a long tube that appear as small round openings on the cuticular surface (sensilla ampullacea), round-shaped porous setae (sensilla placodea) and many others (Borden & Wood, 1966; Dickens et al., 1978; Galizia & Rössler, 2010; Payne et al., 1973; Steinbrecht, 1997; Whitehead, 1981). Usually, morphologically different sensillar types accommodate physiologically different sensory neurons. For instance, sensilla trichodea in many insects, such as flies and moths, mainly house olfactory sensory neurons (OSNs) tuned to detect pheromone components (Khallaf et al., 2021; Ljungberg et al., 1993; Pophof et al., 2005; Steinbrecht, 1997). However, in some species, sensilla trichodea detect other chemicals. For instance, in the tsetse fly *Glossina morsitans* (Diptera: Glossinidae), OSNs housed in the trichodea sensilla respond to a range of diverse chemicals, like 1-octen-3-ol, 2-heptanone, isoamyl acetate, and methyl laurate (Soni et al., 2019). In ambrosia beetles, Biswas et al. (2023) showed that OSNs housed in sensilla trichodea respond to a wide range of volatiles, including host, non-host, and fungal-derived odors. Sensilla basiconica OSNs generally respond to plant volatiles, including various alcohols, aldehydes, esters and, ketones (Cui et al., 2018; De Bruyne et al., 2001). OSNs in coeloconic sensilla are known to be tuned to specific chemosensory stimuli, including acids, aldehydes, ammonia, putrescine, and water vapor (Prieto-Godino et al., 2017; Yao et al., 2005). There are reports of sensilla coeloconica responding to the extent of temperatures and humidity (Ruchty et al., 2010; Schneider et al., 2018).

Antennal morphology and the distribution of different sensillar types have been published for several bark beetle species of the genus *Ips*, including *I. typographus*, *I. sexdentatus*, *I. pini*, *I. subelongatus*, *I. confusus*, and other *Ips* species (Faucheux, 1989, 1994; Hallberg, 1982; Payne et al., 1973; Shi, Zhang, Liu, Xu, et al., 2021; Shi, Zhang, Liu, Zhang, et al., 2021). However, we do not have any literature reporting morphological data about the sensilla types and distribution of *I. duplicatus*. Information about antennal morphology and distribution of the sensillar types is essential for further physiological studies related to the olfaction of *I. duplicatus* that govern the specific behavior and host preferences. We provide external morphology of different sensillar types on *I. duplicatus* antennae and maps of different sensilla on the antennal surface. We compare and discuss our morphological findings with the available literature for other *Ips* species.

2 | MATERIALS AND METHODS

2.1 | Insects

Logs of Norway spruce (*Picea abies*) infested by *I. duplicatus* were collected in the Kostelec nad Černými lesy (49°59′39″, 14°51′33″, Czech Republic) and maintained in insect rearing chambers at the

Faculty of Forestry and Wood Sciences, Czech University of Life Sciences, Prague until beetles developed. Then the logs were debarked, and adult beetles were collected and stored in plastic boxes with small breathing holes in a refrigerator at 4°C until used for experiments. Five individuals of each sex were selected for observations using scanning electron microscopy (SEM).

2.2 | SEM analyses

Beetles were cleaned using an air blower to remove dirt from their surfaces. The antennae of each individual beetle were dissected under a stereomicroscope (Nikon, Japan). At first, antennae were primarily fixed for 24 h in 2.5% glutaraldehyde in 0.5 M cacodylate buffer (pH - 7.2), followed by post-fixation in 2% OsO4 in the same buffer for 4 h. Then, the antennae were washed twice with distilled water for 10 min. Fixed antennae were dehydrated by passing through a series of ethanol with increasing ethanol concentrations in water (35%, 50%, 70%, 96%, and 100%, with 10 min of incubation at each step). Antennae were further dried using a critical point dryer (Bal-Tec CPD 030). Preparations were then sputter-coated with a gold layer (20 nm thickness) in an ion sputter coater (Bal-Tec SCD 050) and observed under a JEOL JSM-IT200 scanning electron microscope and JEOL IT800 high-resolution scanning electron microscope (high-resolution SEM) at 3, 5, 10, and 15 kV with a working distance of 3–5 mm. The antennal morphology and sensilla types, numbers, and distribution were studied on five antennae from adults of both sexes.

2.3 | Sensilla categorization

The general morphology of *I. duplicatus* is described using terminology as per Hulcr et al. (2015). When classifying the sensilla, we combined data from different papers that studied antennal sensillar equipment of different bark beetles of the genus *Ips* (Faucheux, 1989, 1994; Hallberg, 1982; Shi, Zhang, Liu, Xu, et al., 2021; Shi, Zhang, Liu, Zhang, et al., 2021) and other insect species (Chen et al., 2010; López et al., 2018; Payne et al., 1982; Schneider, 1964; Whitehead, 1981). Sensilla categorization was based on external morphological criteria like size, shape, presence or absence of pores, and other features such as the attachment of the sensilla with the cuticle (flexible or inflexible socket) (Nowińska & Brożek, 2017).

2.4 | Statistical analyses

Image J v.1.53q (Schneider et al., 2012) was used to measure and quantify each sensilla type. The software allows you to set a defined scale and measure different parameters of selected region such as length, width, diameter, and area, using the specific tools. The length of the sensilla was measured from the sensilla tip to the base of the sensilla, and basal width was determined at the bottom of the sensilla ($n = 10$ per sensilla type per specimen in each sex). The length, basal width, and total numbers of sensilla of each respective category were compared between the sexes by Bonferroni multi-comparison test using GraphPad Prism v.9.0 trial version for Windows, GraphPad Software (www.graphpad.com).

3 | RESULTS

3.1 | General antennal morphology

The antennae of *Ips duplicatus* are elbowed with seven flexibly connected segments: the scape (on the proximal side), five funicular segments (from proximal to distal: pedicel (F1), F2, F3, F4, F5), and the antennal club (the most distal side) (Figure 1a). The segments between the scape and the antennal club are smaller, flexible, and collectively considered as funicle. The funicle has five bowl-shaped linked segments, with their depth increasing and diameter decreasing distally from F5 to F (Figure 1a and Supplementary Table 1). The segment F1 connected to the scape is also known as the pedicel and is the largest of all funicular segments. The pedicel of the left antenna is slightly bent towards the left side, while the pedicel of the right antenna is curved to the right side. The mean length of the whole antenna is approximately 755.99 ± 1.69 μm. The scape is the longest part of the antenna and is, on average, 354.23 ± 2.05 μm long, followed by the antennal club (220.66 ± 2.43 μm) and the funicular segments. The antennal club is

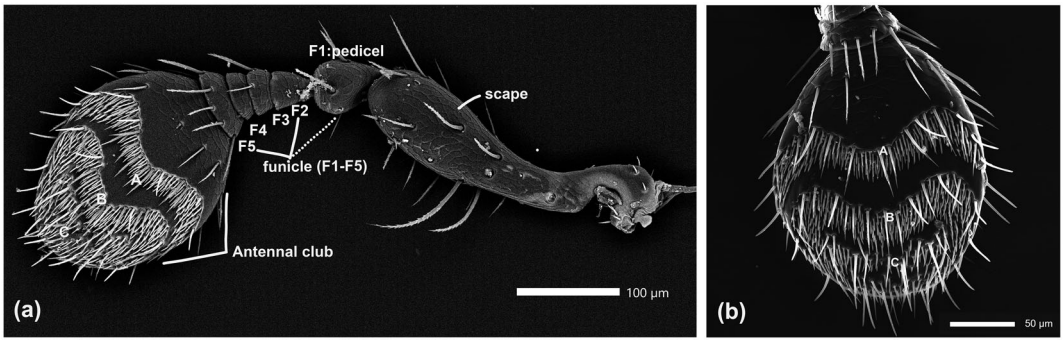


FIGURE 1 The general morphology of *Ips duplicatus* antenna (female). (a) Ventral side of the antennal club showing funicular segments (F1–F5), pedicel (F1) and scape; (b) Three sensory bands on the antennal club (A, B, and C).

TABLE 1 General morphological characteristics based on external appearance and distribution of different sensilla types in *Ips duplicatus*.

Sensilla type	Distribution	Pores	Wall structure	Tip	Shape	Socket
SChI	Antennal club (A, B and C), funicular segments (F1–F5) and scape	Aporous	Longitudinal grooved wall, bilateral branching	Sharp	Straight	Flexible
SChII	Antennal club (C), funicular segments (F1–F5) and scape	Aporous	Longitudinal grooved wall, multi-branching	Sharp	Curved	Flexible
SBI	Antennal club (A, B, C)	Multiporous	Pitted	Blunt	Straight	Inflexible
SBII	Antennal club (A, B and C)	Multiporous	Grooved	Blunt	Straight	Inflexible
SBIII	Antennal club (B and C)	Uniporous	Smooth	Blunt and round	Peg shaped	Inflexible
SBIV	Antennal club (C)	Uniporous	Smooth	Round	Straight	Inflexible
STrII	Antennal club (B and C)	Multiporous	Smooth	Pointed	Slightly curved	Inflexible
STrIII	Antennal club (A, B, and C)	Terminal pore	Smooth	Blunt	Long and curved	Flexible
STrIV	Antennal club (A, B, and C)	Multiporous	Pitted	Pointed	Straight	Inflexible
SCo I	Antennal club (A, B, and C)	Aporous	Grooved	Round	Cone-shaped	Inflexible
SCo II	Antennal club (A, B, and C)	Aporous	Grooved	Sharp	Cone-shaped	Inflexible
BS	Scape	Aporous	Smooth	Blunt and round	Short and straight	Flexible
SP	Club (A, B, and C), funicle segments (F1–F5) and scape	?	Pit on the club surface	—	Oval	—

Abbreviations: SChI, sensilla chaetica type I; SChII, sensilla chaetica type II; SBI, sensilla basiconica type I; SBII, sensilla basiconica type I; SBIII, sensilla basiconica type III; SBIV, sensilla basiconica type IV; STrII, sensilla trichodea type II; STrIII, sensilla trichodea type III; STrIV, sensilla trichodea type IV; SCoI, sensilla coeloconica type I; SCoII, sensilla coeloconica type II; BS, Böhm's sensilla; SP, surface pores.

wide and oval shaped with an average length of 220.66 ± 2.43 μm in the direction of the antennal axis with an average width of 180.70 ± 0.53 μm. The club is slightly bulging (convex) on both sides. The surface of the scape, funicle, and dorsal surface of the club have a scale-like layer on the outer surface. These scales are also visible on the proximal area of the ventral side of the club. The sensilla on the surface of the scape, funicular segments, and dorsal side of the antennal club are sparse, and only a few types are present (Supplementary Figure 1). The majority of sensilla are located on the ventral side antennal club, specifically in its most distal three-fourth area (Figure 1b). The sensilla are systematically organized into three sensory areas, referred to here as A, B, and C bands here (distal sensory band C, middle sensory band B, and proximal sensory band A) (Figure 1b). The sensory bands A and B form two parallel wave-like stripes across antennal club separated by a strip of the plain cuticle. The sensory band B has a deeper curve in the middle compared to sensory bands A and C. A hint of the third distal sensillar band C almost merges with the middle sensory band B area on the antennal club. Oval-shaped pit-like structures scattered randomly among the sensilla, termed here as surface pores (SPs), were observed on both the ventral side and the dorsal part of the club and other antennal segments.

3.2 | Sensilla types and distribution

Two types of sensilla chaetica (SChI and SChII), four types of sensilla basiconica (SBI, SBII, SBIII, and SBIV), three subtypes of sensilla trichodea (STrII, STrIII, and STrIV), two types of fluted cone-shaped sensilla coeloconica (SCoI and SCoII), and Böhm's sensilla (BS) were identified on *I. duplicatus* antennae in both sexes (Table 1). Table 1 summarizes the respective features (length, basal width, socket characterization, presence of pores in the sensillar cuticular wall or at the tip, tip shape, etc.) and numbers of each sensilla type present on the ventral area of the antennal club. On the dorsal surface of the club, only sensilla chaetica type II, sensilla trichodea type III, and Böhm's sensilla were seen (Supplementary Figure 1).

3.3 | Sensillar types and distribution on the antennal surface

3.3.1 | Sensilla chaetica

Sensilla chaetica (SCh) are long aporous sensilla with toothed projections and flexible (deep and wide) sockets (Figure 2). In *I. duplicatus*, sensilla chaetica were generally projected at an angle greater than 45° from the antennal club surface. They comprise 12% of the total observed sensilla. Based on their length and branching pattern, sensilla chaetica were categorized into two subtypes (Figure 2a, b). Shorter and slender sensilla chaetica type I (SChI) with a length of 21.2–46.5 μm in males and 21.9–38.9 μm in females, respectively, had toothed projection oriented in only one plane (saw-toothed with bilateral branching) and visible longitudinal grooves on the wall surface (Figure 2c, f–h).

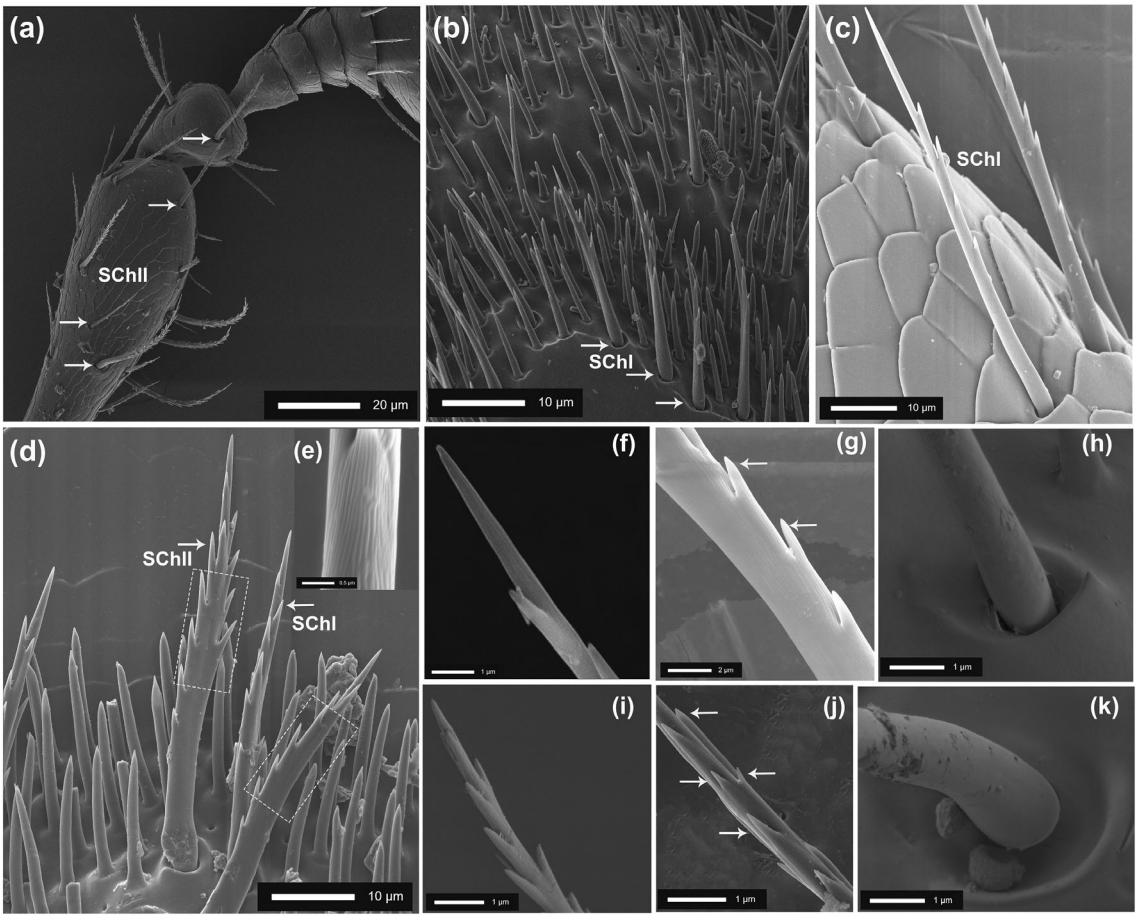


FIGURE 2 *Ips duplicatus* antenna highlighting sensilla chaetica and its subtypes, (a) Sensilla chaetica type II (SChII) (b) Sensilla chaetica type I (SChI), (c) Magnified view of SChI, (d) magnified view of SChII, inset (e) longitudinal grooved wall surface, (f–h) tip shape, branching pattern and, basal socket of SChI, (i–k) tip shape, branching pattern and basal socket of SChII.

SChI are present on all antennal sections, including the dorsal and ventral side of the club. Sensilla chaetica type II (SChII) were longer and thicker, with a length range of 23.9–79.2 µm in males and 29.2–221.3 µm in females, respectively, with multilateral branching (Figure 2d, i–k). The socket shape was different in both types of sensilla chaetica, with SChI having a deeper socket than SChII (Figure 2h, k). SChII on the scape and funicular segments were remarkably longer than those observed on the antennal club surface. SPs were often observed close to both types of sensilla chaetica (Figure 2d).

3.3.2 | Sensilla basiconica

Sensilla basiconica (SB) were the most frequent sensilla type (66% of the total sensilla) observed within the sensory epithelium of the club (Figure 3). All SB have inflexible (fused) sockets. Sensilla basiconica were categorized into four subtypes based on their length, basal width, porosity, tip shape, and wall structure (Figure 3a). The most abundant sensilla basiconica type were sensilla basiconica type I (SBI) (Figure 3b). These sensilla types were straight, multiporous with a pointed tip. The pores of SBI formed longitudinal slit-like depressions on the wall surface (Figure 3c). The pore density was 40 pores/um ^{2}. The SBI length was around 9.3–11.7 µm long in males and 8.6–13.2 µm long in females. Sensilla basiconica (SBII) were also multiporous with pointed tips but comparatively shorter and thicker than SBI (Figure 3d and Table 2). SBII were around 5.8–10.2 µm long in males and 6.3–10.9 µm in females. SBII have a lower pore density of 20 pores/um^2 compared to SBI. The pores collectively resembled pit-like depressions on the sensillar wall surface (Figure 3d). Sensilla basiconica type III (SBIII) were uniporous with peg-like appearance (length and basal width range: 5.8–10.9 µm and 1.1–2 µm, respectively), a slightly tapered tip, and a smooth wall with slight depressions (Figure 3e, f). The range of length of SBIII was around 3.6–6.8 µm in males and 3.3–7.1 µm in females, respectively. Sensilla basiconica type IV (SBIV) were the shortest basiconica type with an inflexible (fused) socket, smooth wall, and an uniporous round tip (Figure 3g) with the length ranging from 3 to 5 µm in males and 3.4–6 µm in females.

3.3.3 | Sensilla trichodea

This category (STr) is rather non-homogeneous in its parameters and covers around 19% of the total sensilla. Considering their length as a

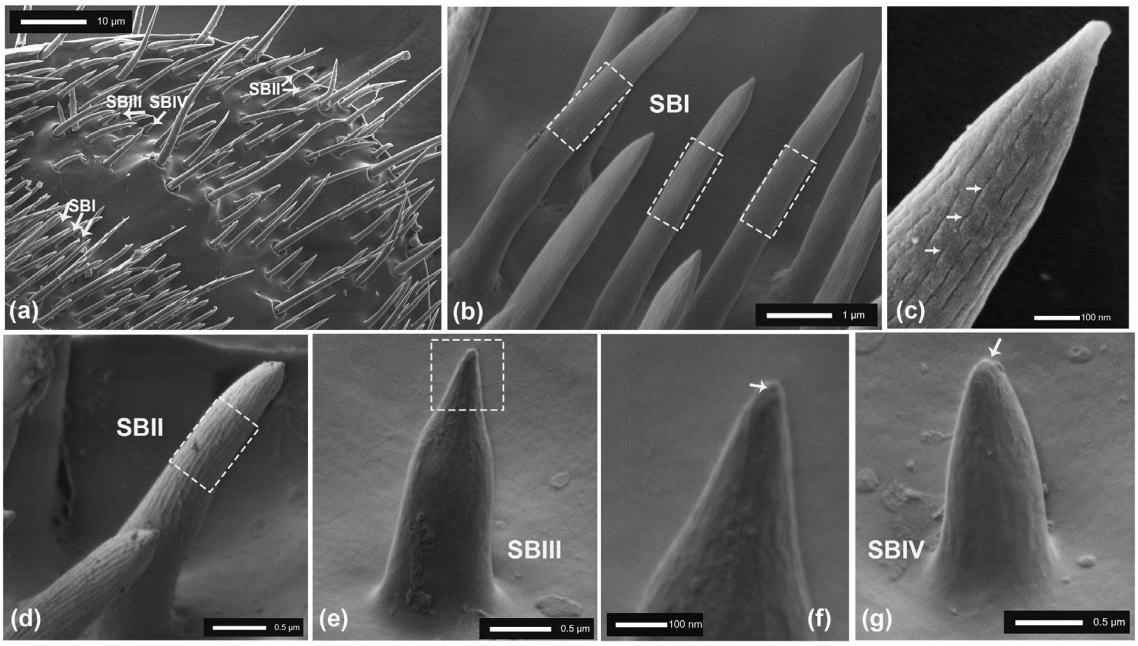


FIGURE 3 *Ips duplicatus* antenna highlighting sensilla basiconica and its subtypes, (a) Antennal sensory band C showing clusters of sensilla showing different types of sensilla basiconica (b) sensilla basiconica type I (SBI) with slit-like depressions on the wall surface and fused socket, (c) magnified SBI tip showing pores (indicated by arrows), (d) sensilla basiconica type II (SBII) with the porous wall surface, (e) peg-shaped sensilla basiconica type III (SBIII) with fused socket and a pointed tip, (f) closer look of SBIII tip with the pore-like structure on the tip and, (g) sensilla basiconica type IV (SBIV) with the pore-like structure on the blunt tip.

classification parameter, ranging from 12.24 to 30.62 µm, they fit between the sensilla basiconica and sensilla chaetica (Table 2). We classified these sensilla types into three categories (Figure 4a). The longest sensilla trichodea type III (STrIII) (length range: 21.5–44.8 µm in males and 18.9–47.6 µm in females) were distinctly curved sensilla with a flexible socket, smooth wall, and a single terminal pore (Figure 4b, g, h). Sensilla trichodea type II (STrII) were shorter and slender than STrIII (length range: 15.7–34.5 µm in males and 13.7–36.4 µm in females, respectively), elongated and tapering towards the tip (Figure 4e), with an inflexible (fused) socket, and a multiporous wall (Figure 4f). The pore density calculated was 30 pores/um^2 for STrII. The shortest sensilla trichodea type IV (STrIV) with length range of 11.1–15.8 µm in males and 9.5–16.2 µm in females, respectively, had a sharp pointed tip, porous wall surface, and fused sockets (Figure 4c, d). STrIV were easily distinguishable from STrII since they bulged in the middle and tapered towards the tip. The pore density was 15 pores/um^2. We did not observe sensilla trichodea type I, which was reported in previous studies in *Ips* species (Supplementary Table 2).

3.3.4 | Sensilla coeloconica

Sensilla coeloconica (SCo) were fluted cone-shaped structures with deep longitudinal grooves on their wall and inflexible (fused) sockets (Figure 5) covering about 2% of the total number of sensilla. Two types of SCo were classified based on the differences in their tip shape. SCo type I had a pointed tip with a length range from 5.9–8.1 µm in males and 5.5–8.3 µm in females (Figure 5a, b), whereas SCo type II had a round and bulgy tip with a length range of 5.8–7.4 µm in males and 5–7.9 µm in females, respectively (Figure 5c, d).

3.3.5 | Böhm's sensilla

The sensilla type, called Böhm's sensilla (BS), were present on the base of the scape and pedicel (Figure 6a). They were short (length range: 3.9–6.9 µm in males and 8.4–15.5 µm in females, respectively), straight or slightly curved hairs with their base in a flexible (deep and wide) socket and a smooth wall surface, typically angled ~45° to the cuticle of the antennal surface (Figure 6b).

3.3.6 | Surface pores

The SPs were present homogeneously on the ventral side of the antennal surface, the funicular segments, and the scape (Figure 6a, d). Some were also observed on the dorsal surface (Supplementary Figure 1). The pore diameter was 0.5 µm. We observed around 28 of these pores in males whereas approximately 18 in females (Supplementary Table 1). The exact number could not be estimated since the pores were difficult to observe since they were often hidden between different sensilla on the antennal surface. The pores were sometimes associated with sensilla with flexible sockets but otherwise

TABLE 2 The length range, mean length, basal width, numbers, and respective *p*-values (mean ± SE) of the different sensilla type in male and female *Ips duplicatus* along with their percent total distribution.

Sensilla type	Sex	*n*	Length range (in µm)	Mean length (in µm) ± SE	*P*-value	Mean basal width (in µm) ± SE	*p*-value	*n*	Number of sensilla (mean ± SE)	*p*-value	% Total distribution
SChI	Males	5	21.2–46.5	35.19 ± 2.02	>.9999	2.15 ± 0.13	>.9999	5	30.4 ± 1.43	.6864	6.50
	Females	5	21.9–38.9	32.49 ± 1.65		2.09 ± 0.11		5	26.2 ± 0.86		5.56
SChII	Males	5	23.9–79.2	44.23 ± 4.78	<.0001****	2.73 ± 0.27	.0058**	5	32.6 ± 1.29	>.9999	6.97
	Females	5	29.2–221.3	66.32 ± 12.84		3.34 ± 0.32		5	29.0 ± 1.00		6.25
SBI	Males	5	9.3–11.7	10.59 ± 0.21	>.9999	1.37 ± 0.04	>.9999	5	265.4 ± 5.45	.0163*	56.80
	Females	5	8.6–13.2	10.64 ± 0.45		1.45 ± 0.07		5	258.2 ± 2.20		55.69
SBII	Males	5	5.8–10.2	8.27 ± 0.23	>.9999	1.49 ± 0.05	>.9999	5	43.2 ± 1.56	>.9999	9.25
	Females	5	6.3–10.9	8.11 ± 0.55		1.56 ± 0.05		5	39.4 ± 1.81		8.49
SBIII	Males	5	3.6–6.8	4.93 ± 0.26	>.9999	1.83 ± 0.06	>.9999	5	4.2 ± 0.49	>.9999	0.89
	Females	5	3.3–7.1	5.68 ± 0.28		1.66 ± 0.08		5	3.8 ± 0.37		0.82
SBIV	Males	5	3.0–5.0	3.86 ± 0.23	>.9999	1.77 ± 0.09	>.9999	5	2.8 ± 0.37	>.9999	0.59
	Females	3	3.4–6	4.47 ± 0.44		1.86 ± 0.24		5	2.6 ± 0.40		0.56
STrII	Males	5	15.7–34.5	26.31 ± 2.36	>.9999	1.89 ± 0.03	>.9999	5	16.6 ± 1.21	>.9999	6.59
	Females	5	13.7–36.4	23.75 ± 2.16		1.99 ± 0.06		5	19.0 ± 1.30		8.45
STrIII	Males	5	21.5–44.8	29.19 ± 0.76	>.9999	2.26 ± 0.09	>.9999	5	28.6 ± 1.36	>.9999	3.55
	Females	5	18.9–47.6	30.62 ± 3.32		2.35 ± 0.13		5	33.0 ± 0.95		4.09
STrIV	Males	5	11.1–15.8	13.68 ± 0.27	>.9999	1.82 ± 0.03	>.9999	5	30.8 ± 1.36	.0026*	6.12
	Females	5	9.5–16.2	12.24 ± 0.30		1.81 ± 0.09		5	39.2 ± 0.86		7.11
SCoI	Males	5	5.9–8.1	7.09 ± 0.22	>.9999	1.70 ± 0.11	.5770	5	5.2 ± 0.58	.5584	1.11
	Females	5	5.5–8.3	7.24 ± 0.27		2.04 ± 0.04		5	5.0 ± 0.32		1.07
SCoII	Males	5	5.8–7.4	6.11 ± 0.18	>.9999	1.69 ± 0.09	>.9999	5	3.6 ± 0.25	>.9999	0.77
	Females	4	5–7.9	6.47 ± 0.32		1.84 ± 0.02		5	4.2 ± 0.37		0.90
BS	Males	2	3.9–6.9	5.21 ± 0.64	>.9999	1.14 ± 0.04	.0058*	5	3.8 ± 0.73	>.9999	0.81
	Females	5	8.4–15.5	10.63 ± 0.14		1.79 ± 0.07		4	5.0 ± 0.91		1.08
SP	Males	5	—	—	Not compared[a]	0.47 ± 0.05	Not compared[a]	5	28.4 ± 3.42	—	—
	Females	5	—	—		0.52 ± 0.05		5	18.2 ± 1.93		

Note: $n = 5$ specimens per sex and 5–20 sensilla of each respective type were measured.

Abbreviations: SChI and SChII, sensilla chaetica types; SBI-SBIV, sensilla basiconica types; STrII-STrIV, sensilla trichodea types; SCoI-SCoII: sensilla coeloconica; BS, Böhm's sensilla; SP, surface pores.

[a]Not compared since in females, the pores were not so easily visible in the dense sensory regions.

*values are statistically significant values showing significant differences where *p*-value was less than 0.05.

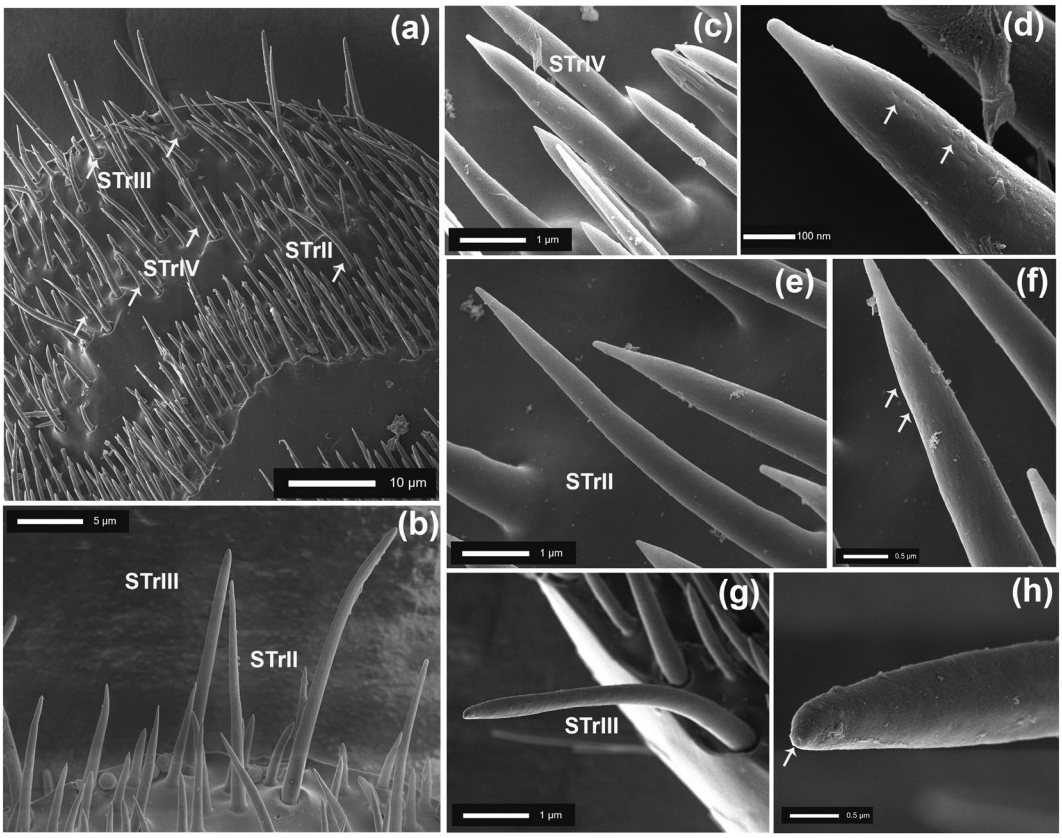
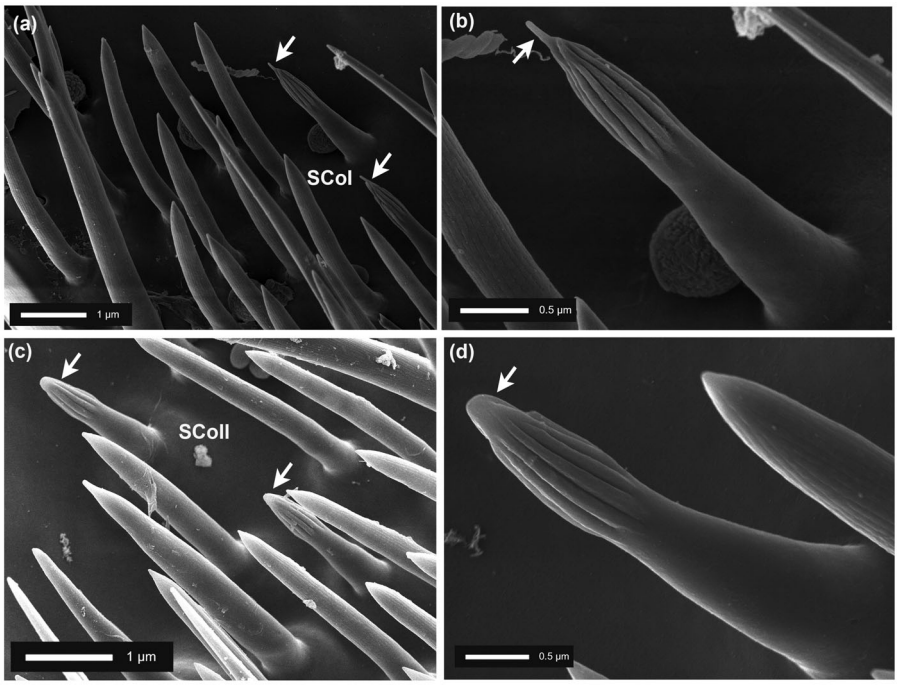


FIGURE 4 *Ips duplicatus* antenna highlighting sensilla trichodea and its subtypes, (a) Group of sensilla trichodea types on the antennal club(indicated by arrows) including (b) sensilla trichodea type II (STrII) and sensilla trichodea type III (STrIII), (c and d) sensilla trichodea type IV (STrIV) with fused socket, bulged middle, porous wall surface and pointed tip, (e) sensilla trichodea type II (STrII) with fused socket, pointed tips and (f) multiporous wall surface, (g) sensilla trichodea type III (STrIII) with deep flexible socket, blunt tip and (h) terminal pore.

FIGURE 5 *Ips duplicatus* antenna highlighting sensilla coeloconica and its subtypes (a) sensilla coeloconica type I (SCoI) with longitudinal grooved wall surface and (b) sharp tip, (c) sensilla coeloconica type II (SCoII) with (d) round tip.



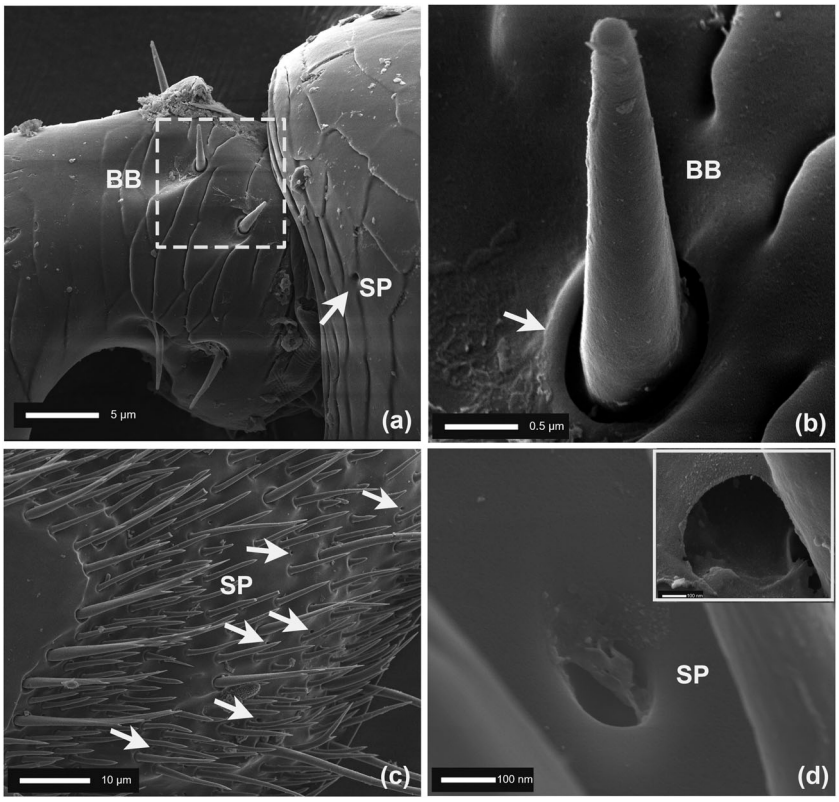


FIGURE 6 *Ips duplicatus* antenna highlighting (a) Böhm's sensilla (BS) on the scape (highlighted square) and surface pore (SP) indicated by an arrow, (b) detailed view of BS with a deep socket and round tip, (c) SPs on the antennal club surface (indicated by arrows) and, (d) magnified view of SPs.

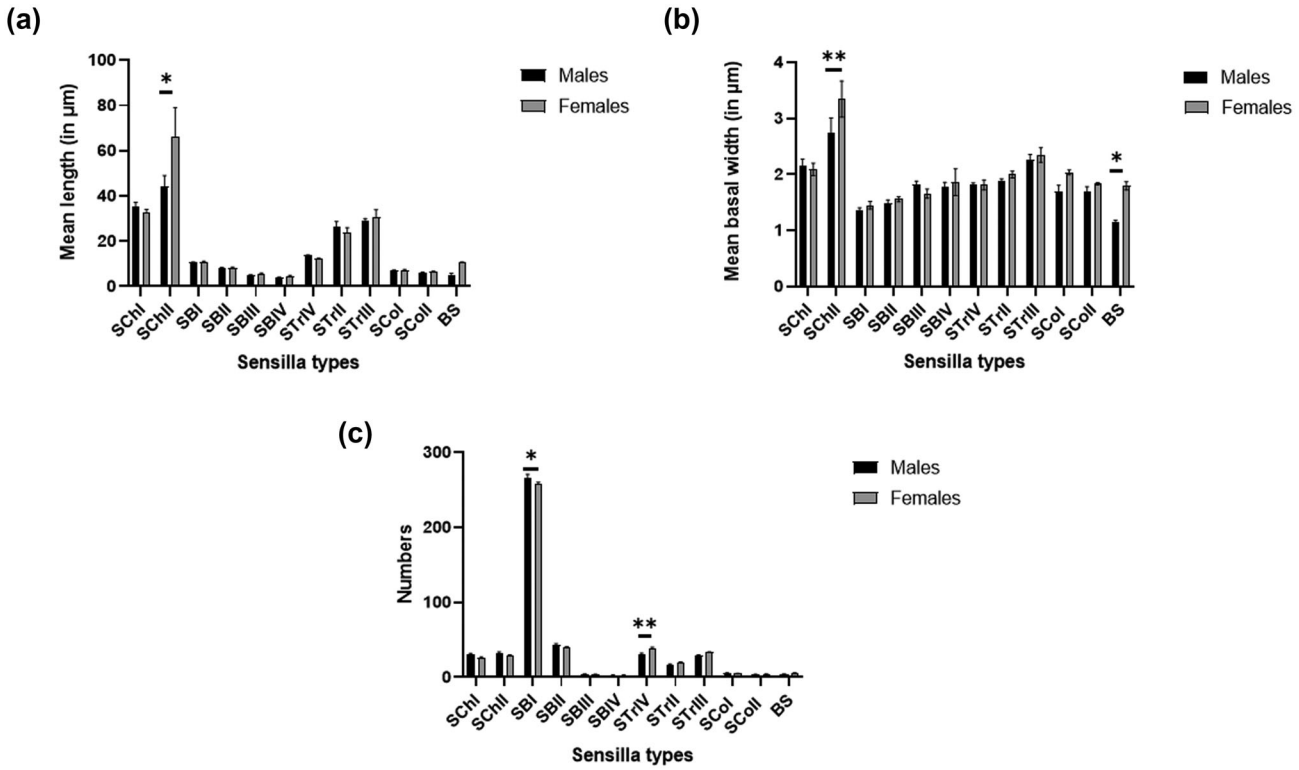


FIGURE 7 Graph showing the comparison of length (a), width (b), and numbers (c) of different sensilla types in *Ips duplicatus* antenna in females and males. Bars represent means and SE. (Bonferroni's multiple comparisons tests; $n = 5$ per sex).

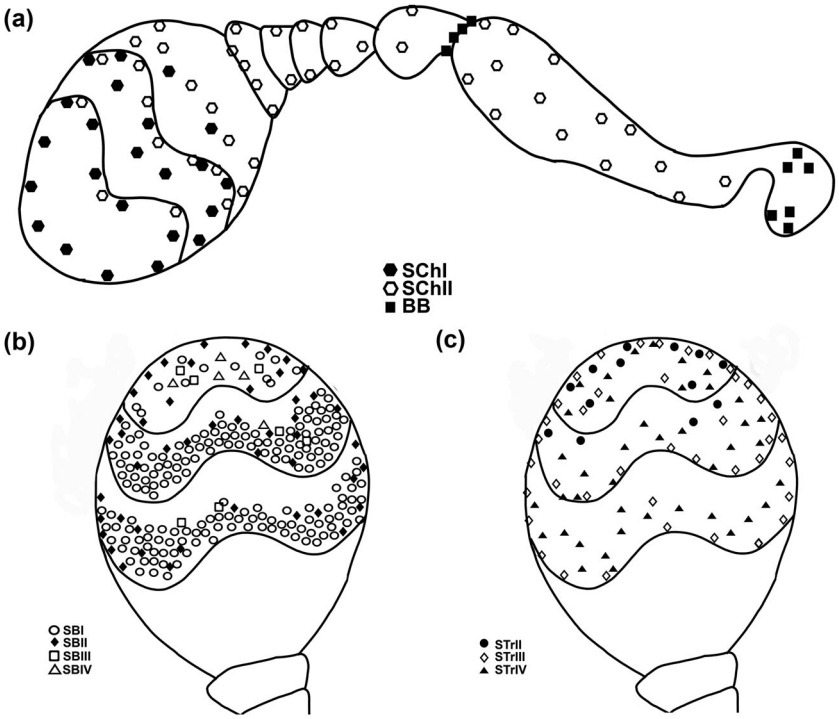


FIGURE 8 Maps of sensillar distribution on ventral side of *Ips duplicatus* antenna. (a) Sensilla chaetica type I (SChI), sensilla chaetica type II (SChII), and Böhm's sensilla (BS); (b) sensilla basiconica type I (SBI), sensilla basiconica type II (SBII) sensilla basiconica type III (SBIII) sensilla basiconica type IV (SBIV); (c) sensilla trichodea type II (STrII), sensilla trichodea type III (STrIII) and sensilla trichodea type IV (STrIV).

diffusely scattered among sensilla on the antennal club surface (Figure 6c).

3.4 | Distribution, dimensions, and numbers among sexes

The structural features and distribution pattern of sensilla on the antennal surface was approximately same in males and females. After performing statistical analyses using Bonferroni's multicomparison test, we noted minor variations concerning the length of a few sensilla types and numbers among the sexes (Table 2). The total length of the antenna and other antennal segments showed no significant differences between the sexes. The mean length of SChII was significantly different when comparing females and males (p-value of <.0001); however, the average length differences in other sensilla types were non-significant (Figure 7a and Table 2). The mean basal width of SChII was significantly higher in females than in males (p-value <.0058). Similarly, the mean basal width of BS was considerably higher in females than in males, with a p-value of .0058 (Figure 7b and Table 2). The average number of SBI was significantly different in males than in females ($p < .0163$), whereas the mean number of STrIV was significantly higher in females than in males ($p < .0026$) (Figure 7c and Table 2).

We mapped the distribution pattern of different sensilla types present on the antennal surface in *I. duplicatus* (Figure 8). The two types of sensilla chaetica, SChI and SChII, often can be seen as forming the boundary within the sensory bands around the sensilla basiconica and sensilla trichodea, whereas SChII is primarily seen on the funicular segments and scape and sometimes on Band A (Figure 8a). BS was exclusively present at the base of the scape and pedicel with only 1% of the total sensilla (Figure 8a). The sensilla basiconica types SBI and SBII, were uniformly distributed on the sensory band A and B. SBI were denser in the middle of the sensory bands A and B. Very few were observed on the distal band C. Shorter types of sensilla basiconica (SBIII and SBIV) were primarily observed in most distal club area C (Figure 8b). Among all the sensilla trichodea types, STrIII is typically present within the proximal boundaries of sensillar bands and at the periphery of the club, and the distribution is quite distinct and uniform. STrII was more abundant in the antennal club's C and B sensory areas, whereas STrIV was spotted more within A and B sensory bands. STrIV were often seen around SBI and SBII forming a peripheral borderline on the sensory band A and B (Figure 8c). Both types of sensilla coeloconica (SCoI and SCoII) were more abundant on the sensory bands B and C and sometimes observed on the sensory band A. These sensilla often occurred in pairs close to each other, and the distribution pattern was quite interesting. Both sensilla coeloconica I and II were more frequently distributed on the middle and to the right side of the left antenna and vice versa; rarely, 1 or 2 sensilla coeloconica were observed on the same side of the antenna (Figure 9a, b).

4 | DISCUSSION

We report the first study focused on the morphological characteristics of the sensory equipment of *I. duplicatus* antenna. Our study shows that the general morphology of *I. duplicatus* antenna is quite similar to other *Ips* species (Faucheux, 1989, 1994; Hallberg, 1982; Payne et al., 1973; Shi, Zhang, Liu, Xu, et al., 2021; Shi, Zhang, Liu, Zhang, et al., 2021). We observed five morphologically distinct sensilla types

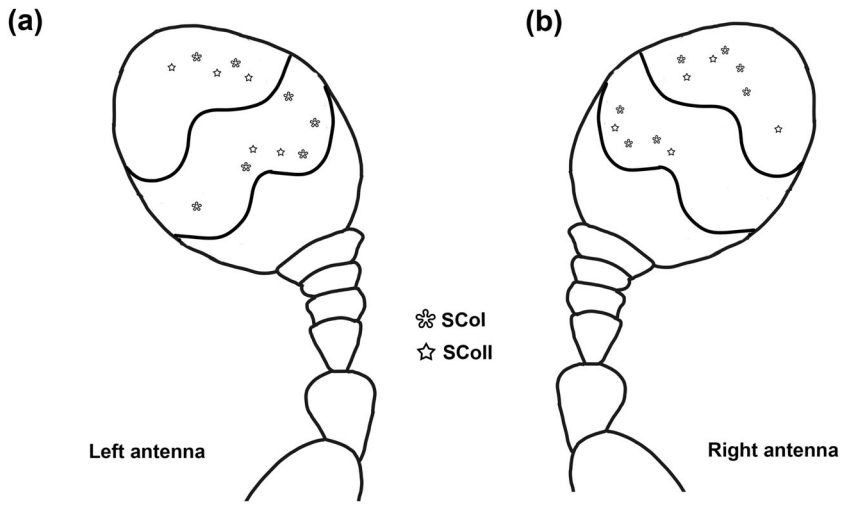


FIGURE 9 Map of sensilla coeloconica distribution on ventral side of *Ips duplicatus*. (a) Sensilla coeloconica type I (SCoI) and sensilla coeloconica type II (SCoII) on the left antenna; (b) sensilla coeloconica type I (SCoI) and sensilla coeloconica type II (SCoII) on the right antenna.

in *I. duplicatus*: sensilla chaetica with two subtypes, sensilla basiconica with four subtypes, sensilla trichodea with two subtypes, sensilla coeloconica with two subtypes, and Böhm's sensilla. SPs were observed occasionally. As it is typical for other species of the genus *Ips*, *I. duplicatus* antenna is also seven-segmented, and the sensilla on the club are arranged in three snake-shaped sensory bands (Payne et al., 1973). Many, but not all sensillar types observed on the antennal surface of *I. duplicatus* were like those described in other *Ips* species (Faucheux, 1989, 1994; Hallberg, 1982; Payne et al., 1973; Shi, Zhang, Liu, Xu, et al., 2021; Shi, Zhang, Liu, Zhang, et al., 2021). However, the sensillar nomenclature in the published studies is inconsistent in all studied species (Supplementary Table 2). We found more sensillar subtypes in the respective categories than previously published studies. Specifically, we observed more subtypes of sensilla basiconica, sensilla coeloconica, and sensilla trichodea in *I. duplicatus*. Based on our study, it is not clear whether these differences reflect specificities related to the technique used in previous studies or whether they indicate a specific adaptation for *I. duplicatus*.

There have been several studies of *Ips* spp. sensilla so far, with different classification and nomenclature (Faucheux, 1989, 1994; Hallberg, 1982; Payne et al., 1973; Shi, Zhang, Liu, Zhang, et al., 2021). Supplementary Table 2 summarizes the classification and nomenclature of sensilla in the present study and previously studied *Ips* species facilitating a clear understanding and avoiding any future confusion. We kept our classification consistent with previous reports and followed a new nomenclature only for those sensilla types which were not reported previously and do not fit within the existing classification.

As also observed for other *Ips* species (Faucheux, 1989, 1994; Shi, Zhang, Liu, Xu, et al., 2021; Shi, Zhang, Liu, Zhang, et al., 2021) and bark beetles in general, sexual dimorphism in *I. duplicatus* was not too prominent, and if present, it generally refers to only minor differences in abundance and length of some sensillar types. This finding indicates that in *I. duplicatus* the sensilla probably have similar functions in both sexes. Interestingly, we observed significantly longer sensilla chaetica with multilateral branching (SChII) in females, the wider multiporous sensilla basiconica (SBI), and their higher number in males. In addition, small sex-specific differences were determined for multiporous sensilla trichodea STrIV, which were present in slightly higher numbers in females. Further experiments are needed to determine whether these differences reflect sex-specific differences related to mating or host selection. Morphologically different sensillar categories are supposed to have specific physiological functions (Hallberg, 1982; Hansson & Stensmyr, 2011; Keil, 1999; Schneider, 1964). Below, we discuss the possible physiological roles of the different morphological types observed in *I. duplicatus*.

4.1 | Sensilla chaetica

This sensillar type have a flexible socket and long, either bilaterally or multilaterally branched hair that project outwards from the antennal surface well above other sensillar types. Two types of sensilla chaetica with the same morphology were also observed in other *Ips* species (Faucheux, 1994; Shi, Zhang, Liu, Xu, et al., 2021; Shi, Zhang, Liu, Zhang, et al., 2021), though there are differences when it comes to nomenclature (Supplementary Table 2). The morphological description and location of this sensilla type in *I. duplicatus* match with the previous studies in *I. typographus* and *I. confusus* (Borden & Wood, 1966; Faucheux, 1989; Hallberg, 1982; Shi, Zhang, Liu, Zhang, et al., 2021).

The cross-sections of sensilla chaetica in *I. typographus* (Hallberg, 1982); shows a basal cuticular ring of articulating membrane, circular solid hair shafts filled with an electron dense material, and a sensory process that terminates as a tubular body in the basal part of the hair. These parameters suggest that sensilla chaetica serves the mechanoreception function (Borg & Norris, 1971; Hallberg, 1982; Moeck, 1968). Though we have not performed the cross-section of sensilla chaetica in *I. duplicatus*, the external morphological similarity between sensilla chaetica in *I. typographus* and *I. duplicatus* allows us to conclude that sensilla chaetica in *I. duplicatus* also have a mechanoreceptive role. Their locations on the scape may enable *I. duplicatus* to determine the positions of the antennae with

respect to its surroundings and detect wind movement (Payne et al., 1973). It may also serve as "displacement detectors" as reported in some beetle species (Borg & Norris, 1971; Henderson & Wadhams, 1981; Moeck, 1968; Wadhams et al., 1982). Alternatively, sensilla chaetica might be involved in fly speed detection during flying as suggested by the electrophysiological recordings from bilaterally branched sensilla chaetica in *Scolytus scolytus* showing that these types of sensilla respond to airflow (Wadhams and Angst unpublished data mentioned in Sivalinghem, 2012). Alternatively, sensilla chaetica might possibly serve as auditory organs (Borden & Wood, 1966) and can be involved in bark beetle acoustic communication that mediates beetle interaction during mating, various social or defensive interactions, and dispersion under the bark (Barr, 1969; Borden & Wood, 1966; Dobai et al., 2018; Hofstetter et al., 2019; Rudinsky, 1979; Rudinsky et al., 1976; Wilkinson et al., 1967). Until now, no tympanal organs in bark beetles have been found (Borden & Wood, 1966). As long sensilla on the antenna can easily vibrate (Yack & Hoy, 2003), sensilla chaetica might be involved in the perception of sound and/or substrate vibrations (Rudinsky et al., 1976; Schmitz, 1972; Sivalinghem, 2012; Swaby & Rudinsky, 1976). Unilateral and bilateral branching may represent specific adaptations with respect to the perception of specific sound/vibration parameters. We noted that in *I. duplicatus*, multilaterally branched sensilla chaetica (SChII) are significantly longer and thicker in females than in males. This may reflect the greater need by ovipositing females to orient under the bark to ensure uniform dispersion. Similar sex-specific differences were reported in *I. typographus*, *I. sexdentatus* (Faucheux, 1989), and *Tryptodendron lineatum* (Moeck, 1968). These differences may reflect the sex-specific differences in premating behavior and/or during oviposition (Hofstetter et al., 2019; Rudinsky, 1979). As compared to other bark beetle species (Bedoya, Brockerhoff, et al., 2019; Bedoya, Nelson, et al., 2019), the genus *Ips* is characterized by a relatively smaller number of sensilla chaetica (Faucheux, 1989; Shi, Zhang, Liu, Xu, et al., 2021; Shi, Zhang, Liu, Zhang, et al., 2021), which can reflect the relative importance in sound communication in different taxonomic categories (Bedoya et al., 2020; Hofstetter et al., 2019).

4.2 | Sensilla trichodea

This morphological category is inconsistent among all sensillar types found in *I. duplicatus*. The only common characteristic of *I. duplicatus* is that they are longer than the sensilla basiconica type and shorter than the sensilla chaetica type. Sensilla trichodea in *I. duplicatus* form two distinct categories. The first category includes two subtypes of multiporous sensilla with inflexible sockets that differ by wall structure (STrII are smooth-walled while STrIV are pitted). The second category represents the long terminal pore sensillum with a flexible socket (STrIII). STrII corresponds to trichodea type II in some *Ips* species (Borden & Wood, 1966; Faucheux, 1989, 1994; Payne et al., 1973) (Supplementary Table 2).

The cross-sections of the multiporous sensilla performed in *I. typographus* (Hallberg, 1982) show single-walled sensilla with numerous pores. The multiporous trichoid sensilla were described in majority of investigated *Ips* species so far (Borden & Wood, 1966; Faucheux, 1989, 1994; Payne et al., 1973). The multiporous sensilla are expected to have an olfactory function (Andersson et al., 2009; Borden & Wood, 1966; Hallberg, 1982; Payne et al., 1973). The wall pores allow volatile molecules to penetrate the sensillar lumen to activate the olfactory receptor neurons. The olfactory function of multiporous sensilla has been confirmed by many electrophysiological investigations performed in *I. typographus* (Andersson et al., 2009; Kandasamy et al., 2019; Schiebe et al., 2019). Sensilla trichodea type IV (STrIV) observed in *I. duplicatus* does not match with any trichodea sensilla types reported previously. Because of its distinct structure and characteristic pore features on the wall surface, the probability of misclassification can be excluded. Further studies are needed to determine the physiology of these two olfactory trichoid sensilla.

The terminal pore trichoid sensilla (STrIII) in *I. duplicatus* corresponds with those described as terminal-pore sensillum by Hallberg (1982) in *I. typographus*. The presence of structural characteristics such as terminal pore and flexible socket suggests bimodal function in chemoreception and mechanoreception (Hallberg, 1982). STrIII observed in *I. duplicatus* corresponds with "TR3" in Shi, Zhang, Liu, Zhang, et al. (2021) in *I. typographus*, "Trichodea III" in *I. sexdentatus*, *I. typographus*, and *I. pini* (Faucheux, 1989, 1994) and in other *Ips* species (Payne et al., 1973) (Supplementary Table 2), and with the "sensilla chaetica" classified in *Ips paraconfusus* (Borden & Wood, 1966).

In our study on *I. duplicatus*, we did not see sensilla trichodea type I, which was observed previously at the base of the scape and pedicel of many *Ips* species (Payne et al., 1973; Shi, Zhang, Liu, Zhang, et al., 2021) and in *T. lineatum* (Moeck, 1968). Sensilla trichodea type I are Böhm's sensilla with proprioceptive function, though no histological information nor the characteristics of the socket are available.

4.3 | Sensilla basiconica

Our study provided evidence for four categories of sensilla basiconica in *I. duplicatus*. This category is also not morphologically homogenous. They are shorter and wider in comparison with sensilla chaetica and trichodea and form two distinct groups. Sensilla basiconica type I (SBI) are the most numerous type occupying about three-fourths of the total area of the antennal club surface. They are highly dense in the sensory bands A and B and represent multiporous sensilla with slit-like depressions suggesting their possible role in olfactory detection. SBI in *I. duplicatus* has features similar to single-walled sensillum type I reported by Hallberg, 1982 in *I. typographus*. The sensilla basiconica type II (SBII) is not described in Hallberg, 1982 in *I. typographus* but is mentioned by Faucheux (1989) in *I. sexdentatus* and *I. typographus*. SBII has lower pore density when compared to SBI. These two types of SB are known to be sensitive to general odors like plant compounds and pheromones validated by electrophysiological studies (Andersson et al., 2009; Biswas et al., 2023; Borden & Wood, 1966; Dickens et al., 1978). Sensilla basiconica type III and IV (SBIII and SBIV) are uniporous peg-shaped hairs with slight depressions on the wall surface present predominantly in the distal club region. Only SBIII was reported

previously in *I. typographus* (Shi, Zhang, Liu, Zhang, et al., 2021). The exact function of these pegs is not known; however, their uniporous nature indicates the contact chemoreception.

4.4 | Sensilla coeloconica

The sensilla coeloconica (SCo) has a distinct shape with longitudinal grooves on the wall surface and have previously been reported in many *Ips* species (Chen et al., 2010; Dickens et al., 1978; López et al., 2018; Whitehead, 1981). In most of the studied species so far, only one type of sensilla coeloconica has been described. Our study is the first to report two different types of sensilla coeloconica.

The cross-section performed on SCo in *I. typographus* (Hallberg, 1982) shows a "double-walled sensilla" with finger-like radial channels connecting the sensillar surface with neuronal sensory processes within the hair lumen. The "double-walled sensilla" are innervated by 2–6 sensory cells with unbranched sensory processes terminating in the apical part of the hair. Below the hair, one of the sensory processes exhibits a lamellar pattern like that of pore less sensilla in which thermoreception has been demonstrated (Altner, 1977). On the other hand, the "double-walled sensilla" of *I. typographus* have a similar structure as certain chemoreceptive sensilla (Altner, 1977; Altner et al., 1977). Thus, in different insect species, SCo may have various functions, such as hygroreception, thermo-hygroreception and olfactory reception. SCo in moths and flies are known to have an olfactory role and olfactory receptor neurons are tuned to compounds like acids, aliphatic aldehydes, amines, and ketones (De Bruyne & Baker, 2008; Pophof et al., 2005; Yao et al., 2005).

We observed the mirror arrangement of SCo on both antennal clubs, with SCo more frequently distributed on the lateral regions of the antennal clubs, indicating a potentially highly specific function. We found no previous literature reporting this kind of arrangement. Further investigation can explain this specific arrangement and modalities of SCo.

4.5 | Böhm's sensilla

BS are usually seen exclusively on the base of the scape and pedicel of the antenna in *I. duplicatus*, also reported as "böhm's bristles" in *I. subelongatus* (Shi, Zhang, Liu, Xu, et al., 2021) and as "böhm sensilla" in *I. typographus* (Shi, Zhang, Liu, Zhang, et al., 2021). In *Curculio nucum*, BS are present on the base of the scape and pedicel (Faucheux et al., 2019). Their location and distribution suggest a propioceptive role (Merivee et al., 1999). They possess a flexible deep socket and smooth wall surface. These are known to monitor the antennal positioning and movements during the flight (Dong et al., 2020).

4.6 | Surface pores

The SPs were about 0.5 um wide and were present on both sides of the club without any association with sensilla. These structures might be similar to the previously reported "glandular pores" in *I. sexdentatus* and *I. typographus* (Faucheux, 1989). However, the glandular pore diameters were not provided, and were associated with sensilla chaetica. Alternatively SP can represent "mechanosensory cuticle sensillum" reported on the antennal club of *I. typographus* (Hallberg, 1982). "Mechanosensory cuticle sensillum" terminates within a cavity of the cuticle with an approximate diameter of 2–2.5 um (Hallberg, 1982). Since *I. typographus* is significantly bigger in overall size than *I. duplicatus*, we can say that the dimensions of SP and "mechanosensory cuticle sensillum" are relatively similar. Alternatively, SP might be glands meant for secretion of the antennal epicuticular layer of the antennae and their sensilla (Bin et al., 1989; Dahms, 1984; Faucheux, 1994; Faucheux & Kundrata, 2015; Romani et al., 2019; Skilbeck & Anderson, 1994; Weiss et al., 2011).

5 | CONCLUSION

Ips duplicatus is a serious conifer pest that shares the same host and has similar biology as compared with *I. typographus*, which is a model bark beetle for studying olfaction. Numerous morphological and electrophysiological investigations have been conducted for *I. typographus*, but we have no information about the sensillar equipment including the typology and functions of different sensilla in *I. duplicatus*. The study addressed the research gap concerning the types of sensilla and their distribution and possible role in *I. duplicatus*. Our microscopic results revealed important information about the general morphology and the distribution of various functionally important sensilla in *I. duplicatus*. We found the sexual dimorphism in *I. duplicatus* is unrelated to the general antennal morphology and concerns only minor variations with the number and length of different sensilla types. This discrepancy in morphological properties can be associated with variation in the biophysical characteristics of different hair types, allowing them to be sensitive to different mechanical stimuli. We also provided comparative information on sensilla typology and its external characteristics in *Ips* species as an attempt to establish the general sensilla nomenclature for future studies in this genus. Overall, the present study provides a map of *I. duplicatus* olfactory equipment and establishes a basis for future olfaction-based and electrophysiological investigations of this destructive forest pest.

AUTHOR CONTRIBUTIONS

Mayuri Kashinath Shewale: Conceptualization; software; data curation; investigation; funding acquisition; writing – original draft; visualization; formal analysis; methodology; writing – review and editing. **Jana Nebesářová:** Formal analysis; validation; methodology; software. **Ewald Grosse-Wilde:** Supervision; writing – review and editing; validation. **Blanka Kalinová:** Funding acquisition; writing – review and editing; supervision; validation; conceptualization.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data of this study and the microscopic images are available from the corresponding author upon reasonable request.

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Paper III

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Manuscript

Comparative descriptive analysis of microscopic morphology and distribution of antennal sensilla in the pine bark beetle, *Ips acuminatus* and the larch bark beetle, *Ips cembrae* (Coleoptera: Curculionidae)

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Simple Summary: Bark beetles are small insects that depend on their antennae to detect odors from host trees, potential mates, and their environment. Two species of concern in European forests, the pine bark beetle (*Ips acuminatus*) and the larch bark beetle (*Ips cembrae*), have been linked to increasing forest damage. Despite their ecological and economic importance, little is known about how these species use their antennal structures to detect sensory cues. In this study, we used scanning electron microscopy to examine the antennae of both species, focusing on the structure and distribution of tiny, hair-like sensory organs known as sensilla. We identified five distinct sensillum types and described their location across the antennal surface. Subtle differences in the distribution and morphology of these structures were observed between the two species, and in some instances, between males and females. By better understanding these beetles' sensory systems, we lay the groundwork for future studies exploring olfactory function and provide morphological insights that could inform the development of environmentally sustainable pest control strategies.

Abstract: Bark beetles of the genus *Ips* rely heavily on olfactory cues for host selection, mate recognition, and orientation in complex environmental landscapes. Among them, *Ips acuminatus* and *Ips cembrae* are significant conifer pests in Europe; however, their antennal morphology and sensory architecture remain poorly documented. This study presents a comparative, descriptive analysis of the microscopic structure and spatial distribution of antennal sensilla in *I. acuminatus* and *I. cembrae* using scanning electron microscopy (SEM). Adult beetles were collected from naturally infested Scots pine (*Pinus sylvestris*) and European larch (*Larix decidua*), then sexed and examined using SEM. Five morphologically distinct sensillum types were identified in both species: sensilla chaetica, basiconica, trichodea, coeloconica, and Böhm's sensilla. These were distributed primarily across the antennal club, organized into three distinct sensory bands (A, B, and C). Although the overall sensilla diversity was conserved, minor interspecific and intersexual variations in sensillar morphology and spatial arrangement were noted. The findings provide a structural basis for studying olfactory-driven behaviors in *I. acuminatus* and *I. cembrae* and lay the groundwork for future electrophysiological studies. A

deeper knowledge of bark beetle antennal sensilla will contribute to more targeted pest management strategies by improving semiochemical-based monitoring and control methods.

Keywords: bark beetles; antennal sensilla; antennal club; sensory structures; insect olfaction; morphology; scanning electron microscopy; *Ips acuminatus*; *Ips cembrae*; conifer pests

1. Introduction

Bark beetles of the genus *Ips* (Coleoptera: Curculionidae) are ecologically and economically important forest pests, responsible for widespread damage to coniferous trees across Europe (Hulcr et al. 2015). Among these, the pine bark beetle (*Ips acuminatus*) and the larch bark beetle (*Ips cembrae*) are emerging concerns due to their expanding geographic distribution and increasing outbreak frequency. *I. acuminatus* is primarily associated with Scots pine (*Pinus sylvestris*), while *I. cembrae* typically infests European and Japanese larch (*Larix decidua* and *L. kaempferi*), but may also colonize Norway spruce (*Picea abies*) under favorable conditions (Pffefer,1955; Postner,1974). Although historically classified as secondary pests, both species have shown increasing potential to attack healthy hosts, particularly under climate-induced stress conditions such as heat and drought (Wermelinger, 2004; Netherer et al. 2021).

The success of bark beetles in locating suitable hosts and coordinating mass attacks is largely mediated through olfactory communication (Byers, 2007). Aggregation behavior is driven by pheromones released by pioneer males, which include components such as *S*-(−)-ipsenol, *S*-(+)-ipsdienol, and host-derived volatiles (Bakke, 1978; Francke et al. 1986),. These chemical cues enable beetles to overcome host defenses collectively and facilitate successful colonization(Byers, 2007). In addition, both species are associated with blue-stain fungi that may support beetle development and influence host tree mortality (Jankowiak et al. 2007, Kirisits, 2004).

The antennae of *Ips* species serve as their primary olfactory organs and are critical for detecting a wide range of chemical signals, including host volatiles, pheromones, and environmental cues. Most olfactory sensilla are localized on the antennal club, arranged in three distinct sensory bands (A, B, and C) along the anterior surface (Payne et al. 1973). These sensilla house olfactory sensory neurons (OSNs) that vary in morphology and function. Structurally, sensilla are categorized into types such as chaetica, trichodea, basiconica, coeloconica, and Böhm's sensilla, based on features like wall thickness, surface pores, and cuticular architecture (Schneider, 1964; Hallberg et al.1982). Single-walled sensilla (e.g., trichodea, basiconica) are often associated with pheromone detection, while double-walled sensilla (e.g., coeloconica) are implicated in sensing humidity, acids, and other environmental cues (Altner et al.1977; Hallberg et al. 1982).

Previous ultrastructural studies have described antennal sensilla in a number of *Ips* species, including *I. typographus*, *I. sexdentatus*, *I. duplicatus* and *I. pini* (Hallberg et al.1982; Shi et al. 2021; Shewale et al.2023; Faucheux et al. 1989, 1994). However, detailed comparative data on *I. acuminatus* and *I. cembrae* remain limited. Understanding the diversity, distribution, and structural features of their antennal sensilla is essential for interpreting species-specific olfactory capabilities and may inform the design of more effective semiochemical-based monitoring tools.

This study presents a qualitative, comparative analysis of antennal sensilla morphology and distribution in *I. acuminatus* and *I. cembrae*, using scanning electron microscopy (SEM). Specifically, we aimed to:

1. Describe the external morphology of the antennae in both species.
2. Identify and classify the types of sensilla present, based on their surface architecture and wall structure.
3. Map the distribution patterns of sensilla across the antennal club, particularly within the sensory bands A, B, and C.
4. Document any observed sex-specific or interspecific differences in sensilla type, number, or location.

By characterizing the antennal sensilla of these two bark beetle species, this study provides essential morphological data to support future electrophysiological investigations and advances our understanding of olfactory specialization in conifer-infesting bark beetles.

2. Materials and Methods

2.1. Study Organisms and Sample Collection:

Logs of European larch (*Larix decidua*) infested with *Ips cembrae* and Scots pine (*Pinus sylvestris*) infested with *Ips acuminatus* were collected in late spring 2024 from the Rouchovany region, Czech Republic. The logs were transported to the Faculty of Forestry and Wood Sciences, Czech University of Life Sciences, Prague, where they were placed in controlled rearing chambers. Following adult emergence, the logs were debarked, and adult beetles of both species were collected.

Sex identification was conducted using morphological characteristics of the second and third elytral spines, following criteria established by Pfeffer (1955) and Zhang and Niemeyer (1992). The beetles were stored at 4°C in sterile plastic containers until examination. For scanning electron microscopy (SEM), five males and five females were randomly selected from each species.

2.2. Sample Preparation and Scanning Electron Microscopy:

Prior to dissection, adult beetles were cleaned using a gentle air blower to remove external contaminants. Antennae were carefully dissected under a NIKON optical microscope (Japan) and processed using the protocol outlined by Shewale et al. (2023). The samples were fixed in 2.5% glutaraldehyde in 0.5 M cacodylate buffer (pH 7.2) for 24 hours, followed by post-fixation in 2% osmium tetroxide in the same buffer. Specimens were then dehydrated through a graded ethanol series, with each step lasting 30 seconds, and dried using a Bal-Tec CPD 030 critical point dryer.

Dried antennae were sputter-coated with a 2 nm layer of gold using a Bal-Tec SCD 050 ion sputter coater. Imaging was carried out using a JEOL JSM-IT200 scanning electron microscope and a JEOL IT800 high-resolution SEM. Micrographs were taken at accelerating voltages of 3, 5, 10, and 15 kV with a working distance of 5 mm. Antennal structures, including overall morphology and sensillar types, were examined from five antennae per sex per species.

2.3. Sensilla Identification and Categorization

Sensilla were identified and categorized according to the criteria established by Schneider (1964), Nowińska and Brożek (2017), and Shewale et al. (2023). Classification was based on external morphology, including overall shape, length, base width, wall structure (single- or double-walled), surface porosity, and socket flexibility (flexible or inflexible attachment to the cuticle). General antennal terminology followed Hulcr et al. (2015).

2.4. Image Analysis

All figures and image-based measurements were generated using ImageJ software (version 1.53q; Schneider et al., 2012). No statistical analysis was conducted, as the study was purely qualitative in nature and aimed at structural characterization rather than hypothesis testing.

3. Results

This study presents the first comparative morphological account of antennal sensilla in *Ips cembrae* and *Ips acuminatus*, two conifer-associated bark beetles of increasing ecological importance in European forests. Using scanning electron microscopy (SEM), we examined the antennal club of both species, focusing on the types and spatial distribution of sensilla. The findings indicate that the general antennal architecture is conserved across the genus *Ips*, aligning with earlier studies in species such as *I. typographus* and *I. duplicatus*.

3.1. General Antennal Morphology

Both *I. cembrae* and *I. acuminatus* possess the typical scolytine antennal structure, composed of four major segments: the scape, pedicel, funiculus, and the terminal club. The antennal club, serving as the principal olfactory organ, is structurally organized into three ventral sensory bands designated A, B, and C. This pattern is consistent with previously documented arrangements in related *Ips* species (Hallberg, 1982a; Shewale et al., 2023).

The majority of sensilla were localized on the anterior (ventral) surface of the antennal club and followed a distribution pattern conforming to the boundaries of the three sensory bands. SEM micrographs (Figs. 1 and 2) provided high-resolution views of the sensillar arrangement and surface morphology, revealing structured groupings across the club. These initial visualizations serve as a morphological reference point for future functional studies. Due to the current qualitative scope of the study, evaluation of sexual dimorphism in sensilla traits was not conducted.

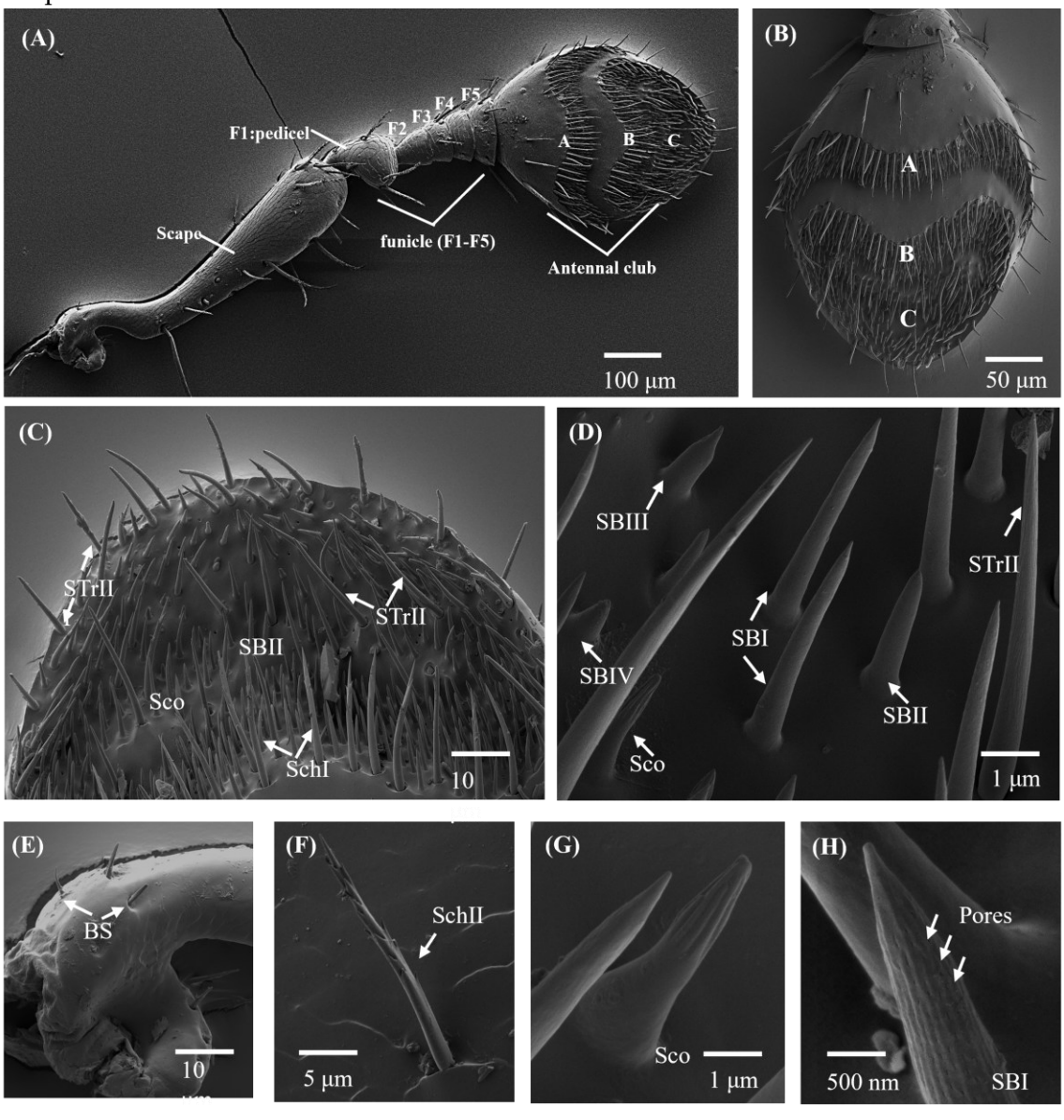

Figure 1. Scanning electron micrographs of the *Ips cembrae* antennal club. **(A)** General view of the antennal club showing its overall morphology. **(B)** Ventral surface of the club indicating the three distinct sensory bands (A–C), with representative sensilla types labeled. **(C)** Higher magnification of sensilla trichodea subtypes STrIII and STrIV. **(D)** Sensilla basiconica, including subtypes SBI, SBII, SBIII, and SBIV. **(E)** Böhm's sensilla (BS) located at the articulation between scape and pedicel. **(F)** Sensilla chaetica, showing both SchI and SchII subtypes. **(G)** Sensilla coeloconica (SCo) with characteristic peg-in-pit morphology. **(H)** Detail of wall pores observed on sensilla basiconica subtype I (SBI).

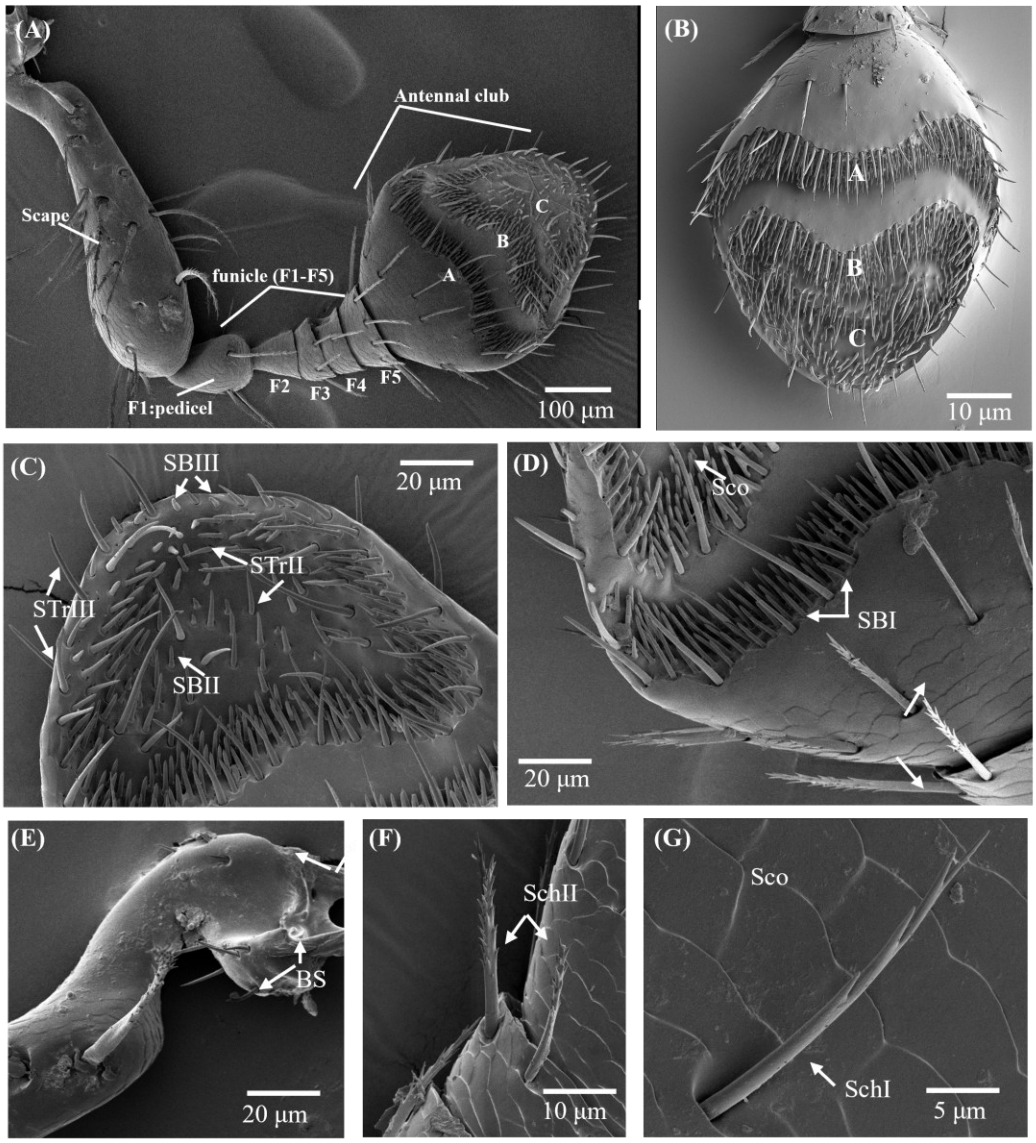

Figure 2. Scanning electron micrographs of the *Ips acuminatus* antennal club. **(A)** Overview of the antennal club showing general structure and segmentation. **(B)** Ventral surface of the club illustrating sensory bands A–C with representative sensilla types labeled. **(C–D)** Surface topography and distribution of key olfactory sensilla, including sensilla trichodea and sensilla basiconica. **(E)** Böhm's sensilla (BS) located near the base of the antenna at the scape–pedicel junction. **(F)** Sensilla chaetica (SchI and SchII) along the club margins. **(G)** Sensilla coeloconica (SCo) with distinct peg-in-pit morphology.

3.2. **Classification of Sensilla Types**

Five major types of antennal sensilla were identified in both species: sensilla chaetica (SCh), sensilla basiconica (SB), sensilla trichodea (STr), sensilla coeloconica (SCo), and Böhm's sensilla (BS). These were categorized based on external morphology, wall structure, and apparent function, in accordance with established taxonomic criteria (Schneider, 1964; Hulcr et al., 2015; Shewale et al., 2023).

- **Sensilla chaetica (SCh):**
  These long, uniporous, hair-like structures were predominantly located along the periphery and lateral margins of the antennal club. Two subtypes were distinguished based on the presence or absence of lateral branching. Their morphology is consistent with a mechanosensory role.
- **Sensilla basiconica (SB):**
  Identified as short, thick, multiporous sensilla, sensilla basiconica exhibited four distinct morphological subtypes. These were concentrated primarily within sensory bands A and B, although the most abundant subtype (SBI) was distributed across all three bands (A, B, and C). Their porous surface and central positioning suggest an important role in olfactory detection.
- **Sensilla trichodea (STr):**
  Slender, hair-like, and porous, sensilla trichodea were observed mainly in sensory band C. Three subtypes were recognized based on variations in length and curvature. Their structure supports a role in pheromone detection.
- **Sensilla coeloconica (SCo):**
  Characterized by a peg-in-pit morphology, these sensilla were sparsely distributed and present in low numbers across the antennal surface. Their typical morphology suggests specialization in detecting environmental cues such as humidity or acidic volatiles.
- **Böhm's sensilla (BS):**
  These short, spine-like structures were located at the articulation between the scape and pedicel. Their rigid morphology and position are consistent with a mechanosensory function related to antennal movement and positioning.

A summary of sensilla types and key morphological characteristics is provided in Table 1. Representative SEM images further illustrate these sensilla across the antennal surface.

Table 1: Morphological characteristics and distribution of sensilla types on the antennae of *Ips acuminatus and I. cembrae.*

Sensilla type	Distribution	Pores	Wall structure	Tip	Shape	Socket
SchI	Antennal club (A, B and C), funicular segments (F1-F5) and scape	Aporous	Longitudinal grooved wall, bilateral branching	Sharp	Straight	Flexible
SChII	Antennal club (A), funicular segments (F1-F5) and scape	Aporous	Longitudinal grooved wall, multi-branching	Sharp	Curved	Flexible
SBI	Antennal club (A, B, C)	Multiporous	Pitted	Blunt	Straight	Inflexible
SBII	Antennal club (A, B and C)	Multiporous	Grooved	Blunt	Straight	Inflexible
SBIII	Antennal club (B and C)	Uniporous	Smooth	Blunt and round	Peg shaped	Inflexible
SBIV	Antennal club I	Uniporous	Smooth	Round	Straight	Inflexible
STrIII	Antennal club (A, B and C)	Terminal pore	Smooth	Blunt	Long and curved	Flexible
Sco	Antennal club (A, B and C)	Aporous	Grooved	Round	Cone-shaped	Inflexible
BB	Scape	Aporous	Smooth	Blunt and round	Short and straight	Flexible
SP?	Club (A, B and C), funicle segments (F1-F5) and scape	?	Pit on the club surface	-	Oval	-

3.3. Observational summary and future directions

This qualitative investigation establishes a foundational antennal sensilla map for *I. cembrae* and *I. acuminatus*, contributing essential baseline data for upcoming single sensillum recording (SSR) studies. The conserved sensilla types and consistent sensory band arrangements observed in both species support the hypothesis that antennal morphology is a shared and evolutionarily stable trait within the genus *Ips*.

While the current analysis focuses exclusively on descriptive traits, future work will expand into quantitative morphometrics, including sensillum dimensions, socket architecture, and potential sex-specific differences. These results will be detailed in a separate manuscript. The structural information provided here enables a better understanding of how bark beetles have adapted olfactory organs in relation to host ecology and offers morphological insights relevant for the development of species-specific pest monitoring strategies.

4. Discussion

This study provides the first comparative morphological assessment of antennal sensilla in *Ips acuminatus* and *Ips cembrae*, two conifer-associated bark beetles of growing importance in European forests. Our scanning electron microscopy (SEM) analysis revealed that both species share a highly conserved antennal architecture, consistent with previous observations in related *Ips* species such as *I. typographus* and *I. sexdentatus* (Hallberg, 1982; Faucheux, 1989). The club-shaped terminal segment in both *I. acuminatus* and *I. cembrae* houses sensilla arranged within three well-defined sensory bands (A, B, and C), a trait considered diagnostic for the genus.

Across both species, we identified five major types of sensilla: chaetica, basiconica, trichodea, coeloconica, and Böhm's sensilla, all of which have been previously reported in other scolytine bark beetles. Despite this general conservation, our observations also indicate interspecific differences in sensillar subtype diversity and distribution patterns, which may reflect ecological specialization or divergence in olfactory function.

4.1 Conserved Antennal Organization with Subtle Differences

The presence and arrangement of sensilla across the antennal surface were largely similar between *I. acuminatus* and *I. cembrae*. In both species, sensilla were densely localized within sensory bands A and B, with band C showing more restricted types such as trichodea. This organization parallels the sensory band pattern seen in *I. typographus* and *I. duplicatus* (Hallberg, 1982; Shewale et al. 2023), suggesting that the peripheral olfactory system in these beetles is evolutionarily stable.

However, minor differences in sensilla subtypes were evident. For example, *I. cembrae* displayed more frequent lateral branching in sensilla chaetica, which could suggest an enhanced mechanosensory function. Similarly, the density and spatial distribution of sensilla basiconica subtypes appeared slightly more uniform in *I. acuminatus* than in *I. cembrae*, although this observation remains qualitative.

This study demonstrates that the peripheral olfactory system in *I. acuminatus* and *I. cembrae* is built upon a structurally conserved antennal basis, with consistent sensilla organization across species. However, subtle morphological differences, such as distinct sensilla subtypes and potentially species-specific olfactory sensory neuron (OSN) arrangements, may reflect adaptations linked to ecological specialization.

4.2 Functional Implications of Sensilla Types

The chaetica sensilla, concentrated along the margins of the antennal club, are likely mechanosensory, assisting in antennal orientation and contact-based navigation. The presence of multibranched subtypes in both species may indicate a conserved role across sexes or potentially a subtle dimorphism that requires further morphometric

investigation. Similar patterns of sexual dimorphism in chaetica sensilla have been reported in *I. sexdentatus* and *T. lineatum* (Moeck, 1968; Faucheux, 1989), often linked to mating or oviposition behavior.

Basiconica sensilla, especially subtype SBI, were the most numerous in both *I. acuminatus* and *I. cembrae*, forming dense clusters in sensory bands A and B. These sensilla are known to contain multiple olfactory sensory neurons and are thought to detect host volatiles and pheromonal cues, as demonstrated electrophysiologically in *I. typographus* (Andersson et al., 2009; Kandasamy et al., 2019, 2023). Their prominence in both species underscores their likely central role in mediating host selection and aggregation behavior.

Trichodea sensilla, primarily located in band C, exhibited three distinct morphological subtypes in both species. Their porous structure indicates an olfactory function, likely tuned to long-range semiochemicals such as sex or aggregation pheromones. Although similar subtypes have been reported in other bark beetles, one particularly elongated variant was more pronounced in *I. acuminatus*, which may reflect differences in communication or host detection strategies.

Coeloconica sensilla, though less abundant, were present in both species and followed a peg-in-pit morphology typical of thermo- and hygroreceptors (Altner et al., 1977; Hallberg, 1982). Their sparse distribution suggests a specialized function, perhaps for detecting microclimatic conditions or volatile cues such as ketones or aldehydes, known to be relevant in host discrimination.

Böhm's sensilla, located at the base of the scape and pedicel, were consistently observed in both species. Their small size and fixed socket indicate a proprioceptive function, likely involved in monitoring antennal position during host exploration or flight behavior (Merivee et al., 1999).

4.3 Evolutionary and Ecological Considerations

The general pattern of sensilla types and their distribution observed in *I. acuminatus* and *I. cembrae* reflects the genus-wide conservation of antennal design. However, the observed morphological nuances such as branching in chaetica sensilla or subtype richness in basiconica and trichodea highlight how structural adaptations may fine-tune olfactory systems to meet species-specific ecological demands.

These findings support the hypothesis that bark beetle olfactory systems balance structural conservation with adaptive plasticity, allowing different species to respond to distinct chemical environments while maintaining core functions. Comparative studies across additional *Ips* species, particularly with functional data such as single sensillum recordings (SSR), will be essential for mapping specific OSN classes to sensilla subtypes and determining their behavioral roles.

5. Conclusion

This study provides the first detailed comparative account of antennal sensilla morphology and distribution in *Ips acuminatus* and *Ips cembrae*, two ecologically important bark beetle species associated with conifer hosts in European forests. Using scanning electron microscopy, we identified five principal sensilla types—chaetica, basiconica, trichodea, coeloconica, and Böhm's sensilla distributed across three distinct sensory bands on the antennal club. While the overall antennal architecture was conserved in both species, subtle

differences in sensilla subtype diversity and spatial arrangement suggest species-specific adaptations related to their ecological niches.

These qualitative findings highlight a structurally stable peripheral olfactory system across the genus *Ips*, with microstructural variation likely supporting functional divergence in odor detection. The antennal sensilla maps generated here provide essential morphological groundwork for future electrophysiological studies aimed at characterizing olfactory sensory neuron (OSN) responses to pheromones, host volatiles, and environmental cues. By enhancing our understanding of antennal sensilla organization, this research contributes to broader efforts in bark beetle sensory biology and supports the development of more targeted, species-specific semiochemical-based pest management strategies.

6. Data availability statement

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

7. Author contributions

MKS: Data curation, Formal analysis, Investigation, Visualization, Validation, Writing—original draft.JD: Methodology, Writing—review & editing. JS: Methodology, Writing—review & editing. JN: Methodology, Supervision. MH: Methodology, Supervision. AJ: Conceptualization, Methodology, Supervision, Validation, Writing—review & editing.

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10. 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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Paper IV

Moliterno, A. A. C. †, **Shewale, M.K.** †, Basile, S.,Synek, J., Jirošová, A.(2025) Size- and dose-dependent behavioral responses to 1,8-cineole and (+)-isopinocamphone: a potential host selection strategy in female Ips typographus L. Submitted to *Annals of Forest Science.* under revision.

†Equal contribution as first author

Title of the article

Size- and dose-dependent behavioral responses to 1,8-cineole and (+)-isopinocamphone: a potential host selection strategy in female *Ips typographus* L.

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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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- **Availability of data and material (data transparency)** Mandatory

The additional data is uploaded as Appendix material as filename: Data_file S1 (sheet A to F)
The raw data is available in**:** http://datadryad.org/stash/share/0VDe7mjUBYWuyw3_XG-NkWnytjtm-T58Z5kwwKKXfV8

- **Code availability (software application or custom code)**

The code and scripts used for this article can be found online at Dryad repository:
URL: http://datadryad.org/share/eMlJtgCcS32RG6YXPuE8Bhnm0CdgOpg3uX2hJlbpiks.

- **Conflicts of interest/Competing interests**

The authors declare no competing interests.

- **Ethics approval (include appropriate approvals or waivers)**

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 to participate (include appropriate statements)**

Not applicable

- **Consent for publication (include appropriate statements)**

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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.

Key message

This study revealed differing behavioral and antennal responses between large and small female *I. typographus* to two bioactive oxygenated monoterpenes, (+)-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 having 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 response to potential host selection decisive volatiles, which can impact reproductive success and bark beetle population dynamics.

Abstract

Context:

Ips typographus, a major pest of Norway spruce (*Picea abies*) 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 was compared. Female *Ips typographus* were sorted based on body length, 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 detection, and had proportionally larger antennal clubs than smaller females. In contrast, smaller females were less repelled by 1,8-cineole added to pheromone but, in contradiction, antennally detected it more strongly than larger females despite having smaller antennal clubs.

Conclusion: The total body length significantly influences 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 detection but also central nervous processing of olfactory signals driving 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 morphology and olfactory cues, with implications for tree–bark beetle interactions.

Keywords: bark beetle, olfaction, pheromone, oxygenated spruce monoterpenes, phenotypic variations, host choice

1. Introduction

The Eurasian spruce bark beetle, *Ips typographus* L. 1758 (Coleoptera: Curculionidae), is a major pest associated with the Norway spruce (*Picea abies*) 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 pheromones (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 host-emitted volatile organic compounds.

Norway spruce releases a range of monoterpenes, including highly abundant compounds such as α-pinene, β-pinene, β-phellandrene, and limonene (Netherer et al. 2021). These compounds have been tested to enhance the attraction of *I. typographus* (Erbilgin et al. 2007*;* Hulcr et al. 2006). 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 monoterpenes, with a few exceptions such as estragole and styrene, which have phenolic character. Oxygenated monoterpenes 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 naturally by air exposure 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 at 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 molecule) 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 and Vite 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 (Frühbort et al. 2023). In parallel, beetle-exosymbiotic ophiostomatoid fungi, which are inoculated into trees by boring beetles during colonization, also metabolize monoterpenes to their oxidative forms (Kandasamy et al. 2023). On the other hand, some oxygenated monoterpenes, 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 pheromones, 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 monoterpenes, 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; Andersson et al. 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 monoterpenes. 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 (Frühbort 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 *Grossmania 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 pheromones 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 more 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 (Muller 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 monoterpenes, 1,8-cineole and (+)-isopinocamphone, tested in combination with aggregation pheromones in a trap-capturing experiment. Additionally, we investigate if their antennae exhibit size-dependent differences in sensitivity to these compounds using electroantennographic analysis, and whether the antennal club shape differs 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 behavioural and electrophysiological responses to the two oxygenated spruce monoterpenes with contrasting ecological roles: 1,8-cineole, which inhibits attraction to unsuitable trees, and (+)-isopinocamphone, which 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 morphological differences in the antennal clubs."

The ecological impact of these size-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) analysis 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 meters 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 2021 experiments, traps were set up approximately 30 meters from the forest edge in a two-

year-old clearing. They were arranged in a row, with a minimum distance of 15 meters between each trap, and were installed on wooden poles 1.5 meters above the ground.

In 2019, seven cross-vane Ecotraps (Fytofarm, Slovak Republic) were used for the 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 pheromone. One trap was baited with pheromone alone and served as a control (for baits 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 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 sorting, and measurement.

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

Chemical	Source	Purity (%)	Dose	Nominal	Field 2019 ±SEM (n=3) †	Field 2022 ±SEM(n=3) †	Dispenser design
1,8-cineole	Sigma-Aldrich	98	L M H	0.1 1 10	0.1 ± 0.04 0.84± 0.09 6.03± 4.78	0.1 ± 0.01 0.92 ±0.12 5.7 ± 6.7	Kartell 731 without hole plus 1 mL of paraffin oil; Glass vial of 2mL, lid hole by syringe (1mm); Kartell 730, lid hole by syringe (2mm)
(+)-isopinocamphone	*	99	L M H	0.1 1 10	0.61 ±0.18 1.95 ±0.41 7.82 ±1.63	0.40±0.11 1.87±0.67 8.21 ±1.41	Foil sachet: hole by syringe (1mm); Kartell 730 without hole; 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): 1mm 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.

2.3. Source and selection of beetles used for body length and antennal size measurement and electroantennographic detection analysis

For further measurement, fifty 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 2021. Selected beetles were dried on tissue paper at 25°C for two hours, 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 x 28 cm) collected in Kostelec nad Černými Lesy from June to July 2024 were used. The beetles were collected 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 millimetres as demonstrated 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 30X magnification. Measurements were taken by the same researcher to ensure consistency, with recorded sizes ranging ranged from 4.2 to 5.3 mm millimetres. Based on this size range, two individuals were classified into two distinct size categories were established for further analysis. Female specimens selected for antennal club measurements and electroantennography were divided into:

1. Large-sized females: Body length ≥ 4.80 mm (n = 30)
2. Small-sized females: Body length ≤ 4.70 mm (n = 30)

To measure the antennal club measurements and electroantennography, 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 micrometres.

2.5. Electroantennographic (EAG) analysis

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 minute 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 minutes. 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) analysis 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-second pulse into the airstream directed at the antennal preparation, ensuring brief and consistent exposure. Control and odor stimuli were presented sequentially, with a one-minute 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-second 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 µg to 1000 µg) 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 sensitivity to each compound.

2.6. Statistical Analysis

We tested normality within each treatment group from 2019 or 2022 using the Shapiro-Wilk test, and homogeneity of variances was assessed using Levene's test. The raw data (total body length of adult female *I. typographus*) were exponentially transformed (Manly 1976), adjusting the assumption toward normality and equal variance. One-way ANOVAs were conducted separately for each year to assess whether insect body size differed significantly among treatment groups. Each ANOVA was followed by Tukey's HSD test for post hoc comparisons, controlling the experiment-wise error rate. The Pearson's Chi-squared test with Yates' continuity correction was applied to check whether female proportion diverge regardless the dosage tested (Zar 2014).

The data obtained from small and large female *I. typographus* were compared using the Wilcoxon signed rank test (Harris and Hardin 2013; Hothorn et al. 2022). The two-sample 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 chosen test deal with non-normality assumption as described before, but also paired-samples (the emerged adults insects collected from the same Norway spruce logs) and repeated measurement from antennal club length and width. 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, 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 indicated isometric relationships, while deviations from <1 indicated allometry (Jolicoeur 1990; Warton et al. 2006). To evaluate the dose-response in electroantennography (EAG) analysis between large (n = 10) and small females (n = 10), the Wilcoxon signed rank test for repeated measurements was applied. All statistical analyses were performed using RStudio version 4.1.1 (Core R Team 2015), with a significance level (alpha) of 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 (supplementary material, figure 1A and table 1A). 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, refer supplementary Table 1E and 1F for more details).

Table 2. Pearson's Chi-squared test with Yates' continuity correction comparing male and female *Ips typographus* catches for two compounds, (+)-isopinocamphone ((+)-IPC) and 1,8-cineole across different dose levels and years.

Group	Absolute catches per females and compound		(+)-IPC vs 1,8-cineole		
	(+)-IPC females/total catches of beetles	1,8-Cineole females/total catches of beetles	Chi-sq	df	p-value
2019 - Low	1267/1508	892/1059	0.008	1	0.9285
2019 - Medium	2329/2875	1682/2103	0.755	1	0.3848
2019 - High	2031/2477	294/370	1.217	1	0.2699
2022 - Low	1052/1267	482/651	21.152	1	<0.001**
2022 - Medium	1125/1406	389/519	5.488	1	0.0191*
2022 - High	1495/1917	183/258	6.028	1	0.0141*

Data represents absolute beetle catches pooled from the respective number of trap rotations per year (2019: 7 rotations; 2022: 8 rotations). Chi-squared values indicate the results of contingency tests comparing female catches across treatments. Df represents degrees of freedom. p-values indicate the significance level of the observed differences between male respectively female catch proportions for respective compound and dose, with significance considered at $p < 0.05$ (*), $p < 0.001$ (**). Refer supplementary Table 1G for more details.

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 contrast, females captured in traps baited with a high dose of 1,8-cineole were smaller, with an average body length of 4.69 mm (SD = 0.26), ($F = 3.15$, p = 0.026) in 2019 and 4.69 mm (SD = 0.23), ($F = 9.59$, p < 0.001) in 2022 (Fig. 1A). For (+)-isopinocamphone, trap catches showed significant differences based on dose. 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) ($F = 3.03$, p = 0.03), while in 2022, the average was 4.75 mm (SD = 0.23) ($F = 2.95$, p = 0.03). Conversely, traps baited with a high dose of (+)-isopinocamphone attracted larger females, with average body lengths of 4.88 mm (SD = 0.19) in 2019 and 4.86 mm (SD = 0.20) in 2022 (Fig. 1B). Detailed data and statistical analyses, including results from ANOVA followed by Tukey's HSD test (supplementary material, Table 1B) and visualized in Fig. 1.

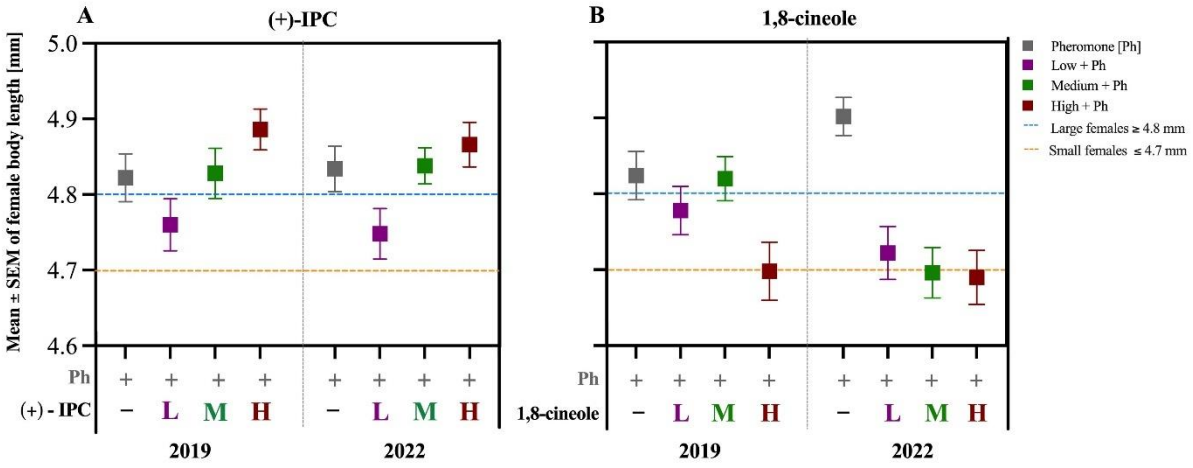

Figure 1. Mean body size of female *I. typographus* captured in response to three doses (low, medium, high) of (A) 1,8-cineole and (B) (+)-IPC is (+)-isopinocamphone, along with a control (pheromone only = Ph), in 2019 and 2022. Colors represent different doses or the control, with a sample size of n = 50 per group. Vertical bars show the standard error of the mean, and (*) indicates significant differences between groups based on Tukey's HSD test (p = 0.05).

3.2. Total body length, antennal club size of large and small females is isometric to body size.

The Wilcoxon signed rank for dependent sample analysis showed total body size (V= 465, p< 0.001), length, antennal club in length (V= 416, p< 0.001), and width (V= 431, p< 0.001) measurements differed significantly between large versus small females (Table 3).

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* was defined as large ≥ 4.80 mm (n = 30) versus small ≤ 4.70 mm (n = 30) and its respective antennal club measurements.

The standardized major axis (SMA) regression focusing on the correlation between length and width indicated significant and positive correlations (Large= R^2= 0.43, p ≤ 0.001) and (Small= R^2= 0.32, p= 0.001). The relationship between antennal club length and width log-transformed indicated that both were scaled isometrically, with slopes close to 1 (Large: 0.99, Small: 1.0)

(supplementary material, Table 1C). This suggests a proportional relationship between length and width in both groups, where the two variables increase at similar rates (Fig. 2).

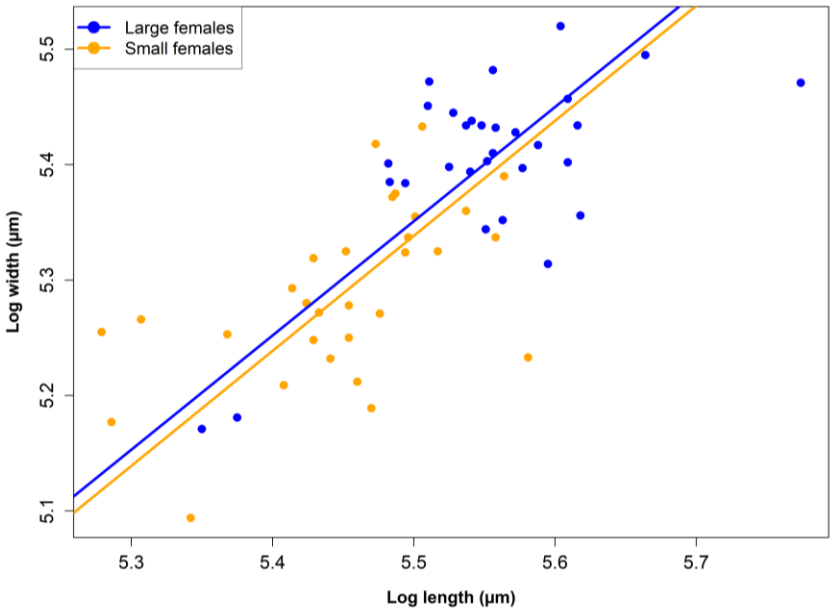

Figure 2. **Standardized Major Axis (SMA) regression analysis representing positive trend in log (ln) length and width of categorized as "large females ≥ 4.80 mm" (*n*= 30) and "small females ≤ 4.70 mm" (*n*= 30) from antennal club of females of *I. typographus*.** Each colour represents the measurements 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.

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

EAG responses to the pheromone blend (MB:cV/ 10:1) did not differ significantly between large (n = 10) and small (n = 10) females (Exact Wilcoxon Rank Sum Test, Fig. 3A). Large females showed significantly stronger responses to four higher doses of (+)-isopinocamphone (1 µg to 1000 µg; log doses 0 to 3) (V= 48, p = 0.048; Fig. 3B). Conversely, small females exhibited significantly stronger EAG responses to three higher doses of 1,8-cineole (10 µg, 100 µg, 1000 µg; log doses 1 to 3) compared to large females (V= 7, p = 0.037; Fig. 3C). Additional details are provided in supplementary material (Table 1D).

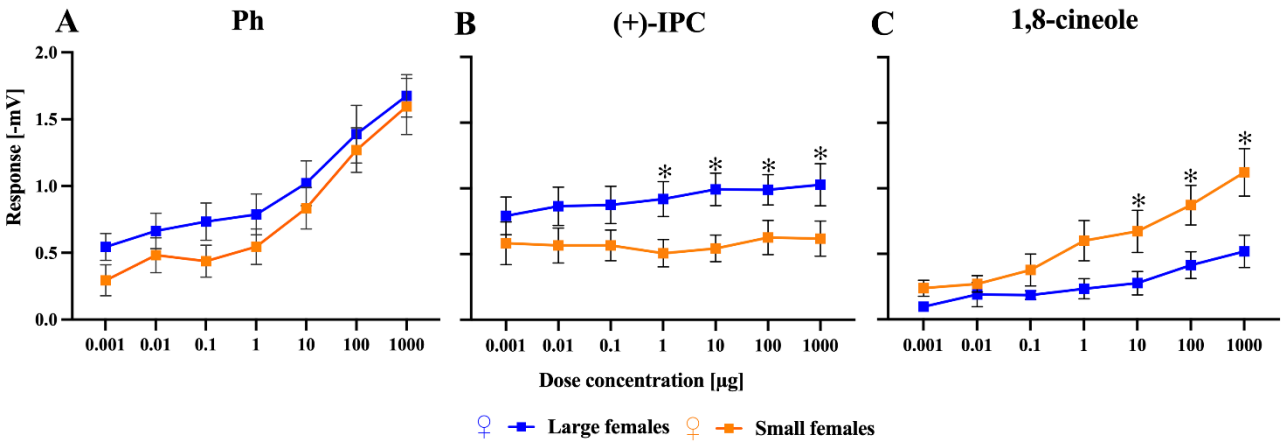

Figure 3. Dose–response curves based on electroantennographic (EAG) responses in female *Ips typographus*, categorized by body size: "large" (≥ 4.80 mm, *n* = 10) and "small" (≤ 4.70 mm, *n* = 10). Responses are shown for (A) Pheromone, (B) 1,8-cineole, and (C) (+)-isopinocamphone. Each compound was tested across a concentration range of 0.001 µg to 1000 µg, 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 size groups at individual doses, based on the Exact Wilcoxon Rank Sum Test ($p < 0.05$).

4. Discussion

Our findings demonstrate that large and small female *I. typographus* respond differently to two ecologically relevant oxygenated spruce monoterpenes, 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.

4.1. 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 females were significantly more attracted to high doses of (+)-isopinocamphone, a compound known to synergize pheromone attraction, when it was presented alongside the aggregation pheromone. This behavioral pattern was supported by electroantennography (EAG) analyses, which showed that larger females exhibited stronger olfactory responses to high doses of (+)-isopinocamphone compared to smaller females. Morphometric analysis further revealed that larger females possess proportionally broader and

longer antennal clubs. This increased antennal surface area likely improves their odour detection capabilities. Across various insect taxa, a correlation between antennal size and odour 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 monoterpenes 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 monoterpenes, 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 size, 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 to treatments, even the mean size of all females caught to pheromone-only was the same in both years. We attribute a shift in the proportion of large females 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 favouring 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.

4.2. The unexpectedly heightened antennal sensitivity of smaller *Ips typographus* females to 1,8-cineole contrasts with their higher behavioral attraction to this anti-attractant

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 to pheromone (Moliterno et al. 2024), a size-dependent pattern in females was observed. Specifically, we observed a higher proportion of smaller females 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 possible explanation for increased antennal sensitivity is that cineole-sensitive olfactory sensory neurons (OSNs) are co-localized with pheromone (*cis*-verbenol)-sensitive neurons, which suppress pheromone detection at high doses of 1,8-cineole (Andersson et al. 2010). Another explanation is that, while differences in peripheral sensitivity at the antennal surface between small and large females are influenced by antennal morphology, 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 internal 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, less competitive during outbreaks, may tolerate trees with high 1,8-cineole levels as a form of 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.

4.3. 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 synthetic oxygenated monoterpenes 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 weren't 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 and 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 to 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, as well as size-dependent behavior and detection abilities in males, is needed.

5. Conclusion

Our study demonstrates how body size 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 morphology, olfactory sensitivity, and host-selection behavior. The de novo spruce-derived oxygenated monoterpene 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 and, 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 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 males.

Supplementary data

The additional data is uploaded as Supplementary material, and available via Dryad Digital Repository: https://doi.org/10.5061/dryad.rxwdbrvn1

URL: http://datadryad.org/share/eMlJtgCcS32RG6YXPuE8Bhnm0CdgOpg3uX2hJlbpiks.

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Paper V

Shewale, M. K., Bláha, J., Synek, J., Schebeck, M., Andersson, M. N., Kandasamy, D., & Jirošová, A. (2025) Comparative analysis of olfactory sensory neurons in two Ips species reveals conserved and species-specific olfactory adaptations. *Frontiers in Forests and Global Change*, *8*, 1588866. doi: 10.3389/ffgc.2025.1588866

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Comparative analysis of olfactory sensory neurons in two *Ips* species reveals conserved and species-specific olfactory adaptations

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Introduction: Bark beetles spend most of their lives under the bark of trees, with some species being economically significant pests that cause widespread tree mortality. Their behavior is primarily driven by olfactory signals, with aggregation pheromones playing a prominent role alongside volatiles from hosts, non-host trees and associated microbes. These signals are detected by olfactory sensory neurons (OSNs) housed in hair-like sensilla on the antennae. In this study, we focused on two *Ips* species with distinct host preferences: *Ips acuminatus*, which infests pine species, and *Ips cembrae*, which primarily attacks European larch. To better understand species-specific adaptations and shared olfactory mechanisms, we compared their olfactory responses with those of *Ips typographus*, a major pest of Norway spruce. By investigating the frequency, specificity, and antennal distribution of various OSN classes, we aimed to uncover both conserved and different olfactory mechanisms across *Ips* species with different host associations.

Methods: We conducted single sensillum recordings (SSR) to examine OSN responses in the antennal olfactory sensilla of *I. acuminatus* and *I. cembrae*. The responses were compared to existing data from *I. typographus* to identify potential species-specific adaptations and conserved olfactory mechanisms. A panel of 57 ecologically relevant odorants was tested, comprising interspecific and intraspecific pheromones, along with compounds associated with host- and non-host trees, as well as symbiotic fungi.

Results and discussion: Based on their response profiles, we identified nineteen OSN classes in both *I. acuminatus* and *I. cembrae*. A few selected OSN classes were further analyzed using dose–response tests to assess their specificity and sensitivity. Three OSN classes in *I. acuminatus* and four in *I. cembrae* were specific to host-related compounds. Two OSN classes responded to non-host volatiles, while one OSN class exhibited strong responses to microbial volatiles in both species. Several OSN classes specific to pheromone compounds, non-host and microbial volatiles showed similar response profiles in both *I. acuminatus* and *I. cembrae* as well as in OSN classes previously reported in *I. typographus*, potentially reflecting close phylogenetic relationships and shared ecological traits among these species.

KEYWORDS

electrophysiology, olfactory sensory neurons, bark beetles, aggregation pheromones, monoterpenes, semiochemicals

1 Introduction

Abiotic disturbances in forests are becoming more frequent and severe due to climate change, leading to increased bark beetle infestations (Jaime et al., 2024). Rising temperatures and prolonged droughts amplify disturbances such as wildfires or windthrow, creating more favorable conditions for bark beetles (Allen et al., 2015; Jakoby et al., 2019; Senf et al., 2018). Warmer climates accelerate beetle development and reproduction, resulting in outbreaks with significant ecological and economic impacts (Biedermann et al., 2019; Dobor et al., 2020; Sommerfeld et al., 2021; Hlásny et al., 2021). The increasing frequency of droughts and extreme weather events will further impact forests, making them more vulnerable to infestations (Netherer et al., 2024). Bark beetles (Coleoptera: Curculionidae: Scolytinae) comprise more than 6,000 species worldwide (Hulcr et al., 2015; Knížek and Beaver, 2007), including some of the most destructive conifer pests, particularly in the northern hemisphere. Bark beetles spend most of their life cycle under the tree bark, where many species feed and develop in the phloem. Many bark beetle species vector symbiotic fungi (particularly ophiostomatoid blue-stain fungi), which further impair tree defenses and contribute to tree decline and potential mortality (Krokene, 2015).

Bark beetles rely on a diverse array of semiochemicals to coordinate their host selection, mass attack, and regulation of colonization density (Byers, 2007). The colonization process typically begins with the detection of host tree volatiles, which help beetles identify and locate suitable coniferous hosts (Jirošová et al., 2022a; Moliterno et al., 2023). In contrast, non-host volatiles (NHVs) emitted by deciduous trees act as repellents, helping beetles avoid unsuitable hosts (Zhang and Schlyter, 2004). Once a host is selected, pioneer beetles release aggregation pheromones, which attract conspecifics and facilitate coordinated mass attacks, a crucial strategy for overcoming tree defenses (Christiansen and Bakke, 1988; Wood, 1982; Raffa et al., 2016; Keeling et al., 2021). As colonization progresses, bark beetles also respond to volatiles produced by their symbiotic ophiostomatoid fungi, which can influence both aggregation behavior and host suitability assessment (Jirošová et al., 2022b; Kandasamy et al., 2019, 2023). To avoid overcrowding and resource depletion, beetles release anti-aggregation pheromones at later stages, which regulate colonization density and promote dispersal to uncolonized trees (Frühbrodt et al., 2024). Beyond intraspecific signaling, bark beetles are also capable of detecting volatiles emitted by other bark beetle species and associated fungi, suggesting a broader role for interspecific chemical communication in mediating competition and spatial distribution (Andersson et al., 2009; Schiebe et al., 2019; Yuvaraj et al., 2024; Zhao et al., 2019; Kandasamy et al., 2019, 2023).

The pine bark beetle, *Ips acuminatus*, and the larch bark beetle, *Ips cembrae*, are both ecologically significant species of coniferous forests in Europe (Papek et al., 2024; Postner, 1974). *Ips acuminatus* mainly infests stressed Scots pine (*Pinus sylvestris*), with outbreaks increasing due to drought and warming (Liška et al., 2021; Wermelinger et al., 2008; Thabeet et al., 2009). It prefers the thin-barked upper trunk and crown, avoiding competition by *I. sexdentatus* (Pettersson, 2000; Pfeffer, 1955; Wood and Bright, 1992), and other bark beetle species such as *Tomicus piniperda* and *T. minor* (Foit and Čermák, 2014; Hlávková and Doležal, 2022). Males release a pheromone blend of *S*-(−)-ipsenol, *S*-(+)-ipsdienol, and (4*S*)-*cis*-verbenol to attract conspecifics (Bakke, 1978; Francke et al., 1986), with mating occurring in a polygynous system (Kirkendall, 1989, 1990). It is associated with ophiostomatoid fungi, including blue-stain species that may support beetle development and survival (Francke-Grosmann, 1965; Villari et al., 2012; Papek et al., 2024).

Similarly, *I. cembrae* primarily infests European (*Larix decidua*) and Japanese larch (*Larix kaempferi*) but can also colonize other conifer species (Postner, 1974). While typically a secondary pest of weakened or felled trees, warming and drought can trigger outbreaks (Grodzki, 2008; EFSA on Plant Health et al., 2017). It colonizes the entire trunk (Pfeffer, 1955) and competes in the crown with bark beetles from several genera, such as *Pityophthorus*, *Pityogenes*, and *Cryphalus* (Postner, 1974). Males emit *S*-(−)-ipsenol, *S*-(+)-ipsdienol, and 3-methyl-3-buten-1-ol to initiate aggregation (Kohnle et al., 1988; Stoakley et al., 1978), followed by mating with 2–4 females in a chamber (Postner, 1974). *I. cembrae* also vectors *Endoconidiophora laricola*, a pathogenic blue-stain fungus that contributes to tree mortality (Redfern et al., 1987; Kirisits, 2004; Jankowiak et al., 2007).

The primary olfactory organ of bark beetles are the club-shaped antennae (Payne et al., 1973), covered with multi-porous sensilla that house olfactory sensory neurons (OSNs) (Hallberg, 1982a). In *Ips* species, the flattened antennal club has the most olfactory sensilla concentrated on the anterior surface, organized into three distinct sensory bands labeled A, B, and C (Hallberg, 1982a; Shewale et al., 2023). Most bark beetle OSNs are narrowly tuned, responding strongly to a single or a few structurally similar compounds, while some exhibit broader tuning (Andersson et al., 2009; Kandasamy et al., 2019, 2023). The dendritic membrane of the OSNs contains chemoreceptor proteins, such as odorant receptors (ORs) (Clyne et al., 1999) and ionotropic receptors (IRs) (Benton et al., 2009), which translate odor information of the environment into electrical signals. These signals can be interpreted by the brain, potentially leading to behavioral responses (Andersson et al., 2015).

Early single sensillum recording (SSR) experiments in bark beetles investigated OSN responses to pheromones and some host volatiles in *I. typographus* (Tømmerås, 1985). Studies on olfactory detection of mainly pheromone compounds are also available for other *Ips* species, such as *Ips pini*, *Ips paraconfusus*, and *Ips grandicollis* (Ascoli-Christensen et al., 1993; Mustaparta et al., 1979; Mustaparta et al., 1980, 1977). *Ips typographus*, a major pest of Norway spruce (*Picea abies*), is the most well-studied *Ips* species in terms of peripheral odor detection, with extensive research reporting the antennal abundance of different OSN classes and the spatial distribution of OSNs tuned to pheromones, host volatiles, NHVs, and microbial volatiles (Andersson et al., 2009; Kandasamy et al., 2019, 2023; Raffa et al., 2016; Schiebe et al., 2019; Yuvaraj et al., 2024). The comprehensive OSN data from *I. typographus* allows for detailed comparison with OSN data from other congeneric species with different host preferences to better understand their olfactory detection mechanisms. Although

semiochemicals are often classified as pheromones, allomones, or kairomones, these classes might be unclear in this study, given their overlapping ecological activities. Therefore, we classify compounds in this study based on their biosynthetic origin, i.e., beetle-produced, host-derived, or microbial, to maintain clarity and avoid confusion. The chemical communication mechanisms that underlie their behavior, including pheromone-mediated aggregation and detection of plant and microbial volatiles, are critical for understanding their success as pests. Addressing these knowledge gaps is especially important given the increasing risks posed by these species under changing environmental conditions.

This study aimed to functionally characterize the OSN classes in two *Ips* species with different host preferences, specifically *I. acuminatus* on pine and *I. cembrae* on larch. By comparing our findings with existing data for the spruce bark beetle, *I. typographus*, we investigated whether the OSN frequencies and response patterns vary between species, and which of these patterns are conserved across *Ips* species. Using SSR, we examined OSN responses to 57 ecologically relevant odorants, including pheromones and volatiles from host trees, non-host trees, and fungi. Gas chromatography-electroantennographic detection (GC-EAD) using essential oils was also performed to investigate whether the antennae of the studied species respond to host volatiles. This study advances our understanding of olfactory adaptations in *Ips* species, particularly in pheromone communication and host detection. Additionally, our findings provide new insights that could be useful for species-specific monitoring and pest management, essential for maintaining forest health under climate change.

2 Materials and methods

2.1 Bark beetle collection

Both bark beetle species were collected from forests near the village of Rouchovany in central Czech Republic (49.0704°N, 16.1076°E) during late spring 2024. Species identification was conducted directly in the field. Branches of *P. sylvestris* (DBH 2–10 cm) infested by *I. acuminatus* were collected, along with logs of *L. decidua* (DBH 20–50 cm) infested by *I. cembrae*. Infested logs were maintained in university rearing facilities (FFWS, CULS) within insect cages (60 × 60 × 110 cm) under controlled laboratory conditions (25°C during the day, 19°C at night, 60% RH, and a 16:8 light/dark photoperiod). Adult beetles began emerging three to 4 weeks after field collection. The emerged beetles were collected and sexed under a stereomicroscope based on external morphology, specifically by the shape of elytral spines (Pfeffer, 1955; Zhang and Niemeyer, 1992). Before use in experiments, adult beetles were individually stored in Falcon tubes lined with moist paper at 4°C for at least 1 week. Each adult beetle was used for ten screenings using single sensillum recordings, whereas each beetle was used only once for dose–response studies. To obtain enough beetles, another batch of *I. acuminatus* was collected in spring 2024 from naturally infested *P. sylvestris* in northeastern Austria (Schönberg am Kamp; 48.5185°N, 15.7322°E) due to unavailability at the original location. Colonized logs (60 cm in length) were transferred to incubators at BOKU University, Vienna, where they were maintained at 25° C with a 16:8 light/dark photoperiod and monitored daily for newly emerged beetles. The emerged beetles were sexed and then express-mailed to Lund University, Sweden, for subsequent SSR experiments.

2.2 Chemical stimuli

The odor panel included 57 ecologically relevant compounds, including beetle pheromones, host-, non-host-, and microbial-related volatiles (Supplementary Table 2). These compounds were selected based on previous studies on *Ips* species, including *I. typographus* (Andersson et al., 2009; Kandasamy et al., 2023). Stock odor solutions (10 μg/μL) were prepared in paraffin oil and further diluted for use in experiments. For stimulation, 10 μL of the solution was applied to a piece of filter paper placed inside glass Pasteur pipettes. Control stimuli consisted of paraffin oil alone. Pipettes were stored at −18°C between experiments and replaced frequently to minimize odor depletion (Andersson et al., 2012b). For GC-EAD experiments, essential oils of *L. decidua* and *P. sylvestris* were purchased from Oshadhi Ltd. (United Kingdom). Stock odor solutions (10 μg/μL) were prepared in hexane and further diluted for use. For GC-EAD experiments, 1 μL of the solution was directly injected into GC.

2.3 Single-sensillum recordings (SSR)

SSR was performed on live adult individuals of *I. acuminatus* and *I. cembrae* to investigate OSN response profiles using previously described procedures (Andersson et al., 2012a). Beetles were immobilized in a 200 μL pipette tip, leaving the antennae and head exposed. One antenna was carefully secured with dental wax onto a microscope slide, ensuring optimal positioning for electrode insertion and light penetration from below. Mounted antennae were observed using a light microscope (Nikon Eclipse E6000FN) at ×500 magnification. Electrophysiological recordings were conducted using tungsten microelectrodes that were electrolytically sharpened with 10% KNO_3. The reference electrode was inserted into a pre-made hole in the beetle's pronotum, while the recording electrode was inserted at the base of an olfactory sensillum. The recording electrode was mounted on a Sensapex micromanipulator (uMp-3, Oulu, Finland) for precise positioning. Signals were amplified and digitized using an IDAC4 interface (Syntech), and real-time recordings were visualized in AutoSpike v. 3.9 (Syntech). A continuous stream of charcoal-filtered and humidified air (1.2 L/min) was directed onto the antenna via a 6 mm inner diameter glass tube positioned 15 mm from the antenna. Odor stimuli were delivered as 0.5 s puffs (0.3 L/min) using a stimulus controller (CS-02, Syntech), allowing the odorant to mix into the continuous airflow and reach the antenna. Odor pipettes for screening experiments were used for a maximum of two consecutive days or ten stimulations per compound. Dose–response pipettes were freshly prepared daily and used for a maximum of two stimulations. To characterize OSN response profiles, a high-dose stimulus (10 μg; 10 μL of a 1 μg/μL solution) was used for initial screening. Odor compounds were tested in random order, and OSNs were allowed to regain basal spontaneous activity between stimulations. OSNs were classified based on their response profiles during the screening experiments. Additionally, five OSN classes for *I. acuminatus* and three OSN classes for *I. cembrae* were selected for dose–response tests. Compounds were tested in increasing concentrations (10 pg. to 10 μg)

to minimize sensory adaptation, starting with the least active compound identified during the screening phase.

2.4 Data analysis

Neuronal responses were quantified offline in AutoSpike v3.9 by calculating spike frequencies during the first 0.5 s of the odor response and subtracting the pre-stimulation activity. Responses to the paraffin oil control were also subtracted. At the screening dose, responses below 20 Hz were considered biologically non-significant. Excitatory responses were categorized as intermediate responses (40–60 Hz) and strong responses (>80 Hz). Poor-quality recordings or incompletely screened neurons were excluded. All data graphs and heatmaps were generated using GraphPad Prism (version 10.1.2, GraphPad Software, San Diego, CA, USA). The venn diagram was created using InteractiVenn (Heberle et al., 2015).

2.5 Gas chromatography coupled electroantennographic (GC-EAD) experiments

For GC-EAD, the head of the beetle with antennae was prepared and connected between glass microelectrodes filled with Ringer's solution (Olsson and Hansson, 2013). Signals from the antenna were recorded using a Universal probe (Syntech) and integrated with IDAC 2 (Syntech). The results were processed using the software GcEad v. 4.6.1 (Syntech). At least five recordings were made for each sample, and a volatile was considered active if at least two antennal responses were recorded in *I. acuminatus* and *I. cembrae*. For the experiments, an Agilent 7890B GC was used, equipped with an HP-5 column (Agilent Technologies, Inc), 30 m in length, 0.32 mm in diameter, and with a film thickness of 0.25 µm, ending in a splitter. From the splitter, 5 m of the same column was led to the FID detector and 1 m of the column on the antenna. At the column outlet, the chemical components were mixed with humidified air at a flow rate of 2 L/min and blown onto the prepared antenna. Samples were injected splitless, and the carrier gas for the GC was helium with a constant column flow rate of 3 mL/min. The inlet temperature was set to 250°C, the initial oven temperature was set to 40°C for 1 min, then increased by 10°C/min to 100°C, held for 0.5 min, then increased by 20°C/min to 150°C, and then increased by 40°C/min to a final temperature of 300°C, with 3 min hold. The FID temperature was set to 300°C.

3 Results

3.1 General classification of OSN types

The responses of OSN in *I. acuminatus* (IAc) and *I. cembrae* (IC) were examined using single sensillum recordings (SSR) from antennal olfactory sensilla. Most sensilla housed two OSNs, distinguishable by their spike amplitudes (A neuron: large amplitude cell; B neuron: small amplitude cell). Some sensilla appeared to house a single OSN, while a few seemed to house three. However, the presence of three neurons was rare and sometimes difficult to confirm due to suboptimal signal quality. The OSNs frequently responded to multiple compounds, but the primary compounds elicited the strongest responses, frequently exceeding 80 Hz. The compounds that triggered weaker secondary responses were often structurally similar to the primary compounds. The OSN response activity generally followed a phasic-tonic pattern with a sharp initial rise in firing rate, followed by a gradual decline to baseline levels (Supplementary Figure 1). However, most responses were rather tonic, with increased firing well beyond stimulus offset, whereas some neurons responded in a phasic manner, with responses quickly returning to baseline activity. The highest response frequencies reached 150 Hz in *I. acuminatus* and 200 Hz in *I. cembrae*. Notably, the compounds that elicited the strongest responses at the high screening dose (10 µg on the filter paper) were also associated with the lowest detection thresholds.

A screening experiment using 57 ecologically relevant odorants at 10 µg revealed that 69 out of 82 contacted sensilla (~84%) in *I. acuminatus* (males, $n = 17$; females, $n = 23$) and 62 out of 85 sensilla (~73%) in *I. cembrae* (males, $n = 28$ and females, $n = 18$), responded to at least one compound. The remaining sensilla (12 in *I. acuminatus* and 23 in *I. cembrae*) did not respond to any of the tested compounds. Additionally, a small number of sensilla (three in *I. acuminatus* and two in *I. cembrae*) were excluded due to poor recording quality or signal loss during the experiment, which prevented OSN classification. OSNs responding strongly (>80 Hz) to at least one compound were categorized into OSN classes based on their response profiles (Figures 1, 2). In contrast, OSNs with weak to intermediate responses (20–80 Hz) were not assigned to any OSN class because their primary compounds were likely missing from the test odor panel (Supplementary Table 1 shows detailed OSN responses).

3.2 Olfactory sensory neuron responses in *I. acuminatus*

3.2.1 OSNs responding to aggregation pheromone components in *I. acuminatus*

Three OSN classes in *I. acuminatus* were strongly activated by its aggregation pheromone components, with distinct ligand specificities and dose-dependent responses. The IAc1 class (*I. acuminatus* OSN class 1) responded strongly (>80 Hz) to (4*S*)-*cis*-verbenol ($n = 5$) which is the major aggregation component in this species. Weaker secondary responses (<60 Hz) were elicited by structurally similar compounds, including (+)-*trans*-verbenol, (−)-*trans*-verbenol, (−)-verbenone, and chalcogran. These A neuron OSNs were co-localized with a B neuron OSN class (IAc2), which strongly responded (>80 Hz) to 1,8-cineole, a host tree defense compound (Figure 3A). Sensilla housing IAc1 neurons were predominantly located on the distal antennal club in the sensory band C (Figure 4A).

The IAc3 class responded primarily (>80 Hz) to *S*-(−)-ipsenol ($n = 6$), which is the naturally occurring enantiomer of ipsenol. These A neurons showed weak secondary responses (<60 Hz) to *R*-(+)-ipsenol and ipsdienol enantiomers. Dose–response tests confirmed the high specificity for *S*-(−)-ipsenol, with a response threshold as low as 100 pg. (Figure 4E). The OSNs of this class were mainly distributed in the sensory band B (Figure 4A). The IAc4 class also exhibited enantiomer-specific detection, responding strongly (>80 Hz) to *R*-(−)-ipsdienol ($n = 3$). These A neurons were

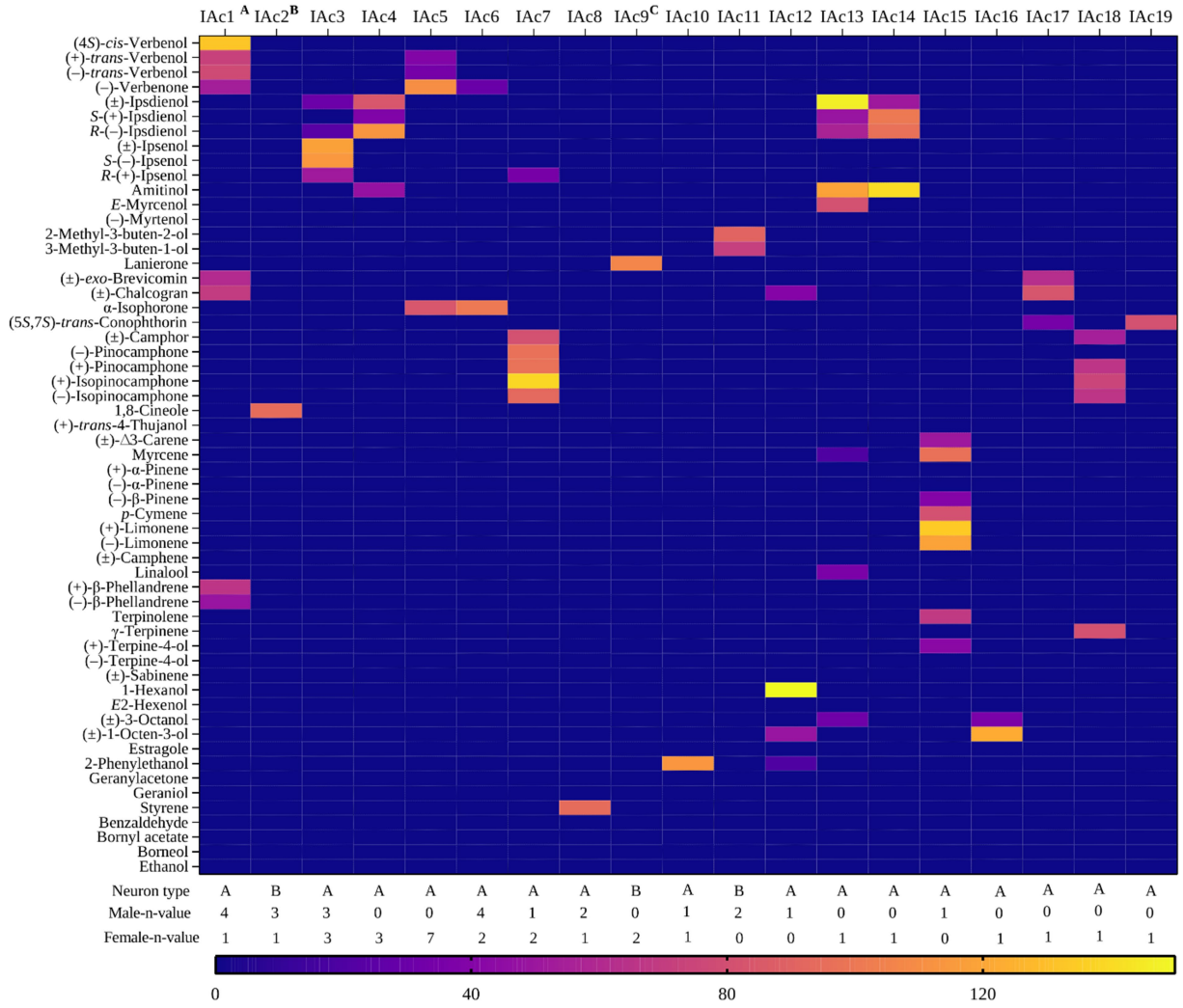

FIGURE 1
Heat map representing response profiles of identified OSN classes responding strongly (>80 Hz) to one or more compounds at a 10 µg stimulus dose in *Ips acuminatus*. The stimulus eliciting the primary response in the 19 OSN classes are as follows: IAc1: (4*S*)-*cis*-verbenol, IAc2: 1,8-cineole, IAc3: (±)-ipsenol, IAc4: *R*-(−)-ipsdienol, IAc5: (−)-verbenone, IAc6: *α*-isophorone, IAc7: (+)-isopinocamphone, IAc8: styrene, IAc9: lanierone, IAc10: 2-phenylethanol, IAc11: 2-methyl-3-buten-2-ol, IAc12: 1-hexanol, IAc13: (±)-ipsdienol, IAc14: amitinol, IAc15: (−)-limonene, IAc16: 1-octen-3-ol, IAc17: (±)-chalcogran, IAc18: *γ*-terpinene, and IAc19: (5*S*,7*S*)-*trans*-conophthorin. [A] and [B] illustrate the co-localization of IAc1 with IAc2, while [C] shows the co-localization of IAc9 with IAc4 and IAc5.

co-localized with lanierone-responsive B neurons (IAc9 class, described below). Weaker secondary responses were observed for the *S*- (+)-enantiomer, the racemic mixture, and amitinol. This class was distributed on the sensory bands A and B on the antennal surface (Figure 4A) and was observed only in females. In contrast to the screening experiments, dose–response studies demonstrated the strongest response to the other enantiomer, to *S*-(+)-ipsdienol, with minimal responses to secondary compounds at lower concentrations (Figure 4E). Interestingly, during initial screening experiments, we did not identify any sensilla that strongly responded to *S*-(+)-ipsdienol. This discrepancy between the screening and dose–response data suggests that two distinct OSN classes likely exist in *I. acuminatus*, each specifically tuned to either *S*-(+)-ipsdienol or *R*-(−)-ipsdienol.

Additionally, OSN class IAc13, ($n = 1$, A neuron) responded strongly (>80 Hz) to racemic ipsdienol, with intermediate secondary responses to amitinol, *E*-myrcenol and ipsdienol enantiomers. Another OSN class, IAc14 ($n = 1$, A neuron), responded most strongly to amitinol with weaker secondary responses to racemic ipsdienol and its enantiomers (Figure 1; Supplementary Table 1).

3.2.2 OSNs responding to other beetle-produced compounds in *I. acuminatus*

Five OSN classes were tuned to additional beetle-produced compounds. The IAc5 class responded strongly (>80 Hz) to (−)-verbenone ($n = 7$) and exhibited weaker secondary responses to *α*-isophorone, (+)-*trans*-verbenol, and (−)-*trans*-verbenol. These A neurons, primarily distributed across the sensory bands A and B, mostly in the middle region of the antennal club, displayed dose-dependent responses with a response threshold at ~1 ng (Figure 4E). This was the most abundant OSN class in this species (Figure 4B) and was found exclusively in females ($n = 7$). The IAc6 class showed

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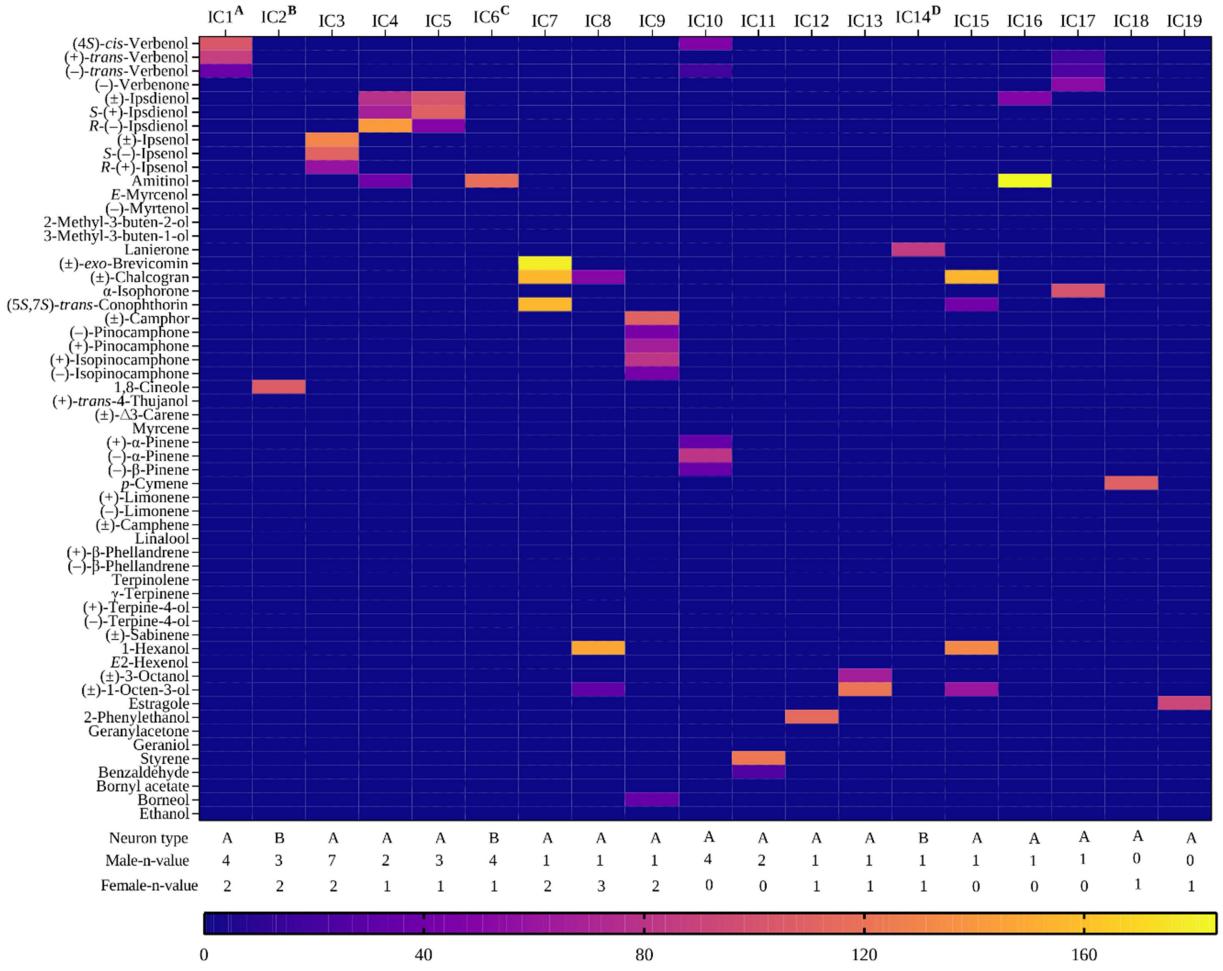

FIGURE 2
Heat map showing response profiles of identified OSN classes responding strongly (>80 Hz) to one or more compounds at the 10 µg stimulus dose in *Ips cembrae*. The stimulus eliciting the primary response in the 19 OSN classes are as follows: IC1: (4*S*)-*cis*-verbenol, IC2: 1,8-cineole, IC3: (±)-ipsenol, IC4: *R*-(–)-ipsdienol, IC5:*S*-(+)-ipsdienol, IC6: amitinol (B neuron), IC7: (±)-*exo*-brevicomin, IC8: 1-hexanol, IC9: (±)-camphor, IC10: (–)-α-pinene, IC11: styrene, IC12: 2-phenylethanol, IC13: 1-octen-3-ol, IC14: lanierone, IC15: (±)-chalcogran, IC16: amitinol (A neuron), IC17: α-isophorone, IC18: *p*-cymene, and IC19: estragole. [A] indicates IC1 co-localized with IC2, [B] illustrates IC2 co-localized with IC1 and IC10, [C] represents IC5 co-localized with IC4 and IC10, and [D] highlights IC14 co-localized with IC8.

high specificity (>80 Hz) to α-isophorone ($n = 6$). These A neurons did not respond to verbenone or any other compounds from the odor panel, and dose–response tests revealed an exceptionally low response threshold at 10 pg., indicating high sensitivity (Figure 4E). This OSN class was distributed mainly in the sensory band B on the antennal club (Figure 4A). Additional pheromone-responsive OSN classes included IAc9, a B neuron class strongly responding (>80 Hz) to lanierone ($n = 2$, both females), with one B-neuron co-localized with an *R*-(−)-ipsdienol-responsive A neuron and the other B-neuron with a non-responsive A neuron. The IAc10 class, also an A neuron, responded strongly (>80 Hz) to 2-phenylethanol ($n = 2$). Another OSN class, IAc11, a B neuron, strongly responded to 2-methyl-3-buten-2-ol ($n = 2$, both males) with secondary responses to 3-methyl-3-buten-1-ol (Figure 4C). These OSNs were co-localized with non-responsive A neurons (Figure 4D). Most of these pheromone-sensitive OSN classes were distributed in all three sensory bands on the antennal surface (Figure 4A). Another A neuron class, IAc17, showed a strong response (>80 Hz) to chalcogran followed by intermediate secondary responses (>50 Hz) to (±)-*exo*-brevicomin and weaker responses (<40 Hz) to (5*S*,7*S*)-*trans*-conophthorin (Figure 4D).

3.2.3 OSN classes responding to host, non-host, and microbial volatiles in *I. acuminatus*

We observed three OSN classes with strong primary responses (>80 Hz) to host volatiles. OSN class IAc2 was a B neuron which showed strong responses to only 1,8-cineole. OSN class IAc15 was an A neuron which responded strongly (>80 Hz) to (−)-limonene and (+)-limonene followed by intermediate (>50 Hz) secondary responses to myrcene, *p*-cymene, terpinolene, and Δ-3-carene, and weak responses (<40 Hz) to (+)-terpine-4-ol and (−)-*β*-pinene (Figure 1). OSN class (IAc18) was an A neuron, which primarily responded strongly (>80 Hz) to γ-terpinene and secondarily to (+)-isopinocamphone, (−)-isopinocamphone, (+)-pinocamphone and racemic camphor (Figure 4D). Most of the OSN classes responding to host volatiles were distributed on the sensory band B (Figure 4A). Notably, pheromone-sensitive and host-specific OSN classes were generally not spatially segregated across the antennal

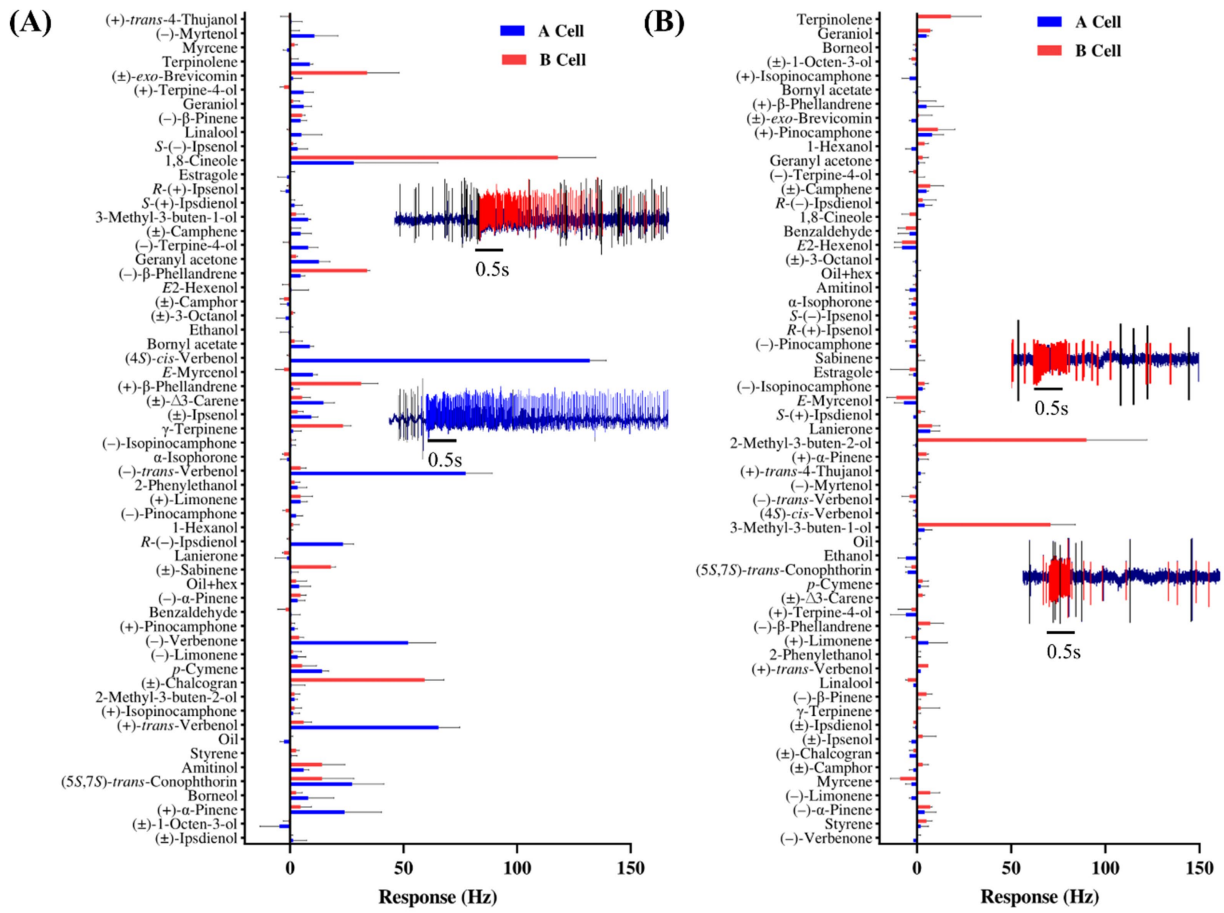

FIGURE 3
Single sensillum recordings from *Ips acuminatus* reveal co-localization of OSN classes with different spike amplitudes and response profiles. **(A)** The response pattern of the large-spiking A neuron with primary response to (4*S*)-*cis*-verbenol, co-localized with a small-spiking B neuron responding primarily to 1,8-cineole (n = 3 sensilla). Response frequencies (Hz) to the 10 μg compound dose are shown as mean ± the standard error of the mean (SEM). Representative action potential traces display the A neuron responses to (4*S*)-*cis*-verbenol and the B neuron responses to 1,8-cineole. **(B)** Responses of B neurons to both 2-methyl-3-buten-2-ol and 3-methyl-3-buten-1-ol (10 μg), co-localized with an unresponsive A neuron in the same sensillum (n = 3 sensilla). Representative action potential traces show the B neuron responses to these compounds.

surface, with the exception of (4*S*)-*cis*-verbenol-responsive neurons, which were exclusively localized within sensory band C. (Figure 4A).

Three OSN classes, all A neurons, exhibited strong responses (>80 Hz) to non-host volatiles. OSN class IAc12 responded strongly to 1-hexanol. Secondary responses (<50 Hz) were observed for other compounds, such as racemic 1-octen-3-ol and chalcogran. OSN class IAc16 displayed strong responses to racemic 1-octen-3-ol and weak responses to racemic 3-octanol (Figure 1). Another OSN class, IAc19, responded strongly (>80 Hz) to the bicyclic ketal (5*S*,7*S*)-*trans*-conophthorin (n = 1). Two OSN classes exhibited strong responses (>80 Hz) to microbial volatiles (Figure 4B). The IAc7 class was an A neuron which responded strongly to (+)-isopinocamphone (n = 3). Secondary responses (60–80 Hz) were observed to related oxygenated monoterpenes from trees and microbes, including (−)-isopinocamphone, (+)-pinocamphone, (−)-pinocamphone, and racemic camphor. These responses suggest broad tuning to structurally similar oxygenated host monoterpenes (Figure 4D). The IAc8 class was an A neuron specific to styrene, with secondary responses to benzaldehyde and racemic camphor (n = 3).

3.3 Olfactory sensory neuron responses in *I. cembrae*

3.3.1 OSN classes responding to aggregation pheromone components of *I. cembrae*

We identified two OSN classes specific to the aggregation pheromone components of *I. cembrae*. OSN class IC1 was an A neuron (n = 6), which responded primarily (>80 Hz) to (4*S*)-*cis*-verbenol with dose-dependent responses and a response threshold of 100 pg. Weaker secondary responses (40–60 Hz) to (+)-*trans*-verbenol and (−)-*trans*-verbenol were observed (Figure 5E). Five out of six of these (4*S*)-*cis*-verbenol-responsive OSNs were co-localized with IC2, which was a B neuron responding to 1,8-cineole (Figure 6A). The distribution was mostly in the distal antennal club region on sensory band C, while few were located on sensory band B (Figure 5A). (4*S*)-*cis*-Verbenol is a male-produced aggregation pheromone component in both *I. typographus* and *I. acuminatus* but not reported as an aggregation pheromone component in *I. cembrae*.

The OSN class IC3 (A neuron) was the most frequently observed class (found in ~14% of the responding sensilla), responding strongly

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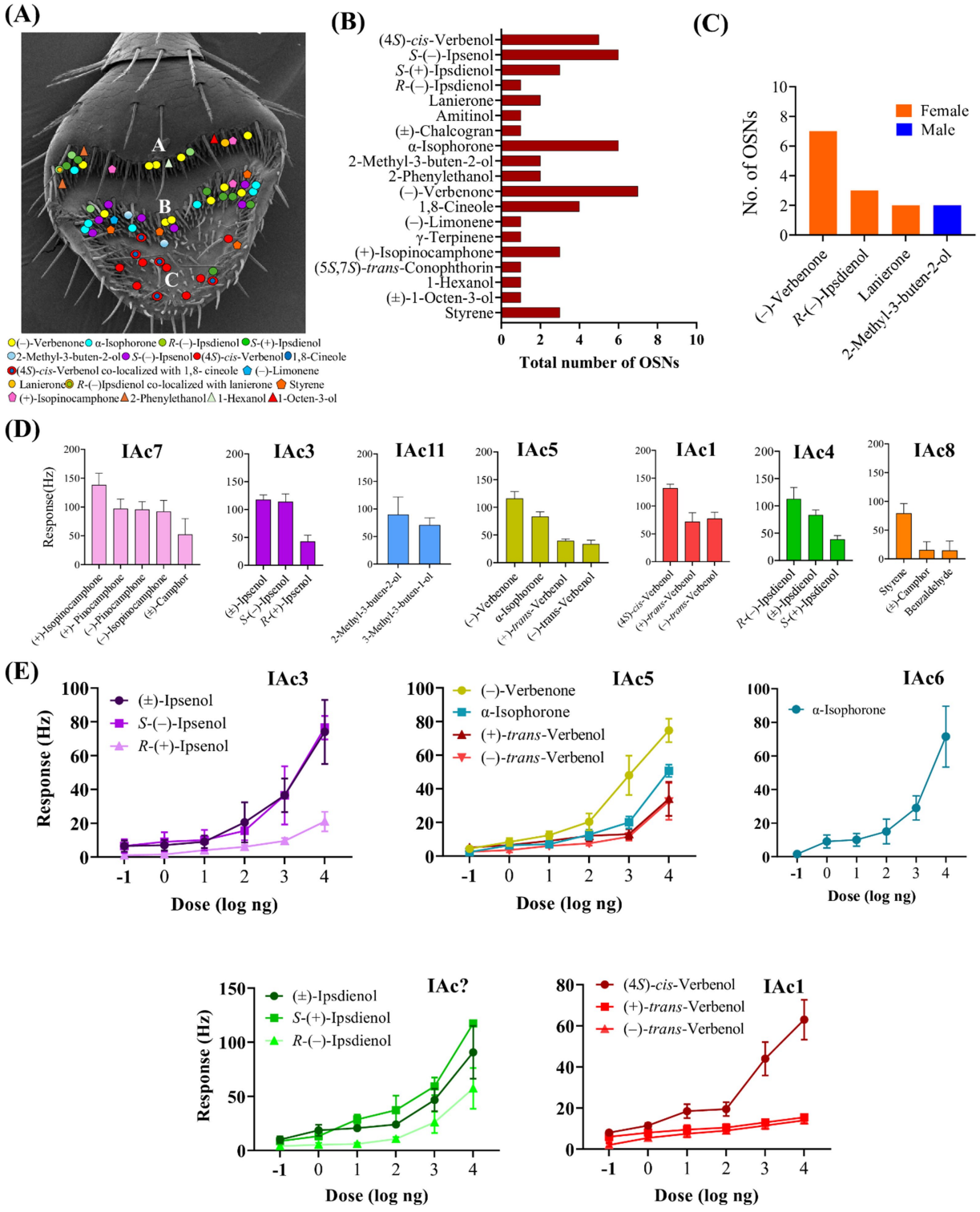

FIGURE 4
(A) Distribution of identified OSN classes in *Ips acuminatus* on the antennal surface with three sensory bands A, B and C. **(B)** Total number of each of the 19 OSN classes identified in *I. acuminatus*, with primary responses to compounds from different ecological origins. **(C)** Total number of OSN that were only found in one of the sexes of *I. acuminatus*. **(D)** Mean response (Hz) of the different OSN classes, including their secondary responses; from left to right: OSN classes IAc7: (+)-isopinocamphone (n = 3), IAc3: *S*-(–)-ipsenol (n = 6), IAc11: 2-methyl-3-buten-2-ol (n = 3), IAc5: (–)- verbenone (n = 6), IAc1: (4*S*)-*cis*-verbenol (n = 3), IAc4: *R*-(–)-ipsdienol (n = 3), and IAc8: styrene (n = 3). Error bars indicate standard error of the mean (SEM). **(E)** Dose–response curves of five OSN classes in *I. acuminatus*; IAc3 class: *S*-(–)-ipsenol (n = 4); IAc5 class: (–)-verbenone (n = 6); IAc? class: *S*-(+)-ipsdienol (n = 3)[the IAc? OSN class was observed only in dose–response tests and not while screening]; IAc1 class: (4*S*)-*cis*-verbenol (n = 4), and IAc6 class: α-isophorone (n = 4). Mean response values are shown, with error bars indicating the standard error of the mean (SEM).

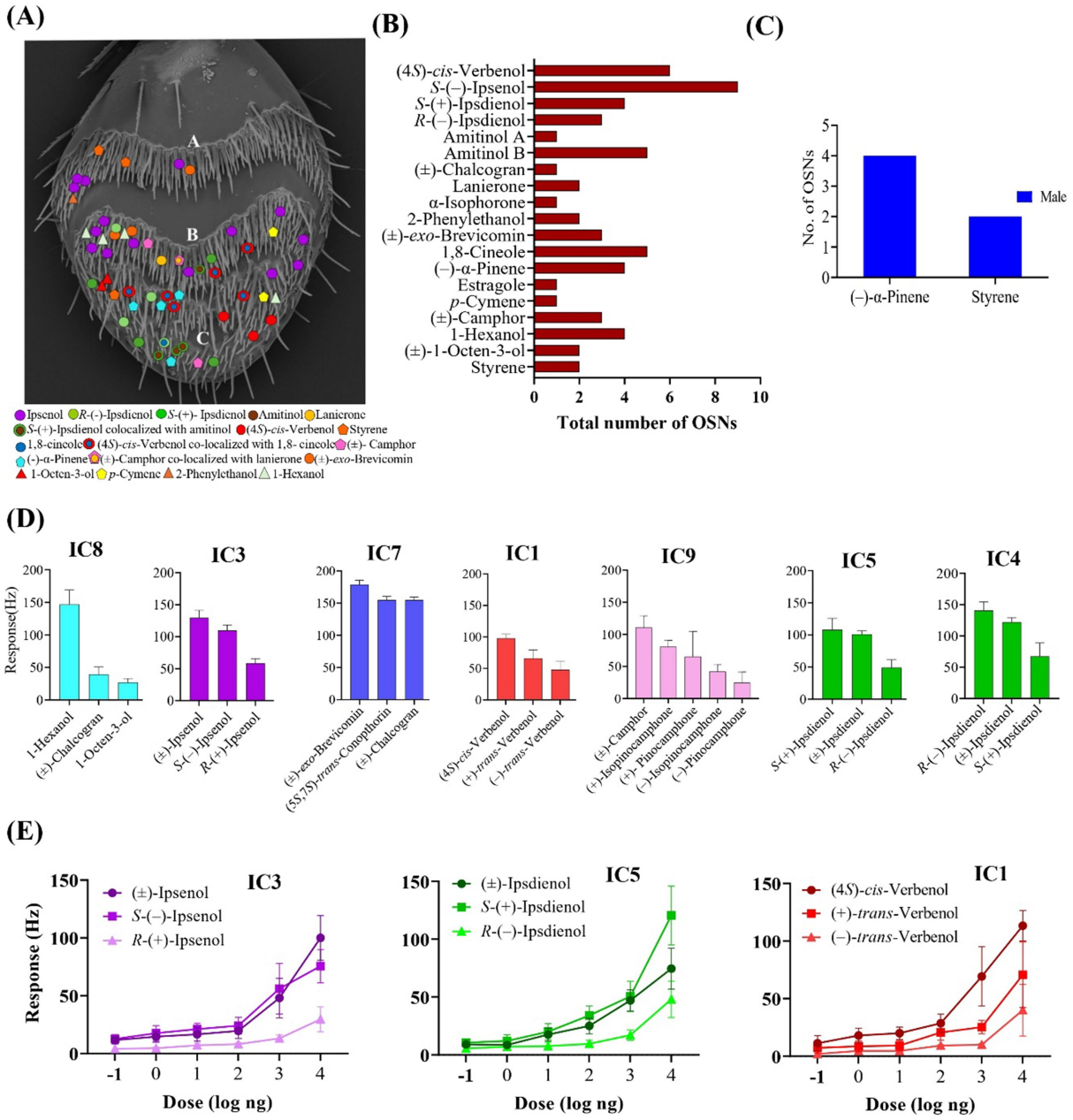

FIGURE 5
(A) Distribution of identified OSN classes in the three sensory bands (A, B, C) on the antennal surface of *Ips cembrae*. **(B)** Total number of each of the 19 OSN classes identified in *I. cembrae*, with primary responses to compounds from different ecological origins **(C)** Total number of OSNs that were only found in males of *I. cembrae*. **(D)** Mean responses (Hz) of different OSN classes, including their secondary responses; from left to right: OSN classes IC8: 1-hexanol (n = 4), IC3: *S*-(−)-ipsenol (n = 9), IAc7: (±)-*exo*-brevicomin (n = 3), IC1: (4*S*)-*cis*-verbenol (n = 4), IC5: *S*-(+)-ipsdienol (n = 4), and IC4: *R*-(−)-ipsdienol (n = 3). Error bars indicate the standard error of the mean (SEM). **(E)** Dose–response curves of three pheromone-specific OSN classes in *I. cembrae*; IC3 class: *S*-(−)-ipsenol (n = 4); IC5 class: *S*-(+)-ipsdienol (n = 4), and IC1 class: (4*S*)-*cis*-verbenol (n = 3). Mean response values are shown, with error bars indicating the standard error of the mean (SEM).

(>80 Hz) to racemic ipsenol and *S*-(−)-ipsenol (n = 9) (Figure 5B). Ipsenol is the major component of the aggregation pheromone in *I. cembrae*. The response threshold of these OSNs was around 100 pg. (Figure 5E). *R*-(+)-ipsenol elicited minimal responses in this OSN class, consistent with its absence in the natural pheromone blend (Stoakley et al., 1978). This OSN class was uniformly distributed across sensory bands A and B (Figure 5A). The OSN class IC4, which was an A neuron, responded strongly to *R*-(−)-ipsdienol (n = 3) and weaker to the *S*-enantiomer and the racemic mixture (Figure 5D). However, we did not find this OSN class during dose–response tests. Additionally, OSN class IC5 (n = 4), also an A-neuron, responded specifically to *S*-(+)-ipsdienol, with a response threshold of 1 ng (Figure 5E). Another OSN class, IC6, was a B neuron responding strongly to amitinol (n = 5); this class was always co-localized with OSN class IC5 (Figure 6B). The *S*-(+)-ipsdienol specific OSN class and *R*-(−)-ipsdienol specific OSN class were distributed across the sensory

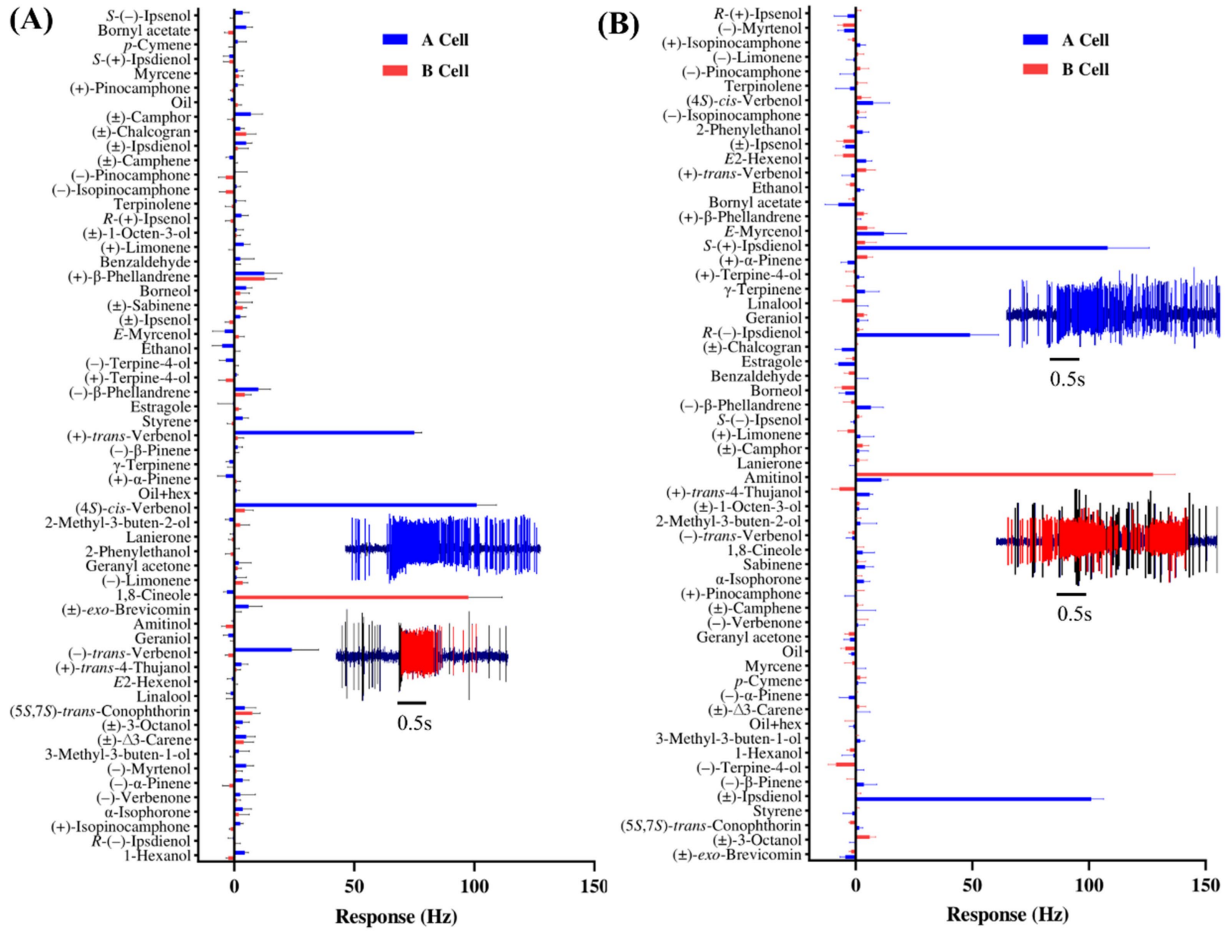

FIGURE 6
Single sensillum recordings from *Ips cembrae*, demonstrating co-localization of large spiking (A cell) and small-spiking (B cell) olfactory sensory neurons (OSNs) with responses to various pheromone compounds, volatiles from host- and non-host plants, as well as fungal symbionts (10 µg stimulus dose). **(A)** The response of A neurons primarily to (4*S*)-*cis*-verbenol, co-localized with B neurons that respond to 1,8-cineole (n = 3 sensilla). The representative action potential traces show A neuron response to (4*S*)-*cis*-verbenol and B neuron response to 1,8-cineole. **(B)** The response profile of A neurons responding to *S*-(+)-ipsdienol, which are co-localized with B neurons responsive to amitinol (n = 3). Representative action potential traces showing A neuron response to *S*-(+)-ipsdienol and B neuron response to amitinol. Response frequencies (Hz) are displayed as mean ± standard error of the mean (SEM).

band C on the antennal club surface and rarely on sensory band B (Figure 5A). It is noteworthy that during our screening, we did not detect neurons that responded to 3-methyl-3-buten-1-ol, which is a pheromone component of *I. cembrae*.

3.3.2 OSNs responding to other beetle-produced compounds in *I. cembrae*

We observed four additional OSN classes strongly responding to different beetle-produced compounds. The OSN class IC7 (A neuron) strongly responded (>80 Hz) to (±)-*exo*-brevicomin (n = 3), with secondary responses to chalcogran and (5*S*,7*S*)-*trans*-conophthorin (Figure 5D). Another OSN class, IC14, a B neuron, responded strongly to lanierone (n = 2). These neurons were co-localized with OSN class IC9 or a non-responsive A neuron. Additionally, OSN class IC12 class (A neuron) responded strongly (>80 Hz) and specifically to 2-phenylethanol (n = 2) with no secondary responses. OSN class IC15 was an A neuron primarily tuned to chalcogran with secondary responses to 1-hexanol, 1-octen-3-ol, and (5*S*,7*S*)-*trans*-conophthorin (n = 1). OSN class IC16 (A neuron) displayed strong responses (>80 Hz) to amitinol with secondary responses to racemic ipsdienol (n = 1). Lastly, OSN class IC17 was an A neuron, which responded strongly to α-isophorone followed by secondary responses to (−)-verbenone, (+)-*trans*-verbenol and (−)-*trans*-verbenol (n = 1). These OSN classes were mostly found in the distal region of the antennal surface (Figure 5A).

3.3.3 OSN classes responding to host, non-host and microbial volatiles in *I. cembrae*

Five OSN classes were specifically tuned to host volatiles, including OSN class IC2, a B neuron specific for 1,8-cineole. The OSN class IC9 showed strong responses (>80 Hz) to camphor (n = 3), with strong secondary responses to (+)-isopinocamphone (>80 Hz) and weaker secondary responses to other related oxygenated monoterpenes, such as (−)-isopinocamphone, (+)-pinocamphone, (−)-pinocamphone and borneol (Figures 4, 5D). This IC9 A neuron was co-localized with B neurons responding specifically (>80 Hz) to lanierone (IAc14). Additionally, the OSN class IC10 was an A neuron specific for (−)-α-pinene (n = 4, all males). This class also showed

weak to intermediate (20–40 Hz) secondary responses to (4*S*)-*cis*-verbenol, (+)-*α*-pinene, (−)-*β*-pinene and (+)-*trans*-verbenol. Another OSN class, IC18 (A neuron), showed strong responses (>80 Hz) to *p*-cymene ($n = 1$). Lastly, OSN class IC19 strongly responded (>80 Hz) to estragole ($n = 1$). The OSN classes corresponding to host volatiles were distributed on the sensory bands B and C on the antennal surface (Figure 5A).

Two OSN classes responded to non-host volatiles. The OSN class IC8 (A neuron) responded primarily to the green leaf volatile 1-hexanol ($n = 4$). This class also showed weak to intermediate secondary responses (<50 Hz) to other compounds, including chalcogran, racemic 1-octen-3-ol and 2-phenylethanol. The IC13 class was also an A-neuron responding to racemic 1-octen-3-ol ($n = 2$), with weaker secondary responses to racemic 3-octanol. These OSN classes were observed on sensory bands B and C on the antennal surface (Figure 5A). Additionally, the OSN class IC11 (A neuron) responded to microbial volatile styrene ($n = 2$, both males) (Figure 4), with weak secondary responses (<40 Hz) to benzaldehyde.

3.4 Comparative analysis of OSN profiles and distribution among *I. acuminatus* and *I. cembrae* with previously characterized *I. typographus*

Our comparative analysis revealed that *I. acuminatus*, *I. cembrae*, and *I. typographus* show both conserved olfactory adaptations and species-specific differences (Table 1, Supplementary Figure 2). Of the 23 OSN classes identified in *I. typographus* (Andersson et al., 2009; Kandasamy et al., 2019, 2023; Yuvaraj et al., 2024) and the 19 OSN classes found in both *I. cembrae* and *I. acuminatus*, 11 were shared among all three species based on similarities in response profiles. These shared OSN classes were tuned to beetle-produced compounds, host or non-host tree, and microbial volatiles (See Table 1 for details on OSN classes). Four OSN classes, specific to 2-methyl-3-buten-2-ol, (−)-verbenone, *α*-isophorone, (+)-isopinocamphone and (5*S*,7*S*)-*trans*-conophthorin, respectively, were found exclusively in *I. typographus* and *I. acuminatus* (Andersson et al., 2009; Kandasamy et al., 2019, 2023), while two OSN classes tuned to p-cymene and estragole were shared between *I. typographus* and *I. cembrae* (Andersson et al., 2009; Raffa et al., 2016). Additionally, OSN classes specific to racemic chalcogran and α-isophorone were shared between *I. acuminatus* and *I. cembrae*. Species-specific OSN differences were particularly evident in responses to host and fungal volatiles. *I. cembrae* had four unique OSN classes tuned to amitinol (B neuron), racemic camphor, racemic *exo*-brevicomin, and (−)-α-pinene, respectively, whereas *I. acuminatus* had two unique OSN classes tuned to (−)-limonene and *γ*-terpinene (Supplementary Figure 2).

3.5 Antennal responses of pine and larch essential oils in *I. acuminatus* and *I. cembrae* using GC-EAD

Since only a few OSN classes specific to host monoterpenes were identified in *I. acuminatus* (OSN classes IAc2, IAc15 and IAc18) and *I. cembrae* (OSN classes IC2 and IC9, IC18 and IC19), we further evaluated the antennal responses of both species to monoterpenes using GC-EAD analysis. We tested the antennae of *I. acuminatus* with pine essential oil and *I. cembrae* with larch essential oil, both with known chemical compositions, to assess their olfactory sensitivity to host-related compounds. GC-EAD analyses with pine essential oil revealed four potential chemical cues that elicited antennal responses from *I. acuminatus,* whereas *I. cembrae* responded to five potential cues in the larch essential oil (Figure 7). The EAD active compounds that elicited antennal response in *I. acuminatus* were identified as *α*-pinene, limonene, linalool and isobornyl acetate (Figure 7A) while *I. cembrae* responded to *β*-pinene, *p*-cymene, linalool, terpinen-4-ol and camphor (Figure 7B). Surprisingly, no EAD responses were observed in *I. acuminatus* to highly abundant pine host volatiles, such as 3-carene, terpinolene, and β-phellandrene. Similarly, *I. cembrae* showed no responses to key larch volatiles, including α-pinene, limonene, *β*-phellandrene, and myrcene.

4 Discussion

This study provides the first electrophysiological characterization of olfactory sensory neuron (OSN) responses in *I. acuminatus* and *I. cembrae*. By testing a comprehensive panel of ecologically relevant compounds, including pheromones, volatiles from the hosts and non-host trees, and associated microbes, we identified 19 OSN classes in both species. Most OSN classes exhibited narrow tuning, responding strongly to only one or a few structurally similar compounds, while fewer were broadly tuned. Furthermore, several of our dose–response tests in both species revealed greater OSN specificity at lower doses, consistent with findings in *I. typographus*, where OSNs exhibited high specificity to either pheromones or to compounds from the host or non-host trees and microbes (Andersson et al., 2009; Kandasamy et al., 2019, 2023).

Ips acuminatus and *I. cembrae* have several OSNs tuned to the enantiomers of ipsenol and ipsdienol, their key aggregation pheromone components (Francke and Vité, 1983; Renwick and Dickens, 1979). Interestingly, the ipsenol-responsive OSNs were highly specific, showing the strongest responses to the naturally occurring enantiomer, *S*-(−)-ipsenol, the main pheromone component in both species. This finding is consistent with previous studies in *I. typographus* and other *Ips* species such as *I. pini* and *I. paraconfusus* (Mustaparta et al., 1979; Mustaparta et al., 1980; Tømmerås, 1985). Also, the ipsdienol-responsive OSNs demonstrated enantiomer-specific tuning, suggesting two distinct OSN classes responding to *R*-(−)-ipsdienol and *S*-(+)-ipsdienol, respectively, in both species. In *I. acuminatus*, initial screenings identified only one OSN class primarily tuned to *R*-(−)-ipsdienol. However, dose–response tests revealed a stronger response to *S*-(+)-ipsdienol, suggesting the presence of distinct OSN classes tuned to each ipsdienol enantiomer. In *I. cembrae*, screenings identified distinct OSN classes specifically tuned to each ipsdienol enantiomer. However, dose–response testing was conducted only for *S*-(+)-ipsdienol, which exhibited high sensitivity. These results align with earlier reports of two enantiomer-specific ipsdienol-responsive OSN classes in *I. typographus* and other *Ips* species (Mustaparta et al., 1980; Tømmerås, 1985). Behavioral studies in *I. acuminatus* suggest that *S*-(+)-ipsdienol and *S*-(−)-ipsenol function as attractants, while *R*-(−)-ipsdienol likely serves as an attraction inhibitor in field (Bakke, 1978; Kohnle et al., 1986).

TABLE 1 Olfactory sensory neurons (OSNs) classified based on their response profiles at a 10 μg screening dose in *I. acuminatus* and *I. cembrae* and comparison to previously characterized OSN classes in *I. typographus*.

Biological origin	OSN class↓/Species→	*I. typographus* (IT)	*I. acuminatus* (IAc)	*I. cembrae* (IC)
Beetle	(4*S*)-*cis*-Verbenol	✓[a,b]	✓	✓
Beetle	*S*-(+)-Ipsdienol	✓[a]	✓	✓
Beetle	*R*-(−)-Ipsdienol	✓[a]	✓	✓
Beetle	*S*-(−)-Ipsenol	✓[f]	✓	✓
Beetle	*R*-(+)-Ipsenol	-	-	-
Beetle	Amitinol	✓[a]	✓	✓(A and B neuron)
Beetle	*2*-Methyl-3-buten-2-ol	✓[a,c] (B neuron)	✓ (B neuron)	-
Beetle	3-Methyl-3-buten-1-ol	-	-	-
Beetle	Lanierone	✓[c](B neuron)	✓ (B neuron)	✓ (B neuron)
Beetle	(±)-Chalcogran	-	✓	✓
Beetle	α-isophorone	-	✓	✓
Beetle/fungi	(−)-Verbenone	✓[a,d]	✓	-
Beetle/ fungi	(±)-*exo*-Brevicomin	-	-	✓
Beetle/fungi	2-Phenylethanol	✓[c]	✓	✓
Host	(+)-3-Carene	✓[a]	-	-
Host	Myrcene	✓[a,b,c]	-	-
Host	(+)-α-Pinene	✓[a]	-	-
Host	(−)-α-Pinene	-	-	✓
Host	*p*-Cymene	✓[a]	-	✓
Host	(−)-Limonene	-	✓	-
Host	γ-Terpinene	-	✓	-
Host	1,8-Cineole	✓[a](B neuron)	✓(B neuron)	✓(B neuron)
Host/fungi	(±)-Camphor	-	-	✓
Host/fungi	(+)-Isopinocamphone	✓[d]	✓	-
Host/fungi	Estragole	✓[g]	-	✓
Host/fungi	(+)-*trans*-4-Thujanol	✓[b,d]	-	-
Non-host	1-Hexanol	✓[a]	✓	✓
Non-host/fungi	(±)-3-Octanol	✓[a]	-	-
Non-host/fungi	(±)-1-Octen-3-ol	✓[a]	✓	✓
Non-host/fungi	Geranyl acetone	✓[c]	-	-
Non-host/fungi	(5S,7S)-*trans*-Conophthorin	✓[a]	✓	-
Fungi	Styrene	✓[b,d]	✓	✓

✓ OSN class identified; – OSN class not found yet.
[a]Andersson et al. (2009).
[b]Schiebe et al. (2019).
[c]Kandasamy et al. (2019).
[d]Kandasamy et al. (2023).
[e]Yuvaraj et al. (2024).
[f]Tømmerås (1985).
[g]Raffa et al. (2016).

(4*S*)-*cis*-Verbenol elicited strong responses in an OSN class in both *I. acuminatus* and *I. cembrae*. While this compound serves as a key aggregation pheromone component in *I. acuminatus*, it does not play a similar role in *I. cembrae*, despite the detection of trace amounts in this species (Kohnle et al., 1988). Interestingly, the presence of (4*S*)-*cis*-verbenol disrupts *I. cembrae* aggregation in field studies, possibly serving as an interspecific signal from *I. typographus*, which relies on this compound as a key aggregation pheromone (Schlyter et al., 1989).

A few examples of OSN co-localization were observed in both *I. acuminatus* and *I. cembrae*. The co-localization patterns of ipsdienol-responsive OSNs differed between the two species. In *I. cembrae*, *S*-(+)-ipsdienol-responsive A neurons were co-localized with amitinol-responsive B neurons. In contrast, in *I. acuminatus*,

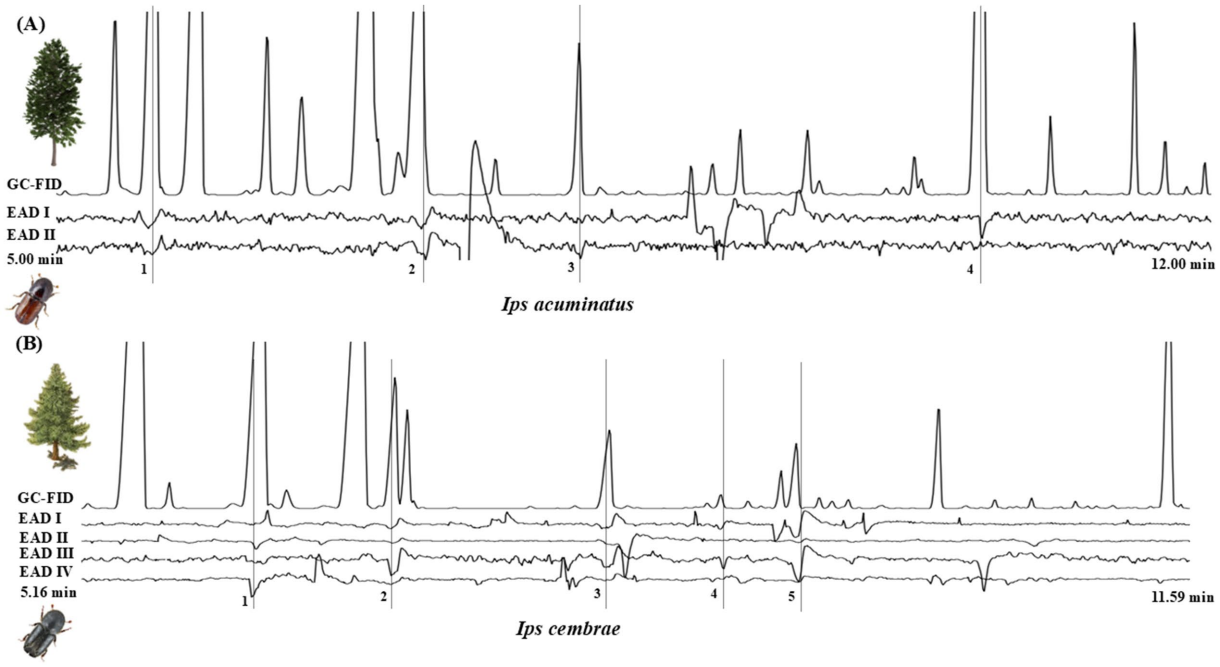

FIGURE 7
GC-EAD antennal responses at 10 ug of essential oil. **(A)** *Ips acuminatus* responses to pine essential oil, with peaks 1, 2, 3, and 4 corresponding to α-pinene, limonene, linalool, and isobornyl acetate, respectively (n = 2). **(B)** Responses of *Ips cembrae* to larch essential oil, with peaks 1, 2, 3, 4, and 5 corresponding to β-pinene, p-cymene, linalool, camphor and, terpinen-4-ol, respectively, (n = 4).

R-(−)-ipsdienol-responsive A neurons were co-localized with lanierone-responsive B neurons, corresponding to the observations in *I. typographus* (Yuvaraj et al., 2024). Additionally, in both species, (4*S*)-*cis*-verbenol- and 1,8-cineole-responsive OSNs were co-localized within the same sensilla, consistent with the co-localization pattern previously reported in *I. typographus* (Andersson et al., 2009). In *I. typographus*, such co-localization is thought to enhance the ability to differentiate odors based on spatial and temporal cues. It may also improve sensitivity to ecologically relevant odor blends by detecting specific ratio differences and regulating olfactory signaling at the peripheral level (Andersson et al., 2010; Baker et al., 1998; Binyameen et al., 2014; Bruce et al., 2005). Similar mechanisms may play a role in *I. acuminatus* and *I. cembrae*. Although the OSN responses in our study were characterized using pure compounds, it is well established that most insect semiochemicals function as multicomponent blends, with specific behavioral roles determined by the precise ratio and combination of constituents (Silverstein and Young, 1976). Such blends can activate distinct combinations of neurons depending on the ecological context, ultimately shaping behavioral outcomes.

Additionally, several OSN classes in *I. cembrae* and *I. acuminatus* responded to pheromone components produced by other *Ips* species, suggesting conserved detection mechanisms (Andersson et al., 2009; Tømmerås, 1985; Yuvaraj et al., 2024). Amitinol is not an aggregation pheromone component in either *I. acuminatus* (Bakke, 1978; Francke et al., 1986) or *I. cembrae* (Kohnle et al., 1986; Stoakley et al., 1978), despite one earlier report mentioning its presence in *I. cembrae* (Kohnle et al., 1986). Amitinol-responsive OSNs were identified in both species, exhibiting strong secondary responses to racemic ipsdienol, likely due to structural similarity and the presence of trace amounts of amitinol in the ipsdienol stimulus, corresponding to OSNs observed in *I. typographus* (Andersson et al., 2012b; Andersson et al., 2009). Interestingly, field studies indicate that amitinol enhances aggregation in *I. cembrae* but reduces attraction in *I. acuminatus*, potentially mediating interspecific interactions (Francke et al., 1986; Kohnle et al., 1986). Lanierone-responsive OSNs were identified in both species studied here, but the frequency of these OSNs was low. This is in absolute contrast to *I. typographus*, in which lanierone-specific OSNs represent the most abundant of all OSN classes (Yuvaraj et al., 2024). In *I. acuminatus*, OSN B neurons responsive to 2-methyl-3-buten-2-ol occurred at relatively low abundance, similar to observations in *I. typographus* (Kandasamy et al., 2019, 2023). Although this compound serves as an aggregation pheromone component in *I. typographus* (Lanne et al., 1989), its ecological role in *I. acuminatus* remains uncertain.

The OSN class responsive to (−)-verbenone, a known bark beetle anti-attractant (Frühbrodt et al., 2024), was the most abundant in *I. acuminatus*. Notably, we observed this OSN class only in female *I. acuminatus* and not at all in *I. cembrae*. *Ips typographus* possesses fewer (−)-verbenone-responsive OSNs than *I. acuminatus*; however, it exhibits strong behavioral avoidance in both sexes in laboratory assays (Yuvaraj et al., 2024) and in field studies (Schlyter et al., 1989). In *I. typographus*, (−)-verbenone-responsive neurons also show secondary responses to α- and β-isophorone, of which the latter compound was reported from hindguts of mated females (Birgersson et al., 1984). However, dose–response tests indicate that this OSN class is sensitive to α-isophorone (Kandasamy et al., 2023). In contrast, *I. acuminatus* possesses two distinct OSN classes: one specifically tuned to (−)-verbenone and another primarily responding to α-isophorone, with weak secondary responses to (−)-verbenone. Similarly, in *I. cembrae*, an OSN class specific to α-isophorone was also

observed, exhibiting secondary responses not only to (−)-verbenone but also to *trans*-verbenol enantiomers, corresponding to an OSN class previously observed in *I. typographus* (Kandasamy et al., 2023). The behavioral role of *α*- and *β*-isophorone remains unclear.

Ips acuminatus and *I. cembrae* exhibited OSN responses to pheromones produced by non-*Ips* bark beetles, differing from OSN responses in *I. typographus*. Both species had an OSN class highly responsive to chalcogran, a pheromone of many *Pityogenes* species (Francke, 1977; Francke et al., 1995). Additionally, *I. cembrae* possessed a separate OSN class tuned to (±)-*exo*-brevicomin, which is a pheromone of *Dendroctonus* species and is also produced by a beetle symbiotic fungus (Zhao et al., 2019). This broader heterospecific pheromone detection in *I. cembrae* may reflect its ability to colonize different hosts, including *Abies*, *Picea*, and *Pinus* trees and frequent interactions with bark beetles from other genera such as *Pityophthorus*, *Pityogenes*, and *Cryphalus* (Postner, 1974; Pfeffer, 1955). In contrast, *I. typographus* OSNs primarily responsive to the non-host volatile (5*S*,7*S*)-*trans*-conophthorin exhibit strong secondary responses to both chalcogran and *exo*-brevicomin, likely due to structural similarities (Andersson et al., 2009).

Major monoterpene hydrocarbons such as *α*-pinene, *β*-pinene, limonene, and myrcene are key volatiles in coniferous trees, the primary hosts of *Ips* bark beetles (Wajs et al., 2007). However, OSN classes responding to monoterpenes were relatively rare in both species. In *I. acuminatus*, one OSN class responded mostly to (−)-limonene, with secondary responses to myrcene, *p*-cymene, terpinolene, and β-pinene. Another distinct OSN class was tuned specifically to *γ*-terpinene. Notably, we did not identify OSN classes for key pine volatiles such as *α*-pinene, 3-carene, β-pinene, and myrcene. In contrast, *I. cembrae* had an OSN class that responded primarily to (−)-α-pinene, with weak secondary responses to β-pinene. Another OSN class was specifically tuned to *p*-cymene. However, no OSN class was identified for major larch volatiles such as β-pinene, 3-carene, limonene, and myrcene. Given the suggested role of monoterpenes in bark beetle behavior (Erbilgin et al., 2007), we conducted GC-EAD analyses using pine and larch essential oils. *Ips acuminatus* antennae exhibited weak responses to *α*-pinene, limonene, linalool, and isobornyl acetate, whereas *I. cembrae* responded to *β*-pinene, p-cymene, linalool, and terpinen-4-ol. The absence or inconsistency of responses to major host volatiles, combined with the finding that *I. acuminatus* does not exhibit attraction to host trees in field studies (Brattli et al., 1998), suggests that these compounds may not play a primary role in host tree attraction for these species. Volatile compounds produced in minor amounts by Norway spruce, such as 1,8-cineole (Jirošová et al., 2022a; Schiebe et al., 2019) and estragole (Moliterno et al., 2023; Joseph et al., 2001), elicit antennal responses in *I. typographus* and function as anti-attractants. In this study, we identified OSNs specifically responsive to 1,8-cineole in both *I. acuminatus* and *I. cembrae*, while estragole-responsive OSNs were observed only in *I. cembrae*. Given that 1,8-cineole has been previously linked to conifer resistance against *I. typographus* attack (Schiebe et al., 2012), its detection by OSNs in the two species examined here suggests a similar ecological role in their host interactions.

Low-abundance oxygenated host monoterpenes, whose concentrations increase in stressed or fungus-infected conifers, likely play a crucial role in beetle discrimination of suitable hosts (Lehmanski et al., 2023). Although present only in trace amounts, these metabolites of monoterpene hydrocarbons can be produced via microbial activity or the tree's own metabolism and may significantly influence bark beetle host selection and colonization strategies (Moliterno et al., 2023; Kandasamy et al., 2023). In our study, *I. acuminatus* exhibited strong OSN responses primarily to (+)-isopinocamphone and secondarily to structurally similar pinocamphone and camphor, closely resembling the OSN responses described in *I. typographus* (Kandasamy et al., 2019, 2023). In contrast, *I. cembrae* had OSNs primarily responsive to racemic camphor, with secondary responses to pinocamphone and isopinocamphone enantiomers. Isopinocamphone, an oxygenated metabolite of pinene (the main component of pine resin), and camphor, a hydroxylated metabolite of borneol from larch-derived bornyl acetate, are produced by beetle-symbiotic fungi (Kandasamy et al., 2023), which can also be associated with stressed host trees (Schiebe et al., 2019).

Ips acuminatus and *I. cembrae* vector different ophiostomatoid fungi (Papek et al., 2024; Jankowiak et al., 2007), whose volatile profiles have not yet been characterized but are likely to differ. Both species exhibited strong OSN responses to fungal volatiles (2-phenylethanol, styrene, 1-octen-3-ol), while (5*S*,7*S*)-*trans*-conophthorin-responsive OSNs were detected only in *I. acuminatus*. These OSN classes have previously been identified in *I. typographus* (Andersson et al., 2009; Kandasamy et al., 2019, 2023). *trans*-Conophthorin has been shown to disrupt aggregation pheromone activity in conifer-infesting bark beetles, including *I. typographus*, in field studies (Huber et al., 2000; Zhang et al., 2002; Zhang and Schlyter, 2004). These compounds, along with oxygenated host monoterpenes (Kandasamy et al., 2023) and (±)-*exo*-brevicomin (Zhao et al., 2019), are also produced by fungi. They likely indicate fungus-colonized or weakened host trees, potentially guiding beetles towards suitable hosts. Additionally, fungi may produce volatiles that elicit a positive response from beetles, as they potentially act as nutritional resources for bark beetles (Kandasamy et al., 2019, 2023). However, further research is needed to clarify their precise ecological roles and the mechanisms by which beetles interpret these chemical cues. Both species exhibited OSN responses to NHVs, helping conifer-feeding bark beetles avoid unsuitable angiosperm trees. OSNs in *I. acuminatus* and *I. cembrae* responded selectively to 1-hexanol, a known anti-attractant emitted by green leaves of non-host trees (Schlyter et al., 1989, 2000; Zhang et al., 1999).

Some OSN classes were only found in one of the sexes of *I. acuminatus* and *I. cembrae*, which may suggest differences in olfactory-driven behaviors between males and females. In *I. acuminatus*, OSN classes for (−)-verbenone, *R*-(−)-ipsdienol, and lanierone were observed in females, whereas 2-methyl-buten-2-ol OSNs were found in males. These female-biased responses may be associated with the species' polygynous mating system, where males form large harems (2–12 females per male), and pseudogamous females breed independently (Kirkendall, 1989, 1990). Thus, these olfactory cues may help females to avoid overcrowded trees and reduce interspecific competition with other conifer bark beetle species (Papek et al., 2024). In *I. cembrae*, OSN classes specific to (−)-*α*-pinene and styrene were found in males, suggesting a role in host location. However, further recordings from additional sensilla and behavioral experiments are needed to determine whether these OSN classes are sex-specific, sex-biased, or simply missed during sampling.

The antennal distribution of OSNs varies among species. Ipsenol-responsive OSNs in *I. cembrae* were located mainly in sensory bands

A and B, similar to *I. typographus* (Andersson et al., 2009), but restricted to band B in *I. acuminatus*, unless we failed to find them in band A. Conversely, ipsdienol-responsive OSNs occurred predominantly in bands B and C in *I. cembrae*, while in *I. acuminatus* they were distributed across bands A and B, resembling the distribution reported in *I. typographus* (Andersson et al., 2009). OSNs responding to (4*S*)-*cis*-verbenol are predominantly located in band C in all three species. These differences in OSN distribution may reflect species-specific olfactory adaptations related to their pheromone detection, distinct host selection, and chemical communication within their distinct ecological contexts. While our study was confined to OSNs housed in the antennae, it is noteworthy to point out that *Ips* beetles also possess chemosensory sensilla on the maxillary palps (Hallberg, 1982b; Hallberg et al., 2003), potentially capable of detecting less volatile or contact-mediated compounds, a subject of interest which needs further investigation.

The olfactory responses we observed in *I. acuminatus* and *I. cembrae* are consistent with broader insect patterns, where selective olfactory systems are shaped by evolutionary pressures. OSNs are frequently specifically tuned to ecologically important stimuli also in non-beetle species, such as moths and *Drosophila*, whereas other neurons may be more broadly tuned (Hallem et al., 2004; de Bruyne and Baker, 2008; Andersson et al., 2015). Additionally, other congeneric species often share several conserved OSN classes, and display a few species-specific ones. This has been shown, for example, in beetles from other families, such as clover seed weevils (*Apionidae*) in the *Protapion* genus (*P. fulvipes* and *P. trifolii*) and scarab beetles (Scarabaeidae) in the *Pachnoda* genus (*P. interrupta* and *P. marginata*) (Bengtsson et al., 2011; Andersson et al., 2012a; Carrasco et al., 2019). At a molecular level, 12 odorant receptors (ORs) have been functionally characterized in *I. typographus* (Hou et al., 2021; Roberts et al., 2021, 2022; Yuvaraj et al., 2021, 2024; Biswas et al., 2024), with responses resembling several of the OSN responses observed in this study. While many OSN classes identified here exhibit response patterns similar to *I. typographus*, it remains unknown whether conserved ORs are responsible. Given that *I. typographus* and *I. duplicatus* share numerous conserved OR orthologs (Johny et al., 2024), similar conservation is likely in *I. acuminatus* and *I. cembrae*. Further OR characterization and comparative genomic analyses across *Ips* species could provide deeper insights into OSN specificity and pheromone detection mechanisms.

Overall, our findings provide valuable insights for improving bark beetle management by refining pheromone-based strategies. Although the pheromone-baited "trap and kill" approach has shown limited success due to spillover infestations and low overall efficacy (Jakuš et al., 2003), pheromone traps remain useful for monitoring beetle activity. Cross-attraction among *Ips* species has been observed (Byers, 1989; Etxebeste et al., 2012), emphasizing the need for species-specific approaches. Several anti-aggregation compounds show potential for spruce protection, including verbenone (Frühbrodt et al., 2024), spruce volatiles like trans-4-thujanol and 1,8-cineole (Andersson et al., 2010; Jirošová et al., 2022a), and others such as hexanol, 1-octen-3-ol, and *trans*-conophthorin (Schiebe et al., 2011; Zhang and Schlyter, 2004; Unelius et al., 2014). These have been repeatedly tested in various combinations against I. typographus (Schiebe et al., 2011; Zhang and Schlyter, 2004; Unelius et al., 2014; Jakuš et al., 2024), though they also suffer from spillover effects due to their repellent nature (Jakuš et al., 2003; Schiebe et al., 2011). Push-pull strategies, which combine anti-attractants with pheromone traps (Jakuš et al., 2022) or baited trap trees (Lindmark et al., 2022), offer a potential improvement. However, their effectiveness decreases under high beetle population density and severe tree stress (Deganutti et al., 2024). The use of anti-attractants for *I. acuminatus* and *I. cembrae* remains untested (Frühbrodt et al., 2024), highlighting the need for further behavioral studies with compound combinations designed according to this study to evaluate their field efficacy.

5 Conclusion

This is the first electrophysiological study to functionally characterize OSNs in *I. acuminatus* and *I. cembrae*, identifying 19 OSN classes in each species. These OSNs exhibited distinct tuning to aggregation pheromones, host monoterpenes, NHVs, and fungal-derived odors, highlighting their crucial role in bark beetle ecology. Comparative analysis with *I. typographus* revealed both conserved and species-specific OSN response patterns. While certain OSN profiles were shared across *Ips* species, suggesting common olfactory strategies for aggregation and host detection, species-specific differences likely reflect adaptations to their respective host tree preferences. The detection of heterospecific pheromones, along with fungal volatiles, further supports the role of multiple chemical cues in species coexistence and host colonization. Although OSN response profiles were generally similar between sexes, further research is needed to determine whether subtle differences influence mate and host selection behaviors. From an applied perspective, our findings support the use of specific compositions of ipsenol and ipsdienol mixtures, including their enantiomeric ratios, in combination with other detected compounds for species-specific *Ips* beetles monitoring and pest management. Integrating NHVs and host volatiles into conifer tree protection strategies could enhance its efficiency. Furthermore, future studies should explore a broader range of volatile compounds to identify additional OSN classes and incorporate molecular analyses of olfactory receptor function to refine our understanding of olfactory coding mechanisms in bark beetles. A deeper disentangling of these mechanisms could enable targeted interventions by disrupting the detection of key compounds by beetles at the gene level. This study lays the groundwork for further exploration of bark beetle olfactory systems, offering insights into ecological interactions and improved pest management strategies.

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

MKS: Data curation, Formal analysis, Investigation, Validation, Visualization, Writing – original draft, Writing – review & editing. JB: Data curation, Investigation, Writing – original draft. JS: Data curation, Writing – review & editing. MS: Methodology, Writing – review & editing. MA: Conceptualization, Methodology, Supervision, Validation, Writing – review & editing. DK: Conceptualization, Methodology, Supervision, Validation, Writing – review & editing. AJ: Conceptualization, Methodology, Supervision, Validation, 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.

Generative AI statement

The authors declare that Gen AI was used in the creation of this manuscript. The English language was edited using ScholarAI (2025), ScholarAI: AI-powered research assistant. OpenAI. Available at: https://notilo.ai. We recognize it in acknowledgement section in the article.

Publisher's note

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/ffgc.2025.1588866/full#supplementary-material

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